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CHARACTERIZING AN IN VITRO MODEL OF SEVERE FOCAL TRAUMATIC BRAIN INJURY IN HIPPOCAMPAL SLICE CULTURES: THE EFFECTS OF ETHANOL AND CALPAIN INHIBITION BY MDL-28170

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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By

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ABSTRACT OF THESIS

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In the United States, 2.8 million people suffer a traumatic brain injury (TBI) annually. Between 25%-50% of TBI injuries happen under alcohol intoxication. It is not understood how alcohol impacts patient outcomes via secondary injury pathways. Secondary injury pathways offer a window for therapeutic interventions, but there has been little success finding effective medications. Slice cultures offer a way to study secondary injury mechanisms in a controlled manner. The transection injury can model excitotoxicy seen following TBI. The current studies examined the effect of alcohol intoxication and withdrawal at the time of injury, and the effect of a calpain inhibitor (MDL-28170) on cell death following a transection injury. Intoxication had no effect on cell death compared to the TBI condition. In the ethanol withdrawal (EWD) study, EWD did not increase cell death following the TBI except at 72 hours. There was no effect of MDL on cell death. The severity of the model may have caused a ceiling effect. Additionally, imaging points may not have been sufficient for proper characterization. Future studies should use a different injury mechanism and other imaging times should be considered.

KEYWORDS: Traumatic Brain Injury, Alcohol, Hippocampal Slice Culture, Calpain

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DEDICATION

To B.F. Skinner, 113, and 213

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CHAPTER 1. INTRODUCTION

1.1 Traumatic Brain Injury

Traumatic brain injury (TBI) is an inclusive term for a wide range of pathologies seen following injury to the brain. One issue with diagnosing TBIs is the lack of a universal definition. For example, the Center for Disease Control (CDC) very generally defines a TBI as being "caused by a bump, blow, or jolt to the head or a penetrating head injury that disrupts the normal function of the brain" (Faul and Coronado, 2015). However, the revised (version 2.0) US Department of Veterans Affairs and the Department of Defense's Clinical Practice Guideline For Management of Concussion-mild Traumatic Brain Injury defines a TBI as a "traumatically induced structural injury and/or physiological disruption of brain function as a result of an external force and is indicated by new onset or worsening of at least one of the following clinical signs immediately following the event: any period of loss of or a decreased level of consciousness, any loss of memory for events immediately before or after the injury (posttraumatic amnesia), any alteration in mental state at the time of injury (e.g., confusion, disorientation, slowed thinking, alteration of conscious/mental state), neurological deficits (e.g., weakness, loss of balance, change in vision, praxis, paresis/plegia, sensory loss, aphasia) that may or may not be transient or intracranial lesion" (VA DoD, 2016). In 2010, part of the International and Interagency Initiative towards Common Data Elements for Research on Traumatic Brain Injury and Psychological Health came up with a definition of what constitutes a TBI: "an alteration in brain function, or other evidence of brain pathology, caused by an external force" (Hawryluk and Manley, 2015). The lack of consensus in definition may contribute to the

underdiagnosing and under treatment of TBIs (Menon, Schwab, Wright, and Maas, 2010; Prince and Bruhns, 2017), and makes comparison of TBI data sets difficult (Faul and Coronado, 2015).

Despite the heterogeneity of TBIs, they are all believed to trigger various pathways that lead to alterations in inflammation, metabolic, axonal, and neuronal function (Shultz et al., 2017). The two most common forces that result in TBIs are a direct impact to the head or the head quickly being accelerated/rotated (Gennarelli, 1994), although penetrative, compressive and blast forces can result in TBIs as well (Shultz et al, 2017). Given the wide range of forces, it is unsurprising that there are a wide range of pathologies that can be seen following a TBI.

