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# CARNOSIC ACID DIFFERENTIALLY MODULATES THE NRF2-ANTIOXIDANT RESPONSE IN MALE AND FEMALE MICE FOLLOWING EXPERIMENTAL TRAUMATIC BRAIN INJURY

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DISSERTATION

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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ABSTRACT OF DISSERTATION

# CARNOSIC ACID DIFFERENTIALLY MODULATES THE NRF2-ANTIOXIDANT RESPONSE IN MALE AND FEMALE MICE FOLLOWING EXPERIMENTAL TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States (U.S.). Each year, an estimated 2.8 million Americans are diagnosed with a TBI due to falling, motor vehicle collisions, gun violence, and sports related concussions. Although inflicted by a single event, the post-traumatic effects of TBI often develop into a life-long disease. Survivors often experience cognitive decline, memory loss, emotional instability, changes in personality, and physical disabilities. A single TBI, and more-so repetitive TBIs, place an individual at a greater risk of developing chronic neurological disorders, such as dementia or Alzheimer's disease, earlier in life. Additionally, the high costs and long-term care associated with treating TBI also strains families, companies, and the health care system. To develop an effective treatment, the underlying neuropathophysiology of TBI has been well studied for decades. Historically, basic research has been conducted more frequently in males, leaving a gap in knowledge about how females may react to a treatment. This may be a contributing factor as to why all TBI clinical trials have failed, leaving us without a treatment for this disease.

One of the central secondary mechanisms associated with TBI is oxidative stress. Within minutes of the initial mechanical injury, the injured neurons and glial cells begin producing toxic amounts of reactive oxygen/nitrogen species (ROS/RNS), free radicals, undergo apoptosis, and initiate other damaging secondary cascades. Oxidative damage typically peaks around 3 days post injury but may persist for weeks depending on the injury severity. Because of the central role, early onset, and prolonged nature of oxidative stress, it has remained a logical avenue for developing treatments for TBI.

Cells are equipped with an innate antioxidant defense system to battle oxidative stress. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper that regulates the expression of several antioxidant proteins within the antioxidant response element (ARE). In healthy cells, Nrf2 is rapidly turned over to maintain a proper redox balance. However, following injury, a rise in oxidative stress, or in the presence of electrophilic species, Nrf2 is shuttled from the cytoplasm to the nucleus to initiate transcription of detoxifying enzymes. Recent understanding of this pathway has led to the development of a class of drugs called "Nrf2 activators". The pro-electrophilic drug carnosic acid (CA), an extract in the common herb rosemary, has been shown to be an extremely effective Nrf2 activator and antioxidant.

The goal of this dissertation was to describe the innate time course of Nrf2-ARE activity following a single controlled cortical impact injury in male and female mice. Previous work in our lab showed that a single controlled cortical impact (CCI) injury increased markers of oxidative stress and consequently, increased the production of Nrf2 mediated phase II enzymes. A single dose of CA at multiple different time points was able to reduce markers of oxidative stress while boosting the Nrf2 response. The goal of this project was to recapitulate this experiment in male and female mice to illuminate potential

sex differences in the Nrf2-ARE response to CCI and test the efficacy of CA as a neuroprotective agent.

In Aim 1, mice were sacrificed at 1, 2, 3, 7 days post injury (DPI). Both the injured cortex and hippocampus were analyzed for Nrf2 protein and mRNA the Nrf2-ARE biomarkers HO-1 and NQO1. In Aim 2, mice received the same injury and were given a single 1.0 mg/kg I.P. dose of CA 1 hr post-injury and sacrificed at 1 and 3 DPI and only the cortex was analyzed. We then examined how CA augmented the cellular localization (nuclear vs cytoplasmic) of Nrf2 and transcription of Nrf2 regulated mRNA biomarkers.

Results from Aim 1 detected notable sex-based differences in the innate Nrf2 response. Male mice had greater amounts of HO-1 mRNA, whereas female mice exhibited higher amounts of HO-1 protein. Results from Aim 2 indicate a sex-based difference in the therapeutic action of CA. Nrf2 nuclear localization was increased in males treated with CA, whereas CA treated females had increased cytoplasmic concentrations of Nrf2. Surprisingly, qRT-PCR analysis revealed that CA treatment drove down transcription of HO-1, NQO1, and Nrf2 in males. Conversely, CA treatment enhanced transcription of HO-1, NQO1, and decreased Nrf2 in females.

From these results, we can conclude that there are minor regional and temporal differences in the Nrf2-ARE pathway in male and female rodents. Also, the therapeutic mechanism of action associated with CA may be different in males and females given that a single dose had the opposite effect on Nrf2 cellular localization, however, with such modest drug effects, this cannot yet be concluded. Future studies may consider a thorough behavioral and histological analysis following a single dose of CA to measure functional recovery and visualize the neuroprotective effect. Additionally, a pharmacokinetic profile of CA clearance would be beneficial in determining the optimal dosing regimen for each sex.

KEYWORDS: TBI, Nrf2, Carnosic Acid, Sex, Mice

Jacob A. Dunkerson

07/27/2021

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DEDICATION

*Dedicated especially to my wonderful wife Jill and amazing son Tenzin for their love, support, and lightheartedness; to my mother Pamela and late father Ronald for instilling values of dedication, hard work, and perseverance; and, to the rest of my family and friends for their encouragement throughout the process.*

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# CHAPTER 1. INTRODUCTION

## 1.1 Traumatic Brain Injury

### 1.1.1 Epidemiology

Traumatic brain injury (TBI) is an overarching term used to describe the disruption of normal brain function acquired from a bump, blow, or jolt to the head or by a penetrating object [1]. It is common for people with a TBI to suffer from a myriad of symptoms and disorders, ranging from seizures, changes in personality, memory loss, limb spasticity or flaccidity, incontinence, and loss of other bodily functions/control [2]. In the United States alone there are an estimated 2.8 million new cases each year, and a recent poll revealed that nearly 23 million adults over 40 years of age reported sustaining at least one head injury at some point in their lifetime [3-5].

TBI-related deaths are most commonly caused by firearms, whereas TBI-related hospitalizations and emergency department visits are primarily the result of falling, motor-vehicle collisions, and being struck by/against an object [1, 3, 6, 7]. In addition to being a major cause of death and disability, with an economic cost of nearly $76.5 billion each year in medical bills and indirect costs, TBI represents significant health care crises in the United States [8, 9].

The severity of head trauma, along with pre-injury lifestyle factors and post-injury medical care all considerably influence the patient's long-term prognosis [10-17]. Although many TBI survivors may seem to achieve a full recovery, after years of research and debate, TBI is now recognized as a chronic neurological disease [9, 18-20]. In fact,

recent data shows that a single TBI increases the chances of developing Alzheimer's disease rises with each subsequent head injury [9, 21]. Moreover, repetitive TBI places individuals at a higher risk of developing neurological disorders, neurodegenerative diseases, neuroendocrine disorders, and psychiatric disease later in life [2, 17, 22].

### 1.1.2 Predictors in TBI Outcomes

Although TBI effects people of all ages, races, but even with all factors considered, males are at a significantly higher risk of acquiring a fatal head injury [1, 5, 6]. There are several factors that contribute to patient prognosis and ability to return to their pre-injury level of function. Studies have indicated that certain demographics, such as age, have been linked to TBI prognosis [7, 17, 23]. The elderly are more prone to falls and are also at greater risk of death and fare worse in the long-term. Another predictor of patient outcome has been directly linked to severity scores on the Glasgow Coma Scale upon admission [24-26]. This predictor has been linked to length of time in coma, with worse outcomes being noted with persons experiencing prolonged comatose states [15, 27-29].

### 1.1.3 Severity Assessment and Glasgow Coma Scale

During the early stages of clinical TBI research, physicians began to appreciate the complex relationship between injury severity and post-injury symptoms [30, 31]. As research gained momentum, they recognized the need for a standard assessment protocol to accurately categorize patients based on injury severity [30]. Over the years, these observations led to the development of key clinical assessment measures that are still widely used today.

The Glasgow Coma Scale (GCS) was one of the first standardized TBI assessment

tools used by clinicians to assess TBI severity in the human population [32, 33]. The initial iteration – The Coma Index – was not intended as a diagnostic tool, rather, it was purposed as means of objectively assessing the level of a person's consciousness while in a comatose state [26, 34]. As it's use gained favor throughout the 1970's, the GCS was eventually assigned an ordinal scale for standardization and to monitor patient recovery [11]. By the 1980's, the GCS had evolved into a prognostic tool that measured the responsiveness of three different modalities – eye opening, motor response, and verbal response – an ordinal scale that corresponded to 3 levels of injury severity (mild, moderate, and severe) [11, 13, 15, 16, 35-37]. With these adaptations, the GCS was quickly adopted as the gold standard evaluation tool due its use of clear terminology and relive ease and quickness to administer [14, 35]. Nowadays, clinicians often take a multimodal approach when evaluating patients that includes bedside assessments in conjunction with neuroimaging techniques and neuromonitoring equipment to achieve a more precise diagnoses [38-42].

## 1.2 Current Treatment Strategies

Though standard of care differs around the world, initial medical care aims to stabilize the patient by re-establishing intracranial pressure (ICP), optimizing cerebral perfusion pressure (CPP) and brain oxygenation [43-47]. Until recently, decompressive craniectomy was a routinely used to decrease ICP pressure, however, advances in monitoring CPP have demonstrated that reducing ICP negatively effects the state of CPP [44]. Regulating CPP during the acute stages of the injury has been shown to decrease ICP thereby reducing the likelihood of ischemia and reperfusion injury [44, 48, 49].

## 1.3 Previously Tested Pharmacological Therapies for TBI

For the past several decades, institutions and pharmaceutical industries have conducted multiple clinical trials testing a variety of synthetic and natural compounds for treating TBI [50-58]. Many of these drugs were developed to target a specific element of the secondary cascade, whereas others possessed a broader range of effects [59-61]. Of these experimental drugs, the following classes made it to phase II and phase III clinical trials classes of drugs ranged from steroids, $Ca^{2+}$ channel blockers, glutamate (NMDA) receptor antagonists, immunosuppressants, inflammatory modulators, sex hormones, and antioxidants [62, 63].

TBI has a long track record of promising therapeutic drugs followed by failed clinical trials and this is just a slight indication to the complex nature of the injury. Another major challenge with successfully translating drugs lies in accurately defining the pharmacodynamics and pharmacokinetics of the drug preclinical animal models [60, 64-66]. Some of the most effective drugs to date are large, highly polar, lipophilic molecules with multiple mechanisms of action, such as the non-glucocorticoid 21-aminosteroid dubbed tirilazad mesylate [52-54]. Studies in rodents demonstrated that tirilazad conferred excellent neuroprotection against cerebrovascular insult, prevented destruction of the lipid membrane by halting lipid peroxidation, and provided antioxidant protection by scavenging iron-catalyzed free radicals [67-71]. In the pre-clinical phases, tirilazad proved to be well tolerated, and during the early clinical phases proved to greatly reduce mortality and improved outcome in a subpopulation of males with SAH [51, 52, 72-76]. Even after showing safety and efficacy, the tirilazad trials were discontinued after failing to show significant therapeutic efficacy in patients with moderate (GCS = 9-12) to severe (GCS =

4-8). The glucocorticoids dexamethasone and methylprednisolone also have a long history as treatments for both spinal cord injury and head trauma [50, 57, 58, 77, 78]. However, poor clinical design, flawed dosing regimes, increased rates of mortality, and lack of post-mortem analysis all contributed to the failure of these drugs [56, 78-80]. The female hormone, progesterone, was one of the largest and most promising to date. In the late 1970's and early 1980's, researchers found that pseudopregnant female rats with elevated levels of progesterone fared better after blunt force trauma to the brain [81]. These findings led to a series of phase II and phase III clinical trials that ultimately determined progesterone use was not effective enough to become a new therapeutic to fight TBI, which was likely due to suboptimal dosing parameters [64, 82-84].

While many promising treatments have been identified in animal models and optimized for human clinical trials, none to date have proven effective enough to become part of the therapeutic regiment for TBI [85]. Reasons for their failure may be linked to differences in biological sex, poor clinical or pre-clinical research design, and an unrealistic therapeutic window of administration [64, 83]. Therefore, elucidating potential sex differences in the pathophysiology of TBI, selecting a drug with a broad therapeutic window, and interpreting pharmacological effects with reliable biomarkers may increase the likelihood of developing a successful treatment for TBI.

## 1.4 Pathophysiology

### 1.4.1 Primary and Secondary TBI

As previously stated, TBI is caused by trauma to the head leading to a loss of consciousness and changes in brain chemistry [35]. To better understand the depth and

consequences of TBI, it has been commonly studied as a two-part injury process. The primary injury is initial damage caused by the forcible impact, penetrating object, coup-countercoup contusion, or percussive force from an explosion [42, 86]. At this stage of the injury, individuals suffer from skin lacerations, cranial fractures, hematomas, swelling, increased intracranial pressure (ICP), contusions, shearing of axons and cell bodies, breeches in the blood-brain barrier (BBB), and loss of brain matter [42, 87, 88].

The sequelae triggered by the primary insult, commonly referred to as the secondary injury, is characterized by the disruption in neurobiological homeostasis [88, 89]. Depending on the severity of the initial injury, certain secondary injury processes may only last a few hours, or can persist for weeks to months after the primary insult [90, 91]. The following section is meant to provide a concise review of the secondary sequela that are related to this body of work.

### 1.4.2 The Blood-Brain Barrier and Cerebral Edema

Cerebral edema (CE) is a well-documented condition that arises following TBI. It is characterized by an excess water from the peripheral vasculature system are able to freely flow across the BBB into the central nervous system (CNS) and accumulate in the brain [92, 93]. The BBB is an intricate network of brain capillary endothelial cells fused together by tight junctions, astrocytes, and pericytes with the purpose of sealing off the peripheral immune response and regulating the exchange of solutes between central and peripheral vasculature [94-96].

Trauma to this protective barrier permits the extravasation of fluid and solutes from the periphery into the interstitial space, causing the cell bodies in the brain to swell [49, 93,

97, 98]. This causes damage to cellular ion exchange pumps, microvascular alterations, and a drop in cerebral perfusion pressure (CPP) [99]. CE is characterized by excess fluid accumulation within brain and leads to an increase in brain volume and intracranial pressure (ICP) [87]. CE also plays a critical role in determining prognosis and mortality in patients with severe TBI [100]. The two common types of edema that frequently occur after TBI are vasogenic edema, localized within the extracellular spaces, and cytotoxic edema, which specially targets brain cells [92].

Vasogenic edema is a consequence of the structural failure of the BBB and separation of endothelial cell lining from adjoining astrocytes, permitting the unregulated passage of ions and proteins into the brain interstitium [101]. This edema causes expansion in brain volume, subsequently raising ICP to dangerous levels [46]. Elevated ICP may initiate a variety of compensatory mechanisms to restore compliance, such as decreasing cerebral spinal fluid (CSF) production, increasing CSF absorption, or shunting CSF out of the cranial vault [92, 102].

Cytotoxic brain edema is characterized by a disruption of the intracellular osmotic balance within vulnerable cells, particularly astrocytes [103]. This process is partially driven by a surge in free extracellular $Na^+$, $K^+$, $Ca^{2+}$, and $Cl^-$ released from cells undergoing apoptosis and necrosis [104]. The central mechanisms contributing to cytotoxic edema are increased intracellular ion concentrations (particularly $Na^+$), sustained absorption of osmotically active solutes, and leaky cell membranes from metabolic exhaustion [105]. Ultimately, ATP production fails to meet the energy demands of the $Na^+/K^+$ ATPases required to restore electrogenic balance and maintain electrochemical stability, generating more neurotoxic positive feedback loops [105, 106].

### 1.4.3 Glutamate excitotoxicity

The excitatory neurotransmitter glutamate is essential for maintaining a host of healthy brain functions, most notably learning and memory formation [107, 108]. Optimal regulation of glutamatergic signaling requires the synchronized effort of both pre-/post-synaptic neurons, ligand receptors activation, and reuptake by neurons and astroglia for signal termination [109-113]. Due to the potent action of glutamate and widespread receptor distribution, a delicate balance between signal transmission and termination must be maintained to prevent neurotoxicity [110, 112, 114-118].

The binding of glutamate, glutamate analogues, and voltage changes regulate synaptic transmissions and several signal cascades in pre-/post-synaptic neuronal populations and amongst glial cells [119-121]. These receptors are classified into two main subfamilies based on structure and the mechanisms regulating signal transduction. Metabotropic glutamate receptors (mGluRs) are seven transmembrane domains G protein-coupled receptors that control several cellular processes [122]. On the other hand, ionotropic glutamate receptors (iGluRs) permit the flow of ions ($Na^+$, $K^+$, and $Ca^{2+}$) through transmembrane ion channels to evoke an excitatory membrane depolarization [112]. There are three subtypes of iGluRs and they slightly vary in function. Both AMPA and Kainate receptors allow $Na^+$ influx into the cells and $K^+$ leakage into the extracellular space upon glutamate-ligand binding, whereas NMDA receptors also require a change in voltage for full activation [123]. This electrogenic process, typically assisted by AMPA receptors, removes a magnesium ion from the channel pore to permit the influx of $Na^+$ and, more importantly, $Ca^{2+}$ influx [115].

Glutamate regulation relies on multiple transporters, enzymes, receptors, and a coordinated effort between neurons and astroglia for reuptake and inactivation [110, 112, 113]. ATP storages become depleted due to the high energy expenditure associated with glutamate removal and inactivation [119, 124]. Following TBI, excess glutamate accumulates within the extracellular space due to increased neuronal firing, loss of inhibition, and impaired astrocyte glutamate uptake, causing persistent glutamatergic signaling, and elevated intracellular concentrations of $Na^+$, $K^+$, and $Ca^{2+}$ within neurons [113, 119, 121, 124]. Specifically, $Ca^{2+}$ overload can initiate several other injury cascades including the activation of caspases, calpains, protein kinases, and mitochondrial uncoupling [125-127].

### 1.4.4 Oxidative Stress

Oxidative stress is reflected by the imbalance between the production of ROS/RNS, lipid peroxidative by-products and the cells ability to neutralize these toxic intermediates [128]. A major source of secondary brain injury is the nearly instantaneous rise in oxidative stress caused by mitochondrial uncoupling, generation of superoxide anions, hydrogen peroxide, and iron-catalyzed hydroxyl radicals [69, 129-132]. Sequestering excess $Ca^{2+}$ causes mitochondria to begin producing excessive amounts of free radicals, reactive nitrogen, and oxygen species (RNS/ROS) [126]. Cells begin swelling and mitochondria succumb to bioenergetic failure from inhibition of the electron transport chain, destabilized membrane potential, and opening of the mitochondrial permeability transition pore (MPTP) [133]. Formation of the MPTP is a critical event that triggers apoptosis and further increases oxidative stress induced cell damage [133]. One mechanism used by the cell to

combat oxidative stress is through upregulating the production of detoxifying enzymes from the antioxidant response element [134].

