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Traumatic brain injury-induced changes of immune/inflammatory response in the context of predisposition of Alzheimer's Disease

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Traumatic brain injury-induced changes of immune/inflammatory response in the context of predisposition of Alzheimer's Disease

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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Abbreviations

3xTg	Triple transgenic mouse model of AD
Aβ	Amyloid beta
AD	Alzheimer's Disease
APOE-e4	Apolipoprotein E4
APP	Amyloid Precursor Protein
ASC	Apoptosis-associated speck-like protein containing CARD
CII	Controlled cortical impact injury
CDC	Centers for Disease Control
cFPI	Central fluid percussion injury
DAI	Diffuse axonal injury
dpi	Day(s) post injury
ELISA	Enzyme-linked immunosorbent assay
GFAP	Glial fibrillary acidic protein
GSDMD	Gasdermin D
HMGB1	High mobility group box protein 1
IACUC	Institution of Animal Care and Use Committee
Il-1β	Interleukin-1β
Mapt	Microtubule-associated protein tau
NFT	Neurofibrillary tangle
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3

TBI	Traumatic Brain Injury
WT	Wild type

Abstract

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Alzheimer's Disease (AD) is a progressive neurogenerative disease that affects millions globally. At this time there are no effective treatment options as the primary cause for AD is not fully understood. In addition to old age, genetic predisposition, and certain health conditions, traumatic brain injury (TBI) has been identified as a risk factor for AD. While the pathway that links TBI to AD is still largely unknown, the neuroinflammatory response to both conditions has been connected to the NLRP3 inflammasome. Due to its role in the innate immune system and proinflammatory cytokine mediation, the NLRP3 inflammasome could be a potential therapeutic target for reducing damage caused by post-TBI inflammation.

In the current study, we examined how reduction of NLRP3 alters post-TBI neuroinflammation in the context of predisposition of AD. Using 3xTg AD mice, a widely used transgenic AD mice model, and 3xTg AD/NLRP3$^{-/-}$ mice, a strain created in our lab with specific

deletion of NLRP3 gene in the 3xTg AD mice, as well as the matched wild type control strain, we measured the TBI-induced neuroinflammatory response related to NLRP3 inflammation following TBI at the acute stage (2 days post injury). The central fluid percussion injury (cFPI) model of TBI was used and protein analysis was done using Western blotting and ELISA methods.

Results suggested that TBI causes a trend of enhanced expression of NRLP3, active caspase-1, IL-1β, and GFAP, however, results only reached significance for GFAP. HMGB1 expression was unaltered by TBI. The 3xTg animals had higher expression of NLRP3 and active caspase-1 compared to 3xTg/KO. This study suggests that a moderate cFPI, the TBI model used for this study, does not cause increased NLRP3 inflammasome activation or microglial activation but does suggest TBI causes enhanced astrogliosis. It is inconclusive whether predisposition to AD leads to enhanced neuroinflammatory and microglial cell response. However, as evidenced by higher caspase-1 expression in 3xTg mice compared to 3xTg/NLRP3$^{-/-}$ mice, there is some data that supports predisposition to AD causes increased formation of the NLRP3 inflammasome and that reduction of NLRP3 in the context of predisposition of AD can mediate this response. Understanding the link between TBI and AD is crucial in developing post-TBI treatments. Future studies should analyze the inflammatory/immune response at the chronic stage post-TBI as well as how different TBI models and intensities would affect inflammatory/immune response.

Chapter 1: Introduction

1.1 Traumatic Brain Injury

1.1.1 Epidemiology

Traumatic brain injury (TBI) refers to a disruption of normal brain function as a result of external trauma. With an estimated 69 million people globally suffering from TBI, it is a significant cause of death and disability (Michael C. Dewan MD, 2018). In the United States alone, the Centers for Disease Control and Prevention (CDC) report that there were almost 224,000 TBI-related hospitalizations in 2017 and 61,000 TBI-related deaths in 2019. TBI is most commonly the result of a fall, firearm-related injury, motor vehicle crash, or an assault. While certain populations have a higher risk of being affected by the injury, anyone can fall victim to TBI.

1.1.2 Mechanism and Biomechanics

TBI can be caused by a variety of external forces. They are commonly caused by a direct impact to the head, but can also be caused by acceleration-deceleration forces, penetrating injury, and concussive forces (e.g., blast from an explosion). A TBI will result if the force causes substantial movement of brain tissue, therefore causing tissue deformation. The effects of a TBI can be broken down to the primary and secondary injury. The primary injury is due to the initial impact that caused a displacement of the brain (Prins, Greco, Alexander, & Giza, 2013). Examples of primary injury include skull fracture, contusion, concussion, lacerations, and diffuse axonal injury. The secondary injury occurs gradually as a result of ongoing cellular events and leads to additional harm (Prins, Greco, Alexander, & Giza, 2013). Examples of secondary injury

include hypoxia, increased intracranial pressure, meningitis, epilepsy, and neurochemical changes.

1.1.3 Classification and symptoms

The effects of TBI vary based on several factors, like age and location of injury. The severity of a TBI can make a significant difference in the outcome of a patient. Using the Glasgow Coma Scale, a TBI can be categorized as mild, moderate, or severe. Patients who have suffered from a mild TBI will likely develop symptoms immediately after or within a day of their injury. Symptoms can be physical (e.g. headaches, nausea, dizziness, fatigue), psychiatric (e.g. anxiety, depression), and/or affect thinking and memory. For mild TBI, symptoms will usually subside within a few weeks. However, for moderate to severe TBI, the effects may be long-term or even life-long.

As one might expect, moderate or severe TBI leads to more severe symptoms. It's not uncommon for victims of moderate or severe TBI to experience extended loss of consciousness or amnesia. They may also have trouble with motor skills, hearing, vision, mood, behavior, thinking, and learning. The CDC notes that the life expectancy of patients who survive a moderate or severe TBI is 9 years shorter on average (CDC, 2022). This is partly due to increased risk of seizures, drug poisoning, infections, and pneumonia (CDC, 2022).

1.1.4 Chronic Condition

While previously TBI may have been thought of as a singular event one can recover from, studies showing the long-term effects support the increasing notion that TBI is an ongoing health issue. In a paper published in 2010, the authors argued that TBI fits the World

Health Organization's definition of a chronic illness, for "having one of more of the following characteristics: it is permanent, caused by non-reversible pathological alterations, requires special training of the patient for rehabilitation, and/or may require a long period of observation, supervision, or care" (Masel & DeWitt, 2010). The paper asserts that TBI is associated with increased rates of seizures, sleep disorders, neurodegenerative disease, neuroendocrine dysregulation, and psychiatric diseases, as well as other non-neurological disorders (Masel & DeWitt, 2010). In the last three decades, studies have shown a link between TBI and Alzheimer's Disease, which will be discussed further in the next section.

1.1.5 TBI animal models

Like with many other scientific research fields, rats and mice are commonly used in TBI research due to their biological similarity to humans, small size, and ease of maintenance. The four most used models for TBI research are fluid percussion injury (FPI), controlled cortical impact injury (CCI), weight drop-impact acceleration injury, and blast injury (Xiong, Mahmood, & Chopp, 2013). FPI uses a rapid injection of a fluid pulse into the epidural space and offers a mixed type injury (Xiong, Mahmood, & Chopp, 2013). CCI uses an air or electromagnetic driven piston to penetrate the brain and offers a mainly focal type injury (Xiong, Mahmood, & Chopp, 2013). In the weight drop model, a weight is released directly on the skull or the exposed dura which can offer either a focal or diffuse type injury. (Xiong, Mahmood, & Chopp, 2013). Finally, the blast brain injury is caused by detonation of a blast or initiation of a shock wave in a shock tube in the vicinity of the mouse causing a mainly diffuse type injury (Xiong, Mahmood, & Chopp, 2013).

1.2 Alzheimer's Disease

1.2.1 Epidemiology

Alzheimer's Disease (AD) is a neurodegenerative brain disease that affects memory, thinking, and behavior. As the most common type of dementia, AD affects millions of people across the globe. The Alzheimer's Association reports that in 2021, 6.2 million Americans aged 65 and older had AD (Alzheimer's Association, 2022). It is estimated that the number of people with AD aged 65 and older will reach 12.7 million by 2050 (Alzheimer's Association, 2022). With no cure, AD is the 6th leading cause of death in the United States according to the CDC. In 2019, 121,499 people died with AD as an underlying cause of death (CDC, 2022).

Once diagnosed with AD, people aged 65 and older will on average live another four to eight years and most will require the aid of a caregiver as the disease progresses (Alzheimer's Association, 2022). Due to the prolonged nature of the illness, AD has a significant burden on family members and the public healthcare system. It is estimated that the loved ones of people suffering with AD provided nearly $257 billion in unpaid care in 2020 (Alzheimer's Association, 2022). In 2021, an estimated $355 billion in total payments went towards caring for individuals with AD or other dementias (Alzheimer's Association, 2022).

1.2.2 Classifications

Alzheimer's disease is a progressive illness and can be divided into three general phases. In the first phase, preclinical AD, individuals do not experience symptoms, but show changes in biomarkers that indicate early signs of AD. The second phase, called mild cognitive impairment (MCI) due to AD, is when subtle changes in memory and thinking begin to emerge, but do not

affect the individual's ability to perform everyday activities. The third phase, dementia due to AD, is characterized by changes in the individual's memory, thinking, or behavior that affect their ability to carry out everyday activities, in addition to evidence of biomarker changes consistent with AD.

The third phase in this progression, dementia due to AD, can further be broken down into three stages: mild, moderate, and severe. These stages reflect how strongly symptoms interfere with everyday activities. Individuals with mild Alzheimer's dementia are largely able to function independently and only need assistance with some activities. In moderate Alzheimer's dementia, individuals begin to struggle with routine daily activities, like dressing and bathing. They may also have difficulty communicating or show changes in personality and behavior. Moderate stage Alzheimer's dementia is typically the longest stage. In the severe stage, individuals are likely to need around-the-clock-care as AD begins to take a toll on their physical health.

In addition to the progressive phases, AD can also be divided by genetic predisposition and age of onset. Sporadic AD accounts for 95% of all AD cases (Bali, Gheinani, Zurbriggen, & Rajendran, 2012). It occurs when diagnosed individuals have no family history of the disorder (Sciences, 2015). Nearly all cases of sporadic AD start after the age of 65 or older, which is classified as late-onset AD (Sciences, 2015). Familial AD occurs in individuals that have two or more family members diagnosed with the disease and appears to be inherited in an autosomal dominant pattern (Sciences, 2015). Individuals diagnosed with AD before the age of 65 are said to have early-onset AD. Nearly 60% of early-onset AD are considered familial (Sciences, 2015).

1.2.3 Pathology

The neurodegenerative processes of AD can be seen with both a macroscopic and microscopic view of the brain. A gross dissection of a human AD brain will typically show cortical atrophy, narrowed gyri, widened sulci, and larger ventricles (DeTure, 2019). Additionally, medial temporal atrophy which includes the amygdala and hippocampus are common in AD brains (DeTure, 2019). While none of these features are specific to AD, they can be highly suggestive and useful in the diagnostic process.

