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Targeting the Subacute Progression of Hippocampal Cellular Senescence Following Repetitive Mild Traumatic Brain Injury

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TARGETING THE SUBACUTE PROGRESSION OF HIPPOCAMPAL CELLULAR SENESCENCE FOLLOWING REPETITIVE MILD TRAUMATIC BRAIN INJURY

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For those facing the everyday challenges resulting from traumatic brain injury, you are not alone.

It is easier to find men who will volunteer to die than to find those who are willing to endure pain with patience.

— Julius Caesar

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LIST OF ABBREVIATIONS

ATP:	Adenosine Triphosphate
Ca^{2+}:	Calcium
CCI:	Controlled Cortical Impact
ChIP-Seq:	Chromatin Immunoprecipitation Sequencing
CA3:	Cornu Ammonis 3
DNA:	Deoxyribonucleic Acid
DPI:	Days Post-Injury
GFAP:	Glial Fibrillary Acidic Protein
HMBG1:	High Mobility Box Group 1
Iba1:	Ionized Calcium Binding Adaptor Molecule 1
K^{+}:	Potassium
mTBI:	Mild Traumatic Brain Injury
Na^{+}:	Sodium
NeuN:	Neuronal Nuclear Antigen
NF-κB:	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
PTD:	Post-Treatment Day
Rb:	Retinoblastoma Protein
rmTBI:	Repetitive Mild Traumatic Brain Injury
rmTBI+T:	Testosterone Treated Repetitive Mild Traumatic Brain Injury

ROS:	Reactive Oxygen Species
SASP:	Senescence-Associated Secretory Phenotype
SIRT1:	Sirtuin 1 / Silent Mating Type Information Regulation 2 Homolog 1
T:	Testosterone
TBI:	Traumatic Brain Injury
UTR:	Untranslated Region

CHAPTER ONE

INTRODUCTION AND SPECIFIC AIMS

As a leading cause of death and disability, traumatic brain injury (TBI) accounts for over 69 million worldwide hospital visits annually, with TBI-related medical costs surmounting $400 billion.[1, 2] Approximately 75-90% of all TBIs are classified as mild TBI (mTBI), sustaining one of which can increase an individual's risk for repetitive mTBI (rmTBI).[2, 3] Though mTBI symptoms generally resolve days to weeks post-injury, up to 30% of patients experience persistent symptoms lasting months to years.[4] These symptoms can be exacerbated and further prolonged in the case of rmTBI.[4, 5] Most molecular studies focus on the acute sequelae following mTBI. However, little is known about the molecular sequelae following rmTBI at subacute and chronic times. **Thus, the cellular and molecular mechanisms by which rmTBI-associated symptoms persist have not been well elucidated**.

Cellular senescence is a dynamic state of cell cycle arrest, implicated in TBI and neurodegeneration, and represents a potential mechanism by which cognitive deficits may persist following rmTBI.[6-8] Testosterone has been shown to be beneficial in the context of TBI and cellular senescence and presents a promising treatment option for rmTBI-associated symptoms.[9-12] Targeting cellular senescence in the hippocampus at a subacute time point with testosterone may prove to be an effective therapeutic strategy for persistent rmTBI-associated cognitive deficits due to hippocampal involvement in cognitive function. **However, subacute systemic testosterone administration has yet to be evaluated as an efficacious**

treatment option for persistent rmTBI-associated cognitive deficits or hippocampal cellular senescence.

The **goal** of this study is five-fold: 1.) to establish a model of persistent rmTBI-associated cognitive deficits 2.) to determine if there is a temporal progression of hippocampal cellular senescence-associated molecular alterations following rmTBI; 3.) to evaluate the effects of subacute systemic testosterone administration on cognition and hippocampal cellular senescence; 4.) to determine if a subacute therapeutic window exists in which intervention ameliorates rmTBI-associated cognitive deficits and hippocampal cellular senescence; 5.) to conduct a preliminary investigation of cell type-specific senescence-associated molecular alterations in the hippocampus at a chronic time point following rmTBI.

Hypothesis

This study hypothesizes that 5-hit closed-head rmTBI induces persistent cognitive deficits and progressive hippocampal cellular senescence-associated molecular alterations and that a subacute treatment window exists in which testosterone administration can alleviate these behavioral and molecular manifestations. The following specific aims are used to test these hypotheses.

Specific Aim 1

Determine if rmTBI induces persistent recognition memory deficits and a progression of hippocampal cellular senescence.

Specific Aim 2

Determine the effects of subacute testosterone treatment on recognition memory and hippocampal cellular senescence following rmTBI.

Specific Aim 3

Gather preliminary data investigating how rmTBI affects cellular senescence in specific hippocampal cell types at a chronic time point.

Experimental Design

The following experimental design was implemented to investigate the aforementioned specific aims. Eight-week-old Long-Evans Hooded rats were randomly divided into three groups: sham, rmTBI, and rmTBI+T. Animals were anesthetized and subjected to five closed-head controlled cortical impact mTBI, spaced 48 hours apart, mimicking the effects of rmTBI. The impact was delivered over the right sensorimotor cortex. Animals were allowed 35 days to recover, representing a subacute time point at which cognitive deficits might persist post-injury. A subset of rmTBI animals had a capsule filled with pure crystalline testosterone powder (rmTBI+T animals), or an empty control capsule (rmTBI animals), implanted in the nape 35 days post-injury (DPI) also referred to as post-treatment day (PTD) 0. Testosterone-filled capsules have been shown to maintain serum levels of testosterone at 4.49 ± 1.40 ng/mL, within the physiological range for rats of 2 – 10 ng/mL.[13, 14]

Multiple cohorts of animals were used for this study utilizing the aforementioned experimental paradigm; the first cohort was behaviorally assayed for recognition memory deficits via novel object recognition (NOR) testing at 7 and 28 DPI. In a second cohort, animals underwent the same injury paradigm; however, animals were sacrificed 35 and 63 DPI with subsequent resection of the ipsilateral hippocampus. The whole ipsilateral hippocampal homogenate was processed in protease and phosphatase inhibitor containing RIPA Buffer or Trizol Reagent for protein or mRNA extraction, respectively. Purified protein and mRNA underwent molecular analysis to evaluate cellular senescence progression following rmTBI.

rmTBI and rmTBI+T animals were compared to investigate the effects of subacute testosterone treatment on senescence-associated markers at PTD 28 (63 DPI). A third cohort of animals was assayed at PTD 7 (42 DPI) via NOR to determine if subacute testosterone treatment affects recognition memory. Animals were sacrificed at 175 DPI and underwent transcardial perfusion with 4% paraformaldehyde to preserve the brains for subsequent immunohistochemistry of the hippocampus for chronic senescence-associated protein alterations.

These experiments characterize the progressive molecular mechanisms contributing to persistent cognitive dysfunction following rmTBI. Furthermore, they determine whether a subacute therapeutic window exists at which systemic testosterone administration mitigates persistent symptoms and prevents potential neurodegenerative cascades. This study is the first of its kind to characterize the subacute progression of cellular senescence and concomitant cognitive deficits in a 5-hit closed-head repetitive mild TBI model. It is also the first of its kind to utilize subacute testosterone treatment as a therapeutic intervention for persistent manifestations of rmTBI. This study also aims to shed light on common mechanisms behind neurodegeneration because of the many similarities with TBI pathology. Future research includes characterizing the progression of cellular senescence at single-cell resolution and developing antibody-directed pharmacological agents to specifically target senescent cells for the treatment of persistent TBI-associated symptoms and potential neurodegeneration.

Significance Statement

TBI affects nearly 69 million people worldwide every year and is associated with over $400 billion in related medical expenses. Up to 90% of TBIs diagnosed in an emergency department setting are classified as mTBI, sustaining one of which can increase the risk for subsequent injury in the form of rmTBI. rmTBI further increases the risk of developing cognitive

dysfunction and neurodegeneration compared to a single mTBI. Cognitive deficits are one of the most common symptoms following rmTBI and generally resolve one to two weeks post-injury. However, a subset of patients will experience persistent symptoms lasting upwards of months to years following the initial injury. The molecular mechanisms by which cognitive symptoms persist and predispose a patient to neurodegeneration are not well defined. Emerging evidence has linked increased cellular senescence to rmTBI, cognitive deficits, and neurodegeneration. Cellular senescence represents a possible mechanism by which persistent cognitive deficits and neurodegeneration might occur. By elucidating the molecular mechanisms behind persistent cognitive dysfunction following rmTBI, it may be possible to treat the persistent symptoms experienced by a large proportion of rmTBI patients and potentially provide insight into therapeutic strategies to retard the progression of neurodegeneration. Work conducted in this study will provide essential insight into the underlying causes of persistent rmTBI symptoms and determine if these symptoms can be mitigated through therapeutic intervention at previously thought to be inefficacious subacute time points, giving hope to individuals suffering the long-term ramifications of rmTBI.

CHAPTER TWO

LITERATURE REVIEW AND BACKGROUND

Traumatic Brain Injury

TBI is a perturbation in neurophysiologic function and/or a structural injury to the brain, caused by the application of an external force to the head or body.[15, 16] The induction of TBI may result from head penetration and direct damage to the brain, exposure to an explosive blast wave, or rapid acceleration/deceleration of the head with subsequent rotation of the brain within and/or impact against the skull.[17] TBI is the worldwide leading cause of mortality, morbidity, and disability across all ages and affects 69 million people yearly, with total medical expenses exceeding $400 billion.[17-19] An estimated 3.5 billion TBIs occur in the United States each year, costing over $40.6 billion in non-fatal TBI-related medical costs, with estimates suggesting that 30% of injuries go undiagnosed or are diagnosed outside of the emergency department.[2, 16, 20] The gross underestimation in the prevalence of TBI is supported by a recent Norwegian study showing that up to 71% of patients diagnosed with TBI were treated outside of the emergency department.[21] Nearly one-third of all injury-related deaths in the United States are TBI-related with approximately 64,000 mortalities occurring in 2020 alone.[22] TBI is often referred to as the “silent epidemic” as a result of the underestimation of its incidence and represents a significant financial and medical burden on modern society. All individuals are susceptible to experiencing a TBI; however, certain populations are more at risk than others.

The definition of elderly varies between studies in the context of TBI from 55 to 75 years old. This population represents the highest risk group of sustaining a TBI and generally has more severe consequences when compared to younger individuals, with approximately 775,00 individuals over the age of 75 living with long-term TBI-related disabilities in the United States.[16, 23-25] Children 0 to 4 years old are the second highest risk group to sustain TBI, likely due to their high fall risk while learning coordinated movement, with adolescents aged 15-19 years being the third most at-risk population.[23] Approximately 145,00 minors were estimated to be living with long-term TBI-related disability as of 2005.[25] Other populations at high risk of TBI include contact-sport athletes, military veterans, and domestic abuse victims.[2, 26] TBI risk disparities are also found across gender and race. A meta-analysis from 2013 demonstrated that men are 2.2 times more likely to sustain a TBI than women due to increased risk-taking behavior.[27] African-American children are 40% more likely to experience a TBI than non-Hispanic white children.[28] TBI data stratified by race and ethnicity are only reported in 14.5% of studies, thus limiting the available literature on racial disparities in TBI.[29] TBI represents a diverse injury with a spectrum of severities regardless of an individual's risk factors.

The Glasgow Coma Scale is most commonly used to measure the severity of TBI and is based on the immediate onset or exacerbation of symptoms including cognitive deficits, memory loss, neurologic deficits, alterations in mental state, and loss or alteration in the level of consciousness.[30] This assessment utilizes a scoring system with the following severity binning: 3-8 as severe, 9-12 as moderate, and 13-15 as mild. Assessments such as the Glasgow Outcome Scale attempt to predict patient outcomes following TBI.[31] These outcome assessments fall short with TBI of mild severity, likely due to the minute but clinically relevant alterations from

baseline, the diverse nature of the injury, and factors specific to the individual such as race, biological sex, and age.[17-19]

Mild Traumatic Brain Injury

mTBI is the least severe form of TBI and can be classified as sub-concussive or concussive. The term "concussion" is generally used interchangeably with mTBI, despite definitions for mTBI varying across professional organizations and concussion being a subclassification of mTBI. A consensus on the distinction between mTBI and concussion is imperative to define the specific injury more accurately; however, concussion and mTBI generally refer to a non-penetrating TBI of mild severity associated with perturbations in normal brain function. mTBI is most studied in the context of contact sports and military service despite the highest risk TBI populations being the elderly and young children. It is estimated that approximately 42 million people receive mTBI each year, making up 90% of all TBI cases diagnosed in an emergency department setting.[24, 32] The true mTBI incidence rate is accepted to be significantly higher as only a small fraction of those impacted by TBI seek medical attention in a hospital setting.[32-34] The underestimation in the prevalence of mTBI is supported by a survey of 486 athletes in which the questionnaire indicated that 30.5% of individuals had an undiagnosed concussion based on experienced symptoms.[35] Furthermore, a retrospective survey of collegiate athletes at the end of their careers indicated that 12% of concussions were unreported and 26% of concussions were potentially unrecognized.[36] The American Medical Society for Sports Medicine reports that an estimated 3.8 million sports-related mTBI occur annually in the United States with up to 50% going unreported.[37] The high rate of incidence in sports has resulted in a focus to improve rules and regulations for player

protection against head injury as well as a movement to improve concussion detection and return-to-play protocols.

Military members represent another population with a high incidence rate of mTBI. Military members are at risk for blast-induced TBI, in addition to impact-induced concussions, which carries inherent mechanistic differences from a concussion that will be discussed later. Return-from-duty surveys suggest that 20% of veterans returning from Iraq and Afghanistan received mTBI while on duty regardless of the mechanical cause of mTBI.[38] A more recent retrospective cohort study of 46,309 service members having sustained TBI between 2001 and 2018 showed that of the 9,412 hospitalized, 83% were diagnosed with mTBI. Furthermore, 54% of these service members with mTBI returned to duty.[39] Most mTBI studies utilize male model systems due to the disproportionate number and higher likelihood of males sustaining mTBI in sports and combat settings.

An unfortunate and underreported cause of mTBI is intimate partner violence and child abuse. Intimate partner violence is experienced by one in three women and one in four men.[40] This form of domestic violence is an attempt for one partner to exert power or control over the other partner through various means including physical abuse. Up to 50% of women of African descent reported TBI symptoms with intimate partner violence in a convenience sample survey.[40] Child Protective Services classifies over 650,000 children as victims of child abuse every year, 17.6% of whom are physical abuse victims.[41] Estimates suggest that approximately 30% of adults have been physically abused at some point in their lifetime.[42] Studies report that the highest incidence of child abuse-inflicted TBI occurs in infants with 32 cases per 100,000 infants per year with incidence rates decreasing with age.[43] However, the estimation of women and children that have sustained TBI because of intimate partner violence or child abuse is likely

underrepresented as the women surveyed were those that sought medical attention and the children diagnosed were those discharged from the hospital post-injury. Many individuals are unlikely to receive medical attention for such injuries due to parental neglect or for fear of retaliation by their partner and the intimate nature of such injuries. Intimate partner violence and child abuse victims represent a particularly fragile population due to the unpredictability of sustaining mTBI by their partner or parent. Further research is imperative to identify the prevalence of mTBI in the case of domestic and child abuse and to assist individuals experiencing symptoms associated with TBI.

Repetitive Mild Traumatic Brain Injury

rmTBI refers to the acquisition of one or more subsequent mTBIs following the initial injury. Studies show that sustaining a single TBI increases an individual's risk of experiencing subsequent TBI later in life, regardless of age at the time of the first injury, likely due to the molecular events following the initial injury that contribute to sustained brain and cellular dysfunction.[44] However, the true incidence of rmTBI is not well known. rmTBI research has primarily focused on contact-sport athlete and military service member populations. Up to 42% of individuals report rmTBI throughout their lifetime regardless of the at-risk population.[45] A surveillance study supports these findings and shows that up to 22% of collegiate athletes report at least 3 concussions by the end of their career.[36] A retrospective cohort analysis of United States athletes 12 to 22 years of age indicates that young adults with a history of previous concussion had a significantly higher incidence of head injury when compared to individuals experiencing their first concussion (436.7/1,000 person-years versus 194.4/1,000 person-years, $P < 0.0001$).[46] Another retrospective cohort study of patients 5 to 15 years old showed that within two years of the first concussion, one in six patients was diagnosed with at least one

subsequent concussion.[47] This study also showed that patients with a clinical course greater than 30 days had a higher risk of subsequent injury than those with symptoms resolving zero to seven DPI, highlighting the importance of targeting persistent mTBI symptomology.[47] Patients with rmTBI are also twice as likely to have increased symptom burden when compared to those with a single mTBI.[46, 47] Further research is needed to determine how mTBI and rmTBI contribute to acute and persistent patient symptomology.

mTBI Symptomology

The clinical manifestation of mTBI varies from person to person, due to the heterogeneous nature of the injury. The presentation of mTBI-associated symptoms generally occurs at acute to subacute time points and includes affective, somatic, and cognitive disturbances, with some symptoms persisting for months to years post-injury.[48] mTBI-associated affective disturbances may include increased depression, anxiety, irritability, and decreased behavioral inhibition. Somatic disturbances following mTBI may include increased sensitivity to light and sound, headaches and migraines, dizziness and nausea, and problems falling or staying asleep. Cognitive symptoms associated with mTBI include decreased processing speeds, difficulty maintaining attention and multitasking, and problems with memory formation and recall.[49, 50] Though these symptoms generally resolve days to weeks post-injury, even a single mTBI can result in long-term perturbations.[51-53] Up to 30% of patients that experienced mTBI have persistent symptoms referred to as persistent post-concussion syndrome.[4, 54] A 2008 study supports this data by reporting that in 2005 an estimated 3.2 million individuals were living with long-term TBI-related disability.[25] Reports suggest that 40% of military members with a history of mTBI experience the persistence of one or more

symptoms post-injury.[55] Furthermore, studies show that sustaining rmTBI further increases an individual's risk of experiencing persistent and exacerbated symptoms.[48]

Memory Deficits Following mTBI

Cognitive impairment is the hallmark complaint following mTBI.[16] Cognitive impairment can range from an inability to maintain attention to memory deficits. Deficits in cognition can severely impact an individual's quality of life by affecting memory formation and recall. The limbic system is a major center for learning and memory in the brain.[56] A limbic structure known as the hippocampus is of particular interest due to its role in memory formation. The hippocampus is responsible for organizing sensory and cognitive experiences prior to memory consolidation by the cerebral cortex.[56] Hippocampal damage and dysfunction are associated with cognitive and memory deficits and are found in cases of neurological disorders and TBI.[57] Severe lesions to the hippocampus result in anterograde amnesia, or the inability to form new memories, as exemplified by the famous 1953 case of Henry Molaison (H.M.), who had both hippocampi removed.[58, 59] Both sub-concussive and concussion injuries have been associated with impairments in working memory and memory formation that persist following mTBI.[60-62] Deficits in working memory have been shown to worsen with each subsequent mTBI.[63] Increased risk and severity of memory deficits associated with rmTBI necessitates the elucidation of the pathophysiologic cause of such symptoms.

TBI Mechanisms of Injury

An important consideration when reading TBI literature is the mechanism by which the primary injury is sustained. Three mechanisms of injury can induce TBI: direct impact, blast, and acceleration-deceleration. A direct impact TBI generally occurs with cranial penetration resulting in blunt force to or penetration of the brain. Blast TBIs may occur when explosives are detonated

near an individual, resulting in a percussive wave that inflicts force upon the brain as it propagates through the skull. Acceleration-deceleration injuries can occur when a force is applied to the skull or body resulting in the impact of the brain against the skull. This initial impact of the brain against the skull is referred to as the coup injury. The contrecoup injury occurs subsequently as the brain rebounds to the other side of the skull, contributing to the diffuse nature of mTBI. However, Newtonian mechanics-based biophysical studies suggest that the contrecoup injury may occur first as the higher-density cerebrospinal fluid displaces the lower-density brain in the contrecoup direction upon rapid deceleration of the skull.[64] A further complication arises when the different types of TBI occur simultaneously or in tandem. Studies have shown that the brain is affected differentially based on the mechanism of injury. The physical differences between mechanisms of injuries can range from gross pathological differences to differences in the molecular cascades following injury.[65] Therefore, careful consideration must be taken when choosing the appropriate model for the experimental questions to be investigated.