## 1.5 The Nrf2-ARE Pathway

The antioxidant response element (ARE) encodes many powerful detoxifying enzymes to combat oxidative stress and reduce oxidative damage [134]. Transcription from the ARE by the transcription factor nuclear factor erythroid-2 related factor 2 (Nrf2) can be induced by endogenous signalling metabolites, dietary compounds, pharmaceuticals, and cell damage [135-144]. The main purpose of these cytoprotective enzymes is to prevent lipid peroxidation, quench free radicals, reduce oxidized molecules, and purge the cell of xenobiotics. For example, heme oxygenase 1 (HO-1) is a detoxifying enzyme that catalyzes the degradation of heme groups into bilirubin, and subsequently biliverdin and carbon monoxide [145-147]. It has also been shown to be quickly upregulated through Nrf2 activity in several models of brain injury, making it a prime candidate for studying Nrf2 activation [145, 146, 148-151]. Another well-known phase II enzyme under Nrf2 regulation is NAD(P)H: quinone oxidoreductase 1 (NQO1) [152, 153]. NQO1 catalyzes the two-electron reduction of dietary quinones to less harmful hydroquinones, detoxifies chemotherapeutic compounds, and reduces glutamate excitotoxicity [152, 154-157].

Under quiescent conditions, cells rapidly degrade Nrf2, exhibiting a half-life of around 20 minutes [158, 159]. In this case, Nrf2 is bound by its repressor protein, Keap1 and shuttled into the cytoplasm for degradation via the Cul3-based E3 ligase and 26S proteasome [158, 160-163]. When cells are stressed from either an excessive build-up of oxidants or electrophilic species, Nrf2 is stabilized and undergoes nuclear translocation

[158, 161, 164-168]. Recently, researchers have identified molecules with certain properties that can effectively modulate Nrf2 transcriptional activity. Since this discovery, these molecules have been tested as a treatment for many types of neurological disorders, including TBI. These molecules all act under similar mechanisms, which is by triggering redox sensitivity cysteines on Keap1 to block Nrf2 degradation [169, 170]. Figure 1.1 shows a schematic of the Nrf2-ARE.

### 1.5.1 Carnosic Acid, a Nrf2 Activator

Carnosic acid (CA), an ortho-diphenolic abietane diterpene found in *rosmanris officinalis* (rosemary), is a pro-electrophilic molecule known to upregulate Nrf2 transcription from the ARE [171-173]. It contains a catechol ring that has been shown to scavenge free radicals, and after accepting an electron, it is able to activate the Nrf2 pathway through S-alkylation of cysteine 151 on Keap1 [171, 174, 175]. Several studies have shown that CA-induced activation of Nrf2 leads to a dose-dependent upregulation in transcriptional activity within the ARE, which has been recorded by the luciferase assay [174, 175]. *In vivo* studies have shown that CA elicits neuroprotective effects in models of stroke and closed head injury [174, 176, 177]. Possessing both a pro-electrophilic state and an electrophilic state, CA has been considered a potent antioxidant that can also upregulate endogenous cytoprotective enzymes effect and Nrf2 has made CA an attractive candidate for treating TBI [173, 174, 178].

## 1.6 Addressing Biological Sex as a Variable in TBI

Biological sex is considered a binary term to identify males and females based on genetic makeup, sexual reproductive organs present at birth, and hormonal profiles [179].

Although gender is sometimes used interchangeably with biological sex, it is a social construct used for self-identification, forming relationships, and defining masculine or feminine characteristics [179]. Indeed, gender may also impact patient outcomes and deserves consideration when developing personalized medicine [180, 181]. Nevertheless, the term biological sex will be used to discuss the pathophysiological consequences associated with TBI in both humans and animals in this body of work.

### 1.6.1 Biological Sex Differences in Outcomes

Both males and females can sustain a life-altering TBI at any point in life, yet men comprise the majority of TBI patients [4, 5, 182]. This could be for various reasons, such as, men more often work dangerous jobs, take greater risks, play high contact sports, and make up a greater population of the military. Although TBI is more prevalent amongst men, the reports on patient outcome are not as straightforward.

Recently, an extensive report attempted to decipher the complex relationship between biological sex in the realm of TBI [183]. In this review, the authors cited multiple studies with contradicting results, however, a closer analysis revealed certain trends related to biological sex and TBI. For instance, conditions such as injury severity, study size, mechanism of injury, and age were all identified as potential factors contributing to patient outcomes and preclinical research. Out of each of these conditions, the authors identified injury severity as a major contributing factor when determining outcomes related to biological sex differences [183].

Numerous studies that evaluated patient outcomes following a mild to moderate TBI found that women had worse outcomes than men. Women were at a higher risk of

depression and reported more post-concussive symptoms [184-191]. Other studies found that women reported poorer quality of life, had greater mortality rates, and that young girls (≤ 19 years) took longer to recover in the hospital [192-198]. Furthermore, women also tended to suffer more long-term post-injury complications, such as headaches, migraines, anxiety, post-traumatic stress, and higher rate of unemployment [199-206]. These conclusions, however, are controversial due to the substantial amount of evidence supporting the complete opposite outcomes following a moderate to severe TBI. In these cases, women were more likely to be successfully employed, report a better quality of life, have superior emotional processing skills, and had a lower risk of mortality [207-212].

To complicate matters even further, there are also numerous studies that found no significant sex differences regardless of injury severity. These studies evaluated duration of post-concussive symptoms, length of hospital stay, numerous neurocognitive exams, psychological well-being, and mortality [213-222]. After many years of promising preclinical research and failed clinical trials, the National Institutes of Health (NIH) amended its guidelines for more rigorous research. In 2001, the NIH introduced a mandate forcing the inclusion of females and minorities in clinical research. Another revision in 2015 called for biological sex to be incorporated as a variable into the research design for NIH-funded animal research [223].

Based on the NIH guidelines and a plethora of evidence to suggest that biological sex may impact antioxidant buffering capacity, we aimed to first describe the endogenous Nrf2-ARE in male and female mice. After defining the time course, we aimed to augment the Nrf2-ARE with CA.

Table 1.1 Glasgow Coma Scale

Representation of a simplified version of the Glasgow Coma Scale. Adapted from [224].

| **Eye Opening** | **E =** |
|---|---|
| *Spontaneous* | *4* |
| *To Speech* | *3* |
| *To Pain* | *2* |
| *No Response* | *1* |
| **Best Motor Response** | **M =** |
| *Obeys* | *6* |
| *Localizes* | *5* |
| *Withdraws* | *4* |
| *Abnormal Flexion* | *3* |
| *Extends* | *2* |
| *No Response* | *1* |
| **Verbal Response** | **V =** |
| *Oriented* | *5* |
| *Confused Conversation* | *4* |
| *Inappropriate Words* | *3* |
| *Incomprehensible Sounds* | *2* |
| *No Response* | *1* |
| **Total GSC Score** | **3 – 15** |

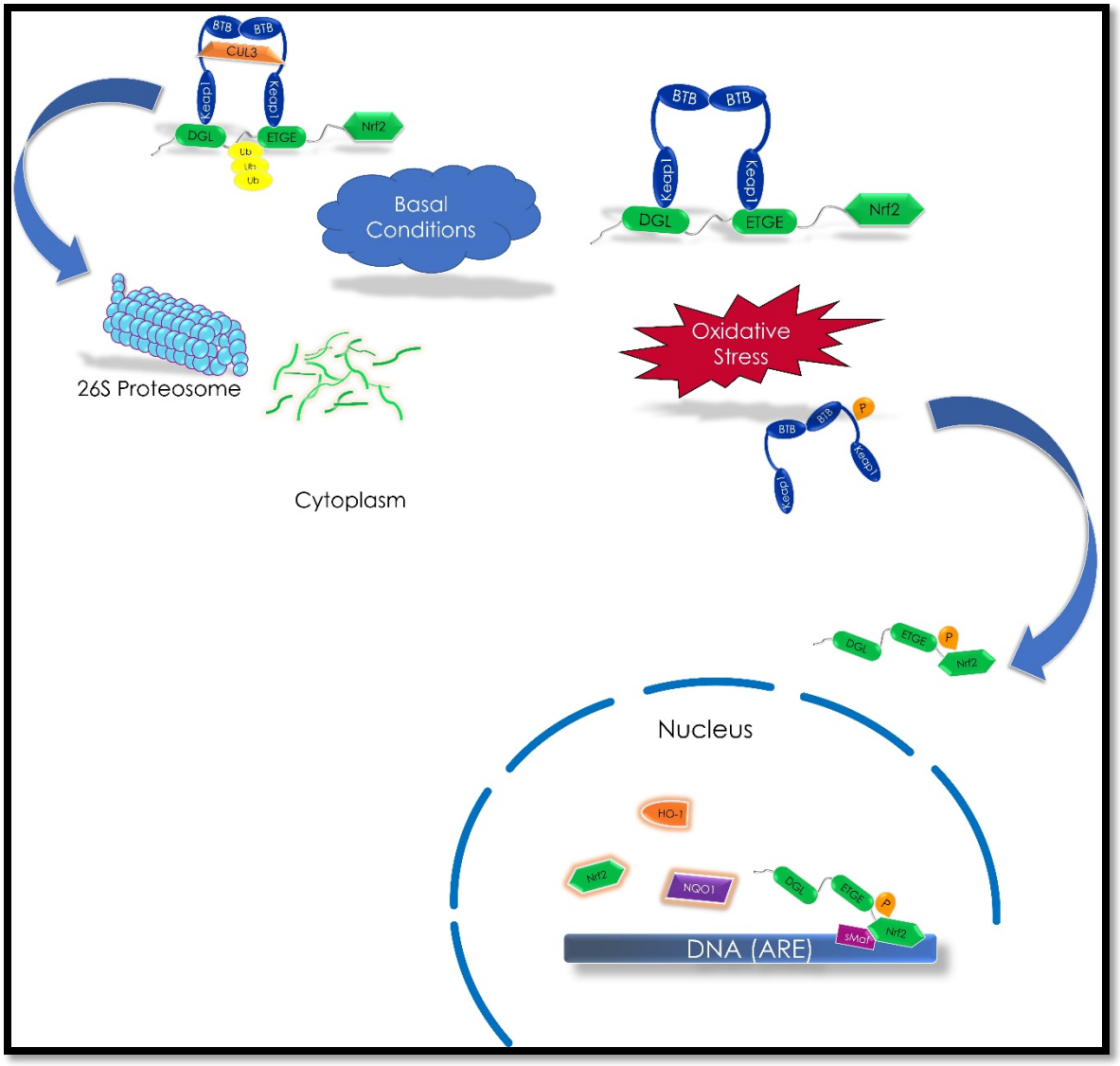


Figure 1.1 Schematic of the Nrf2-ARE

Nrf2 is bound by Keap1 and shuttled into the cytoplasm. Under basal conditions, Keap1 presents Nrf2 for ubiquitination and subsequent proteasomal degradation by the Cul3-based E3 Ligase and 26S proteasome. In the presence of oxidative stress, Keap1 cysteine residues are modified, releasing Nrf2 and allowing it to accumulate within the nucleus. In the nucleus, sMaf proteins bind to Nrf2 and initiate transcription of cytoprotective enzymes from the antioxidant response element (ARE).

## CHAPTER 2. TIME COURSE OF THE ENDOGENOUS NRF2-ANTIOXIDANT RESPONSE ELEMENT AFTER CONTROLLED CORTICAL IMPACT INJURY IN MALE AND FEMALE MICE

## 2.1 Introduction

Nearly 5.3 million Americans currently suffer from chronic conditions associated with TBI and, without an effective therapy, will remain a health safety concern for the entire population. TBI accounts for approximately 2.8 million related hospitalizations, emergency room visits, and more than 55,000 deaths each year [20, 225, 226]. It is widely known that men are more likely to suffer from TBI at a rate nearly 3 times higher than women, and are more likely to die because of the injury up until 65 years of age [180, 207]. This may be the only justification for why preclinical studies used almost exclusively male mice when testing and developing drugs. Until recently, it was widely accepted that females performed better in animal studies, whereas human studies report women as having worse outcomes [189, 193, 198, 210].

Clinical experimental design has not always stratified the patient population by sex, potentially confounding the results. Unfortunately, there is one case where an ad hoc analysis revealed that the drug tirilazad mesylate was effective in a subpopulation of male subjects with subarachnoid haemorrhage (SAH), but ineffective in female patients [72]. Interestingly, a human pharmacokinetic evaluation of tirilazad previously showed that women metabolized the drug at a higher rate than men, yet they received the same dose based on their weight and were not stratified by sex during later clinical testing [52, 75, 227].

One of the most substantiated secondary processes that contributes to TBI-induced neurodegeneration is the neurochemical pathway giving rise to oxidative stress [228]. Damaged mitochondria become a significant source of oxidative stress in the form of excess free hydroxyl radicals, reactive oxygen/nitrogen species (ROS/RNS), and

superoxide radicals [229, 230]. Overproduction of these free radical species occurs within minutes of the initial insult and initiates several other secondary cascades, including lipid peroxidation (LP) [129-131, 231]. LP is initiated when reactive oxygen species (ROS) attack polyunsaturated fatty acids found within lipid membranes, triggering membrane destabilization and generation of neurotoxic aldehydes (e.g., 4-HNE, acrolein) [129, 232, 233]. These neurotoxic by-products then form adducts to proteins, RNA, and DNA, perpetuating LP and further compromising the structural components and functional integrity of the cellular membrane [234]. Consequently, toxic amounts of $Ca^{2+}$ and chemical neurotransmitters are released, causing mass depolarization, metabolic dysfunction, apoptosis and necrosis [69, 125, 150].

The Nrf2-ARE pathway provides cells with innate protection from abrupt increases in free radical production and lipid peroxidation. Under homeostatic conditions, Nrf2 is quickly bound by its repressor protein and redox/electrophile sensor, Keap1, and targeted for proteasomal degradation [158, 161, 235, 236]. A sudden increase in oxidative stress or electrophiles rapidly inhibits Keap1-mediated ubiquitination, triggering Nrf2 liberation, stabilization, and subsequent nuclear accumulation [158, 161]. Nuclear Nrf2 heterodimerizes with small Maf proteins and begins transcription from the antioxidant response element (ARE), quickly elevating the production of phase II enzymes, such as heme oxygenase 1 (HO-1) and NAD(P)H: quinone oxidoreductase 1 (NQO1) [134, 142, 164, 237-239]. The highly reactive nature of the Nrf2/ARE axis and potential for neuroprotection against a variety of neurodegenerative diseases has led to the discovery of Nrf2 activating compounds, such as sulforaphane, tert-butylhydroquinone, and CA [142,

240]. These agents typically possess electrophilic properties that interrupt Keap1/Nrf2 interaction, thus freeing Nrf2 for transcription within the ARE [142, 178].

There is evidence to support that biological sex does play a role in the functional recovery process [188, 241-243]. Preclinical studies have attributed differences to the female sex hormones estrogen and progesterone, mitochondrial respiration rate, and the inflammatory response [244-247]. There are some reports indicating that certain aspects of the secondary pathogenic processes of TBI are differentially regulated in males and females [242, 247-249]. There is evidence of brain-region specific biological sex differences in the response to oxidative stress, suggesting that Nrf2/ARE pathway may be differentially regulated in males and females [230, 250-254]. Furthermore, HO-1 knock-out is neuroprotective in a male specific manner [251, 255]. Due to growing interest in developing effective Nrf2 activating drugs, it is essential that we define the time course of Nrf2-ARE following TBI in both male and female mice. To our knowledge, this is the first study to investigate potential sex differences in the time course of the Nrf2 response following CCI injury.

## 2.2 Materials and Methods

### 2.2.1 Animals

This study used weight-matched male (9 weeks) and female (12 weeks) CF-1 mice (Charles River Labs, USA) weighing 28–32g at the time of surgery. Weight matched mice were chosen to ensure that the brain sizes of both sexes were as similar as possible, resulting in a similar initial injury depth and volume. Each sex was housed separately in groups of 4–5 and allowed to acclimate for 7 days on a 12 hr light/dark cycle with *ad*

*libitum* access to food and water. All animal procedures and housing conditions were conducted in accordance with the University of Kentucky Institutional Animal Care and Use Committee.

### 2.2.2 Surgical Procedures and Injury

Surgical procedures are previously described in detail [232]. Briefly, all mice were initially anesthetized with 3% isoflurane and maintained at surgical plane with 2.5% isoflurane during the surgical procedure. Mice were secured in a stereotaxic device and a sagittal incision (~2.0 cm) was made to expose the skull. A 4.0 mm diameter craniotomy centered between bregma and lambda over the left parietal lobe was performed with a hand trephine, taking care to leave the dura intact. Brain-injured mice were rotated 20° clockwise before contusion. A pneumatic controlled cortical impact (CCI) device (Precision Systems Instrumentation, PSI TBI0300, Fairfax Station, VA) equipped with a 3.0 mm stainless steel beveled impactor tip was used to create the injury. Cortical deformation was -1.0 mm and inflicted at velocity of 3.5 m/s with a dwell period of 500 ms. Following injury, a 6.0 mm sterilized plastic disc was secured in place over the craniotomy with quick-bonding liquid cyanoacrylate and the incision was sutured closed. Mice were placed in a temperature-controlled chamber for 20–30 min to safely regain consciousness before returning to their previous housing assignments. Shams underwent all surgical procedures excluding the injury. Normal feeding and grooming behavior was exhibited soon after recovering from anesthesia and monitored daily. Mice in the experimental group were sacrificed at 1, 2, 3, and 7 days post-injury (DPI).

### 2.2.3 Quantitative Real-Time PCR and Gene Expression Analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine

mRNA expression of Nrf2 (Thermo Fisher Scientific, Assay ID Mm00477784_m1) and the Nrf2-ARE mediated targets HO-1 (Thermo Fisher Scientific, Assay ID Mm00516005_m1), NQO1 (Thermo Fisher Scientific, Assay ID Mm00500821_m1). Briefly, mice were asphyxiated in a $CO_2$ filled chamber. Following decapitation, brains were rapidly removed and dissected on ice using caution to prevent contamination between samples. A 4.0 mm disc of the cortex (penumbral tissue and injured core) and the ipsilateral hippocampus were carefully dissected and immediately placed in RNAlater solution (Thermo Fisher Scientific) for 24 hrs at 4°C to minimize cellular disruption and then stored at -80°C until analysis.

For total RNA isolation, the TRIzol Reagent protocol (Thermo Fisher Scientific, Cat# 15596018) was used per manufacturer specifications. Homogenization was achieved using Lysing Matrix D tubes (MP Biomedicals, San Diego, CA, Cat# 116913050) filled with TRIzol Reagent in conjunction with the FastPrep®-24 homogenizer (MP Biomedicals). Lysates were phase-separated in bathocuporine (BCP); RNA was precipitated in 2-propanol, washed in 75% ethanol and decontaminated of residual DNA using the DNA-*free* kit (Thermo Fisher Scientific, Cat# AM1906).