To achieve a definitive diagnosis of AD, the brain must be examined at the microscopic level. The two "hallmarks" of AD diagnosis are amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs). Aβ plaques are extracellular accumulations of Aβ peptides which form from the abnormal processing of amyloid precursor protein (APP) by β and γ-secretase and an imbalance in the production and clearance pathways (DeTure, 2019). These plaques are believed to trigger the processes of inflammation, tau-tangle formation, synapse dysfunction, and cell death (Makin, 2018). NFTs are intracellular accumulations of microtubule-associated protein tau (Mapt). It is believed that hyperphosphorylation causes Mapt to dissociate from microtubules leading to microtubule destabilization and subsequent neuron death (Metaxas & Kempf, 2016).

1.2.4 Risk Factors

There are several risk factors that contribute to the development of AD, including environmental and genetic influences. In sporadic or late-onset AD, the greatest risk factor is age. The Alzheimer's Association reports that 11.3% of individuals aged 65 and older have Alzheimer's dementia. Additionally, the prevalence is shown to increase with age: 5.3% of

people aged 65-75, 13.8% of people aged 75-84, and 34.6% of people aged 80 and older have Alzheimer's dementia (Alzheimer's Association, 2022).

In addition to age, there is also a genetic risk factor that can contribute to late-onset AD. The APOE-e4 gene has been implicated as a major risk factor for AD. The gene codes for apolipoprotein E which facilitates binding and internalization of soluble Aβ. (Liu, Kanekiyo, Xu, & Bu, 2013). Carriers of APOE-e4 have more Aβ deposition in the form of senile plaques compared to non-carriers (Liu, Kanekiyo, Xu, & Bu, 2013). In contrast, APOE-e2 is associated with decreased risk of AD (Liu, Kanekiyo, Xu, & Bu, 2013).

Risk factors for familial or early-onset AD include mutations in the genes PSEN1, PSEN2, and APP which code for presenilin 1, presenilin 2, and amyloid precursor protein, respectively. Presenilin 1 and presenilin 2 are subunits of γ-secretase, which cleaves APP leading to the formation of Aβ. APP gene mutations lead to overproduction of Aβ (Kowalska, 2003). Due to its location on the 21st chromosome, APP can be overexpressed in individuals with Trisomy 21, which leads to increased Aβ deposition and increased risk of early-onset AD (Doran E, 2017).

In addition to genetic risk factors, several health conditions have also been linked to increased risk of AD. These acquired risk factors include cerebrovascular diseases, hypertension, type 2 diabetes, obesity, dyslipidemia, marital status, stress, depression, inadequate sleep, and smoking (Silva, 2019). Apart from the above-mentioned diseases, epidemiological studies have strongly suggested that TBI is a significant risk factor for AD.

1.2.5 TBI as a risk factor to the development of AD

Growing research shows TBI is a risk factor for dementia and AD (Gardner, et al., 2014) (Fleminger, Oliver, Lovestone, Rabe-Hesketh, & Giora, 2003) (Plassman, et al., 2000) (Sivanandam & Thakur, 2012). Studies also suggest that the magnitude of risk for developing dementia/AD is dependent on severity of the TBI. While both moderate and severe TBI have been associated with increased risk of AD, mild TBI does not show the same result (Gardner, et al., 2014). Another study also included that head injury with loss of consciousness (LOC) corresponds to higher incidences of AD compared to head injury without LOC (Guo, et al., 2000).

A history of TBI has been associated with accelerated age of onset of AD. In a study of people diagnosed with AD, those that had a history of TBI with LOC had a mean age of AD onset 2.5 years earlier than participants without a history of TBI (LoBue, et al., 2016). In another study, autopsy-confirmed AD individuals with a history of TBI with LOC that occurred at least one year before their diagnosis were associated with an approximately 3 year earlier age of onset (Schaffert, et al., 2018). Furthermore, the accelerated cognitive decline in subjects with a history of TBI showed to be independent of sex, race, education, and APOE genotype (Iacono, Raiciulescu, Olsen, & Perl, 2021).

In addition to accelerating age of AD onset, TBI has also showed to accelerate the hallmarks of AD pathology: Aβ deposition and tau abnormalities. Aβ deposits have been observed in approximately 30% of patients dying acutely from head injury (Roberts, et al., 1994) (Sivanandam & Thakur, 2012). In a study comparing the post-mortem brains of long-term TBI survivors to uninjured controls, NFTs were rarely found in the uninjured controls, but were

abundant in about a third of TBI survivors and Aβ plaques were found in greater density in TBI survivors than the controls (Johnson, Stewart, & Smith, 2012).

TBI causes a myriad of biologic responses including: brain swelling, axonal injury, disruption of the blood brain barrier, increased inflammatory responses, and neurodegeneration (Sivanandam & Thakur, 2012). The cerebrovascular response to TBI is one aspect that connects TBI and AD. TBI alters blood vessel cells and maintenance leading to long-term changes in brain physiology (Jullienne, et al., 2016). Cerebrovascular dysfunction following TBI has showed to be a potential contributor to Aβ/tau deposition, neurodegeneration, and early initiation of AD-like pathology (Ramos-Cejudo, et al., 2018). Microbleeds detected in chronic stages of TBI cause persistent inflammation which may lead to activation of microglia, stimulation of gliosis, and apoptosis, all of which are associated with AD (Ramos-Cejudo, et al., 2018).

The neuroinflammatory response to TBI has also been implicated in connecting TBI and AD, as it is seen in both diseases. In a study analyzing neuroinflammatory changes following TBI, an AD transgenic mice model (APP/PS1 mice) showed a delayed neuroinflammatory response compared to wild-type mice, with a peak at 7 days post-injury (Webster, Van Eldik, Watterson, & Bachstette, 2015). However, the APP/PS1 mice had a more persistent neuroinflammatory response leading to chronic neuroinflammation (Webster, Van Eldik, Watterson, & Bachstette, 2015). Additionally, after injury, the APP/PS1 mice showed a decrease in memory capability compared to sham and injured wild type mice (Webster, Van Eldik, Watterson, & Bachstette, 2015). Another study using a mouse model of cerebral amyloidosis found that inflammatory cytokine expression was enhanced in the AD-model compared to non-transgenic mice

regardless of injury group (Kokiko-Cochran, et al., 2016). This study suggested that accumulating Aβ leads to an altered post-TBI immune response (Kokiko-Cochran, et al., 2016).

1.2.6 AD animal models

The most commonly used experimental animal models for AD are transgenic mice that overexpress genes associated with familial AD (Drumond & Wisniewski, 2018). There are several different models of AD mice that express one or more of the hallmark pathological signs of AD. Expression of *APP* and/or *PSEN1* results in the formation of amyloid plaques while the expression of *MAPT* results in the expression of neurofibrillary tangles (Drumond & Wisniewski, 2018). It stands to reason that a more complete model of AD would account for both amyloid plaques and NFTs. The triple transgenic model of AD (3xTg-AD or 3xTg) expresses three mutations, specifically: β-amyloid precursor protein (βAPPSwe), presenilin-1 (PS1M146V), and tauP301L (Oddo, Caccamo, Kitazawa, Tseng, & LaFerla, 2003). The 3xTg mice develop extracellular Aβ around 6 months and NFTs around 12 months (Drumond & Wisniewski, 2018). The 3xTg model is the most widely used and regarded as the most complete transgenic mouse model for AD pathology (Drumond & Wisniewski, 2018). The 3xTg model is however limited in that it is not representative of sporadic AD and production of Aβ and NFTs is over-expressed in a non-physiologic manner (Drumond & Wisniewski, 2018).

1.3 NRLP3 Inflammasome

As previously stated, various studies have shown a strong connection between TBI and the development of AD, however the pathway that links them together is still largely unknown.

In recent years, studies have shown both TBI and AD are associated with the NLRP3 inflammasome.

As part of the innate immune system, inflammasomes are cytosolic multiprotein complexes that mediate proinflammatory cytokines. They typically consist of a sensor protein, an adaptor protein containing a caspase recruitment domain, and pro-inflammatory caspase. In the case of the NLRP3 inflammasome, the respective components are NLRP3, ASC, and pro-caspase-1. As seen in Figure 1.1, formation of the inflammasome is triggered by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Upon formation, active caspase-1 is released and promotes maturation of pro-inflammatory cytokines IL-1β and IL-18. This process leads to inflammation and an amplified immune response.

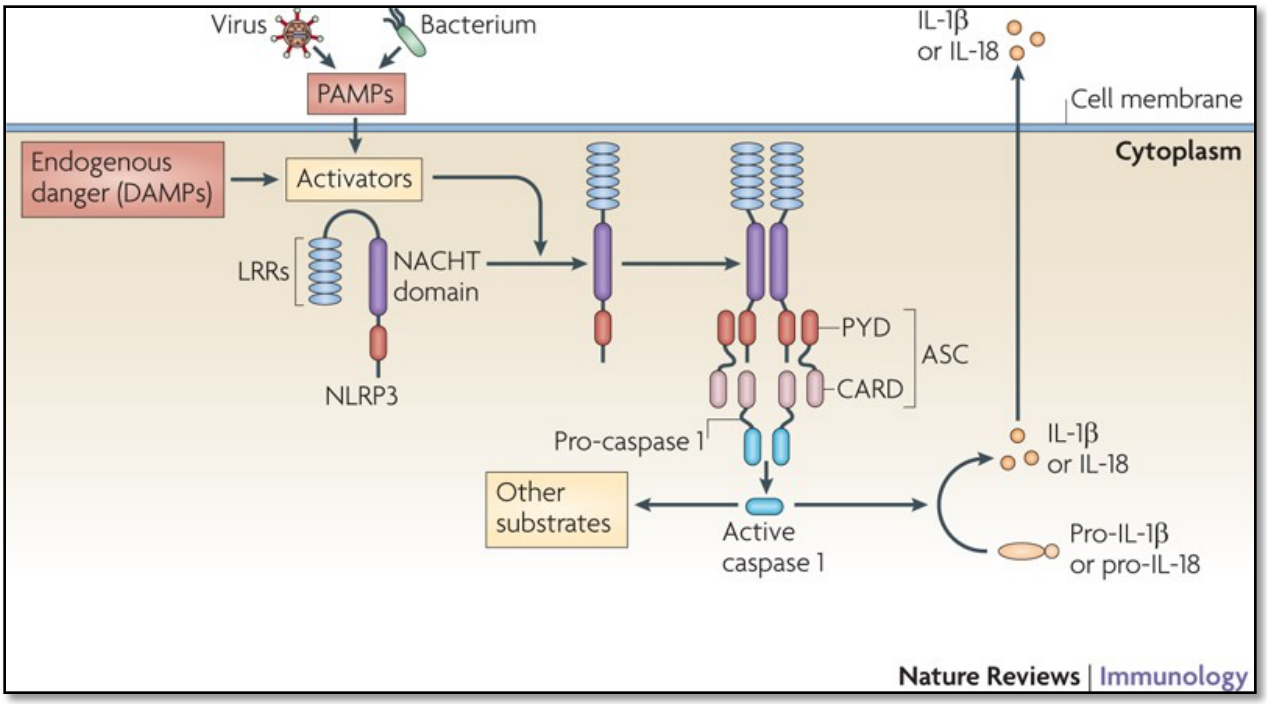

Figure 1.1 Mechanism of NLRP3 inflammasome complex formation.
Image extracted from (Tschopp & Schroder, 2010). PAMPs and DAMPs trigger the association of the NLRP3 inflammasome. The inflammasome is composed of NLRP3, ASC, and pro-caspase-1. The activated inflammasome releases active caspase-1 which promotes maturation of pro-inflammatory cytokines IL-1β and IL-18, which subsequently get released from the cell.