Experimental Models of mTBI

The field of experimental TBI research has significantly expanded over recent decades, with multiple injury induction methods having been developed. Experimental models of TBI most commonly utilize mice and rats regardless of the injury induction method. Open-head and closed-head are the two categories of experimental mTBI at present. An open-head model provides the investigator direct access to the brain through a cranial window, allowing the induction of focal lesions to a precise region of the brain in a controlled and reproducible manner. A more clinically relevant category of experimental mTBI is the closed-head model, due to the nonpenetrating nature of the injury. Two of the most used closed-head models for inducing

mTBI are the weight-drop and the controlled cortical impact (CCI) model, though both have been utilized in open-head models. The most utilized and oldest closed-head injury system is the weight-drop model in which a defined mass is allowed to fall from a specific height to deliver an impact of calculated force to the skull. This mass can be guided vertically along a rail to deliver an impact from above or fixed to a pendulum to allow impact to the side of the animal's skull. The weight-drop model has proven to be less consistent than the CCI model as drop parameters vary between investigators. The CCI model is the most recently developed mTBI paradigm and consists of a pneumatic, hydraulic, or magnetic piston of a defined diameter delivering an impact to the skull at a known velocity for a predetermined distance.[66]

Acceleration-deceleration forces are the mechanism by which mTBI and concussion are sustained and therefore the most experienced mechanism of TBI. Both the weight-drop and CCI models can be used on animals fixed in a stereotaxic device for increased reproducibility; however, more clinically relevant designs allow investigation of the acceleration-deceleration and rotational forces experienced in mTBI by delivering the impact to an unrestrained skull. The allowance of such forces provides investigators with a method to study the complex and diffuse nature of mTBI caused by coup and contrecoup injuries.[66] *In vitro* and *in silico* methods for exploring mTBI do not currently exist, making simplification of such a complex injury quite difficult. However, employing animal models of mTBI allows pathological and molecular interrogation of the injury and serves to test potential therapeutic strategies *in vivo*.

mTBI Sets the Stage for Cellular Senescence Induction in the Hippocampus

The overall pathophysiology of mTBI results from a myriad of cellular and molecular alterations post-injury. Neurotransmitters and ions are indiscriminately released immediately following the primary mechanical portion of mTBI due to diffuse axonal shearing and

widespread cell membrane disruption, leading to dysregulated neuronal depolarization.[67, 68] Further depolarization occurs as the released neurotransmitters, predominantly glutamate, bind to their respective receptors and contribute to initial excitotoxic conditions.[69] The metabolic changes described below are especially increased in the cerebral cortex and hippocampus, both of which are essential to cognitive function and memory.[70]

The unregulated ion flux due to neuronal depolarization results in a dramatic increase in glycolysis as the adenosine triphosphate (ATP)-dependent sodium (Na^+)-potassium (K^+)-ATPases attempt to restore ionic homeostasis.[71] A cellular energy crisis ensues as available glucose is diminished in the context of reduced cerebral blood flow.[72] Large cellular influxes of calcium (Ca^{2+}) occur as voltage-gated Ca^{2+} channels are aberrantly activated, leading to intracellular Ca^{2+}-dependent Ca^{2+} release and a dramatic increase in intracellular Ca^{2+} concentrations.[73] Excess intracellular Ca^{2+} activates apoptotic pathways directly, further contributing to the acute tissue damage following mTBI.[74] The initial period of hypermetabolism is followed by a period of hypometabolism as an increasing amount of free Ca^{2+} is sequestered by mitochondria.[69] Electrons begin to leak from the electron transport chain as the mitochondrial membrane is depolarized from Ca^{2+} sequestration.[69] Consequentially, oxidative phosphorylation uncouples and ATP production is reduced while ROS production increases.[69, 71] This increased ROS production is further exacerbated in a positive autoregulatory fashion.[71] Widespread neuroinflammation and cellular dysfunction occur as increased ROS production leads to protein, carbohydrate, lipid, and deoxyribonucleic acid (DNA) damage.[72, 75]

A resulting shift in metabolic activity away from oxidative phosphorylation toward glycolysis increases lactate production; however, this metabolic shift concurrently leads to

decreased lactate metabolism.[76-80] An accumulation of lactate contributes to disruptions in blood-brain barrier permeability, cerebral edema, cell membrane damage, and neuronal acidosis following the initial injury.[76-80] Lactate accumulation is therefore believed to contribute to the vulnerability of subsequent mTBI.[69] Excess extracellular K^+ ions induce diffuse spreading depressions after dysregulated neuronal excitation and are thought to contribute to loss of consciousness and acute cognitive deficits following mTBI.[69] The initial neurometabolic cascade of mTBI results in long-lasting alterations in brain cells following the primary mechanical injury.[72] Ultimately, metabolic dysfunction increases oxidative stress, subsequently resulting in irreparable DNA damage, metabolic dysfunction, and neuroinflammation.[81-84] These intrinsic and extrinsic factors create an environment conducive to a phenomenon known as cellular senescence.

Cellular Senescence

Cellular senescence is a complex stress response resulting in the stable arrest of the cell cycle at the G_1/S-Phase or G_2/M-Phase checkpoints, thus leading to replicative stasis while maintaining cell viability and avoiding cell death. The idea of cellular senescence was first conceptualized by the German biologist August Weismann in 1881 who suggested that "death takes place because a worn-out tissue cannot forever renew itself, and because a capacity for increase by means of cell division is not everlasting but finite."[85] The term "senescence" was first coined and experimentally demonstrated in 1961 by Hayflick and Moorhead while determining serial cultivation limits for 25 different fibroblast cell strains originating from various tissues of the human fetus. Cells lost their replicative capabilities, accumulated debris about the cell surface, lost their cell-to-cell contact inhibition, and became sparse after repeated passaging. However, the observed cells were still considered viable, as they were metabolically

active, as demarcated by drops in the pH of the culture media. If these cells continued to be fed, acid production continued for months following cessation of mitosis before they began to degenerate.

Hayflick and Moorhead found that the tissue origin of fibroblasts plays a role in the presumed degeneration rates of passaged cells as the skin fibroblast strain continued to proliferate while the lung fibroblast strain did not despite identical conditions.[6] Thus, Hayflick and Moorhead divided the progressive differences within diploid cell strains into three distinct phases. Phase I was considered the early growth phase in which primary cells are establishing themselves on their growth surface which generally lasts one to three weeks. Phase II begins as the first confluent sheet of cells is formed, indicating the need for the first passage. Phase II is characterized by the exponential growth rate of the cells and acid production as the cells are passaged at least twice a week for two to ten months. Phase III, also referred to as the terminal phase, begins as the cultured cells undergo observed degeneration, which is characterized by debris accumulation, significantly longer times to confluency, and ultimately a reduction in mitosis. They reported that “bizarre nuclear forms and sizes become more frequent and the appearance of these nuclei is reminiscent of irradiated cultures.” Irradiation was later shown to be a senescence-inducing factor when plant cells were exposed to infrared or ultraviolet light.[86] Multiple unsuccessful attempts were made by Hayflick and Moorhead to reverse Phase III.[6] These data suggested that a finite limit to replication of diploid cell strains exists, that is specific to the tissue of origin, and leads to presumably irreversible mitotic stasis and eventual degeneration of the cell, opening the door for a potential explanation behind aging and cellular degeneration.

Cellular senescence has been more recently redefined as “a mechanism whereby a dividing cell enters a stable cell cycle arrest upon a damaging or stressing stimulus and generally exerts a complex secretion of factors that impacts the nearby tissue while remaining metabolically active and unresponsive to mitogenic and apoptotic signals.”[87] Cellular senescence can occur in a variety of situations and is beneficial or detrimental depending on the context. Tissue remodeling during embryonic development and wound healing is promoted by cellular senescence and generally occurs in the absence of DNA damage.[88, 89] Senescence is also an antitumorigenic response to oncogenic or mitogenic stimuli,[90-96] though specific factors secreted by senescent cells have been shown to promote tumorigenesis.[97, 98] Cellular senescence has been implicated in aging and numerous disease states.[99] This complex stress response can be induced by a variety of factors, both intrinsic and extrinsic to the cell. Furthermore, the molecular mechanisms by which different stressors induce cellular senescence vary in a cell type- and tissue-dependent manner, making the characterization of senescent cells and the identification of a single, highly specific senescence-associated marker quite difficult.

Types and Causes of Cellular Senescence

The form of cellular senescence observed by Hayflick and Moorhead has since been termed “replicative senescence.” Replicative senescence or replicative exhaustion refers to the cessation of the cell cycle because of repeated mitosis. This finite number of cell divisions before senescence is referred to as the Hayflick Limit and generally occurs after 40-60 mitotic events. Repeated cell division and DNA replication result in the progressive shortening of telomeres and the eventual loss of the telomere t-loops which cap and protect the ends of DNA strands.[100] Telomerase prevents telomere attrition under normal circumstances; however, the activity of this enzyme is diminished with age and disease.[101] The loss of t-loop capping is recognized by the

cell as a double-strand DNA break (DSB) and triggers the DNA damage response resulting in the initiation of cellular senescence.[102] Replicative senescence is distinct from the other forms of DNA damage response-induced senescence as shown in a 2019 study by Sanokawa-Akakura et. al.[103] This study compared replicative senescence to bleomycin-caused oxidative DNA damage-induced senescence. No distinguishable differences were noticed between the two types of senescence using conventional senescence-associated markers. However, it was found that the 18S, 5.8S, and 28S ribosome promoter regions in the DNA were hypermethylated in the case of replicative senescence but absent in the bleomycin-caused oxidative DNA damage-induced senescence. This hypermethylation of ribosomal promoter regions resulted in the reduced expression of the corresponding ribosomal RNA in replicative senescence alone.[102, 103]

Similarities and differences between senescent cells are dependent on the initiating cause of cellular senescence, the environment in which the cell becomes senescent, and the cell type undergoing senescence, all of which contribute to observed senescence-associated molecular alterations.[6, 103] Aside from developmental-, nutrient deprivation-, and some forms of tissue repair-induced senescence, DNA damage is a common underlying feature of stress-induced senescent cells.[104-106] Non-telomere attrition-associated DNA damage occurs in oxidative stress-, oncogene-, irradiation-, and chemically-induced senescence.[107] The distinguishing factors are the mechanisms by which DNA damage was accrued and the type of DNA damage detected by the cell. Furthermore, cellular senescence can result from both nuclear and mitochondrial DNA damage.[108]

Oncogene-induced senescence is activated due to tumor-suppressor gene inactivation or oncogenic mutation and serves as a preventative measure against neoplasm formation within an organism.[109] Irradiation-induced senescence is a result of direct damage to the DNA.[110]

Irradiation can cause single- and double-strand breaks in the DNA, DNA crosslinking that prevents DNA replication, and alterations to nucleotide bases such as thymine-thymine dimer formation.[111-113] Chemically-induced senescence occurs as DNA damage accumulates as a direct or indirect result of genotoxic agents.[114] Doxorubicin is thought to cause DNA damage-associated senescence by directly intercalating into DNA and preventing topoisomerase-II-mediated repair or by increasing reactive oxygen species (ROS) and indirectly damaging DNA.[114] Oxidative stress and increased ROS production are well-established molecular consequences of mTBI.[72] ROS act as regulators of various cellular processes under homeostatic conditions such as metabolic regulation; however, exogenous application or aberrant endogenous production of ROS causes dysfunction in a multitude of cellular processes, many of which can be senescence-initiating factors.[115] Interestingly, senescent cells further increase ROS production.[116] Increased ROS production can lead to irreparable DNA damage, mitochondrial and metabolic dysfunction, and protein misfolding, all of which are characteristics and inducers of cellular senescence.[106]

Complex Interplay of Stress-Induced Cellular Senescence Mechanisms and Markers

The diverse nature of senescent cells and the elusion of a single identifying biomarker is due to the highly context-specific circumstances in which senescence-associated molecular alterations occur.[117] The complex nature of cellular senescence necessitates the identification of multiple senescence-associated characteristics when evaluating senescence levels within a given tissue. There are many diverse characteristics to senescent cells, but the defining characteristic of all senescent cells is the cessation of the cell cycle at the G_1/S-Phase or G_2/M-Phase checkpoints with the release of proinflammatory molecules.[118] As previously mentioned, stress-induced senescent cells generally have chronic or irreparable DNA

damage.[107] p53 is widely recognized as “the guardian of the genome” and indirectly halts cell cycle progression or induces apoptosis in response to DNA damage.[119] Two positive p53 regulatory mechanisms have been identified in the context of cellular senescence. Mdm2 negatively regulates p53 under homeostatic conditions by promoting its proteasomal degradation to ensure cell cycle continuation.[119] This negative regulatory mechanism is circumvented when ATR phosphorylates p53 in rapid response to DNA damage acquisition.[120] Degradation of p53 can also be avoided by $p19^{ARF}$ ($p14^{ARF}$ in humans)-mediated inhibition of Mdm2 in the context of chronic or irreparable DNA damage.[121] p53 can then act as a transcription factor and/or protein binding partner to ultimately activate numerous genes and proteins that result in cell cycle arrest and DNA damage repair.[122]

Cellular senescence subsequently arises when DNA damage is chronic or beyond repair.[122] Two senescence-associated genes, *Cdkn1a* and *Cdkn2a*, encode the cyclin-dependent kinase (CDK) inhibitors that directly prevent cell cycle progression known as $p21^{Waf1/Cip1}$ and $p16^{INK4a}$, respectively.[123] The direct binding of p53 to the promoter region of the *Cdkn1a* gene results in the upregulation of $p21^{Waf1/Cip1}$.[124, 125] This mechanism is known as the p53-$p21^{Waf1/Cip1}$ pathway and has been associated with the induction of senescence when DNA damage accrues.[126] A p53-independent mechanism of cellular senescence is the $p16^{INK4a}$ pathway.[126] $p16^{INK4a}$ is encoded by the *Cdkn2a* gene that also encodes $p19^{ARF}$ through an alternate reading frame.[127] The maintenance of cellular senescence has been associated with the $p16^{INK4a}$ pathway in the context of chronic or irreparable DNA damage but can also be activated by ROS sensing mechanisms.[128] The $p21^{Waf1/Cip1}$ and $p16^{INK4a}$ proteins prevent cell cycle progression by inhibiting the association of cyclins with their respective cyclin-dependent kinases, preventing the phosphorylation of the Retinoblastoma (Rb) protein.[129]

Hypophosphorylation of Rb results in the sequestration of E2F transcription factor 1, ultimately preventing the transcription of genes necessary for DNA replication and the progression of the cell cycle.[106] Increases in p53, $p21^{Waf1/Cip1}$, and $p16^{INK4a}$ are consequentially the most commonly used identifiers when evaluating senescence.

There is substantial cross-activation between the p53-p21Waf1/Cip1 and p16INK4a pathways despite the ability of these two pathways to induce senescence independently. For instance, p53 can stabilize Lamin A/C to promote the degradation of Polycomb repressor complex 1 components, resulting in the derepression of *Cdkn2a* and a subsequent increase in $p16^{INK4a}$.[130] $p21^{Waf1/Cip1}$ has been shown to induce $p16^{INK4a}$ expression by promoting Sp1 transcription factor association with the $p16^{INK4a}$ promoter region.[131] Conversely, $p16^{INK4a}$ can stabilize $p21^{Waf1/Cip1}$ mRNA and prevent Mdm2-mediated p53 degradation, leading to subsequent increases in both proteins.[132, 133] The complex inter-regulation of senescence-associated markers thus contributes to the difficulty in identifying a single, definitive senescent marker.

The complex role of p53 in cellular senescence is not limited to DNA damage and cell cycle arrest. The senescence-associated secretory phenotype (SASP) is the release of proinflammatory cytokines, chemokines, and proteases by senescent cells.[134] Senescent cells generally release the SASP to recruit immune cells to the site of injury for debris clearance and subsequent destruction of the senescent cells.[134] However, senescent cells may become detrimental and pathological when these cells evade immune clearance.[135] The complex and diverse nature of senescent cells has prevented the identification of ubiquitously expressed SASP molecules. However, proteins such as high mobility box group 1 (HMGB1) serve as proxies for SASP production due to their regulation of SASP-encoding genes. HMGB1, for instance, resides within the nucleus under normal conditions and represses SASP gene transcription by interacting

with DNA and histones.[136] Cytoplasmic p53-mediated translocation of HMGB1 from the nucleus occurs during senescence initiation, allowing the transcription of SASP genes.[136, 137] HMGB1 is then secreted from the cell and acts as a SASP component.[137] p53 has also been shown to downregulate HMGB1 promoter activity, though this concept has not been investigated in the context of cellular senescence.[138] Cellular release of HMGB1 results in paracrine SASP induction of surrounding cells by stimulating activity of the SASP master regulator, nuclear factor kappa B subunit p65 (NF-κB).[136] NF-κB then promotes further SASP production while contributing to cell replication suppression and senescence maintenance.[139-141] Downregulation of HMGB1 gene expression, therefore, serves as a proxy for SASP transcription.

Nicotinamide adenosine dinucleotide-dependent deacetylase sirtuin-1 (SIRT1) regulates a myriad of cellular processes including cellular senescence, metabolism, aging, and inflammation, through its ability to deacetylate a diverse range of target proteins.[142-144] It has been shown that SIRT1 is downregulated by multiple mechanisms in the context of senescence and that overexpression of SIRT1 can prevent various forms of senescence.[145-147] SIRT1 is further associated with the regulation of senescence genes through its inherent histone deacetylase activity.[148] Senescence studies have shown that SIRT1 regulates the SASP by directly repressing the gene promoter sequences and indirectly by deacetylating p53 at lysine 382 to limit the SASP-promoting p53 transcriptional activity.[146, 149] p53 binding to the *Sirt1* promoter lowers SIRT1 levels in response to DNA damage and results in the derepression of SASP genes.[146, 150] Increased lysosomal activity further decreases SIRT1 protein levels through selective autophagy.[147] Implications of SIRT1 overexpression ameliorating senescence and

aging along with the observed decreases in gene and protein expression in senescence make SIRT1 a prominent marker of senescence.

Lamin B1 is also degraded through selective autophagy in senescent cells.[147] Under homeostatic conditions, Lamin B1 functions as a nuclear structural protein that assists in maintaining the epigenetic configuration of DNA and therefore indirectly regulates gene expression by controlling gene sequence accessibility.[151] The maintenance of heterochromatin by Lamin B1 helps to suppress senescence-associated gene transcription.[152, 153] Senescence-associated degradation of Lamin B1 results in the rearrangement of genetic material and the loss of heterochromatic DNA conformation.[153] The transition of Lamin B1-bound loci to euchromatin allows the transcription of senescence-associated genes.[153] Observing decreases in Lamin B1, therefore, serves as a proxy for senescence-associated epigenetic alterations. The increased lysosomal activity of senescent cells, specifically at pH 6, can be observed through senescence-associated β-galactosidase assays and is among the most used senescent cell markers.[154] The pitfall of the senescence-associated β-galactosidase assay is that fresh tissue or cell samples are required.

As described briefly above, cellular senescence is a complex cellular process in which no single molecular marker results in positive senescence identification. A wide range of senescence-associated markers have been identified and may be employed for various reasons. Other markers include various senescence-associated cell surface proteins, microribonucleic acid expression or protein associations, and the phosphorylation state of specific proteins. This wide range and complex interplay between the many senescence-associated markers makes it difficult to determine a single, stable cellular senescence marker.

Implications of Cellular Senescence in TBI-Associated Neurodegeneration

Cellular senescence is thought to be the underlying mechanism behind organismal aging; however, the proportion of senescent cells within a given tissue is marginal when compared to healthy cells.[155, 156] The sparse abundance of senescent cells can have a widespread impact on surrounding tissue through the release of the SASP.[157] The SASP acts in an autocrine and paracrine manner to reinforce senescence within a cell and induce senescence in surrounding cells.[106] A pro-inflammatory environment is created by senescent cells via the chemokines and cytokines comprising the SASP.[106] This can induce neuroinflammation in the brain which is thought to contribute to the development of neurodegenerative diseases.[158-160] Senescent cells have been associated with various neurodegenerative diseases including Alzheimer's Disease, Parkinson's Disease, and Frontotemporal Dementia.[159, 161-163] Protein aggregates are characteristic of many neurodegenerative diseases and studies have associated senescent cells with these insoluble clusters.[7] Ablation of senescent cells by senolytic compounds results in a reduction of these pathological aggregates and improves cognitive functioning. However, little is known about the long-term ramifications of widespread senescent cell ablation.[164, 165]

Emerging evidence has implicated senescence-associated alterations at acute time points following mTBI.[8, 166-168] Alterations peak at roughly seven DPI and generally resolve by 14 DPI.[168] Initial peaks in senescence-associate changes may be an attempt to prevent further cell death during the acute phase of mTBI; however, increased cellular senescence has also been reported at chronic time points following the initial injury.[169] No studies to date address cellular senescence at subacute time points following mTBI at which the resolution of initial senescence-associated alterations transitions to the chronic progression of cellular senescence. Treatment of persistent cognitive deficits following mTBI may therefore be possible if a

subacute therapeutic window can be identified to halt the progression of cellular senescence. Research of this nature may also provide insight into the mechanisms driving neurodegenerative and age-associated diseases.

Therapeutic Potential of Testosterone in TBI and Cellular Senescence

Few studies have investigated the effects of testosterone administration in the context of TBI. However, studies to date show that acute testosterone treatment improves functional cognitive outcomes and mitigates many of the molecular consequences induced by TBI.[10, 170] Acute testosterone administration has been shown to reduce reactive astrogliosis and reactive microgliosis thereby ameliorating neuroinflammation.[9] Furthermore, benefits of testosterone administration following severe TBI include prevention of Ca^{2+} uptake by the mitochondria and reactive oxygen species production, promotion of oxidative metabolism, and attenuation of neurodegenerative biomarker expression.[10] The reduction of these factors limits the presence of stimuli that are known to induce cellular senescence. The sparse senescence-specific testosterone studies support this claim as testosterone has been shown to antagonize senescence induced chemically by doxorubicin and paraquat in cardiomyocytes and retard the progression of senescence in vascular smooth muscle cells.[11, 12, 171] In 1988, Goudsmit et al. demonstrated that testosterone administration restored vasopressin innervation in a senescent rat model.[172] Testosterone treatment has also been demonstrated to increase synaptic plasticity and improve learning and memory in senescence-accelerated mouse models through the androgen receptor.[173, 174] Therefore, testosterone represents a novel therapeutic strategy to target the persistent cognitive deficits and the progression of cellular senescence associated with rmTBI.

CHAPTER THREE

RATIONALE AND EXPERIMENTAL DESIGN

Rationale

This thesis serves to investigate the effect of rmTBI on hippocampal cellular senescence and recognition memory while determining if a subacute timepoint exists in which delayed testosterone administration mitigates these manifestations. An animal model of rmTBI was employed in this study due to the significant proportion of TBIs that are classified as mTBI.[2, 3] This research was conducted strictly on male rats since males are twice as likely as females to experience TBI due to risk-taking behavior.[27] Animals were subjected to repetitive closed-head mTBI because a single mTBI increases the risk for subsequent mTBI and concomitant persistent and exacerbated symptoms.[36, 44-47] Injuries were delivered via a controlled cortical impactor allowing for free head swing and rotation to ensure well-defined parameters and reproducibility, and to model the clinical representation of rmTBI.

Acute and persistent cognitive deficits are one of the most common symptoms reported following rmTBI.[46, 47] rmTBI increases the risk of developing various neurodegenerative diseases with concomitant cognitive deficits [175-179]; however, the mechanism by which these consequences occur remains to be elucidated. The hippocampus plays a role in behavior, emotion, and cognition, all of which are known to be impacted by rmTBI [180, 181]; thus, the hippocampus was the region of interest in this study. Cellular senescence has been associated with all severities of TBI and multiple neurodegenerative disease pathologies, and results in

progressive functional and structural changes in the brain associated with cognitive impairment.[7, 159, 168, 169, 182-191] Therefore, the progression of senescence-associated alterations in the hippocampus following rmTBI was interrogated throughout this work.

The clinical relevance of testosterone as a neurotherapeutic is multifaceted. Testosterone administration has been shown to promote neurogenesis, neuroplasticity, and synapse formation/maintenance.[192-196] Testosterone has been shown to reduce oxidative stress, prevent apoptosis, reduce neuroinflammation, and improve mitochondrial function when administered immediately following TBI.[10] Testosterone administration has also been shown to inhibit cellular senescence induced by various methods, though limited studies are focused on the brain.[11, 12, 171] However, the use of testosterone as a subacute therapeutic intervention for rmTBI-associated cellular senescence with concomitant cognitive deficits has not been characterized. Testosterone was the therapeutic agent examined in this study for the aforementioned reasons. Most mTBI-associated cognitive symptoms resolve days to weeks post-injury; however, up to 30% of these patients experience persistent symptoms lasting months to years, affecting an individual's quality of life.[197-199] Patients with unresolved symptoms are likely to seek further medical consultation approximately one-month following injury, after completing the recommended recovery period of one to two weeks. This study aimed to determine if subacute testosterone administration attenuates the progression of hippocampal cellular senescence and cognitive deficits following rmTBI. Animals underwent subacute testosterone treatment (rmTBI+T animals) 35 DPI (0 PTD) to target persistent symptoms after the clinically recommended recovery period.