In preparation for qRT-PCR analysis, purified total-RNA concentrations were determined using a NanoDrop (Thermo Fisher Scientific) with 260/280 ratios of 1.8–2.2 were considered satisfactory. A total of 1.0 $\mu$g of purified total-RNA was then reverse transcribed to produce cDNA for subsequent qRT-PCR analysis. The StepOne-Plus Real-Time PCR System (Thermo Fisher Scientific, SCR_015805) in conjunction with commercially available TaqMan® RT-PCR primers and probes (Thermo Fisher Scientific) were used to detect gene amplification.

PCR reactions were run in duplicates in a 96 well format at a final volume of 25.0 µL using a standard amplification protocol (2.5 hrs, 40 cycles). Each reaction for a target gene contained 3.0 µL of stock cDNA plus 22.0 µL of a TaqMan RT-PCR Master Mix and gene-specific primers. Each reaction used for normalization purposes contained 3.0 µL of 1:10 diluted total cDNA plus 22.0 µL of the TaqMan PCR Master Mix and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer. The resulting amplification curves were analysed by the well-established $2^{(-\Delta\Delta Ct)}$ method, with GAPDH used as the reference gene and the sham groups as controls. All fold change values reported were log2 normalized. Methods used for total RNA extraction, purification, and complimentary DNA (cDNA) preparation, and qRT-PCR analysis have been previously validated in our lab [233].

### 2.2.4 Immunoblotting and Protein Analysis

Western blotting technique was used to determine protein levels of the phospho-Nrf2 (pNrf2) and Nrf2-ARE mediated targets HO-1 and NQO1. Briefly, mice were asphyxiated in a $CO_2$ filled chamber and brains were rapidly removed and dissected on ice. A 4.0 mm disc of the cortex (penumbral tissue and injured core) and the ipsilateral hippocampus were immediately transferred to Triton lysis buffer (1.0% Triton, 20.0 mM Tris HCL, 150.0 mM NaCl, 5.0 mM EGTA, 10.0 mM EDTA, and 10.0% glycerol) containing protease inhibitors (Sigma Aldrich, St. Louis, MO, Cat# 11836153001). Tissue samples were sonicated and then vortexed every 15 min for 1 hr. The whole-cell lysate emulsifications were centrifuged for 30 min (13,000 rpm at 4°C), the resulting supernatant was transferred to fresh tubes, and the debris pellet was discarded. Protein concentrations

were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Cat# 23225). Samples were kept on ice throughout all of the procedures.

Equal amounts of protein (35.0 $\mu$g aliquots) were separated on either 12% (for HO-1 and NQO1) or 10% (for pNrf2) Bis-Tris SDS-PAGE precast gels (Bio-Rad, Hercules, CA, Cat# 3450118; 3450112) using XT-MOPS running buffer. A semi-dry electro-transferring unit set to 15 V for 45 min was used to transfer proteins onto nitrocellulose membranes. Membranes were then placed in blocking solution (5% milk/TBS for HO-1, NQO1; 5% BSA/TBS w/v for pNrf2) for 1 hr at room temperature. Next, membranes were incubated with the appropriate primary antibody solutions at 4°C overnight (HO-1) or 72 hr (NQO1 and pNrf2). Finally, membranes were washed 3x5 min in TBST, incubated in the appropriate secondary solution for 2 hr at room temperature, and washed again 3x10 min in TBST prior to imaging. A LI-COR Odyssey-CLx InfraRed Imaging System (LI-COR Biosciences, Lincoln, NE, SCR_014579) and Image Studio software (version 5.2, Li-Cor Biosciences, SCR_015795) were used to scan and quantify the band intensities of the membranes.

Primary antibodies used for Western blotting were rabbit monoclonal for anti-Heme-oxygenase-1 (HO-1) (Abcam, Cambridge, MA, ab68477), anti-NAD(P)H: quinone oxidoreductase 1 (NQO1) (Abcam); rabbit polyclonal for anti-Phospho-Nrf2 (Ser40) (pNrf2) (Thermo Fisher Scientific, PA5-67520), and mouse monoclonal for anti-Alpha Tubulin (α-tubulin) (Abcam) was used as the internal control. The fluorescent secondary conjugate antibodies were either goat anti-rabbit (1:5,000; IRdye-800CW, Rockland, Limerick, PA) or goat anti-mouse (1:5000, IRdye-700CW, Rockland). With exception to the detection of pNrf2, blocking solution was prepared as TBS/5% milk and all primary

and secondary antibodies were diluted in TBST/5% milk. (Note: blots probing for pNrf2 were blocked in TBS/5% BSA; primary and secondary antibodies were diluted in only TBST only). A more detailed list of the primary antibodies and vendors is provided in Table 2.2.

### 2.2.5 Statistical Analysis

GraphPad Prism (version 9.0, GraphPad Software Inc., San Diego, CA, SCR_002798) was used for statistical analysis and graphing for data obtained from qRT-PCR and immunoblot quantifications. An ordinary two-way ANOVA was used to detect significant main effects of time and of sex and an interaction of time and sex in both followed by a Tukey's post hoc multiple comparisons test. Differences between the mean (+/-) were considered significant at $\alpha = 0.05$. Statistical data were reported as the mean standard deviation (SD). For all multiple comparisons, the sham mice were used as the control group.

## 2.3 Results

For this study a total of 120 rodents were organized into two cohorts, one for qRT-PCR (N = 60) and a second for immunoblot analysis (N = 60). Each cohort was comprised of an equal number of males (n = 30) and females (n = 30) and underwent the same surgical techniques and experimental model of CCI. At the time of surgery, mice were randomly assigned to receive sham operation or to an experimental group (1, 2, 3, and 7 DPI). For each sham and experimental group, there were an equal number of males (n = 6) and females (n = 6). Full statistical values are reported in the figure captions.

### 2.3.1 Time course of Nrf2

Focal TBI is known to cause an increase in oxidative damage for up to one-week post-injury [256, 257]. Cells respond to these sudden increases in oxidants by stabilizing the transcription factor Nrf2 to ramp up production of detoxifying enzymes from the antioxidant response element [145, 164, 230, 253, 254, 258]. To date, the post-injury time course of Nrf2 has yet to be described in both males and females. To determine if biological sex differences exist in the Nrf2-ARE pathway following experimental TBI, we measured the gene expression of Nrf2 and two of its key downstream targets, HO-1 and NQO1 in the cortex and hippocampus. Here, we report that CCI injury altered Nrf2-ARE mediated gene expression and protein concentration of HO-1 and NQO1 in a region and sex specific manner.

### 2.3.2 Nrf2 Gene Expression and Protein in the Cortex and Hippocampus

Nrf2 mRNA was significantly elevated in brain-injured male mice at 2, 3, and 7 DPI (Figure 2.1A). Similarly, Nrf2 mRNA levels also elevated at 2, 3, and 7 DPI in female mice (Figure 2.1A). The gene expression of cortical Nrf2 was not found to be significantly different between males and females at 1, 2, 3, or 7 DPI (Figure 2.1A). Analysis of the hippocampus revealed that Nrf2 gene expression increased from sham levels at 1 DPI and remained elevated for up to 7 DPI in both sexes (Figure 2.2B). However, at 7 DPI, hippocampal Nrf2 was significantly lower in females compared to males (Figure 2.2B). To determine if upregulation in Nrf2 mRNA corresponded with changes in Nrf2 protein, we probed whole cell lysates for pNrf2. We chose to analyze pNrf2, specifically the pSer40 version, because this would indicate activation related to PKC pathway by the lipid peroxidation by-product 4-HNE [259]. In the cortex, pNrf2 levels were highest in shams

regardless of biological sex (Figure 2.1C). In the male cortex, we observed a steady decrease in pNrf2 that was significant at 3 DPI (Figure 2.1C). In the female cortex, pNrf2 was also significantly downregulated, but this occurred at 7 DPI (Figure 2.1C). We did not observe an injury effect in pNrf2 in the hippocampus for either sex (Figure 2.3D).

### 2.3.3 Time course of NAD(P)H dehydrogenase; quinone 1

NAD(P)H dehydrogenase; quinone 1 (NQO1) is a potent 2-electron reductase capable of quenching free radicals in the cytoplasm by forming a homodimer and binding to the redox-active coenzyme FAD [152]. NQO1 is also a known target gene for Nrf2 and, recently, it has been shown to have a sex-based divergent expression in the liver of rats [152, 260, 261]. In the present study we found cortical NQO1 mRNA significantly increased in male mice at 3 DPI and 7 DPI and female mice at 3 DPI and 7 DPI (Figure 2.2A). A significant injury effect was also observed with NQO1 gene expression in the hippocampus. While male mice exhibited a significant increase in NQO1 mRNA at 7 DPI, female mice had significantly higher expression at 3 DPI and 7 DPI (Figure 2.2B). In good accordance with our gene expression analysis, we observed a similar injury effect in the cortex and hippocampus with respect to NQO1 protein. Although not significant, cortical NQO1 protein was elevated with respect to shams at 3 DPI and 7 DPI in both male and females (Figure 2.2C). NQO1 hippocampal protein was significantly elevated at 3 DPI in females only (Figure 2.2D).

### 2.3.4 Time Course of Heme Oxygenase 1

Heme oxygenase 1 (HO-1) is a highly inducible enzyme essential for catabolizing heme groups into biliverdin, and subsequently into bilirubin and carbon monoxide [262]. When analysing the induction of HO-1 mRNA following CCI, we found a sex-specific

temporal pattern in both the cortex and hippocampus. In the cortex of both sexes, HO-1 mRNA induction followed an inverted-U shaped curve, peaking at 3 DPI in both sexes, a time point when oxidative stress is at the highest. In the male cortex, HO-1 expression was significantly elevated at 1, 2, 3 DPI to 7 DPI relative to shams (Figure 2.3A). In the female cortex, HO-1 mRNA was significantly elevated at 3 DPI (Figure 2.3A). In the male hippocampus, HO-1 induction was elevated relative to shams at 1, 2, and 3 DPI (Figure 2.3B). In the female hippocampus, HO-1 mRNA was elevated 1 DPI and 3 DPI (Figure 2.3B). Interestingly, hippocampal HO-1 was significantly elevated in males compared to females at 3 DPI (Figure 2.3B). For both males and females, HO-1 protein was significantly elevated at 3 DPI, the corresponding peak for both sexes (Figure 2.3C). In the hippocampus, only female HO-1 protein was significantly elevated compared to shams, and this occurred at 2 DPI and 3 DPI (2.3D).

## 2.4 Discussion

Studies examining the effect of biological sex in brain injury have reported sexually dimorphic outcomes dating back to the 1970's. These studies report regional differences, where males tend to recover better when injured in the frontal cortex and septal nucleus, while females recovered better from hippocampal lesions [263-265]. Other studies have found that female gonadal hormones, progesterone and estrogen, provided enhanced neuroprotection [81, 246, 266]. Furthermore, the neuroinflammatory profile of female mice tends to be less pronounced in CCI models insult [247, 266], and there is even evidence to suggest sex-dependent differences in mitochondria and other metabolic processes that contribute to neurodegeneration [250, 253, 267]. A recent study also found that the Nrf2 regulated proteins HO-1 and NQO1 and the multidrug resistance associated protein 4

(MRP4) efflux transporter were more plentiful in the female choroid plexus [253]. Due to a growing mass of literature supporting that multiple biological systems are influenced by sex-dependent factors, we aimed to directly compare the spatial and temporal dynamics of the Nrf2-ARE pathway in both male and female mice. To the best of our knowledge, this is the first attempt to investigate the endogenous antioxidant response in a mouse model of CCI induced brain injury that included a sex-based comparison of the Nrf2-ARE molecular pathway.

It is well known that CCI injury accelerates the production of free radicals, increases lipid peroxidation and results in cell death [90, 130, 268, 269]. Previously, our lab conducted a time course study which showed that levels of 4-hydroxy-2-nonenal (4-HNE), a by-product of lipid peroxidation, coincided with Nrf2 activation following CCI injury in male mice [233]. Increases in oxidative stress, such as 4-HNE, or electrophilic compounds, such as sulforaphane and CA, triggers the dissociation of the Keap1/Nrf2 complex by modifying Keap1 cysteine residues or by direct phosphorylation of Nrf2 [161, 270]. It has been shown that phosphorylation of Nrf2 at the serine 40 site is a critical event for releasing Nrf2 from the repressor protein during high levels of oxidative stress [166].

First, we evaluated the injured cortex and ipsilateral hippocampus for Nrf2 mRNA and relative protein quantities. In the present study, Nrf2 mRNA increased equally in males and females at each of the recorded time points. On the other hand, hippocampal Nrf2 mRNA was significantly decreased in females compared to males at 7 DPI. Temporal, regional and cell-specific sex differences have been identified regarding the inflammatory response as well as the extent of protein carbonylation, both factors that impact the transcription of Nrf2, following CCI injury [247, 271]. A previous study showed that at 7

DPI males showed enhanced IBA1 immunoreactivity in the dentate gyrus compared to females [247]. Another study showed that at 5 DPI, females had less carbonylation near the dorsal third ventricle and median eminence, structures near the hippocampus [230].

Next, we prepared total cell lysates from the injured cortex and ipsilateral hippocampus and tested for the presence of the serine 40 phosphorylated form of Nrf2 (pNrf2). This post-translational modification occurs via activation of the atypical PKC (aPKC) pathway by 4-HNE, a by-product of lipid peroxidation and known Nrf2 activator [259]. Interestingly, male and female mice had similar basal levels of pNrf2, which were highest in shams and decreased at each post-injury time point. Although we observed a steady decline in the levels of pNrf2, there were signs of increased transcriptional activity, represented by the amplified production of Nrf2, HO-1 and NQO1 mRNA. This may seem paradoxical, however, can be easily explained. The phosphorylation of the serine 40 site on Nrf2 is required to release Nrf2 from Keap1 but is not required to enhance transcriptional activity [166, 272 ]. Nrf2 can also be modified through many alternative pathways and mechanisms [160]. Therefore, pNrf2 represents a specific pool of cellular Nrf2, as it excludes newly transcribed, or otherwise unaltered by post-translational modifications. Furthermore, this suggests that the newly transcribed Nrf2 may be targeted through alternative activating pathways thus enhancing the efficiency of Nrf2 transcriptional activation [135, 143, 273, 274]. Future studies should consider measuring both pNrf2 and total Nrf2 levels after injury.

Our current study aligns with previous studies, verifying that CCI injury does lead to the induction of Nrf2 mediated effectors, specifically HO-1 and NQO1 [137, 233]. The HO-1 enzyme catalyzes the breakdown of heme into biliverdin, ferrous iron, and carbon

monoxide and is a highly inducible antioxidant that is activated in response to brain injury, especially following the breakdown of the blood-brain barrier [275]. Recently, it was found that CCI injury leads to significantly elevated levels of HO-1 protein in female mice compared to male mice at 1 DPI, but not 7 dpi [245]. We did not see a significant increase in HO-1 protein until 3 DPI, however, this difference could be attributed to differences in injury severity produced by our CCI model. In our study, a post-hoc analysis revealed that cortical HO-1 mRNA levels were significantly higher in males compared to females at 2 and 3 DPI, indicating a sex-dependent mechanism involved in the secondary pathophysiology. Interestingly, neurodegeneration induced by TBI has been shown to peak in males within 3 days, while maximum neurodegeneration occurred 14 days post-injury in females [90]. Other reports provide evidence that male mice exhibit greater HO-1 protein induction over females following ferrous iron-induced injury within the mouse striatum [251]. However, when injured castrated male HO-1+/– mice were treated with estradiol (E2), HO-1 expression fell to injured female levels. Interestingly, greater HO-1 expression in males was associated with larger lesions and worse behavioural outcomes, while HO-1 suppression, castration, and E2 treatment attenuated these results. Another study examining the effects of E2 on HO-1 induction during intracerebral haemorrhage (ICH) supported these outcomes. After experimental ICH, E2 treatment in males abolished HO-1 induction and significantly reduced brain edema [276]. HO-1 activation in male mice has been shown to be detrimental during the early stages of ICH but aided in hematoma clearance and improved neurological outcomes during the later stages of recovery [151]. Keeping that in mind, there are studies citing improvements associated with HO-1

induction in males using Nrf2 inducers, suggesting a more complex role in the regulation of this pathway [277, 278].

The phase II enzyme NQO1 plays a direct role in the catalytic detoxification of toxic quinones to benign hydroquinones and is tightly regulated by the Nrf2-ARE pathway [233, 277, 279]. In the current study, we found that NQO1 mRNA and protein induction coincided with Nrf2 mRNA production in both the male and female cortex and hippocampus. Interestingly, the hippocampal fractions of females tended to have a more robust expression of NQO1 protein and an earlier mRNA induction profile than that of males. Although NQO1 can be detected in all areas of the brain including the cortex, hippocampus, striatum, and cerebellum, it is predominately located in endothelial cells and lateral walls of the choroid plexus (CP) [253, 255, 280]. Interestingly, when examining the cytoprotective gene composition of the CP, researchers found that females possessed greater concentrations of NQO1, HO-1, and certain ATP-binding cassette transporters associated with the passage of NQO1 and HO-1 metabolites and other compounds when compared to males [253]. Collectively, these data indicate that, to a certain degree, Nrf2 activity may also contribute to sex-specific variations in the detoxification of molecules and the transportation of metabolites between blood and cerebrospinal fluid.

Effectively managing the acute neuropathological consequences of TBI inflicted by oxidative insult is critical for maximizing the patient's potential of recovery [85, 240, 281]. The Nrf2-ARE pathway is the cell's main emergency defense against rapid destabilization in redox homeostasis and can be manipulated using electrophilic compounds. Mounting literature supports that targeting the endogenous Nrf2-ARE

antioxidant pathway with Nrf2 stabilizers may confer neuroprotection in multiple neurological diseases [140, 144, 173, 232, 233, 258, 281].

## 2.5 Conclusion

The present study aimed to analyze and compare the spatial and temporal characteristics of the male and female Nrf2-ARE axis. Our findings indicate that there is no difference in the basal expression of the Nrf2-ARE pathway between young-adult male and female mice, however, we did find sex-based differences in the spatial and temporal profiles of Nrf2-ARE activity within the first week following CCI injury. These data suggest that there are other underlying factors influencing the regulation of Nrf2-ARE response. Contributing factors may be linked to circulating gonadal hormones, mitochondrial makeup, differences in neuroinflammatory profiles, and potential structural and functional differences in communicating across the BBB, however, the specific mechanisms have yet to be elucidated. Ongoing studies are comparing the influence of sex differences in response to the Nrf2-ARE activating drug CA. Prior work from our laboratory has observed that in male mice subjected to CCI TBI, CA treatment is impressively neuroprotective with at least an 8-hour post-injury therapeutic efficacy window [232]. Currently, we are comparing the findings and the responsiveness to CA in female vs. male mice.

