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Lujain Almuhanna

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Traumatic brain injuries (TBIs) are a common form of head injury, with mild TBIs (mTBIs) making up 75-90% of the TBI severity scale. It’s been suggested that repetitive mild TBIs (rmTBIs) may enhance effects of Alzheimer’s Disease (AD). With dementia linking TBI and AD together, we investigated the memory hub of the brain - the hippocampus. Astrocytes and microglia are two glial cells that respond to neuroinflammation and therefore imaged in hippocampal regions of the 3xTg-AD mouse brain. We hypothesized that AD brain pathology (i.e., neuroinflammation) would be accelerated in 3xTg-AD mice following rmTBIs early in life. Analyses revealed that rmTBIs didn’t enhance the extent of gliosis in AD mice. We conclude that an increased risk of neurodegeneration is not a predetermined outcome from rmTBIs early in life. A future investigation of the expression of specific receptors (e.g., TREM2) and neuronal loss can better illustrate the role of glial cells.

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Traumatic brain injuries (TBIs) are a common form of head injury, with mild TBIs (mTBIs) making up 75-90% of the TBI severity scale. It's been suggested that repetitive mild TBIs (rmTBIs) may enhance effects of Alzheimer's Disease (AD). With dementia linking TBI and AD together, we investigated the memory hub of the brain - the hippocampus. Astrocytes and microglia are two glial cells that respond to neuroinflammation and therefore imaged in hippocampal regions of the 3xTg-AD mouse brain. We hypothesized that AD brain pathology (i.e., neuroinflammation) would be accelerated in 3xTg-AD mice following rmTBIs early in life. Analyses revealed that rmTBIs didn't enhance the extent of gliosis in AD mice. We conclude that an increased risk of neurodegeneration is not a predetermined outcome from rmTBIs early in life. A future investigation of the expression of specific receptors (e.g., TREM2) and neuronal loss can better illustrate the role of glial cells.

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Chapter One: Introduction

1.1 Neurodegenerative Disease and Aging

Clinical literature has long established that with increasing age comes cognitive impairments. The severity of the decline depends on health, genetics, head injuries, and other biological factors (Barrientos et al., 2015). Age-related neurodegenerative diseases affect the central nervous system (CNS) due to proteins misfolding, causing aggregation and disease-specific defects in protein synthesis. Cellular mechanisms that change with age are linked with increases in mitochondrial dysfunction and reactive oxygen species (ROS) in the brain. A decline in repair mechanisms and antioxidant neutralization promotes the accumulation of oxidative damage and yield age-related neurodegenerative disorders (Hung et al., 2010).

Progressive age-related cerebral atrophy has neurodegenerative hallmark features that include weak rates of neuronal death replacement via loss of neuroprogenitors, ventricular enlargement, volume loss, hippocampal atrophy, and an overall reduction in gray and white matter (Rigby Dames et al., 2023). While these symptoms are mostly associated with pathological changes relative to disease, some of these changes are also associated with typical aging, such as volume loss and ventricular enlargement (Anderton, 2002). Although cognitive loss is a normal part of healthy aging, there are intrinsic physiological key regulators that preserve and promote synaptic plasticity and neurogenesis in the healthy brain (Dahan et al., 2020). An unimpaired mitochondrial

metabolism is a natural rescuer that improves adult neurogenesis by proliferating progenitor cells, synthesizing cellular building blocks, and generating reactive oxygen species that signaling pathways can utilize in a redox-dependent dynamic (Beckervordersandforth, 2017). Furthermore, a human brain can harness young immature neurons preserved in areas such as the dentate gyrus to arrest the damages caused from aging (Dahan et al., 2020). Temporal regions of the brain, such as the hippocampus, are responsible for harboring memory and cognitive pathways; the gradual disease-driven deterioration of these regions is what causes memory deficits and shrinkage, leading to the accumulation of intracellular aggregates such as tangled tau protein (Rigby Dames et al., 2023).

1.2 Role of Traumatic Brain injuries (TBI) in Alzheimer's Disease (AD) Development

Traumatic brain injuries (TBIs) and AD are two neurological conditions that, with age, can trigger neuroinflammatory pathways, ultimately disrupting brain function. Alzheimer's disease (AD) affects over 10% of the elderly population worldwide, making it the most common cause of dementia (Zysk et al., 2019). AD is a condition whose severity and progression depend on variables such as aging, head injury, environment, and genetic mutations of the amyloid precursor protein (APP), presenilin (PSEN) genes, and p-Tau (tau). In an AD patient, the combination of these factors results in the gradual accumulation of beta-amyloid protein fragments that eventually form senile plaques, and p-Tau proteins coalesce to form neurofibrillary tangles (NFT), which can extensively spread into memory-dependent regions of the brain such as the hippocampus (Armstrong,

2013). Multiple theories as to what exacerbates the pathological features of AD have been proposed, with one factor being TBIs.

TBIs can be categorized in a varying scale ranging from mild to severe. Mild traumatic brain injuries (mTBIs) are traditionally classified as blunt head traumas, such as concussions. It has been reported as the most common type of TBI diagnosis, making up 75%-90% of total TBI cases due to sports, wars, transportation, and fall-related injuries (Hiskens et al., 2023). Repeated events of mTBIs have been acutely studied and assumed to exacerbate neuroinflammatory pathways in the brain. As a result, inflammatory sites activate prominent markers associated with neurodegenerative disorders such as AD, including reactive astrocytes and microglia (Hiskens et al., 2023). Axonal injuries are a form of such damages that occur with repetitive mild traumatic brain injuries (rmTBIs), which potentially contribute to the neurodegenerative pathological processes underlying AD due to synaptic dysfunction (Blennow et al., 2016).

It is important to note that normal aging is accompanied by a natural decline in cognitive function, where individuals biologically inherit a non-pathological or pathological profile associated with cognitive impairment. TBIs can accelerate brain atrophies in a way that prematurely manifests age-associated neurodegenerative conditions. Consequently, this may lead to an increase in “brain age”, suggesting a potential acceleration of age-related neurodegenerative processes. Although the extent of this effect depends on the severity of the trauma, it does not entirely dismiss the possibility of such outcomes for individuals with a history of rmTBIs (Cole et al., 2015).

A study conducted by the Natural Health Research Institute revealed that TBI and AD are possibly interlinked, with TBI being a risk factor for early onset AD symptoms such as dementia. The participants involved had a confirmed diagnosis of AD revealed in autopsies, while the control group had no history of head injury. Results confirmed that the average age of symptom onset and diagnosis of dementia due to AD was 3.6 years earlier ($p < 0.001$) for TBI + patients with loss of consciousness compared to TBI- patients (NHRI, 2019). These results, along with countless studies have indicated a correlation between TBIs and AD, suggesting that TBIs may play a role as a catalyst for AD symptoms.

1.3 Pathological Processes Involved in TBI that increase risk for developing AD

Thus far, studies support the idea that the multidirectional pathophysiological mechanisms that TBI induces may increase the advancement and acceleration of neurodegenerative diseases such as AD; however, this process has not been heavily investigated. The initial biochemical and molecular damages that manifest from any form of brain injury include neuronal cell overstimulation, glutamate excitotoxicity, and damage in the blood-brain barrier (BBB). Overstimulated neuronal cells cause a calcium influx that later manifests into DNA damage due to perturbed mitochondrial functions and free radicals. Subsequently, neuroinflammation and apoptosis caused by tissue and DNA damage leads to neurodegeneration. Glutamate excitotoxicity and calcium influx can cause axonal stress due to fluid build-up and the accumulation of inflammatory proteins (Gupta & Sen, 2015). As a result, axonal preservation becomes jeopardized due to a loss of myelin. Animal models have revealed that the disruption of neuronal

networks in impacted areas later progresses to behavioral abnormalities and memory loss (Lu et al., 2024).

Axonal damage is a key feature that associates TBI with AD. Axonal focal swelling has been linked to impaired transport of essential proteins that mediate neuronal function and health (Sivanandam & Thakur, 2012). This has been concluded due to changes observed in cytoskeletal structures post TBI, inducing a series of neuroinflammatory receptors, proteins, and caspases (Gupta & Sen, 2015). APP is cleaved into certain peptides, such as Amyloid-β (Aβ), at the cell membrane and is internalized by cell receptors. The accumulation of Aβ aggregates reaches organelles such as the mitochondria and begins impairing complex functions due to abnormal interactions (Asik et al., 2021).

Secondary neuronal injuries of neurodegenerative disorders and TBI are both accompanied with inflammatory mediators. Because there is an increase in calcium influx and Aβ accumulation, inflammatory responses are activated due to the production of free radicals and reactive oxygen species (ROS). A disturbed blood-brain barrier immediately activates a microglial response, which are fundamental immune cells in the CNS. The release of pro-inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), and IL-6 are all mediated by the dramatic activation of microglia (Gupta & Sen, 2015). These cytokines are also expressed in the pathogenesis of AD, making it possible for such responses to accelerate the effects of AD pathology. Such cascades mediated by active microglia can have dual effects. On one end, the persistence and prolonged period of microglial activation triggers the exudation of pro-inflammatory cytokines and

upregulation of several inflammatory cascades, resulting in tissue damage and neurodegenerative progression. On the other end, microglia work to reduce and phagocytose Aβ deposits to prevent plaque formation through the expression of certain anti-inflammatory properties (Wang et al., 2015). Microglial activation responds to brain injury by increasing levels of anti-inflammatory cytokines, IL-10 and TGF-β. This response induces necessary cytokines with pro-phagocytic properties that protect existing neurons, mediate neuronal regeneration, and reverse the effects of Aβ species in the beginning stages of disease. Anti-inflammatory microglial proteins associate with such species to assist in β-amyloid plaque clearance. However, microglial activation upregulates several inflammatory responses and mediators, such as APP and the pro-inflammatory cytokines mentioned, as a response to the Aβ deposition cycle and tau accumulation. The persistence and long-term functional abnormalities of such pathological pathways result in chronic neuropathology (Gupta & Sen, 2015).