Experimental Design

Eight-week-old Long-Evans Hooded rats were randomly divided into three groups: sham, rmTBI, and rmTBI+T. Long-Evans Hooded rats were chosen over Sprague Dawley and Fischer 344 rats due to their higher propensity for motility during behavioral assays and decreased risk for cell cycle dysregulation presented as neoplasm formation, respectively.[200, 201] Following sedation, five mTBIs were administered to the rats with each impact spaced 48 hours apart. Sham rats acted as a control and did not receive an injury. rmTBI rats received five mTBIs using a CCI device with a metal piston, and rmTBI+T rats received five mTBIs and were allowed to recover until 35 DPI (PTD 0) before surgical implantation of pure crystalline testosterone-filled capsules into the animals' napes. Sham and rmTBI animals were implanted with phosphate-buffered saline-filled capsules to serve as surgical controls for the rmTBI+T group. All rats received equal doses of isoflurane for anesthesia, regardless of group. 35 DPI was chosen to represent a time point at which the window of natural symptom resolution has passed, and symptoms may be considered persistent.

Multiple cohorts of animals were used for this study utilizing the aforementioned experimental paradigm; the first cohort was behaviorally assayed for recognition memory deficits via NOR testing at 7 and 28 DPI. A second cohort was used for molecular interrogation of hippocampal cellular senescence progression at subacute time points. Euthanasia of animals from each group occurred at 35 and 63 DPI. Brains were harvested and stored at -80^{o}C for future dissection. The whole ipsilateral hippocampus was resected and homogenized for molecular analysis. Ipsilateral hippocampal homogenates from each animal were subsequently divided for extraction and purification of mRNA and protein using Trizol and protease and phosphatase containing RIPA buffer, respectively. Purified protein was analyzed via Western Blotting and

mRNA was analyzed via RT-qPCR. rmTBI and rmTBI+T animals were compared to investigate the effects of subacute testosterone treatment on senescence-associated markers at PTD 28 (63 DPI). A third cohort of animals underwent NOR at 42 DPI (PTD 7) to examine the effects of subacute testosterone treatment on recognition memory. These animals were subsequently sacrificed 175 DPI for immunohistochemical probing to examine chronic differences in senescence-associated markers between cell types in the hippocampus following rmTBI.

CHAPTER FOUR

METHODOLOGY

Animals

All experiments were conducted on male eight-week-old Long-Evans Hooded rats obtained from Envigo Laboratories. Upon arrival, animals were initially housed in groups of three in Edward Hines Jr. Veterans Administration Hospital's Veterinary Medical Unit. Animals were exposed to a 12-hour light-dark cycle and acclimated to the new environment in temperature and humidity-controlled rooms for approximately one week. Animals were handled by experimenters after three days of environmental acclimation to reduce animal stress levels during manipulation. Animals had access to standard chow, water, and enrichment *ad libitum*. Rats were later housed in groups of two. Experimental procedures were authorized by Edward Hines Jr. Veterans Administration's Institutional Animal Care and Use Committee.

Closed-Head Repetitive Mild Traumatic Brain Injury via Controlled Cortical Impactor

Animals were randomized into sham, rmTBI, and rmTBI+T groups prior to the first injury. All five impacts were administered using the following procedure at intervals of 48 hours to model rmTBI with sham rats receiving identical experimental manipulations apart from the insult. All rats were placed into an induction chamber and anesthetized using 3% isoflurane, USP (Baxter) with a flow rate of 1 L/min. The right side of the rat's skull was shaved between the eye and the ear to expose the site of impact. The rats then received a constant supply of anesthesia via nosecone and were placed prone on a 5 cm thick foam pad attached to a Plexiglas® frame

with a side wall angled at 89^{o} toward the impact device to support the animal's body throughout the mTBI procedure. The foam pad allowed a free range of head movement replicating the rotational and horizontal acceleration observed in many mTBI cases. Closed-head repetitive mild TBI was induced by controlled cortical impact (CCI) via the Impact One Stereotaxic Impactor Device (Leica Biosystems).

The CCI device was positioned over a region of the unexposed skull corresponding to the right sensorimotor cortex at an angle of 20^{o} from vertical to place the 5 mm diameter steel tip of the actuator flat against the site of injury. The area of the skull corresponding to the right sensorimotor cortex was determined experimentally in reference to bregma (0.5 mm anterior and 4.0 mm lateral) by a collaborator, providing a general estimate of the impact site.[202] The nosecone was removed before impact, and the animal's body was stabilized by the experimenter's hand while allowing free movement of the head. The mTBI was then administered by firing the actuator 10 mm from the surface of the rat's skin at a speed of 6.5 m/s with a dwell time of 300 msec. This paradigm is well-established to induce mild TBI and has been replicated by our laboratory in the past. The animal was then inspected for lacerations and palpated for skull fractures at the site of injury. The skin at the site of injury was marked with a permanent marker to ensure subsequent injuries were delivered to the same approximate location. The rats were then weighed and placed in a recovery cage to regain consciousness. Animals were subsequently placed back in their home cage while the remaining animals underwent the mTBI procedure. All mTBIs were administered by a single investigator to ensure consistency and reproducibility of the closed-head mTBI.

Novel Object Recognition Testing

NOR testing was performed to assess recognition memory following rmTBI and was conducted in a 50 cm by 50 cm arena. NOR testing consisted of a habituation phase, a familiarization phase, and a testing phase. Each NOR phase was conducted 24 hours apart. The arena and objects were thoroughly cleaned with 70% ethanol between each animal to eliminate lingering odors. Animals were placed in the arena for 10 minutes during the habituation phase to become accustomed to the environment. The familiarization phase was conducted by placing two identical objects at the arbitrarily named North and South ends of the arena. Animals were then placed into the center of the arena facing either the East or West wall and allowed to freely explore for 5 minutes. Animal exclusion was implemented if the exploration time for an individual animal during the familiarization phase fell two standard deviations below the mean exploration time across groups for each respective time point. Under these exclusion parameters, no animals were eliminated from the analysis. During the testing phase, all parameters were kept consistent with the familiarization phase except that one familiar object was replaced by a novel object. Novel objects were the same color as the familiar object to eliminate potential color preference. Animals were allowed to freely explore the arena with familiar and novel objects for 5 minutes. Different objects were used between repeated NOR testing periods. The time spent exploring the familiar and novel objects was recorded. The percentage of exploration time with the novel object was calculated by dividing the novel object exploration time by the total time spent exploring both the familiar and novel object. Graphs depict the percentage of total exploration time spent with the novel object ± standard error of the mean (SEM).

Collection and Processing: Fresh Frozen Hippocampal Brain Tissue

Rats were anesthetized using isoflurane, USP (Baxter) on the day of sacrifice. Rats were then decapitated, and the brains were harvested. Whole brains were flash-frozen in a dry ice-cooled biphasic solution of 1-bromobutane and 2-methylbutane. Brains were then stored in a -80^{o}C freezer until brain dissection. The hippocampus was dissected by a single investigator to ensure the consistency of tissue collection. Each hippocampus was isolated and manually homogenized using a curved dental pick and divided with one half placed into 1 mL of protease and phosphatase inhibitor containing RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) for total protein extraction and analysis, and the other half placed into 1 mL Trizol Reagent (Thermo Fisher Scientific) for total RNA extraction and analysis. Tissue lysis was performed according to each respective manufacturer's protocol. All lysates were stored at -20^{o}C until biomolecule extraction and analysis.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Trizol Reagent was used to extract and purify total RNA according to manufacturer instructions. Extracted RNA purity and concentration were determined via NanoDrop 2000 (Thermo Fisher Scientific). SuperScript IV VILO Master Mix with ezDNase (Thermo Fisher Scientific) was used following protocol instructions to generate cDNA from 250 ng purified RNA via reverse transcription. Assuming 100% reverse transcription efficiency, cDNA samples were diluted to 25 ng/µL using RNase-free water. The expression of eight senescence-associated genes was analyzed at 35 and 63 DPI to account for the multifaceted complexity of cellular senescence. The genes analyzed are listed in Table 1.

Table 1: Senescence-Associated Genes of Interest. List of primers acquired from BioRad Laboratories for qRT-PCR assessment of senescence-associated gene expression.

Gene-Associated Protein	Gene Symbol	Alterations in Senescence	Unique Assay ID
Hypoxanthine phosphoribosyl-transferase 1 (HPRT1)	*Hprt1*	Reference Gene	qRnoCED0057020
p53	*Tp53*	Cell Cycle Arrest	qRnoCED0004065
$p21^{Waf1/Cip1}$	*Cdkn1a*	Cell Cycle Arrest	qRnoCID0007165
$p16^{INK4a}$	*Cdkn2a*	Cell Cycle Arrest	qRnoCED0004217
Nicotinamide adenosine dinucleotide-dependent deacetylase sirtuin-1 (SIRT1)	*Sirt1*	Lysosome Function	qRnoCID0002838
High mobility group box 1 (HMGB1)	*Hmgb1*	Cytokine Production	qRnoCID0006047
Lamin B1	*Lmnb1*	Nuclear Structure	qRnoCID0051561

To assay the genes of interest listed above, experimentally validated primers specific to the respective gene's rat mRNA sequence were purchased from BioRad Laboratories. No primer sequences were attained due to their proprietary nature. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SsoAdvanced™ Universal SYBR Green Supermix (BioRad Laboratories) according to manufacturer protocols using a total of 100 ng cDNA template for each qRT-PCR reaction. qRT-PCR was conducted as 20 µL reactions, performed in triplicate for each sample, on a 96-well plate. BioRad Laboratories CFX96 Touch Real-Time PCR detection system was used for thermocycling and generating qRT-PCR data. CFX Manager software (BioRad Laboratories) was used to analyze the data and set detection thresholds. A common pooled sample was included in triplicate on each plate to allow threshold standardization for inter-plate comparison of each respective gene. Outlier analysis of technical triplicate cycle threshold (Ct) values was performed using the Grubbs' Test with a significance level of $\alpha = 0.05$. If a Ct value was identified as an outlier ($Z \geq |1.15|$) it was excluded from

further analysis while the other two values were retained to generate the average Ct value for that sample. Average Ct values were normalized to the reference gene hypoxanthine phosphoribosyl transferase 1 (*Hprt1*) and relative target gene expression was determined using the $2^{-\Delta\Delta Ct}$ method.[203, 204] Statistical analysis was performed on $2^{-\Delta\Delta Ct}$ values. rmTBI and rmTBI+T values were standardized to age-matched sham animals to eliminate the effect of age-associated senescence on gene expression. Graphs depict relative gene expression ± SEM.

Total Protein Extraction and Western Blotting

Homogenate-containing RIPA Lysis and Extraction Buffer was removed from -20°C storage and allowed to thaw. Samples were vortexed at 1300 rpm for 10 minutes to ensure thorough mixing and lysis of the homogenate. Samples were centrifuged at 4°C for 15 minutes at 14,000 g to pellet tissue fragments per the manufacturer's instructions. The protein-containing supernatant was transferred into a fresh tube and stored at -20°C. Protein concentrations were determined via the Lowry method by using the commercially available DC Protein Assay (BioRad Laboratories).

Samples were prepared by adding 10 µg of total protein to Fluorescent Compatible Sample Buffer (4X) (Thermo Fisher Scientific) and Bolt Reducing Agent (10X) (Thermo Fisher Scientific). Samples were heated at 70°C for 10 minutes to reduce the proteins. Samples were loaded into Bolt 4-12% Bis-Tris Plus Mini Gels (Thermo Fisher Scientific). Gel electrophoresis was run at 200 volts for 20 minutes in a Mini Gel Tank with Bolt MES SDS Running Buffer (Thermo Fisher Scientific). Proteins were subsequently transferred to low fluorescence polyvinylidene difluoride (PVDF) membranes with a pore size of 0.2 µm pore size using the iBlot 2 Gel Transfer System (Thermo Fisher Scientific). Protein transfer was conducted using the P4 setting (15 volts for 7 minutes). Protein transfer efficiency was assessed using Ponceau S

staining solution (Research Products International) during transfer optimization. TotalStain Q (PVDF) (Azure Biosystems) was used to stain for total protein according to the manufacturer's instructions. The total protein stain was imaged prior to immunoblotting using the Azure 600 Imaging System (Azure Biosystems) under the Cy3 filter. Total protein was used as the normalization factor for all immunoblots.

Immunoblotting was conducted using the iBind Flex Western Device (Thermo Fisher Scientific) according to the manufacturer's instructions. The following primary antibodies were used: mouse anti-p53 (1:500; ab26, Abcam), mouse anti-$p21^{CIP1/WAF1}$ (1:500; NBP2-29463, Novus Biologicals), rabbit anti-$p16^{INK4a}$ (1:500; 10883-1-AP, ProteinTech), mouse anti-SIRT1 (1:500; ab110304, Abcam), and rabbit anti-LAMIN B1 (1:500; ab16048, Abcam). Anti-mouse HRP-conjugated secondary antibody (1:1000; 7076S, Cell Signaling Technologies) and anti-rabbit HRP-conjugated secondary antibody (1:1000; 7074S, Cell Signaling Technologies) were used to detect primary antibody immunolabelling following application of SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Immunoblot imaging was performed using chemiluminescence detection via the Azure 600 Imaging System (Azure Biosystems). Relative protein quantity was analyzed using Image Lab software (BioRad Laboratories).

Testosterone Capsule Implantation

Testosterone and control capsules were produced by loading 1.0 cm of ≥ 98% pure testosterone powder (Sigma) or phosphate buffered saline vehicle phosphate-buffered saline (Sigma), respectively, into 1.5 cm long silastic tubes with an inner diameter of 1.57 mm (Dow Corning). 0.25 cm wooden plugs sealed the ends of the silastic tube. Medical-grade silicone-based adhesive was applied over the capsule's plugged ends to create a smooth, waterproof seal.

Previous rat studies have shown that these capsules maintain an average serum testosterone concentration of 4.49 ± 1.40 ng/mL.[13] Capsules were submerged in sterile phosphate-buffered saline (Sigma) to prime the testosterone for diffusion through the pores of the silastic tubing before implantation. After 35 days following the fifth rmTBI (PTD 0), animals were anesthetized with 3% isoflurane, USP (Baxter), and a single capsule was implanted in the nape of the animals. Animals that received the vehicle capsule served as a surgical control.

Collection and Processing: Fixed Hippocampal Brain Tissue

Animals were anesthetized and preserved via transcardial perfusion with 4% paraformaldehyde (Electron Microscopy Sciences) at 175 DPI. Brains were collected and stored in 30% sucrose overnight as a cryoprotective measure then stored at -80^{o}C until sectioning. Brains were acclimated to -20^{o}C in a Leica CM 1850 UV CryoStat (Leica Biosystems) and mounted in Optimal Cutting Temperature compound (Sakura Finetek) to a cryostat chuck on the day of sectioning. Superfrost Plus Microscope slides (Fisher Scientific) were submerged in and cleaned with 100% ethanol and wiped dry with a Kimwipe (Kimberly-Clark Professional) before mounting the tissue on the slides. Coronal sections of 40 µm thickness were mounted onto the Superfrost Plus Microscope slides and allowed to air dry for 30 minutes before storing the slides at -80^{o}C. Heat-induced antigen retrieval technique using an autoclave was employed to resolve antigen masking caused by protein crosslinking. Slides were acclimated to room temperature for 90 minutes prior to heat-induced antigen retrieval. An antigen retrieval solution of 10 mM sodium citrate buffer (Sigma), 0.05% Tween-20 (Sigma) at a pH of 6.0 was preheated to 90^{o}C to ensure maximum exposure time to the autoclave's set temperature of 121^{o}C. Slides were submerged in the heat-induced antigen retrieval solution perpendicular to the bottom of the buffer-containing vessel and exposed to 121^{o}C for 15 minutes. The autoclave was naturally

depressurized to prevent the boiling of the heat-induced antigen retrieval solution and the potential detachment of tissue from the microscope slide. Slides were removed from the heat-induced antigen retrieval solution and allowed to come to room temperature before immunohistochemistry.

Immunohistochemistry

The tissue was permeabilized for 5 minutes using 0.04% Triton X-100 (Sigma) in 10mM phosphate-buffered saline (Sigma), 0.05% Tween-20, pH 7.4, followed by two 5-minute washes with 10mM PBS, 0.05% Tween-20, pH 7.4. Non-specific antibody binding was blocked by incubating the tissue in 10% donkey serum albumin (Sigma), 10% normal goat serum (Millipore) in 10 mM PBS, and 0.05% Tween-20, pH 7.4 for one hour. The primary antibodies were applied to the tissue and incubated overnight at 4^{o}C in a humidified slide chamber. The primary antibodies used were as follows: mouse anti-p16^{INK4a} (1:500; ab54210, Abcam), mouse anti-p21$^{CIP1/WAF1}$ (1:500; NBP2-29463, Novus Biologicals), rabbit anti-neuronal nuclear protein (NeuN) (1:500; ABN78, Millipore Sigma), rabbit anti-ionized calcium binding adaptor molecule 1 (Iba1) (1:500; 019-19741, Wako Inc.), and rabbit anti-glial fibrillary acidic protein (GFAP) (1:500; ab7260, Abcam). Primary antibodies were washed from the tissue samples with 10mM PBS, 0.05% Tween-20, pH 7.4 for 5 minutes, three times. Secondary antibodies were applied and incubated at room temperature for one hour followed by three 5-minute washes with 10mM PBS, 0.05% Tween-20, pH 7.4. The following Invitrogen Alexa Fluor® secondary antibodies were used to visualize primary antibody targets through fluorescent laser confocal imaging: goat anti-mouse IgG (H+L) 647 conjugate (1:500; A32728, Thermo Fisher Scientific) and donkey anti-rabbit IgG (H+L) 555 conjugate (1:500; A31572, Thermo Fisher Scientific). Tissue was subsequently coated with Vectashield Vibrance Antifade Mounting Media with 4’,6-diamino-2-

phenylindole (DAPI) (Vector Laboratories, Inc.) and covered using Microscope Cover Glass (Fisher Scientific). Immunohistochemically stained slides were left in the dark to dry at room temperature for 5-7 days before imaging.

Confocal Imaging

All micrographs were taken on a Leica TCS SPE confocal imaging system (Leica Biosystems) under 63X oil immersion with an ACS APO 63X/1.30 Oil CS objective (Leica Biosystems) and rendered via the Las X Life Science Microscope Software (Leica Biosystems). Hippocampal samples were imaged as Z-stacks with sequential laser scanning, in descending wavelength values, to avoid cross-excitation of fluorophores. Each Z-plane scan had a frame average set to 6 to enhance the signal-to-noise ratio. Fluorophore emission data was collected via PMT detectors with a pinhole size set to 1AU for each respective emission wavelength. Laser scanning of samples generated images 512 x 512 pixels at a speed of 400 Hz with a zoom factor of 1.50 and pixel dwell time of 1.44 µs. Z-depth was set to 44 µm with a step size of 0.5 µm. Alexa Fluor Plus 647 acquisition parameters were: 635 nm (excitation laser), 16.3% (laser power), 650-685 nm (emission wavelength range), and 1061.0 V (gain). Alexa Fluor 555 acquisition parameters were: 561 nm (excitation laser), 14.4% (laser power), 570-600 nm (emission wavelength range), and 999.9 V (gain). DAPI acquisition parameters were: 405 nm (excitation laser), 11.5% (laser power), 445-465 nm (emission wavelength range), and 900.2 V (gain). Z-stack files were exported as .tiff files and converted to max intensity 2D images using the open-source image processing software Fiji (Fiji is just ImageJ) and saved as .tiff files. 2D max intensity images were imported to the open-source digital pathology image analysis software QuPath to ensure that all images to be compared were processed in the same way. The

consistency between immunohistochemical staining, imaging, and image processing parameters allowed for direct comparison between samples.

Behavioral and Molecular Power Analysis

An *a priori* power analysis was performed to determine the sample size for the behavioral aspect of this study using an alpha of 0.05 and a power of 0.80, based on standard practices across behavioral research. An effect size of 0.40 was used based on findings from a previous study and literature review. Unpaired t-tests were planned to be used for statistical analysis. These parameters determined that 64 animals were required for the total sample size. Unexpected animal death was accounted for with a ~10% attrition rate, yielding 72 animals required for this study. These 72 animals were divided into three groups across four time points, yielding six animals per group at each time point, remaining consistent with published studies utilizing a rmTBI animal model to investigate delayed treatments for behavioral deficits.

Statistical Analysis

GraphPad Prism Version 9.2.0 was used to conduct all statistical analyses using $p \leq 0.05$ as the statistical significance threshold. Data normality was assessed upon collection. Grubbs' Test ($\alpha = 0.05$) was used to remove outliers prior to data processing. Unpaired t-tests were used to determine the variance between specific factors, i.e., the effects of injury over time (35 DPI rmTBI versus 63 DPI rmTBI) or the effects of treatment (rmTBI versus rmTBI+T) at individual time points. Graphical representation of data is displayed as the mean ± the standard error of the mean (SEM). Statistical significance is represented as $***p \leq 0.001$ $**p \leq 0.01$; $*p \leq 0.05$.

CHAPTER FIVE

SPECIFIC AIM 1

Specific Aim 1

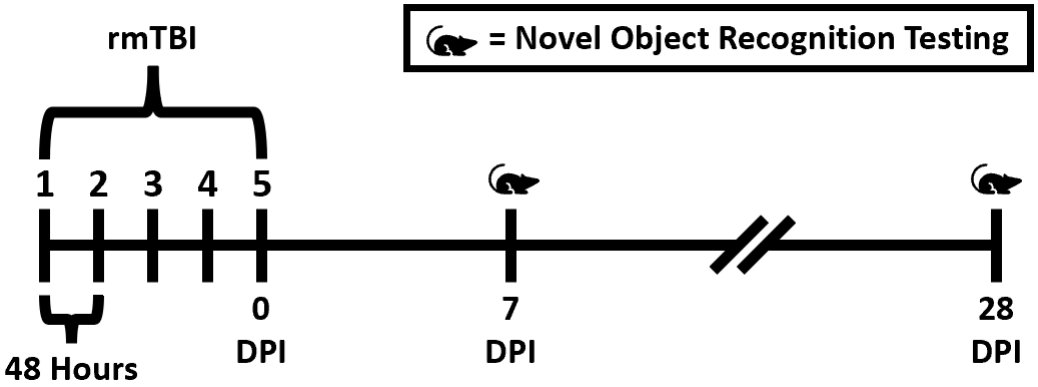
Determine if rmTBI induces persistent deficits in recognition memory and a progression in hippocampal cellular senescence.

