Diffuse Brain Injury Incites Sexual Differences and Hypothalamic-Pituitary-Adrenal Axis Disruptions

by

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A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved June 2019
by the Graduate Committee

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ARIZONA STATE UNIVERSITY

August 2019

ABSTRACT

Of the 2.87 million traumatic brain injuries (TBI) sustained yearly in the United States, 75% are diffuse injuries. A single TBI can have acute and chronic influences on the neuroendocrine system leading to hypothalamic-pituitary-adrenal axis (HPA) dysregulation and increased affective disorders. Preliminary data indicate TBI causes neuroinflammation in the hippocampus, likely due to axonal damage, and in the paraventricular nucleus of the hypothalamus (PVN), where no axonal damage is apparent. Mechanisms regulating neuroinflammation in the PVN are unknown. Furthermore, chronic stress causes HPA dysregulation and glucocorticoid receptor (GR)-mediated neuroinflammation in the PVN. The goal of this project was to evaluate neuroinflammation in the HPA axis and determine if GR levels change at 7 days post-injury (DPI).

Adult male and female Sprague Dawley rats were subjected to midline fluid percussion injury. At 7 DPI, half of each brain was post-fixed for immunohistochemistry (IBA-1) and half biopsied for gene/protein analysis. IBA-1 staining was analyzed for microglia activation via skeleton analysis in the hypothalamus and hippocampus. Extracted RNA and protein were used to quantify mRNA expression and protein levels for GRs. Data indicate increased microglia cell number and decreased endpoints/cell and process length in the PVN of males, but not females. In the dentate gyrus, both males and females have an increased microglia cell number after TBI, but there is also an interaction between sex and injury in microglia presentation, where males exhibit a more robust effect than females. Both sexes have significant decreases of endpoints/cell and process length. In both regions, GR protein levels decreased for injured males, but in the hippocampus, GR levels increased for injured females. Data indicate that diffuse TBI causes alterations in microglia morphology and GR levels in the hypothalamus and hippocampus at 7 DPI, providing a potential mechanism for HPA axis dysregulation at a sub-acute time point.

ACKNOWLEDGEMENTS

A puzzle piece. It's small and jagged and it may be out of time, but somewhere – it fits. That's how I've felt for most of my life: out of place, out of step, fighting to do something worthwhile. This has been a long and tedious road, with many professional and personal changes. It is far from over as I continue onto a path where I will take what I have learned and use it in a new way – hopefully to change the world (because has always been my ambition). But I don't feel alone anymore. I don't feel out of place.

Dr. Theresa Currier Thomas. I almost quit my third year as a PhD student, but I didn't because of you. You almost refused to let me give up – not because I was a great scientist, but because you could see how much I was hurting, and you knew how much it meant to me to persevere. We've had our bumps and we've learned from each other along the way as we challenged each other to grow and view the world from different perspectives. In these last 18 months, I've accomplished things I never thought possible. My confidence has grown, my writing has metamorphosized, and I've even managed to learn (some) statistics – and that's just the surface. I am so grateful that we met, and you took me under your wing. I couldn't have asked for a better mentor. I am excited to see where this data takes the Thomas lab and I am so honored to have been a part of it!

Dr. Heather Bimonte-Nelson – Along with Dr. Currier Thomas, you're another reason why I didn't give up. Just being around you is like taking a spiritual and emotional bath in sunshine – you wash all of the negativity, worry, and doubt away instantly. I was honored to be a part of one of your courses and from the moment I realized I wanted to teach like you. But being around you outside of class, I have realized something much more – I want to affect people like you. You are never easy on me. If someone on my committee is going to tell me "the hard thing" I know it will be you, but I welcome it because I know it will be out of instruction and because you want me to grow. You have always guided me and encouraged me – knowing I could push myself further. I am excited to take this next step in Science Education Research after having your example as a professor. It's given me something to strive for and I hope I can make you proud.

Dr Jason Newbern – One of the main reasons I wanted you on my committee is because I knew that when the time came for me to take exams or defend, you wouldn't ask the easy, predictable questions. When I told many of my friends I had Dr. Newbern on my committee, they all looked at me with sympathy because reaching the goal wouldn't be *easy*. They were right. However, I didn't want easy. I wanted to be ready. I remembered so many of the questions you asked me during my PhD qualifying exams that I couldn't answer, and I made sure I was ready for the second time. You challenged me and made me better. I appreciate you and thank you. You have molded me into the student I wanted to become – even if it took the hard way.

To my partner in crime – Caitlin. We make mischief in the lab. Thank you for always having my back. For teaching me – sometimes the same thing more than 10 times. You've never lost your patience. When I felt alone, you were the first to make me feel included. I am grateful to have met you and I am so honored to call you a friend. None of this would have been possible without you.

To everyone else in my wonderful lab – Emily, GK, Bret, Erum – thank you for helping me with my craziness always. It's been a crazy ride and I can't wait to see what you all do!

To my wife – We've been like two ships passing in the night lately. I am ever grateful for your patience and your attentiveness. For your kind words and encouragement. For never getting angry or upset when I have to work or study or write. You are always there. I wouldn't be half this far without you and I know it. You are my teammate and I am so thankful and so blessed to have you in my life. I love you with all my heart.

To my son – I have fallen more times than I can count trying to get here. This has been a 12-year adventure (so far). It has not been easy, but it is worth it. I have cried. I have been exhausted. I have worked almost 48 hours straight some days (I do not recommend this). You can be and you can do anything in this world you set your mind to – it will be hard, and it will be strenuous. You

will not have a lot of free time. But in the end, it will be worth it. I believe it you, my son. I love you.

To my mom – This is dedicated to you. You have never given up on me. We have fought for 33 years together. You are my hero. Everything I do is because of you. Everything I am is because of you. You are my superhero. I don't need an Ironman or a Wonder Woman. I have you. You are my rock. You are my example. You are my everything. When I'm faltering, I ask myself "what would mommy do" and then I have my answer. This thesis is your success as much as it is mine. It would not be written without your influence over my life.

Lastly, none of this would have been possible without the help of two very important people: Chasity Bojorquez and Tara Furcini. I am Deaf and neuroscience is not for the faint of heart. I would not be anywhere without my team. These two women have given their lives for me. They have missed ball games, trips, dinners, time with family, sleep, meals… for me. We have spent countless hours together. Each of them has listened to me vent, each of them has helped me fight, each of them has studied terminology and developed signs with me. I am where I am because of the two of you. I love you two with all of my heart. "And because I knew you, I have been changed…For Good."

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LIST OF ABBREVIATIONS

ABBREVIATION	***FULL NAME***
ACTH	adrenocorticotropic hormone
ATM	atmospheres of pressure
AUC	area under the curve
BBB	blood brain barrier
CCI	controlled cortical impact
CDC	Center for Disease Control
CNS	central nervous system
CORT	cortisol/corticosterone
CRH	corticotropin-releasing hormone
DAI	diffuse axonal injury
DPI	days post injury
dTBI	diffuse traumatic brain injury
FSH	follicle stimulating hormone
GR	glucocorticoid receptor
HPA axis	hypothalamic pituitary adrenal axis
IBA1	ionized calcium-binding adapter molecule 1
LH	luteinizing hormone
mFPI	midline fluid percussion injury (diffuse injury)
MR	mineralocorticoid receptor
PCS	post-concussive symptoms
PVN	paraventricular nucleus of the hypothalamus
RBCs	red blood cells
RT-qPCR	real-time quantitative PCR
SA	skeleton analysis
TBI	traumatic brain injury
Wes	automated capillary western

1.0 INTRODUCTION: TRAUMATIC BRAIN INJURY

1.1 Epidemiology of a Traumatic Brain Injury

There are 2.87 million reported traumatic brain injuries (TBI) every year in the United States. Of these, over 75% include diffuse axonal injury (DAI)[1, 2]. Yet, the true incidence of TBI is unknown as many do not seek medical care. According to the National Women's Health Network, it is estimated that overall 20 million women have sustained at least one TBI from domestic violence in the United States - exceeding the numbers for athletes and military combined[3]. As a result, TBI has been carrying the moniker of a "silent epidemic." TBI has been indicated in chronic neurological and endocrine dysregulation, for which there are limited treatment options.

According to the Center for Disease and Control (CDC), falls accounted for 48% of all reported TBIs, followed by "being struck by or on the head", motor vehicle accidents, and reported assaults[2]. Children ages 0-4 and older adults over 75 years of age have the highest rates for TBI-related ER visits [2]. As such, TBI carriers a wide economic burden, costing the United States approximately $48.3 billion dollars annually in ongoing medical, socioeconomic (disability payments/vocational rehab), and lost wage costs[4]. There is a 12-70% chance of TBI survivors returning of work, dependent upon injury severity [5]. The World Health Organization predicts that by 2020, TBI will be the third leading cause of death and disability in the world[6, 7], leading to an even greater economic burden.

1.2 Diffuse Axonal Injury is Characterized by Axonal Shearing

There are three main types of TBIs – focal, diffuse, and blast – each characterized by the hallmarks of the injury. Focal injury is characterized by an injury in a localized part of the brain[8]; blast injury is brain trauma characterized by three main effects: shockwaves, shrapnel, and acceleration/deceleration[9]. Lastly, diffuse traumatic brain injury (dTBI) is characterized by rapid acceleration and deceleration of the head[8] often with side-to-side head rotation, which leads to

DAI. The hallmark pathology of DAI is axonal shearing and tearing[10], causing disruptive circuits, thus leading to a secondary injury cascade and subsequent post-concussive symptoms (PCS)[11].

1.3. Brain Injury Leads to Post-Concussive Symptoms

Roughly 20% of TBI survivors report PCS after one month of recovery, and 15% continue to report persisting or permanent symptoms. PCS are typically categorized into three categories: somatic, affective, and cognitive which include symptoms such as headaches or migraines, seizures, neuro-ocular dysfunctions, depression, memory problems, dizziness, fatigue, and anxiety[12-16]. Table 1 provides a more comprehensive, but not complete list of the symptoms within these categories.

Somatic	**Cognitive**	**Affective**
Endocrine dysfunction	Inability to concentrate	Anxiety
Speech changes	Communication problems	Depression
Headaches/migraines	Memory problems	Mood swings
Loss of motor skills	Inability to plan	Impulsivity
Seizures	Impaired problem solving	Apathy
Visual issues	Difficulty processing	Disinhibition
Sleep changes		Aggression
Problems with coordination		Impaired social skills
Stress disorders		Stress disorders

Table 1 Post-Concussive Symptoms

Stress disorders and other endocrine-related symptoms are interspersed throughout the PCS list as the hypothalamic-pituitary-adrenal (HPA) axis is particularly susceptible to damage from primary and secondary injury. After injury, there is an initial, short-lived cortisol (CORT) surge followed by a drawn-out decrease, causing dysregulation the HPA axis[17]. Damage to or dysregulation of the HPA axis leads to increased affective disorders (up to 50% of survivors) and to endocrinopathies (between 30-50% of survivors)[18].

1.4 TBI is a Diseased State

1.4.1 Every TBI is Initiated by a Mechanical Event

Every TBI begins with mechanical forces initiating the primary injury. Such damages are irreversible and are only amenable to prevention. The primary injury is considered to be the damage that occurs during the moment of the event, including cell damage or loss, axonal shearing or tearing, broken synapses, subarachnoid hemorrhage, contusion, epidural or subdural hematoma, neurovascular damage, and blood-brain-barrier breakdown[16, 19, 20]. Primary injury leads to a secondary injury cascade which can last for days to weeks to months to years[19].

1.4.2 The Primary Injury Leads to a Secondary Injury Cascade

Secondary signaling continues over the ensuing days to weeks in an attempt to restore homeostasis in injured tissue, which, if not controlled, can exacerbate the injury[4]. Cellular and humoral inflammation are principle among the responses and a hallmark of both clinical and experimental brain injury. Post-traumatic responses are communicated by robust increases in pro- and anti-inflammatory cytokines, which are below detectable levels in healthy tissue[21, 22]. Cytokines induce structural and functional changes in the resident central nervous system (CNS) cells, particularly microglia. Primarily, microglia remove cellular debris to attenuate inflammatory processes and promote tissue remodeling[23, 24]. At the same time, activated microglia release neurotoxic substances (e.g., free radicals, glutamate) that can exacerbate injury. Therefore, neuroinflammation is currently viewed as a double-edged sword with the potential to both promote and/or repair injury[25].

2.0 MICROGLIA

2.1 Microglia are the Brain's Resident Immune Cells

Microglia are the brain's resident immune cells. Microglia are a type of neuroglia found throughout the CNS[26] comprising of about 10-15% of all cells in the brain[27]. The primary duty of microglia is to remove cellular debris and promote tissue remodeling [23, 24]; however, different morphologies of

microglia have specific and designated functions [28, 29]. Microglia are typically described in one of six morphologies: ramified, hyper-ramified/bushy, activated, amoeboid, rod, or dystrophic [30-32, 33].

2.2 Microglia Activate Following Injury

The morphology of a microglia cell reflects its function[11-12,33]. **Figure 1** is a representative image that depicts the typical morphologies of microglia[33]: Under normal physiological conditions, *ramified (resting)* microglia have a small cell body with fine, long processes (Fig. 1A). Ramified microglia continually survey the CNS and respond to stimuli to maintain homeostasis[13-15]. When ramified microglia encounter a stimulus, it will activate, proliferate, and migrate to the site of injury. *Hyper-ramified* microglia retract and thicken their processes, creating a "bushy" like appearance (Fig. 1B). Hyper-ramified and activated morphologies initiate either neurotoxic or neurotrophic signaling, depending on the nature of the stimulus[16] with continued signaling through fractalkines, glycoproteins, and cytokines[34]. *Activated* morphologies retract and thicken their processes even more (Fig. 1C). Activated morphologies are presumed to secrete cytokines and migrate to sites of injury[11,12]. Amoeboid microglia are fully activated, are indistinguishable from infiltrating macrophages (fully retracted processes), and function to phagocytose cellular debris[9,11-12] (Fig. 1D). *Rod* microglia are elongated, polarized, and morphologically distinguishable from other forms of microglia [31, 35] (Fig. 1E). The distinct rod microglia morphologies have been identified in numerous disease states [31, 35-50]. Although most of their function remains unclear, it is proposed that rod microglia act as scaffolding to axons and dendrites to the injured, ailing, or aging brain as they consistently align themselves with injured neurons[51].