Table 2.1 Primary Antibodies

| Host-Target | Dilution | Band | Vendor | Catalog | RRID |
|---|---|---|---|---|---|
| Rb anti-phospho-Nrf2(Ser40) | 1:500 | 100/110 kDa | Thermo Fisher Scientific | PA5-67520 | AB_2691678 |
| Rb anti-HO-1 | 1:2,000 | 33 kDa | Abcam | ab68477 | AB_11156457 |
| Rb anti-NQO1 | 1:500 | 31 kDa | Abcam | ab80588 | AB_1603750 |
| Ms anti-α Tubulin | 1:10,000 | 52 kDa | Abcam | ab7291 | AB_2241126 |

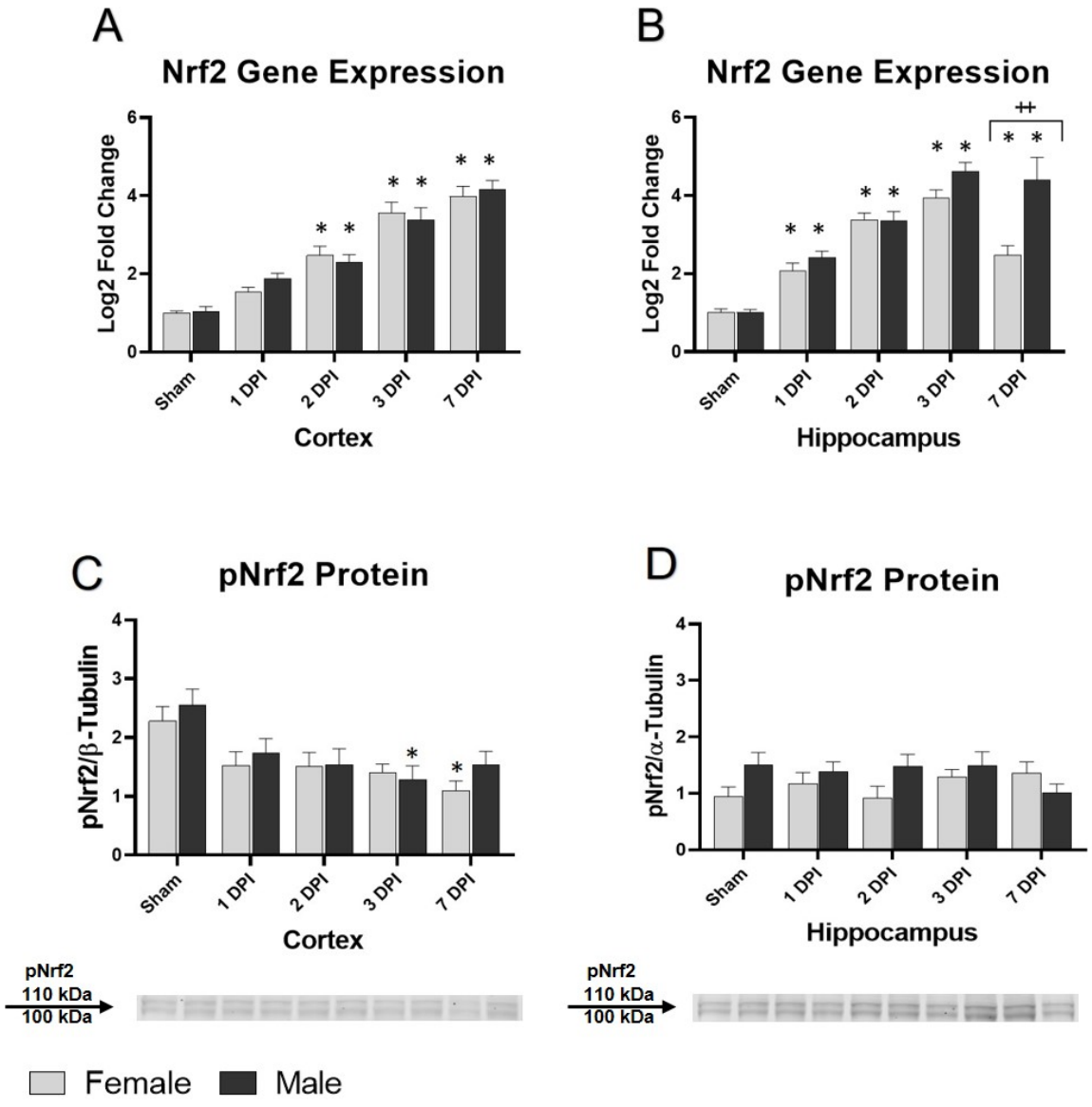


Figure 2.1 Nrf2 gene expression and pNrf2 protein quantities in the cortex and hippocampus.

A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. Some sex-dependent changes were found in cortical Nrf2 gene expression and protein concentrations in cortical tissue from male and female CF-1 mice in response to a controlled cortical impact (CCI) injury. A. Gene expression in the cortex revealed no sex differences in Nrf2 mRNA upregulation 2, 3, and 7 DPI compared to shams (Time: $F_{4, 50} = 70.84$, $p < 0.0001$; Sex: $F_{1, 50} = 0.075$, $p = 0.78$; Interaction $F_{4, 50} = 0.57$; $p = 0.785$). B. Sex differences were found in Nrf2 gene expression in the hippocampus (Interaction: $F_{4, 50} = 4.85$, $p = 0.002$; Time: $F_{4, 50} = 48.50$, $p < 0.0001$; Sex: $F_{1, 50} = 12.68$, $p = 0.0008$). Nrf2 was signficantly upregulated for both sexes at 1,

2, 3, and 7 DPI. Expression was significantly higher in males at 7 DPI. C. Protein quantities of pNrf2 in the cortex decreased over time in males and females, but revealed no significant sex differences (Interaction: $F_{4, 50} = 0.43$, $p = 0.12$; Time: $F_{4, 50} = 7.55$, $p < 0.0001$; Sex: $F_{1, 50} = 1.29$, $p = 0.2602$;). D. In the hippocampus, pNrf2 protein levels did not fluctuate significantly in response to injury (Interaction: $F_{4, 50} = 1.88$, $p = 0.1277$; Time: $F_{4, 50} = 0.39$, $p = 0.8144$; Sex: $F_{1, 50} = 3.67$, $p = 0.0623$).

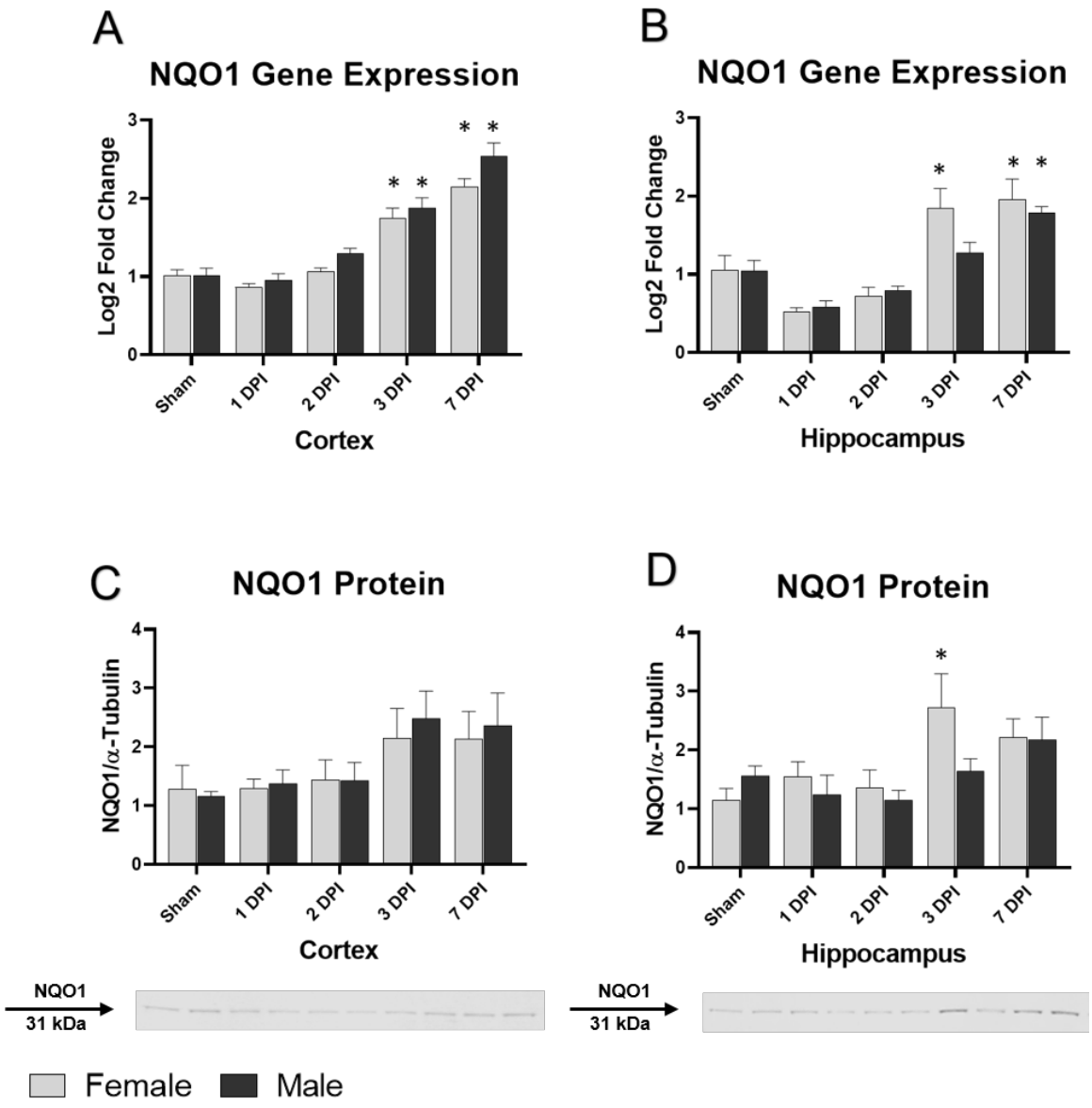


Figure 2.2 NQO1 gene expression and protein quantities in the cortex and hippocampus. A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. Post-injury upregulation of NQO1 gene expression and protein concentration is brain region and sex-specific. A. When compared to shams, NQO1 gene induction in the cortex was significantly higher at 3 DPI and 7 DPI in males and females, no sex differences were detected (Interaction: $F_{4, 50} = 1.09$, $p = 0.3673$; Time: $F_{4, 50} = 72.09$, $p < 0.0001$; Sex: $F_{1, 50} = 7.15$, $p = 0.0100$). B. In the hippocampus, when compared to shams, females had significantly higher levels of NQO1 mRNA at 3 DPI and 7 DPI, whereas NQO1 mRNA peaked in males 7 DPI. (Interaction: $F_{4, 50} = 1.49$, $p = 0.2172$; Time: $F_{4, 50} = 26.08$, $p < 0.0001$; Sex: $F_{1, 50} = 1.73$, $p = 0.1934$). C. Cortical NQO1 protein appeared to increase, but results were not signficant (Interaction: $F_{4, 50}$

= 0.10, $p$ = 0.9785; Time: $F_{4, 50}$ = 3.81, $p$ = 0.0088; Sex: $F_{1, 50}$ = 0.18, $p$ = 0.6661). D. In the hippocampus, NQO1 protein quantities significantly increased from sham levels at 3 DPI in females (Interaction: $F_{4, 50}$ = 1.49, $p$ = 0.2185; Time: $F_{4, 50}$ = 4.49, $p$ = 0.0035; Sex: $F_{1, 50}$ = 1.52, $p$ = 0.2226).

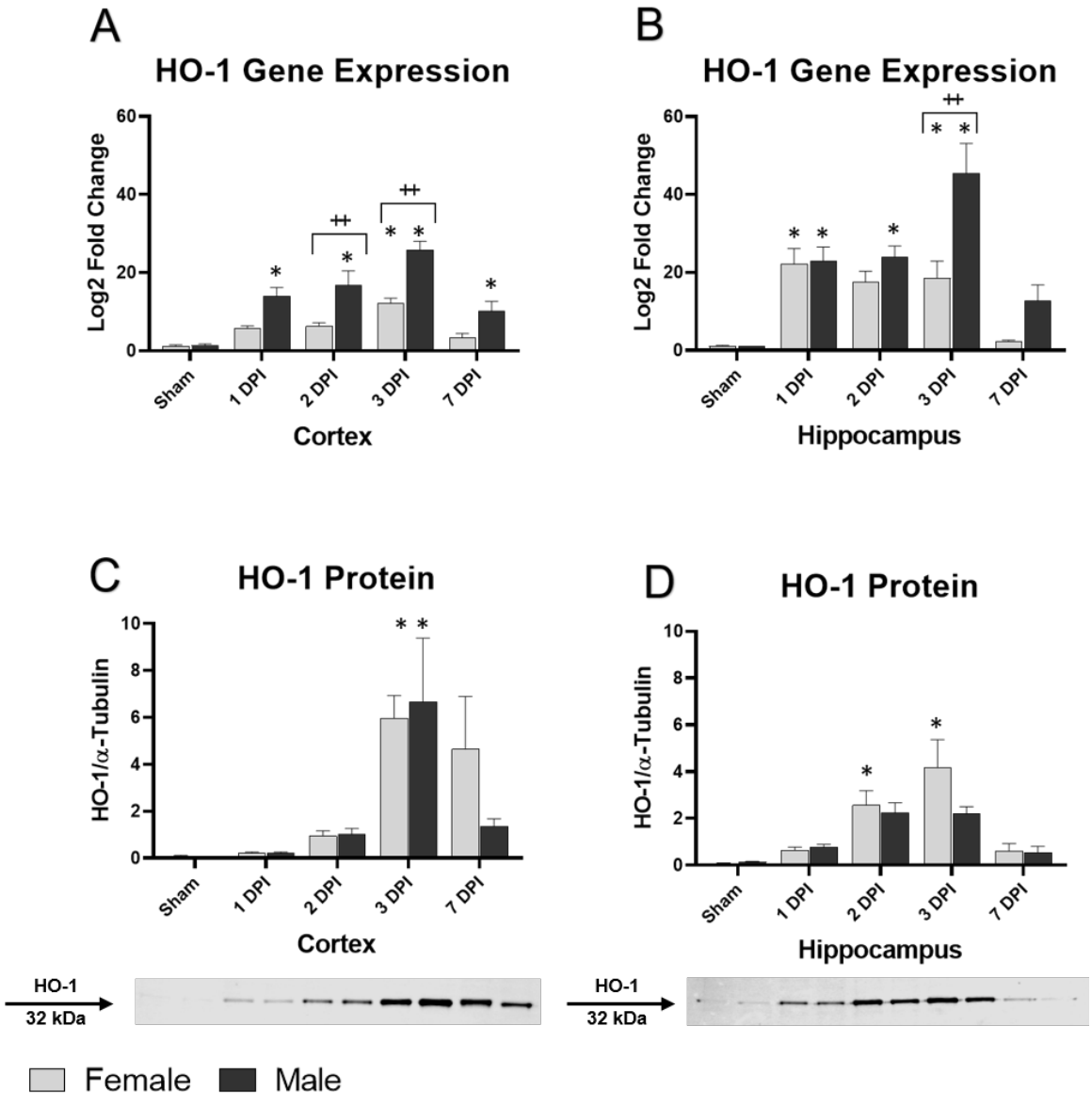


Figure 2.3 HO-1 gene expression and protein quantities in the cortex and hippocampus. A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. Post-injury upregulation of HO-1 gene expression and protein concentration is brain region and sex-specific. A. When compared to shams, HO-1 gene induction in the cortex was significantly higher at all recorded post-injury time points in males, and at 3 DPI in females. Sex differences were detected at 2 and 3 DPI (Interaction: $F_{4, 50} = 3.67$, $p = 0.0106$; Time: $F_{4, 50} = 24.35$, $p < 0.0001$; Sex: $F_{1, 50} = 45.19$, $p < 0.0001$). B. When compared to shams, males had significantly higher levels of HO-1 mRNA at 1, 2, 3, and 7 DPI, whereas HO-1 mRNA peaked in females at 2 and 3 DPI. Males had significantly higher HO-1 gene expression than females at 3 DPI (Interaction: $F_{4, 50} = 4.32$, $p = 0.0044$; Time: $F_{4, 50} = 22.24$, $p < 0.0001$; Sex: $F_{1, 50} = 14.28$, $p = 0.0044$).

C. Cortical HO-1 protein significantly increased from sham levels at 3 DPI in both males and females (Interaction: $F_{4, 50} = 0.911$, $p = 0.4644$; Time: $F_{4, 50} = 10.09$, $p < 0.0001$; Sex: $F_{1, 50} = 0.48$, $p = 0.4906$). D. In the hippocampus, HO-1 protein quantities significantly increased from sham levels at 2 and 3 DPI in females (Interaction: $F_{4, 50} = 1.682$, $p = 0.1688$; Time: $F_{4, 50} = 15.10$, $p < 0.0001$; Sex: $F_{1, 50} = 1.99$, $p = 0.1641$).

# CHAPTER 3. CARNOSIC ACID DIFFERENTIALLY EFFECTS THE NRF2-ARE PATHWAY RESPONSE IN MALE AND FEMALE MICE FOLLOWING EXPERIMENTAL BRAIN INJURY

## 3.1 Introduction

Traumatic brain injury is a serious global health problem. The heterogeneity of TBI has presented unique hurdles to both the preclinical research process and clinical trial methodology. In order to develop an effective treatment for TBI, researchers developed several animal models of head injury to mimic the various mechanisms of injury seen in humans [256, 269, 282-287]. Rigorous testing had led to the development of several promising neuroprotective agents and clinical trials. Although these drugs had striking results in animals, that same therapeutic efficacy has not yet been achieved in human patients.

Over the years, there has been much debate about why these drugs failed to make the transition from the bench to the bedside, however, one of the prevailing arguments lies in the preclinical research process. Most of these drugs were designed to act with high specificity, targeting a single secondary cascade or cell receptor. For instance, the NMDA receptor antagonists were designed to combat excessive glutamate signalling, a known secondary insult that occurs in the acute stages of secondary injury. Although demonstrating a very clean mechanism of action, these drugs ultimately failed because of their high degree of receptor binding. NMDA antagonists outcompeted glutamate binding, and because glutamate signalling is essential for life, this led to several complications, including death. A silver-bullet drug with a single mechanism of action does sound appealing, but it may not be a logical approach to treating such a complicated injury process.

Basic experimental research has typically overlooked the use of females in TBI research, however, there mounting evidence to support that biological sex should be considered a factor in both in preclinical and clinical TBI research. Preclinical experimental TBI studies have demonstrated biological sex differences in the antioxidant capacity of mammalian in the injured CNS [90, 245, 254, 288]. For example, one study showed that CCI injury lead to a greater degree of protein carbonylation near the ependymal zones of the dorsal third ventricle of the lesion in male mice [230]. In corroboration, another study found that aging male mice generated a greater degree of protein carbonylation and reduced catalytic activity of glutathione peroxidases in compared to aging females [254]. On the other hand, some studies find that lipid peroxidation also to occur at higher rates in the female brain, whereas other studies have found that the female brains tend show a decrease in antioxidant activity [289]. Sex differences have also been noted in the catalytic activity of NQO1 in the liver as well as HO-1 mediated iron catabolism activity in the brain [290, 291].