Experimental Question 1.1

Does rmTBI result in persistent recognition memory deficits?

Aim 1: Experimental Question 1.1 – Rationale

The purpose of this experimental question is to determine if a 5-hit closed-head rmTBI model produces persistent recognition memory deficits following injury. It is well documented that cognitive deficits, such as memory impairment, can be induced following mTBI and that a portion of these patients report cognitive symptoms persisting months to years post-injury, ultimately affecting an individual's quality of life.[197-199] An rmTBI model provides the ideal system to study the cellular and molecular mechanisms altered following rmTBI that may contribute to persistent cognitive deficits because mTBI predisposes an individual to increased risk for cognitive symptoms and rmTBI. No therapeutic interventions have been approved for clinical use to treat rmTBI concomitant persistent symptoms thus far. Further research is imperative to elucidate the molecular mechanisms governing cognitive deficits following mTBI. Persistent deficits in cognition must first be established following 5-hit closed-head rmTBI to effectively study the molecular basis of cognitive dysfunction. Animals were assessed for



recognition memory deficits via the NOR test at 7 and 28 DPI (Fig.1).**Figure 1: Experimental Timeline for the Establishment of Persistent Recognition Memory Deficits following rmTBI.** Animals were divided into two groups: sham and rmTBI. Sham animals served as a control group and did not receive an injury. rmTBI animals received five mTBIs spaced 48 hours apart. The days following the last impact are denoted as DPI. Animals underwent the testing phase of NOR at 7 DPI and again at 28 DPI.

Aim 1: Experimental Question 1.1 – Results

Novel object recognition following rmTBI at 7 and 28 DPI. Recognition memory was assayed via NOR at 7 and 28 DPI to determine if a 5-hit closed-head rmTBI model induces persistent cognitive deficits and data are represented as the percentage of total object exploration time spent investigating the novel object (Fig. 2). The sample size for sham and rmTBI groups were 7 animals per group at each time point. Unpaired two-tailed t-tests were performed at each time point to determine if differences in recognition memory exist between sham and rmTBI groups. A significant decrease in the percentage of time spent investigating the novel object was observed in rmTBI animals when compared to sham animals at 7 DPI, indicating significant impairment in recognition memory (sham 72.09 ± 4.46; rmTBI 45.16 ± 5.89; $p = 0.0034$) (Fig. 2). Similarly, a significant decrease in novel object exploration was observed in rmTBI animals

at 28 DPI when compared to sham animals (sham 60.89 ± 4.54; rmTBI 47.36 ± 4.05; p = 0.0461) (Fig. 2).

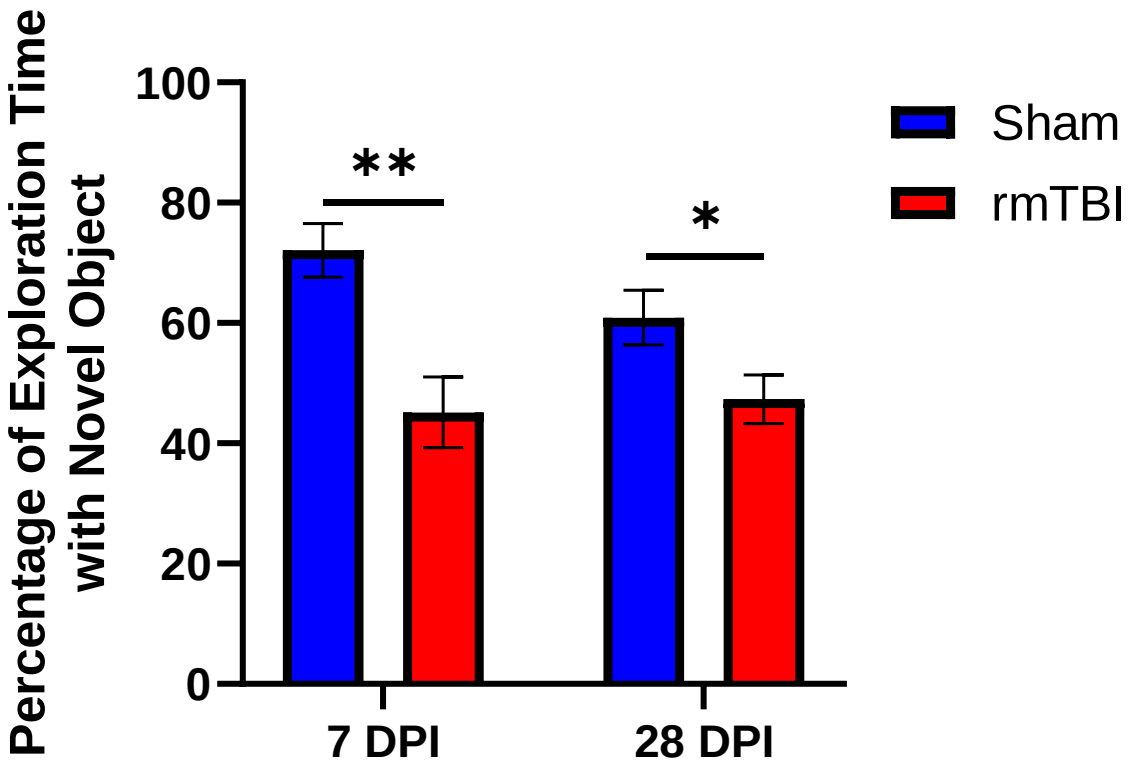


Figure 2: 5-Hit Closed-Head rmTBI Induces Persistent Deficits in Recognition Memory. Novel object recognition data is expressed as the percentage of total object exploration time spent investigating the novel object. Data are presented as mean ± SEM. N = 7 animals per group. **p ≤ 0.01, *p ≤ 0.05.

Experimental Question 1.2

Does 5-hit closed-head rmTBI induce progressive subacute molecular alterations associated with cellular senescence in the hippocampus?

Aim 1: Experimental Question 1.2 – Rationale

This experimental question serves to 1.) examine the subacute progression of cellular senescence in the hippocampus following a 5-hit closed-head rmTBI model, and 2.) determine if there is a window of time where the progression of hippocampal cellular senescence diverges between sham and rmTBI animals. Cellular senescence has been implicated in mTBI, age-related cognitive deficits, and various neurodegenerative diseases.[159, 182-188, 197, 199] Cellular senescence thus provides a potential mechanism behind the persistent deficits in recognition memory that an individual might experience following rmTBI. Single-hit mTBI models report increases in senescence-associated gene expression and protein alterations peaking at 7 DPI and returning to baseline by 14 DPI.[8, 168] Long-term studies show increased levels of senescence-associated molecular alterations at chronic time points.[7, 169] Currently, no interim studies have been conducted to identify at what point the resolution of initial senescence-associate alterations transitions to the differential progression of senescence following injury. It is therefore necessary to determine at what point levels of cellular senescence might diverge following injury to better understand potential therapeutic intervention windows for persistent cognitive symptoms.

Animals were sacrificed at 35 and 63 DPI to examine the subacute progression of cellular senescence following 5-hit closed-head rmTBI. Whole ipsilateral hippocampi were collected, homogenized, and subsequently extracted for protein or mRNA. Molecular analysis was conducted via qRT-PCR and Western Blotting to determine the effects of the injury on the

progression of cellular senescence by examining multiple senescence-associated markers at 35 and 63 DPI (Fig. 3). This study is the first of its kind to examine the progression of cellular senescence at subacute time points following 5-hit closed-head rmTBI and will hopefully identify a window of time in which the progression of cellular senescence can be targeted before differentiation from sham levels.

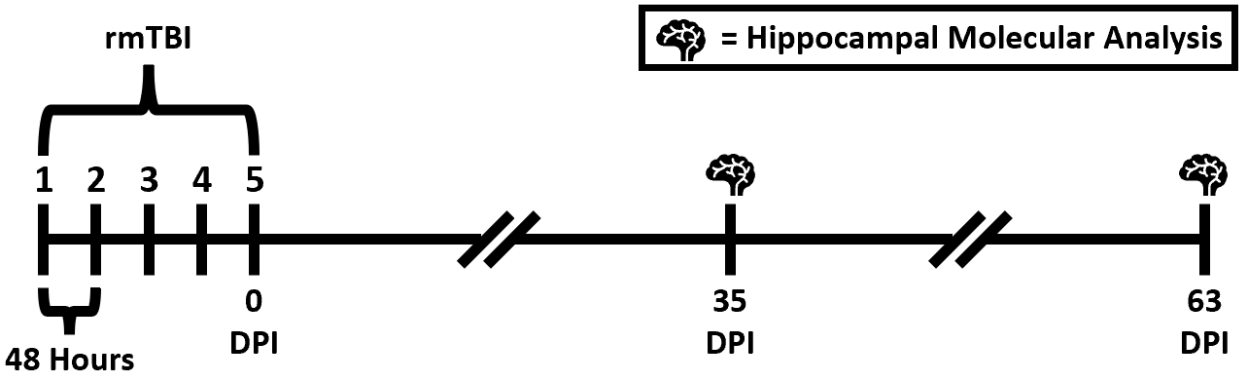


Figure 3: Experimental Timeline to Examine the Progression of Cellular Senescence following rmTBI. Animals were divided into two groups: sham and rmTBI. Sham animals served as a control group and did not receive an injury. rmTBI animals received five mTBIs spaced 48 hours apart. The days following the last impact are denoted as DPI. Animals were sacrificed at 35 and 63 DPI and the whole ipsilateral hippocampus was collected for molecular analysis of senescence-associated markers.

Aim 1: Experimental Question 1.2 – Results

Senescence-associated molecular alterations between 35 and 63 days following rmTBI. Senescence-associated molecular alterations between 35 and 63 DPI following 5-hit closed-head rmTBI were assessed via Western Blot analysis and RT-qPCR to examine progressive hippocampal senescence-related protein and gene expression changes, respectively (Fig. 3). Data for 35 and 63 DPI rmTBI groups were normalized to age-matched sham animals for each molecular target to eliminate the effect of natural aging on senescence-associated

molecular alterations. Western Blot and RT-qPCR data are represented as relative protein quantity and relative gene expression, respectively. A sample size of 12 animals was used for the 35 DPI rmTBI group and 6 animals for the 63 DPI rmTBI group at each time point for both Western Blot analysis and RT-qPCR. Progressive senescence-associated molecular alterations were analyzed via unpaired two-tailed t-tests between 35 and 63 DPI rmTBI groups for each molecular target. Significant elevation in relative p53 protein quantity was observed in the 63 DPI rmTBI group when compared to 35 DPI rmTBI animals (35 DPI rmTBI 0.96 ± 0.13; 63 DPI rmTBI 1.84 ± 0.32; $p = 0.0080$) (Fig. 4A). Interestingly, a significant decrease in relative *Tp53* (p53) gene expression was observed in the 63 DPI rmTBI animals when compared to the 35 DPI rmTBI group (35 DPI rmTBI 1.07 ± 0.03; 63 DPI rmTBI 0.91 ± 0.07; $p = 0.0204$) (Fig. 4B), possibly due to a negative autoregulatory mechanism when p53 protein levels are sufficiently high. A trending upregulation of *Cdkn1a* ($p21^{Waf1/Cip1}$) was detected in 63 rmTBI animals compared to the 35 DPI rmTBI group (35 DPI rmTBI 0.81 ± 0.03; 63 DPI rmTBI 0.96 ± 0.09; $p = 0.0696$), though no difference in relative protein quantity of $p21^{Waf1/Cip1}$ was observed (Fig. 5B). A trending increase in both $p16^{INK4a}$ relative protein quantity and gene expression was observed in 63 DPI rmTBI animals, though no statistical significance was reached (Fig. 6A, Fig. 6B). A significant downregulation of *Sirt1* relative gene expression was observed in the 63 DPI rmTBI group (35 DPI rmTBI 1.11 ± 0.04; 63 DPI rmTBI 0.83 ± 0.02; $p = 0.0006$) (Fig. 7B) with similar trends in SIRT1 relative protein quantity when compared to 35 DPI rmTBI animals (35 DPI rmTBI 1.64 ± 0.29; 63 DPI rmTBI 0.79 ± 0.21; $p = 0.0976$) (Fig 7A). Whole hippocampal relative *Hmbg1* gene expression was significantly reduced in 63 DPI rmTBI animals when compared to the 35 DPI rmTBI group (35 DPI rmTBI 1.05 ± 0.04; 63 DPI rmTBI 0.84 ± 0.03; p

= 0.0042) (Fig. 7C). No statistical difference was observed between time points for Lamin B1 protein (Fig. 8).

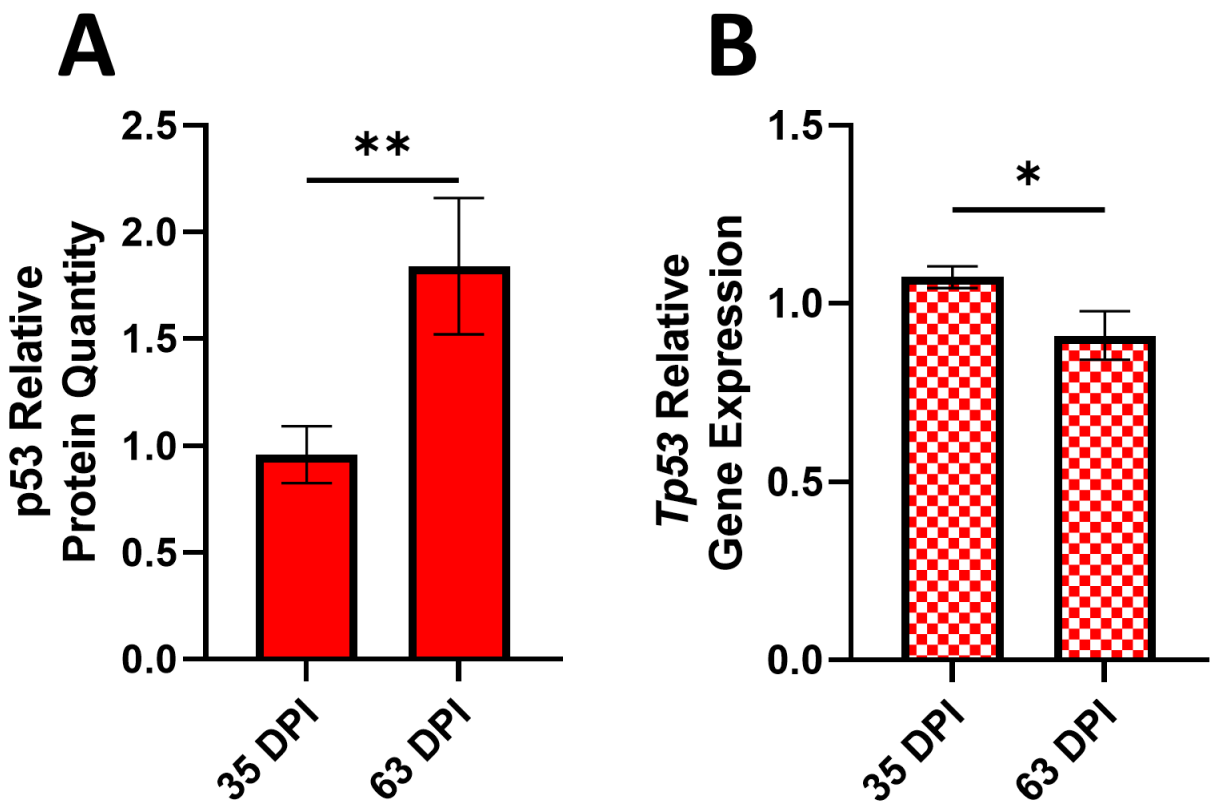
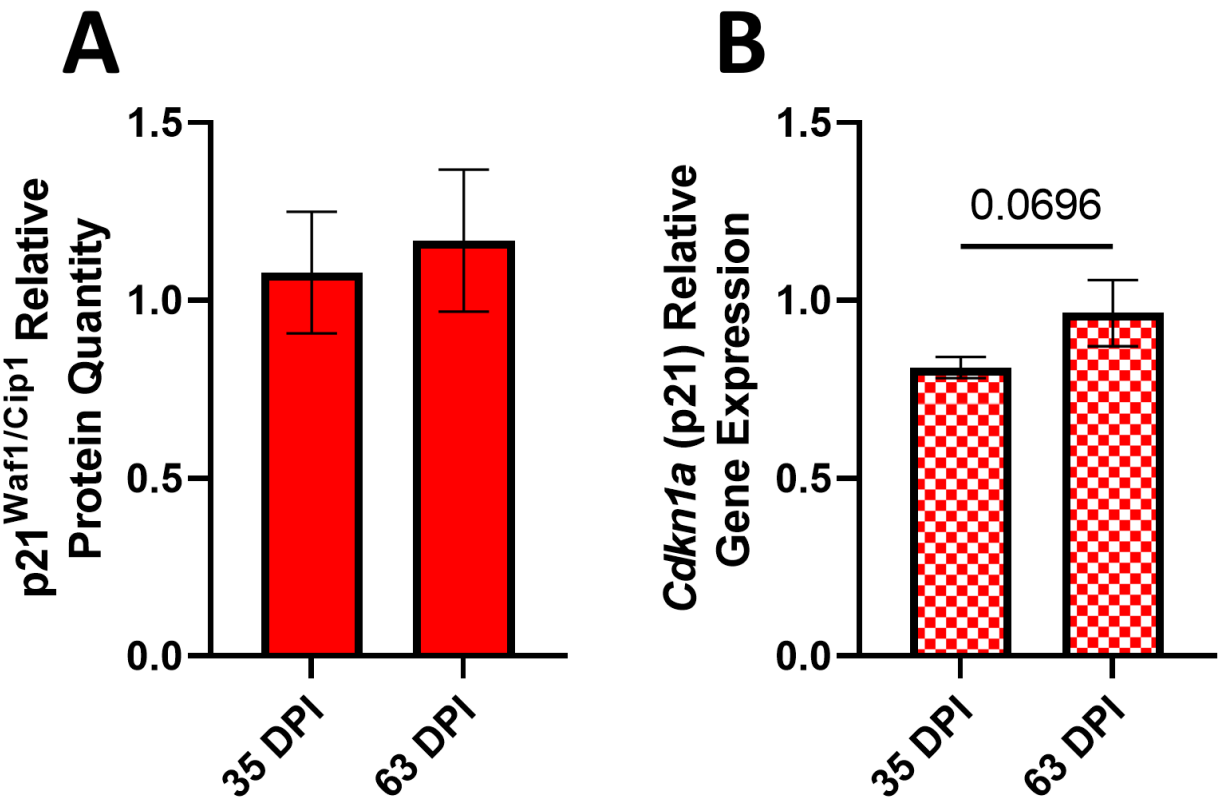


Figure 4: Cell Cycle Regulator p53 Protein Levels Significantly Increase in the Ipsilateral Hippocampus between 35 and 63 DPI. Relative protein quantity and gene expression of senescence-associated marker p53 in the ipsilateral hippocampus at 35 and 63 DPI following rmTBI normalization to age-matched sham animals. (A) Relative p53 protein quantity. (B) Relative *Tp53* (p53) gene expression. All protein data were normalized to total protein. All gene expression data were normalized to the reference gene *Hprt1*. Data are presented as mean ± SEM. N = 12 for 35 DPI rmTBI, N = 6 for 63 DPI rmTBI. **$p \leq 0.01$, *$p \leq 0.05$.



p21waIpsilateral Hippocampus between 35 and 63 DPI. Relative protein quantity and gene expression of senescence-associated marker $p21^{Waf1/Cip1}$ in the ipsilateral hippocampus at 35 and 63 DPI following rmTBI normalization to age-matched sham animals. (A) Relative $p21^{Waf1/Cip1}$ protein quantity. (B) Relative *Cdkn1a* (p21) gene expression. All protein data were normalized to total protein. All gene expression data were normalized to the reference gene *Hprt1*. Data are presented as mean ± SEM. N = 12 for 35 DPI rmTBI, N = 6 for 63 DPI rmTBI.

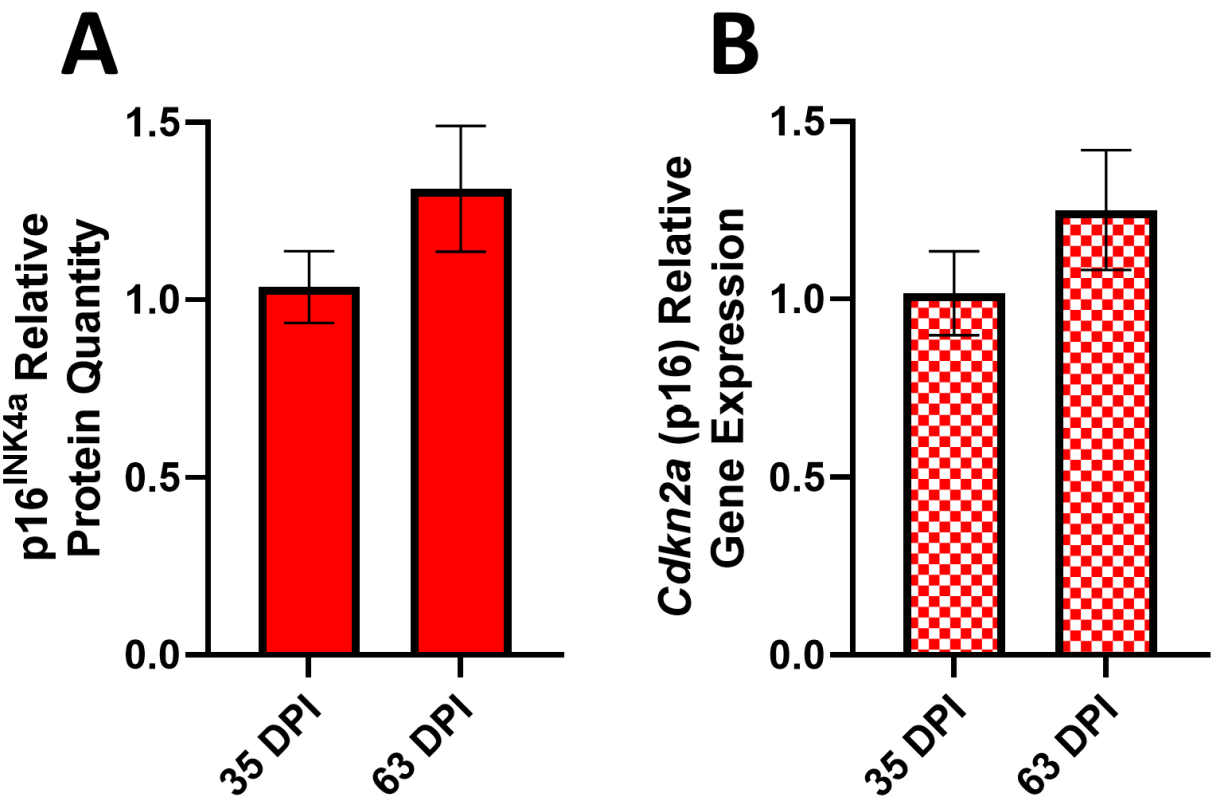


Figure 6: Senescence Maintenance-Associated p16^{INK4a} Increases in the Ipsilateral Hippocampus between 35 and 63 DPI. Relative protein quantity and gene expression of senescence-associated marker p16^{INK4a} in the ipsilateral hippocampus at 35 and 63 DPI following rmTBI normalization to age-matched sham animals. (A) Relative p16^{INK4a} protein quantity. (B) Relative *Cdkn2a* (p16) gene expression. All gene expression data were normalized to the reference gene *Hprt1*. Data are presented as mean ± SEM. N = 12 for 35 DPI rmTBI, N = 6 for 63 DPI rmTBI.