1.4 Neuroinflammatory biomarkers: Astrocytes and Microglia in the Hippocampus

Neuronal insults that include effects of AD pathogenesis are considered to jeopardize necessary metabolic functions and cause oxidative and cellular stress due to neuroinflammation (De Felice and Lourenco, 2014). This outcome results from axonal swelling and dysfunction, causing protein aggregation and neurotransmission deficits (Sivanandam & Thakur, 2012). Studies suggest that rmTBIs may cause the progression of cognitive dysfunction resulting from axonal impairment, a hallmark associated with age-driven neurodegeneration (Liu et al., 2024). Research has shown that lesions and metabolic deficiencies extend to the hippocampus of animal models. The framework that

follows the detection of injured sites in the hippocampus is comprised of residential glial cells, such as astrocytes and microglia, found in the CNS that activate and induce a plethora of inflammatory responses that recruit to those sites to restore neuronal integrity (Batlle et al., 2015).

The hippocampus is a structure found in the temporal lobe of the brain that's responsible for memory and spatial learning; the systemic complexity that goes into the crosstalk between neurons and other cells makes it crucial for trafficking systems and homeostatic conditions to remain at sustained and regulated (Barrientos et al., 2015). This map is comprised of uniquely positioned regions that represent specific roles when contributing to the patterns of synaptic networks that make up recognition and memory uptake: the dentate gyrus (DG) region, and the CA1 and CA3 fields. Animal model research has implicated that the CA3 region of the hippocampus is involved in pattern recognition, the CA1 region is involved in environmental dynamics, and the DG collaborates with CA3 for spatial sensory input (Guzowski et al., 2004). It is suggested that the neuronal density of CA1 and CA3 regions of the brain make them highly sensitive to glial activation and plaque formation, but this association is indefinite (Ugolini et al., 2018). Glial cell abundance and activation in the different hippocampal regions will be explored later in this paper to determine which fields are most affected when subjected a rmTBI on a background of genetic AD.

1.4.1 Glial Fibrillary Acidic Protein (GFAP) Biomarker for Astrocyte Activation

Astrocytes are glial cells in the CNS that aid in neuronal support and transmission using pre- and post-synaptic nerve terminals. Because of their synaptic plasticity, they

play a crucial role in regulating CNS and cognitive processes, protecting the integrity of the BBB, and enhancing memory via gap junctions (Cragnolini et al., 2018). When subjected to CNS damage and immune cell activation from inflammatory threats or pathology, astrocytes overexpress glial fibrillary acidic proteins (GFAP). GFAP is a cytoskeletal protein and a downstream biomarker of astrogliosis that becomes upregulated in response to dysfunctional synapses. This structural marker protein can detect the morphology of astrocytes, which acquire branching and scaffolding properties that embody neuronal network and trafficking integrities. This neurotransmission feature is especially important when responding to inflammation. Astrocytes modulate inflammation by cooperating with other glial cells, making them reactive vehicles for pro- and anti-inflammatory intermediaries (Jurga et al., 2021). In normal conditions, GFAP expression is reduced when hippocampal neurogenesis increases; subsequently, active gliosis decreases due to a sustained environment (Bettcher et al., 2021). However, when immune cells are directed to sites of inflammation or injury in the brain, cytokines are released which activate pro- and anti-inflammatory pathways. This feedback loop consists of the constant release of proteins and stimulates prolonged astrocyte activation and GFAP upregulation, ultimately changing the morphology and gene expression of astrocytes in response to stress (Cekanaviciute & Buckwalter, 2016).

Astrocytes and microglia upregulate specific proteins to protect cells against neuronal insults in several areas of the brain, such as the hippocampus. In patients with AD, a calcium binding protein called S100B appeared to be largely expressed and released by astrocytes (Reeves et al., 1994). In vitro studies have shown that S100B

maintains homeostasis levels, cell metabolism, growth, and structure (Zimmer et al., 1995). Therefore, this protein is released in large amounts in response to brain damage. The long-term potency in the brain causes an exacerbation in inflammatory response due to persistent astrocyte and microglia activation (Rao et al., 2022). When it comes to AD, the damage imposed on the CNS is due to prolonged effects of abnormal protein accumulation and aggregation. As a response, astrogliosis is more prevalent due to astrocytes conforming into a more reactive state to increase expression in neuronal support, homeostatic, and metabolic maintenance to ensure neuronal survival (Kamphuis et al., 2012). The brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5) are significant proteins that promote the survival of neuronal cells (Rao et al., 2022). The signaling uptake of such proteins are typically dependent on their ability to interact with their respective receptors found in astrocytes (Cragnolini et al., 2018). The expressions of such genes exist in regions of the brain responsible for memory and cognition: the cortex, the hippocampus, and basal forebrain regions. While the relationship between the neuotrophin family and astrocyte activation is not properly understood, a study conducted at the National University of Singapore measured BDNF serum levels in both AD and mild cognitive impairment (MCI) subjects in comparison to healthy controls to better understand the role of neurotrophins when operating in a neuro-compromised environment. They found that patients with AD showcased lower levels of BDNF than other subjects in the study, suggesting that the extensive neuronal damages were not able to maintain the effect of neuroprotective factors in the brain (Ng et al., 2018).

Changes in synaptic plasticity and neurotransmission deficits that occur in the hippocampus are signs of chronic neuroinflammation that are associated with neurodegenerative disorders (Mathias et al., 2004). Although studies have shown that animal models with administered rmTBIs exhibit hallmark cognitive impairment behaviors, such as memory deficits, this association suggests that astrogliosis may be an accelerator of dementia for those with a history of TBIs. However, this has not been investigated on a cellular level (Aungst et al., 2014).

1.4.2 Ionized Calcium-binding adaptor Molecule (Iba1) Biomarker for Microglia Activation

Microglia are cells in the CNS that are part of the innate immune response, making them the first responders against neuronal insults. These cells undergo and adapt to morphological changes when a threat is detected, signaling their activation (Szalay et al., 2016). They play a huge role in synaptic development and plasticity in the healthy brain by protecting extracellular spaces through phagocytosis (Calcia et al., 2016). Microglia preserve neuronal conditions through continuous crosstalk with neurons; their neurotransmitter receptors make this stimulation possible. This type of stimulation allows microglia to signal the release and activation of other molecules and mediators to sustain homeostasis (Marinelli et al., 2019).

While active microglia serve to protect the brain, persistent activation can lead to irreversible damage in the CNS. Prolonged activation can result from the consequence of neuroinflammation; microglia respond to tissue damage by increasing rates of proliferation through the release of cytokines and chemokines that can later contribute to

neurodegenerative diseases with age (Muzio et al., 2021). The production and release of pro- and anti-inflammatory responses are induced by microglia activation through the downstream activation of toll-like receptors (TLRs) pathways. TLRs are extracellular transmembrane receptors on microglial cells that detect pathogens-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Piccinini and Midwood, 2010). Tissue damage and inflammation trigger the neuronal release of molecules like PAMPs or DAMPs, which are recognized by pattern recognition receptors (PRR) such as TLRs on microglia immune cells; their engagement with such receptors mediates downstream effects that are associated with neurodegenerative mechanisms, such as the production of pro-inflammatory cytokines and type I interferons (IFN-I) (Thundyil and Lim, 2015).

In the beginning stages of brain-related disease, microglia adapt a classic activation profile (M2). This phenotype is associated with the release and production of anti-inflammatory cytokines (IL-4, IL-10, and IL-13), and neurotrophic factor BDNF. Macrophages in the M2 phenotype are the first to be directed to sites of injury and inflammation to restore homeostasis and utilize anti-inflammatory cytokines for the repairing process (Tang and Le, 2016). The conversion of M2 microglia to M1 microglia, cells that produce pro-inflammatory cytokines and exhibit phagocytic characteristics, happens later in the disease when M2 microglia are dampened (Nakagawa and Chiba, 2014).

Microglial activation is a nuanced system that exerts a complex balance of neurotoxic or neuroprotective functions in response to pathological events. The

deposition of β-amyloid signals microglia to defend the brain against pathogens by stimulating the production of pro-inflammatory cytokines and tumor cells, such as IL-1β, TNF-α, STAT3, IL-6, IL-12, IL-23 and free radicals such as reactive oxygen species (ROS) (Wang et al., 2015). To better understand the role of pro-inflammatory responses, scientists use lipopolysaccharide (LPS) to induce inflammation and mimic cellular stress in *in vitro* and *in vivo* models. LPS is an endotoxin found on the outer membrane of gram-negative bacteria that has a resistant profile to enzymatic degradation (Zakaria et al., 2017). This endotoxin binds to microglia membranes by interacting with its TLR-4, which activates microglia and signals transduction cascades that release the pro-inflammatory cytokines mentioned and chemokines such as CCL2, CCL5, and CXCL8 (Sun et al., 2015). To further confirm the effects of LPS, immunostaining for Iba1 in the presence of LPS revealed an increase in microglia activation in a study conducted by Sheppard et al. (Sheppard et al., 2019) This feature provides a persistent inflammatory stimulus and sabotages homeostatic conditions, resulting in an increase of mitochondrial dysfunction and the upregulation of proteins that enhance AD pathology (Ghosh et al., 2015). Inflammatory cytokines have been shown to amplify APP expression and Aβ accumulation by transcriptionally upregulating β-secretase, an enzyme that promotes Aβ production from APP (Walter et al., 2001). Ultimately, the amplification of Aβ in the hippocampus results from altered transportational uptake at the BBB, making the effects of LPS-induced inflammation mimic AD pathology (Jaeger et al., 2009).