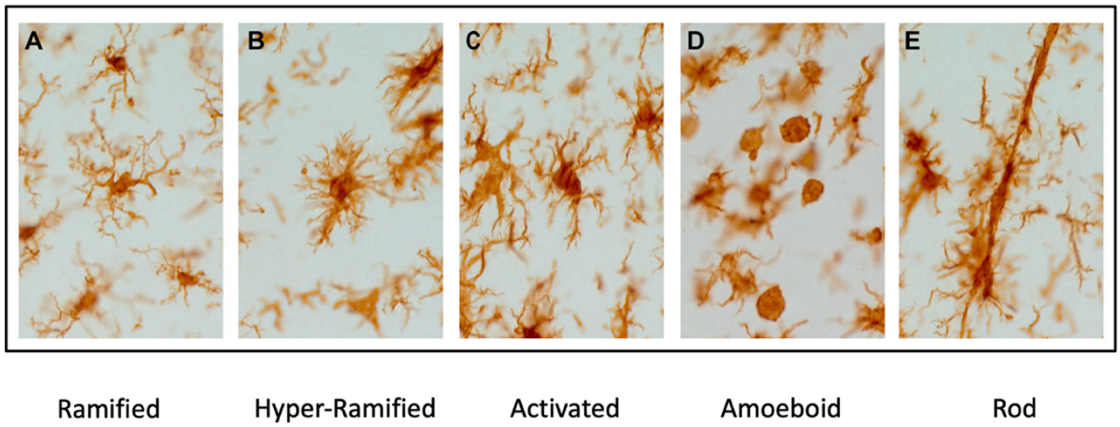


Figure 1 Representative Figure Depicting the Most Common Microglia Morphologies Following Injury (Ziebell et.al, 2016).

Lastly, dystrophic *(senescent)* microglia are termed to be "diseased" microglia as they cannot respond to any stimulus as their processes are beaded, fragmented, and twisted. Dystrophic microglia are typically found in the aging brain, but have appeared after experimental brain injury in non-aged adult mice[52, 53]. See Streit et.al, 2009 for images and characteristic information.

2.2 Microglia Can Be Detected by Several Different Markers

Microglia are typically detected by the ionized calcium binding adaptor molecule-1 (IBA-1) marker for general inflammatory investigation, however, they can also be detected by the fractalkine receptor CX3CR1. Additionally, different morphologies of microglia can be detected by different markers. For instance, MHCII+ and CD80+ identify activated microglia[54], CD86+ identifies amoeboid microglia[55], and $CD45^{low}$ identifies ramified microglia. Additionally, F4/80 and CD14 markers distinguish infiltrating macrophages/monocytes from resident microglia[56, 57]. Lastly, the dystrophic morphology has a phenotype all of their own with increased microglia expression of IL-1α, HLA-DR, and MHCII [32].

2.3 Microglia are Usually Found in Areas of Neuropathology

Neuroinflammation and neuropathology tend to overlap after injury. Neuropathology, defined for the scope of this study and for evaluation of TBI, is dead or dying neurons typically evaluated by

silver staining[58]. When a ramified microglia encounters debris, it proliferates, differentiates, and migrates to the site of injury[33]. Proliferation and migration increases the microglia cell number; differentiation causes microglia endpoints/cell and process length to decrease, indicating microglial activation[59, 60]. Activated microglia are critical for cleaning up the debris after neuronal death.

3.0 Neuropathology is Not the Only Trigger for Altered Microglia Presentation

Neuroinflammation also occurs when an animal or human is exposed to stress[61]. In different studies, rodents that undergo differing stress paradigms see an increase in microglia number and activation and an increase in proinflammatory cytokines[62-64].

In a restraint stress mouse model, after 4 days of exposure to stress, whole brain flow cytometry indicated elevated levels of microglia cell number[65]. In a repeated social defeat mouse model, microglia cell numbers and activation increased in the paraventricular nucleus (PVN) of the hypothalamus and the amygdala. When coupled with varied unpredictable stress, repeated social stress increases microglia cell numbers and activation in the hippocampus[62].

In a chronic mild stress model, proinflammatory cytokines were elevated in the cortex (TNF-α/IL-10) and hypothalamus (TNF-α/IL-10, IL-6/IL-10) in males[66]. In females, restraint stress increased circulating IL-6 after two weeks of prolonged stress. In the cortex, IFN-γ and TNF-α were increased; in the hippocampus, IFN-γ was increased[67].

There is limited information about sex differences in stressed animals and microglia presentation in the HPA axis. In the hippocampus, amygdala, and prefrontal cortex, unstressed females had a higher activated to ramified ratio than unstressed males. However, when females were exposed to stress, activated microglia in the prefrontal cortex decreased. Additionally, microglia in females had increased expression of CX3CR1 fractalkine receptors but decreased MHCII and CD40[68]. In a sex-differences study looking at 1 (acute) and 10-day (chronic) restraint stress outcomes, male

and females had several regional differences in the orbitofrontal cortex, amygdala, and hippocampus. In the hippocampus, male activation decreased acutely and chronically. There were no significant differences in microglia presentation or activation for females. Acutely CD40 and CX3CL1 expression increased in females while chronically CD40, iNOS, CX3CL1, and CD200 increased in males[69]. In the hypothalamus, acute restraint stress activated more CRH-producing neurons in female rats than in male rats, which could lead to increased levels of CORT production[70].

Microglia express glucocorticoid receptors (GR) on their surface, therefore, the drastic increase of the stress hormone, cortisol (CORT), may affect the microglia's response[62]. However, to fully understand the dynamics, the circuitry behind the HPA axis and its relationship with inflammation must first be explored.

4.0 THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

4.1 The Hypothalamus Maintains a Homeostatic State

The hypothalamus is located inferior to the thalamus and is comprised of several nuclei, each specializing in a particular function. The hypothalamus receives neural information from several different areas in the brain – including the spinal cord, the amygdala, the hippocampus, and the prefrontal cortex. The hypothalamus is responsible for keeping the body at homeostasis. The hypothalamus is comprised of several specific nuclei all designed to regulate specific functions geared towards maintaining homeostasis: growth, temperature control, metabolism and body weight, fear and anger responses, circadian rhythm, reproduction, fluid balances, hunger, thirst, sexual arousal, aggression, regulates calcium balance, respiratory control, immune system influences through the thymus, and stress response regulation[71, 72].

The vascular of the hypothalamus is critical as the hypothalamus sends and receives information by hormones – which travel through the circulatory system. The hypothalamus receives its blood

supply from the circle of Willis, which branches into an eventual fenestrated capillary network. Additionally, the hypothalamus lies above a venous circle, providing drainage for several smaller vein[73]. The hypophyseal portal system is a circulatory system of blood vessels that connects the hypothalamus and anterior pituitary. This system contains several fenestrated capillary networks enabling the exchange of hormones, particularly growth hormone releasing hormone, thyrotropin-releasing hormone, and corticotropin-releasing hormone (CRH)[74]. However, these fenestrated capillary networks leave the hypothalamus vulnerable to damage by large molecules that cannot cross the blood brain barrier (BBB) at other locations[75, 76].

The PVN is a specialized nucleus in the hypothalamus that regulates the stress response. It is responsible for synthesizing a 41-amino acid peptide, CRH, which is released in the presence of a stimulus. CRH travels through fenestrated capillaries of the hypophyseal portal system to the anterior pituitary and binds to the CRH receptor, a G-protein coupled receptor, initiating intracellular signaling to stimulate the release of adrenocorticotropic hormone (ACTH)[77].

4.2 The Pituitary Gland is Known as the “Master Gland”

The pituitary gland is attached to the base of the hypothalamus and has been dubbed “the master gland” because its hormones cause a neuroendocrine cascade influencing other regions, particularly the thyroid gland, adrenal gland, ovaries, and testes to synthesize and release hormones of their own. The hypothalamus sends signals to the pituitary gland to either inhibit to release pituitary hormones[71, 78].

The pituitary is divided into two parts: the anterior pituitary and the posterior pituitary. The anterior pituitary gland, also known as the *adenohypophysis*, is the glandular part of the pituitary and mediates many processes including stress, growth, reproduction and lactation by synthesizing and releasing specialized hormones: ACTH, thyroid-stimulating hormone (TSH), follicle-stimulate hormone (FSH), luteinizing hormone (LH), prolactin, and growth hormone (GH). The posterior pituitary gland, or *neurohypophysis*, is comprised of neural tissue and axons of neurons whose

cell bodies reside in the supraoptic nucleus and PVN. These projections stem from the PVN, run through the infundibulum, and terminate into the posterior pituitary to release hormones oxytocin and vasopressin[71, 78].

In the anterior pituitary, CRH stimulates ACTH release. ACTH is formed from proopiomelanocortin (POMC), a 241 amino acid protein. POMC undergoes several cleavages, yielding many peptides, including ACTH, a 39 amino acid peptide. ACTH travels through systemic circulation to the adrenal gland and binds to G protein-coupled receptors in the fasciculata and reticularis zone of the adrenal cortex[79].

4.3 The Adrenals Release Corticosterone

The adrenal glands are located superior and medial to the superior pole of each kidney. Each adrenal gland is divided into the cortex and the medulla. The adrenal cortex consists of three distinct layers: the glomerulosa (15% of the cortex), the fasciculata (75% of the cortex), and the reticularis (10% of the cortex). ACTH initiates the production of cortisol (CORT) in humans (corticosterone in rats), which is released within minutes of a stimulus[80] – a fact that drives translational research methodology when measuring CORT.

4.4 The Hippocampus Provides Primarily Inhibitory Signals to the HPA Axis

The hippocampus is a part of the limbic system and is well known for its role in memory and learning; however, it also has a lesser-known, yet equally critical role in HPA axis regulation. In humans, the hippocampus is located under the cerebral cortex in the medial temporal lobe. In rats, the hippocampus is located at the dorsal part of the brain. Moving posteriorly, the CA1 forms dorsal horns that wrap laterally around the brain on either side. Additionally, Ammon's Horn (pyramidal layers), otherwise known as the “hippocampus proper”, and the dentate gyrus (granule layer) structures look like two interlocking “Cs”.

There are several subsections to the hippocampus including the CA1-4 and the dentate gyrus. Each subsection has differing inputs and outputs and thus different roles. The hippocampus, including the dentate gyrus, is inhibitory to the HPA axis in circadian and stressed conditions by way of negative feedback when subjected to CORT[81]. The dentate gyrus has the highest concentration of GRs in comparison to the rest of the hippocampus, suggesting a more integral role in the regulation of the HPA axis[82, 83].

Neuronal death and atrophy of the hippocampus cause an increase in CORT production[84, 85]. This is important to note as the hippocampus is susceptible to neuronal damage after DAI[86], where the degree of injury correlates to the degree of atrophy[87, 88].

4.5 The HPA Axis Circuit Is Regulated by Negative and Positive Feedback Loops

The HPA axis is a highly-regulated circuit between the hypothalamus, the pituitary gland, and the adrenal glands. Indirect interactions with the hippocampus, amygdala, and prefrontal cortex also play a pivotal role in regulation. This system sends and receives communication - both neurally and hormonally - and is the primary system for stress regulation[89]. Stress can stimulate CRH into blood from the PVN. CRH then travels to and stimulates the anterior pituitary which releases ACTH into the blood which binds to the receptors on the adrenal glands and stimulates the release of CORT. Differential regulation can occur because MRs and GRs are not co-localized [90]. When CORT binds to GRs or MRs in the hippocampus, it initiates a positive and negative feedback loop respectively[91]. However, the hippocampus' primary purpose is to inhibit the release of CRH through trans-synaptic inhibition using glutamatergic efferent projections to the PVN[92].

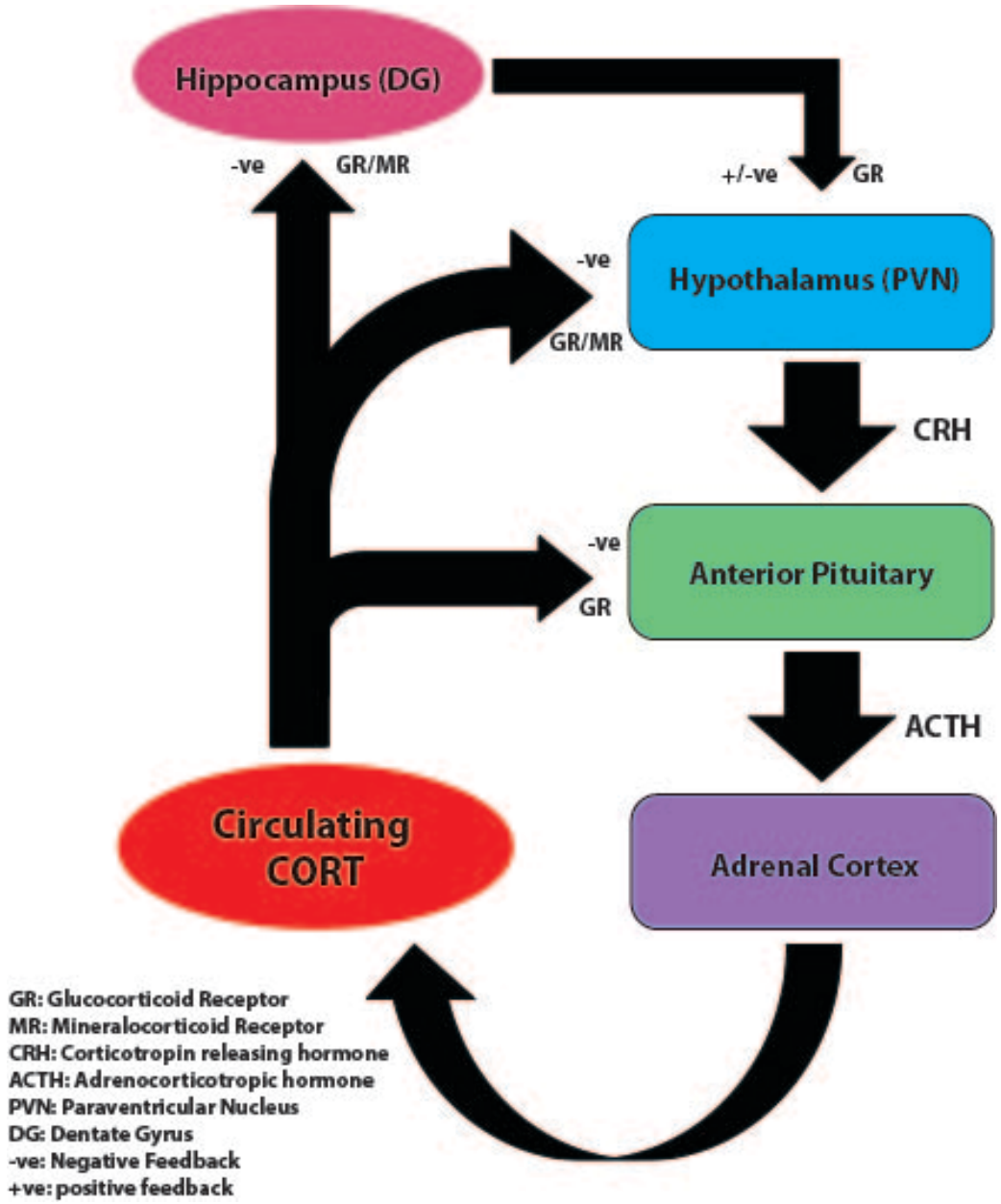


Figure 2 Schematic of CORT Regulation by The HPA Axis. The PVN Releases Corticotropin-Releasing Hormone (CRH). CRH Travels to The Anterior Pituitary and Triggers Release of Adrenocorticotropic Hormone (ACTH), Which Travels Through the Blood Stream to the Adrenal Cortex on Top of The Kidneys. ACTH Stimulates the Release of Cortisol/Corticosterone (CORT). CORT Circulates in the Blood and Travels to the Anterior Pituitary, Binds to Glucocorticoid Receptors (GR) And Through Negative Feedback Mechanisms, Slows the Production Of ACTH. CORT Also Travels to the PVN And to the Hippocampus, Binds to Grs and Mineralocorticoid Receptors (MR) and Slows the Production of CRH by Way of Negative Feedback. There is Some Positive Feedback Through Indirect Hippocampal-Hypothalamic Signaling, However, the Hippocampal-Hypothalamic Signals are Mostly Inhibitory.