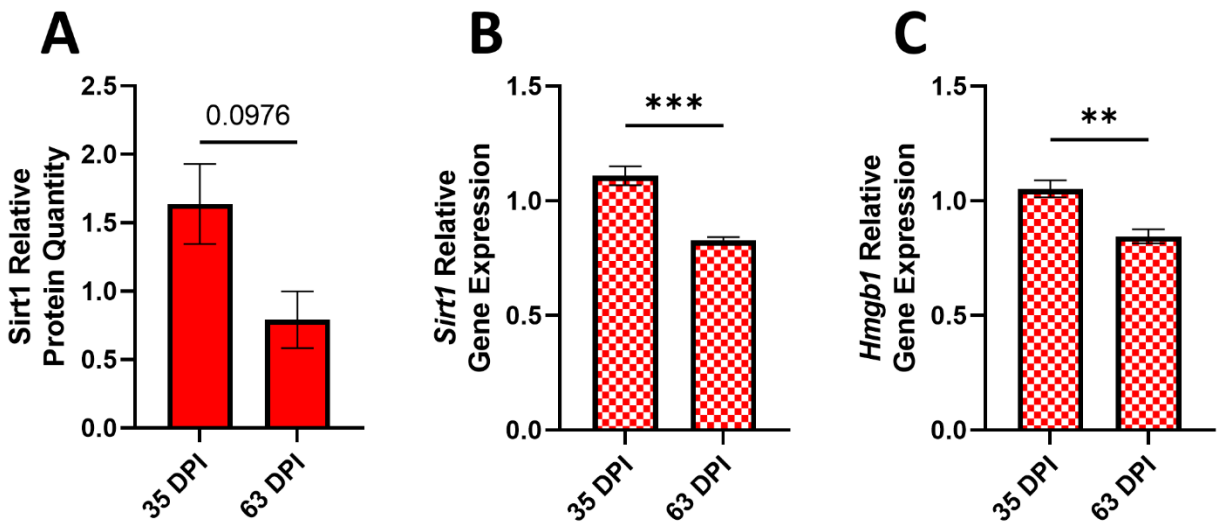


Figure 7: Senescence-Associated Secretory Phenotype Suppressors SIRT1 and HMGB1 are Significantly Downregulated in the Ipsilateral Hippocampus between 35 and 63 DPI. Relative protein quantity and gene expression of senescence-associated markers SIRT1 and HMGB1 in the ipsilateral hippocampus at 35 and 63 DPI following rmTBI normalization to age-matched sham animals. (A) Relative SIRT1 protein quantity. (B) Relative *Sirt1* gene expression. (C) Relative *Hmgb1* gene expression. All gene expression data were normalized to the reference gene *Hprt1*. Data are presented as mean ± SEM. N = 12 for 35 DPI rmTBI, N = 6 for 63 DPI rmTBI. ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$.

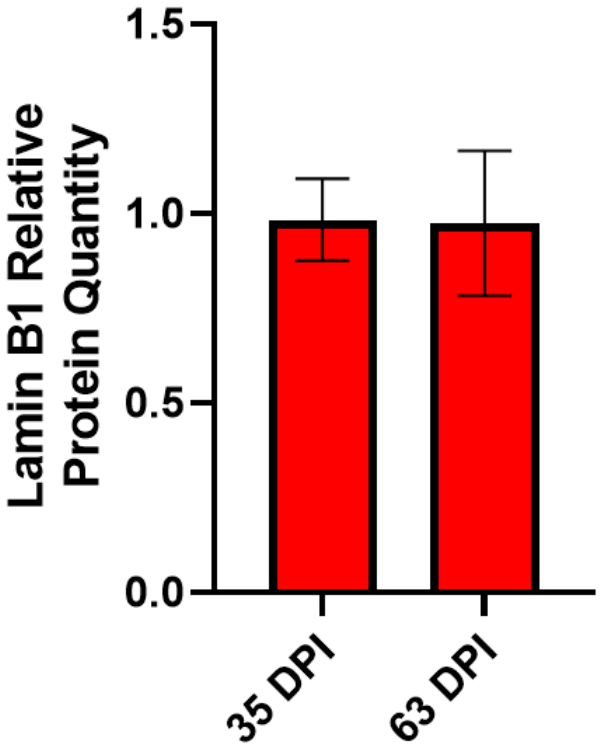


Figure 8: Senescence-Associated Nuclear Integrity Marker Lamin B1 Remains Constant in the Ipsilateral Hippocampus between 35 and 63 DPI. Relative protein quantity of senescence-associated marker Lamin B1 in the ipsilateral hippocampus at 35 and 63 DPI following rmTBI normalization to age-matched sham animals. All protein data were normalized to total protein. Data are presented as mean ± SEM. N = 12 for 35 DPI rmTBI, N = 6 for 63 DPI rmTBI.

CHAPTER SIX

SPECIFIC AIM 2

Specific Aim 2

Determine the effects of subacute testosterone treatment on recognition memory and hippocampal cellular senescence following rmTBI.

Experimental Question 2.1

How does subacute testosterone treatment affect recognition memory following rmTBI?

Aim 2: Experimental Question 2.1 – Rationale

This experiment aims to 1.) determine if persistent rmTBI-induced cognitive deficits can be ameliorated through pharmacological intervention at a subacute time point, and 2.) determine if subacute systemic testosterone delivery ameliorates persistent deficits in recognition memory following rmTBI. Hypogonadotropic hypogonadism has been demonstrated to occur in a significant portion of male patients following TBI.[205-208] Studies have shown that reduced sex hormones can have detrimental effects on cognition and memory.[209-212] Testosterone administration has been shown to promote synaptic formation, maintenance, and plasticity while improving cognitive outcomes in hypogonadal model systems.[213-215] However, in the context of 5-hit closed-head rmTBI, no studies to date have investigated the potential neurotherapeutic role of testosterone when administered at a subacute timepoint following injury. This study is the first of its kind to examine the effects of testosterone administration at a subacute time point with the intent to ameliorate persistent rmTBI-associated cognitive deficits. The effects of subacute

testosterone administration on persistent recognition memory deficits were evaluated by utilizing the NOR test and comparing rmTBI to rmTBI+T animals at PTD 7 (42 DPI) (Fig. 9).

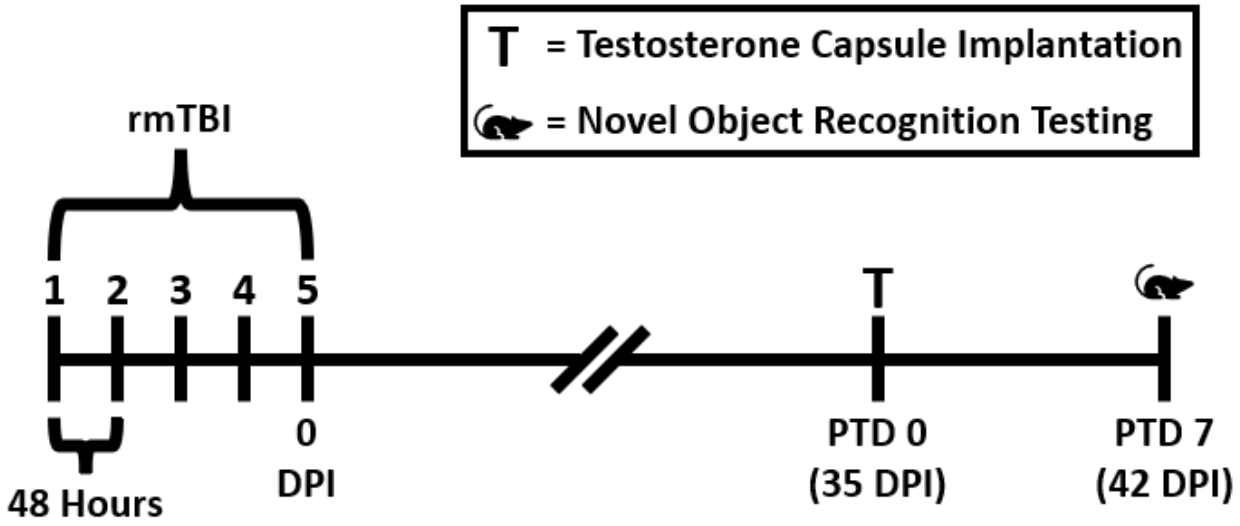


Figure 9: Experimental Timeline to Examine the Effects of Subacute Testosterone Treatment on Persistent Recognition Memory Deficits following rmTBI. Animals were divided into three groups: sham, rmTBI, and rmTBI+T. The days following the last impact are denoted as DPI. The days following testosterone treatment are denoted as PTD. Sham animals served as a control group and did not receive an injury. rmTBI animals received five mTBIs spaced 48 hours apart and had a vehicle-filled capsule implanted in the nape at PTD 0 (35 DPI) to serve as a surgical control. rmTBI+T animals received five mTBIs and had a crystalline testosterone-packed capsule implanted in the nape at PTD 0 (35 DPI). Animals underwent the testing phase of NOR at PTD 7 (42 DPI).

Aim 2: Experimental Question 2.1 – Results

Novel object recognition after subacute testosterone administration following rmTBI at PTD 7 (42 DPI). A subset of rmTBI animals described in Specific Aim 1: Experimental Question 1.1 were administered systemic testosterone at PTD 0 (35 DPI) (rmTBI+T), and recognition memory was assessed via NOR at PTD 7 (42 DPI). Data are represented as the percentage of total object exploration time spent investigating the novel object. Unpaired two-tailed t-tests were performed at each timepoint to determine if the persistent rmTBI-associated recognition memory deficits demonstrated in Figure 2 could be

mitigated via subacute testosterone treatment (Fig. 9). The sample size for rmTBI and rmTBI+T groups was 3-4 animals per group at each time point. A significant increase in the percentage of total object exploration time spent investigating the novel object was observed in the rmTBI+T group at PTD 7 (42 DPI) when compared to the rmTBI animals (PTD 7 (42 DPI) rmTBI 53.13 ± 5.28; PTD 7 (42 DPI) rmTBI+T 74.87 ± 0.75; p = 0.0180) (Fig. 10).

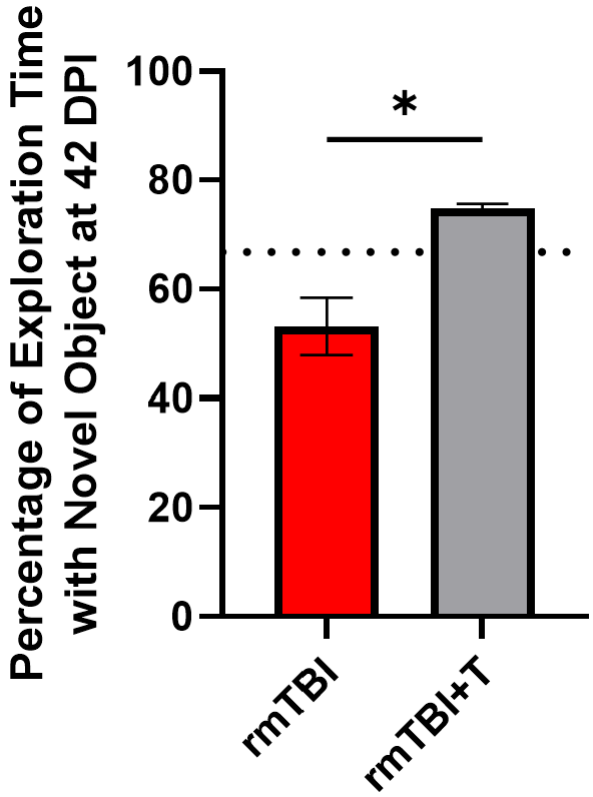


Figure 10: Subacute Testosterone Administration Mitigates Persistent rmTBI-Associated Deficits in Recognition Memory. Novel object recognition data at PTD 7 (42 DPI) is expressed as the percentage of total object exploration time spent investigating the novel object. The dashed line represents the sham animal's mean percentage of exploration time spent with the novel object. Data are presented as mean ± SEM. N = 3-4 animals per group. *p ≤ 0.05.

Experimental Question 2.2

How does subacute testosterone treatment affect senescence-associated markers in the hippocampus following rmTBI?

Aim 2: Experimental Question 2.2 – Rationale

The purpose of this experiment is two-fold: 1.) investigate the effects of subacute testosterone administration on rmTBI-induced hippocampal senescence-associated molecular alterations, and 2.) determine if senescence-associated molecular alterations in the hippocampus can be pharmacologically mitigated at a subacute time point following rmTBI. Testosterone has been shown to be protective against chemically-induced and aged-associated senescence.[11, 12, 171] Acute testosterone treatment following a single-hit severe TBI has also been shown to improve molecular outcomes that have been widely recognized as senescence-inducing factors in other studies.[10] However, no studies have directly investigated the role of subacute testosterone treatment in cellular senescence following rmTBI. It is therefore imperative to determine if testosterone administered at a subacute time point influences senescence-associated molecular alterations following rmTBI.

At 35 DPI following 5-hit closed-head rmTBI (PTD 0), animals had crystalline testosterone- or vehicle-filled capsules implanted in their nape. Animals were subsequently sacrificed at PTD 28 (63 DPI) and whole ipsilateral hippocampi were collected, homogenized, and extracted for protein and mRNA (Fig. 11). Molecular interrogation via Western Blot and qRT-PCR analysis was performed to determine if delayed testosterone treatment influences senescence-associated markers following 5-hit closed-head rmTBI. This study is the first of its kind to utilize systemic testosterone administration at a subacute time point following 5-hit closed-head rmTBI to attenuate rmTBI-associated alterations in senescence markers.

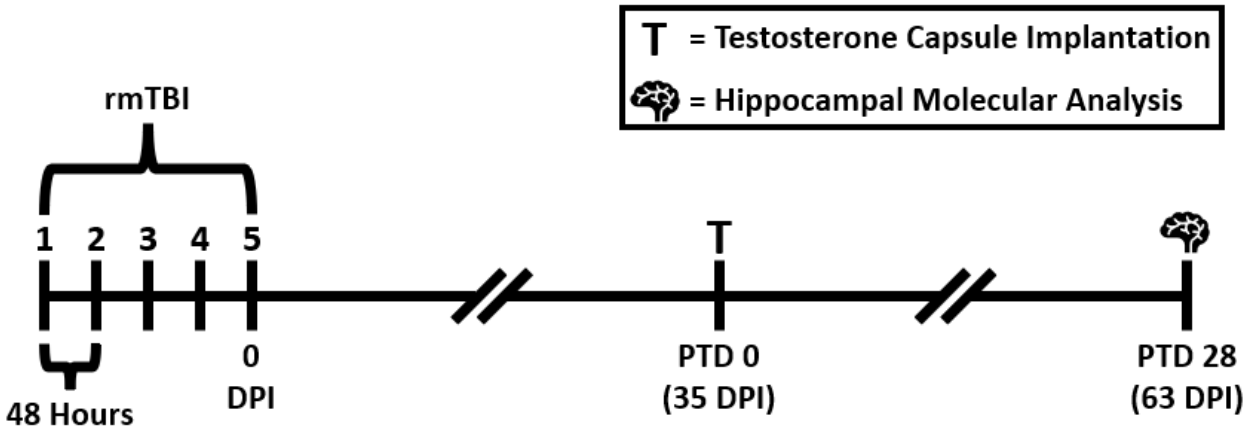


Figure 11: Experimental Timeline to Examine the Effects of Subacute Testosterone Treatment on Senescence-Associated Molecular Markers at PTD 28 (63 DPI). Animals were divided into three groups: sham, rmTBI, and rmTBI+T. The days following the last impact are denoted as DPI. The days following testosterone treatment are denoted as PTD. Sham animals served as a control group and did not receive an injury. rmTBI animals received five mTBIs spaced 48 hours apart and had a vehicle-filled capsule implanted in the nape at PTD 0 (35 DPI) to serve as a surgical control. rmTBI+T animals received five mTBIs and had a crystalline testosterone-packed capsule implanted in the nape at PTD 0 (35 DPI). Animals were sacrificed at PTD 28 (63 DPI) and the whole ipsilateral hippocampus was collected for molecular analysis.

Aim 2: Experimental Question 2.2 – Results

Molecular analysis of senescence-associated markers following subacute testosterone treatment at PTD 28 (63 DPI). A subset of animals described in Specific Aim 1: Experimental Question 1.2 were administered systemic testosterone at PTD 0 (35 DPI) and molecularly interrogated at PTD 28 (63 DPI) to determine the effects of delayed testosterone administration on hippocampal cellular senescence-associated markers (Fig. 11). Molecular analyses of senescence-related markers via Western Blot and RT-qPCR were conducted at PTD 28 (63 DPI) to ensure that any testosterone-induced changes were not transient. Data for PTD 28 (63 DPI) rmTBI and rmTBI+T groups were normalized to age-matched sham animals for each molecular target. Western Blot and RT-qPCR data are represented as relative protein quantity and relative gene expression, respectively. A sample size of 6 animals was used for both the

rmTBI and rmTBI+T groups for both Western Blot analysis and RT-qPCR. Potential testosterone-induced senescence-associated molecular alterations were analyzed via unpaired two-tailed t-tests between PTD 28 (63 DPI) rmTBI and rmTBI+T groups for each molecular target. A decrease in relative p53 protein quantity was observed in the rmTBI+T group when compared to rmTBI animals (rmTBI 1.84 ± 0.32; rmTBI+T 1.12 ± 0.15; $p = 0.0674$) (Fig. 12A), though no difference was observed in p53 gene expression (Fig. 12B). Additionally, relative *Cdkn2a* ($p16^{INK4a}$) gene expression was significantly reduced with delayed testosterone treatment when compared to untreated injured animals (rmTBI 1.25 ± 0.17; rmTBI+T 0.76 ± 0.14; $p = 0.0486$) (Fig. 13B), however; no difference was detected with relative $p16^{INK4a}$ protein quantity following testosterone treatment (Fig. 13A). No notable differences were seen amongst the other senescence-associated markers following delayed testosterone administration.

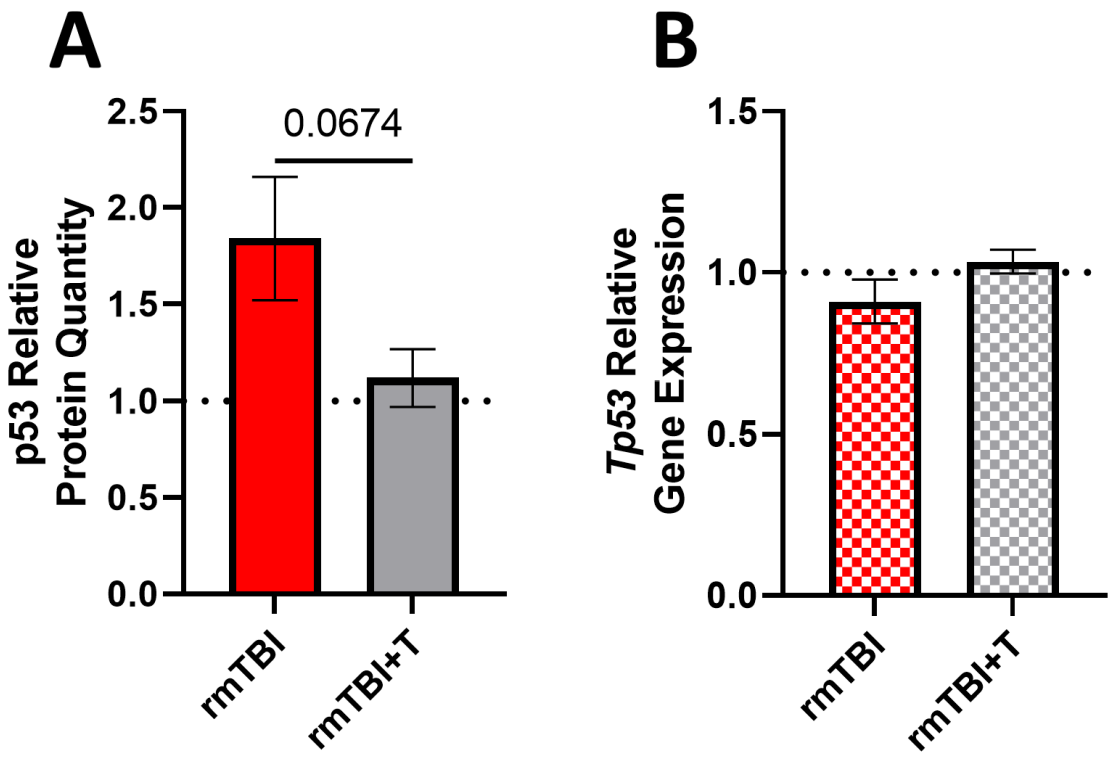


Figure 12: Subacute Testosterone Treatment Decreases p53 Protein Levels in the Ipsilateral Hippocampus at PTD 28 (63 DPI). Relative protein quantity and gene expression of senescence-associated marker p53 in the ipsilateral hippocampus at PTD 28 (63 DPI) following rmTBI and rmTBI+T normalization to age-matched sham animals. (A) Relative p53 protein quantity. (B) Relative *Tp53* (p53) gene expression. All protein data were normalized to total protein. All gene expression data were normalized to the reference gene *Hprt1*. The dotted lines represent sham animal mean relative protein quantity and gene expression. Data are presented as mean ± SEM. N = 6 animals per group.