There is increasing evidence of sex differences in brain drug metabolism and BBB penetrability. The presence and abundance of multidrug resistant associated proteins (MRPs) transporters, which can affect the bioavailability of drug metabolites, were also found to be distributed differently throughout the male and female brain. Males exhibited a greater expression of MRP5 whereas females showed greater expression of MRP 4 [253]. Moreover, researchers identified Nrf2 as a mediated the expression of MRP2, MRP 3, and MRP4 expression through use of a Nrf2-KO model [292]. These gross differences in antioxidant capacity and regulation suggest that the endogenous antioxidant pathway may also be under differential regulation.

The Nrf2-Keap1 antioxidant pathway is responsible for protecting cells against oxidative damage. Under redox stable conditions, Nrf2 is constitutively expressed, bound by Keap1, a Cullin-3 E3 ubiquitin ligase adapter protein, shuttled between the nucleus and cytoplasm for ubiquitination and subsequent proteasomal degradation [143, 178, 293]. Keap1 also facilitates the nuclear accumulation of Nrf2 with the aid of several redox sensitive cysteine moieties [235, 274, 294-296]. When triggered, ceases the ubiquitination and degradation of Nrf2, increasing translocation into the nucleus subsequently upregulating transcription of cytoprotective genes [135, 144, 274, 297].

Two such mechanisms by which Nrf2 is stabilized occurred in the presence of oxidative stress and through electrophilic modification of the Keap1 cysteine residues [143, 158, 161, 235, 239, 270, 274, 294, 296, 298-302]. Since its discovery, it has been considered an attractive pharmaceutical target for CNS disorders due to its ability to cleanse cells of oxidative stress and xenobiotics [142, 281]. In fact, research involving this pathway has led to an entire class of several neuroprotective compounds termed Nrf2 activators, which have been screened in various models of models of CNS injury and neurodegeneration [140, 141, 303]. These acquired their name based on their ability upregulate Nrf2 transcriptional activity. One common characteristic shared among this class of drugs is the preferentially selection and modification of key cysteine residues on Keap1, which interrupts the ubiquitination pathway and facilitates Nrf2 nuclear translocation [239, 270, 299, 300].

One of the more potent Nrf2 activating compounds is carnosic acid (CA), an ortho-diphenolic abietane diterpene found in Rosmanris officinalis (the common herb, rosemary). It contains a catechol moiety that has been shown to scavenge free radicals and activate the

Nrf2 pathway through S-alkylation of cysteine 151 on Keap1 [171, 174, 175]. CA-induced activation of Nrf2 leads to and upregulation in transcriptional activity of within the ARE, which has been recorded by luciferase assay. *In vivo* studies have shown CA bestows neuroprotective effects in models of stroke and closed head injury. Possessing both an antioxidant effect and Nrf2 stabilizing properties has made CA an attractive candidate for TBI. Figure 3.1 provides a depiction of the proposed mechanism by which CA exerts its neuroprotective effect *in vivo* following traumatic CNS injury.

Several studies to date have demonstrated the mechanisms associated with the therapeutic potential of manipulating the Nrf2-Are pathway in males [136, 140, 277, 304, 305], however, to date, no study has evaluated the subcellular localization of Nrf2 or demonstrated neuroprotective effects in CNS trauma in female mice. To determine if the subcellular distribution of Nrf2 is different between sexes, we first compared the levels of cytoplasmic Nrf2 to nuclear Nrf2 for each sex. In line with other studies, we found that Nrf2 was preferentially localized in the cytoplasmic fraction in male mice. Interestingly, the females had significantly higher amounts of nuclear Nrf2. Furthermore, after a thorough literature review, we could not find studies that described Nrf2 cytoplasmic and nuclear distributions in the female mouse brain. To the best of our knowledge, this is the first study to report the subcellular distribution of Nrf2 in the female cortex; the first sex comparison of subcellular Nrf2 in the mouse cortex; and the first to determine if the effects of an electrophilic compound on Nrf2 localization is sex-dependent in the cortex of mice.

## 3.2 Methods and Materials

### 3.2.1 Animals

All animal procedures and housing conditions were conducted in accordance with the University of Kentucky Institutional Animal Care and Use Committee. This study used weight-matched male (9 weeks) and female (12 weeks) CF-1 mice (Charles River Labs, USA) weighing 28–32g at the time of surgery. Each sex was housed separately in groups of 4–5. The mice were allowed to acclimate for 7 days on a 12 hr light/dark cycle with *ad libitum* access to food and water in the vivarium. Following surgery, mice were placed back in freshly prepared cages in their original groups before being returned to the vivarium.

### 3.2.2 Mouse Model of Controlled Cortical Impact Injury (CCI)

To ensure that the male and female mice did not come into contact on the day of surgery, the female cohort underwent surgical procedures first, followed by the male. To further ensure no contact, each sex was initially anesthetized in a designated Plexiglas chamber. Mice were initially put under anesthesia using 4.0% isoflurane, heads shaved, and placed in a stereotaxic instrument (David Kopf, Tujunga, CA, USA). Mice were secured in a stereotaxic device and a sagittal incision (~2.0 cm) was made to expose the skull. A 4.0 mm diameter craniotomy centered between bregma and lambda over the left parietal lobe was performed with a hand trephine, taking care to leave the dura intact. Brain-injured mice were rotated 20° clockwise before contusion. A pneumatic controlled cortical impact (CCI) device (Precision Systems Instrumentation, PSI TBI0300, Fairfax Station, VA) equipped with a 3.0 mm stainless steel beveled impactor tip was used to create the injury. Cortical deformation was -1.0 mm and inflicted at velocity of 3.5 m/s with a

dwell period of 500 ms. Following injury, a 6.0 mm sterilized plastic disc was secured in place over the craniotomy with quick-bonding liquid cyanoacrylate and the incision was sutured closed. Mice were placed in a temperature-controlled chamber to safely regain consciousness before returning to their previous housing assignments. Shams underwent all surgical procedures excluding the injury. Normal feeding and grooming behavior was exhibited soon after recovering from anaesthesia and mice were monitored daily. Mice were allowed to survive for either 1 or 3 days post injury (DPI).

### 3.2.3 Drug Preparation and Administration

The dose, preparation, route of administration, and therapeutic window of efficacy were previously established in our lab [232]. Carnosic acid (Millipore Sigma, USA) was prepared at a concentration of 1 mg/kg in a 10% ethanol/90% PBS vehicle solution. Animals were treated with CA or vehicle solution (10% ethanol/90% PBS) via intraparatoneal (I.P.) injection 1 hr following the CCI injury.

### 3.2.4 Tissue Collection

At either 1 or 3 DPI, mice were asphyxiated in a $CO_2$ filled chamber. Following decapitation, brains were rapidly removed and dissected using caution to prevent contamination between samples. A 5.0 mm disc of the injured cortex (penumbral tissue and injured core ~40 mg) was isolated and prepared for cellular fractionation using the following procedure. In Chapter 2, we also assessed the ipsilateral hippocampus, however, we wanted to focus on how CA altered the Nrf2-ARE pathway specifically at the contusion site.

### 3.2.5 Cell Fractionation: Nuclear and Cytoplasmic Isolation

Separation and purification of the cytoplasmic and nuclear contents of cortical samples was achieved using the NE-PER Nuclear Cytoplasmic Extraction Reagent kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's directions. Optimization to increase protein yield and concentration were done per manufacturer's recommendations. Briefly, cortical samples were washed with PBS and then transferred to a 1.5 mL microcentrifuge tube (SP Bel-Art, Wayne, NJ, USA) filled with 400 µL ice cold reagent 1 (CERI). Samples were homogenized using a cordless homogenizer (SP Bel-Art, Wayne, NJ, USA) equipped with a sterile plastic pestle (SP Bel-Art, Wayne, NJ, USA). The homogenate was then vortexed on the highest setting for 15 s and placed on ice for 10 min followed by the addition of 22 µL of reagent 2 (CERII), vortexed for 5 s, incubated another 1 min on ice, and centrifuged for 15 min at 16,000 g at 4°C. The cytoplasmic supernatant was transferred to a clean pre-chilled tube and stored at -80°C. The nuclear pellet was resuspended in 100 µL of reagent 3 (NER) and vortexed for 15 s, incubated on ice for 10 min, 9 times for a total of 90 min. The nuclear homogenate was centrifuged for 15 min at 16,000 g at 4°C. The resulting supernatant containing the nuclear contents was then transferred to a clean pre-chilled tube and stored at -80°C.

### 3.2.6 Western Blot for Nuclear and Cytoplasmic Nrf2

Protein concentration of the cytoplasmic fraction and nuclear fractions were determined using the BCA Protein Assay kit (Pierce; Rockford, IL, USA). A 10 µg aliquot of each cell protein fraction was diluted in Milli-Q© water with 4x protein sample loading buffer (Licor; Lincoln, NE, USA) and 20x reducing agent (Bio-Rad; Hercules, CA, USA)

to a total volume of 20 µL. Samples were vortexed for 5 s, then placed in a 60°C water bath for 10 min and vortexed again. Equal volumes (15 µL) of each cell fraction were loaded on a precast 3-8% Criterion™ XT Tris-Acetate gel (Bio-Rad) in a Criterion™ Vertical Electrophoresis Cell (Bio-Rad) filled with ice cold XT Tricine Running Buffer (Bio-Rad). Electrophoresis was conducted on ice using a Power Ease 500 power supply (Invitrogen/Thermo Fisher Scientific; Waltham, MA) at 80 V for 15 min followed by 150 V for 90 min. Gels were removed from the cassette and equalized in ice cold Towbin transfer buffer (Table 3.1) for 5 min then placed between filter paper and transferred to nitrocellulose using a Trans Blot Semi-Dry Transfer Cell (Bio-Rad) at 18 V (constant), 2.00 mA for 40 min at room temperature (RT). The gel sandwiches were moistened with Towbin transfer buffer prior to mounting the cathode plate.

After the transfer, blots were briefly rinsed in TBS and then blocked in TBS/5% milk for 1 hr at RT on a lab rotator. It should be noted that each step involving an incubation period took place while gently rotating on a lab rotator. After being rinsed 1x in TBS, the primary antibody (Table 3.2) was added for overnight (~16 hr) incubation at 4°C. The following day, blots were allowed to equilibrate to RT for 10 min, washed 3x5 min in TBS-T. After the final wash, the blot was incubated for 1hr at RT in the secondary antibody (Table 3.2) diluted in TBS-T/5% milk. Blots were washed 3x5 min in TBS-T at room temperature and then scanned on the LI-COR Oddessy-CLx (Licor).

### 3.2.7 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Briefly, mice were asphyxiated in a $CO_2$ filled chamber, brains were removed from the skull, and dissected on ice using caution to prevent contamination between samples. A 5.0 mm disc of the cortex, including the penumbral tissue and injured core, was carefully

dissected out and immediately placed in RNAlater® solution (Thermo Fisher Scientific) for 24 hr at 4°C to minimize cellular disruption. The cortical samples were then stored at -80°C until further processing.

The TRIzol® Reagent protocol (Thermo Fisher Scientific) was used per manufacturer specifications to isolate total RNA from the cortical tissue samples. Tissue was homogenized using Lysing Matrix D tubes filled with beads (MP Biomedicals) and 800µL of TRIzol® Reagent in conjunction with the FastPrep®-24 homogenizer (MP Biomedicals). Lysates were incubated at RT for 10 min then centrifuged at 18,000 g for 10 min at 4°C. Without disturbing the beads, the supernatant was carefully transferred to tubes filled with 100 µL of BCP and gently mixed. After 15 min of incubation at RT, the phase-separated samples were centrifuged at 18,000 g for 20 min at 4°C. The clear aqueous top layer was carefully transferred to fresh tubes containing 500 µL 2-propanol, vortexed for 10 s, and incubated at RT for 15 min. The solution containing precipitated RNA was centrifuged at 18,000 g for 15 min at 4°C, leaving a clear supernatant and white pellet of RNA. The supernatant was discarded, and the RNA pellet was subsequently washed in 75% ethanol (100% ethanol diluted in Nuclease-Free Water (Applied Biosystems; Waltham, MA, USA)), and decontaminated of residual DNA using the DNA-*free* kit (Thermo Fisher Scientific). The RNA samples were treated with 6 µL of the DNase and buffer enzyme cocktail, heated in a 37°C water bath for 30 min, then treated with 5 µL of the DNAse Inactivator cocktail. After centrifuging at 10,000 g for 5 min, the supernatant was removed, and resuspended in 20-30 µL of Nuclease-Free water (Applied Biosystems).

Purified total-RNA concentrations were determined using a NanoDrop (Thermo Fisher Scientific) with 260/280 ratios of 1.8–2.2 were considered to be acceptable. A total

of 1.0 $\mu$g of purified total-RNA in a 40 µL reaction was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per the directions and the C1000 Touch™ Thermal Cycler (Bio-Rad) to produce complimentary DNA (cDNA) for subsequent qRT-PCR analysis. The lid of the cycler was set to 105°C, and cycle protocol was 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min.

The StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) in conjunction with commercially available TaqMan® RT-PCR primers and probes were used to detect gene amplification. Duplicate PCR reactions were ran in in a 96 well format at a final volume of 25.0 µL using a standard amplification protocol (2.5 hr, 40 cycles). Each reaction for a target gene contained 3.0 µL of stock cDNA plus 22.0 µL of a TaqMan® RT-PCR Master Mix and gene-specific primers (Table 3.3). For normalization and quantification, each plate had a set of control reactions containing 3.0 µL of 1:10 diluted total cDNA plus 22.0 µL of the TaqMan® PCR Master Mix and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer (Table 3.3). The resulting amplification curves were analysed by the well-established $2^{(-\Delta\Delta Ct)}$ method, with GAPDH used as the reference gene and the sham groups as controls. All fold change values reported were log2 normalized. Methods used for total RNA extraction, purification, and complimentary DNA (cDNA) preparation, and qRT-PCR analysis have been previously validated in our lab [233].

### 3.2.8 Sample Size and Statistical Analysis

Experimental groups for Western blot analysis were comprised of an equal number of male and female mice. Each cytoplasmic and nuclear fraction came from a single mouse from one of the following groups: Sham (n=4); 1 DPI + Veh (n=7); 1 DPI + CA (n = 7); 3

DPI + Veh (n=7); 3 DPI + CA (n = 7). qRT-PCR analysis experimental groups were as follows. Males: Sham (n=4); 1 DPI + Veh (n=7); 1 DPI + CA (n = 7); 3 DPI + Veh (n=6); 3 DPI + CA (n = 7). Females: Sham (n=4); 1 DPI + Veh (n=6); 1 DPI + CA (n = 8); 3 DPI + Veh (n=6); 3 DPI + CA (n = 7). GraphPad Prism (version 9.0, GraphPad Software Inc., San Diego, CA, SCR_002798) was used for statistical analysis and to create the graphs. A Two-way analysis of variance (ANOVA) with Tukey's post hoc was used for all multiple comparison tests, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the standard error of the mean (SEM).

## 3.3 Results

### 3.3.1 Carnosic Acid Differentially Effects the Distribution of Nuclear and Cytoplasmic of Nrf2 Protein in Male and Female Mice

To determine the subcellular localization of Nrf2, we isolated the nuclear and cytoplasmic protein contents from the lesion core of the injured cortex. In line with other studies, we found that the nuclear concentration of Nrf2 was lower than the cytoplasmic concentration of Nrf2 in male shams (Figure 3.2) [158, 167, 302]. Interestingly, in female shams, the nuclear concentration of Nrf2 was not significantly different in shams (Figure 3.3).

To determine the effect of CA on the stabilization and translocation of Nrf2, we divided the total nuclear concentration by cytoplasmic concentration. In males, we found that CA treatment led to a significant increase in the ratio of nuclear to cytoplasmic Nrf2 at both 1 DPI and 3 DPI (Figure 3.4A). In complete opposition, we found that CA treatment

led to an decreased cytoplasmic concentration of Nrf2 in female mice at 1 DPI (Figure 3.4B).

Next, we directly compared the effect of CA treatment between males and females to determine if this effect was significant between sexes. We found that males had significantly small ratio of nuclear to cytoplasmic Nrf2 as compared to female shams and 1 DPI + Veh treated groups (Figure 3.5).

### 3.3.2 qRT-PCR Analysis of Nrf2 Transcriptional Targets

#### 3.3.2.1 Nrf2 Gene Expression following Delayed CA Treatment

Nrf2 is known to target transcription of Nrf2, therefore, we analysed mRNA to determine the effect of CA or Veh treatment. We found significantly elevated levels of Nrf2 mRNA in male mice at 1 DPI and 3 DPI in both Veh and CA treated groups (Figure 3.6). In females, Nrf2 mRNA levels were found to be elevated at 3 DPI in female in both Veh and CA treated groups (Figure 3.6). Though there were no significant differences between the magnitude change of Nrf2 between males and females, it was interesting to observe that the increase in Nrf2 gene expression occurred at an earlier time point in males compared to females.

#### 3.3.2.2 NQO1 Gene Expression following Delayed CA Treatment

NQO1 is another well-cited target for Nrf2 transcription, therefore, it was chosen for mRNA analysis to determine the effect of CA or Veh on Nrf2 transcription [153, 280]. We found NQO1 mRNA was significantly elevated in male mice at 3 DPI in both Veh and CA treated groups (Figure 3.7). Similarly, in females, NQO1 mRNA levels were found to

be elevated at 3 DPI in both Veh and CA treated groups (Figure 3.7). There were no significant differences between the magnitude change of NQO1 between males and females.

#### 3.3.2.3 HO-1 Gene Expression following Delayed CA Treatment

Transcriptional regulation of HO-1 is a highly cited target for Nrf2 transcription, therefore, it was chosen for mRNA analysis to determine the effect of CA or Veh on Nrf2 transcription [145, 146, 150]. HO-1 mRNA was significantly elevated in male mice at 1 DPI and 3 DPI in both Veh and CA treated groups (Figure 3.8). In females, HO-1 mRNA levels were found to be elevated at 1 DPI and 3 DPI in both Veh and CA treated groups (Figure 3.8). At 3 DPI, Veh treated males had significantly higher levels of HO-1 compared to females, but this difference was not present in the CA treated groups at 3 DPI.

### 3.3.3 qRT-PCR Analysis of Nrf2 Negative Regulators

#### 3.3.3.1 NF-κB Gene Expression following Delayed CA Treatment

There are studies to suggest that the pro-inflammatory transcription factor NF-κB is a negative regulator of Nrf2 transcriptional activity. To determine if CA possessed anti-inflammatory properties, we analysed the total NF-κB content. We found that NF-κB mRNA was significantly elevated in male mice at 3 DPI in both Veh and CA treated groups (Figure 3.9). Similarly, in females, NF-κB mRNA levels were found to be elevated at 3 DPI in both Veh and CA treated groups (Figure 3.9). There were no significant differences between the magnitude change of NF-κB between males and females.