4.6 CORT Follows a Diurnal Rhythm That Can Be Disrupted

ACTH and CORT follow a diurnal rhythm controlled by the suprachiasmatic nucleus of the hypothalamus. In humans, both hormones peak in the early morning followed by a slow descent until reaching the nadir at approximately midnight. ACTH and CORT increase slowly after the nadir, and just before peaking, increase abruptly, simulating a hormone surge[80, 93]. ACTH typically peaks between 4:00AM and 6:00AM, whereas CORT peaks at approximately 8:00AM[80]. As rats are nocturnal animals, their diurnal rhythm of ACTH and CORT is reversed; under normal light conditions, ACTH and CORT are peaked in the evening, not the morning[93].

Sometimes the diurnal cycle becomes unbalanced. Trauma – including blood loss, surgery, infection, and stress – can increase the secretion of ACTH and CORT into the blood as well as contribute to increased inflammation. Activated microglia -the hallmark of neuroinflammation- release proinflammatory cytokines including IL-1, IL-6, IL-2, and TNF-α [94, 95]. IL-1 and IL-6 stimulate the release of CRH from the hypothalamus, which in turn stimulates the release of ACTH and eventually, CORT. IL-2 and TNF-α can stimulate the release of ACTH from the pituitary, thus stimulating the release of CORT[96].

4.7 CORT Binds to the Adrenoreceptors

There are two types of adrenocorticoid receptors, type I (MR) and type II (GR). While both receptors bind CORT, each differ in structure, function, and concentration levels throughout brain regions. MR's are composed of 984 amino acids while the two main isoforms of GR's, GRα and GRβ are composed of 777 and 742 amino acids, respectively[97]. CORT has a higher affinity for MR's, thus saturating MR's before binding to GR's. CORT's affinity for each receptor parallels with the receptor's functions: MR's are tasked with several different functions that are considered to be proactive, maintaining a basal HPA homeostatic state, including diurnal rhythms, while also binding aldosterone, a mineralocorticoid that plays a critical role in regulating the cell concentration of sodium and potassium by stimulating the transcription for sodium-potassium pumps[98]. When CORT binds to MR's, it creates proactive feedback to maintain basal HPA axis

activity. Once all MR's are saturated, CORT will bind to GR's which are reactive in functioning – responding to increased CORT from stress or stimulus exposure and providing negative feedback to slow down production of CRH and ACTH.

GRs and MRs are both expressed on microglia, however, the two receptors are not always co-localized[99]. GR's have the highest expression in the PVN, then the dentate gyrus, the CA1 of the hippocampus respectively, and finally the central and cortical amygdala. MR's have the highest expression in the hippocampus[100].

4.8 Cortisol is the Main Glucocorticoid

CORT is the main glucocorticoid and is produced by the adrenal cortex of the adrenal gland. Although CORT is involved in the regulation of energy, immune reactions, and most notably, the stress response, the cellular targets and mechanisms of CORT are largely unknown. CORT binds to both MRs - influence proactive feedback in order to maintain basal HPA axis activity- and GRs – which regulates the reactive feedback of the HPA axis in response to stress. There is evidence that TBI is capable of influencing gene expression, protein levels, and signaling of both receptors[101-103].

4.9 CORT Can Be Proinflammatory

The production of CORT can either inhibit inflammation, under low level stress conditions, or activate inflammation responses under chronic, intense levels of stress[104, 105]. CORT's classic anti-inflammatory response inhibits MHCII expression, thus disabling dendritic cells from interacting with immature T-cells while also promoting the expression of anti-inflammatory cytokines (IL-10, TGF- β) and restricting the expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α)[106]. Alternate studies have shown that exposure to stress, after LPS injection, increases TNF-α[63], MHCII[107], F4/80[65], and activates microglia[63, 107]. Additionally, in a restraint-stress mouse model, after exposure to 15 hours of restraint stress for 4 days, serum CORT levels in mice significantly increased and microglia cell counts in the brain significantly increased[65]. Increased

stress, and thus CORT levels, reflect increased activity of NFκB and expression of NFκB target genes[106]. Furthermore, exogenous CORT administration can also up-regulate Toll-like Receptor 2/4 (TLR2/4)[40] which increases microglia activation[41].

4.10 CORT is Influenced by the Estrous Cycle

Additionally, circulating CORT is different amongst males and females while also indicating a plausible relationship with the estrous cycle in females. Circulating CORT levels increase at the peak times of estrus and proestrous rats; nadir levels remain unchanged[108]. In cholinergic stimulation, females in proestrous and estrus have a higher ACTH and CORT response than females in diestrous or metestrous. Males had a higher ACTH and CORT response than the females after stimulation, but females in all cycle stages had higher baseline levels of ACTH and CORT than males[109]. In males and estrus females, there was a minimal slope in the concentration of plasma CORT per unit concentration of immunoreactive ACTH, however, the slope reached its maximum for rats in proestrous[110].

5.0 THE HPA AXIS AND TBI

5.1 THE HPA AXIS is Highly Susceptible to Primary Injury

The anatomical location the pituitary gland, stalk, and the hypothalamus makes the HPA axis highly susceptible to damage after DAI[111]. The rapid acceleration or deceleration of the head can injure the pituitary by fracturing the bony sella or shearing the pituitary stalk, effectively disrupting the circuits of the axis[112]. Additionally, as there is much vascular in and around the hypothalamus and pituitary, DAI can cause direct damage to the hypophyseal portal veins which supplies blood to the pituitary causing possible infarction or hypoxia. Additionally, due to fenestrated capillaries, the hypothalamus is also vulnerable to damage by circulating molecules that cannot cross the BBB in other locations[75, 76].

5.2 Secondary Injury of the HPA Axis Leads to Dysfunction

As mentioned earlier, the HPA axis is particularly susceptible to damage from secondary injury contributing to increased affective disorders and to endocrinopathies[18]. TBI is one of the leading causes of hypopituitarism – the severity of the hormonal dysfunction correlating to the severity of the injury[113]. In one-third of autopsy reports, pituitary necrosis was documented[114]. Gonadotropin and GH are the two most common disrupted hormones after TBI[113, 115], however recent studies have indicated that TBI may disrupt vasopressin release, inciting diabetes insipidus development among survivors[116]. Data describing prognosis is conflicting, with some studies describing that symptoms improve over time and others describing that symptoms will worsen overtime[117, 118].

6.0 EXPERIMENTAL INJURY

6.1 There are Several Models of Experimental Injury

Rodent models are a useful tool to replicate symptoms, understand mechanisms, and develop treatments for TBI as the physiological circuitry is translational to humans. There are several experimental injury models, each designed to mimic a type of brain injury and the injury's pathology. For example, controlled cortical impact (CCI) and weight drop for focal injury[119], blast simulators and blast overpressure for blast injury[120, 121], and fluid percussion for diffuse injury[122, 123].

6.2 Midline Fluid Percussion Injury is a Clinically Relevant Model

There are multiple variations to the fluid percussion injury model (FPI): lateral, parasagittal, and midline. Each of these variations were designed to develop unique pathology and symptomatology at the acute and chronic time points[88]. The lateral FPI model produces a more focal-type injury and the parasagittal FPI model produces a focal and diffuse injury. The midline FPI (mFPI) model chosen for these experiments produces a theoretical evenly distributed bilateral diffuse injury[23, 88, 122]. However, mFPI is heterogeneous as there is neuronal death next to healthy tissue with some areas being affected more than others when evaluated by silver stain. Regardless, mFPI is a clinically relevant model of dTBI as it is the most translational of the three

variations of FPI models with over 30 years of implementation and supporting literature. See Lifshitz et. al, 2016 for more information on clinical relevance.

6.3 Midline Fluid Percussion Injury

To deliver a mFPI, a craniectomy is performed along the sagittal suture, approximately half-way between bregma and lambda. Then, a pendulum is dropped from a pre-determined height that delivers a single fluid pulse through a fluid-filled chamber over the dura, producing a bilateral diffuse injury without cavitation or contusion[88, 122]. Determinants of injury severity for mFPI include the fencing response and righting reflex time. The fencing response is a lateral or bilateral forearm posturing upon the moment of impact that is proposed to coincide with neurochemical and pathological cascades in the lateral vestibular nucleus of the brainstem. In the mFPI model, 100% of rats fence[88, 122, 124]. The righting reflex time is the second determinant of injury severity. After impact, the rat is laid supine on its back and its suppression of reflexes is timed. The timer is stopped when the rat rights itself. The righting reflex time correlates to injury severity: the longer it takes for the rat to right, the more severe the injury. For this study, all animals fell between a 6-12-minute righting reflex time (average RR time) which is indicative of a mild-moderate injury.

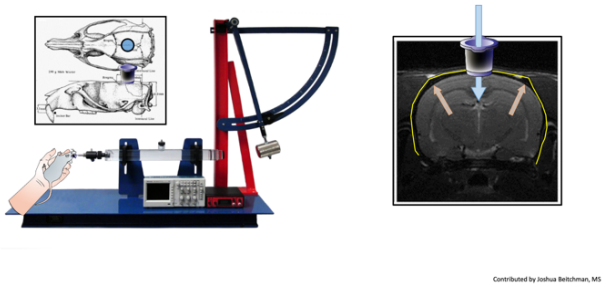


Figure 3 FPI Device. An Implanted Cranial Hub is Attached to the Injury Device Where an Adjustable Pendulum Falls and Strikes the Plunger, Generating a Fluid Pulse That Impacts the Dural Surface, Resulting in a DAI Without Cavitation or Contusion

6.4 Histopathology and Neuropathology of mFPI

The mFPI model reproduces many of the expected results of human dTBI including metabolic dysregulation, hypotension, and subdural hematoma[125]. Additionally, mFPI induces a histopathological state similar to concussion particularly in regions of greatest strain, such as blood vessels, neuronal processes and at gray-white matter interfaces[123, 126-128]. Astrocytes - whose main function is neuronal maintenance, synapse formation, and extracellular formation - react and proliferate after mFPI for up to 28 DPI[129, 130]. In response to mFPI, microglia react in a

highly coordinated time-course consisting of pro- and anti-inflammatory cytokines, chemokines, and reactive oxygen species[33].

Neuropathology has been identified chronically up to 28 DPI, occurred primarily in the sensory barrel fields of the cortex and lateral sections of the midbrain, specifically ventral thalamus and the CA3 of the hippocampus[131]. Additionally, dentate gyrus becomes excitable, likely contributing to seizure disorders and cognitive deficiencies[132]. The proximity of these regions to the temporal ridge makes them particularly susceptible to damage by mFPI (Lifshitz et al., in preparation).

6.5 Motor and Cognitive Deficits From mFPI

Specifically, mFPI results in morbidities related to: motor performance using rotarod and beam walk on days 1-5 following injury[133], post-injury learning using the Morris water maze (MWM) 1-2 weeks following injury[134], late-onset sensory hypersensitivity using the whisker nuisance task (WNT) [135], and anxiety-like behavior using the open field test[130]. As such, mFPI results in a reproducible, clinically relevant model of dTBI.

6.6 Limits of mFPI

In humans, TBIs are like fingerprints – each unique and distinct to the individual and no two exactly alike - even though there are some similarities and overlap. Contrary to initial assumption, a harder impact does not always equate to a more severe injury or increased number of symptoms[136, 137]. In mFPI, this variability is difficult to replicate as mFPI is performed under controlled conditions. mFPI surgical procedures creates a craniectomy on the midline of the skull, which releases cranial pressure upon impact; in humans and rats, elevated intracranial pressure after TBI is associated to increased deficits and prolonged or more severe symptoms[138-140]. While mFPI is a great translational and clinically replicable model, sleep disorders, vestibular symptoms, migraines, and deficits among females have not been replicated or measured in this model, yet[88].

7.0 SEXUAL DIFFERENCES

7.1 Sexual Differences in Physiology and Anatomy

The gonadal hormonal differences between males and females can influence several downstream effects. While males and females both have testosterone, estrogen, and progesterone, the levels for the hormones vary depending on sex: males have higher testosterone; females have higher estrogen and progesterone[141]. Males and females have some anatomical differences in the brain, many of these differences in specific nuclei of the hypothalamus and the medial nucleus of the amygdala[142]. Additionally, there are many neurochemical differences between the sexes. Males and females differ in their levels of norepinephrine, serotonin, vasopressin, acetylcholine, and dopamine[142]. In humans, females tend to have less neck muscles than men, leading to a lower neck to head strength ratio and creating an opportunity for an increase in acceleration at the neck at the time of impact[143]. In rats, females generally tend to weigh less than their male age-matched counterpart[144]. Our laboratory has also noted that a female Sprague Dawley's skull is much thinner than an age-matched male. Weight and skull thickness must be taken into consideration when conducting mFPI, as the females will need to be hit at a lower atmospheres of pressure (atm) to equate the same injury severity as the males (calculated by righting reflex time and survival rate as a function of atm carried out in both male and female rats).

7.2 Female Estrous Cycle

Females have four different stages of estrous - proestrus, estrus, metestrus, and diestrus, each characterized by differing hormone levels and differing vaginal cells that can be analyzed by vaginal swabbing or flush. Humans and rats' cycles are not identical. Humans have a 28-day cycle. The LH surge occurs around day 14-15 of the cycle. Estrogen and FSH are peaked at the same time as the LH surge and progesterone peaks about 5-6 days later. Female rats have a 4.8 day cycle average[145]. Estrogen peaks just before the near-simultaneous LH surge, progesterone, and FSH peaks[146]. While a limitation of these studies was that estrous was not tracked, the

influence of the estrous cycle and its influence is critical for determining the role of gonadal hormones, PCS, and HPA axis dysregulation.