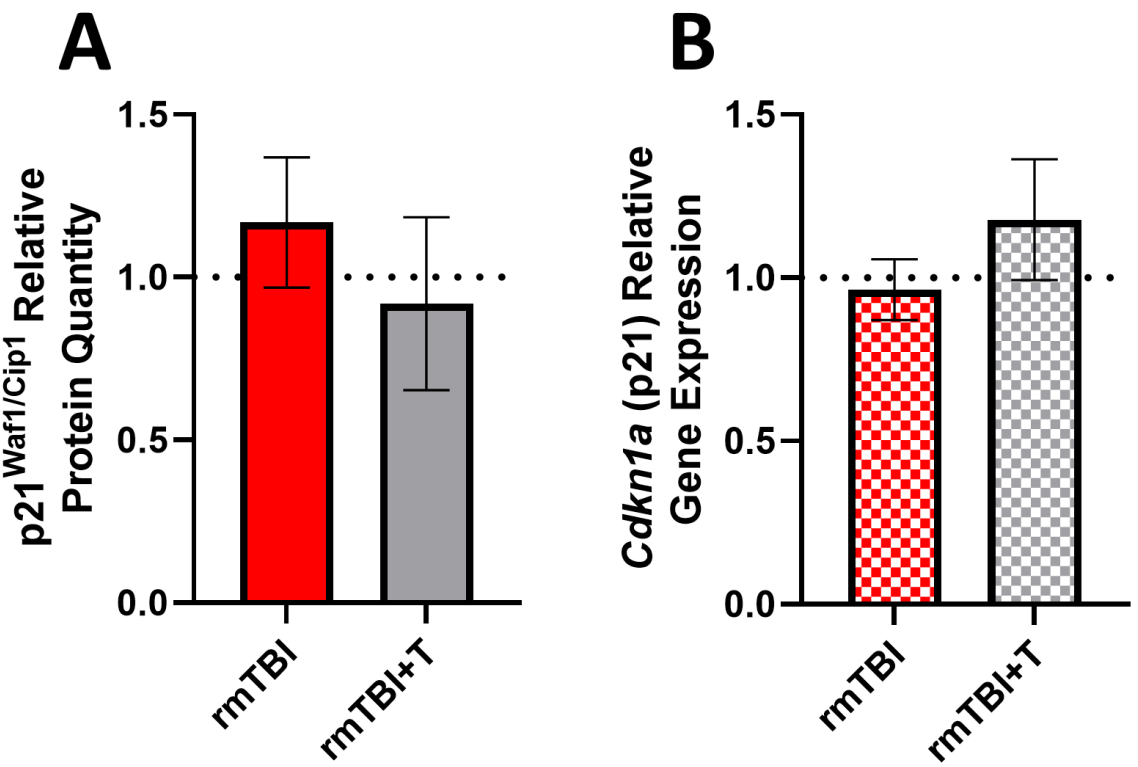


Figure 13: Subacute Testosterone Treatment has no Effect on p21$^{Waf1/Cip1}$ in the Ipsilateral Hippocampus at PTD 28 (63 DPI). Relative protein quantity and gene expression of senescence-associated marker p21$^{Waf1/Cip1}$ in the ipsilateral hippocampus at PTD 28 (63 DPI) following rmTBI and rmTBI+T normalization to age-matched sham animals. (A) Relative p21$^{Waf1/Cip1}$ protein quantity. (B) Relative *Cdkn1a* (p21) gene expression. All protein data were normalized to total protein. All gene expression data were normalized to the reference gene *Hprt1*. The dotted lines represent sham animal mean relative protein quantity and gene expression. Data are presented as mean ± SEM. N = 6 animals per group.

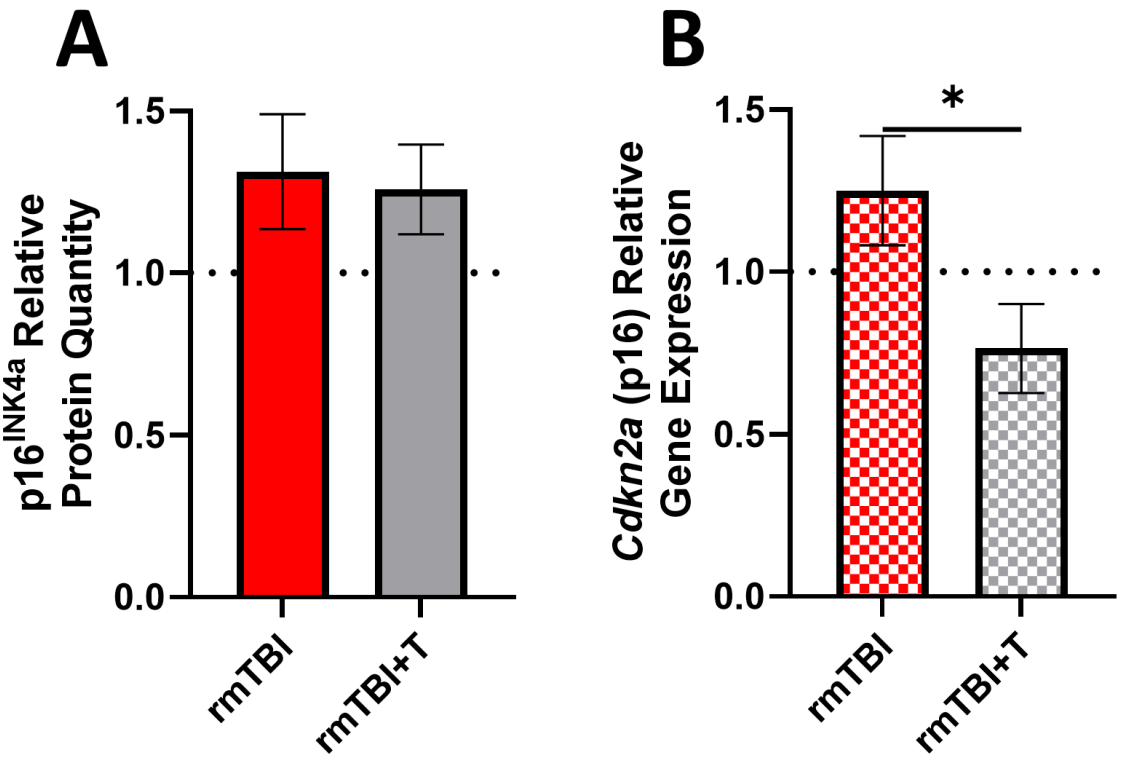


Figure 14: Subacute Testosterone Treatment Downregulates *Cdkn2a* (p16) Expression in the Ipsilateral Hippocampus at PTD 28 (63 DPI). Relative protein quantity and gene expression of senescence-associated marker $p16^{INK4a}$ in the ipsilateral hippocampus at PTD 28 (63 DPI) following rmTBI and rmTBI+T normalization to age-matched sham animals. (A) Relative $p16^{INK4a}$ protein quantity. (B) Relative *Cdkn2a* (p16) gene expression. All gene expression data were normalized to the reference gene *Hprt1*. The dotted lines represent sham animal mean relative protein quantity and gene expression. Data are presented as mean ± SEM. N = 6 animals per group. *$p \leq 0.05$.

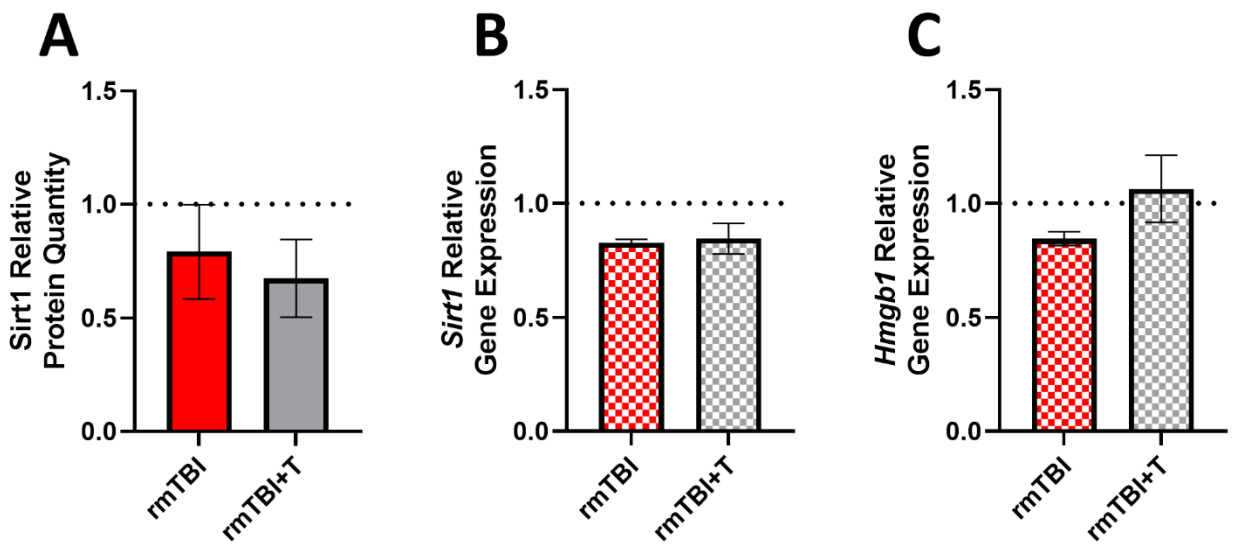


Figure 15: Subacute Testosterone Treatment has no Effect on SIRT1 or HMGB1 in the Ipsilateral Hippocampus at PTD 28 (63 DPI). Relative protein quantity and gene expression of senescence-associated markers SIRT1 and HMGB1 in the ipsilateral hippocampus at PTD 28 (63 DPI) following rmTBI and rmTBI+T normalization to age-matched sham animals. (A) Relative SIRT1 protein quantity. (B) Relative *Sirt1* gene expression. (C) Relative *Hmgb1* gene expression. All gene expression data were normalized to the reference gene *Hprt1*. The dotted lines represent sham animal mean relative protein quantity and gene expression. Data are presented as mean ± SEM. N = 6 animals per group.

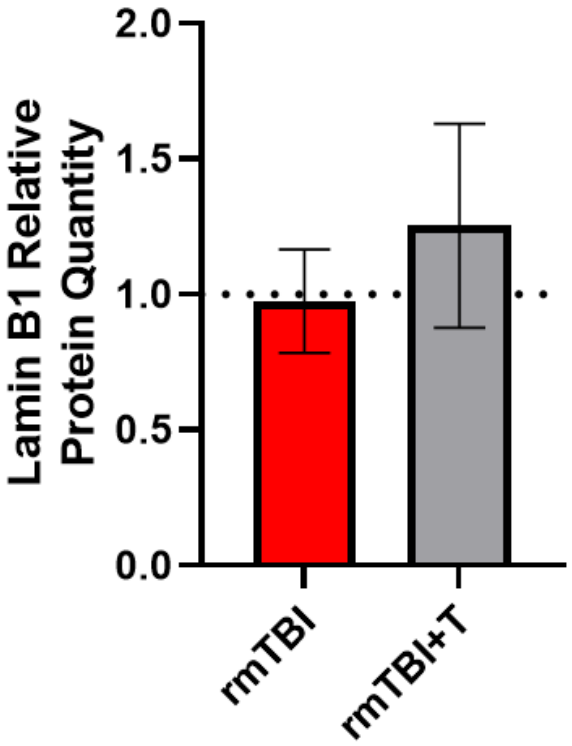


Figure 16: Subacute Testosterone Treatment has no Effect on Lamin B1 in the Ipsilateral Hippocampus at PTD 28 (63 DPI). Relative protein quantity of senescence-associated marker Lamin B1 in the ipsilateral hippocampus at PTD 28 (63 DPI) following rmTBI and rmTBI+T normalization to age-matched sham animals. All protein data were normalized to total protein. The dotted lines represent sham animal mean relative protein quantity. Data are presented as mean ± SEM. N = 6 animals per group.

CHAPTER SEVEN

SPECIFIC AIM 3

Specific Aim 3

Gather preliminary data investigating how rmTBI affects cellular senescence in specific hippocampal cell types at a chronic time point.

Experimental Question 3.1

Are there differences in senescence-associated markers between cell types of the hippocampus at a chronic time point following rmTBI?

Aim 3: Experimental Question 3.1 – Rationale:

The goal of this experiment is to provide preliminary data justifying more in-depth studies to identify how senescence-associated markers are differentially influenced in specific cell types of the hippocampus following 5-hit closed-head rmTBI. Numerous studies have shown that age-related diseases such as dementia, Alzheimer's Disease, Parkinson's Disease, and frontotemporal dementia are associated with increased cellular senescence within the brain.[159, 182-188, 197, 199] Few studies have evaluated differences in cellular senescence between rmTBI and uninjured subjects at chronic time points. However, a 2019 study by Schwab et. al. showed evidence of increased cellular senescence in post-mortem human brains with a history of brain injury when compared to humans with no reported TBIs.[169] With few studies investigating chronic senescence-associated alterations following rmTBI, animal studies must be conducted to characterize the progressive deviation of senescence-associated markers with

rmTBI when compared to uninjured control animals. Doing so will allow the determination of which cell types initially become senescent, allowing targeted elimination of those cells and prevention of paracrine recruitment of surrounding cells into a senescent state. Animals were sacrificed 175 DPI and perfused with 4% paraformaldehyde to preserve the tissue for immunohistochemical analysis following 5-hit closed-head rmTBI. Brains were collected and the hippocampus was sectioned at 40 µm. Heat-induced antigen retrieval was performed prior to duplex fluorescent immunolabelling. Two hallmark senescence-associated proteins, $p16^{INK4a}$ and $p21^{Waf1/Cip1}$, were chosen to identify senescence-like alterations in hippocampal cells. The previously mentioned markers were duplexed with one of the following antibodies: NeuN to identify neurons, Iba1 to identify microglia, or GFAP to identify astrocytes, allowing identification of the cell types with senescence-associated protein alterations. The results from these data provide the basis to conduct future studies investigating senescence-associated molecular alterations in a cell-type-specific manner following 5-hit closed-head rmTBI.

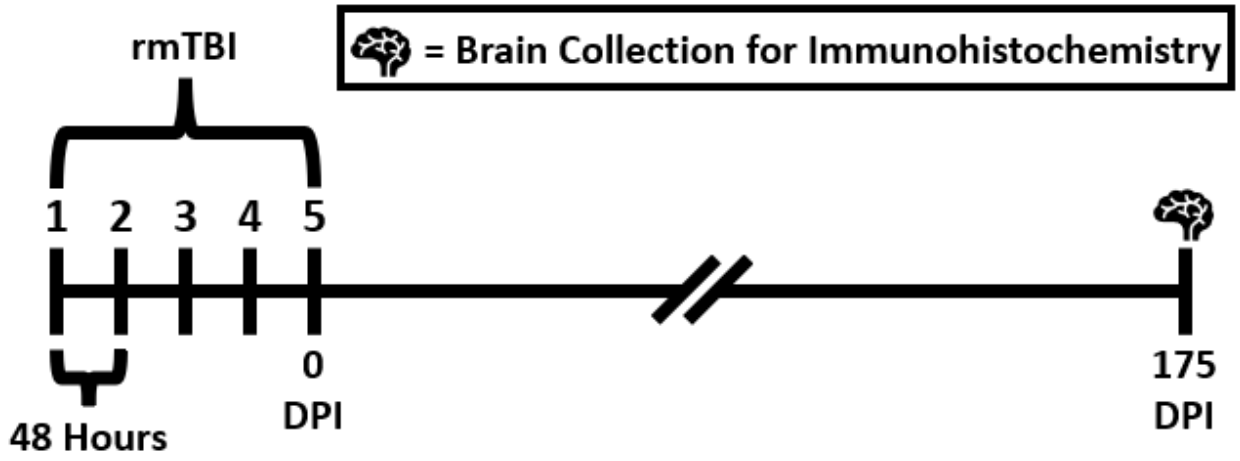


Figure 17: Experimental Timeline to Immunohistochemically Examine the Chronic Effects of rmTBI on Senescence-Associated Markers $p16^{INK4a}$ and $p21^{Waf1/Cip1}$. Animals were divided into two groups: sham and rmTBI. Sham animals served as a control group and did not receive an injury. rmTBI animals received five mTBIs spaced 48 hours apart. The days following the last impact are denoted as DPI. Animals were sacrificed at 175 DPI and brains were collected for immunohistochemical analysis.

Aim 3: Experimental Question 3.1 – Preliminary Results

Animals were sacrificed at 175 DPI in a preliminary investigation to assess alterations in senescence-associated markers between neurons, astrocytes, and microglia of the hippocampus at a chronic time point following rmTBI (Fig. 17). Heat-induced antigen retrieval with subsequent co-immunolabelling of $p16^{INK4a}$ or $p21^{Waf1/Cip1}$ with NeuN, GFAP, or Iba1 allowed preliminary assessment of hippocampal senescence-associated marker expression between cell types via fluorescent confocal microscopy. Quantification of relative fluorescence intensity at 10X suggests that $p16^{INK4a}$ is potentially increased in the dentate gyrus and cornu ammonis 3 (CA3) regions of the hippocampus following rmTBI (Fig. 18). Visual inspection of $p16^{INK4a}$ and $p21^{Waf1/Cip1}$ was performed under 63X oil immersion to investigate cell-type-specific expression patterns. $p16^{INK4a}$ fluorescence intensity did not appear to change in NeuN positive cells with rmTBI (Fig. 19). Interestingly, NeuN positive cell density may have been reduced by rmTBI (Fig. 19). No discernable difference in $p16^{INK4a}$ labeling was detected between rmTBI and sham GFAP positive cells, though an apparent reduction in GFAP staining is depicted in rmTBI animals (Fig. 20). $P16^{INK4a}$ immunostaining seemed to decrease in Iba1 positive cells of rmTBI animals when compared to sham (Fig. 21). An increase in $p21^{Waf1/Cip1}$ fluorescence intensity was observed in NeuN positive cells of rmTBI animals (Fig. 22). GFAP positive cells appeared to have an increase in $p21^{Waf1/Cip1}$ fluorescence intensity following rmTBI (Fig. 23). No $p21^{Waf1/Cip1}$ was observed in Iba1 positive cells regardless of injury status (Fig. 24).

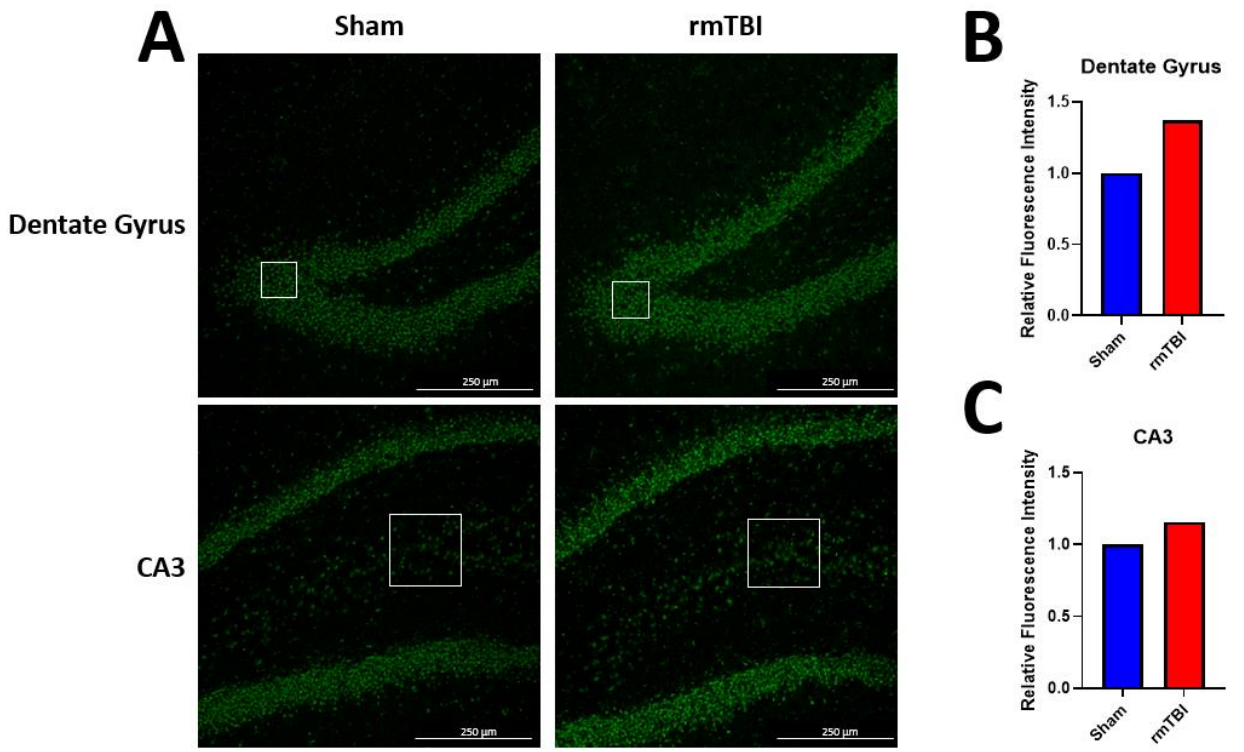


Figure 18: Preliminary Immunohistochemical Data Suggests a Chronic Increase in Senescence-Associated p16^{INK4a} in the Dentate Gyrus and CA3 Regions of the Hippocampus following rmTBI. Fluorescent immunolabeling of p16^{INK4a} in the Dentate Gyrus and CA3 region of the hippocampus at 175 DPI. (A) 10X confocal micrographs comparing p16^{INK4a} immunolabeling between sham and rmTBI animals in the Dentate Gyrus and CA3 region of the hippocampus. (B) Relative fluorescence intensity quantification of p16^{INK4a} immunolabeling in the Dentate Gyrus. (C) Relative fluorescence intensity quantification of p16^{INK4a} immunolabeling in the CA3 region of the hippocampus. N = 2 animals per group.