#### 3.3.3.1 GSK-3β Gene Expression following Delayed CA Treatment

There are studies to suggest that the serine-threonine kinase GSK-3β is a negative regulator of Nrf2 transcriptional activity. We found that GSK-3β mRNA was significantly elevated in female mice compared to male mice at 1 DPI in Veh treated groups, but CA treatment abolished this effect in females at 1 DPI (Figure 4.0).

## 3.4 Discussion

Several studies to date have demonstrated the mechanisms associated with the therapeutic potential of manipulating the Nrf2-ARE pathway in males [136, 140, 277, 304, 305], however, to date, no study has evaluated the subcellular localization of Nrf2 or demonstrated neuroprotective effects in CNS trauma in female mice. To determine if the subcellular distribution of Nrf2 is different between sexes, we first compared the levels of cytoplasmic Nrf2 to nuclear Nrf2 for each sex. In line with other studies, we found that Nrf2 was preferentially localized in the cytoplasmic fraction in mice. Unexpectedly, female mice did not show preferential location of Nrf2 to the cytoplasm or nucleus when comparing the purified extracts. After a thorough literature review, we could not find studies that described Nrf2 cytoplasmic and nuclear distributions the female mouse brain. To the best of our knowledge, this is the first study to report the subcellular distribution of Nrf2 in the female cortex and a sex comparison of subcellular Nrf2 in the mouse cortex.

Carnosic acid is a potent Nrf2 inducer that has demonstrated neuroprotective effects by enhancing nuclear translocating and upregulating Nrf2 transcription of the ARE [174-177, 306]. We have previously shown that a 1.0 mg/kg I.P. dose of CA administered 1 hr post-TBI significantly reduced oxidative damage and cytoskeletal breakdown in the cortex and hippocampus of male mice, and this dose remained effective when delayed for up to 8

hr post-injury [232]. Evidence from our study recapitulated these findings, with males showing increased Nrf2 nuclear localization after a single I.P. dose of CA 1 hr post injury. To date, however, all neuroprotective studies using Nrf2-inducers, including CA, have been conducted *in vitro* or used exclusively male animals. Therefore, we based our therapeutic dose of CA based on our previous studies. Unexpectedly, CA treatment in female mice significantly decreased concentrations of nuclear Nrf2 at 1 DPI. The reason for this differential effect so far is unclear, however, we speculate that the dosing paradigm for females did not lie in the therapeutic range. Nrf2 activators tend to have different effects on the repressor protein Keap1, which regulates Nrf2 subcellular location and degradation, when administered in high vs low concentrations [174, 307, 308]. CA treatment may have induced a nuclear efflux of Nrf2 at 1 DPI due to not receiving the optimal therapeutic dose, as by 3 DPI, the ratio had returned to baseline. It also must be noted that females started out with significantly more nuclear Nrf2 as compared to males and that the Veh treated group also experienced a drop in nuclear concentration of Nrf2 at 1 DPI. These data indicate that while transcriptional activity may not differ at baseline, females may be primed for Nrf2-mediated transcription or other factors are influencing Nrf2 localization.

Several studies corroborated that both experimental brain injury and CA upregulate gene expression of Nrf2, HO-1 and NQO1 [137, 174, 258, 259]. To determine if the subcellular distribution of Nrf2 was associated with synergistic upregulation of Nrf2 transcriptional activity with CA treatment, we conducted qRT-PCR targeting gene expression of Nrf2, and the Nrf2-ARE modulated effectors HO-1 and NQO1. Nrf2 is a known regulator of Nrf2 expression and, indeed, we found that Nrf2 mRNA was upregulated in males at 1 DPI, and both sexes at 3 DPI. These findings were likely related

to an injury effect and not a treatment effect. However, it should be noted that the increase in Nrf2 mRNA was only significantly elevated in males at 1 DPI. Expression of NQO1 is known to be modulated via the Nrf2-ARE pathway in response to oxidative stress and electrophilic induction [153, 309, 310]. In the present study, we did not find a significant treatment effect in NQO1. This may be due to an overall a lower induction rate of NQO1 and because NQO1 is primarily localized to vascular tissue [153, 255, 280], and a closer look at tissue-specific increases in NQO1 may reveal a different effect. We did detect a major sex difference in the induction of HO-1 at 3 DPI in our Veh treated groups, with males showing elevated levels of HO-1 in comparison to females. The effect was abolished with the administration of CA at 3 DPI. Lower levels of HO-1 have been associated with a neuroprotective effect in males when compared to females [251, 276, 311]. It appears that CA may be exerting neuroprotective effects via the Nrf2 pathway in tandem with other cell signaling pathways.

Nrf2-ARE activity is also known to be negatively regulated by the pro-inflammatory transcription factor NF-κB and the serine-threonine kinase GSK-3β [171, 312-317]. Previous studies have demonstrated that CA has anti-inflammatory properties, which were linked to a decrease in NF-κB expression [176, 318]. In the present study, we did not detect a significant anti-inflammatory treatment effect regarding NF-κB. We did detect significantly higher levels of GSK-3β at 1 DPI in Veh treated females compared to males, but this effect was abolished with CA treatment. Interestingly, a single dose of CA attenuated GSK-3β levels in females, but also led to a smaller Nrf2 nuclear/cytoplasmic ratio. Though there is a paucity of information on the subject, we believe that CA may be

acting on different cell signaling pathways in upstream of Nrf2 in males and females or be exerting minimal anti-inflammatory effects.

## 3.5 Future Experimental Considerations

Due to a lack of treatment effect, future studies should consider conducting a dose response experiment as well as a therapeutic window of efficacy in females to determine the therapeutic range of CA. Considering the rather futile dose-response 1.0 mg/kg produced in male mice, higher doses of CA should also be tested, as well as repeat dosing procedures. In Chapter 2, we found regional sex differences in Nrf2 activity, specifically in the hippocampus. Because we chose to focus our efforts on the cortex in this study, future experiments should also assess the regional differences in Nrf2-ARE activity. Also, in Chapter 2 we measured pNrf2, whereas in the current Chapter we assessed unaltered Nrf2. We chose to assess unmodified Nrf2 to have a better correlation between newly transcribed Nrf2 and freshly translated Nrf2. Future studies should consider probing for several post-translationally modified forms of Nrf2 to gain a better understanding of the signaling pathways involved regarding the CCI injury, CA, or other Nrf2 activators, and to illuminate potential sex differences.

## 3.6 Conclusion

In the present study, we found that CA increased nuclear localization of Nrf2 in males and reduced it in females. We also found that as opposed to increasing gene expression of downstream Nrf2 effectors, it did not influence NQO1 mRNA and in fact, it reduced the expression of HO-1 mRNA in males, while increasing it in females. Due to the lack of information on behavioral measures and histological data, we cannot determine if

these changes were either beneficial or detrimental. Future studies should consider investigating the role of negative Nrf2 regulators, such as NF-κB as well as GSK-3β.

Table 3.1 Towbin Transfer Buffer

| Reagent | Per 1 Liter | Concentration |
|---|---|---|
| Trizma | 3.03 g | 25 mM |
| Glycine | 14.4 g | 192 mM |
| SDS | 0.025 g | 0.087 mM |
| Methanol | 200 mL | |
| Milli-Q $H_2O$ | 850 mL | |

Towbin transfer buffer was made fresh prior to Western blot analyses and stored at room temperature for up to 7 days. The recipe for this buffer can be found on the Bio-Rad website [319].

Table 3.2 TaqMan® Assay Primers and Probes.

| Gene Symbol (RefSeq) | Gene Alias | Assay ID | Amplicon Length | Vendor (Catalog #) | Catalog # |
|---|---|---|---|---|---|
| Hmox1 (NP_034572.1) | HO-1 | Mm00516005_m1 | 69 | Thermo Fisher Scientific | 4331182 |
| NQO1 (NP_032732.2) | NQO1 | Mm00500821_m1 | 74 | Thermo Fisher Scientific | 4331182 |
| Nfe2l2 (NP_035032.1) | Nrf2 | Mm00477784_m1 | 61 | Thermo Fisher Scientific | 4331182 |
| NFKB (NP_032715.2) | NFκB | Mm00476361_m1 | 70 | Thermo Fisher Scientific | 4331182 |
| GSK3B (NP_062801.1) | GSK3β | Mm00444911_m1 | 72 | Thermo Fisher Scientific | 4331182 |
| GAPDH (NM_008084.2) | GAPDH | Mm99999915_g1 | 107 | Thermo Fisher Scientific | 4352932E |

Table 3.3 Western Blot: Cytoplasmic and Nuclear Nrf2 Antibodies.

| Primary | Dilution | Band | RRID | Vendor | Product ID |
|---|---|---|---|---|---|
| Rb-Nrf2 | 1:2,000 | 110 kDa | AB_2687540 | Abcam | ab137550 |
| Ms-βIII Tubulin | 1:5,000 | 50 kDa | AB_2691678 | Abcam | ab78078 |
| Chx-Lamin A/C | 1:2,000 | 74 kDa/65 kDa | AB_2892106 | Novus | NBP2-25152 |

| Secondary | Dilution | Signal | Target | Vendor | Product ID |
|---|---|---|---|---|---|
| Goat anti-Rb | 1:7,5000 | IR800 | Nrf2 | Licor | 926-32211 |
| Goat anti-Ms | 1:15,000 | IR680 | βIII Tubulin | Licor | 925-32210 |
| Donkey anti-Chx | 1:10,000 | IR680 | Lamin A/C | Licor | 926-68075 |

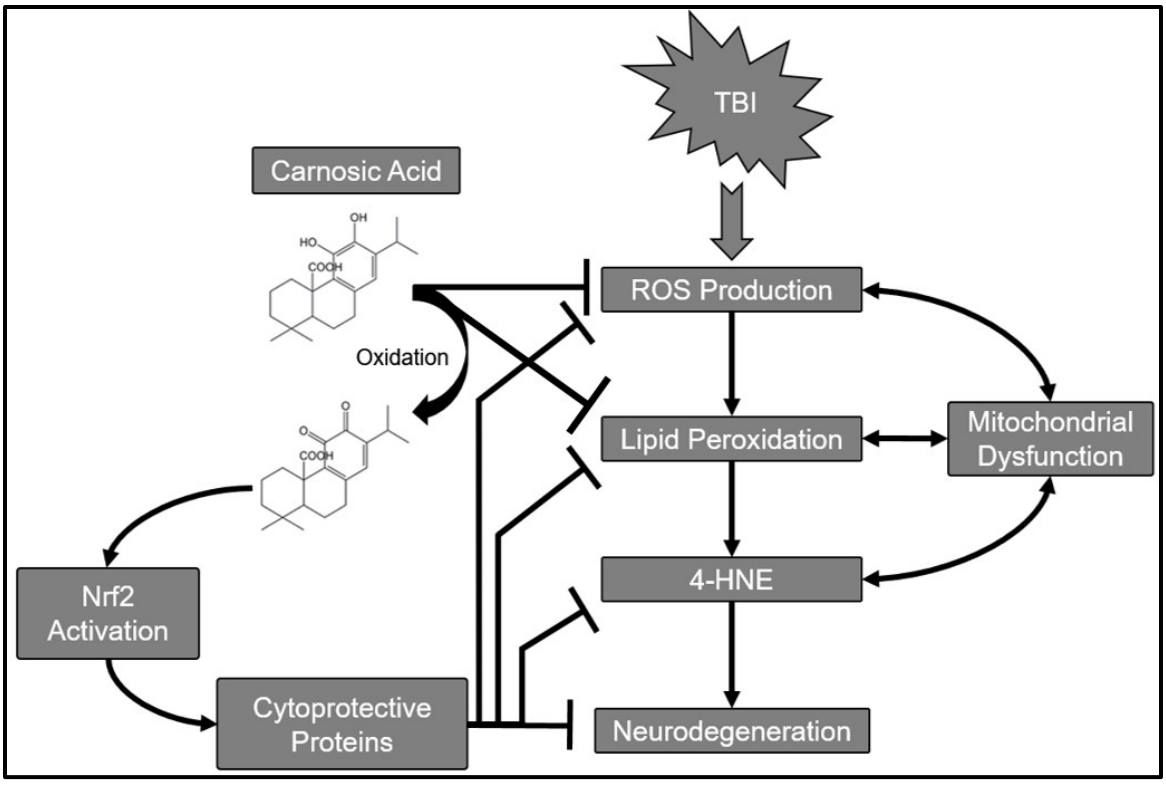


Figure 3.1 Proposed Pleiotropic Mechanism of Carnosic Acid in TBI.

Prior to oxidization, carnosic acid possesses a pro-electrophilic catechol nucleus that renders it as an antioxidant. Following the acceptance of an electron via free radical, or quenching lipid peroxide species, the catechol nucleus performs an electrophilic attack on cysteine 151 of Keap1, stabilizing Nrf2 and facilitating nuclear translocation. Nrf2 then upregulates the production of cytoprotective gene to combat multiple secondary injury pathways.

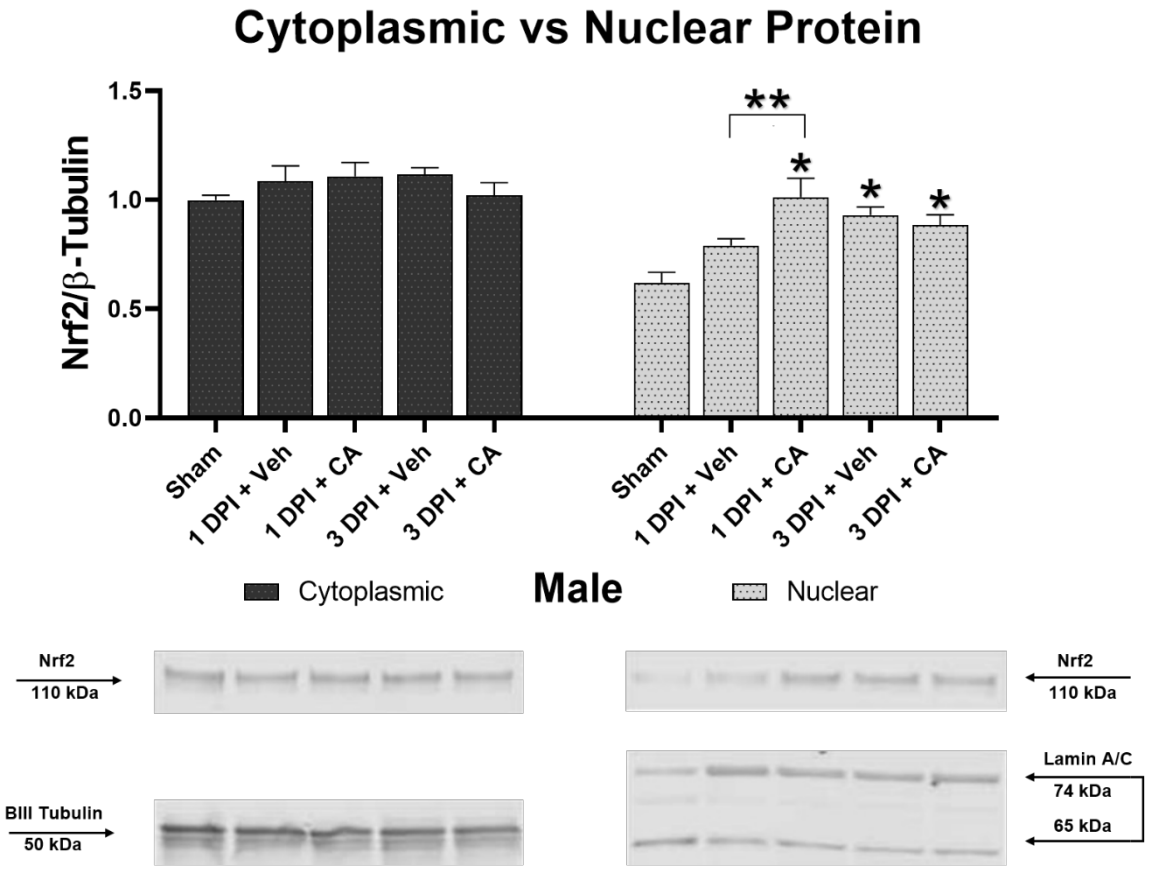


Figure 3.2 Male Cytoplasmic and Nuclear Nrf2 Protein.

Western blot analysis to determine the effect of CA on distribution of Nrf2 in purified cytosolic and nuclear fractions from the injured cortex of male mice. A single dose of CA significantly increased nuclear Nrf2 concentration compared to vehicle treatment 1 DPI. By 3 DPI, both CA and vehicle treated groups had significantly elevated Nrf2 nuclear concentrations compared to sham controls. Comparison of cytoplasmic to nuclear concentration indicated that Nrf2 was preferentially localized to the cytoplasm in sham and 1 DPI vehicle treated mice. A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. (Interaction: $F_{4, 54} = 1.86$, $p = 0.1302$; Treatment: $F_{4, 54} = 4.72$, $p = 0.0024$; Cell localization: $F_{1, 54} = 36.19$, $p < 0.0001$).

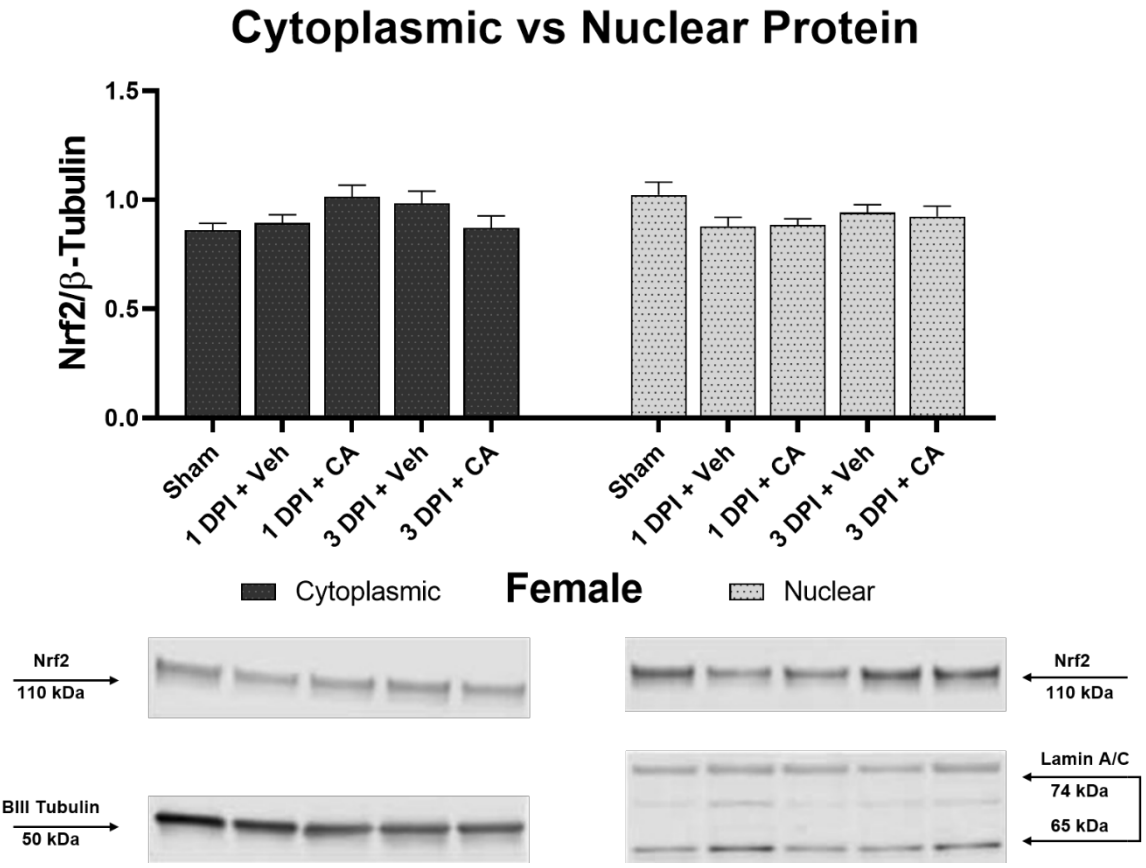


Figure 3.3 Female Cytoplasmic and Nuclear Nrf2 Protein.