1.5 CCI Model Relevance/Efficacy for TBI Treatment

The controlled cortical impact (CCI) was used to administer controlled TBIs on mice. This is a well-known method used to give close headed injuries and cause BBB disruption, a prominent secondary injury that occurs after TBIs (Koza et al., 2023). This mechanical injury can precisely inflict mild, moderate, or severe reproducible impacts by centering the target in a stereotaxic frame and adjusting parameters, such as the piston speed, depending on the impact severity desired (Alluri et al., 2018). This model can cause axonal injuries that resemble those of a human's post-TBI, making it an effective tool used to investigate the effects of neuroinflammation induced by TBI using histopathological processes and other experimental designs (Dixon et al., 1991).

1.6 Hypothesis and Rationale

Recent research suggests that the correlation between football players and the number of years they partake in concussive related sports leads to more severe dementia-related pathology (Schneider, 2019). This suggests that the aging process may contribute to brain pathology of repeated sports-related mTBIs. We will test the hypothesis that age-dependent and genetics-driven cognitive decline and brain pathology are accelerated following repeated mTBIs. In particular, the focus of this study is to assess the extent of neuroinflammation, measured as astrogliosis and microgliosis, in the hippocampus of AD model mice subjected to rmTBIs early in life.

The interaction between genetic predisposition and mTBIs has not been investigated using transgenic mouse models of AD combined with multiple mTBIs. This will be examined by assessing the effects of repetitive mTBIs administered early in life.

Previous research suggests that mTBIs can also accelerate neuropathology and cognitive deficits in mouse models of AD, including 3xTg-AD mice. However, this question has only been examined acutely post-injury (24 hours to 30 days post-TBI). We propose to utilize WT and transgenic (Tg) mice which are homozygous for expression of human mutant APP (Swe), Tau (P301L), and PSen1 (M146V) (3xTg-AD mice, Mutant Mouse Resource & Research Centers (MMRRC) supported by NIH). This mouse model develops age-dependent amyloid and tau pathology, and cognitive deficits; this includes astrogliosis and microgliosis, which are activated astrocytes and microglia that respond to sites of injury and inflammation. The rationale for using this mouse model is that it replicates both Tau and amyloid pathology in the AD brain but with a less aggressive approach than the 5XFAD mouse model.

1.7 Summary of Findings

A 3xTg-AD mouse model was used to study the effects of rmTBIs early in life and observe if TBI is an accelerator for AD pathology. Brains were stained with either GFAP antibodies that identified active astrocytes in the hippocampus or Iba1 antibodies that identified active microglia in the hippocampus; images of each brain section displayed results that were averaged among the population of mice of each treatment group; images were displayed and coded on FIJI for average mean fluorescent intensities (MFI) and glial cell counts. Ultimately, both qualitative histopathology and quantification results revealed that mice with rmTBIs given at 3-months-old did not showcase any signs of accelerated or enhanced astrogliosis or microgliosis in comparison to the naïve or sham AD mice. Some inflammation and immunoreactivity

are existent in AD TBI mice, particularly in Iba1 stained brain sections, since there is a statistically significant difference in microglia activation in AD TBI vs. WT groups across all hippocampal regions; however, this also applies for naïve AD mice. This suggests that TBIs do not affect the acceleration and reactivity of astroglia branching and microglia migration. Mouse brains stained for GFAP biomarker revealed no statistical difference of astrogliosis in AD TBI mice in comparison to WT mice; the only group that exhibited a significant difference in MFI values corresponding to astrocyte activation were naïve AD vs. WT mice. The most hippocampal region affected in brains stained for astrocyte immunoreactivity was in the CA3 region; on the other hand, microglia immunoreactivity and cell count were the most abundant in the CA1 region of the hippocampus.

Chapter Two: Materials and Methods

2.1 TBI procedure and flow diagram

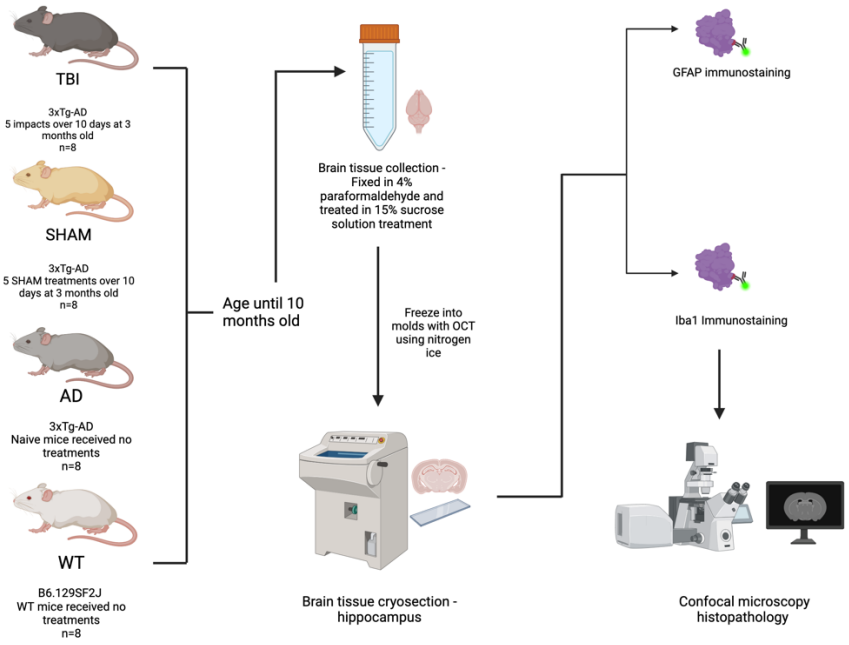

Figure 1: Mouse treatment groups, and post-euthanasia tissue processing and imaging experimental design. Mice were divided into four groups: TBI 3xTgAD mice, SHAM 3xTgAD mice, naïve 3xTgAD mice, and wild type B6. 129SF2J mice. Mice were subjected to 5 rmTBIs at 3 months old, with an inter-concussion interval of 48 hours. TBI treatment mice were anesthetized with isoflurane and given impacts administered at 5 m/s with a 200 msec dwell time. SHAM treatment mice were given sham treatments at the same time with no impact administered. SHAM treatments resemble placebo effects in which mice receive similar care and are centered in the stereotaxic frame of the CCI device regardless of treatment to ensure that effects of the study are not driven by stress and mishandling animals. At 10 months of age, mice were euthanized, and brains were collected and preserved in sucrose solution. Brains were then centered in square molds

and covered with OCT to protect structural integrity while freezing in nitrogen ice. Frozen brain molds were stored in -80°C freezer then cryosectioned for hippocampal regions with four 35 μm sections per slide. Slides were stored in -20°C freezer prior to staining with GFAP and Iba1. After staining, brain sections were imaged in OLYMPUS FV30-ILSW confocal microscope under the 10x objective lens for astrogliosis and microgliosis. (Created with BioRender.com)

A 3xTg-AD mouse model was used for this study, which is a popular model used for AD research to track effects of interventions on mice with mutations found in genes of AD patients. Four experimental groups were compared throughout this study: naïve 3xTg-AD mice, SHAM 3xTg-AD mice, rmTBI 3xTg-AD mice, and wild type B6.129SF2J mice (Fig. 1). Four groups of mice will be compared [WT, AD naive, AD SHAM, AD TBI]. Males and females in each group will receive multiple mTBIs at 3 months of age or sham treatments. An mTBI protocol consisting of an electromagnetically controlled cortical impact device (Impact One Stereotaxic Impactor, Leica Biosystems, 5.5 m/s impact velocity and 1.5 mm impact depth) in a closed head injury paradigm that has been used extensively and produces reproducible pathology of the cortex and subcortical regions of the brain. This protocol does not result in overt hippocampal neuronal cell loss but does elicit significant axonal injury, glial pathology, and deficits in motor and cognitive function. At 3 months old, the rmTBI and SHAM treatment groups were subjected to rmTBIs and sham treatments. Mice were anesthetized with isoflurane and then subjected to close-head injuries (CHI). In the rmTBI group, the administration of impacts was centered at the bregma of the mice's skulls using a controlled cortical impact device (CCI). Five repetitive mild impacts were given in ten days, with a 48-hour inter-concussion interval between each impact and a dwell time of 200 msec per hit. The CCI device consists of specific parameters that apply this kind of

force: a piston that shoots down at a velocity of 5 m/s^2, with a probe that's 5 mm in diameter, resulting in an impact depth of 1 mm. SHAM mice were anesthetized and centered in the stereotaxic frame of the CCI device; however, no impact was administered. Both groups of mice were monitored for post-impact apnea and reflex times to detect any unusual responses; WT and Naïve AD mice were excluded as they received no treatments.

2.2 Euthanasia and Tissue Preparation

At 10 months old, the mice were euthanized, and their brains were ready to collect for histopathology and quantitative analysis. Each mouse was euthanized by an inhalation overdose of isoflurane, followed by a secondary decapitation. This euthanasia protocol is approved by the American Veterinary Medical Association (AVMA) and by the Institutional Animal Care and Use Committee (IACUC) – protocol approval #1774485 at the University of Denver. The skull was then cracked open, and the brain was extracted to use for histopathology. Brain tissue was fixed with 4% paraformaldehyde for 24 hours, to ensure optimum preservation of the brain to later prepare for immunohistochemistry (Gage et al., 2012). Brains were then treated with a 15% sucrose solution cryoprotectant for 48 hours, to minimize the formation of ice crystal artifacts when freezing brain sections (Rosene & Rhodes, 2013). 35 μm tissue sections of the hippocampus were then sliced using a cryostat and mounted on positively charged glass slides.