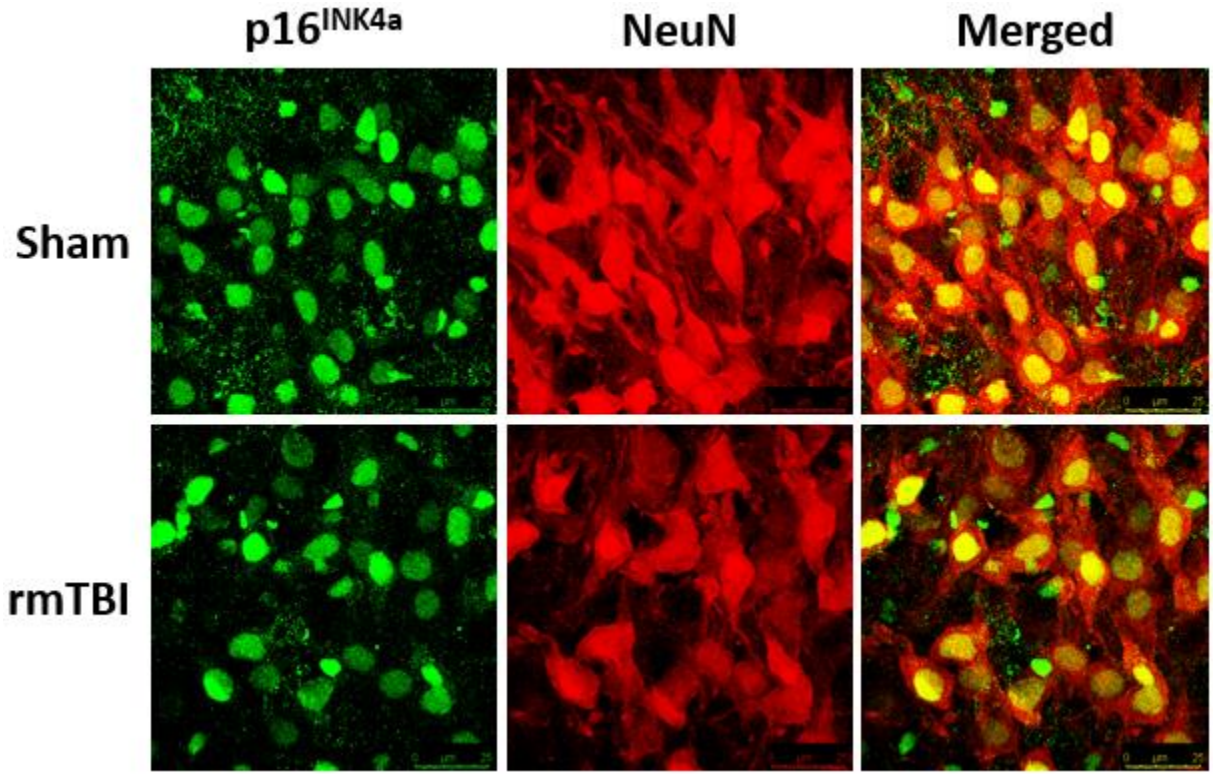


Figure 19: Senescence-Associate p16^{INK4a} in Neurons of the Hippocampus. Fluorescent 63X oil immersion confocal micrographs comparing p16^{INK4a} immunolabeling in NeuN positive cells between sham (top) and rmTBI (bottom) animals in the CA3 region of the hippocampus at 175 DPI. N = 2 animals per group.

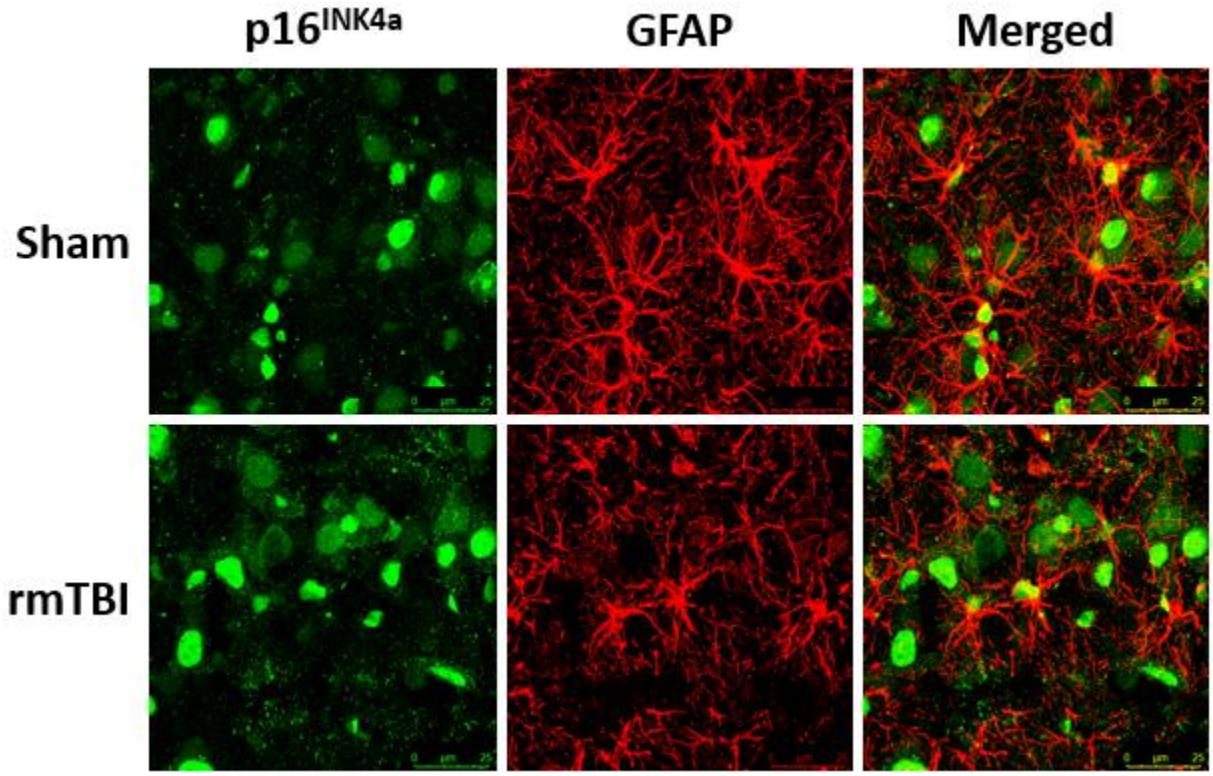


Figure 20: Senescence-Associate p16^{INK4a} in Astrocytes of the Hippocampus. Fluorescent 63X oil immersion confocal micrographs comparing p16^{INK4a} immunolabeling in GFAP positive cells between sham (top) and rmTBI (bottom) animals in the CA3 region of the hippocampus at 175 DPI. N = 2 animals per group.

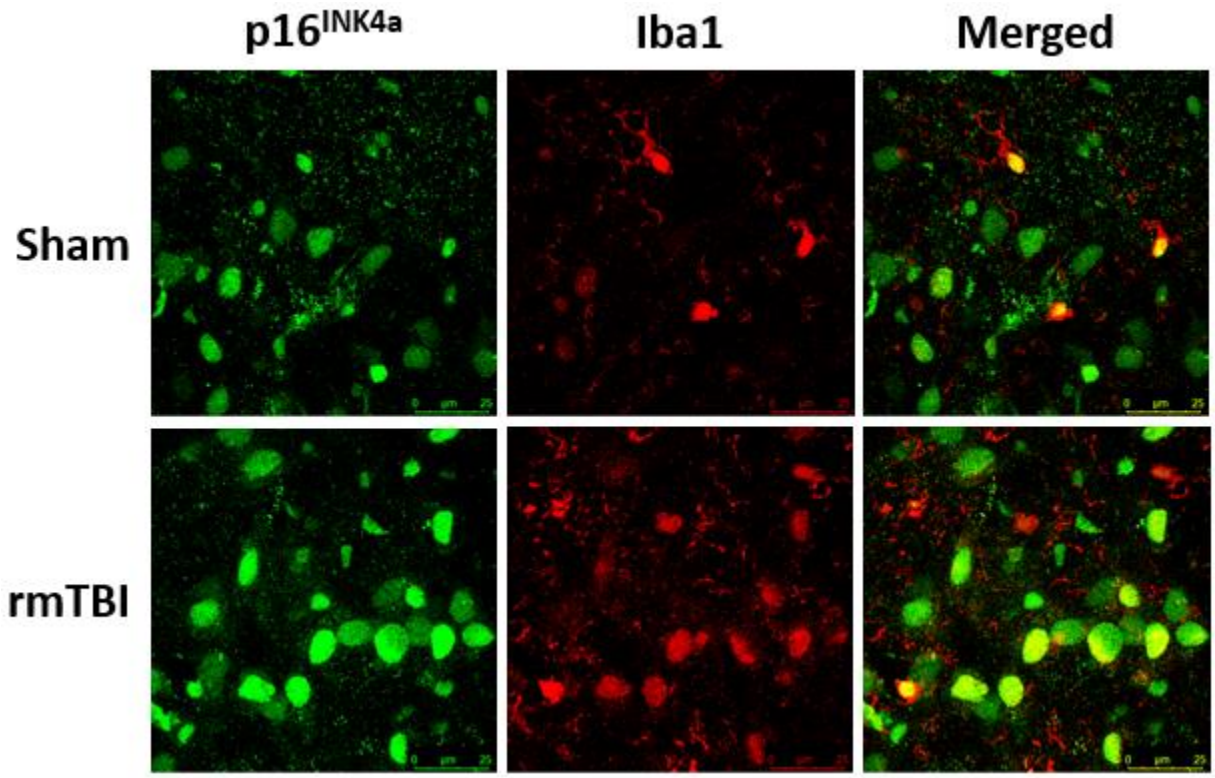


Figure 21: Senescence-Associate p16^{INK4a} in Microglia of the Hippocampus. Fluorescent 63X oil immersion confocal micrographs comparing p16^{INK4a} immunolabeling in Iba1 positive cells between sham (top) and rmTBI (bottom) animals in the CA3 region of the hippocampus at 175 DPI. N = 2 animals per group.

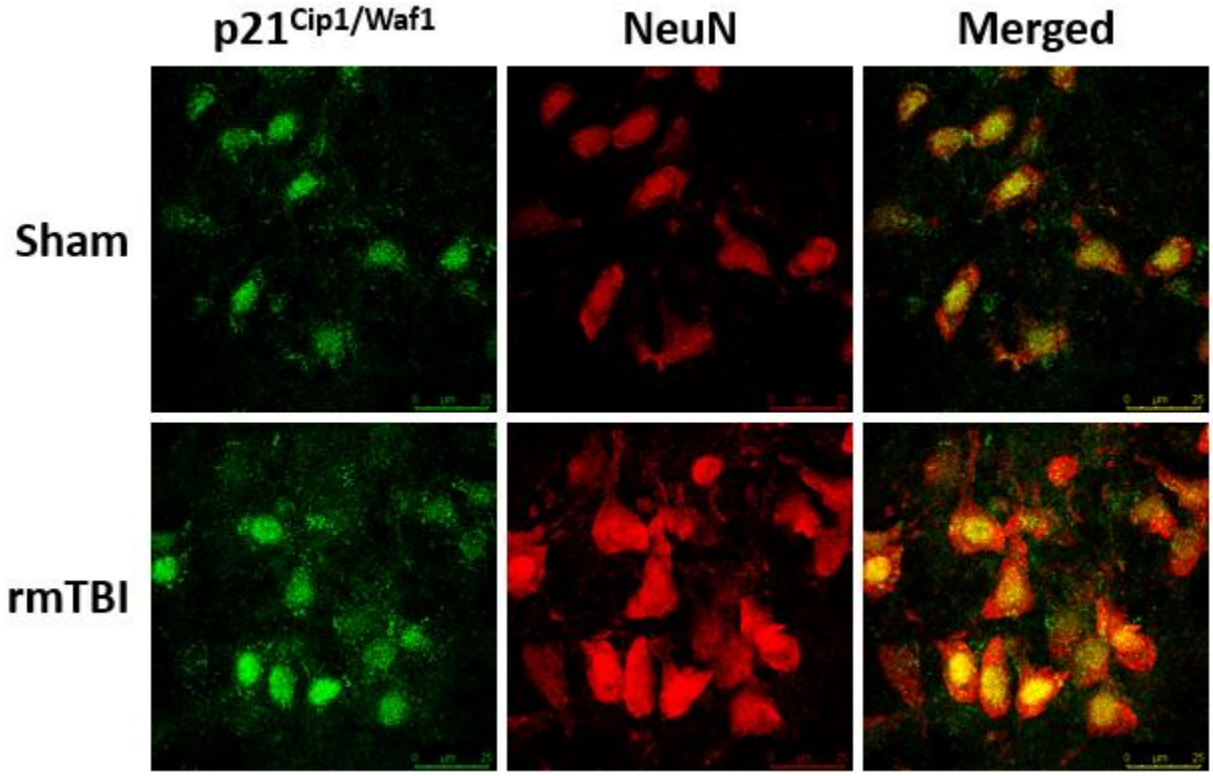


Figure 22: Senescence-Associate p21$^{Waf1/Cip1}$ in Neurons of the Hippocampus. Fluorescent 63X oil immersion confocal micrographs comparing p21$^{Waf1/Cip1}$ immunolabeling in NeuN positive cells between sham (top) and rmTBI (bottom) animals in the CA3 region of the hippocampus at 175 DPI. N = 2 animals per group.

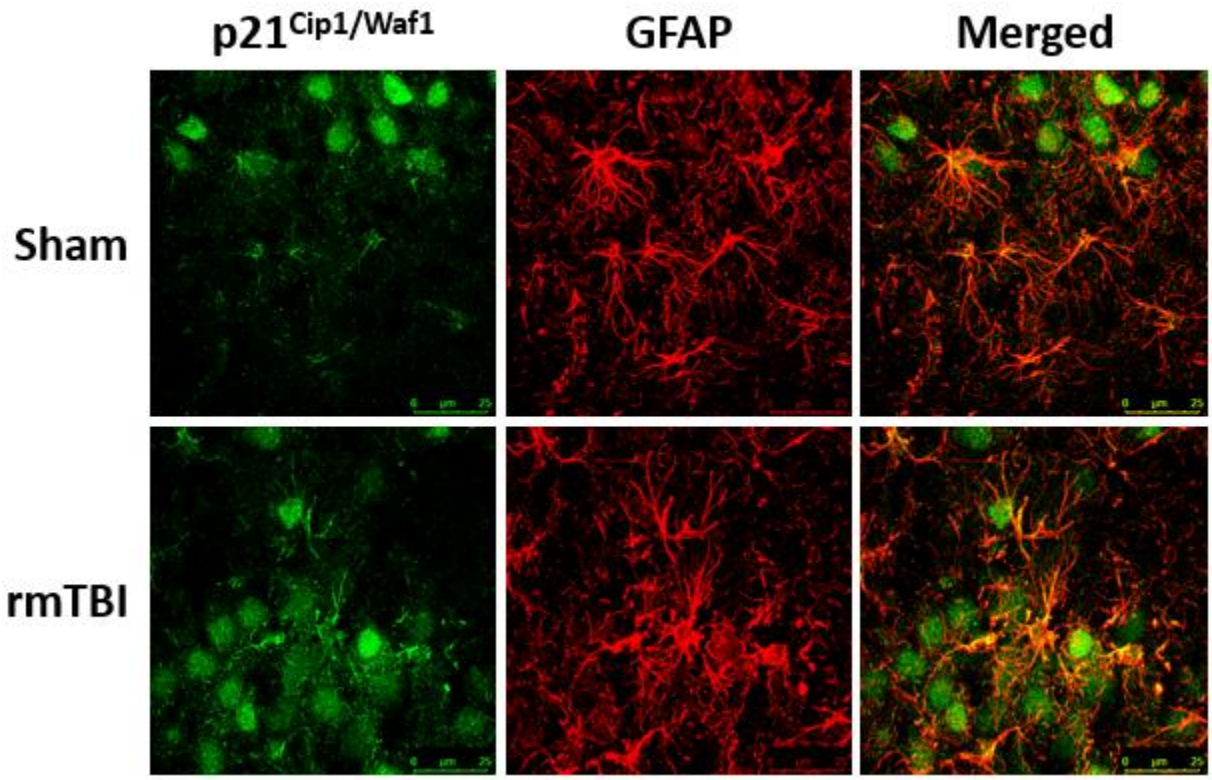


Figure 23: Senescence-Associate p21$^{Waf1/Cip1}$ in Astrocytes of the Hippocampus. Fluorescent 63X oil immersion confocal micrographs comparing p21$^{Waf1/Cip1}$ immunolabeling in GFAP positive cells between sham (top) and rmTBI (bottom) animals in the CA3 region of the hippocampus at 175 DPI. N = 2 animals per group.

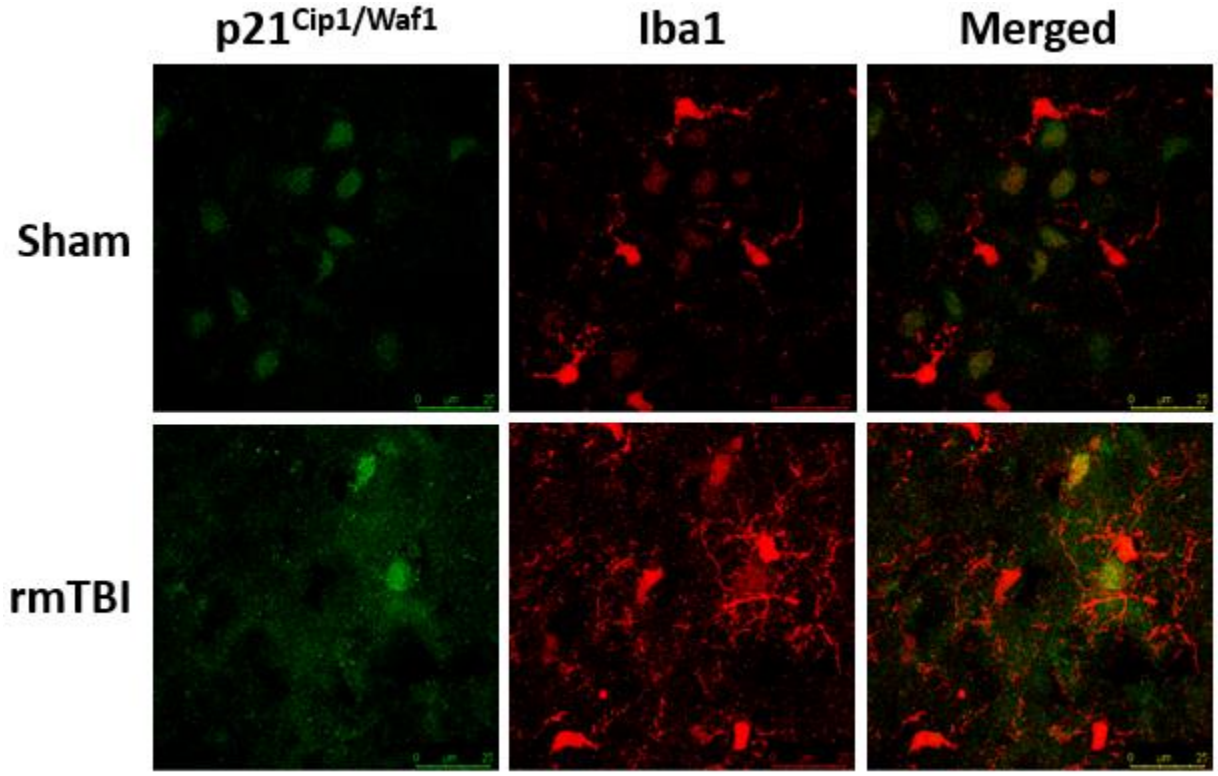


Figure 24: Senescence-Associate p21$^{Waf1/Cip1}$ in Microglia of the Hippocampus. Fluorescent 63X oil immersion confocal micrographs comparing p21$^{Waf1/Cip1}$ immunolabeling in Iba1 positive cells between sham (top) and rmTBI (bottom) animals in the CA3 region of the hippocampus at 175 DPI. N = 2 animals per group.

CHAPTER EIGHT

DISCUSSION

Cognitive deficits are one of the most commonly reported mTBI symptoms and sustaining rmTBI has been shown to exacerbate and increase the likelihood that symptoms will persist.[16, 57, 60-63] mTBI initially disrupts brain homeostasis by physically shearing tissue and transiently increasing plasma membrane permeability, resulting in a pathophysiological cascade of events that can lead to cognitive deficits.[67-75] Although the initial cellular and molecular events following mTBI may be similar to those of rmTBI, inherent differences may arise as repetitive impacts occur within the recovery period, also referred to as the "window of vulnerability." The differences between a single mTBI and rmTBI during the window of vulnerability may contribute to the persistence and exacerbated symptomology displayed following rmTBI. A plethora of hippocampal and cortical research has been conducted to elucidate the long-term pathophysiology behind cognitive dysfunction during the acute and chronic phases of rmTBI with limited success. Few studies have examined the cellular and molecular alterations contributing to the persistence of these symptoms at more distal, subacute time points. Cellular senescence has recently been implicated as a potential mechanism underlying acute symptoms following rmTBI; however, these studies are limited and further research is required to elucidate the role of cellular senescence in rmTBI-induced persistent symptoms.[8, 166, 167, 169, 216] The research presented in this thesis evaluates the use of 5-hit losed-head rmTBI in rats to model persistent deficits in recognition memory and is the first study

of its kind to examine the progression of senescence-associated molecular alterations in the hippocampus between approximately one- and two months post-injury.

Memory deficits are a common cognitive symptom reported following mTBI, which generally resolve one- to two weeks post-injury.[16, 60-62] A portion of individuals that sustained mTBI report symptoms that persist over a year following the incident.[4] However, the attempt to develop a single-hit injury paradigm to model persistent cognitive deficits in animals has had limited success. rmTBI has been well established to increase the likelihood of persistent cognitive deficits and even exacerbate them, providing the justification to employ rmTBI models in the study of persistent cognitive symptoms.[5] Studies to date have successfully demonstrated that persistent deficits in memory can be achieved through a variety of rmTBI paradigms.[217-221] The findings of this thesis corroborate the use of rmTBI models to achieve persistent memory deficits by demonstrating that recognition memory impairment occurs in injured animals at one week and continues one-month following 5-hit closed-head rmTBI via NOR testing. A 5-hit closed-head rmTBI study with a 24-hour impact latency demonstrated recognition memory deficits continuing eight weeks following injury.[221] Though the various rmTBI models have successfully demonstrated persistent cognitive deficits, it is rare for two injury paradigms to be the same. rmTBI models can differ by a variety of factors such as the number of mTBI administered, latency between impacts, and impact force.[217-221] The model of rmTBI in this thesis differs from other studies by using a closed head system and allowing free head swing with a 48-hour latency between impacts. Due to these differences, the model used in this thesis must first be validated. The inconsistencies between injury paradigms argue a limited ability to contextualize one study's findings with the results from a separate study; however, the inherent differences between injury models likely allow a better collective

representation of the heterogeneity observed with rmTBI. Translatability to the diverse nature of human rmTBI is thus supported by validation of the induction of persistent deficits produced by the injury paradigm used in this thesis. Henceforth, the findings of this study will be discussed within the context of TBI severity and rmTBI as opposed to the differences between rmTBI paradigms.