7.3 Sexual Differences in TBI

The research studying sexual differences in TBI is limited and data conflicts[147-149], likely due to variables of estrous/menstruation at the time of injury, which must be taken into consideration[150]. Furthermore, research assessing the differences in outcomes between women in different stages of estrous/menstruation is even less conclusive[150, 151]. In addition to higher rates of loss of consciousness[152], several studies report that women of child-bearing age are more likely to have continuing symptoms of anxiety and depression after mild TBI[147, 153-156]. Recent data suggest that in sports with similar rules, women athletes sustain more concussions than men athletes. Likewise, women athletes experience or report a higher number of concussions, increased severity of symptoms, and report a longer duration of recovery than men athletes[157-159]. As previously stated, cycle at the time of injury is an import factor of injury outcome. Women in their high-progesterone luteal phase when sustaining a TBI showed significantly worse outcomes, including quality of life and neurological functioning in comparison to women in the follicular phase or who took oral contraceptives[150]. When compared with controls, women with TBI have an increased risk of giving birth to a stillborn, more severe postpartum depression, lower mental health, increased number of abnormal papsmears, and increased problems with menstrual cycles[160]. In experimental models of TBI, anxiety and depression have also been identified[161].

8.0 PREVIOUS STUDIES

There are changes in microglia cell number after TBI in the PVN overtime. Skeleton Analysis (SA) was conducted on sham, 1, 2, and 7 male Sprague Dawley rats by taking six 40x images of the PVN per animal using standard protocol through ImageJ. Data were analyzed and summarized. Data showed a decrease in microglia cell number at 1 and 2 DPI in comparison to sham animals, which was not expected. There was an increase at 7 DPI in comparison to 2 DPI. There was no significant difference in endpoints or in branch length **(Fig 4).** These data indicate the microglia cell

counts change over time, indicating some influence of TBI on microglia in the PVN in the absence of neuropathology or astrocytosis.[162] The endpoint data was used to conduct a power calculation where n=8 was necessary to achieve >90% power when comparing sham and 7 DPI rats.

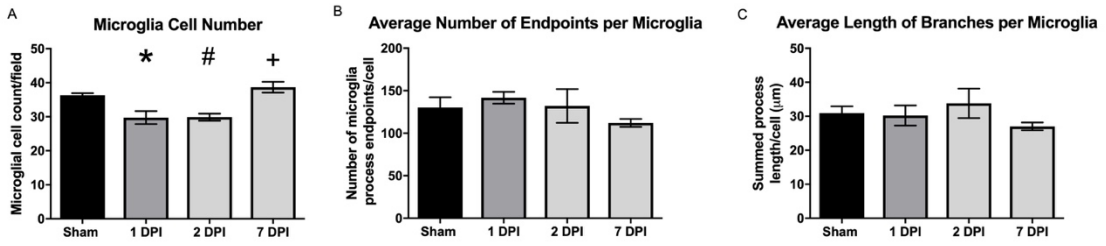


Figure 4 Preliminary Data of Skeleton Analysis of Microglia Changes in the PVN. Skeleton Analysis Was Conducted at Sham, 1, 2, And 7 DPI in the PVN. A) The Number of Microglia Significantly Decreased at 1 and 2 DPI Compared to Sham. Microglia Cell Number Significantly Increased From 2 DPI to 7 DPI. B) There Were No Significant Differences in Average Number of and Points Per Microglia. C) There Were No Significant Differences Between Average Lengths of Branches Per Microglia.

There are changes in glucocorticoid receptor gene expression after TBI in the pre-optic hypothalamus over time in males. Our laboratory previously used real-time (RT) qPCR to analyze gene expression in GRs in Sprague Dawley rats on sham and at 1, 3, 5, and 7 DPI. Gene expression for GRs changed significantly over time $F(1, 12)=4.6$; $p=0.015$), first increasing at 5 DPI and then significantly decreasing between 5 and 7 DPI indicating a disruption in the HPA axis after TBI, albeit there was no significant difference between 7 DPI and sham (**Fig 5).**

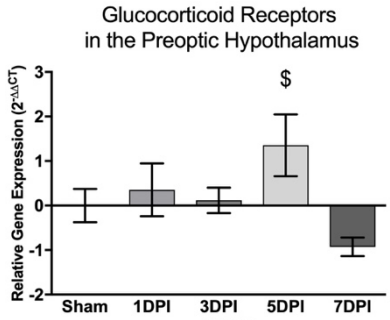


Figure 5 Glucocorticoid Relative Gene Expression in the Pre-Optic Hypothalamus in Males. Relative Gene Expression of Glucocorticoid Receptors Was Significantly Decreased Between 5 DPI and 7 DPI ($P<0.05 Between 5 And 7 DPI).

At 7 DPI significance is not reached between CORT levels in male sham and injured rats. In these experiments, terminal trunk blood from live decapitated rats was collected between 8-10AM and centrifuged to separate plasma from red blood cells (RBCs). CORT was quantified in plasma samples (diluted 1:50) using ELISA kits. Due to variability in sham animals CORT did not reach

significance in males (**Fig 6)**. Animal numbers are being increased due to variability for each group.

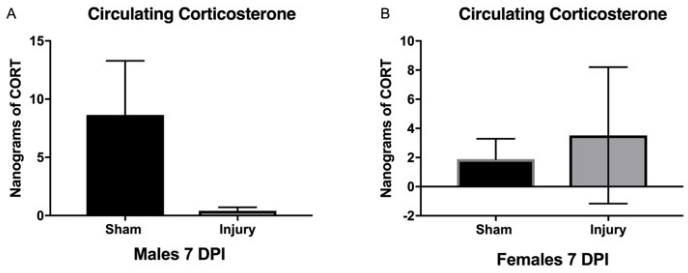


Figure 6 Circulating Corticosterone Levels in Males (A) and Females (B) at 7 DPI.

At 2 months post-TBI, basal/resting levels of CORT are lower than sham and blunted response to restraint stress indicates CORT dysregulation. In these experiments, baseline tail vein blood was taken prior to surgery for all rats between 8-10AM and centrifuged to separate plasma from RBCs. CORT was quantified in plasma samples (diluted 1:50) using ELISA kits. Then at 2 months post-injury, injured and sham rats were placed in a cylindrical restrainer tightly for 30 minutes and then loosened. Blood was taken by tail vein at 0, 30, 60, and 90 minutes. These results indicate that TBI causes CORT dysregulation that does not resolve by 2 months[162] (**Fig 7 and 8).**

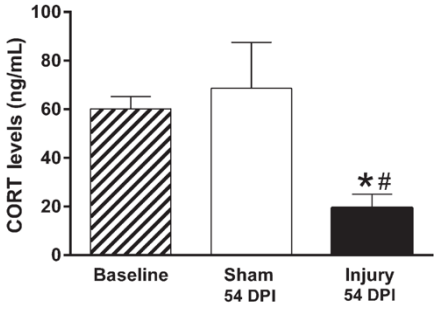


Figure 7 At 2 Months Post-Injury, CORT Levels Are Significant Decreased Compared to Compared to Baseline Levels and Compared to Sham Rats (Rowe et. al, 2016)

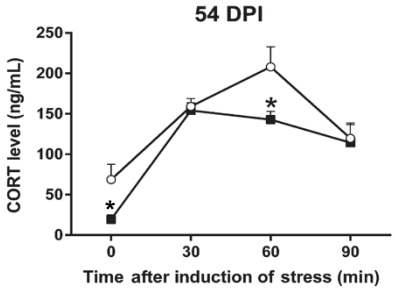


Figure 8 At 2 Months Post-Injury, There is HPA Axis Dysregulation as Indicated by Blunted CORT Response in Injured Rats (Rowe et. al, 2016)

9.0 SCOPE OF THE PROJECT AND HYPOTHESIS

Our laboratory's preliminary data have indicated changes in microglia cell number in the PVN across time with a peak at 7 DPI in comparison to other times points and with a significant

decrease in cell number at 1 and 2 DPI in comparison to sham. Our laboratory's preliminary data have also shown that after dTBI, GR gene expression changes over time and it coincides with microglia presentation. GRs are present on microglia (and other cell types) and have been indicated to regulate microglial proliferation, differentiation, and activation state [163-165].These data indicate that TBI has some influence over microglia in the PVN. Previous studies have shown drastic differences in neuroinflammation between males and females after focal TBI where males were more likely to have increased neuroinflammation at several DPI, yet no studies have investigated these changes in dTBI[166, 167]. Additionally, in mouse restraint stress models, stress incites CORT dysregulation, microglia activation, and proinflammatory cytokine release[155]. The purpose of this project was to evaluate neuroinflammation in an area with no quantifiable neuropathology and an area with neuropathology in the HPA axis and determine if GR levels were changing in those regions, providing evidence that GR expression may be driving microglial activation after TBI. Therefore, these experiments were designed to test the hypothesis that **diffuse experimental TBI incites sexual differences in the HPA axis, influencing hypothalamic and hippocampal microglia presentation as well as glucocorticoid receptor levels at 7 DPI.**

9.0 METHODS

9.1 Subjects

For these experiments, **previously collected tissue was utilized** from, young, adult, age-matched, male and female Sprague-Dawley rats (9-12 weeks old when ordered; 300-350 g vs 200-250 g). Rats were pair housed in a 12-hour light/12-hour dark cycle (6:00/18:00) at a constant temperature (23°C ± 2° C) with food and water *ad libitum*. Rats acclimated to the environment for at least three days before surgery.

9.2 Midline Fluid Percussion Injury

Rats were randomized into sham or brain-injured groups and were prepared for sham surgery or mFPI to induce a 6-12 minute righting reflex time using previously published methods[161, 168] .

Rats were anesthetized using 5.0% isoflurane (3L/minute oxygen) and then were maintained during surgery using 2.5% isoflurane (3L/minute oxygen). Rats were placed on a heating pad during and immediately following surgery to maintain body temperature. In a stereotaxic frame, a midline scalp incision exposes the skull and then 4.8mm craniotomy was made positioned on the sagittal suture halfway between bregma and lambda without disrupting the underlying dura mater. The injury hub – which was created prior to surgery from the female end of a Luer-lok – was then set on the craniotomy site and secured using glue and dental acrylic. Rats were returned to a warmed holding cage and monitored until injury induction (about 90 minutes) at which time they were re-anesthetized using 5% isoflurane. The injury-hub assembly was filled with physiological saline and attached to the male end of the fluid percussion device (Custom Design and Fabrication, Virginia Commonwealth University, Richmond, VA). As the rat's reflexive responses return, a moderate injury (1.8- 2.1atm) was administered by releasing the pendulum onto the fluid-filled cylinder. Rats were monitored for the return of the righting reflex. Sham rats were connected to the FPI device, but the pendulum was not released. The injury-hub assembly was removed *en bloc*, integrity of the dura observed, and bleeding controlled prior to the incision being stapled. Lidocaine and bacitracin were applied to the incision site. After injury, animals were monitored for apnea, return of righting reflex, and presence of the fencing response.[169] **Inclusion** criterion for the injury group included a righting reflex time of 6-12 minutes and a fencing response[169] (>90% occurrence). Rats had post-ops for at least 3 days' post-surgery. Rats that lost 10% or more body weight in the first 3 days' post-injury were **excluded** from the study (<5% occurrence).

Discomfort, distress, pain, and injury were limited to that which is unavoidable in the conduct of scientifically sound research. Injured rats were monitored for three days post-injury for signs indicative of distress: lack of activity, vocalizations, lack of appetite, dehydration, excessive weight loss, and excessive gnawing or scratching at the injury site. Any rats demonstrating persistent visible signs of pain, distress or discomfort (vocalizations, absence of eating or drinking, etc.) were immediately euthanized (<5% occurrence). Typically, rats subjected to these

procedures in our laboratory recover, eat, drink, and show no outward signs of pain or distress. Post-surgery analgesics are not given for pain management aside from topical lidocaine as they can alter functional and pathophysiological outcomes of the injury, thus skewing results[170]. Rats were assessed for their post-operative condition at 1-3 hours and 24 hours after injury and daily thereafter. Post-operative reports noting locomotion, behavior, and appearance of incision site were recorded.

9.3 Live Decapitations and Dissections

At 7 DPI, between 8:00 and 10:00AM, rats were prepared for live decapitation, trunk blood collection, organ, and brain dissections. Rats were taken from the home room and the home cage was temporarily held outside of the necropsy room while the first rat was anesthetized. Rats were live decapitated with a two-minute delay. Rats were anesthetized with 5.0% isoflurane (3L/minute oxygen) for 2.0 minutes. Rats were measured from the tip of the nose to the base of the tail, weighed, and then immediately decapitated. Trunk blood was centrifuged to separate plasma from RBCs. Adrenal glands, kidneys, ovaries, testes, thyroids, and pituitaries were harvested and weighed. Brains were harvested and halved. One half of each brain was post-fixed in 4% paraformaldehyde for immunohistochemistry analysis. The other half was dissected using a chilled rat brain matrix. Coronal sections were 2mm apart and tissue biopsies were 2mm in diameter. Brain regions includes the hypothalamus, hippocampus, amygdala, thalamus, striatum, cortex, ventral tegmental area, and nucleus acumens. Plasma was stored in -20 °C. Half brains were sent to Neuroscience Associates in Knoxville, TN for professional IBA-1 staining. Brain biopsies were stored in -80 °C.

The original study was designed to have multiple samples across time at 7, 56, and 168 DPI. However, some of these samples were stored incorrectly. Trial experiments were performed to test the viability of the samples. All 7 DPI samples were stored correctly. See **Appendix B** for further details.

9.4 Skeleton Analysis Quantifies Microglia

SA is the only method available to quantify microglia morphologies. SA demonstrates the required sensitivity to identify small changes in microglia ramification status among experimental groups. It is flexible and adaptable to: whole image or single cell analysis, high or low magnifications, and immunofluorescence or non-fluorescence immunohistochemistry protocols[59, 60]. Lastly, this analysis method uses readily available software (ImageJ).

SA performs 3 quantifications on photomicrographs of labeled microglia: 1) number of soma/cell bodies, 2) number of processes, and 3) summed length of processes. From these numbers, we can quantify the average number of processes per cell and the length of processes per cell. To quantify population morphology, a previously published ImageJ based SA protocol will be used. SA uses a 1-pixel wide backbone of an image and provides relevant data such as the number of microglia processes and process length that reflect cell ramification. Photomicrographs will be prepared for skeletal analysis using plugins, available in ImageJ (unsharp mask filter, thresholding, and remove outliers), in order to visualize all microglia processes. Image processing protocols were pre-determined and remained consistent for all image processing. Resulting images were converted to binary, skeletonized, and the number of processes per skeleton, and the branch lengths are then generated by the Skeletonize (2D/3D) plugin, and summarized or averaged, respectively[59, 60].