2.3 Reagents

The primary antibody for GFAP staining, Anti-GFAP Goat Polyclonal antibody (ab53554), was purchased from Abcam (Cambridge, MA). The primary antibody for Iba1

staining, Anti-Iba1 Rabbit Polyclonal (for Paraffin Section) was purchased from Fujifilm (Richmond, VA). For secondary antibody staining, the Nucleic Acid Stain Fluorescent Dye Hoechst 33342 20 mM was purchased from Thermo Scientific™ (Rockford, IL). The secondary antibody used for GFAP, Alexa Fluor® 488-AffiniPure Donkey Anti-Goat IgG (H+L), was purchased from Jackson ImmunoResearch (West Grove, PA). The secondary antibody used for Iba1, Cyanine Cy3 Anti-Rabbit IgG (H+L) Donkey, was purchased from Jackson ImmunoResearch (West Grove, PA).

2.4 Immunostaining

After brains were cryosectioned, the tissue was ready to be stained with specific antibodies to reveal astrocyte and microglia biomarkers. Four males and four females of each group were chosen for quantification, resulting in a total of 8 subjects per treatment (n=8), and a total population of 32 mice across all treatments (n=32) (Table 1).

Experimental Cohort Summary

Ear-tag ID	Sex	Genotype	Treatment

Naive AD n=8	AD019	M		
	AD112	M		
	AD276	M		
	AD277	M	3xTgAD	GFAP
	AD275	F		
	AD130	F		
	AD146	F		
	AD260	F		
	AD112	M		
	AD164	M		
	AD276	M		
	AD279	M	3xTgAD	Iba1
	AD130	F		
	AD151	F		
	AD275	F		
	AD146	F		
AD TBI n=8	AD077	M		
	AD171	M		
	AD173	M		
	AD264	M	3xTgAD	GFAP
	AD083	F		
	AD178	F		
	AD262	F		
	AD271	F		
	AD173	M		
	AD280	M		
	AD011	M		
	AD171	M	3xTgAD	Iba1
	AD186	F		
	AD178	F		
	AD262	F		
	AD271	F		

AD SHAM n=8	AD008	M		
	AD30	M		
	AD073	M		
	AD159	M	3xTgAD	GFAP
	AD013	F		
	AD080	F		
	AD175	F		
	AD284	F		
	AD159	M		
	AD259	M		
	AD008	M		
	AD073	M	3xTgAD	Iba1
	AD004	F		
	AD013	F		
	AD080	F		
	AD284	F		
WT n=8	WT031	M		
	WT034	M		
	WT227	M		
	WT229	M	B6129SF2J	GFAP
	WT010	F		
	WT016	F		
	WT038	F		
	WT234	F		
	WT227	M		
	WT031	M		
	WT229	M		
	WT034	M	B6129SF2J	Iba1
	WT010	F		
	WT016	F		
	WT234	F		
	WT368	F		

Table 1: Experimental Cohort Summary. Each treatment had a total of eight mice (n=8), made up of four females and four males. Ear-tag ID, sex (M/F), genotype

(3xTgAD/B6.129SF2J), and the antibody stained (GFAP/Iba1) are displayed to show different groups of mice were stained for.

A 3-day protocol was conducted for GFAP and IBA1 antibody staining. Slides containing brain tissue were prepared in large Petri dishes, containing napkins dampened by DI water to ensure a stable humid environment for brain tissue. A Phosphate Buffered Saline (PBS) solution was first used to wash the tissue 3 times to rinse away frozen OCT. A 5% Bovine Serum Albumin (BSA) blocking solution composed of 0.2% Triton 100-X and Bovine Serum Albumin was then carefully pipetted onto the brain tissue for the purpose of limiting background and blocking non-specific binding. The slides were then placed in the cold room (4°C) overnight.

2.4.1 GFAP staining

For slides stained for astrocytes (GFAP), the blocking solution was aspirated the following morning, and the brain tissue was then ready for the primary antibody. The GFAP primary antibody solution had a 1:500 dilution; a 1 mL tube composed of 5% BSA blocking solution, 0.2% Triton 100-X, and Anti-GFAP polyclonal goat antibody was pipetted onto the slides and left overnight at 4°C. The primary antibody solution is then aspirated off the brain tissue and washed with a 1X PBS buffer solution 3-5 times before adding the secondary antibody, which is a 1 mL aliquot composed of 5% BSA blocking solution, 0.2% Triton 100-X, anti-goat conjugated Alexa Fluor 488 dye, and Hoechst 33342. The incubation time for the secondary antibody on brain tissue was 90 minutes.

2.4.2 Iba1 staining

For slides stained for reactive microglia (anti-IBA1), the same protocol was used, except the primary and secondary antibody solutions had different polyclonal antibodies derived from different species. IBA1's primary antibody solution used on brain tissue was a 1:500 dilution composed of 5% BSA blocking solution, 0.2% Triton 100-X, and the Anti-IBA1 Rabbit antibody. The secondary antibody solution was a 1 mL aliquot composed of 5% BSA blocking solution, 0.2% Triton 100-X, anti-rabbit conjugated Cy3 dye, and Hoechst 33342.

2.5 Fluorescence microscopy

All brain tissue was examined and imaged using the OLYMPUS FV30-ILSW confocal microscope under the 10x objective lens. Each of the mounted glass slides contained 4 slices of coronally sectioned hippocampus region of the brain; this was to ensure the ability to capture the proteins of interest's abundance, and to make sure that tissue is intact for at least 2 brain slices to avoid imaging hippocampal regions with too much background or tissue breakage. For tissue stained for GFAP, the scans had parameters that include: an HV of 700 Volts, a Gain of 1.000, and a 3% Offset, with a laser ND filter of 10%; these parameters ensured maximum background reduction and a clear GFAP fluorescent foreground. For tissue stained for Iba1, the parameters stayed the same, except the Offset setting was set to 25% to ensure that only activated microglial cells are captured. Channels 1 and 2 in the confocal microscope displayed each of the different stains consisting of the nuclei and antibody, while channel 3 displayed merging stains of both. The confocal aperture was set at 125 μm. Two hippocampal regions are

displayed in each hemisphere of the brain due to its bilateral structure, which constitutes for 2 of each hippocampal region per brain slice. Since each slide contained 4 brain slices of the hippocampus, the total amount of each hippocampal region captured per mouse was either a minimum of four or a maximum of eight.

2.6 Quantification and Statistics

Brain images captured on the confocal microscope were displayed and coded on a FIJI/ImageJ-based plugin. This program can validate and detect cell quantification and morphology through image thresholding techniques (Vosatka et al., 2022). The plugin traces objects of interest and collects data respective of the macroscript code used; it does this by immunolabelling and undergoing a segmentation process that pixelates the image and assigns each pixel a value of either background or object of interest (Healy et al., 2018). The measurements set standards across all images by initially subtracting background then calculating intensities and counts of pixel values assigned to the object. The plugin locates local maximum and local minimum intensities, which is a computational analysis that sets a threshold range for pixelated values that are considered the objects of interest and background noise values that should be excluded (Klemm, 2020).

For GFAP-stained brain sections, an average mean fluorescent intensity (MFI) is obtained per image due to its branching morphology and rate of activation depicted by its fluorescent brightness. The script provides a result table containing these final outputs per image: area, MFI, a standard deviation value, a minimum threshold value, and a

maximum threshold value. These values are limited to the area of the brain region it analyzes.

For Iba1-stained brain sections, active microglia in sites of injury appear as individual cells; therefore, both an average MFI value and a total glial cell count are provided in the output per brain region to ensure a fluorescent intensity threshold that eliminates background noise and counts Iba-1 tagged cells. As a result, the result table contains a final output containing values of both MFI and cell count measurements: cell count, total area, average size, % area the object of interest occupies in relation to the total area, mean, perimeter, circumference, solidity, average MFI, and a standard deviation value.

The average MFIs and cell counts were analyzed in four different comparative analysis data sets between mouse treatments: total hippocampus, the dentate gyrus (DG) brain region, the CA1 brain region, and the CA3 brain region. Each data set had an n value of 8, which constituted for the four males and four females in that treatment group.

Using GraphPad Prism, the data points were compiled into graphs that plotted for means and margins of error. A one-way ANOVA with post-hoc Tukey's multiple comparisons test was used to determine significant differences between groups using p-values: *$p<0.5$, **$p<0.01$, and ns=nonsignificant.

Chapter Three: Results

3.1 rmTBIs at 3-months-old had no effect on extent of astrogliosis

Different regions of the hippocampus were imaged after the 35µm tissue sections were stained for GFAP using the confocal microscope; the same parameters were used to ensure quality control and consistent imaging. The images selected showcase the brain region with highest astrogliosis reactivity in AD TBI and Naïve AD mice: CA3. This selection is based on the data set values displayed in Figure 3. Each column represents a different dye selector channel: brain nuclei were stained with Hoechst 33342 dye (Fig. 2A), astrocytes were stained with Alexa Fluor 488 for the GFAP marker protein (Fig. 2B), and merged channels of both stains show astrocytes associated with nuclei of the brain region (Fig. 2C).

Based on observations, active astrocytes appear to be the most fluorescent in naïve AD mice compared to the other three treatment groups, while the rest of the treatment groups don't show alarming differences in fluorescence (Fig. 2). Staining for GFAP revealed that there is no significant difference between 3xTg-AD mouse groups, suggesting that astrogliosis is not accelerated in AD mice with a history of rmTBIs.