Western blot analysis to determine the effect of CA on distribution of Nrf2 in purified cytosolic and nuclear fractions from the injured cortex of female mice. A single dose of CA or vehicle did not significantly alter the cytoplasmic concentration or nuclear concentration of Nrf2 compared to sham controls. Results from a Two-way ANOVA with Tukey's post hoc for multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. (Interaction: $F_{4, 54} = 2.25$, $p = 0.075$; Cell localization: $F_{1, 54} = 0.038$, $p = 0.84$; Treatment: $F_{4, 54} = 1.06$, $p = 0.3812$)

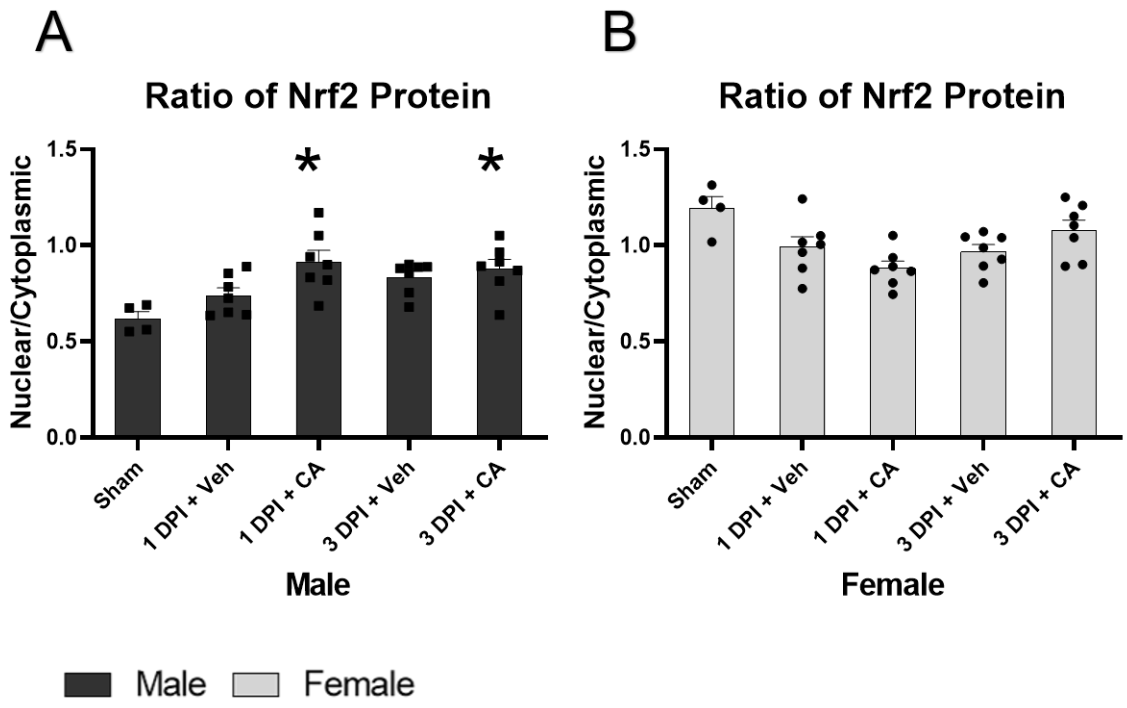


Figure 3.4 Male and Female Nuclear to Cytoplasmic Nrf2 Protein Ratio.

Analysis of subcellular protein fractions to determine the effect on the preferential location of Nrf2 and if the effect of CA on Nrf2 cellular concentration is sex dependent. Cytoplasmic and nuclear fractions were normalized to Beta-III Tubulin. Then, to determine the preferential subcellular localization of Nrf2, the ratio of nuclear to cytoplasmic concentration was determined by dividing the normalized nuclear fraction by the normalized cytoplasmic fraction for each sex. A. Delayed administration of CA significantly increased the nuclear/cytoplasmic concentration of Nrf2 in male mice at 1 DPI and 3 DPI with respect to male shams. (Treatment: $F_{4, 21} = 6.23$, $p = 0.0018$) B. In contrast, CA significantly decreased the nuclear/cytoplasmic concentration in female mice compared to female shams 1 DPI. (Treatment: $F_{4, 21} = 5.30$, $p = 0.0041$). A One-way ANOVA with Tukey's post hoc was used for multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM.

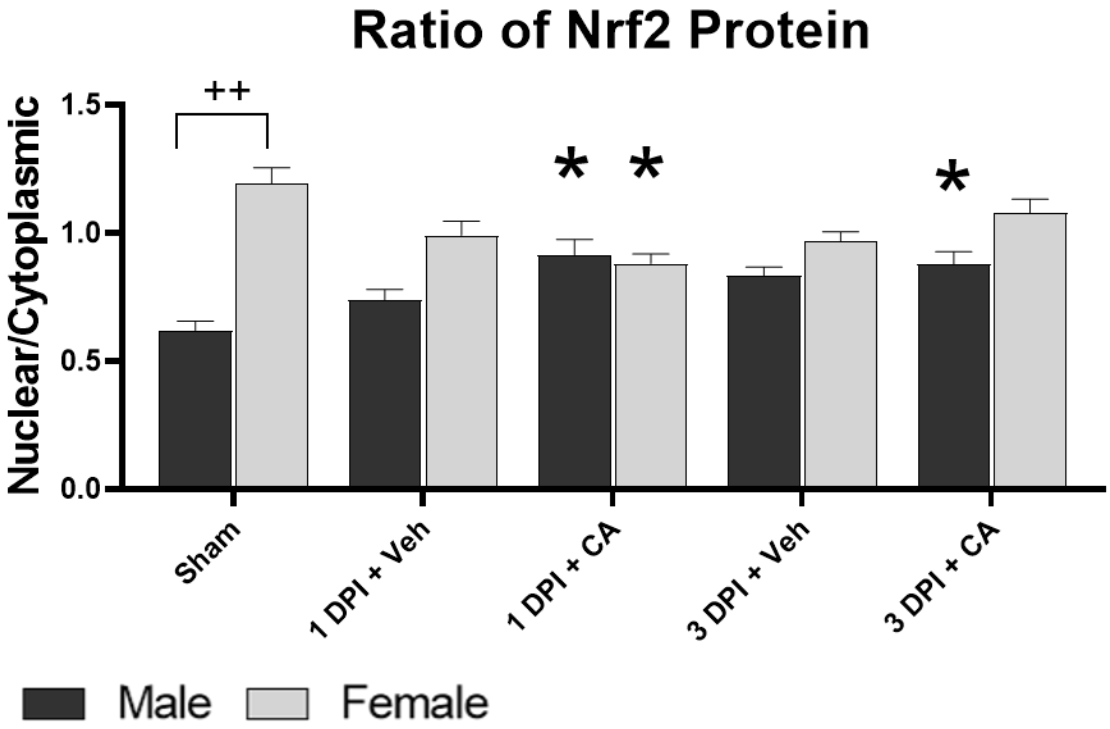


Figure 3.5 Male and Female Nuclear to Cytoplasmic Nrf2 Protein Ratio.

Analysis of subcellular protein fractions to determine the effect on the preferential location of Nrf2 and if the effect of CA on Nrf2 cellular concentration is sex dependent. Delayed administration of CA significantly increased the nuclear/cytoplasmic concentration of Nrf2 in male mice at 1 DPI and 3 DPI with respect to male shams. In contrast, CA significantly decreased the nuclear/cytoplasmic concentration in female mice compared to female shams at 1 DPI. Comparison of nuclear/cytoplasmic fractions between sham males and females revealed that Nrf2 is preferentially localized to the cytoplasm in sham males and nucleus of sham females. A Two-way ANOVA with Tukey's post hoc was used for multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. (Interaction: $F_{4, 54} = 8.427$, $p < 0.0001$; Sex: $F_{1, 54} = 52.66$, $p < 0.0001$ Treatment: $F_{4, 54} = 1.64$, $p = 0.1767$).

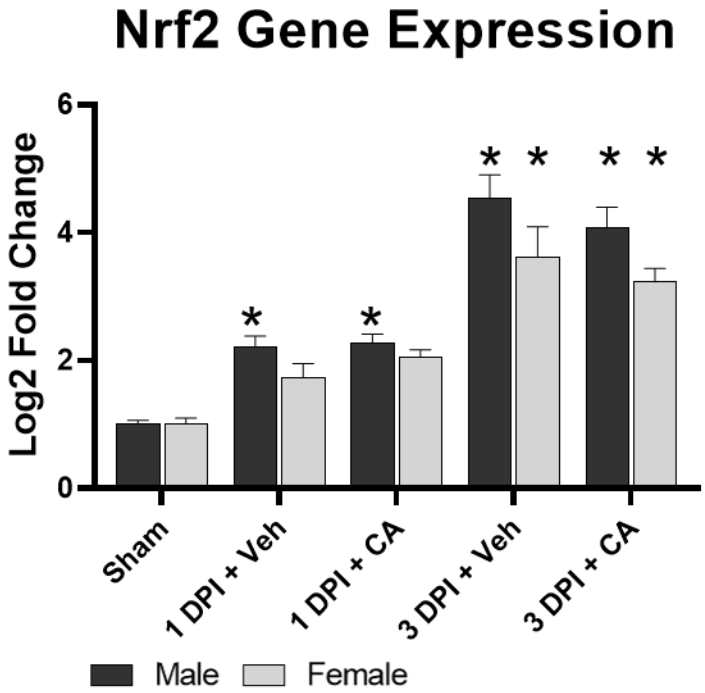


Figure 3.6 Male and Female Nrf2 Gene Expression.

The post-injury effect of CA or Veh on the induction of Nrf2 gene expression in the cortex of male and female mice. Nrf2 gene expression was significantly upregulated in males at 1 and 3 DPI regardless of Veh or CA treatment. Nrf2 gene expression in females was significantly elevated at 1 DPI as a result of CA treatment. Nrf2 expression remained elevated in females at 3 DPI in the presence of Veh and CA treatment. A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. (Interaction: $F_{4, 55} = 1.388$, $p = 0.2503$; Sex: $F_{1, 55} = 0.0018$, $p < 0.0001$; Treatment: $F_{4, 55} = 54.23$, $p < 0.0001$).

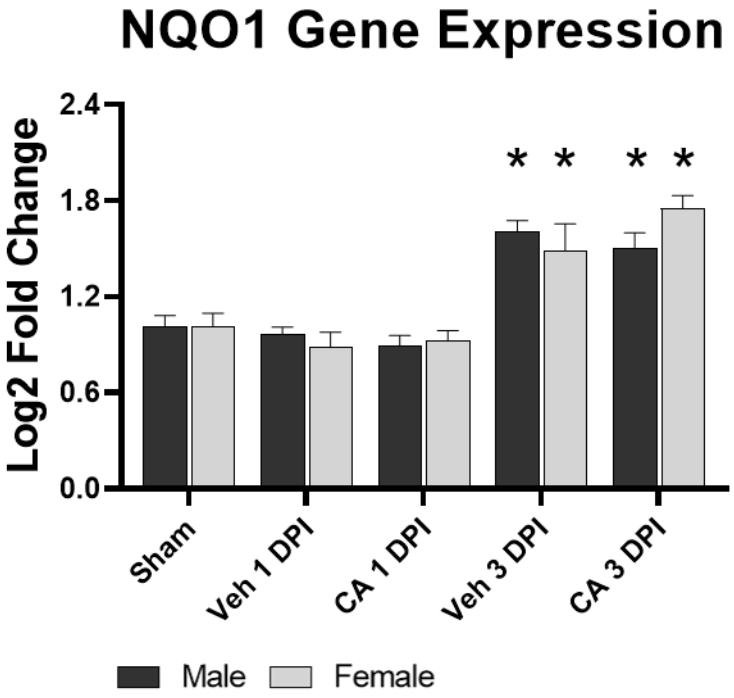


Figure 3.7 Male and Female NQO1 Gene Expression.

The post-injury effect of CA or Veh on the induction of NQO1 gene expression in the cortex male and female mice. NQO1 gene expression was significantly upregulated in males at 3 DPI regardless of Veh or CA treatment. NQO1 gene expression was also upregulated as a result of injury at 3 DPI in both Veh and CA treated female groups. A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. (Interaction: $F_{4, 55} = 0.070$, $p = 0.5941$; Sex: $F_{1, 55} = 0.2366$, $p = 0.6286$; Treatment: $F_{4, 55} = 36.01$, $p < 0.0001$).

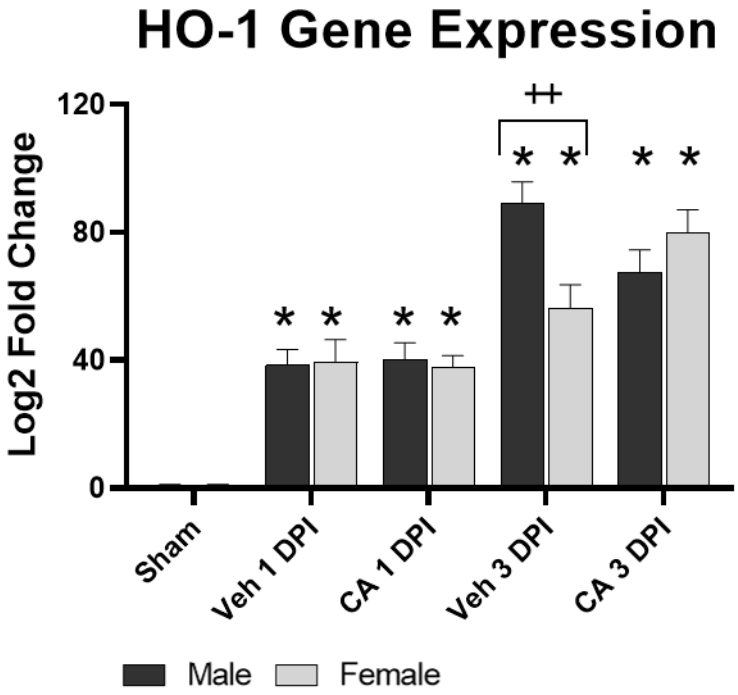


Figure 3.8 Male and Female HO-1 Gene Expression.

The post-injury effect of CA or Veh on the induction of HO-1 gene expression in the cortex male and female mice. HO-1 gene expression was significantly upregulated in males 1 and 3 DPI regardless of Veh or CA treatment. HO-1 gene expression was also upregulated as a result of injury in females at 1 and 3 DPI. At 3 DPI. Interestingly, Veh treated males had significantly higher HO-1 mRNA expression compared to Veh treated females at 3 DPI. A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. (Interaction: $F_{4, 55} = 4.403$, $p = 0.0.0037$; Sex: $F_{1, 55} = 1.46$, $p = 0.2314$; Treatment: $F_{4, 55} = 55.93$, $p < 0.0001$).

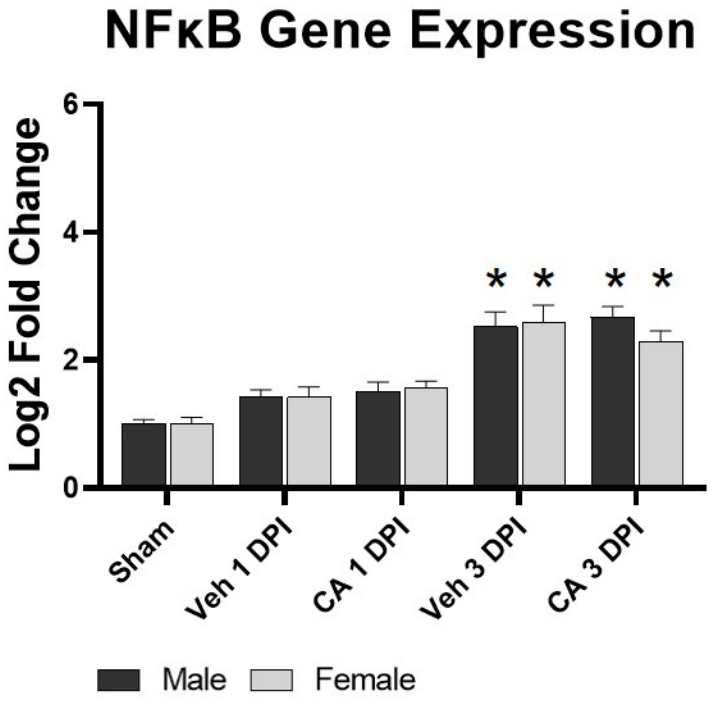


Figure 3.9 Male and Female NFκB Gene Expression.

The post-injury effect of CA or Veh on the induction of NFκB gene expression in the cortex male and female mice. NFκB gene expression was significantly upregulated in males at 3 DPI regardless of Veh or CA treatment. In females, NFκB gene expression was also upregulated as a result of injury at 3 DPI in both Veh and CA groups. A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. (Interaction: $F_{4, 55} = 0.070$, $p = 0.5941$; Sex: $F_{1, 55} = 0.2366$, $p = 0.6286$; Treatment: $F_{4, 55} = 36.01$, $p < 0.0001$).