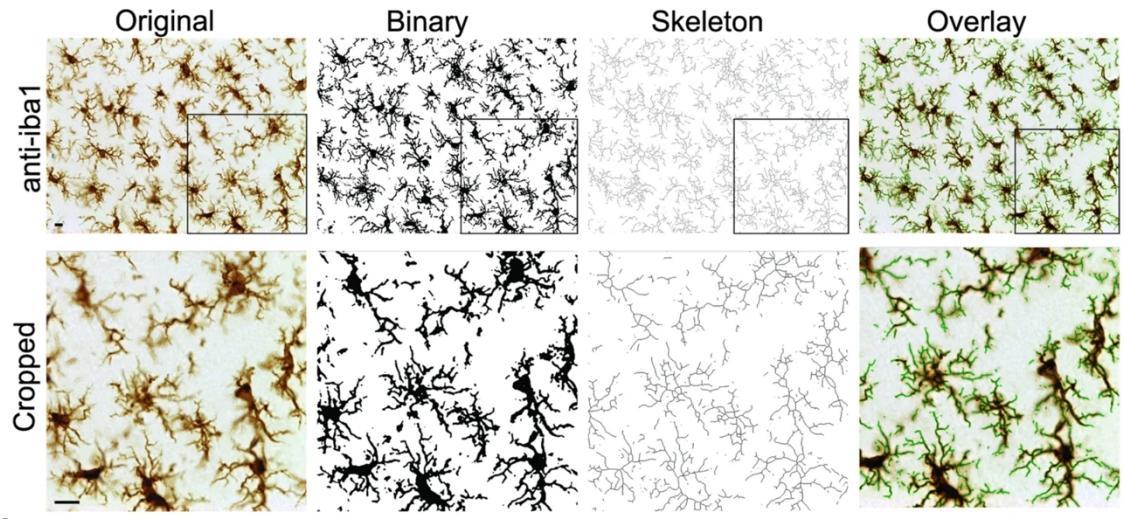


Figure 9 Representative Images of the ImageJ Conversation From an Original IBA-1 Image to a Binary and Finally to a Skeletonized Image (Morrison et.al, 2017)

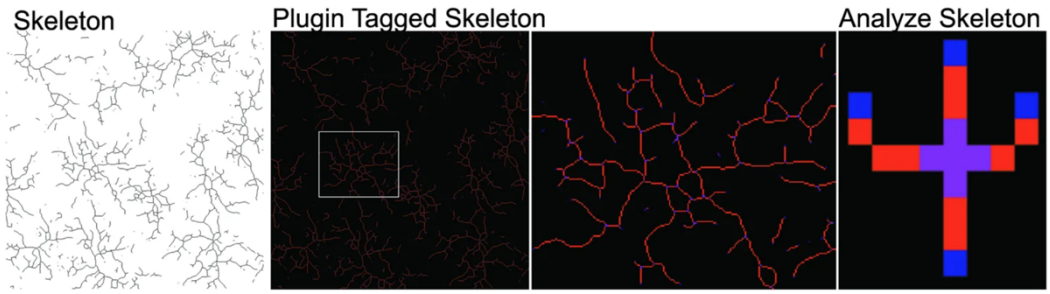


Figure 10 Representative Images of Skeletonized Microglia Being Converted Into a Quantifiable Form Where Processes (Orange), Junctions (Purple) and Endpoints (Blue) Are Differentiated (Morrison et.al, 2017).

9.5 RNA and Protein Extraction

An optimized Qiagen Total All-Prep (catalog # 80004) protocol was used to extract RNA and protein from hypothalamus and hippocampal biopsies. Biopsies were homogenized in 600mL of a 1:100 ratio BME: Buffer RLT ratio using a FisherBrand Pellet Pestle mixer for 3 minutes. Lysate was centrifuged at 4°C for 3 minutes at full speed before the supernatant was transferred to an AllPrep DNA spin column and centrifuged again for 30 seconds at 8000g. The flow-through was combined with 100% ethanol and mixed well. The new mixture was transferred into a RNeasy spin column and centrifuged for 20 seconds at 8000g. The flow-through was poured into a new 2ml tube and placed on ice for protein purification. Several buffer washes were added to the

RNeasy spin column: Buffer RW1, Buffer RPE, and 80% EtOH. The RNeasy spin column was then centrifuged at full speed for 5 minutes to dry before adding 30µl of RNase-free water and centrifuging again at 8000g for 1 minute to elute the RNA. See **Appendix A** for the full optimized protocol.

RNA purity and quality were immediately checked (Nanodrop, Thermo-Scientific 2000) by the 260/280nm absorbance ratio. Quantity of RNA (ng/µl) was also checked and recorded. All RNA was within the established range of purity (1.9-2.1) and were at least 25ng/µl. RNA was stored at -80°C.

Buffer APP was added to the “protein” flow-through, mixed vigorously, and incubated at room temperature for 10 minutes before being centrifuged for 10 minutes at full speed. The supernatant was decanted and 500µl of 70% ethanol was added. The tubes were centrifuged for 1 minute at full speed, then all liquid was decanted, and the protein pellet was left to dry for at least 10 minutes. The protein pellet was resuspended in SDS and incubated for 5 minutes at 95°C to completed dissolve and denature the protein. Protein concentrations were determined using the Bicinchoninic acid assay (BCA) using manufactures instructions (Pierce, Rockford, IL). Protein was stored at -80°C.

9.6 Quantitative RT qPCR

Total RNA was converted to cDNA using the High Capacity RNA-to-cDNA Kit from Life Technologies™ (catalog # 4387406), then diluted to 5ng for RT qPCR use. Applied Biosystems TaqMan® Gene Expression Assays for GR (Rn00561369_m1) and CRH (Rn00578611_m1) were evaluated. Assays were run in multiplex along with a biological control of Eukaryotic 18S ribosomal RNA (rRNA) (4310893E) and the TaqMan® Fast Advanced Master Mix (catalog # 4444963) in a ratio of 9µl of master mix: 1µl of gene: 1µl 18s rRNA per well. Samples were run in triplicate. TaqMan® Fast Advanced Master Mix thermocycling protocols were used. Eukaryotic 18S rRNA was used as a biological control as it shows less variance in expression across a

variety of treatment conditions than β-actin or GAPDH in addition to quick amplification. **See Appendix C** for sample plate design.

For relative gene expression analysis, each sample was normalized to the 18S rRNA biological control and then to gene expression levels in the sham group using the $2^{-\Delta\Delta CT}$ method[171].

9.7 Automated Capillary Western –ProteinSimple (Wes)

The Automated Capillary Western (Wes) by ProteinSimple ® is an advanced technology that can analyze up to 25 samples of proteins by size from 2-440 kDa using 0.5-5 µg of protein in approximately 3-4 hours. Through this novel technology, small tissue biopsies (2x2mm) from discrete brain regions can be used to perform multiple assays. This enables more specific nuclei to be assessed, albeit with some error relevant to the size of the nuclei within hypothalamus and hippocampus. The associated software, Compass (ProteinSimple®), provides results that are quantifiable and reliable[172]. Further technical properties are described by Rustandi et. al, 2012 and Loughney et. al, 2014.

Protein expression was evaluated using the Wes. The primary antibodies and plate conditions are listed in **Table 2.** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the biological control for the Wes as it is dispersed throughout the brain, albeit with some variability. Our laboratory conducted several optimizations with different biological controls, including β-actin, and due to the properties of the Wes, including chance of chemiluminescence blow out, GAPDH was the most accurate control for these experiments.

Secondary antibodies, streptavidin HRP, dithiothreitol (DTT), molecular weight fluorescent standards (internal control), luminol, hydrogen peroxide, sample buffer, antibody diluent, running buffer, wash buffer, capillaries and plates (stacking matrix, separation matrix, wash buffer, matrix removal buffer) were purchased from ProteinSimple (Santa Clara, CA). The Simple Western™ was also purchased from ProteinSimple.

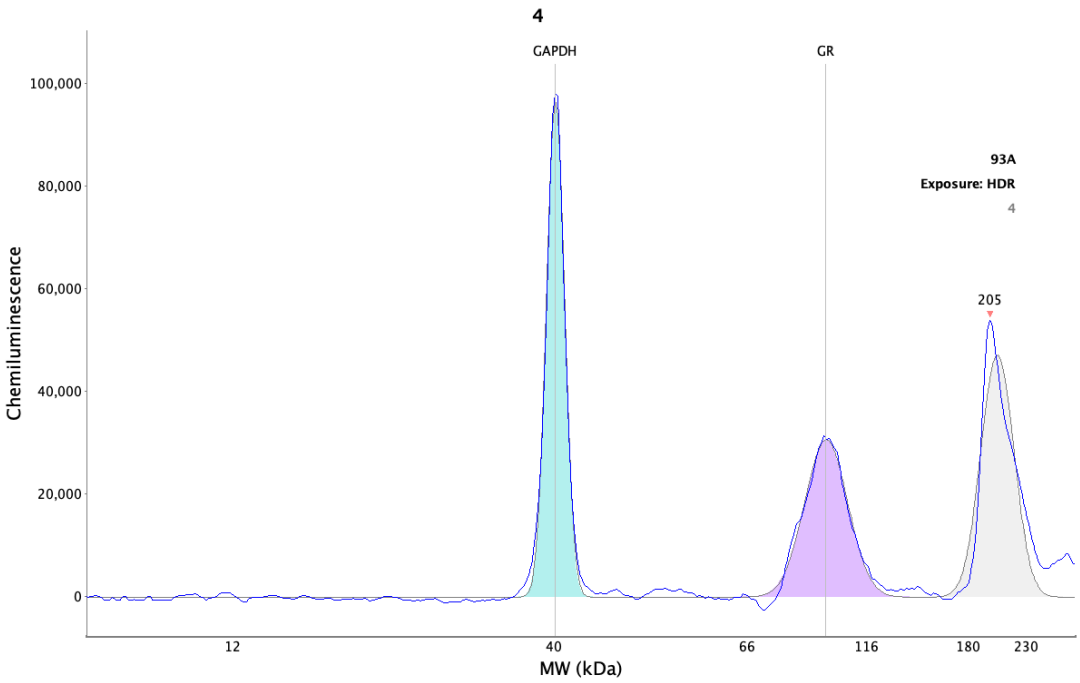
Antibody	Species	Ig company, catalog #	Denaturing Temp (celcius)	[Protein] Ug/ul	[Ig]	Biological control
NR3C1 (GR)	Rabbit	AbClonal, A2164	37°C X 30 mins	0.25	1/50	GAPDH
GAPDH	Mouse	Novus, NB600-502			1/50	

Table 2 Simple Western Plate Properties

Following protein concentration determination by BCA assay, samples were prepared according to the manufacturer's recommendations. Samples were combined with sample buffer and master-mix (40mM DTT, 0.1x ProteinSimple Sample Buffer, and 1x Fluorescent Standards) to achieve the desired concentration. Samples were then denatured via heating block at 37°C for 30 minutes. The ladder, samples, primary and HRP-conjugated secondary antibody, wash buffer, antibody diluent and chemiluminescent were then placed in the designated wells per experimental design. Each plate was centrifuged at 2500 RPM for 5 minutes and placed into the automated capillary western machine where proteins were separated by size (electrophoresis), immobilized and immunoprobed in individual capillaries. Once loaded into the instrument, the following metrics were utilized for separation and detection: Separation matrix loading time 200 seconds, stacking matrix loading time 15 seconds, sample loading time 9 seconds, separation time 25 minutes at 375V. After separation, samples are the exposed to UV light for 4sec and capillaries are immobilized for 200 seconds to allow for cross-linking between the sample and wall of the capillary. The matrix is removed with matrix removal buffer and 3 consecutive wash buffer cycles. Sample were then blocked with antibody diluent for 5 min followed by primary antibody (incubation times indicated in Table 1). Secondary antibodies were incubated for 30 minutes followed by detection with chemiluminescence (luminol/ hydrogen peroxide solution). Capillaries were then imaged at the following times: 5, 15, 30, 60, 120, 240 and 480 seconds.

Each sample is run in duplicate. To lessen variability, the same sham samples are run on every plate, in addition to a positive control from another cohort. **See Appendix D** as a sample plate design. For analysis, Compass (ProteinSimple®) generates an electropherogram containing peaks that correspond to the expression of all proteins of interest, including a peak for the

biological control protein (see Figure 12). The area under the curve (AUC) is calculated for each peak. To calculate relative protein expression, the AUC for the protein of interest is divided by the AUC for the biological control protein for all samples. All sham samples are averaged then each sample was normalized to the average of the shams.



Sample	Primary	Cap	Peak	Name	Position	MW (kDa)	Height	Area	% Area	Width	S/N	Baseline
93A	1/25 GA...	4	1	GAPDH	481	40	97134.7	808034	56.8	7.8	300.0	9339.1
93A	1/25 GA...	4	2	GR	583	99	30714.4	614901	43.2	18.8	54.8	14259.0
93A	1/25 GA...	4	3		644	205	47241.8	722820		14.4	131.9	15692.1

Figure 11 Sample of the Electropherogram Generated by Compass (ProteinSimple®) for Target Protein Analysis. The Biological Control, GAPHD, is at 42 kDa, While the Target Protein, NC3R1 (GR), is Known to be Around 103 kDa.

9.8 Statistical Analysis

For statistical analysis, the primary outcome measure was to compare sham rats to injured rats in each sex (Student's t-test). The secondary outcome measure was to measure sex differences between sham (Student's t-test). The tertiary outcome measure was to assess for interactions between injury and sex using a two-way ANOVA with a Fisher's LSD post-hoc comparison. All bar graphs represent the mean ± SEM, where *$p<0.05$ unless otherwise specified. For unequal

variance, we used non-parametric equivalents. All statistical analyses were performed using Prism 7 (Graph Pad, La Jolla, CA).

10.0 RESULTS

10.1 Skeleton Analysis of Microglia

dTBI results in neuroinflammation, as indicated by cell number increase, endpoint decrease, and branch length decrease – which is supported by our preliminary data. As such, the hypothesis for these experiments was cell numbers for males would be increased in both the PVN (a region with no pathology) and the dentate gyrus (a region with overt pathology), but endpoints and branch length would decrease. Female microglia presentation for the PVN is unknown, but per Villapol et. al, 2017, female microglia presentation is expected to be increased after injury in the dentate gyrus, but less so than males[166].

In the PVN, the average microglia cell numbers were significantly increased at 7 DPI in males but not in females (t_8=5.797), p=0.001; n=5. The number of microglia process endpoints per cell were significantly decreased at 7 DPI in males but not in females in the hypothalamus (t_8= 2.480), p=0.038; n=5. There were no significant differences in process branch length for males or females in the hypothalamus. The endpoint data from this cohort was used to conduct a power calculation and 80% power was achieved with n=5.

In the dentate gyrus, the average of microglia cell numbers was significantly increased at 7 DPI in males (t_8= 7.717), p<0.001 and in females (t_8= 2.754), p=0.025 compared to respective shams (n=5/group). There was a robust interaction between sex and injury for microglia cell number (F(1,16)=16.60, p<0.001). Post-hoc analysis with an uncorrected Fisher's LSD revealed a significant decrease in microglia cell number between injured males and injured females (t_{16}= 4.955, p<0.001. The number of microglia process endpoints per cell were significantly decreased at 7 DPI in males (t_8= 4.020, p=0.004) and in females (t_8= 2.635, p=0.029) compared to

respective shams. There was a significant decrease in process branch length in males (t_8= 3.417, p=0.009) and females (t_8= 3.726, p=0.006)

My hypothesis was supported for males and females in both the hypothalamus and dentate gyrus. These data indicate that microglia morphology is significantly altered in males, not females, in the hypothalamus at 7 DPI. In the hippocampus, TBI induced a robust response in cells, branch length and endpoints in both males and female, however, there was a significant interaction between injury and sex for the number of microglia cells.