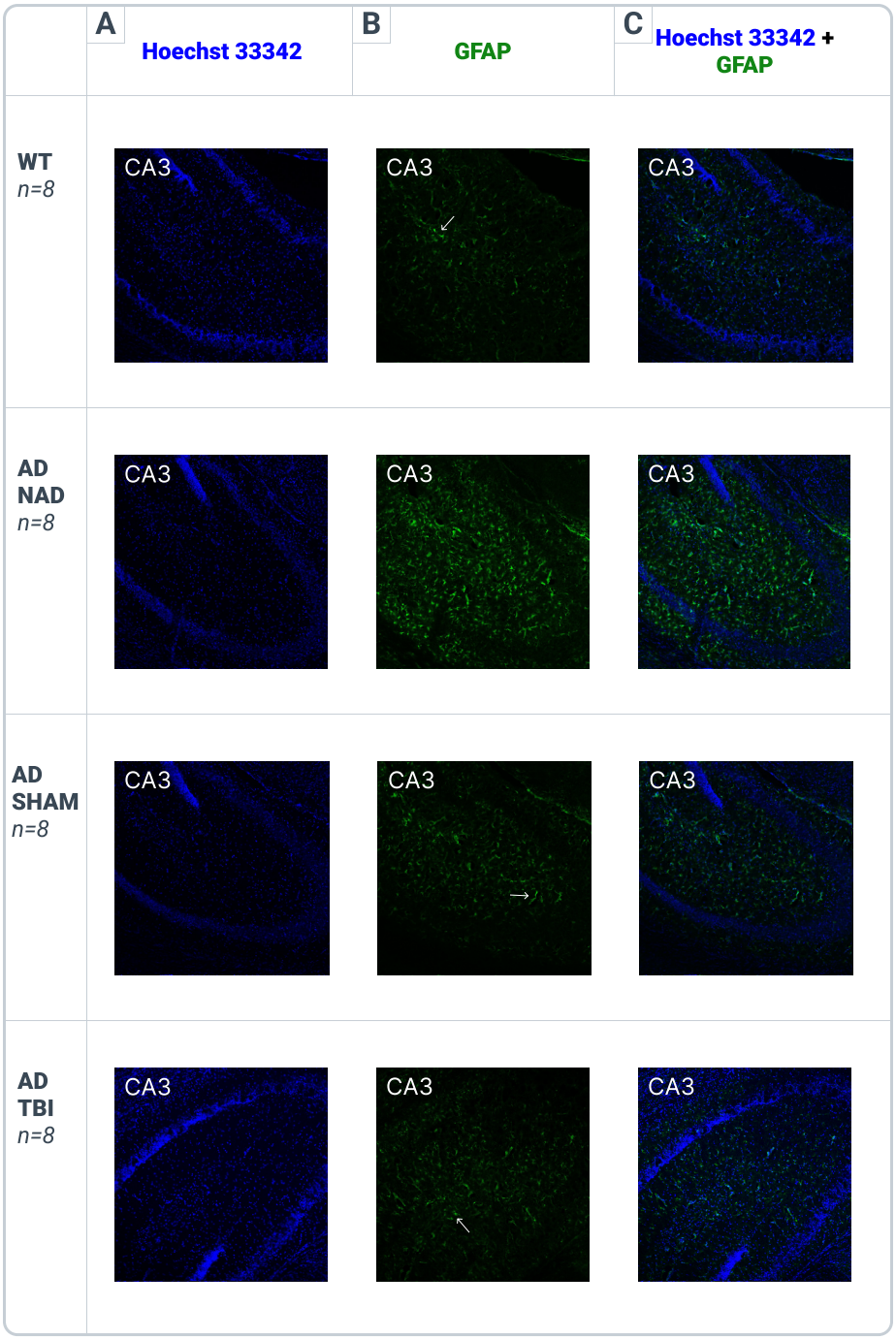

Figure 2: Confocal microscopy images of astrogliosis in CA3 region of the hippocampus of 10-month-old 3xTgAD and wild type mice. 35µm tissue sections of the hippocampus were stained for GFAP. Arrows indicate active astrocytes expressing GFAP. Representative images of each treatment group reveal the most affected region of the brain following GFAP staining taken with a spinning disk confocal microscope. (A) Hoechst 33342 shown in blue, for nuclear staining, (B) GFAP, shown in green, for

astrocytes, and (C) Hoechst 33342 + GFAP merged channels. Images analysis and display was done using the software FIJI. NAD and TBI mice had highest astrogliosis activity in CA3 region of hippocampus. NAD mice appear to have highest fluorescence in GFAP tagged astrocytes compared to other treatment groups. rmTBIs do not seem to accelerate gliosis inn 3xTg-AD mice.

A one-way ANOVA with post-hoc Tukey's multiple comparisons test compared mean values of every possible treatment group by using pairwise post-hoc testing (Lee and Lee, 2018). This method revealed that MFIs of active astrocytes expressing GFAP in 10-month-old mice with a history of rmTBIs appear to be nonsignificant (ns) to WT mice (Fig 3. A-D). An expected increase in GFAP astrogliosis immunoreactivity in 10-month-old naïve AD mice compared to WT mice is apparent across all brain regions DG, CA1, and CA3. Astrocytes in AD TBI mice do not exhibit activation levels that are significantly different than the WT treatment. Astrogliosis appears to be the most reactive in naïve AD mice in all regions and total hippocampus, resulting in a significant difference in MFI than mice in the WT group ($p<0.5$). The AD SHAM treatment group also has a nonsignificant p-value when compared to the WT treatment group across all brain regions. Bar plots contain data analysis with mean (± SEM) assessments and an asterisk comparing groups that show statistical significance. Since the AD naïve and WT treatment groups show statistical differences, mean differences were collected per brain region (DG, CA1, and CA3) with an average of 56.71 in comparison to the total hippocampal mean difference of 56.58.

GFAP Immunostaining in Mouse Hippocampus

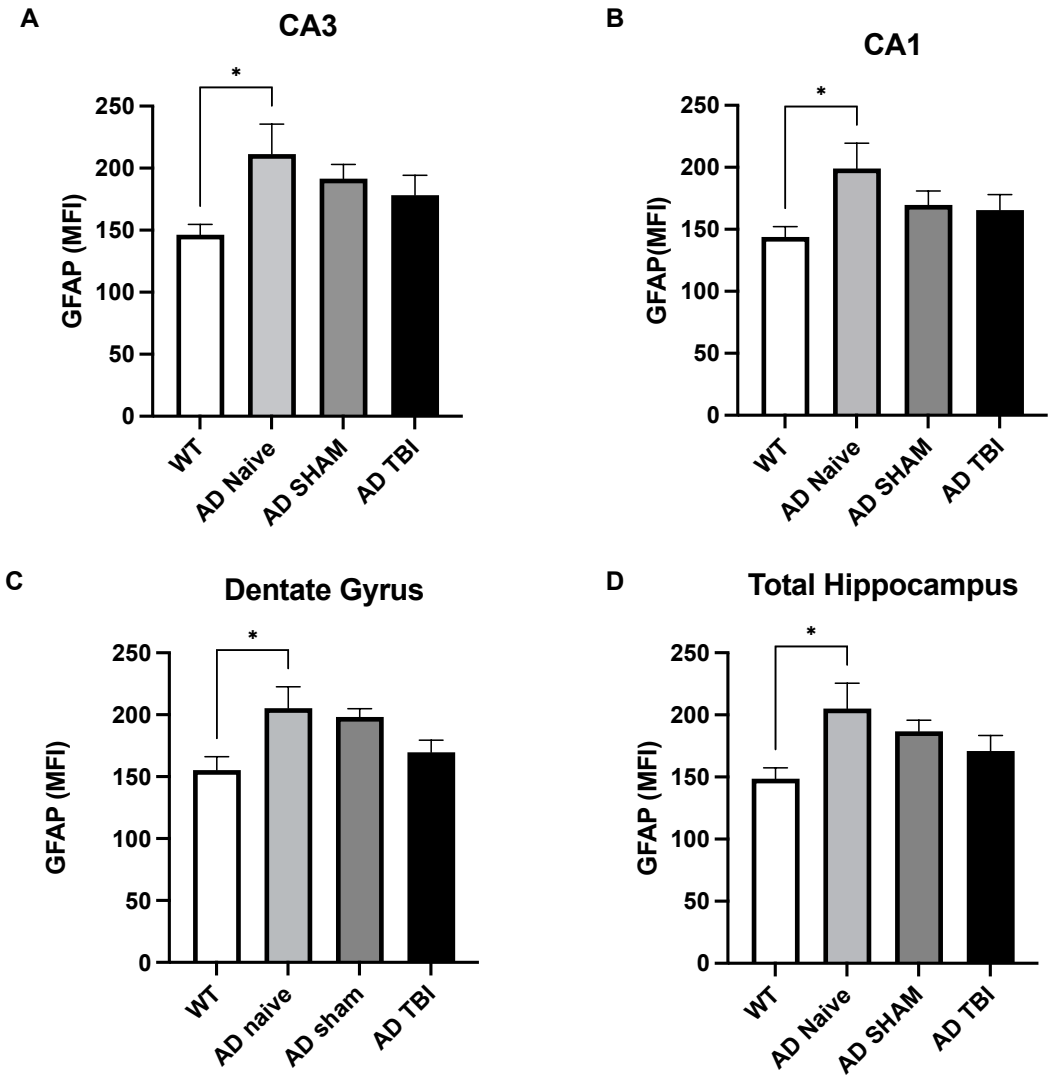

Figure 3: Average GFAP MFI values in hippocampus across all treatment groups. One-Way ANOVA with post-hoc Tukey's test bar plots (n=8 mice per group; 4 males and 4 females). (A) Avg. GFAP MFI in CA3 region of the hippocampus in naïve AD mice is significantly different than WT mice. Mean (± SEM) assessment revealed an MFI mean difference of -65.13 for WT vs. AD Naïve (*$p<0.05$). (B) Avg. GFAP MFI per treatment group in CA1 region of the hippocampus in naïve AD mice is significantly different than WT mice. Mean (± SEM) assessment revealed an MFI mean difference of -55.13 for WT vs. AD Naïve (*$p<0.05$). (C) Avg. GFAP MFI per treatment group in DG region of the hippocampus in naïve AD mice is significantly different than WT mice. Mean (± SEM) assessment revealed an MFI mean difference of -50.00 for WT vs. AD Naïve (*$p<0.05$). (D) Avg. GFAP MFI per treatment group in total hippocampus in naïve AD

mice is significantly different than WT mice. Mean (± SEM) assessment revealed an MFII mean difference of -56.58 for WT vs. AD Naïve (*p<0.05). Overall, AD SHAM and AD TBI treatment groups have MFIs that are ns to WT treatment.