The most common ways of classifying pathologies are by focal vs. diffuse injury and by primary vs. secondary injury. Focal injuries include skull fracture, contusions, hematomas, and hemorrhages, whereas edema, diffuse axonal injury (DAI), oxidative stress, and excitotoxicity are categorized as diffuse pathologies (McKee and Daneshvar, 2015). Primary injuries are considered to be "direct" and are incurred as a result of the injuring force. They are then believed to cause secondary injury (Hawryluk and Manley, 2015; McKee and Daneshvar, 2015). Secondary injuries include mitochondrial dysfunction, excitotoxicity, increased production of free radicals (Hawryluk and Manley, 2015), secondary hemorrhage, hypotension, hypoperfusion, hypoxia, ischemia, neuroinflammation, and edema (McKee and Daneshvar, 2015). It is important to acknowledge that these pathologies do not happen in isolation from one another, do not occur in every incident of a TBI, nor do they occur with identical frequencies.

TBIs can also be categorized based on severity from mild to moderate to severe by assessing symptoms and physiological signs post injury (Barlow, 2013). The Glasgow Coma Scale (GCS) assesses patient function post TBI and helps classify the severity of the injury (Barlow, 2013). The GCS quantifies patients' ability to open their eyes, their motor and verbal responses on a scale from 3-15 with 3 indicating no response and 15 indicating no noticeable deficits (Barlow, 2013). GCS scores of 13-15, 9-12, and 3-8 indicate a mild, moderate, and severe TBI, respectively. The GCS is a commonly used assessment as it can be conducted quickly and easily (Barlow, 2013). However, there are concerns with using the GCS as it shows poor inter-rater reliability, especially when given by inexperienced administrators (Barlow, 2013; Teasdale et al., 2014; Zuercher, Ummenhofer, Baltussen, and Walder, 2009). Andriessen et al. (2011) suggest that GCS scores taken at the injury site may contradict emergency department scores up to 15% of the time. This is especially concerning since GCS scores play a major role in determining the best facility for patient admission (Sasser et al., 2012). The GCS is time sensitive, so any delay in assessment can inappropriately impact the scores (Barlow, 2013). GCS scores can also be influenced by patient inebriation and facial swelling at the time of the assessment (Faul and Coronado, 2015). In fact, GCS scores can be influenced by a wide range of patient factors and conditions including age, presence of other psychological disorders, and facial or nerve damage, as well as factors that may be instigated by the treatment facility such as sedation, intubation, and administration of blockers of neuromuscular transmission (Middleton, 2012; Teasdale et al., 2014; Zuercher et al., 2009). The GCS does not have an animal equivalent for use in research, which hinders translatability of TBI research to clinical settings (Faul and Coronado, 2015). Other scales

have been utilized but have their own limitations. One such assessment is based on the length of time a patient experiences a loss of consciousness (LOC) post injury, however, this has been shown to be an unreliable indicator of a patient even having a TBI (Faul and Coronado, 2015). Another measure assesses the length of time a patient experiences post-trauma amnesia. The problem with this measure is that not all TBI patients experience amnesia following injury (Faul and Coronado), however, when patients do experience post-trauma amnesia, this assessment is indicative of the severity of impairment (Khan, Baguely, and Cameron, 2003). Not surprisingly, individuals that have just received a TBI may struggle with accurately reporting their symptoms, so measures of TBIs that rely on self-report pose validity concerns (Faul and Coronado, 2015).

1.2 Prevalence and Impact

It is estimated that 2.8 million people in the United States (U.S.) sustain a traumatic brain injury (TBI) every year, and 50,000 of these individuals die annually (Taylor, Bell, Breiding, and Xu, 2017). These numbers, however, are believed to underestimate the prevalence of both TBIs and TBI-related deaths. Prevalence of TBIs is reported as the number of cases per 100,000 of the population using emergency department visits, and varies by age. TBIs are most prevalent in the elderly (age 75+) with 1701.7 cases per 100,000, followed by children aged 0-4 years old with 1541.1 cases per 100,000 (Taylor et al., 2017). TBI patients in the 75+ age group also experience the highest rates of hospitalization and death due to TBIs, followed in these categories by patients in the 65-74 age range (Taylor et al., 2017). While the elderly have the highest rate of TBIs based on the population, patients aged 15-24 make up 17.9% of TBI-related emergency department visits, which is more than any other age group (Taylor et al., 2017).