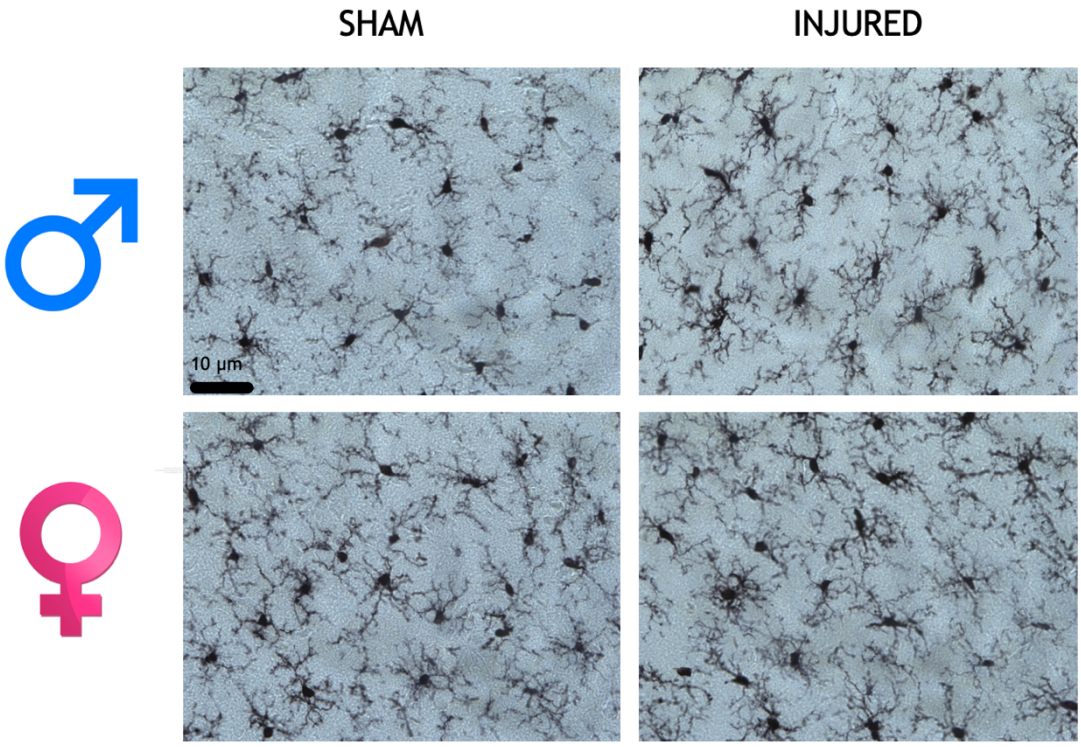


Figure 12 Representative 40x Images Showing IBA-1 Stained Microglia in the PVN. The Top 2 Panels are Representative Images of Male Sham (Left) and Male Injured (Right). The Bottom 2 Panels are Representative Images of Female Sham (Left) and Female Injured (Right).

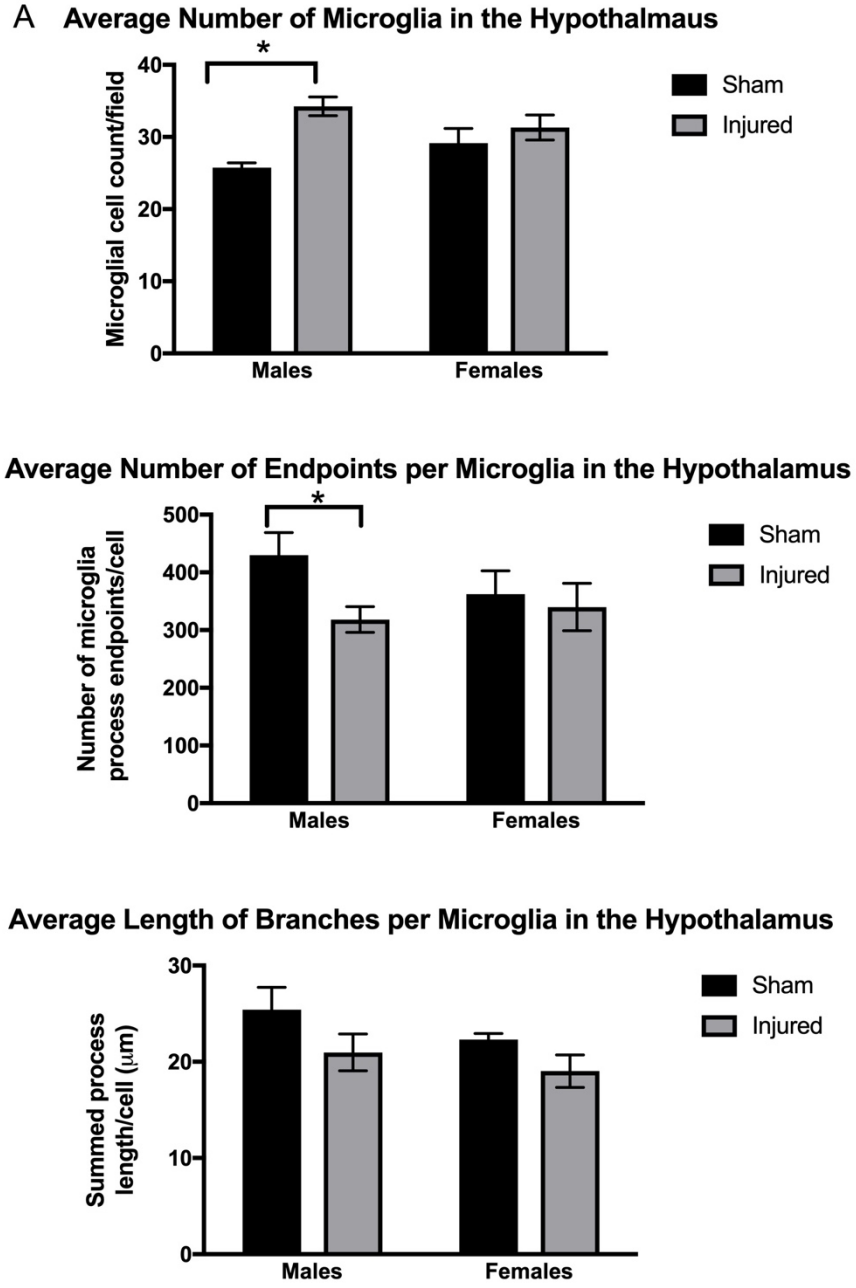


*Figure 13 A) The Average of Microglia Cell Numbers Was Significantly Increased at 7 DPI in Males but Not in Females in the PVN (n=5). B) The Number of Microglia Process Endpoints Per Cell Were Significantly Decreased At 7 DPI in Males but Not in Females in the Hypothalamus (n=5). C) There Were No Significant Differences in Process Branch Length for Males or Females in the Hypothalamus (n=5) Bars Represent the Mean±SEM, *P<0.05.*

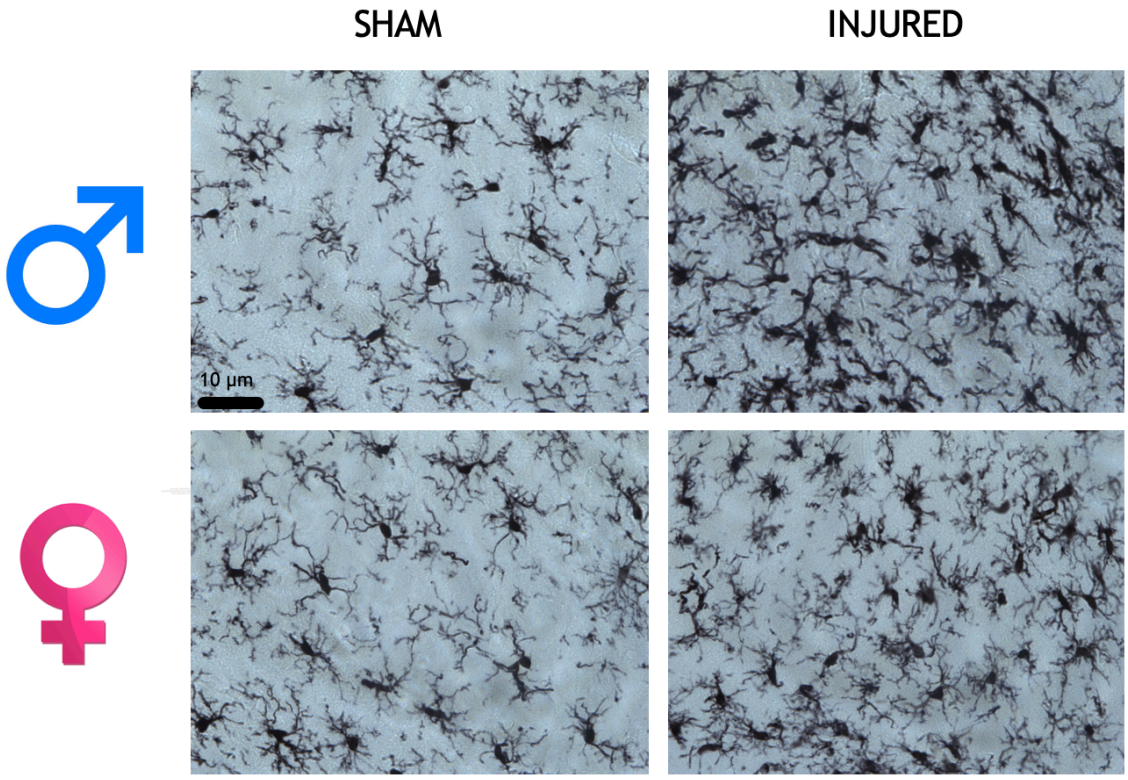


Figure 14 Representative 40x Images Showing IBA-1 Stained Microglia in the Dentate Gyrus. The Top 2 Panels are Representative Images of Male Sham (Left) and Male Injured (Right). The Bottom 2 Panels are Representative Images of Female Sham (Left) and Female Injured (Right).

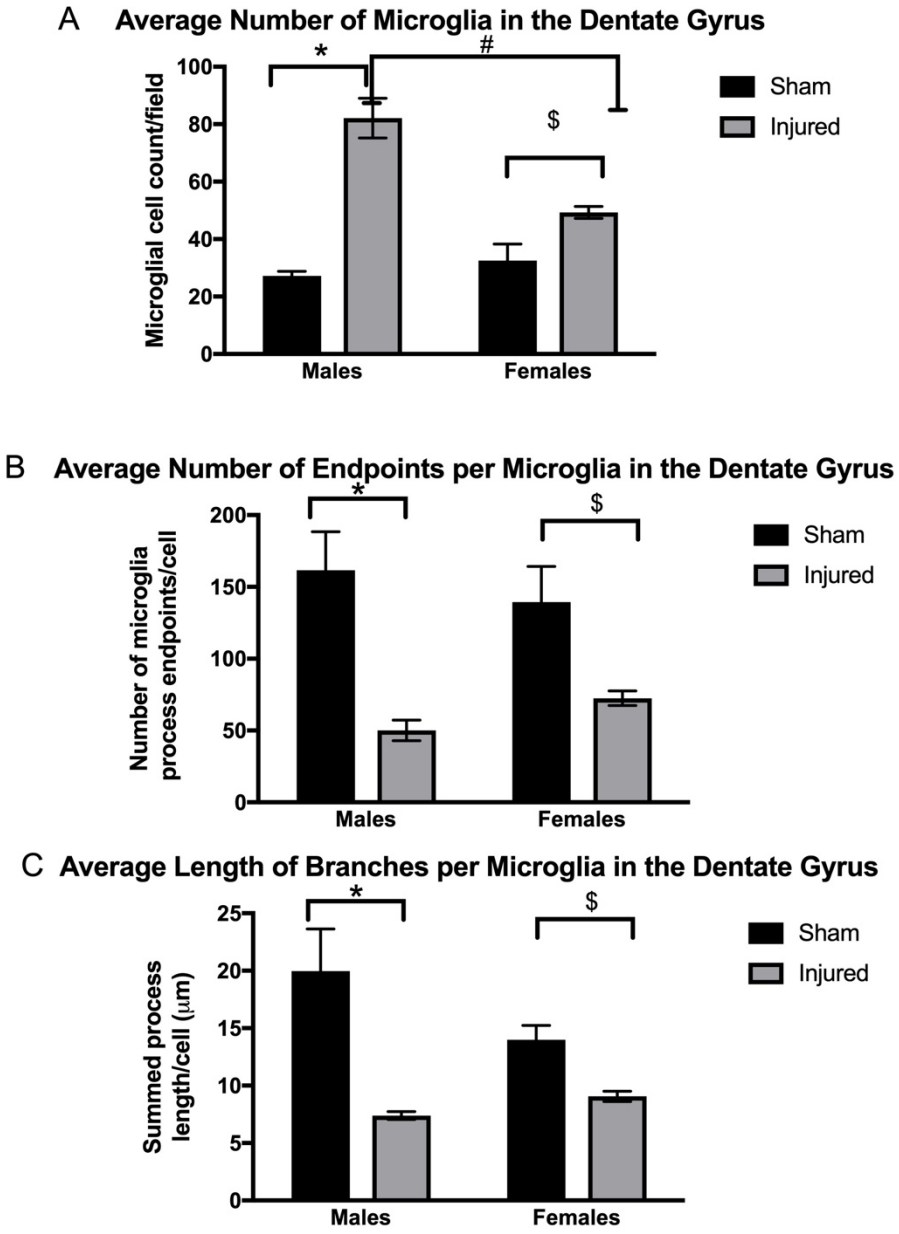


*Figure 15 A) The Average of Microglia Cell Numbers Was Significantly Increased at 7 DPI in Males (*p<0.05; n=5) and in Females ($p<0.05; n=5) Compared to Respective Shams in the Dentate Gyrus. Additionally, There is a Significant Decrease in Microglia Cell Number Between Injured Males and Injured Females in the Hippocampus (#p<0.05; n=5). There Was an Interaction Between Sex and Injury. B) The Number of Microglia Process Endpoints Per Cell Were Significantly Decreased at 7 DPI in Males (*p<0.05; n=5) and in Females ($p<0.05; n=5) Compared to Respective Shams in the Hippocampus. C) There was a Significant Decrease in Process Branch Length in Males (*p<0.05; n=5) and Females ($p<0.05; n=5) in the Hippocampus. Bars Represent the Mean±SEM.*

10.2 Gene Expression of Glucocorticoid Receptors

In these experiments, relative gene expression of GRs from extracted hypothalamus and anterior hippocampus were quantified. GR's have a lower affinity for CORT but are present in higher quantities. CORT only binds to GR's when there is an overabundance, inciting pathways to bring CORT levels back to homeostatic levels. Based on the preliminary data for GR expression in males, we hypothesized that GRs would be decreased at 7 DPI in both the hypothalamus and hippocampus. Based on the high variability of plasma CORT levels in injured females vs. injured males we predict more variability in females.