3.2 rmTBIs at 3-months-old had no effect on extent of microgliosis compared to naïve AD mice

Different regions of the hippocampus were imaged after the 35µm tissue sections were stained for Iba-1 using the confocal microscope; the same parameters were used to ensure quality control and consistent imaging. Images of the CA1 brain region were selected because that part of the hippocampus appeared to be the most affected across all treatment groups when accounting for mean fluorescent intensity (MFI) + cell count shown later in Figure 5 and Figure 6. The columns exhibit different dye detectors: brain nuclei were stained with Hoechst 33342 dye (Fig. 4A), microglia were stained with Cy3 for the Iba-1 marker protein (Fig. 4B), and merged channels of both stains show microglia associated with nuclei of the brain region (Fig. 4C).

Based on observations, microglia appeared to be the most fluorescent and abundant in naïve AD mice compared to the other three treatment groups. AD TBI mice did exhibit a slightly higher microglia cell count and immunoreactivity compared to WT and AD SHAM mice in the CA1 region of the hippocampus. WT and AD SHAM treatment groups appeared to have the most similar microglia abundance and visibility (Fig. 4).

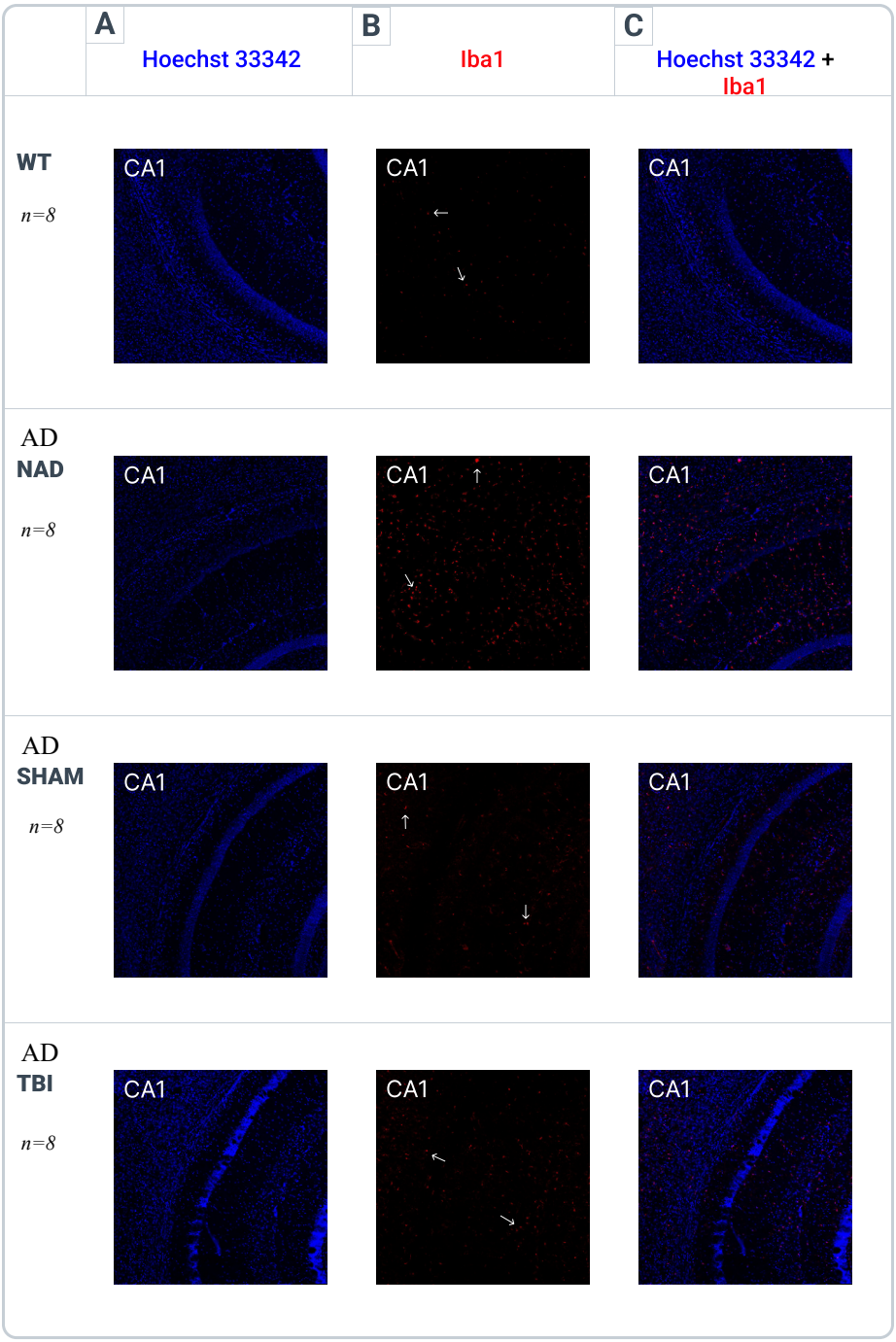

Figure 4: Confocal microscopy images of microglia in the most affected regions of the hippocampus of 10-month-old 3xTgAD and wild type mice. 35µm tissue sections of the hippocampus were stained for Iba1. Arrows indicate individual microglia cells expressing Iba-1. Representative images of each treatment group reveal the most affected region of the brain following Iba1 staining taken with a spinning disk confocal microscope. (A) Hoechst 33342 shown in blue, for nuclear staining, (B) Iba1, shown in red, for microglia, and (C) Hoechst 33342 + Iba1 merged channels. Image analysis and display was done

using the software FIJI. All treatment groups exhibited high counts of microglia cells in the CA1 region of the hippocampus. NAD mice appear to have highest fluorescence and individual glial cell count in Iba1 tagged microglia compared to other treatment groups.

3.2.1 Iba1 Avg. MFI shows significant difference in MFI values in CA1 region of hippocampus for AD TBI mice compared to WT mice

Data sets in mean (± SEM) bar plots were analyzed using one-way ANOVA with post-hoc Tukey's multiple comparisons test. The data revealed that Iba1-tagged microglia in 10-month-old mice with a history of rmTBIs appear to have a significant difference in MFI values in the CA1 region (Fig. 5B), and the total hippocampus (Fig. 5D) when compared to the mean values of the WT treatment group ($p<0.05$). An increase in Iba1 immunoreactivity in 10-month-old naïve AD mice compared to WT mice is apparent across all brain regions DG, CA1, and CA3 ($p<0.05$) (Fig. 5A-D). The AD SHAM treatment group has a nonsignificant p-value when compared to the WT treatment group across all brain regions. Since the AD naïve and AD TBI treatments show statistical differences when compared to WT, mean differences were collected in affected brain regions respectively. The average mean difference in MFI values across all brain regions for AD naïve vs. WT treatment groups is 32.3 in comparison to the total hippocampus mean difference of 32.5. The average mean difference in MFI values in the CA1 region for AD TBI vs. WT treatment groups is 27.66. This brings the p-value of the total hippocampus between the two groups equal to 0.04 and an MFI mean difference of 27.45.

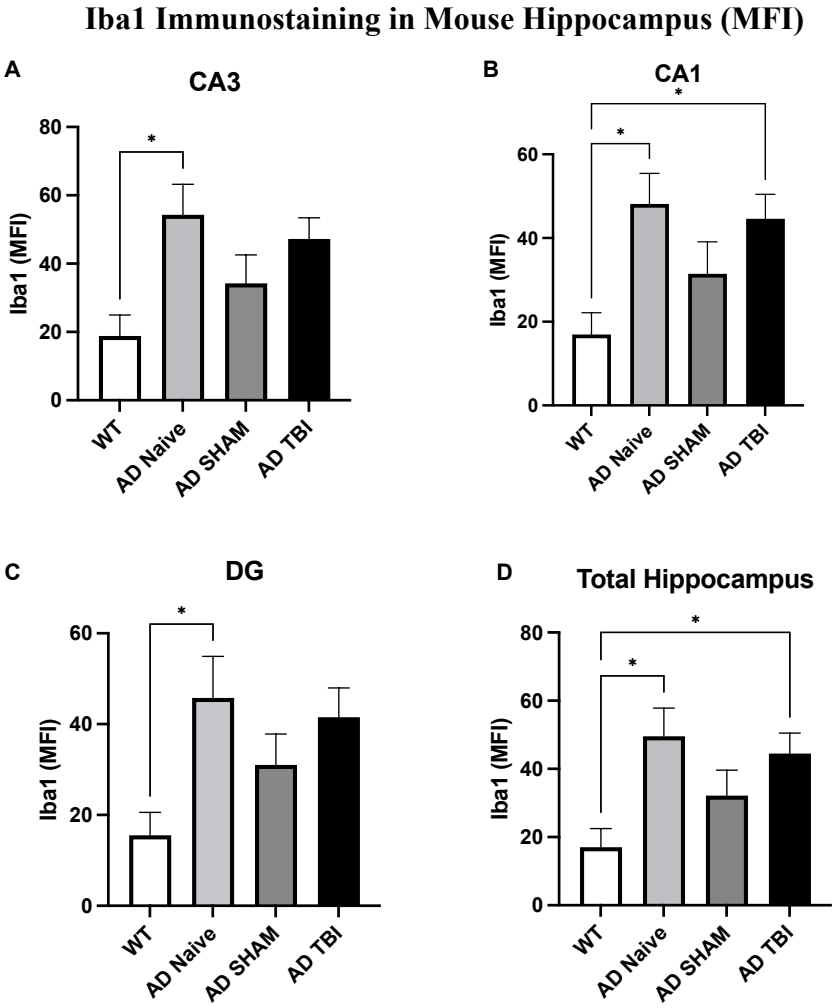

Figure 5: Average Iba1 MFI values in hippocampus across all treatment groups. Quantification of Iba1 MFIs reveal a significant difference between mice with history of rmTBIs compared to WT mice in CA1 region of the hippocampus, and a significant MFI difference in all hippocampal regions for naïve AD mice vrs. WT mice**.** (A) Avg. Iba1 MFI per treatment group in CA3 region of the hippocampus in naïve AD mice is significantly different than WT mice, revealing an MFI difference of -35.44 for WT vs. AD Naïve (*$p<0.05$). (B) Hippocampus CA1 regions of both naïve AD and AD TBI mice have high fluorescent intensity differences than WT mice, with MFI differences of -31.23 and -27.66 respectively (*$p<0.05$). (C) Avg. Iba1 MFI per treatment group in DG region of the hippocampus in naïve AD mice is significantly different than WT mice, with an MFI difference of -30.25 for WT vs. AD Naïve (*$p<0.05$). (D) Significant differences in MFIs exist in total hippocampus for WT vs. AD Naïve (*$p<0.05$) Δ: -31.54 and WT vs. AD TBI (*$p<0.05$) Δ: -27.45. Overall, rmTBIs showed no influence on 3xTg-AD mice, with ns differences between 3xTg-AD mice in all treatment groups (naïve AD, SHAM,