1.3.1 TBI induced inflammasome up regulation

Following a TBI, neuroinflammation is a significant concern as it can lead to lasting brain damage. Due to their role in the innate immune system and proinflammatory cytokine mediation, inflammasomes have been considered as a potential therapeutic target for reducing damage caused by post-TBI inflammation. Several studies have shown there is a link between TBI and the NLRP3 inflammasome.

A study published in 2009, showed that a moderate fluid percussive injury promoted formation of the NLRP1 inflammasome, which is similar in structure to the NLRP3 inflammasome (de Rivero Vaccari, et al., 2009). Additionally, activation of downstream proteins caspase-1 and IL-1β was also observed following injury (de Rivero Vaccari, et al., 2009). One study showed that administration of anti-ASC neutralizing antibodies immediately after injury resulted in reduced caspase-1 activation and IL-1β processing (de Rivero Vaccari, et al., 2009). In 2013, a similar study showed that TBI also caused formation of the NLRP3 inflammasome as well as increased expression of downstream proteins (Liu, et al., 2013). NLRP3 protein levels have also showed to increase in CSF following severe TBI in infants and children (Wallisch, et al., 2018). These studies support that TBI induces inflammasome upregulation and frames inflammasomes as a potential therapeutic target for post-TBI care.

Our lab has been studying how inhibiting the NLRP3 inflammasome affects the neuroinflammatory response following TBI. Adult rats given a novel drug with specificity for the NLRP3 inflammasome after TBI had decreased injury-induced degenerating neurons, inflammatory cell response, and cortical lesion volume compared to non-treatment groups (Kuwar, et al., 2019). The treatment group also had reduced protein expression levels of NLRP3,

ASC, IL-1β, TNFα, iNOS, and caspase-1 (Kuwar, et al., 2019). In these studies, animals were subjected to a moderate cortical impact injury and were sacrificed 2 days post injury (Kuwar, et al., 2019). This pharmacologic approach to inhibiting the NLRP3 inflammasome suggests that NRLP3 reduction has an anti-inflammatory effect that can be used to protect the brain following TBI.

1.3.2 Link to Alzheimer's Disease

In addition to TBI, NLRP3 and the NLRP3 inflammasome have been linked to AD. Components of the inflammasome and its downstream effectors showed to be upregulated in severe and mild AD cases (Saresella, et al., 2016). Accumulation of Aβ and NFTs as well as neuroinflammation are key pathological signs of AD, however the cellular events that lead to these anomalies are not fully understood. Because inflammatory mediators like IL-1β contribute to the neuroinflammation observed in AD, it is reasonable to suspect inflammatory regulators, like the NLRP3 inflammasome, may also be involved in the pathogenesis of AD.

The NLRP3 inflammasome is involved in the innate immune response to Aβ (Halle, 2008). Activated microglia are recruited to phagocytose Aβ and secrete cytokines. IL-1β released by activated microglia has been observed in higher concentrations surrounding Aβ plaques in animal models of AD (Griffin, et al., 1989). IL-1β is dependent on the activity of caspase-1 which is controlled by inflammasomes. The NLRP3 inflammasome showed to be activated by Aβ phagocytosis and subsequent lysosomal damage as well as shown to be a requirement for Aβ-induced activation of caspase-1 and the subsequent release of mature IL-1β

(Halle, 2008). These findings suggest that activation of the NLRP3 inflammasome by Aβ may play a vital role in the inflammatory response of AD.

Additionally, a reduction of the NRLP3 inflammasome showed to cause an increase in differentiation of microglia cells to an M2 (anti-inflammatory) phenotype leading to decreased Aβ deposition in the APP/PS1 model of AD (Saresella, et al., 2016). Analysis of NLRP3 inflammasome associated genes and proteins in individuals with either AD or mild cognitive impairment showed the NRPL3 inflammasome is activated in individuals with AD but not in those with only mild cognitive impairment (Saresella, et al., 2016).

A reduction of NLRP3 and caspase-1 via knockout in APP/PS1 mice showed animals were largely protected against loss of spatial memory and other AD associated deficiencies (Heneka, et al., 2013). Reduction of NLRP3 and caspase-1 via knockout in APP/PS1 mice has also showed to result in reduced caspase-1 and IL-1β activation and an increase in Aβ clearance (Heneka, et al., 2013).

In addition to microglia, Aβ has showed to activate astrocytes (Couturier, 2016). Down regulation of inflammasome activity via ASC knockout increases phagocytosis in astrocytes (Couturier, 2016). Down regulation of inflammasome activity may be a reason for the decrease in amyloid load and rescue of memory deficits in AD mice. These studies suggest that activation of inflammasome complexes could explain AD-associated neuroinflammation.

1.4 Hypothesis

As mentioned above, TBI is a leading risk factor for development of AD; however, the underlying mechanisms are unclear. Thus far, published studies have found that 1) TBI-induced NLRP3 inflammasome activation is critical for subsequent neuroinflammatory response; 2) NLRP3 inflammasome plays an important role in AD pathological development; 3) in AD animal models, TBI induces an abnormal immune response. Based on these observations, we hypothesize that TBI induced activation of the NLRP3 inflammasome mediates an abnormal neuroinflammatory response in the brain in the predisposition of AD and knock down of NLRP3 inflammasome could mitigate this response. Using 3xTg AD mice, a widely used transgenic AD mice model, and 3xTg AD/NLRP3$^{-/-}$ mice, a strain created in our lab with specific deletion of NLRP3 gene of the 3xTg AD mice, as well as the matched wild type control strain, this study is set to examine TBI-induced neuroinflammatory response related to NLRP3 inflammation at the acute stage following TBI (2 days post-injury) using Western Blotting and ELISA methods.

Chapter 2: Methods and Materials

2.1 Experimental Animals

For this study, three genotypes of mice were included: wild type (WT), 3xTg-AD (3xTg), and 3xTg-AD/NLRP3$^{-/-}$ (3xTg/KO). The 3xTg mice are a widely used triple-transgenic AD model that carry three mutant genes: β-amyloid precursor protein (βAPPSwe), presenilin-1 (PS1M146V), and tauP301L (Oddo, et al., 2003). These mice exhibit amyloid plaques, neurofibrillary tangles, synaptic dysfunction, and hyperphosphorylated tau (Oddo, Caccamo, Kitazawa, Tseng, & LaFerla, 2003). The 3xTg/KO mice are a novel line of mice created by crossing 3xTg mice with NLRP3 knockout mice and therefore lack NLRP3 gene expression. The 3xTg/KO mice were generated and bred by VCU's Transgenic Mice Core and their genotype was confirmed through PCR genotyping. Animals were divided by sex and genotype and then randomly selected to be either injured or sham. The selected 3xTg, 3xTg/KO and age and sex matched wild type controls received a central fluid percussive injury at 4-months of age, before the onset of AD. A total of 22 male and 26 female mice were used. Experimental cohorts are outlined in Table 1. Animals were sacrificed two days post-injury. Prior to sacrifice, animals were housed in the animal facility with a 12-hour light/dark cycle at room temperature with water and food provided ad libitum. All procedures were approved by the Institution of Animal Care and Use Committee (IACUC).

Males (n=22)		
	Sham	TBI
WT	n=3	n=3
3xTg	n=4	n=4
3xTg/KO	n=3	n=5
Females (n=26)		
	Sham	TBI
WT	n=3	n=4
3xTg	n=3	n=5
3xTg/KO	n=5	n=6

Figure 2.1 Experimental Cohorts.

2.2 Surgical Procedure - Central Fluid Percussion Injury

Animals were subjected to a moderate central fluid percussion injury (cFPI). This model causes diffuse axonal injury with minimal focal tissue damage. The surgical instruments were sterilized and aseptic procedures were followed. Mice were first anesthetized in a plexiglass chamber with 4% isoflurane. Mice were then fixed to a stereotaxic frame and ventilated with 2.5% isoflurane mixed with 30% oxygen and room air for the duration of the surgery. A continuously heated water pad was placed under the mice during surgery to protect from hypothermia. A midline incision on a shaved head exposed the skull and a 2.7mm trephine was used for craniotomy to expose the brain without breaching the dura mater. A Luer-lock syringe hub was affixed to the craniotomy site. Animals were then brought out of anesthesia and allowed to rest for one hour. All mice, apart from sham animals, were subjected to cFPI one hour after the surgical preparation for placement of the hub. Mice were anesthetized again using 4% isoflurane and secured to the fluid percussion instrument. In order to minimize the effect anesthesia may have on mice during injury, mice were not injured until there were signs the anesthesia was beginning to wear off. The height of the pendulum was adjusted to administer an injury with the range of 1.6-1.8 atmospheres. Mice were attached via a saline-filled hub to the Luer-Lock fitting on the fluid percussive device (VCU Biomedical Engineering Facility, Richmond, VA, USA). Injury was produced by releasing the pendulum resulting in force applied to the saline-filled reservoir, transmitting a pressure wave into the closed cranial cavity. The peak pressure displayed on the oscilloscope and righting time for the mouse were recorded. Righting time ranged from 5-10 minutes. After another period of rest, mice were re-anesthetized and the Luer-lock syringe hub was removed. After suturing the incision, triple anti-

biotic ointment and Lidocaine were applied to the skin incision area. Animals were returned to a clean cage on a warm blanket until fully recovered before being returned to the animal care facility. The general condition of the animals was observed daily until they were sacrificed.

2.3 Sacrifice and Tissue Processing

Animals were sacrificed 2 days post-injury. Mice were deeply anesthetized with an overdose of isoflurane inhalation followed by a transcardial perfusion with 30mL of ice-cold PBS. The brains were then quickly dissected on ice. Half of the brain was dissected for protein analysis and the other half for histology. For protein analysis, samples from the cortex and hippocampus were separated and homogenized with RIPA buffer (Stock 10x RIPA, EMD Millipore, MA), containing 10% TritonX-100, 10% SDS solution, protease inhibitor, and 0.5 M EDTA. Homogenates were centrifuged at 14,000 rpm for 25 minutes and supernatants were collected and stored at -80°C until use. The total protein concentration was determined using BCA method (Pierce, Rockford, IL).