Rodriguez, Mallonee, Archer, and Gofton (2006) conducted a study examining deaths certificates in Oklahoma in 2002 for individuals that had multiple causes of death listed. They found that found that 212 of the 952 deaths that included TBIs were classified as false negatives (i.e. a TBI was a cause of death but was not reported as such), making death certificate surveillance only 78% sensitive for TBIs (Rodriguez et al., 2006). The majority of death certificates that fell into the false negative category (69%) had the cause of death listed as "multiple trauma," mainly as the result of automobile accidents (86%). Many of the death certificates were incomplete or completed incorrectly, accounting for 91% of the false negatives (Rodriguez et al., 2006). While this study only encompassed Oklahoma, it is plausible that other states may suffer similar inadequacies in reporting. A small scale study in Finland found that 74.2% patients that were admitted to a rehabilitation center for spinal cord injury (SCI) also presented symptoms of TBIs which went undiagnosed (Tolonen, Turkka, Salonen, Ahomiemi, and Alarant, 2007). Unsurprisingly, a SCI patient also suffering from an undiagnosed (and therefore untreated) TBI can encounter additional concerns and have worse patient outcomes than if the TBI is diagnosed and treated (Tolonen et al., 2007). Schootman and Fuortes (2000) suggest that underreporting may occur for other reasons as well. Since the annual number of TBIs is taken from emergency departments (ED), it cannot take into account TBIs that were treated in outpatient facilities, which may result in as many as 89,000 unreported TBIs per year. TBIs, particularly mild TBIs (mTBI), may go undiagnosed and untreated with estimates suggesting that 11-75% of TBIs are not diagnosed (reviewed in Faul and Coronado, 2015). Possible explanations for this discrepancy include a lack of proper education regarding TBIs as well inaccessibility of health care resources (Faul and Coronado, 2015).

Traumatic brain injuries are responsible for an economic burden of $76 billion in the U.S every year (CDC, 2019; Finkelstein, Corso, and Miller, 2006). The cost of productivity loss from TBIs is higher than productivity loss associated with any other injury (Faul and Coronado, 2015). In addition to the cost to society, the economic burden of TBIs to patients and their families is also extensive. It is difficult to truly measure lifetime costs following a TBI, however, one study found that from the time of TBI to six years post injury, TBI patients had significantly higher health care costs than their matched controls (Leibson et al., 2012). Unsurprisingly, more severe TBIs were associated with higher medical costs than more mild TBIs (Leibson et al., 2012). Lifetime costs depends on the severity of the injury but the estimated lifetime cost of caring for a person with a TBI is between $600,000 and $1,875,000 ("Brain Injury Costs", 2017). Compounding this financial burden is that TBI patients have very high rates of unemployment, with studies estimating the rate of unemployment for up to two years post injury is between 42% (Doctor et al., 2005) and 60% (Cuthbert et al., 2015). This is especially concerning given that employment has been associated with better patient outcomes and improved quality of life (reviewed in Wehman, Targett, West, and Kregel, 2005).

While individuals can receive a TBIs through a number of different means, falling, being struck by or striking against an object, and motor vehicle accidents are the three most common causes of TBI-related emergency room visits, hospitalizations and deaths (Taylor et al., 2017). TBIs induced by falls make up the majority of TBIs for the youngest age group (0-4 years old) and the oldest age group (65+ years old), accounting for 72.8% and 81.8% of TBIs in the age groups, respectively (CDC, 2016). However, in both the age groups of 15-24 and 25-44 year olds, the percentage of TBIs induced by automobile

accidents, assaults and falls are similar, with each making up ~20% of the TBIs in the age group (CDC, 2016). Using emergency department visit information, men are more likely than women to experience a TBI, although the ratio varies between age groups (Faul, Xu, Ward, and Coronado, 2010). There have been a few studies looking at distribution of TBIs across race and these data also show that black individuals had the highest reported rate of TBI (Faul et al., 2010), but the racial categories used were far from inclusive. It has also been shown that emergency room care given to racial minorities following mTBIs can vary from care given to white patients including increased time to see a health care provider, departure from the emergency room department prior to being seen, increased likelihood of being seen by a resident instead of a staff physician or emergency medicine tech, while being subjected to over testing compared to white patients (Bazarian, Pope, McClung, Cheng, and Flesher, 2003). These discrepancies in care may play a role in the why African Americans have been reported to experience worse psychological outcome compared with white patients (Jorge, Robinson, Starkstein, and Arndt, 1994).