TBI). Results shown as bar plot mean (± SEM) assessments for n=8 mice per group: 4 females and 4 males.

3.2.2 Iba1 Avg. Glial Cell Count Highest in CA1 regions

Data sets in mean (± SEM) bar plots were analyzed using one-way ANOVA with post-hoc Tukey's multiple comparisons test for total microglia cell count per region in the brain. The data revealed that there is no significant difference between AD TBI and AD naïve groups, but these two treatment groups did exhibit differences when compared to WT mice (Fig. 6). Active microglia expressing Iba1 in 10-month-old mice with a history of rmTBIs appear to have a significant difference in average glial cell count values in all regions of the brain (Fig. 6A-C), and the total hippocampus (Fig. 6D) when compared to the mean values of the WT treatment group. In the CA3 region, WT mice had an average of 56 immunoreactive cells, naïve AD mice had an average of 149 immunoreactive cells, AD SHAM had an average of 99 immunoreactive cells, and AD TBI had an average of 134 immunoreactive cells. Therefore, naïve AD vs. WT treatment groups has the highest statistically significant difference ($p<0.01$) and AD TBI vs. WT treatment groups has the second highest difference ($p<0.05$); the rest of the comparisons has nonsignificant differences (Fig. 6A). In the CA1 region, WT mice had an average of 71 immunoreactive cells, naïve AD mice had an average of 166 immunoreactive cells, AD SHAM had an average of 98 immunoreactive cells, and AD TBI had an average of 158 immunoreactive cells. Therefore, naïve AD vs. WT treatments and AD TBI vs. WT treatments have statistically significant differences ($p<0.05$), while the rest of the comparisons have nonsignificant differences (Fig. 6B). In the DG region, WT mice had an average of 57 immunoreactive cells, naïve AD mice had an average of 122

immunoreactive cells, AD SHAM had an average of 98 immunoreactive cells, and AD TBI had an average of 139 immunoreactive cells. Therefore, AD TBI vs. WT treatment groups have the highest statistically significant difference ($p<0.01$) and naïve AD vs. WT treatment groups have the second highest difference ($p<0.05$); the rest of the comparisons had nonsignificant differences (Fig. 6C). Overall, naïve AD and AD TBI treatment groups each hold a significant difference when compared to WT mice ($p<0.01$) (Fig. 6D).

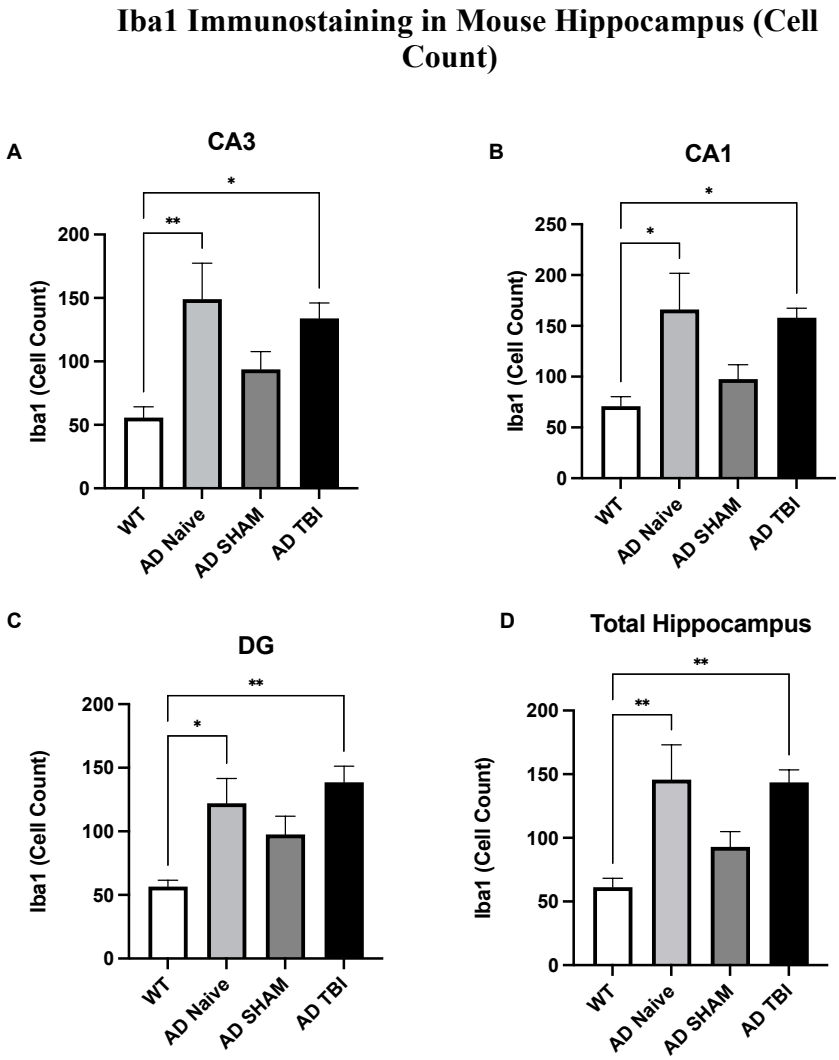

Figure 6: Average Iba1 cell count values in hippocampus across all treatment groups. Results showed that rmTBIs early in life showed no effects on microglia activation in

3xTg-AD mice. (A) Naïve AD and AD TBI mice had significant differences in # of activated microglia compared to WT mice in CA3 region of the hippocampus; however, ns differences across all 3xTg-AD groups. Quantified values revealed differences of -93 cells for WT vs. AD Naïve (**$p<0.01$) and -78 cells for WT vs. AD TBI (*$p<0.05$). (B) Avg. Iba1 cell count per treatment group in CA1 region of the hippocampus revealed significant differences for WT vs. AD Naïve (*$p<0.05$) Δ: -95 active microglia, and WT vs. AD TBI (*$p<0.05$) Δ: -87 active microglia. (C) Avg. Iba1 MFI per treatment group in DG region of the hippocampus. Mean (± SEM) assessment revealed a difference of -66 for WT vs. AD Naïve (*$p<0.05$) and a difference of -82 for WT vs. AD TBI (**$p<0.01$). (D) Avg. Iba1 cell count per treatment group in total hippocampus; Mean (± SEM) assessment revealed a mean difference of -85 for WT vs. AD Naïve (**$p<0.01$) and a mean difference of -82 for WT vs. AD TBI (**$p<0.01$). Overall, ns differences in active microglia between 3xTg-AD groups across all regions of hippocampus.

To observe the effects that rmTBIs have on active microglia proliferation and abundance, an unpaired two-tailed t-test quantified the mean differences of AD SHAM and AD TBI groups in each hippocampal region and the total hippocampus (Table 2). Mice in WT and Naïve AD groups were compared to ensure that reactive microglia are significantly different in abundance when comparing healthy mice to 3xTg-AD mice, which establishes the effects of AD pathology. AD SHAM groups were compared with AD TBI groups to see if effects of TBI do induce some reactivity in microglia and to better understand the cellular manifestations that separate a SHAM treatment from TBI administrations. According to the results in Table 2, there is a significance in mean differences for active microglia present in the hippocampus when comparing AD SHAM and AD TBI groups. A higher significant difference is present in the CA1 region of the hippocampus (**$p<0.01$), confirming that this region seems to be slightly more affected. Overall, rmTBIs do affect the integrity of the hippocampus in the context of a SHAM treatment, where mice do not receive actual impacts but are treated like they are about to, versus actual impact administration. Therefore, TBIs must play a role in triggering

inflammatory responses, which recruits the proliferation and migration of microglia to sites of injury in the brain.

Unpaired T-Test for TBI Effect

Region	WT (A) *n=8*	Naïve AD (B) *n=8*	(B - A) ± SEM	P value	AD SHAM (C) *n=8*	AD TBI (D) *n=8*	(D - C) ± SEM	P value
DG	56.5	122	65.50 ± 20.28	**0.006	97.5	138.6	41.13 ± 19.08	*0.049
CA1	70.86	166.2	95.32 ± 36.84	**0.0215	97.54	158	60.49 ± 17.12	**0.0033
CA3	55.83	149.1	93.29 ± 29.50	**0.0069	93.81	133.9	40.11 ± 18.48	*0.0477
Total HC	61.25	145.8	84.50 ± 28.17	**0.0096	92.88	143.6	50.75 ± 15.57	**0.0057

Table 2: Avg. Iba1 microglia cell-count mean difference for AD SHAM vrs. AD TBI. Unpaired two-tails t-test with Mean ± SEM to compare Iba1 microglia cell count reveals significant mean differences between AD SHAM (n=8) and AD TBI (n=8) treatment groups. For (D - C) ± SEM, DG and CA3 regions reveal a significant difference of *$p<0.05$; CA1 region and total hippocampus reveal a significant difference of **$p<0.01$. WT (n=8) vrs. Naïve AD (n=8) groups were compared to confirm effects of AD pathology, revealing significant differences in active microglia abundance across all regions of the hippocampus (**$p<0.01$). Overall, rmTBIs do affect the integrity of the hippocampus in the context of a SHAM treatment.