In the hypothalamus, significance for GR gene expression was not reached in males (t_{10}=1.220, p=0.218; n=5-7). There were no significant differences in GR gene expression in females (t_{10}=0.356, p=0.729; n=4-8). In the hippocampus, significance for GR gene expression was not reached in males (t_7=1.371, p=0.218; n=4-6) or females (t_{12}=1.651, p=0.125; n=6-8). (See Figure 16).

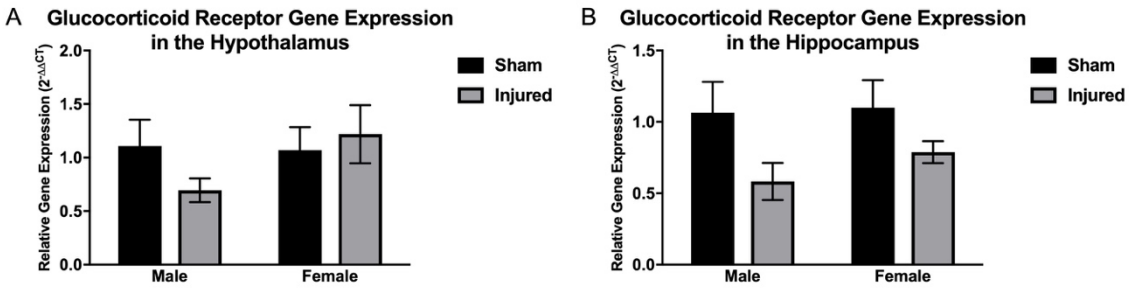


Figure 16 A) Significance is Not Reached for GR Gene Expression Between Sham and Injured Males and There are No Significant Differences for GR Gene Expression Between Sham and Injured Females. (n=4-8). B) Significance is Not Reached for GR Gene Expression in Males or Females Between Sham and Injured Rats in the Hippocampus (n=3-7). Bars Represent the Mean±SEM.

10.3 Protein Expression of Glucocorticoid Receptors

Our laboratory's preliminary data indicated that relative GR gene expression was significantly increased at 5 DPI but significantly decreased at 7 DPI in comparison to sham. We hypothesized that in both regions, the hypothalamus and hippocampus, protein expression would be decreased in comparison to sham at 7 DPI in males. Female expression was unknown.

In the hypothalamus, injured males were significantly decreased in GR protein expression compared to sham males (t_{10}= 3.397, p=0.007; n=5-7), however, injured females were significantly increased in GR protein expression (t_{12}= 2.562, p=0.025; n=5-9). When comparing GR expression of sham males to GR expression of sham females, significance was not reached (t_7= 1.371, p=0.213; n=4-5). In the hippocampus, injured males had a significant decrease in GR protein expression compared to sham males (t_{10}= 2.977, p=0.014; n=4-8). There were no significant differences between sham females and injured females (t_{11}= 0.817, p=0.436; n=4-9). When comparing GR expression of sham males to GR expression of sham females, sham males expressed significantly more GR than sham females (t_6= 6.552, p=0.001; n=4).

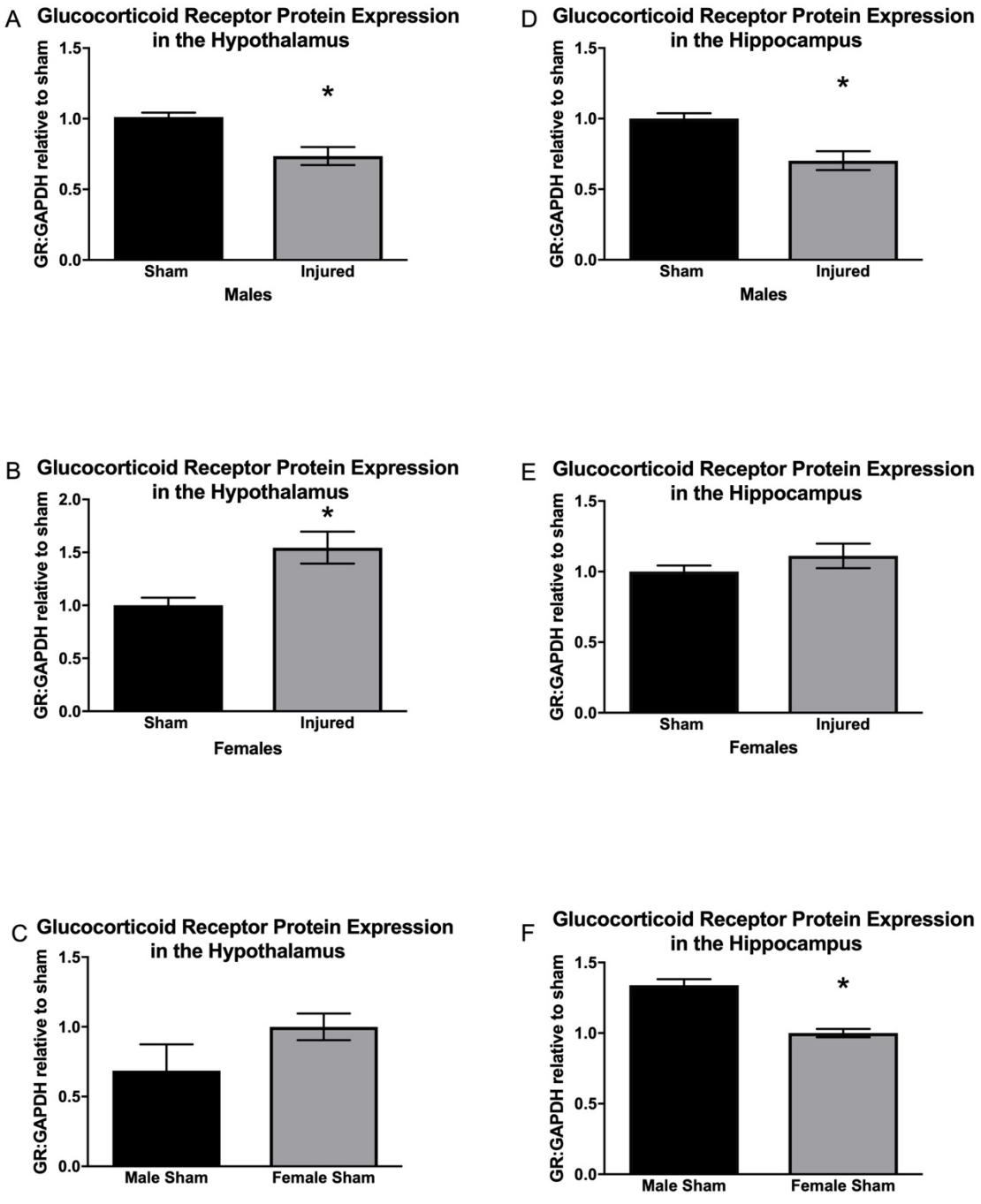


*Figure 17 Glucocorticoid Protein Expression in the Hypothalamus and Hippocampus. A) and B) In the Hypothalamus, There Was Significant Decrease in GR Gene Expression in Males (N=5-7) Yet Significant Increase in GR Expression in Females (n=5-9). C) Significance Was Not Reached Between Hypothalamus Sham Male and Sham Female GR Expression (n=4-5). D) and E) In the Hippocampus, There Was Significance Decrease in GR Gene Expression in Males (n =4-8), But No Significant Differences in Females. F) In the Hippocampus, Sham Females Expressed Significantly Less GR Protein Expression Than Sham Males (n=4). Bars Represent the Mean±SEM. *P<0.05*

10.4 Gene Expression of Corticotrophin-Releasing Hormone

In these experiments, we quantified the relative gene expression of CRH from extracted hypothalamus and hippocampus. It is widely known that CRH is released from the PVN of the hypothalamus, however, when the hippocampus is exposed to CORT, hippocampal neurons, particularly those in the CA1 and CA3, also release CRH[173]. Previous preliminary data indicated that circulating CORT was lower, but not quite significant in males in our laboratory's studies. However in a lateral model of FPI, basal levels of CORT at 7 DPI were significantly lower than shams[174]. With a decrease in CORT and a decreased expression of GR in males, there is a decrease of negative feedback for CRH. Therefore, we hypothesized that CRH would be higher in the injured animals in both males but not in females.

There were no significant differences in relative CRH gene expression in either the hypothalamus or hippocampus. My hypothesis was not supported by relative CRH gene expression.

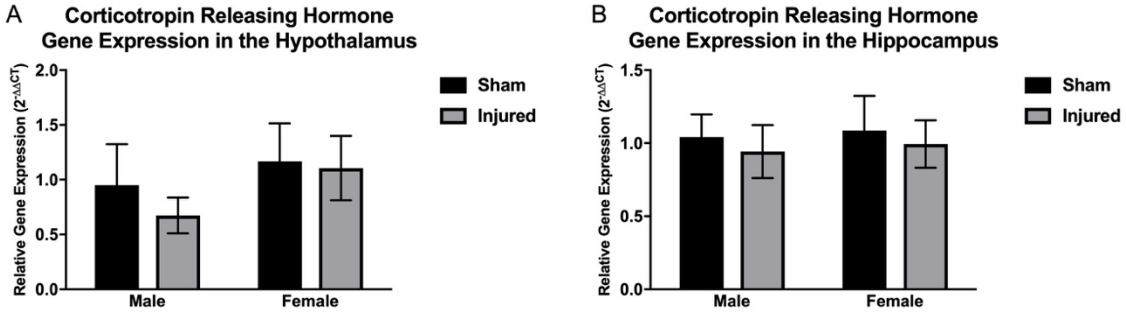


Figure 18 Relative Gene Expression Analysis of CRH. A) and B) There Were No Significant Differences in CRH Gene Expression in Either the Hypothalamus (n=4-7) or the Hippocampus (n=4-6). Bars Represent the Mean±SEM.

11.0 DISCUSSION

The purpose of this project was to evaluate neuroinflammation in an area with no quantifiable neuropathology and an area with neuropathology in the HPA axis and determine if GR levels were changing in those regions, providing evidence that GR expression may be driving microglial activation after TBI. Therefore, these experiments were designed to test the hypothesis that **diffuse experimental TBI incites sexual differences in the HPA axis, influencing hypothalamic and hippocampal microglia presentation as well as glucocorticoid receptor levels at 7 DPI.**

11.1 Summary of Results

Hypothalamus	Inflammation	GR Gene Expression	GR Protein Expression
Males	↑	—	↓
Females	—	—	↑
Male to Female	—	—	—

Table 3 Summary of Results for The Hypothalamus. Microglia Presentation and Activation Is Indicative of Inflammation in Males, but Not in Females. There Were No Significant Changes in GR Gene Expressions in Males or Females. GR Protein Expression Significantly Decreased in Males, Yet Significantly Increased in Females.

Hippocampus	Inflammation	GR Gene Expression	GR Protein Expression
Males	↑	—	↓
Females	↑	—	—
Male to Female	M > F	—	M > F

(shams)

Table 4 Summary of Results for the Hippocampus. Microglia Presentation and Activation is Indicative of Inflammation in Males and Females, with Microglia Presentation Being Greater in Males Than in Females. There Were No Significant Differences in GR Gene Expression. GR Protein Expression Significantly Decreased in Males, but Not Females. Sham Females Expressed Significantly Less GR Protein Than Sham Males.

We found significant increases in PVN microglia cell number and decreases in endpoint/cell and branch length per cell in males, which is indicative of microglia morphology during neuroinflammation. We saw no significant differences in females, but also saw no interaction between sex and injury in our two-way ANOVA. See Table 3. In 2016, Rowe et.al published that males did not indicate any quantifiable pathology in the PVN at 7 DPI, suggesting that another mechanism may influence microglia activation in the PVN, possibly through GR-related mechanisms[175].

In the dentate gyrus, an area of overt neuropathology, we found a significant increase in microglia cell numbers in both injured male and injured females compared to respective shams, however, injured males had significantly more microglia cell numbers than injured females. Both injured males and injured females had a significant decrease in endpoints/cell and injured males had a significant decrease in branch length/cell. A two-way ANOVA indicated an interaction between sex and injury, with a main effect that sex influences injury outcome See Table 4.

There are few studies that have evaluated microglia and females. In this study, activated microglial presentation differed between males and females, where both showed evidence of microglial activation – where males responded more than females. Mechanisms that could be mediating this difference have been indicated by sex-specific genes and possibly maintained by circulating sex hormones, including 17β-estradiol[176]. Another study indicated that naïve males exhibited an increased amount of phagocytotic activity compared to naïve females; while naïve females secreted higher levels of IL-1β and TNF-α compare to naïve males. In another model of TBI (CCI), Villapol et al. demonstrated that neuroinflammation in males is much greater than females, not only in the dentate gyrus, but also in a the cortex and thalamus[166]. In the cortex, Villapol et al. found that females exhibited an increase in activated microglia morphology (ramified with rough prolongations) at 4h, 1 DPI, and 30 DPI, where males predominantly exhibited amoeboid microglia until 3 DPI. Also, at 1 DPI in CCI, injured males exhibited an increase of peripheral myeloid cells (precursor cells; amoeboid morphology) followed by proliferation of resident microglia at 3 DPI, however female myeloid influx and microglia activation is diminished[177]. Villapol's data was similar to our results in the dentate gyrus, where the number of microglia in males increased by 200% and females increased by 52%. In the hypothalamus, the number of microglia in males increased by 33%, while no differences were discovered in females. Amoeboid cells in the dentate gyrus were not evaluated in this study but could potentially also demonstrate sex-differences. Together, these data compliment previously reported sex-differences in neuroinflammatory response after brain injury.

Estrogen and progesterone[178, 179] are noted to play a protective role against injury including decreasing neuroinflammation in the brain as a whole[107]. In a stroke model, when males were given a 17β-estradiol and progesterone treatment after stroke, microglia activation and lessen proinflammatory cytokine release[180]. The literature suggests that differences in circulating estradiol, ILs, and myeloid cells could have contributed to the differences observed in this study. A limitation of this study did is we did not track female estrous cycles, as menstrual cycle might influence outcome of TBI[150, 181], potentially adding variability to data from females.