2.4 Western Blotting

Cortical and hippocampal tissue lysate was processed for Western blotting. Samples were loaded onto an SDS-PAGE Criterion TGX Stain-Free gel (Bio-Rad, USA). Depending on sample concentration and protein expression, the total protein load and gel percentage varied. Gels were activated with UV light using the Chemidoc MP Imaging System (Bio-Rad, USA) for 45s before transfer to a PVDF membrane using a Trans-Turbo Blot transfer system (Bio-Rad, USA). Following transfer, a stain-free image was obtained to be used for normalization. Blots were blocked for 1 hour at room temperature before being incubated with a primary antibody

overnight at 4°C. The following primary antibodies were used: NLRP3 (1:800; #15101S, Cell Signaling Technology, MA, USA), ASC (1:800; AG-25B-0006-C100, Adipogen Life Sciences, CA, USA), caspase-1 (1:1000; Cell Signaling Technology, MA, USA), IL-1β (1:300; ab200478, Abcam, MA, USA), GSDMD (1:1000; #10137, Cell Signaling Technology, MA, USA), HMGB1 (1:2000; NB100-2322, Novus Biologicals, CO, USA), and GFAP (1:1000; Dako Z0334). After primary antibody incubation, membranes were washed 5 times for 5 minutes, before being incubated with the secondary antibody for 1 hour at room temperature. The secondary antibody used was horseradish peroxidase-conjugated anti-rabbit IgG (1:5000; Cell Signaling, MA, USA). The membranes were then washed again 5 times for 5 minutes and developed with Clarity Western ECL Substrate (Bio-Rad, USA). Chemiluminescent images were obtained using the Chemidoc MP Imaging system. Finally, ImageLab software (Bio-Rad, USA) was used to obtain the normalized volume intensity for each sample.

2.5 ELISA

The level of IL-1β in cortical brain tissue lysate was measured using ThermoFischer ELISA Mouse IL-1β Kit (MBS6002), following the manufacturer's instructions. Limits of detection were 7.8 – 500 pg/mL. Microwell strips were washed twice with approximately 400μL of wash buffer before 100μL standard dilution and 100μL of each diluted sample were added. Samples were run in duplicate with 100μg of protein loaded to each well. Next, 50μL of Biotin-Conjugate was added to each well before incubating on a shaker for two hours at room temperature. Following incubation, wells were washed 4 times with approximately 400μL of wash buffer. Next, 100μL of diluted Streptavidin-HRP was added to each well before covering and incubating on shaker for one hour at room temperature. Wells were then washed following the protocol

above. Then 100μL of TMB Substrate Solution was added to each well before incubating for 10 minutes at room temperature with care to avoid direct lighting. Finally, the reaction was stopped by adding 100μl of stop solution to each well. Absorbance was read at 450nm using a spectro-photometer.

2.6 Immunoprecipitation

In order to increase signal detection of NLPR3, samples from each group were pooled and immunoprecipitated. ThermoScientific's Pierce Protein A/G Agarose kit and instructions were used. Samples of same genotype and injury were equally combined for a total of 180μg per group. Antigen samples were combined with the optimized amount of antibody and incubated overnight at 4°C. The following day, 100μL of Immobilized Protein A/G resin in slurry was added and supernatant discarded after a brief centrifuge. Next, the samples were washed with 0.5mL of IP buffer 3 times with the supernatant discarded after each wash. The antigen-antibody complex was added to the resin and incubated with gentle mixing for two hours at room temperature. Next, the samples were washed again following the same procedure listed above. Finally, the complex-bound resin was washed with 0.5mL water, centrifuged, and supernatant discarded. The samples were then heated for 10 minutes at 95°C and then subjected to Western blot protocol.

2.7 Statistical Analysis

The volume intensity values obtained from Image Lab following Western Blotting were normalized to the control average. Values obtained from ELISA remained in their densitometric units. All data was run through GraphPad's online Outlier Calculator which performs a Grubb's

test. Outliers were removed from data pool for groups where n > 3. Data was then analyzed using GraphPad Prism 8 software to determine the significance of genotype and injury. A two-way analysis of variance (ANOVA) test was performed with Tukey's multiple comparisons test. A p value less than or equal to 0.05 was considered statistically significant. Data is presented as mean ± SEM in all figures.

Chapter 3: Results

3.1 TBI induced changes in the NLRP3 signaling pathway

3.1.1 Confirmation of NLRP3 gene knock down in 3xTg/NLRP3 KO Mice

We first assessed the expression of the NLRP3 protein in our mice strains to confirm the knock down of NLRP3 gene in our 3xTg/KO mice using Western blotting. The protein expression level of NLRP3 in the cortex was assessed in a group of 3xTg and 3xTg/KO female mice at two days post injury in both sham and TBI conditions. As shown in Figure 3.1, NLRP3 protein (110Kd band) was detected in the 3xTg cortex samples in both the sham and TBI groups and was absent in the 3xTg/KO mice cortex samples in both sham and TBI groups. A two-way ANOVA was performed to analyze the effect of genotype and injury on NRLP3 expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury ($F(1, 9) = 1.491$, $p=0.2530$). Simple main effects analysis showed that genotype did have a statistically significant effect on NLRP3 expression ($F(1, 9) = 33.78$, $p=0.0003$), but injury did not ($F(1, 9) = 1.491$, $p=0.2530$). Post-hoc multiple comparisons analysis revealed there was significantly lower NLRP3 expression in 3xTg/KO mice compared to 3xTg mice in both the sham ($p=0.0484$) and injured groups ($p=0.0028$). Additionally, there was a slight increase of NLRP3 expression in the injured 3xTg group indicating some degree of injury effect.

After confirming the successful generation of 3xTg/NLRP3 KO mice line, I performed WB on all samples collected for this study to assess the changes in protein expression level of NLRP3 in association to TBI in the context of AD. Due to technical issues in Western blotting,

the expression of NLRP3 in other samples was not detected despite all efforts in trouble shooting. In attempt to boost signal detection for NLRP3, samples from each group were pooled for immunoprecipitation. Unfortunately, as seen in Figure 3.2, no signal was detected in any group from the female cortex samples.

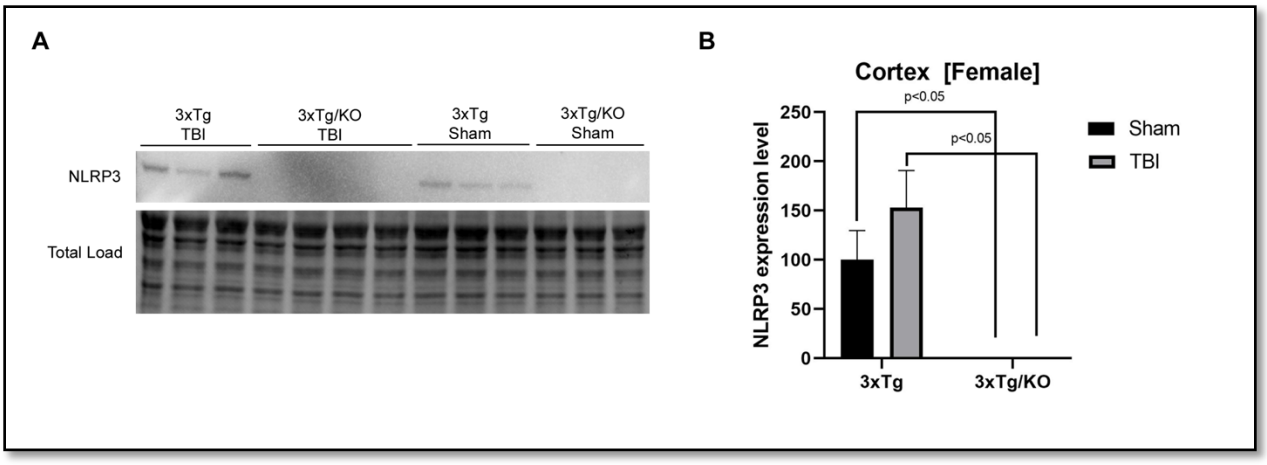

Figure 3.1 NLRP3 protein expression level in the cerebral cortex of female mice.

A) Western blot image showing 110kD NLRP3 detected in the cortex of female 3xTg-Sham and 3xTg-TBI groups (60ug protein, 4-15% gel), NLRP3 is not detected in the 3xTg/KO animals in both sham and TBI groups. B) The densitometry values in the bar graph showed 3xTg animals had significantly higher expression of NLRP3 compared to 3xTg/KO-TBI animals in both sham and TBI groups.

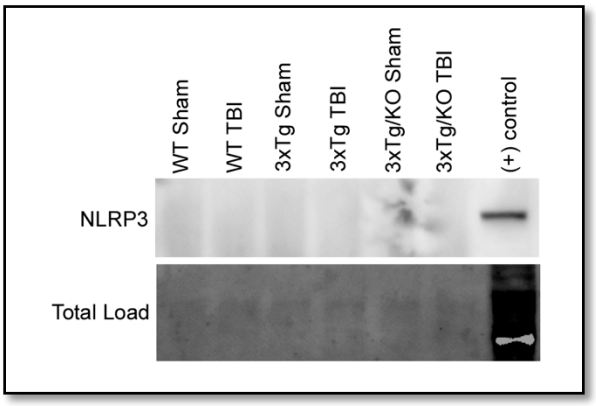

Figure 3.2 Immunoprecipitation to detect NLRP3 expression in the cerebral cortex

Western blot image for 110kD NLRP3 in the cortex of female WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups after immunoprecipitation (180μg protein, 4-15% gel). No bands were detected in any of the sample groups. The NLRP3 band was detected in the positive control sample (J774A.1 cells-macrophages that overexpress NLRP3).

Subsequently, to assess changes in the NLRP3 signaling pathway, the expression of NLRP3 inflammasome associated proteins was analyzed. Using Western blotting, the expression of NLRP3 adaptor protein ASC, downstream effector caspase-1, IL-1 beta, and GSDMD was analyzed in WT, 3xTg, and 3xTg/KO samples of both cortex and hippocampus in both sham and TBI groups.

3.1.2 ASC

ASC is an adapter protein that is required for the formation of the NLRP3 inflammasome. Due to technical issues in Western Blotting, the expression of ASC was not detected despite all efforts in trouble shooting. Trouble shooting efforts to detect ASC included testing of different antibodies, gel percentages, transfer procedure, denaturing procedure, and total protein load. None of the efforts were successful to detect the 22kD band.

3.1.3 Caspase-1

Caspase-1 is synthesized as a pro-protein and undergoes autoproteolysis within inflammasomes which results in the generation of the active enzyme subunits, p20 and p10 (Broz, von Moltke, Jones, Vance, & Monach, 2010). Activate caspase-1 is required for processing and release of IL-1β and GSDMD. In this study, the p20 form of activated caspase-1 was measured using Western Blotting.

In male cortex tissues, as shown in Figure 3.3, expression of caspase-1 (p20) (20kD band) was detected across all male cortex samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on caspase-1 expression. The results revealed that there was a statistically significant interaction between the effects of genotype and injury ($F(2, 15) = 6.712$,

p=0.0083). Simple main effects analysis showed that genotype did have a statistically significant effect on caspase-1 expression (F (2 ,15) = 5.091, p=0.0205), but injury did not (F (1, 15) = 0.08106, p=0.7798). Post-hoc multiple comparisons analysis showed significantly lower caspase-1 expression in 3xTg/KO-TBI compared to 3xTg-TBI (p=0.0122). In 3xTg mice, higher expression was found in the TBI groups compared to the sham, however, no statistical significance was found.

In male hippocampal tissues, as shown in Figure 3.4, expression of caspase-1 (p20) (20kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on caspase-1 expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury (F (2, 15) = 3.390, p=0.0610). Simple main effects analysis showed that genotype did not have a statistically significant effect on caspase-1 expression (F (2, 15) = 1.705, p=0.2151) and neither did injury (F (1, 15) = 0.3134, p=0.5839).