1.3 Animal Models of TBI

There are a variety of animal models of TBI that can be used in numerous species as well as in cell culture methods. Rodent models of TBI are the most commonly used due to the reduced expense and increased convenience over larger animals (Johnson et al., 2015; Xiong, Mahmood, and Chopp 2013). This has allowed the outcome measures of the rodent models to become more standardized than those for other animals (Xiong et al., 2013). Despite this, a major concern of using rodent models is that the physiology and size of the species is further removed from humans than is the case for larger animals (Xiong et al., 2013) which may hinder translatability of these models to clinical populations.

Additionally, there are concerns that attempting to scale the injury to a brain significantly smaller than a human's may also hinder translatability (Johnson et al., 2015).

Animal models of TBI can be categorized based on the type of injury they induce: focal, diffuse, mixed, or combined (Morales et al., 2005). Animal models that induce a focal injury include models such as the weight-drop models, controlled cortical impact (CCI), and midline fluid percussion injury (FPI) (Morales et al., 2005).

1.3.1 Weight Drop Models

The weight-drop induced TBI is very commonly used and is believed to be the original experimental model (Morales et al., 2005). It can be varied to produce a range of severities based on the weight dropped and the height from which it is dropped (Xiong et al., 2013). Feeney, Boyeson, Linn, Murray, and Gail (1981) adapted a spinal cord injury device to induce a TBI, in brief, by dropping a weight down a thin tube onto a footplate placed on the exposed of dura of rats (Feeney et al., 1981). Unfortunately, although the Freeney weight-drop model is capable of producing consistent injuries, it can have a high rate of mortality (Xiong et al., 2013). A variant of this model was later developed in which the weight was dropped onto a closed headed rat (Shapira et al., 1988). Due to the increased protection this model offers the skull, it is sometimes referred to as a model of mTBI (Shultz et al., 2017). This weight-drop model (commonly referred to as the Shohami model) is easy to perform, but may not produce consistent injuries across animals or laboratories (Xiong et al., 2013). Although the Shohami weight-drop model is usually classified as a focal injury model (Morales et al., 2005; Xiong et al., 2013), it is sometimes included as model of mixed injury (Shultz et al., 2017). Weight-drop models are easy to perform and are clinically relevant, but there are concerns about the weight rebounding and

inducing a second injury as well as concerns with the accuracy of the injury induced (Morales et al., 2005).

1.3.2 Controlled Cortical Impact Model

The CCI model relies on a small pneumatic impactor to deliver a calculated blow to the exposed dura that deforms the brain tissue (Dixon, Clifton, Lighthall, Yaghami, and Hayes, 1991). The CCI model can be used to give a range of TBI severities (mild to severe) and is considered to be give a more accurate and consistent injury than weight drop and fluid percussion models (Romine, Gao, and Chen, 2014; Xiong et al., 2013). The impactor can be placed over the midline or the lateral cortex to model injuries sustained at different sites (Romine et al., 2014). While the CCI model is believed to model human TBIs well, it can also cause pathologies that are not typically seen in the clinical population (Romine et al., 2014). There is some concern that the CCI model can lead to high rates of mortality (Romine et al., 2014), but others report the CCI model has a low rate of mortality (Xiong et al., 2013). Additionally, CCI models requires a craniotomy, opening of the skull, which decreases the translatability to the clinical population (Xiong et al., 2013).

1.3.3 Fluid Percussion Model

Early FPI experiments were primarily performed on cats, rabbits, and nonhuman primates but the FPI model was adapted for the rat in the 1980s (Dixon et al., 1987; McIntosh et al., 1989). A few labs worked to thoroughly characterize the FPI model for rats at a range of severities (Dixon et al., 1987; McIntosh, Noble, Andrews and Faden, 1987). FPI, in brief, induces injury when a weight on a pendulum is dropped from a predetermined height and strikes a piston on a Plexiglas cylinder filled with 37º C saline.