The changes in microglia presentation and activation in both the PVN and dentate gyrus indicate that TBI influences inflammation in the HPA axis at 7 DPI. Additionally, the dentate gyrus has overt neuropathology, however as previously stated, the PVN does not. These data led us to evaluate GR gene and protein expression in both regions. In the hypothalamus, male GR protein expression decreased while female GR protein expression increased, however there were no changes in gene expression. In the hippocampus, GR protein expression is decreased in injured males, but no differences in gene expression. There are no differences in GR gene expression or protein expression in females. These data indicate that other mechanisms may be regulating protein expression downstream from gene transcription. Additionally, these data support the notion that males and females respond to TBI differently.

In the hypothalamus, there are differences between gene expression and protein expression in both sexes, but in the hippocampus when gene expression decreases, protein expression decreases and when gene expression stays constant, protein expression stays constant. Vogel and Marcotte (2012) explain that, in a homeostatic steady state, mRNA and protein expression only correlate about one to two thirds of the time, depending on the organism, however studies attempting to investigate the correlation in disrupted states, have acquired conflicting results[182]. Additionally, when CORT is low, GR's will downregulate their receptors through reduced mRNA transcription, protein degradation, ubiquitin-dependent degradation, receptor inactivation or modifications[183] CORT is low in males 7 DPI, if GRs are reduced by degradation or inactivation, may explain why our data indicated no changes in gene expression but decreases in protein expression .

Literature suggests that GR expression in the HPA axis is critical for CORT regulation and affective disorder management. Decreased GR expression in the hypothalamus is associated with higher levels of circulating CORT, indicative of HPA axis dysregulation[184]. Decreased GR expression in the hippocampus is associated with increased affective disorders[81, 185].

Understanding how TBI affects GR receptors post-injury and thus how GRs influence HPA axis regulation can help provide therapeutic targets for clinical treatment.

In normal conditions, CORT binds to GRs in the PVN which slows CRH release – a standard negative feedback loop. Other models of TBI in males indicate acute increases in CORT and CRH. For at least 6 hours after experimental TBI, CORT is increased[186, 187], then decreases for up to 7 DPI before slowly increasing at 14 DPI[174]. However, our model of TBI indicates that in males, CORT is decreased at 54 DPI, suggesting long-term HPA axis dysregulation[162]. Concurrently, CRH levels increase for up to 4 hours post-injury[188]. The mechanisms behind the acute CRH increase are still unclear.

At 7 DPI CORT is dysregulated in the CCI model[174] – and is trending towards a decrease in males with no change in females in our preliminary data in mFPI. While our data indicates downregulated GRs in males and upregulated GRs in females, CRH gene expression showed no significant differences in either males or females. It is expected that CRH expression would not change in females, as GR levels did not change. However, in males, this could be indicative of another influencing mechanism. CRH is also regulated by diurnal rhythms through MR's and may possibly reach homeostasis. However, dysregulation can occur downstream with ACTH in the pituitary. As previously stated, DAI causes circuit disruption between the hypothalamus and pituitary, leading to the increase in risk for hypopituitarism[111] and ACTH deficiencies[189]. An ACTH deficiency will reduce the level of CORT production, keeping the HPA axis in a state of dysregulation.

11.2 Future Studies and Implications

TBIs are not solely mechanical events, but rather, they are a complex cascade of molecular mechanisms that involve several intertwining systems. TBI incites neuroinflammation, which, if

left unattenuated, can become neurotoxic. Stress, environmental or physiological, has also been shown to incite neuroinflammation. What occurs when TBI and stress are combined?

This project was designed to provide the answers to basic questions before more complex studies could be designed. The next step is to conduct colocalization of GRs on microglia in the hypothalamus and dentate gyrus. The dentate gyrus was chosen for SA as it is the brain region with most dense concentration of GRs next to the PVN. However, biopsies contained the entire anterior hippocampus. Colocalization will determine if GR levels are changing on microglia in the PVN and dentate gyrus specifically.

Glucocorticoids receptors have many different isoforms as a result of mRNA alternative splicing: GRα and GRβ. GRα is the most expressed of the GR isoforms[190]. Little is known about the functions and expression levels of GRβ, however there is evidence that GRβ contributes to glucocorticoid resistance. When CORT binds to GRβ, it increases GR resistance [10] and can also up-regulate Toll-like Receptor 2/4 (TLR2/4)[40] which increases microglia activation[41]. Future studies are needed to determine which isoform has increased expression. It is expected that with an increase in inflammation but a decrease in GR protein expression, that the GR isoforms remaining are GRβ.

This project used previously harvested tissue. During tissue collection, rats of the same cage were live decapitated with a two-minute lapse between rats. CORT rises after 4 minutes of disturbance from the home-room. The second animal may have had yielded atypical results. As such, we have amended our protocol to reflect home-room to live decapitation under 4 minutes for both rats in the cage. We are currently expanding our animal numbers all groups for the later time points.

This study is among the first to investigate sex differences in the HPA axis after TBI. Further, there is a paucity of information on how estrous cycle influences general injury outcomes. However, the estrous cycle has been reported to influence CORT levels, GR expression,

neurological outcomes after injury (in humans)[108, 150, 152, 191]. Future translational experiments would do well to track estrous, which may possibly explain why the outcome measures between female sham and injured rats differed in comparison to male sham and injured rats. Additionally, it would be beneficial to add a behavioral component for anxiety, such as the open field test, as several studies report that women are more likely to have continuing symptoms of anxiety and depression after mild TBI[147, 153-156].

11.3 Conclusion

The HPA axis is a highly regulated system and disruption of that system provokes several hormone imbalances that can contribute to reported endocrinopathies as well as persisting anxiety and/or stress disorders. Taken together, these data indicate HPA axis dysregulation after TBI at 7 DPI in males and females. Further investigation is needed into the mechanism behind microglia presentation in the PVN in males and the GR isoform presentation on microglia in both the PVN and dentate gyrus. Additionally, further investigation is needed into the mechanisms behind the sexual differences in inflammation and GR expression between males and females. These data are among the first in the field to look at females and the HPA axis after injury and as such will serve as a launchpad for future, mechanistic experiments.

Most importantly, these data are clinically translational and can be applied to patient care. Patients who sustain a DAI have an abrupt increase in CORT levels, taken together, these data can help physicians asses treatment plans based on the fundamental knowledge of how inflammation increases in males verses females and how the HPA axis dysregulates over the first week of injury. Translational research is imperative as it increases the standard for personalized medicine, increasing the quality of life for each individual patient.

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APPENDIX A

DATA COLLECTED JANUARY 2019 – JULY 2019

Optimization of Qiagen DNA/RNA/Protein Protocol

Homogenization:

1. Add **1ul** of **BME** to **100ul** of **Buffer RTL** in a fume hood (can store up to 1 month)
2. In a 2mL tube, add **600ul** of **Buffer RLT**

#Samples _____ + 1 _____ X 600ul of Buffer RLT/BME = _____ Total ul

1:100 ratio $\frac{1ul\ BME}{100\ ul\ RLT} = \frac{x}{\_\_\_\_\_}$ $100x =$ ___ $x =$

3. Transfer sample to tube with buffer
4. Use homogenizer to homogenize sample (2 minutes)

Procedure - *RNA is centrifuged at 4C; protein at 13C*

1. Centrifuge the lysate for 3 min at full speed
2. Remote the supernatant and transfer to an AllPrep DNA spin column/collection tube
3. Centrifuge for 30 seconds at 8000g. Repeat. Discard (not using DNA).

RNA Protocol

1. Take the flow-through from previous step and add **430ul of 100% ethanol**. Mix well by pipetting.
2. Transfer 700 µl of the sample to an RNeasy spin column placed in a 2 ml collection tube.
3. Centrifuge for 15 seconds at 8000g
4. **Transfer the flow-through to a 2 ml tube for protein purification**. Repeat and flow-through on ice.
5. Add **700 µl Buffer RW1** to the RNeasy spin column
6. Centrifuge for 30 seconds at 8000g. Discard the flow-through
7. Add **500 µl Buffer RPE** to the RNeasy spin column.
8. Centrifuge for 30 seconds at 8000g. Discard the flow-through
9. Add **500 µl Buffer RPE** to the RNeasy spin column
10. Centrifuge for 1 minutes at 8000g
11. Add **500 µl of 80% EtOH** to the RNeasy spin column
12. Centrifuge for 2 minutes at 8000g
13. Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through. Leave lid open. Centrifuge at full speed for 5 min.
14. Place the RNeasy spin column in a new 1.5 ml collection tube. Add **30 µl RNase-free water** directly to the spin column membrane. Centrifuge for 1 minute at 8000g to elute RNA.
15. Do Nano-drop → then place on ice/freeze at -80 until can do cDNA steps.

Sample	Ng/ul	260/280 ratio

Total Protein Precipitation Protocol

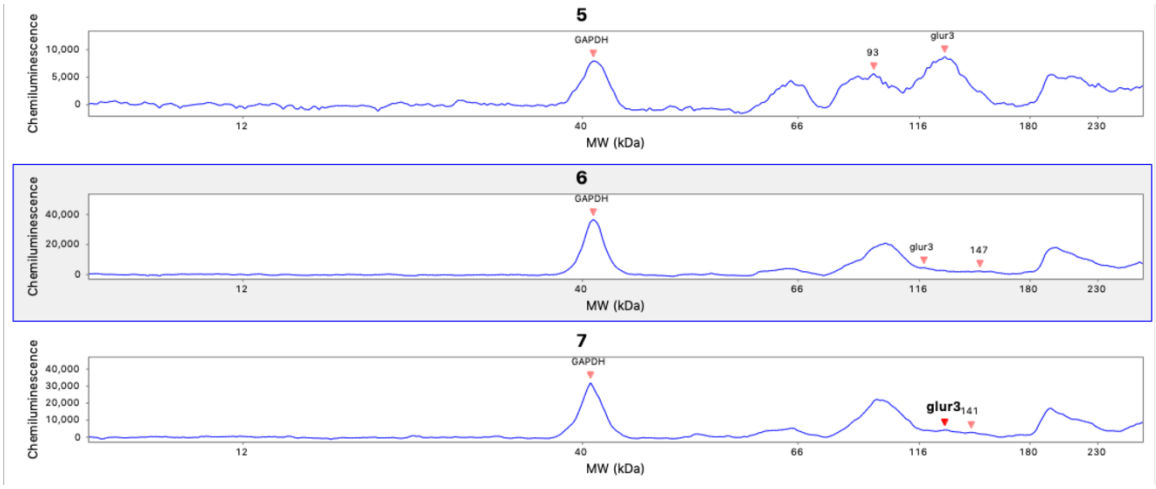
1. Take flow through from **RNA Step 4** and add **600ul of Buffer APP**. Mix vigorously and incubate at room temperature for 10 min to precipitate protein.
2. Centrifuge at full speed for 10 min.
3. Carefully decant the supernatant.
4. Add **500 µl of 70% ethanol** to the protein pellet.
5. Centrifuge at full speed for 1 min.
6. Remove the supernatant by using a pipet or by decanting as much liquid as possible.
7. Dry the protein pellet for 10 min at room temperature.
8. Add up to **100 µl SDS** and mix vigorously to dissolve the protein pellet. Vortex for several minutes or disaggregate the pellet by pipetting up and down several times.
9. Incubate for 5 min at 95°C to completely dissolve and denature protein. Then cool the sample to room temperature.
10. Centrifuge for 1 min at full speed to pellet any residual insoluble material.
11. Do protein assay or freeze at -80 until can do protein assay.

APPENDIX B

DATA COLLECTED JANUARY 2019 – JULY 2019

20/80 Optimization

The original study design included time points at 56 and 168 DPI, however, original samples from the 2017 study were stored incorrectly. Almost half of the brain punches of 56 and 168 DPI samples were stored, either entirely or temporarily, in -20°C conditions. As the length of time the samples were -20°C conditions were unknown, and the total effect on qPCR and protein analysis was unknown, I ran several samples through the full protocol. For samples stored in the -80°C, the average ng/ul was ~90 and the 260/280 ratio average was ~2.02. For samples stored in the -20°C, average ng/ul was ~15 and the 260/280 ratio average was ~1.88. Protein concentration values on the BCA assay were inconsistent and not associated to freezer storage. However, the protein assay showed many inconsistencies, including atypical protein levels and atypical protein bands for the target protein.



APPENDIX C

DATA COLLECTED JANUARY 2019 – JULY 2019

RT-qPCR Plate Design

Multiplex RT-PCR Gene expression assay protocol for 96 well

Brain region: HYPO
Conc smaple: 5 ng
Gene control: 18s RNA
Gene testing: GR

Synaptophysin		TSP1	
GAP-43		TSP2	
PSD-95		α2δ-1	
Ezrin		arc	
		GR	x

Plate set up 2/8/19

	1	2	3	4	5	6	7	8	9	10	11	12
	Gene			18s rRNA			Gene		18s rRNA			
A	00B			84B			0			0		
B	01B			85B			0			0		
C	04B			92B			0			0		
D	05B			119A			0			0		
E	10B			0			0			0		
F	11B			0			0			0		
G	78B			0			0			0		
H	83B			0			0			Water NTC		

Gene / 18s rRNA / NTC

Reagent using NTC = get DEPC 9ul
1) 20x Taqman Fast Advance Universal Master Mix
Part #: 4444963

2) TaqMan Gene Expression Assay
Part # Rn00561369_m1

Procedure
1) Mix Primer and Master Mix as next page, centrifuge, mix and centrifuge
2) use defrosted diluted cDNA. Vortex and centrifuge
3) Load reagents on well. Then mix for 500 rpm, 30 sec. Then centrifuge 1000rpm, 3 min
9ul cDNA (1:10) + 11ul Master Mix/ Primer
4) Use RTPCR machine, use template for 96 well.
Notes

RTPCR reaction for one wll 20ul total

(Gene) TaqMan Gene Expression Assay	1.0 µl	→ make 11ul Master Mix/ Primer (both Tagmans)
18S TaqMan Gene Expression Assay	1.0 µl	
2X TaqMan Universal Master Mix	9.0 µl	
10x dilution of cDNA (40 µl stock)	9.0 µl	Keep the sample always the same.

***need 13*3*9 µl = 351 µl per plate

Reactoins

For both 18s and Gene of interest

2X TaqMan Universal Master Mix	386.1 ul	
20X TaqMan Gene Expression Assay	42.9 ul	**for each - taqman AND 18s**

Sample number	Samples	
00B	3	
01B	3	
04B	3	
05B	3	
10B	3	
11B	3	
78B	3	
83B	3	
84B	3	
85B	3	
92B	3	
119A	3	Positive control
Water NTC		

This amount can be optimized for each different gene

APPENDIX D

DATA COLLECTED JANUARY 2019 – JULY 2019

Wes Plate Design

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
ladder		120A		121B		123B		126B		093A		098B		117A		117B		119A		CTX		Sample Buffer		119A	
A.D.		0.25		0.25		0.25		0.25		0.25		0.25		0.25		0.25		0.25		0.25		0		0.25	
blocking		GluR 1/25+**GAPDH 1/25**																				A.D.	A.D.	ERK 1/10	
strep		mRTU+rbRTU																						rbRTU	
		luminol																							