In female cortex tissues, as shown in Figure 3.5, expression of caspase-1 (p20) (20kD band) was detected across all female cortex samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on caspase-1 expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury (F (2, 17) = 2.845, p=0.0860). Simple main effects analysis showed that genotype did have a statistically significant effect on caspase-1 expression (F (2, 17) = 10.29, p=0.0012), but injury did not (F (1, 17) = 0.0236, p=0.8797). Post-hoc multiple comparisons analysis revealed significantly higher caspase-1 expression in 3xTg-TBI compared to WT-TBI (p=0.0091) and significantly lower caspase-1 expression in 3xTg/KO-Sham compared to 3xTg-Sham (p=0.0459).

In female hippocampal tissues, as shown in Figure 3.6, expression of caspase-1 (p20) (20kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on caspase-1 expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury ($F(2, 15) = 0.6697$, $p=0.5265$). Simple main effects analysis showed that genotype did have a statistically significant effect on caspase-1 expression ($F(2, 15) = 4.505$, $p=0.0294$), but injury did not ($F(1, 15) = 0.0203$, $p=0.8885$). Post-hoc multiple comparisons analysis did not reveal any significant comparisons.

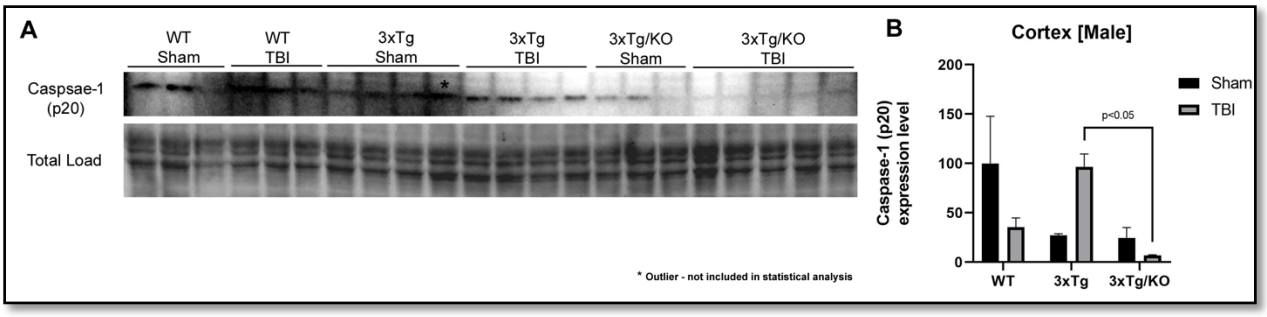

Figure 3.3 Caspase-1 expression level in the cerebral cortex of male mice.

A) Western blot image showing 20kD band of caspase-1 detected in the cortex of male WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (60μg protein, 12% gel). B) The densitometry values in the bar graph showed a significant decrease in caspase-1 expression in 3xTg/KO-TBI compared to 3xTg-TBI.

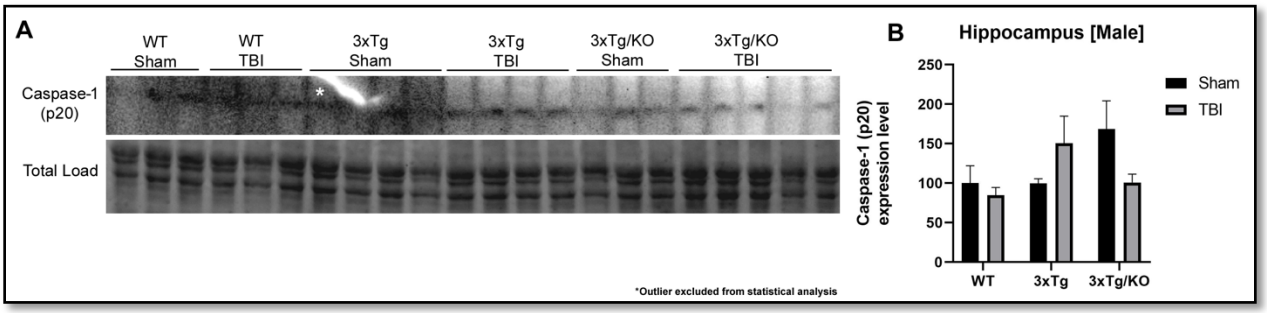

Figure 3.4 Caspase-1 expression level in the hippocampus of male mice.

A) Western blot image showing 20kD band of caspase-1 detected in the hippocampus of male WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (40μg protein, 12% gel). B) The densitometry values in the bar graph did not show any statistical significance.

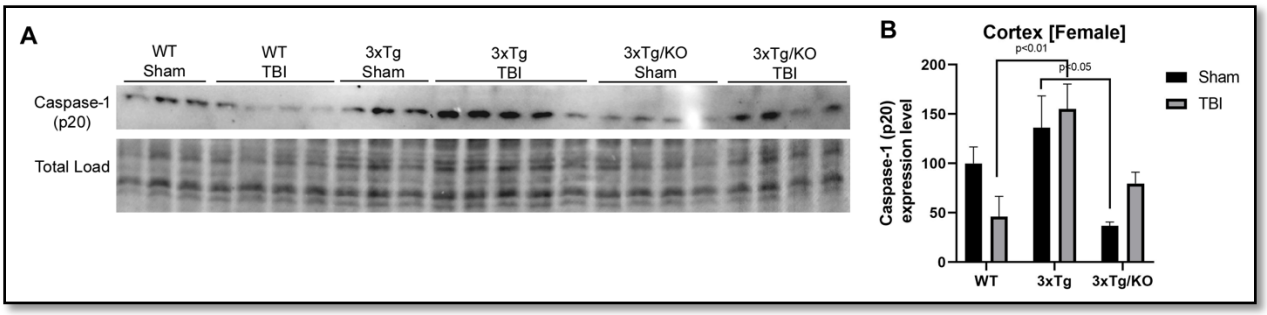

Figure 3.5 Caspase-1 expression level in the cerebral cortex of female mice.

A) Western blot image showing 20kD band of caspase-1 detected in the cortex of female WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (60μg protein, 12% gel). B) The densitometry values in the bar graph showed a significantly higher caspase-1 expression in 3xTg-TBI compared to WT-TBI. Additionally, there was a significantly lower caspase-1 expression in 3xTg/KO-Sham compared to 3xTg-Sham.

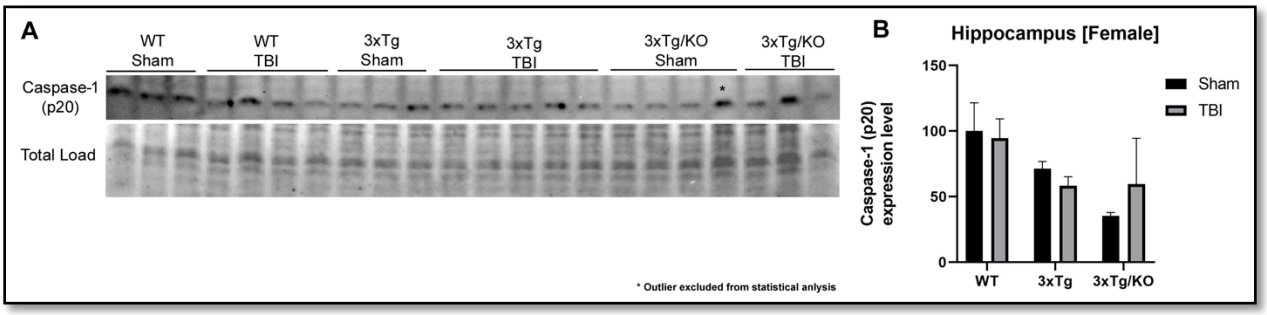

Figure 3.6 Caspase-1 expression level in the hippocampus of female mice.

A) Western blot image showing 20kD band of caspase-1 detected in the hippocampus of female WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (30μg protein, 12% gel). B) The densitometry values in the bar graph did not show any statistically significant data.

3.1.4 IL-1β

Il-1β is a key downstream cytokine of the NLRP3 inflammasome. The expression level of IL-1β was initially measured with Western blotting without success. Efforts then switched to ELISA using the same cerebral cortex homogenate samples that were used for Western blotting.

As seen in Figure 3.7, IL-1β expression was detected in all male cortex samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on IL-1β expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury (F (2, 14) = 0.1996, p=0.8214). Simple main effects analysis showed that genotype did not have a statistically significant effect on IL-1β expression (F (2, 14) = 2.100, p=0.1593). Simple main effects analysis did show injury had a statistically significant effect on IL-1β expression (F (1, 14) = 26.41, p=0.0002). Post-hoc multiple comparisons analysis did not reveal any significant comparisons.

As seen in Figure 3.8, IL-1β expression was detected in all female cortex samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on IL-1β expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury (F (2, 14 = 1.707, p=0.2171). Simple main effects analysis showed that genotype did have a statistically significant effect on IL-1β expression (F (2, 14) = 14.88, p=0.0003), but injury did not (F (1, 14) = 4.230, p=0.0588). Post-hoc multiple comparisons analysis showed lower expression of IL-1β in 3xTg-Sham compared to WT-Sham (p=0.0131) as well as lower expression of IL-1β in 3xTg/KO-Sham compared to WT-Sham (p=0.0076).

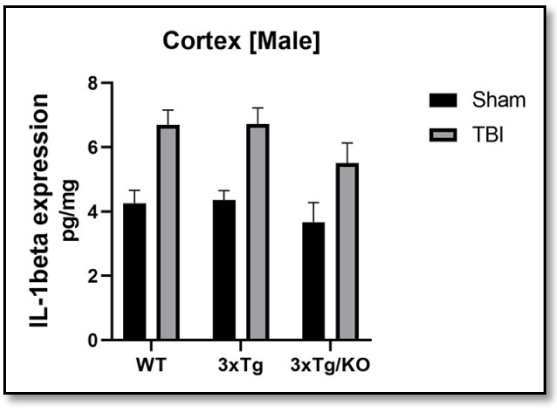

Figure 3.7 IL-1β expression level in the cerebral cortex of male mice.

Bar graph showing expression of IL-1β detected in the cortex of male WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (100μg protein) using ELISA. Data analysis did not show any statistical significance across groups.

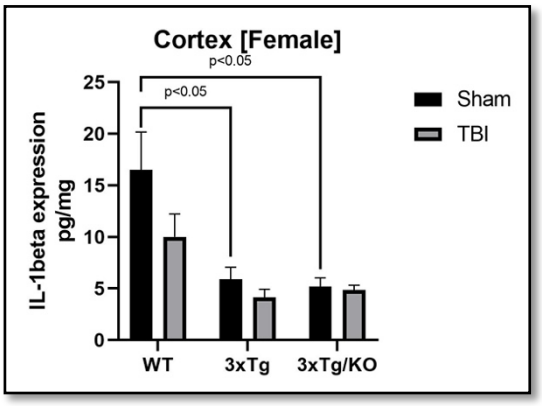

Figure 3.8 IL-1β expression level in the cerebral cortex of female mice.