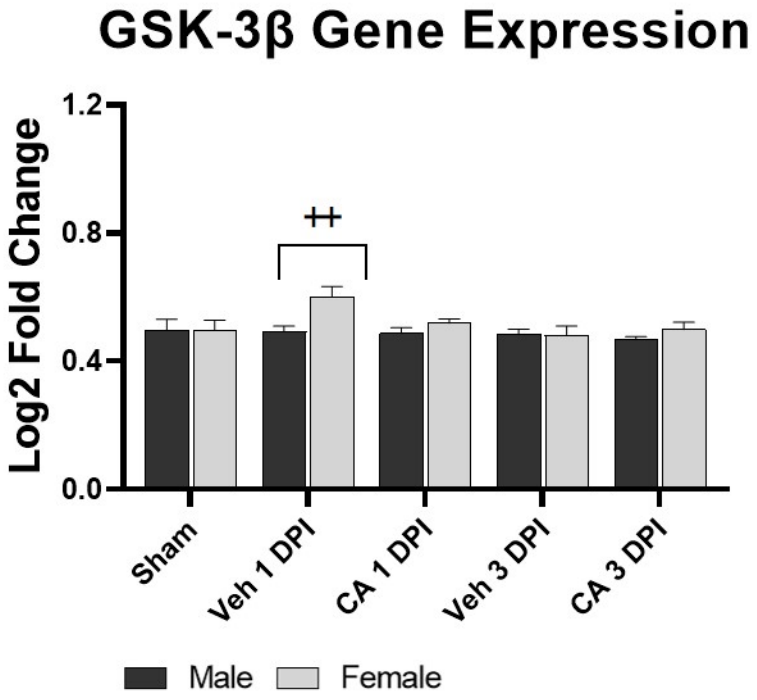


Figure 4.0 Male and Female GSK-3β Gene Expression.

The post-injury effect of CA or Veh on the induction of GSK-3β gene expression in the cortex male and female mice. GSK-3β gene expression did not significantly change from sham levels in males as a result of injury, CA or Veh treatment. At 1 DPI, Veh treated females had significantly higher levels of GSK-3β compared to Veh treated males at 1 DPI. A Two-way ANOVA with Tukey's post hoc was used for all multiple comparisons test, with statistical significance level set to $\alpha = 0.05$. Error bars on graphs represent the SEM. (Interaction: $F_{4, 55} = 1.88$, $p = 0.1266$; Sex: $F_{1, 55} = 5.643$, $p = 0.0210$; Treatment: $F_{4, 55} = 2.57$, $p = 0.0475$).

# CHAPTER 4. DISCUSSION

## 4.1 The Secondary Injury of TBI in Males and Females

Considering the heterogeneity of TBI and myriad of secondary consequences that arise from the injury, it comes with little surprise that a successful treatment has been so elusive over the years. Although science has developed several animal models of TBI to recapitulate the human heterogeneity condition, no single model alone can be used to truly predict the potential success or failure of a treatment. Furthermore, most of these animal models have historically used exclusively male mice, thus our understanding of the secondary pathology that arises from these injury models has been skewed towards the male sex. Evidence keeps mounting that biological sex contributes to numerous differences in the secondary pathology of TBI in not just humans, but also in animal models [230, 243, 245, 247, 249, 250, 254, 271, 320, 321]. There have been numerous drugs tested and dubbed a success or failure in the preclinical stages throughout the years based on using a single model of TBI and only male rodents. These drugs, showing great success in preclinical research, failed to elicit a therapeutic response, and sometimes even worsened outcomes during clinical trials, or therapeutic efficacy was shown in only one sex [53, 54, 72, 73, 75, 322]. Therefore, we must consider the heterogeneity of TBI in the light of both sexes as well as the model of TBI being used to test and develop drug treatments. A thorough understanding of the pathophysiology associated with a single model has led to the development of many potent drugs with a single mechanism of action. Furthermore, it is becoming more apparent that males and females metabolize drugs differently and these differences may extend to the blood-brain barrier BBB [244, 253, 323-325].

### 4.1.1 The Nrf2-ARE Pathway following Experimental Brain Injury and Delayed CA Treatment

The Nrf2-ARE pathway is ubiquitously expressed in mammalian cells and acts as the master regulator of redox homeostasis in response to sudden increases in oxidative stress, as well as xenobiotic and electrophilic molecules [135, 163, 164, 238, 302]. Not only does the Nrf2-ARE pathway combat oxidative stress, but it has also been shown to modulate the inflammatory response [143]. This pathway has been well characterized in males following CCI injury and considered a potential therapeutic target for certain drugs called Nrf2 activators [141, 142, 162, 178, 326, 327]. However, there is a paucity of information to date on studies that have examined this pathway in the female uninjured brain or following TBI to determine if it holds the same therapeutic potential in females. Despite the relative lack of information on the Nrf2 regulation in the brain of both sexes, previous work has demonstrated sex-based differences in brain and hepatic antioxidant capacities, cortical revascularization, and cytoskeletal degradation following CCI [90, 230, 252]. Therefore, we set forth to characterize this pathway in both sexes to determine if biological sex played a role in its modulation following a CCI injury.

The cytoplasmic phase II enzyme NQO1 is critical for the two electron reduction reaction of FAD and has been shown to protect against glutamate toxicity [156, 328]. There are also studies demonstrating significant sex differences in the catalytic activity and induction of NQO1 being higher in females [255, 260, 329]. In chapter 2, we provided data corroborating these findings, with females showing significantly elevated levels of NQO1 protein in the hippocampus whereas males did not demonstrate a rise in NQO1 following injury. Interestingly, it has been shown that NQO1 deactivates electrophiles and in doing

so, increases their excretion [330]. The catechol ring on CA is indeed a potential substrate for NQO1 and given that females have a higher NQO1 metabolic activity, this may decrease the availability of the electrophilic from of CA, thus reducing the therapeutic effect. A more thorough dose-response study with direct male to female comparisons will be required to determine why CA decreased nuclear total Nrf2 concentration in females.

HO-1 is critical for mediating the catalytic reduction of heme groups and the abundance of this detoxifying enzyme is heavily dependent on both the presence of heme and Nrf2 activity [145, 146, 148, 275, 331]. Another study has demonstrated the neuroprotective effect of HO-1 upregulation is present in both sexes, though in this case it was not Nrf2 mediated [332]. In Chapter 2, we found that the endogenous transcriptional response was more sensitive in males, whereas females a greater abundance of HO-1 protein. This could be due to the presence of catechol estrogens, which are known to modify cysteine 288 of Keap1, potently increasing the Nrf2/HO-1 signaling [333]. In Chapter 3, we found that CA treatment increased the ratio of nuclear/cytoplasmic Nrf2 in males at 1 DPI and 3 DPI. These results confirm that CA successfully increased nuclear localization in males, but unexpectedly HO-1 transcription was higher in the Veh treated males compared to CA treated males at 3 DPI. Furthermore, at 3 DPI, CA treated females had higher levels of HO-1 than Veh treated levels. This indicates that other mechanisms are implicated in the Nrf2-regulated control of HO-1. Since catechol estrogens modify cysteine 288 and CA modifies cysteine 151 of Keap1, it is possible that females benefit from dual activation of Nrf2. Some studies have shown that HO-1 is differentially regulated in the male and female brain, indicating that downregulation of HO-1 elicits neuroprotective effects in males but not females [251, 276]. This suggests that high levels

of HO-1 may be a pathological sign in males, but not females. Moreover, the coordinated regulation of HO-1 appears to be more complicated than we initially predicted, and CA may be interacting with other pathways to control the downstream effects of Nrf2.

## 4.2 Limitations and Considerations

One of the major limitations in TBI research is accurately modeling the heterogeneous nature of TBI. This poses multiple challenges for researchers. In the lab setting, all variables must be controlled for to be sure that the treatment is having an effect. However, over the years, several models of TBI have been developed to mimic different aspects, to a certain degree, found in the human condition. More recently, while more emphasis has been placed on sex as a potential factor in treating TBI, it is becoming more apparent that sex differences are common in these animal models as well. For example, the CCI model which produces a focal lesion, has shown clear sex differences in the rate of cytoskeletal degradation, variances in oxidative stress, and a divergence in the brain vasculature repair process [245, 256, 269, 283]. Studies of closed head injury produce less severe anatomical disturbances, but still result in lasting metabolic changes and altered microvasculature [91]. Sex differences have also been found in diffuse models of closed head injury, with females showing more severe metabolic disturbances than males, especially in mitochondrial function [334]. Other diffuse models of animal TBI, such as the lateral fluid percussion (LFP) injury, have also demonstrated sex differences in the pathophysiology of the secondary injury. A group found that male mice exhibited a less severe neuroinflammatory profile as compared to females using the same parameters of LFP injury [335]. More interestingly, another group found that males had more severe

physiological consequences following LFP injury, and phenylephrine aggravated this injury males but was found to be beneficial in females [336].

Another consideration is whether what we believe is a beneficial response as measured by biomarkers translates to functional recovery. One of the limitations we were presented with in Chapter 3 is we did not conduct a behavioral analysis to determine if changes in biomarkers conferred functional benefits or functional deficits. Furthermore, we still have yet to determine the subpopulation of brain cells as to which CA effects *in vivo* in both males and females at these time points. A recent study demonstrated the dynamic temporal tissue specific expression of Nrf2 following diffuse head injury in rats [337]. CA has been shown to be neuroprotective in neurons and astrocytes *in vitro* [171, 174]. However, studies on the subcellular population that are protected, and if they are protected, in males and females has yet to be elucidated. Considering the progressive changes in cell expression of Nrf2 following a single diffuse injury, it would be worth knowing if CA targets a specific subpopulation of brain cells, how it changes over time, and whether biological sex effects the temporal activation of Nrf2.

## 4.3 Future Directions

The relative paucity of information on the secondary processes of TBI in the female is becoming more obvious. Therefore, more studies should be conducted to determine the secondary effects of these animal models in females alone. A better description of these injury models is necessary to determine the efficacy of certain drugs. It is also becoming more apparent that certain TBI treatments are going to be efficacious in males whereas some will be more efficacious in females. It is possible that the severity of injury, secondary

pathology, as well as other circumstances, such as age, and time of admission post-injury, are all going to be factors to consider when administering a certain drug therapy. To develop TBI treatments that will successfully translate from the bench to the bedside, drugs should be tested in several different models of TBI. These models should also include both males and females. Lastly, more emphasis should be placed on whether these drugs improve functional outcomes, and then determine their mechanisms of action. *In vitro* models are great for defining the mechanisms, whereas *in vivo* studies allow for behavioral analysis to determine therapeutic efficacy in the whole organism as well as anatomical evidence of neuroprotection.

## 4.4 Conclusion

The Nrf2-ARE pathway seems to be different in male and female mice following a single CCI injury. Furthermore, a single 1.0 mg/kg I.P. dose of CA 1 hr following CCI injury produces differential effects in male and female mice. Based on the literature, our results indicate that this dose and therapeutic window of administration have a greater therapeutic effect in male mice, compared to female mice. A dose response study may help conclude that the therapeutic dose of CA for females is different than that of males or reveal that CA does not hold the same therapeutic benefits in females. A thorough behavioural analysis as well as histological analysis would also describe the potential for functional recovery and neuroprotection. Lastly, testing CA in several other TBI injury models may be beneficial in identifying potential subpopulations that should be stratified during clinical testing.

# APPENDICIES

## APPENDIX 1. ABBREVIATIONS

| | |
|---|---|
| BBB | Blood-brain barrier |
| BCP | Bathocuporine |
| CE | Cerebral edema |
| Chx | Chicken |
| CT | Computer tomography |
| Dk | Donkey |
| DTI | Diffusion tensor imaging |
| Gt | Goat |
| HO-1 | Heme Oxygenase 1 |
| I.P. | Intraparatoneal |
| MRI | Magnetic resonance imaging |
| Ms | Mouse |
| NQO1 | NAD(P)H: quinone oxidoreductase 1 |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| qRT-PCR | Quantative real time polymerase chain reaction |
| GSK-3β | Glycogen synthease kinase 3-beta |
| NFκB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| Rb | Rabbit |
| RT | Room temperature |
| SDS | Sodium dodecyl sulfate |
| TBS | Tris buffered saline |
| TBS-T | Trist buffered saline with Tween® 20 (0.1%) |
| CA | Carnosic acid |
| Veh | Vehicle |

APPENDIX 2. EQUIPMENT

| PRODUCT | MANUFACTURER | CATALOG NUMBER |
|---|---|---|
| Nuclease-Free Water | Applied Biosystems, Thermo Fisher Scientific | AM9937 |
| High Capacity cDNA Reverse Transcription Kit | Applied Biosystems, Thermo Fisher Scientific | 4366814 |
| Cordless Homogenizer | Bel-Art | F65000-0000 |
| Power Pac 200 (Semi-Dry Transfer Power Supply) | Bio-Rad | 165-5052 |
| C1000 Touch Thermal Cycler | Bio-Rad | 1851148 |
| Tans-Blot Sd Semi-Dry Transfer Cell | Bio-Rad | 1703940 |
| 3-8% Criterion™ XT Tris-Acetate Gel | Bio-Rad | 345-0130 |
| XT Tricine Buffer | Bio-Rad | 161-0790 |
| 20x Reducing Agent | Bio-Rad | 161-0792 |
| Bovine serum albumin | Bio-Rad | 5000206 |
| Tween® 20 | Bio-Rad | 1706531 |
| Synergy HTX Multi-Mode Reader | Bio-Tek | |
| Methanol | Fischer Scientific | A412-500 |
| Glycine | Fischer Scientific | BP381-500 |
| NaCL | Fischer Scientific | BP358-212 |
| HCL | Fischer Scientific | A142P-19 |
| Power Ease 500 (Electrophoresis Power Supply) | Invitrogen | EI8600 |
| LI-COR Oddessy CLX Infrared Scanner | LI-COR | |
| 4x Protein Sample Loading Buffer | LI-COR | 928-40004 |
| BCA Protein Assay Kit | Pierce | 23225 |
| Trizma® | Sigma-Aldrich | T1503 |
| NE-PER kit | Thermo Scientific | 78833 |

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VITA

## Education

**Doctor of Philosophy, Neuroscience** **2016 – 2021**
University of Kentucky, Lexington, KY
Dissertation Advisor: Edward D. Hall, PhD

**Graduate Certificate in Anatomical Sciences Instruction** **2018 – 2019**
University of Kentucky
Teaching Certificate in Gross Anatomy

**Bachelor of Science, Health Science** **2006 – 2015**
Saginaw Valley State University, Saginaw, MI
Academic Advisor: Jeff Smith, PhD

**Masters of Science, Occupational Therapy** **2011 – 2013**
Saginaw Valley State University, Saginaw, MI
Academic Advisor: Donald Earley, OTD, MA, OTRL

## Research Experience

**Graduate Research Assistant, University of Kentucky** **2016 – Present**
Research Advisor: Edward Hall, PhD
Carnosic acid differentially modulates the Nrf2-Antioxidant Response Element Following Experimental Traumatic Brain Injury

**Undergraduate Research, Saginaw Valley State University** **2011 – 2015**
Research Advisor: Jeff Smith, PhD
Investigating the efficacy of transplanting stem cells combined with enriched environment as a therapy for rats with frontal brain contusions.

## Funding and Awards

**Travel Award $3,495.00** **2014**
A proposal to partially cover the member and conference registration, abstract submission, and travel costs associated with the 2014 National Neurotrauma, San Francisco, CA.
Student Research and Creativity Proposal Review Committee,
Saginaw Valley State University

**Travel Award $1,000.00** **2014**
A proposal to partially cover the member and conference registration, abstract submission, and travel costs associated with the Faculty for Undergraduate Neuroscience in Washington, D.C.
Student Research and Creativity Proposal Review Committee,
Saginaw Valley State University

**Research Award $9,994.40** **2014**
A proposal to study the effects of enriched environment and delayed transplantation of embryonic neural stem cell therapy on functional recovery from chronic traumatic brain injury.
Student Research and Creativity Proposal Review Committee,
Saginaw Valley State University

**Travel Award $1,666.64** **2013**
A proposal to partially cover the member and conference registration, abstract submission, and travel costs associated with the National Neurotrauma Symposium in Nashville, TN.
Student Research and Creativity Proposal Review Committee,
Saginaw Valley State University

**Conference Presentations**

2019 Joyce Massey TBI Summit III, Ann Arbor, MI. "Towards a Novel Neuroprotective Approach for Traumatic Brain Injury: Harnessing the Nrf2-Antioxidant Response Element." Platform Speaker

2019 Kentucky Spinal Cord and Head Injury Research Trust Symposium, Louisville, KY "Towards a Novel Neuroprotective Approach for Traumatic Brain Injury: Harnessing the Nrf2-Antioxidant Response Element." Platform Speaker

2019 National Neurotrauma Symposium. Pittsburgh, PA. "Characterizing the endogenous Nrf2 response after controlled cortical impact injury in female and male mice." (Cortical and hippocampal protein and mRNA) Poster Presenter

2018 National Neurotrauma Symposium. Toronto, Ontario, Canada. "Characterizing the endogenous Nrf2 response after controlled cortical impact injury in female and male mice." (Cortical protein and mRNA only) Poster Presenter

2014 Faculty for Undergraduate Neuroscience. Washington, D.C. "Does chronically placed embryonic neural stem cell therapy induce restoration of function following cortical contusion impact in adult rats reared in an enriched environment?" Poster Presenter

2014 National Neurotrauma Symposium. San Francisco, CA. “Combining enriched environment and induced pluripotent stem cell therapy results in restoration of cognitive and improved motor function following traumatic brain injury.” Poster Presenter

2013 National Neurotrauma Symposium. Nashville, TN “The impact of enriched environment and transplantation of induced pluripotent stem cells on recovery from controlled cortical contusion injury.” Poster Presenter

2012 Michigan Chapter Society for Neuroscience. Ann Arbor, MI. “The impact of enriched environment and transplantation of murine cortical embryonic stem cells on recovery from controlled cortical contusion injury.” Poster Presenter

**Publications**

Furman, J. L., Sompol, P., Kraner, S. D., Pleiss, M. M., Putman, E. J., **Dunkerson, J**., & ... Norris, C. M. (2016). “Blockade of Astrocytic Calcineurin/NFAT Signaling Helps to Normalize Hippocampal Synaptic Function and Plasticity in a Rat Model of Traumatic Brain Injury.” The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 36(5), 1502-1515. doi:10.1523/JNEUROSCI.1930-15.2016

**Dunkerson, J**., Moritz, K.E., Young, J., Pionk, T., Fink, K., Rossignol, J., Dunbar, D., Smith, J.S., (2014). “Combining enriched environment and induced pluripotent stem cell therapy results in restoration of cognitive and improved motor function following traumatic brain injury.” Restorative Neurology and Neuroscience, 32(5), 675-687. doi: 10.3233/RNN-140408

Peruzzaro, S., Gallagher, J., **Dunkerson, J**., Fluharty, S., Mudd, D., Hoane, M., & Smith, J. (2013). “The impact of enriched environment and transplantation of murine cortical embryonic stem cells on recovery from controlled cortical contusion injury.” Restorative Neurology and Neuroscience, 31(4), 431-450. doi:10.3233/RNN-120299

**Dunkerson, J.A.,** Wang, J., Hill, R.L., Hall, E.D., “A time course of the endogenous Nrf2-antioxidant response element after controlled cortical impact injury in male and female mice.” *In progress*

**Dunkerson, J.A.,** Wang, J., Hall, E.D., “Carnosic acid differentially effects the Nrf2-ARE in male and female mice following experimental brain injury.” *In progress*