The other end of the cylinder is a transducer with a Leur-Loc that can be fastened to a Leur-Loc implanted over a rat's exposed dura. When the weight hits the piston, it creates a pulse that briefly increases intracranial pressure that deforms the brain tissue (Dixon et al., 1987; McIntosh et al., 1989). The severity of the TBI can be altered by adjusting the height from which the weighted pendulum is dropped (Dixon et al., 1987). The craniotomy can be done either on the midline or laterally, which is considered a mixed injury model (Morales et al., 2005), allowing it to translate to a wider variety of clinical cases (McIntosh et al., 1987; McIntosh et al., 1989).

1.3.4 Impact Acceleration Model

Some animal models of TBI induce a more diffuse pattern of injury, thus broadening the scope of injuries that can be investigated. One of the most commonly used diffuse injury models is the impact acceleration model. In brief, a steel plate is affixed to the rat skull and the anesthetized rat is then placed on a foam pad with the steel plate directly under a thin Plexiglas tube. A known weight is dropped down the tube from a known distance and strikes the steel plate on the rat's skull. The steel plate allows the force to disperse more widely across the skull, which works to prevent skull fracture as well as produce a more diffuse pattern of injury. Once the weight has struck the steel plate, the pad is moved to prevent a secondary impact of the weight (Marmarou et al., 1994), which is a drawback of other weight-drop models. Another advantage of the foam pad in the Marmarou model is that it allows for a rotational injury, which is believed to be critical to produce a loss of consciousness (Schultz, 2017). This is especially clinically relevant as some definitions of TBI require a loss or alteration of consciousness (VA DoD, 2016) and diagnoses of mTBI cannot be made without LOC or altered consciousness (National Center

for Injury Prevention and Control, 2003). However, due to the nature of this model, the injuries produced are not highly consistent between animals and there is a high mortality rate for the animals due to respiratory depression if they do not receive ventilation post injury (Marmarou et al., 1994; Xiong et al., 2013). A newer variation of this model utilizes an air-driven impactor, guided with the use of a laser, to hit a steel plate affixed to the rat's skull while the rat's head is placed over a gel base which slows the head down post impact while allowing the head to rotate (Cernak et al., 2004).

1.3.5 Blast TBI Model

Due to the high rates of TBIs in military populations, a model of blast-induced TBI was created (Faul and Coronado, 2015; Xiong et al., 2013). There are various animal models used to mimic blast TBIs in use (e.g. Cernak et al., 2011; Cheng et al, 2010; Reneer et al., 2011; Rubovitvh et al., 2011), causing blast TBI models to suffer from a lack of standardization (Xiong et al., 2013). Briefly, this model uses a variety of mechanisms (from explosives to shock tubes) to model injuries received from explosions. Explosions release a large amount of energy, causing an increase in temperature, followed by a drop in pressure resulting in a shockwave (Ning and Zhou, 2015). The initial injury from the blast can be followed by injuries from flying debris, burns, the acceleration of the body, etc. (Ning and Zhou, 2015). Real world blast incidents can induce multiple TBIs including acceleration injuries, penetrating injuries and injuries from being struck (Johnson et al., 2015). Unfortunately, due to the nature of real world blast injuries, there is not a clinical population that has experienced a pure blast injury, which hinders the development of animal models of blast TBIs (Johnson et al., 2015).

Combined models of TBI include the original TBI injury using one of the previously described models, followed by a secondary injury such as ischemia or temporary hypoxia that serves to exacerbate the initial insult (Morales et al., 2005). These models may be especially beneficial as hypotensive and hypoxic episodes are common following TBIs in clinical populations (Marshall et al., 1975).

1.4 In Vitro Models

In addition to the *in vivo* models, there is also a variety of *in vitro* models of TBIs. *In vitro* models can utilize either dissociated cell cultures or slice cultures. Cell cultures provide a highly controlled and repeatable way to study various diseases and disorders (Morrison, Elkin, Dollé, and Yarmush, 2011). Additionally, use of culturing methods can help to lower the number and distress of laboratory animals used (Humpel, 2015). Dissociated cell cultures allow for the study of a specific type of cell, such as neurons or astrocytes; however, while they can provide information about cell survival, morphology and responses to various toxins, chemicals and pharmaceuticals, etc. they also suffer from a lack of translatability because they exist in isolation outside of their natural environment (Humpel, 2015). This isolation can be beneficial for studying effects of a manipulation on a specific type of cells without systematic confounds that would exist in whole animals (Morrison et al., 2011). While dissociated cell cultures can be beneficial for isolated populations of cells, organotypic slice cultures have the advantage that the majority of the cellular structure is preserved. Because the structure is maintained, information about cellular and molecular workings of the different population of cells naturally interacting in the brain can be gathered (Humpel, 2015). Organotypic slice cultures can be a middle ground between dissociated cell cultures and *in vivo* studies.