Bar graph showing expression level of IL-1β detected in the cortex of female WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (100μg protein) using ELISA. Data analysis showed a significantly lower level of IL-1β expression in 3xTg-Sham and 3xTg/KO-Sham compared to WT-Sham.

3.1.5 Gasdermin D (GSDMD)

GSDMD is cleaved into its active form by caspase-1 and acts as an effector for pyroptosis. Due to low signal detection in Western blotting, the expression of GSDMD was not reported here.

3.2 Assessment of neuroinflammatory glial cell response

3.2.1 High mobility group box 1 protein (HMGB1)

HMGB1 is a nonhistone chromatin-associated protein that is released by activated monocytes and macrophages as well as passively released by damaged cells (Scaffidi, Misteli, & Bianchi, 2002). Inflammasome activation has showed to trigger release of HMGB1 from microglia, which goes on to act as an initiator of neuroinflammation (Paudel, et al., 2018). HMGB1 has showed to be a mediator for neurological conditions like TBI, neuroinflammation, and cognitive dysfunction (Paudel, et al., 2018). During TBI, HMGB1 acts a DAMP and is released from necrotic neurons (Richard , Min, Su, & Xu, 2017). Once released, HMGB1 binds with RAGE and TLR4, which promotes macrophages and endothelial cells to release TNF-α, IL-1, and IL-6 and also promotes further upregulation of HMGB1 and pro-inflammatory mediators (Paudel, et al., 2018). HMGB1 has also showed to be a mediator of cognitive impairment caused by the NRLP3 inflammasome in late stage TBI (Tan, et al., 2021). Using Western blotting, the protein expression level of HMGB1 in the cerebral cortex and hippocampus was measured.

In male cortex tissues, as shown in Figure 3.9, expression of HMGB1 (29kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on HMGB1 expression. The results revealed that there was a statistically

significant interaction between the effects of genotype and injury ($F(2, 16) = 10.85$, $p=0.0011$). Simple main effects analysis showed that genotype did have a statistically significant effect on HMGB1 expression ($F(2,16) = 25.28$, $p<0.0001$), but injury did not ($F(1, 16) = 2.621$, $p=0.1250$). Post-hoc multiple comparisons analysis showed HMGB1 expression was significantly higher in WT-Sham animals compared to WT-TBI ($p=0.0073$), 3xTg-Sham ($p<0.0001$), and 3xTg/KO-Sham ($p=0.0016$).

In male hippocampal tissues, as shown in Figure 3.10, expression of HMGB1 (29kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on HMGB1 expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury ($F(2,16) = 2.226$, $p=0.1403$). Simple main effects analysis showed that genotype did have a statistically significant effect on HMGB1 expression ($F(2,16) = 9.126$, $p=0.0023$), but injury did not ($F(1,16) = 0.8476$, $p=0.3709$). Post-hoc multiple comparisons analysis showed 3xTg/KO-TBI animals had significantly higher expression of HMGB1 compared to WT-TBI ($p=0.0045$) and 3xTg-TBI ($p=0.0212$) animals.

In female cortex tissues, as shown in Figure 3.11, expression of HMGB1 (29kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on HMGB1 expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury ($F(2,16) = 0.5321$, $p=0.5974$). Simple main effects analysis showed that genotype did have a statistically significant effect on HMGB1 expression ($F(2, 16) = 6.146$, $p=0.0105$), but injury did not ($F(1, 16) = 0.0021$,

p=0.9637). Post-hoc multiple comparisons analysis did not show any statistically significant changes in HMGB1 expression.

In female hippocampal tissues, as shown in Figure 3.12, expression of HMGB1 (29kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on HMGB1 expression. The results revealed that there was a statistically significant interaction between the effects of genotype and injury ($F(2, 18) = 4.007$, $p=0.0363$). Simple main effects analysis showed that genotype did have a statistically significant effect on HMGB1 expression ($F(2, 18) = 4.836$, $p=0.0209$), but injury did not ($F(1, 18) = 0.1796$, $p=0.6767$). Post-hoc multiple comparisons analysis did not show any statistically significant changes in HMGB1 expression.

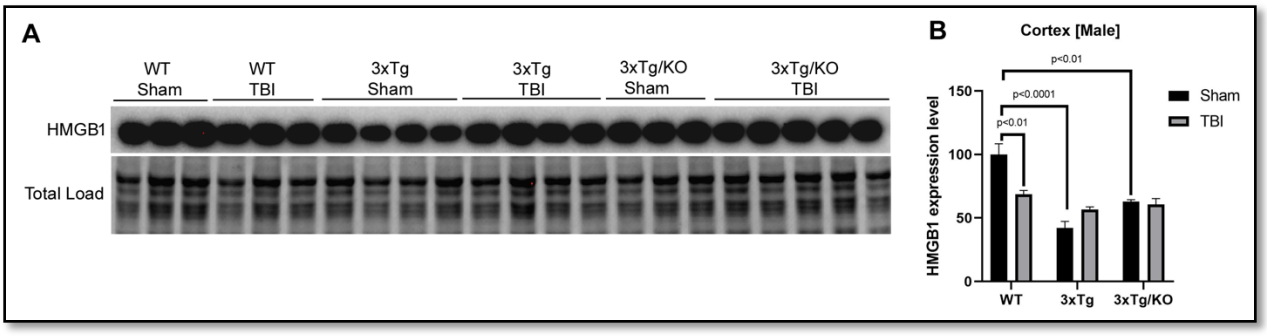

Figure 3.9 HMGB1 expression level in the cerebral cortex of male mice.

A) Western blot image showing 29kD HMGB1 detected in the cortex of male WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (20μg protein, 4-15% gel). B) The densitometry values in the bar graph showed WT-Sham animals had significantly higher expression of HMGB1 compared to WT-TBI, 3xTg-Sham, and 3xTg/KO-Sham animals.

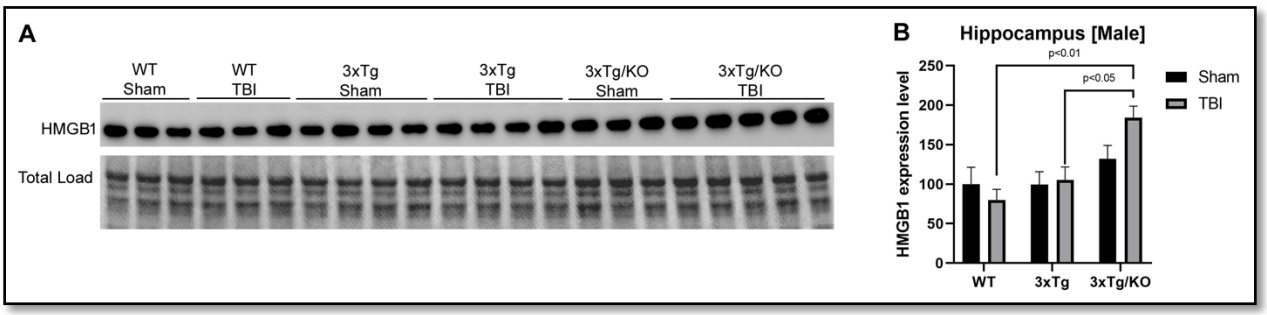

Figure 3.10 HMGB1 expression level in the hippocampus of male mice.

A) Western blot image showing 29kD HMGB1 detected in the hippocampus of male WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (20μg protein, 4-15% gel). B) The densitometry values in the bar graph showed 3xTg/KO-TBI animals had significantly higher expression of HMGB1 compared to WT-TBI and 3xTg-TBI animals.

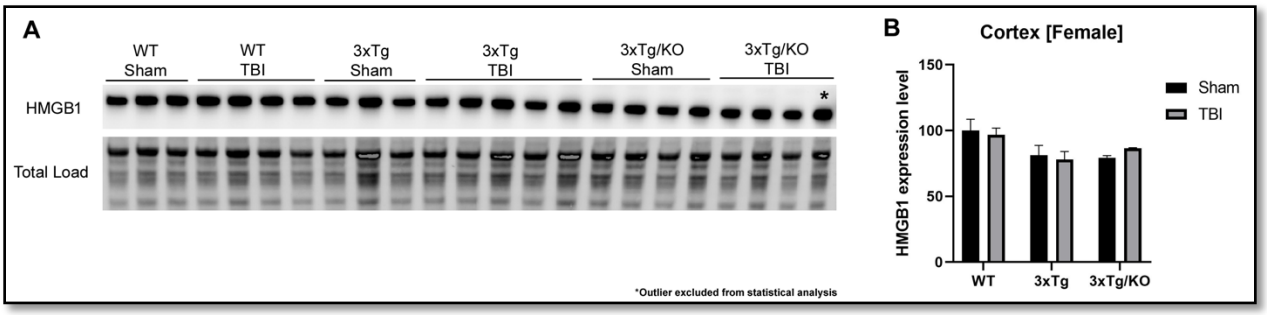

Figure 3.11 HMGB1 expression level in the cerebral cortex of female mice.

A) Western blot image showing 29kD HMGB1 detected in the cortex of female WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (20μg protein, 4-15% gel). B) The densitometry values in the bar graph did not show any statistical significance.

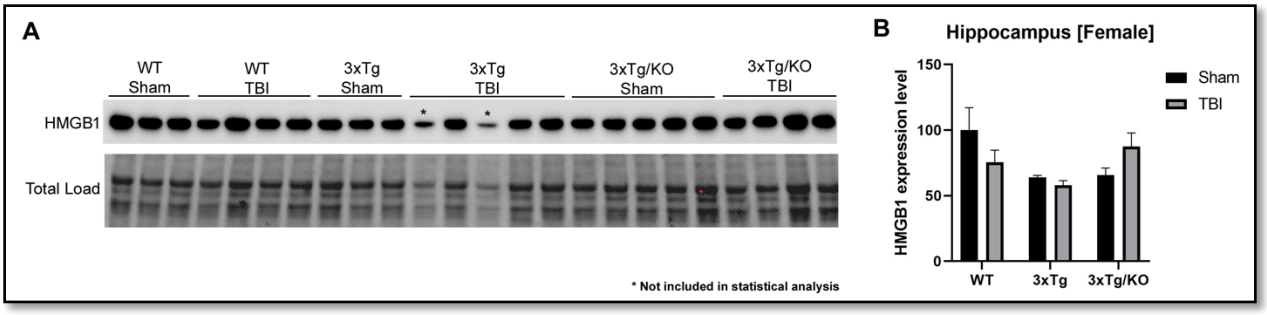

Figure 3.12 HMGB1 expression level in the hippocampus of female mice.

A) Western blot image showing 29kD HMGB1 detected in the hippocampus of female WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (20μg protein, 4-15% gel). B) The densitometry values in the bar graph did not show any statistical significance.

3.2.2 GFAP

Glial fibrillary acidic protein (GFAP) is a type III intermediate filament protein that is expressed in astrocytes. Following a CNS injury, GFAP expression is increased as reactive astrocytes undergo astrogliosis, a process to repair the initial damage from a CNS injury. Overexpression of GFAP in reactive astrocytes has showed to be correlated with AD progression (Serrano-Poza, et al., 2011). Additionally, density of GFAP-positive astrocytes has showed to be correlated with microglial activation and up-regulation of NLRP3 inflammasome-related proteins (Li, Ayaki, Maki, Sawamoto, & Takahashi, 2018). Using Western blotting, the protein expression level of GFAP in the cerebral cortex and hippocampus was measured.