Chapter Four: Discussion

It has been suggested that a history of TBIs can exacerbate the effects of dementia development later in life, but the link between experimental models used thus far and human TBI patients has yet to be investigated (Godbolt et al., 2014). TBI and AD have been epidemiologically associated with each other based on previous performance- and brain image-based research. Although studies suggest that the effects of TBIs can linger years after the impact and cause tissue damage and hippocampal atrophy, most have only investigated this cause-and-effect the first couple of weeks following the injury as opposed to measuring neuroinflammatory responses in the brain at a later stage (Wilson et al., 2017).

Astrocytes and microglia are two glial cells found in the CNS and are associated with Aβ pathology in neurodegenerative diseases by detecting tissue damage in the brain. These cells respond to inflammation in the brain and are reported to stay active for as long as brain tissue is damaged, causing detrimental outcomes for neuronal integrities and structures (Zysk et al., 2019). The ability of these different cells to adapt the morphological changes of its environment overtime makes them excellent biomarkers that can track the progression of neurodegeneration and atrophy. Therefore, this paper investigated the prevalence of astrogliosis and microgliosis 7 months post-impact and observed if rmTBIs accelerated age-dependent and genetics-driven cognitive decline and brain pathology.

The hippocampal structure of the brain oversees memory, spatial, and pattern recognition. It harbors a network of neurons that function through mediated mechanisms in delicate homeostatic environments (Ugolini et al., 2018). This crosstalk is affected when confronted with neuronal insults that cause protein aggregation and deposition resulting from age-related neurodegenerative disease; although it is suggested that the effects of TBI may contribute to axonal damage and synaptic plasticity that can lead to hippocampal atrophy, experimental models have yet to adapt protocols to investigate this further (Barrientos et al., 2015).

The results revealed that repetitive mild TBIs (rmTBIs) are not enough to amplify AD pathology due to nonsignificant results 3xTg-AD treatment groups across all brain regions of the hippocampus (CA3, CA1, and DG). This is also reflected in histopathological analysis, showcasing a higher fluorescence and abundance in astrocyte and microglial cell immunoreactivity in naïve AD mice; however, gliosis across all 3xTg-AD mice, regardless of treatment, had non-significant differences. This concludes that rmTBIs early in life did not accelerate the rates of gliosis immunoreactivity, suggesting that it is not enough to enhance risk and/or accelerate age-related and genetics-driven cognitive decline. Overall, AD TBI mice only exhibited some statistically significant differences when compared to WT mice but did not exacerbate the effects of AD and had nonsignificant differences with the SHAM treatment group for both stains. This can mean a couple of things on a molecular level: A) the CNS innate immune system may have not exhibited extensive damage due to a potentially delayed activation, meaning that inflammatory pathways involving pro- and anti-inflammatory mediators were not

continuously recruited and dampened, B) the extent of BBB disruption depends on impact severity, in which the homeostatic condition of the BBB was not detrimental enough to over-excite receptors and introduce a plethora of pathogens; subsequently, phagocytic properties in microglia can keep up more easily than if it were to be more moderate or severe impacts administered. This would supposedly limit free radicals and oxidative stress, making inflammatory sites more manageable, C) a more complex system of neuro-immunoregulatory mechanisms exist that modulate inflammatory responses and glial cell activation, ultimately reducing tissue degradation for more regulated repair (Griffiths et al., 2010), D) pro-inflammatory products, such as TNF-α, are excessively produced and induced in the naïve 3xTg-AD mice subjects used, resulting to neurotoxicity that can trigger rapid rates of apoptosis; this can promote higher levels of astrogliosis and microgliosis (Batlle et al., 2015). While TBIs do produce slightly higher gliosis activity compared to SHAM groups, which was further proved in an unpaired t-test quantification analysis, overall analyses still indicate that mTBIs are not strong enough of an intervention to exacerbate effects of AD pathology. This outcome did not match our expectations and hypothesis since we predicted that MFIs and microglia cell counts of 3xTg-AD mice subjected to rmTBIs early in life would have significant differences in mean values compared to other 3xTg-AD groups that did not receive impacts. We conclude that the brain may be resilient to rmTBIs early in life and an increased risk of neurodegeneration is not a predetermined outcome.

Giving rmTBIs to 3xTg-AD mice at 3-months-old did not have an effect on astrocyte activation. In fact, mice that received rmTBIs displayed no significant

difference in astrocyte immunoreactivity when compared to WT mice. Naïve AD mice appeared to have the highest GFAP MFI in the CA3 region of the brain, but it is not statistically significant in comparison with the rest of the brain regions; however, there was a slightly higher fluorescent intensity of astrocyte immunoreactivity in the CA3 region of the brain.

Naïve AD and AD TBI mice showed statistical significance in Iba1 glial cell count and MFI differences compared to WT mice across all hippocampal regions. The crosstalk sequence that occurs between both types of glial cells has not been established yet in terms of the order of signaling and detection. Because microgliosis in AD TBI mice seems to be more frequent than astrogliosis, it may be possible that microglia cells initiate the inflammatory response and facilitate the activation of astrocytes (GFAP) (Liu et al., 2011). Microgliosis has also consistently shown to be the most active and present in the CA1 region of the hippocampus. Signaling failure occurs in the CA1 when oxygen and glucose levels are altered from interfering neuronal insults, making its neurons the most vulnerable to this kind of deprivation (Lana et al., 2020). Researchers suggest that CA1 pyramidal neurons are more sensitive under stressful environments due to oxidative and mitochondrial stress; therefore, a more sensitive reaction to ROS species may influence cell abundance in that region (Ouyang et al., 2007).

The AD model and CCI device used are the most popular and controlled method used for animal research when studying neurodegenerative disorders and chronic neuroinflammation from TBIs. Although this approach is currently understood to best reflect age-related brain pathology, the limitation of research surrounding TBIs and

animal models are still not completely reflective on humans. Biological mechanisms surrounding the effect of impact-induced neuroinflammation on AD pathology such as astrogliosis, microgliosis, Aβ deposition, and p-tau pathology have only been acutely measured, yielding different results and expectations due to the different histopathological collection time frames and aging techniques. As a result, little research utilizes experimental designs that inspect the idea of TBI causing dementia on a molecular level; otherwise, the theory has not progressed. Also, a larger sample size (higher n-value) can deliver more promising results and decrease error margins, giving a more accurate count that properly represents each treatment group.

Chapter Five: Conclusions and Future Directions

TBI research can be explored through various angles depending on the nature of its background and severity. The focus of this paper prioritized cells and pathways that respond to neuroinflammation by initially saving damaged tissue, but exhibiting persistent activation that may result in a neurotoxic environment and potentially increase the risk of hippocampal integrity atrophy in 3xTg-AD mice. Repetitive mTBIs are thought to accelerate and amplify age-related neurodegeneration, particularly exacerbating effects of neuroinflammation, axonal injury, and protein aggregation. GFAP and Iba1 marker proteins were used to reveal expressions of intermediate filament proteins in active astrocytes and microglia. These are different forms of glial cells in the CNS that undergo separate mechanisms by upregulating and recruiting proteins to sites of injury, as well as activating and scaffolding neuronal plasticity to protect the integrity of cell crosstalk.

Although results indicate that TBIs do not seem to induce effects of gliosis in AD mice, AD pathology involves a broader family of genetic mutations that each jeopardize the neuronal viability and homeostatic conditions of nuclei in the brain. The hippocampus can be subjected to Aβ deposition of amyloid plaques resulting from APP aggregation, and NFTs resulting from microtubular mutations in tau. Comparing the rate and immunoreactivity of gliosis with β-amyloid plaque and NFT presence can offer a better understanding of whether TBIs do accelerate hallmarks of AD pathology.

With the current knowledge we have about first responders recruited from astrocytes and immune cells in the CNS when subjected to neuroinflammation in the hippocampus, longitudinal studies that involve diverse experimental designs on animal models that best resemble human TBIs are recommended. Also, to better understand all elements that drive microglia and astrocyte activation, a deeper look into the roles certain receptors play in the innate immune system is beneficial. An example of an AD risk variant is TREM2, a trigger receptor that has been observed to ambush microglia signaling and worsen β-amyloid plaque clearance. A deficiency in this variant is suspected to protect the brain against neuronal insults and mitigate neurotransmission deficits (Leyns et al., 2017). Therefore, staining for TREM2 can offer more insight as to whether rmTBIs trigger this variant or not when comparing to AD pathology.

Neuronal death can also be accounted for when it comes to inflammation and BBB disturbance. NeuN is a neuronal nuclear marker that can be used to stain for neural cell counts. Neuronal cell death may be a driver for the upregulation of inflammatory pathways glial cell recruitment to sites of injury; apoptotic patterns have the potential to tie AD pathology mediated pathways with effects of neuroinflammation induced by rmTBIs. Ultimately, accounting for all proteins, cells, and receptors that upregulate inflammatory pathways or exhibit deleterious behaviors in longitudinal studies, along with models and aging protocols that best resemble the effects of human TBIs, would help better associate if a history of traumatic brain injury can manifest accelerated effects of neurodegenerative decline.

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