Cellular senescence has recently been proposed as a pathophysiological consequence of rmTBI that may contribute to persistent cognitive deficits.[8, 166, 167, 169, 216] The complex and diverse nature of cellular senescence has prevented the identification of a single definitive marker and necessitates the use of multiple molecular identifiers to evaluate senescence levels in tissue homogenate; however, the presence of multiple senescence markers merely suggests that senescence is occurring, as these alterations can manifest in other cellular processes.[106, 126, 128] Investigators must therefore use caution when making definitive claims about the occurrence of cellular senescence and generally use three to four markers to increase confidence in senescence detection. This study aims to further increase confidence in evaluating hippocampal cellular senescence by analyzing the senescence-associated alterations of five protein and six gene expression markers. The markers discussed throughout this thesis are among the most widely recognized identifiers of senescence-associated cellular processes including cell cycle arrest, SASP production, and epigenetic alterations.

The investigation of cellular senescence in the context of rmTBI has only been evaluated at acute and chronic time points following rmTBI.[8, 166, 168, 169, 216] An acute resolution of various gene expression and protein senescence-associated markers by 14 DPI has been established with chronic increases in senescence. Though understanding the acute molecular sequelae and chronic changes following rmTBI is undoubtedly important, determining the

ramifications of acute events as they transition from acute to chronic alterations at both the physiological and functional levels would allow the study and development of targeted therapies to mitigate persistent rmTBI symptomology. It has been shown that senescence-associated markers in the hippocampus peak seven days post-injury and resolves by 14 days following rmTBI.[8, 168] Given the physiological role of the SASP in tissue repair-induced senescence, this transient induction is likely an attempt to recruit immune and glial cells to the site of injury followed by subsequent clearance of these pro-inflammatory senescent cells to undetectable levels through conventional methods of molecular interrogation.[106, 157] A subset of these senescent cells may become pathological by avoiding immune clearance, slowly inducing senescence in surrounding cells via continued SASP and production over an extended period. However, the minuscule fraction of brain cells undergoing senescence poses a problem when attempting to quantify cellular senescence in whole hippocampal homogenate.[155] Alterations in senescence-associated markers are therefore expected to be relatively small. Despite this consideration, this thesis demonstrates for the first time that a molecular progression occurs between approximately one- and two months following rmTBI, suggesting the hippocampal reinduction of cellular senescence. These findings corroborate chronic studies in which post-mortem analysis of human brains with a history of rmTBI showed increases in senescence-associate molecular alterations when compared to age-matched controls, along with persistent DNA damage, a known cause and characteristic of cellular senescence.[169] Furthermore, the evidence of persistent DNA damage in post-mortem human brains is supported by the results of this thesis as demonstrated by increases in the DNA damage- and cell-cycle arrest-associated markers discussed below.[169]

DNA is assessed for damage at two checkpoints during the cell cycle to ensure the proper replication of genetic material. These checkpoints occur at the G_1/S-phase and G_2/M-phase transitions, and the cell cycle is arrested at either of these checkpoints depending on when the DNA damage is detected by the cell.[118] The p53 protein is continually produced and subsequently degraded during the cell cycle.[119] However, this constitutive expression allows for the rapid increase of p53 protein in the canonical response to DNA damage and cellular insult.[222] p53 then acts as a protein binding partner and transcription factor to initiate the DNA damage response and halt cell cycle progression, leading to cellular senescence under conditions of persistent DNA damage.[102, 122, 130, 137, 222, 223] Senescence-associated cell cycle arrest is mediated by the CDK inhibitors $p21^{WAF1/CIP1}$ and $p16^{INK4a}$, which comprise the p53-$p21^{WAF1/CIP1}$ and $p16^{INK4a}$ axes, respectively.[126] p53 directly induces $p21^{WAF1/CIP1}$ transcription upon the detection of DNA damage in the p53-$p21^{WAF1/CIP1}$ axis.[124, 125] The results of this thesis demonstrate for the first time that p53 protein quantity is significantly increased and $p21^{WAF1/CIP1}$ gene expression increases between approximately one- and two months following rmTBI. These exciting findings corroborate the known mechanism by which the p53-$p21^{WAF1/CIP1}$ axis induces senescence.[124, 125] However, future studies utilizing techniques such as chromatin immunoprecipitation sequencing (ChIP-Seq) must be performed to establish the p53 induction of $p21^{WAF1/CIP1}$ gene expression under these experimental conditions. Relative protein quantity analysis found no increase in $p21^{WAF1/CIP1}$ protein, though protein increases may lag behind initial gene upregulation which would further support the initial activation of the p53-$p21^{WAF1/CIP1}$ axis.[124, 125] Interestingly, a significant decrease in p53 gene expression was observed over the course of this study. Because the p53 protein is no longer being rapidly degraded and is significantly increased, transcription of p53 is likely reduced through negative

feedback inhibition. This suggested mechanism is supported by a study in which the p53 protein was found to tightly bind to the 5'-untranslated region (UTR) of its mRNA, resulting in p53 mRNA degradation.[224] Cumulatively, the findings from this study suggest the initiation of cellular senescence occurring between approximately one- to two months following rmTBI through the p53-$p21^{WAF1/CIP1}$ axis.

An independent mechanism by which cellular senescence can be induced is through the activation of the $p16^{INK4a}$ axis; however, this pathway can also be activated by the p53-$p21^{WAF1/CIP1}$ axis.[130, 131] The $p16^{INK4a}$ axis has thus been associated with the maintenance of stress-induced cellular senescence regardless of whether it is activated by the p53-$p21^{WAF1/CIP1}$ axis.[128] Slight but proportional increases in both $p16^{INK4a}$ protein and mRNA expression were observed but did not reach significance. These findings suggest that $p16^{INK4a}$-mediated maintenance of cellular senescence may begin between approximately one- and two months post-rmTBI. This claim is supported by the known temporal activation of the p53-$p21^{WAF1/CIP1}$ and $p16^{INK4a}$ axes in the induction and maintenance of cellular senescence, respectively.[128] The p53, $p21^{WAF1/CIP1}$, and $p16^{INK4a}$ data presented in this thesis suggest for the first time that senescence-associated cell cycle inhibitory mechanisms are upregulated to detectable levels between one- and two months following rmTBI.

The SASP is a critical component of cellular senescence.[134] This release of pro-inflammatory factors recruits immune and glial cells to the sight of tissue injury and promotes the clearance of senescent cells.[134] It is believed that senescent cells become detrimental when they escape immune clearance and continue releasing the pro-inflammatory SASP into the extracellular milieu where paracrine factors induce senescence in surrounding cells while autocrine factors contribute to the maintenance of cellular senescence.[135] One mechanism by

which SASP regulation occurs is through chromatin accessibility and epigenetic modification.[130, 136, 146, 153] Two epigenetic modifiers, HMGB1 and SIRT1, have been shown to repress SASP transcription by maintaining SASP genes in a heterochromatic state.[136, 137, 146, 225] Downregulation of HMGB1 and SIRT1 gene expression is associated with cellular senescence; however, the fate of their protein products differs.[145-147, 225] SIRT1 protein has been demonstrated to undergo degradation through selective autophagy during senescence, whereas the HMGB1 protein is exported from the nucleus and subsequently released into the extracellular environment in a p53-dependent manner.[136, 147] The molecular analyses performed in this thesis utilized whole ipsilateral hippocampal homogenate and prevented the quantification of nuclear or extracellular HMGB1 protein release. Consequentially, the gene expression for both HMGB1 and SIRT1 was assessed and only the SIRT1 protein quantity was analyzed. This study found a marked reduction in the gene expression of both HMGB1 and SIRT1 between approximately one- and two months post rmTBI, suggesting derepression of SASP transcription.[136, 146] The reduction in SIRT1 gene expression is corroborated by a near significant reduction in SIRT1 protein and the significant increase in p53 protein, as p53 is known to directly bind and suppress SIRT1 transcription.[150] The downregulation of HMGB1 gene expression that occurs in senescent cells is corroborated by the findings of this study.[225] Furthermore, the increased gene expression of $p21^{WAF1/CIP1}$ is corroborated by the HMGB1 gene expression findings since HMGB1 gene silencing results in $p21^{WAF1/CIP1}$ gene upregulation.[225] The exciting work presented in this thesis demonstrates for the first time that negative regulators of the SASP are significantly reduced between one- and two months following rmTBI.

Lamin B1 acts as a structural protein that maintains the peripheral orientation of senescence-associated genes within the nucleus and contributes to gene repression.[151] Spatial

reorganization of chromatin through the autophagic degradation of Lamin B1 has been associated with cellular senescence.[152, 153] This study used relative protein quantification to assess if the senescence-associated spatial reorganization of chromatin occurs between approximately one- to two months after rmTBI, and no change was observed. These findings contradict other senescence studies because there is usually a marked decrease in Lamin B1 protein when cellular senescence occurs.[153] One possible explanation for no change is that Lamin B1 may not be degraded during the process of senescence initiation as suggested by the previously discussed data. A more plausible rationale is that because senescence is such a complex cellular process, it is highly improbable that senescence-associated molecular alterations will be observed with every marker investigated.

Studies have demonstrated that the use of testosterone as an acute treatment for moderate to severe TBI results in functional cognitive improvement and mitigation of factors known to induce senescence, like oxidative stress and neuroinflammation.[9, 10, 170] However, no studies to date have examined the effects of administering testosterone at subacute time points on cognitive deficits and cellular senescence following rmTBI. The investigations in this thesis are the first of their kind to examine the effects of systemic testosterone administration approximately one-month post-injury on cognition and senescence-associated molecular markers out to two months following rmTBI. The innovative design of this study examines the cognitive and molecular ramifications of rmTBI from a perspective clinically relevant to persistent symptomology and treatment beyond acute time points. The results of this thesis are the first to demonstrate that persistent deficits in recognition memory can be mitigated when testosterone is administered approximately one-month following rmTBI by utilizing NOR testing. These findings not only support the usage of testosterone to treat persistent cognitive deficits but also

successfully demonstrate that persistent symptoms can be targeted at time points distal to the injury.

Testosterone has been shown to mitigate metabolic dysfunction, oxidative stress, and neuroinflammation when administered at acute time points following severe TBI, effectively reducing factors that can induce senescence.[10] This study is the first of its kind to investigate the delivery of systemic testosterone approximately one-month following rmTBI and analyze senescence-associated molecular alterations at two months post-injury. The data presented in this thesis is the first to demonstrate that subacute testosterone treatment reduces the senescence-associated cell cycle arrest markers, p53 and $p16^{INK4a}$/$p19^{ARF}$. A marked decrease was observed in p53 protein quantity at PTD 28 (63 DPI); however, this trend did not reach statistical significance. No difference in p53 gene expression was observed, suggesting that the decrease in p53 may result from the disinhibition of p53 degradatory mechanisms.[119, 226] These findings corroborate other studies indicating that testosterone ameliorates and protects against senescence-inducing insults; however, these data refute other studies that indicate activation of the androgen receptor can drive cellular senescence.[11, 12, 171, 227] However, limited literature exists discussing the presence of androgen and estrogen response elements within the promoter regions of the senescence-associated markers evaluated in this thesis. Therefore, it is likely that testosterone may be acting through noncanonical pathways to induce changes and may contribute to the limited therapeutic potential of subacute testosterone treatment. Significant downregulation of $p16^{INK4a}$ gene expression was observed at PTD 28 (63 DPI), whereas no difference was observed in $p16^{INK4a}$ protein levels. The expected decrease in p16INK4a protein may be delayed relative to the downregulation of $p16^{INK4a}$ gene expression. An alternative hypothesis relies on the specificity of the *Cdkn2a* primer used for RT-qPCR. Because the

Cdkn2a gene encodes both $p16^{INK4a}$ and $p19^{ARF}$, the primer may be unable to distinguish between the two transcriptional products.[127] It therefore must be considered that $p16^{INK4a}$ protein levels remain high and the detected decrease in *Cdkn2a* expression corresponds with a decrease in $p19^{ARF}$ protein levels. This decrease in $p19^{ARF}$ protein would disinhibit the Mdm2-mediated degradation of p53, corroborating the p53 decrease observed in this study.[121] However, further research is required to elucidate the mechanism by which testosterone lowers p53 protein and *Cdkn2a* gene expression. Though no detectable differences in the other senescence-associated molecular markers were observed in this study, this thesis demonstrates for the first time that subacute testosterone treatment can alter the cellular composition of senescence-associated biomolecules following rmTBI. The exciting findings presented in this work indicate for the first time that, when administered approximately one-month post-rmTBI, testosterone improves recognition memory deficits and alters cell cycle arrest-associated cellular processes. This work encourages further investigation of subacute pharmacological intervention to improve the quality of life for individuals suffering the long-term ramifications of rmTBI.

Post-mortem studies of human brains with a history of rmTBI indicate increased cellular senescence and DNA damage relative to age-matched individuals with no rmTBI history.[169] The work in this thesis presents a preliminary study using immunohistochemical investigation to evaluate senescence levels at a chronic time point following 5-hit closed-head rmTBI. Preliminary work suggests that an increase in the senescence-associated marker, $p16^{INK4a}$, remains in the dentate gyrus and CA3 regions of the hippocampus approximately six months following rmTBI. These findings corroborate the post-mortem human brain work conducted by Schwab et. al.[169] However, as demonstrated by Tominaga et. al., senescence-associated molecular alterations may differ between cell types following rmTBI and are corroborated by the

preliminary IHC of $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ presented in this thesis.[168] The presence and localization of the $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ appear to differ between cell types and between injured and naïve hippocampi. For instance, $p21^{WAF1/CIP1}$ was observed to increase in astrocytes following rmTBI, whereas no $p21^{WAF1/CIP1}$ was detected in microglia. These preliminary data inform future investigations by necessitating the characterization of cell type-specific responses to rmTBI. The characterization of rmTBI-induced cellular senescence via single-cell approaches would allow the differentiation between cell populations undergoing primary senescence and those recruited into senescence through paracrine SASP signaling. By identifying primary senescent cells, investigators may be able to determine the mechanisms by which senescent cells evade immune clearance and become pathological, allowing targeted pharmacological intervention to clear these cells and prevent senescence induction in surrounding tissue.

The experiments conducted in this thesis are the first to evaluate hippocampal cellular senescence as a potential cellular and molecular mechanism underlying persistent cognitive deficits between one- and two months following rmTBI. This thesis validates the use of a 5-hit closed-head rmTBI rat model in the investigation of persistent rmTBI-induced recognition memory deficits. Proteomic and transcriptional profiling of the whole ipsilateral hippocampus indicates that rmTBI results in a progressive increase in senescence-associated molecular alterations that are suggestive of cellular senescence induction between one- and two months post-injury. Testosterone demonstrates a mitigation of persistent recognition memory deficits and a reduction in senescence-associated cell cycle arrest markers when administered approximately one-month following rmTBI. Preliminary immunohistochemical investigation suggests a chronic increase in hippocampal cellular senescence and a need to investigate the induction of senescence following rmTBI at the single-cell level. In summary, the findings

presented throughout this thesis increase our understanding of cellular senescence as a possible mechanism by which rmTBI symptomology persists and provide further evidence that subacute testosterone administration is an effective therapeutic intervention for enduring symptoms.

CHAPTER NINE

EXPERIMENTAL LIMITATIONS

The most apparent limitation of this study is the ability to deduce cellular senescence using molecular markers. The presence of a single senescence-associated molecular alteration is not sufficient to claim that senescence is occurring because cellular senescence is such a complex cellular response. This study uses a multitude of senescence-associated gene expression and protein markers to identify senescence more confidently; however, the presence of these senescence-associated alterations simply suggests that senescence-like changes are occurring. A multi-targeted probe approach is an optimal method to identify potential cellular senescence since no senescence-specific markers have been identified. This study will more confidently suggest whether molecular alterations occurring following rmTBI within the hippocampus are senescence-related by utilizing numerous senescence-associate markers. Additionally, the type of cellular senescence induced by rmTBI will be difficult to identify in this study due to the complexity of cellular senescence, its nature of propagation, and the multitude of molecular pathways that can induce senescence. This last limitation presents an obstacle to targeted therapy development, as the pathways involved in the different forms of senescence show similarities yet are distinct from each other in certain respects. Furthermore, the accumulation of senescent cells within the brain is a gradual process and it is possible that senescence-associated molecular alterations may not reach detectable levels by the chosen time points. To account for this confounding factor, future studies should characterize progressive senescence-associated

alterations beyond 63 DPI. An alternative option is to use a senescence-accelerated rat line, such as the OXYS rats, in hopes that rmTBI-induced changes would be accelerated, thus shortening the timeline of the overall study. It is possible that extending the course of this study or utilizing a senescence-accelerated rat model would allow an increased number of senescence-associated changes to become detectable, allowing a more confident determination that cellular senescence increases following rmTBI.

Utilizing whole hippocampal homogenate to perform gene expression and protein analysis presents another limitation to this study. By using whole ipsilateral hippocampal homogenate, the ability to detect hippocampal region and cell-specific alterations in the hippocampus is lost. Furthermore, small alterations in senescence utilizing the whole hippocampal homogenate approach may be difficult to identify since such a small proportion of cells undergo senescence. However, findings from this study would provide the foundation to conduct a more intensive single-cell analysis of cellular response to rmTBI in the future. Certain populations of cells are likely to respond to injury before others or these cells may respond differentially since the secondary injury associated with TBI is a propagating molecular cascade. The highly context-dependent nature of cellular senescence may further complicate findings with the possibility that certain populations of cells may undergo senescence while others retain normal function. Progressive analysis should be conducted on a single-cell level to characterize which cells are initially undergoing senescence so that those cells might be targeted to prevent the propagation of senescence through the senescence-associated secretory phenotype. To optimally evaluate the temporal response of cell types to rmTBI, tissue should be collected at multiple time points following injury, enzymatically dissociated, and cells should be sorted by type through methods such as fluorescent-activated cell sorting. Once major cell types have been

separated, cell subtypes should be isolated using a similar method. After cell subtype enrichment, single-cell transcriptomic, proteomic, and epigenomic approaches would allow the identification of senescent cells with high confidence. A single-cell analysis would be beyond the scope of a master's thesis due to the large volume of data that would need to be analyzed.

The sample size used in this study presents another experimental limitation. Despite the power analysis determining that 6 animals from each group, at each time point, would be sufficient for statistical analysis of molecular alterations, there is inevitable variability in the response of individual animals to rmTBI given that this model mimics the diverse nature of rmTBI observed in the human population. Furthermore, when studying cellular senescence, one must consider that an extremely small fraction of cells within the brain are truly senescent. Though statistical significance was only found in a few senescence-associated markers, the findings of this study provide insight into the recommended sample size should the molecular portion of this study be repeated. For instance, the suggested sample size increases to approximately 35 animals per group due to the low effect size and high variability between animals when examining the progressive differences in $p16^{INK4a}$ gene expression and protein data collected from whole ipsilateral hippocampal tissue. Though the use of this many animals is feasible, it suggests that there may be a better approach when investigating senescence-associated alterations as previously outlined by the proposed single-cell omics study.

A perceived limitation to this study is inconsistencies between each hit and between each animal since the injuries were not delivered using a stereotaxic animal restraint apparatus such as ear bars nor was a consistent impact site determined relative to Bregma. However, the inconsistencies between injuries allow for a more accurate representation of rmTBI clinical manifestation because the likelihood of an individual experiencing multiple mTBIs in the same

location is highly improbable. Furthermore, the impact inconsistencies are expected to better represent the diffuse nature of rmTBI. Inconsistencies in behavioral data may arise due to various investigators of different sexes conducting the assays.[228, 229] Unfortunately, behavioral assays were conducted during the COVID-19 pandemic, and the sex of the individual conducting the behavioral assays was unable to be controlled. Future behavioral assessment should ideally be conducted by a single investigator that remains consistent through repeated testing periods.

CHAPTER TEN

CONCLUSIONS

The aim of this body of work was multifaceted. First, it was necessary to establish a rat model of persistent cognitive deficits following 5-hit closed-head rmTBI and determine if subacute testosterone treatment influences recognition memory post-injury. The successful demonstration that our model induces persistent deficits in recognition memory justifies the use of this model in future studies evaluating the effects of rmTBI on persistent cognitive deficits. The exciting and novel findings indicating that subacute testosterone administration mitigates recognition memory deficits support the notion that pharmacological intervention to effectively treat persistent symptoms following rmTBI is possible.

This work next aimed to determine if progressive rmTBI-induced cellular senescence-associated molecular alterations occur approximately one to two months following injury in the hippocampus. This aim was investigated through molecular interrogation of ipsilateral hippocampal tissue by analyzing transcriptional and proteomic profiles of numerous senescence-associated biomolecules. Though not all biomolecules analyzed exhibited alterations expected with increased cellular senescence, these data indicate that detectable molecular alterations corresponding with the onset of cellular senescence progressively increase between one and two months following rmTBI in the hippocampus. These novel findings demonstrate for the first time that acute resolution of senescence-associated molecular profiles observed in other studies transitions toward a subacute reinduction of these markers, potentially contributing to

pathological senescence demonstrated chronically following rmTBI and in various neurodegenerative diseases. The surprising findings that delayed administration of testosterone reduced biomolecules associated with cell cycle arrest and cellular senescence provide evidence that senescence-associated molecular alterations can be targeted to potentially mitigate the progression of cellular senescence observed following rmTBI and in neurodegenerative diseases. Collectively, these findings challenge the traditional paradigm of acute therapeutic intervention following mTBI and provide hope for individuals suffering from the long-term cognitive ramifications of rmTBI. This work establishes a clinically translatable rmTBI model of persistent cognitive deficits, allowing this thesis to interrogate the effects of delayed testosterone treatment on persistent cognitive deficits and their potential underlying cellular and molecular mechanisms following rmTBI. This clinically translatable work encourages novel studies to elucidate the cellular and molecular mechanisms underlying long-term symptoms that diminish the quality of life for those affected by rmTBI.

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