In male cortex tissues, as shown in Figure 3.13, expression of GFAP (50kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on GFAP expression. The results revealed that there was a statistically significant interaction between the effects of genotype and injury ($F(2, 15) = 4.207$, $p=0.0355$). Simple main effects analysis showed that genotype did not have a statistically significant effect on GFAP expression ($F(2, 15) = 3.073$, $p=0.0761$), but injury did ($F(1, 15) = 21.55$, $p=0.0003$). Post-hoc multiple comparisons analysis showed that GFAP expression was significantly higher in the 3xTg-TBI group compared to 3xTg-Sham ($p=0.0029$). GFAP expression was also higher in 3xTg/KO-Sham compared to 3xTg-Sham ($p=0.0272$). Injury appeared to cause an increase in GFAP expression in WT mice, but not in the 3xTg/KO mice.

In male hippocampal tissues, as shown in Figure 3.14, expression of GFAP (50kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on GFAP expression. The results revealed that there was not a statistically

significant interaction between the effects of genotype and injury (F (2, 16) = 1.930, p=0.1774). Simple main effects analysis showed that genotype did have a statistically significant effect on GFAP expression (F (2, 16) = 8.136, p=0.0037) and injury also had an effect (F (1, 16) = 11.56, p=0.0037). Post-hoc multiple comparisons analysis showed that GFAP expression was significantly higher in the 3xTg-TBI group compared to 3xTg-Sham (p=0.0181). Slightly higher GFAP expression was also detected in the injured groups in WT and 3xTg/KO animals, however, the difference was not statistically significant.

In female cortex tissues, as shown in Figure 3.15, expression of GFAP (50kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on GFAP expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury (F (2, 17) = 0.5239, p=0.6015). Simple main effects analysis showed that genotype did not have a statistically significant effect on GFAP expression (F (2, 17) = 0.0541, p=0.9475), but injury did have an effect (F (1, 17) = 7.318, p=0.0150). Post-hoc multiple comparisons analysis did not show any statistically significant changes in GFAP expression. GFAP expression appeared to be higher in the injured groups in WT, 3xTg, and 3xTg/KO compared to the genotype matched sham group, however, the difference was not statistically significant.

In female hippocampal tissues, as shown in Figure 3.16, expression of GFAP (50kD band) was detected across all samples. A two-way ANOVA was performed to analyze the effect of genotype and injury on GFAP expression. The results revealed that there was not a statistically significant interaction between the effects of genotype and injury (F (2, 16) = 1.255, p=0.3116). Simple main effects analysis showed that genotype did not have a statistically significant effect

on GFAP expression ($F(2, 16) = 1.918$, $p=0.1791$), but injury did have an effect ($F(1, 16) = 6.515$, $p=0.0213$). Post-hoc multiple comparisons analysis did not show statistically significant changes in GFAP expression. Similar to female cortex samples, GFAP expression appeared to be higher in the injured groups in WT, 3xTg, and 3xTg/KO compared to the genotype matched sham group, however, the difference was not statistically significant.

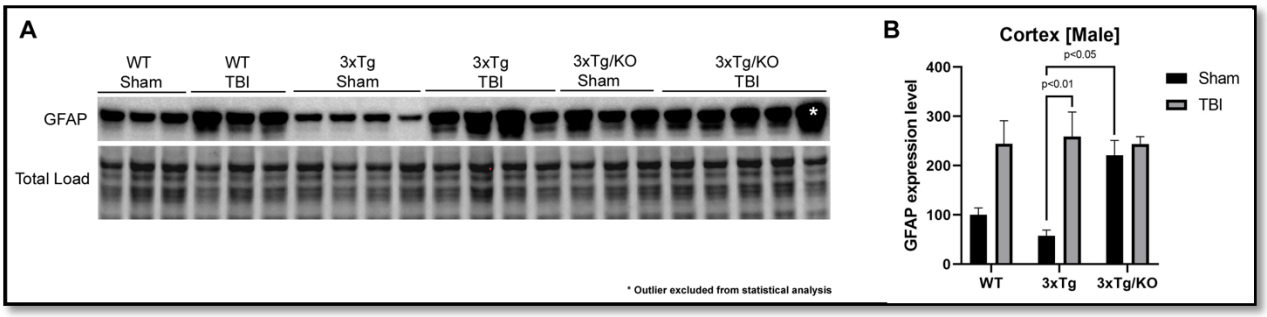

Figure 3.13 GFAP expression level in the cerebral cortex of male mice.

A) Western blot image showing 50kD GFAP detected in the cortex of male WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (20μg protein, 4-15% gel). B) The densitometry values in the bar graph showed GFAP expression was significantly lower in 3xTg-Sham compared to 3xTg-TBI and 3xTg/KO-Sham.

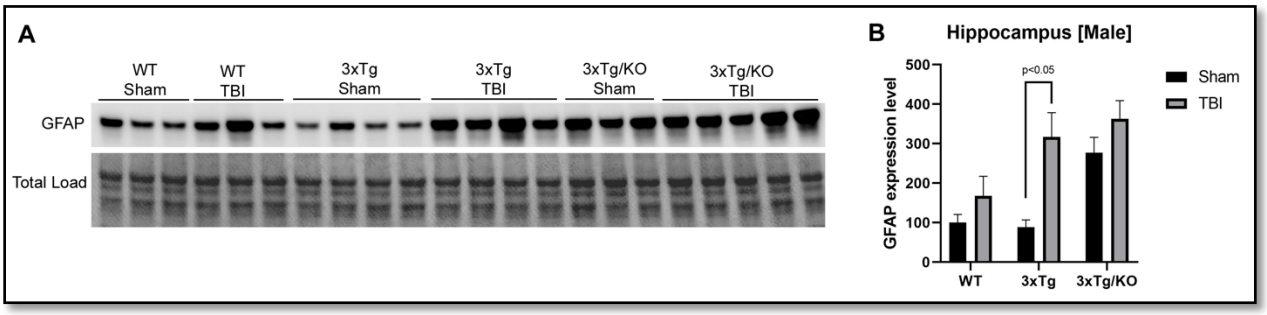

Figure 3.14 GFAP expression level in the hippocampus of male mice.

A) Western blot image showing 50kD GFAP detected in the hippocampus of male WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (20μg protein, 4-15% gel). B) The densitometry values in the bar graph showed 3xTg-Sham animals had significantly lower expression of GFAP compared to 3xTg-TBI animals.

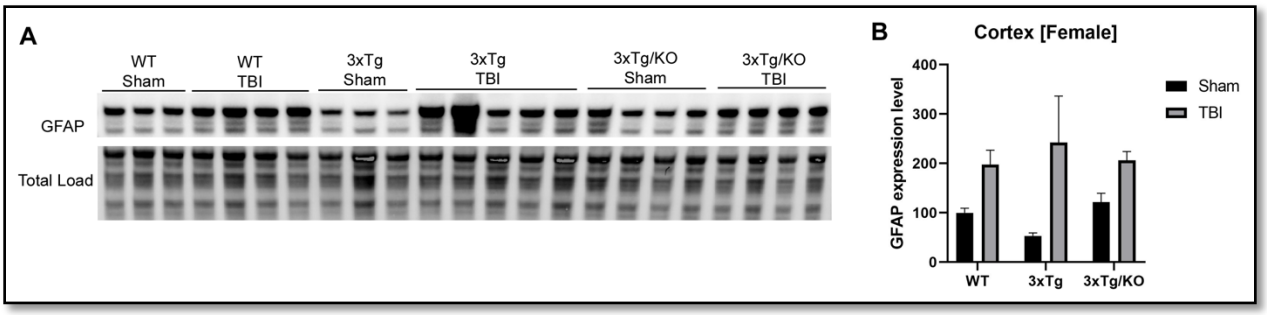

Figure 3.15 GFAP expression level in the cerebral cortex of female mice.

A) Western blot image showing 50kD GFAP detected in the cortex of female WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (20μg protein, 4-15% gel). B) The densitometry values in the bar graph did not show any statistical significance.

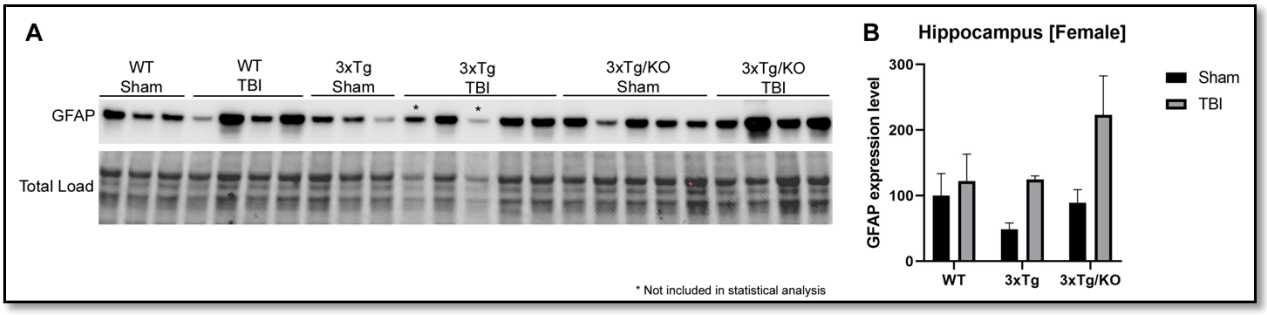

Figure 3.16 GFAP expression level in the hippocampus of female mice.

A) Western blot image showing 50kD GFAP detected in the hippocampus of female WT-Sham, WT-TBI, 3xTg-Sham, 3xTg-TBI, 3xTg/KO-Sham, and 3xTg/KO-TBI groups (20μg protein, 4-15% gel). B) The densitometry values in the bar graph did not show any statistical significance.

Chapter 4: Discussion

4.1 Summary of Results

TBI causes activation of the NLRP3 inflammasome which is critical for the propagation of subsequent neuroinflammatory cascades. Using a novel line of AD mice with NLRP3 knockout we tested if this abnormal neuroinflammatory response following TBI could be mitigated in the context of predisposition of AD. In this study, we measured changes in NRLP3 inflammasome related immune/inflammatory protein expression following TBI in 3xTg mice and 3xTg mice with NLRP3 knockout as well as a genetic background matched wild type mice. Samples from the hippocampus and cortex were collected at two days post-injury and divided by sex and brain region for protein analysis using a combination of Western blots and ELISA.

4.1.1 Summary of NLRP3 Inflammasome Cascade Response

NRLP3 inflammasome cascade proteins did not show statistically significant injury difference; however, a trend of an injury effect was found in several data sets. Most notably, IL-1β expression was elevated in TBI samples across all genotypes in the cerebral cortex of male mice (**Figure 3.7**). Caspase-1 (p20) expression was elevated in cerebral cortex in TBI samples of the 3xTg male and female mice (Fig. 3.3 and 3.5). NLRP3 expression was also elevated in TBI samples for the 3xTg group in the cerebral cortex of female mice (Fig. 3.1).

There were a few notable differences in NLRP3 inflammasome cascade protein expression patterns in male versus female samples. IL-1β expression in the cerebral cortex of male mice showed a consistent increase with injury in all genotypes, whereas in the cerebral

cortex of female mice, injury appeared to have almost the opposite effect in WT samples and minimal affect in the other genotypes.

When looking at the effect of genotype, NRLP3 inflammasome cascade proteins did show some statistically significant changes. NLRP3 expression was significantly decreased in 3xTg/KO samples compared to 3xTg samples in both the sham and injured groups. This confirmed that the NLRP3 knockout was successful. Caspase-1 (p20) expression was significantly lower in the cortex in male mice in the injured 3xTg/KO group compared to injured 3xTg animals. In the cortex of the female mice, higher Caspase-1 (p20) was found in the 3xTg-Sham group compared to 3xTg/KO-Sham, whereas the 3xTg-TBI group had higher expression than the WT-TBI group. Finally, IL-1β expression in female mice, WT-Sham mice had significantly higher expression compared to sham 3xTg and 3xTg/KO groups. Of note, this unusually high expression in WT-sham samples was also noted with caspase-1 (p20) expression.

4.1.2 Summary of Glial Cell Response

Glial cell response to injury was measured by assessing protein expression of HMGB1 and GFAP. HMGB1 is a nuclear protein released following TBI and plays a role in initiating neuroinflammation. Results did not show a significant change in HMGB1 expression with injury across any of the genotypic groups. However, a genotype difference was found in HMGB1 expression. Both male and female cortex and hippocampus samples had a significant main genotype effect. In the cortex of male mice, WT-sham group had higher expression than the 3xTg-sham and 3xTg/KO-Sham. Whereas in the hippocampus of male mice, higher HMGB1 expression was found in the 3xTg/KO-TBI group than the WT-TBI and 3xTg-TBI group.

For GFAP, both male and female cortex and hippocampus samples had a significant main injury effect. Expression was increased in injured groups in WT and 3xTg mice in both regions in both sexes, although statistical significance was only found in the 3xTg-TBI group compared to 3xTg-Sham in male mice. A genotype difference of GFAP expression was found in the male mice with the 3xTg/KO-Sham group showing higher expression than WT and 3xTg sham groups. This genotype difference was not observed in female mice.

4.2 Discussion

TBI has showed to induce the formation of the NLRP3-inflammasome complex, activate caspase-1, and lead to the processing of IL-1β (Liu, et al., 2013). TBI also induces a glial cell response. Following TBI, astrocytes have showed to change morphology and increase production of GFAP (Mira, Lira, & Cerpa, 2021) (Li , et al., 2009). HMGB1 released from activated microglia and/or necrotic neurons following TBI act on microglia to mediate chronic neuroinflammation (Gao, et al., 2011). In AD mouse models, TBI has showed to induce an altered neuroinflammatory response (Webster, Van Eldik, Watterson, & Bachstette, 2015) (Kokiko-Cochran, et al., 2016).

We predicted that TBI-induced NLRP3 inflammasome activation mediates the altered neuroinflammatory response in the predisposition of AD and that genetic NRLP3 knockout could mitigate this abnormal response. Based on these published studies, we expected to see TBI-induced increased expression of NLRP3, caspase-1, and IL-1β as well as increased glial cell response proteins, HMGB1 and GFAP. However, our results only partially supported this prediction. In this study, no statistical significance was found in our data supporting a TBI-

induced increase in expression of NLRP3, capase-1, IL-1β, or HMGB1. GFAP was the only protein that showed a statistically significant increase in expression following TBI. While not statistically significant, a trend of TBI-induced higher expression was found in some of these proteins such as caspase-1 and IL-1β . Our data not supporting the hypothesis may be due to several factors.

First of all, the TBI model used in this study. The TBI model used for this study was cFPI, which causes diffuse axonal injury (DAI) without focal tissue contusion resulting from rapid acceleration/deceleration force on the brain (Ehmark-Lewen, et al., 2013). In parallel to the current study, in another cohort of animals with the same TBI model, other investigators in our lab evaluated immune cell phenotypes using FAC sorting and mRNA expression using real time PCR and the results suggested that TBI induced an abnormal immune response and NLRP3 knockout mediated this. The cFPI model induces DAI in white matter tracts and causes a bilateral neuroinflammatory response and an astroglia response (Ehmark-Lewen, et al., 2013). The inflammatory response to cFPI has showed to be restricted to the cortex and subcortical white matter (Ehmark-Lewen, et al., 2013), which could explain the difficulty detecting inflammatory related proteins in hippocampal samples. Studies have also shown secretion of HMGB1 requires severe injury (Richard , Min, Su, & Xu, 2017). One of the disadvantages of the cFPI model used in this study is that only a limited injury severity can be achieved (Marklund & Hillered, 2011). The lack of main injury effect on NLRP3 inflammasome activation seen in our study could be due to our TBI model not providing a severe enough injury to cause focal tissue damage as previous published TBI studies including our own, which showed activation of the NLRP3 inflammasome was mostly reported in focal cortical contusion injury models (Kuwar, et al., 2019). The diffuse nature of the cFPI model may not cause significant tissue damage to

induce NLRP3 inflammasome complex formation, in a way that is detectable with the methods used. Compared to the cFPI, the CCI model of TBI models mechanical contusion injury in the cortex and resulted in a focal, rather than diffuse tissue damage. The CCI model has showed to cause astrocyte and microglial activation and hippocampal neuronal injury (Niesman , et al., 2014). Perhaps the CCI model would have provided a more quantifiable change of neuroinflammatory response in the region subjected to the focal injury.

Secondly, the timeline for sample collection after injury also contributes to quantifiable changes in protein expression. In a study using rats and a weight drop model TBI, levels of NRLP3, ASC, and caspase-1 were enhanced in the injured condition with a peak at 7 days post injury (Liu, et al., 2013). In the same study, cortical levels of IL-1β rapidly increased 6 hours post injury and steadily declined after 24 hours. Our study collected protein samples at 2 days post injury. It's possible that protein expression for the NLRP3 inflammasome complex proteins had not reached its peak and therefore differences in expression were not significant. Additionally, IL-1β expression could already have declined by the time our samples were collected to a point where changes in expression were no longer significant. It's also possible that the time related variations of protein expression is dependent on the severity and injury model.

Thirdly, issues with the Western blotting technique could also be a contributing factor. The NLRP3 inflammasome and its downstream proteins including ASC and caspase-1 are difficult to work with using Western blotting due to the available antibodies and the proteins' molecular sizes. Additionally, repeated freeze/thaw cycles of tissue samples may have led to protein degradation, which would affect their detectability. During my study, I have experienced tremendous difficulty getting Western blotting to work on these proteins. The

protein bands presented in this dissertation were not the clearest, thus the qualitative data may not be accurate as it should be. Furthermore, the relatively low sample number for each group means decreased statistical power. Had more samples been included in each group, the subsequent increase in statistical power may have led to more data significance.

Contrary to our prediction, higher expression of caspase-1, IL-1β, and HMGB1 was found in some WT-Sham groups compared to WT-TBI. It is possible this is due to artifacts from the detection technique that affected protein normalization. It is also possible that the wild mice were not true wild type representations and therefore not suitable controls.

In our study, we reported injury induced changes of these proteins in both male and female mice. Sex differences in neuroinflammatory response in animal models of TBI are highly variable and model dependent. A review of TBI studies using both male and female animals showed that females have a protective advantage following TBI (Rubin & Lipton, 2019). When ovariectomized female mice were supplemented with exogenous estrogen, there was a reduction of proinflammatory IL-6 and MCP-1 as well as enhanced anti-inflammatory IL-4 compared to male mice (Rubin & Lipton, 2019). Additionally, after TBI, studies have shown female rats expressed more cortical IL-6 compared to males, while males expressed more cortical TNFα and IL-1β (Rubin & Lipton, 2019). Compared to females, male mice subjected to CCI showed more activated microglia at 1 dpi, greater peripheral macrophage infiltration at 1 and 3 dpi, as well as more astrogliosis as seen by GFAP expression at 1 and 7 dpi (Rubin & Lipton, 2019). However, another study showed greater GFAP expression in female mice at 1 dpi, but no sex difference at 7 dpi (Rubin & Lipton, 2019). There are also studies suggesting no sex differences in neuroinflammatory response. One study showed CCI caused both males and

females to have increased expression of IL-1β, TNFα, MCP-1 and activated microglia (Rubin & Lipton, 2019). Another study indicated that TBI-induced microglial activation was not significantly affect by sex (Bruce-Keller, et al., 2007).

Our results showed that cFPI caused increased expression of IL-1β in male mice but had very little effect on IL-1β expression in female mice. This is in agreement with the studies suggesting males have higher expression of cortical IL-1β post-TBI. As mentioned above, cortical IL-1β levels have shown to decrease after 24 hours and in GFAP expression, female mice initially have higher expression, but by 7dpi expression is equal among the sexes. The lack of injury effect seen in IL-1β expression in our female mice may be due to the time-dependent variables that were not detected in our single time study design. In terms of microglial response, our study coincided with the studies suggesting there is no sex difference in TBI-induced microglial activation. In terms of astrocyte response measured by GFAP expression, both male and female mice showed TBI enhanced expression of GFAP, however male 3xTg mice showed a more significant increase of GFAP expression following TBI compared to female 3xTg mice. Our research coincides with the study suggesting males have more post-TBI astrogliosis than females.

In the 3xTg/KO mice, we expected to see lower expression of the NRLP3 inflammasome complex proteins and its downstream effectors. While we did see a lack of NLRP3 expression in the 3xTg/KO mice, we did not see a corresponding decrease of caspase-1 or IL-1β expression. This could be due to compensation from other inflammasomes. Other Nod-like receptor proteins, such as NLRP1 and NLRC4, in combination with ASC and pro-casepase-1 could have

formed inflammasomes and thus caused caspase-1 activation and subsequent maturation of IL-1β. This could explain why we did not see a decrease in active caspase-1 or IL-1β in the 3xTg/KO mice.

4.3 Conclusion and Future Direction

The association between TBI and AD has been well documented, but the mechanism that connects them is still largely unknown. In this study we evaluated the NRLP3 inflammasome as a potential link between the two conditions. Although some data supported our prediction that NLRP3 knockout would mediate the abnormal neuroinflammatory response caused by TBI-induced NLRP3 inflammasome activation in the context of predisposition of AD, the overall results did not support this prediction. In this model system, TBI-induced NLRP3 inflammasome activation is not apparent based on the data collected. The outcome of this experiment may have been limited by severity of TBI model, study time frame, and challenges with method of detection. Future studies should consider expanding study time frame and using other models of TBI at more severe levels. This study focused on the acute effect of TBI (2 dpi). Our lab is already working on a similar study that evaluates neuroinflammatory response at longer time points: 7 dpi and 30 dpi. Additionally, this study used a genetic approach to reduce NLRP3 expression and our lab is also evaluating a pharmaceutical approach to reduce NLRP3. Further analysis of NLRP3 and the NLRP3 inflammasome's effect on inflammatory/immune response is needed to better evaluate its role as a link between TBI and AD.

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