1.4.1 Compression Models

Some *in vitro* models mimic the *in vivo* models, such as the compression models (Morrison et al., 2011). The compression model of TBI mirrors the weight drop model of TBI in live animals onto brain tissue cultures (Morrison et al., 2011). In the compression model, a stylus is positioned over the tissue and dropped onto the impact site and is controlled with electromagnets (Sieg, Wahle, and Pape, 1999). Similar to weight drop models, the severity of the injury impacted by the compression models can alter the severity of the injury by varying the height of the drop, the weight dropped, the duration of the injury as well as the depth of the injury (Morrison et al., 2011). Unfortunately, the force of injury and the deformation of the brain tissue are hard to measure, making these models hard to standardize (Morrison et al., 2011).

1.4.2 Stretch Injury Models

Stretch injury models are another common method of inducing TBI-like injuries in vitro. These models were designed to mirror the tissue deformation seen in TBIs (Morrison et al., 2011). One mechanism of inducing this injury on the cells/tissue is with the use of compressed air. Cells are placed on a flexible membrane and compressed gas is used to distort the membrane, stretching (and therefore damaging) the cells for a set duration (Ellis, McKinney, Willoughby, Liang, and Povlishock, 1995). In a variation on the stretch model, slices are again placed on a flexible membrane, but instead of using compressed gas, the flexible membrane is stretched over an indenter (Morrison, Cater, Benham, and Sundstrom, 2006). A drawback to this model is that the injury is not uniform and therefore not well characterized (Morrison et al., 2011).

1.4.3 Transection Models

Another method of inducing traumatic injury is with transection models (Morrison et al., 2011). The transection models are beneficial for modeling primary axotomy which results in cell death. These can work on single cells or multiple cell cultures (Morrison et al., 2011). There are variations of the transection models including a device that rotates needle(s) to create concentric circles of damage in a mixed culture of neuronal and glial cell cultures (Mukhin, Ivanova, Knoblach, and Faden, 1997). This particular model results in primarily neuronal cell death while glia remain relatively unharmed (Mukhin et al., 1997). Mukhin et al. (1997) also found glutamate antagonists were neuroprotective in this injury model, indicating that this model may be especially beneficial for studying glutamate-dependent secondary injury. A year later, the same group devised a variant on this model, utilizing an injury device (mechanical punch device) that was composed of 28 parallel blades that are pressed into either glial or neuronal/glial cell cultures (Mukhin, Ivanova, Allen, and Faden, 1998). Glial cultures showed cell death isolated to points of injury; however, this injury mechanism showed a spread of cell death from the points of injury in neuronal/glial cell cultures and, as in the previous model, NMDA antagonists were neuroprotective (Mukhin et al., 1998).

Transection models can also be used with organotypic hippocampal slice cultures (OHSC). Organotypic slice cultures may provide more translatable models than dissociated cell cultures since the culturing of tissues allows for the preservation of the structural and organizational integrity of the tissue (Humpel, 2015). One transection model in OHSCs induces injury by slicing through the cornu ammonis (CA) 2 region of the hippocampus with a scalpel to transection the Schaffer collateral fibers (Laskowski,

Schmidt, Dinkel, Martinez-Sánchez, and Reymann, 2005). Schaffer collateral fibers run from the CA3 to the CA1 region of the hippocampus and are believed to play a role in plasticity and memory formation (Sevilla, Cabezas, Prada, Sánchez-Jiménez, and Buño, 2002; Teixeira et al., 2018). Our lab has also looked at the effect of different transections of OHSCs. Mulholland and Prendergast (2003) measured the neurotoxic effects of transecting different areas of OHSCs, specifically the mossy fibers, Schaffer collateral fibers, and alveus fibers. This model of TBI may be more applicable for specific types of TBIs (i.e. penetrating injuries/bullet wounds) or for modeling specific features of TBIs, such as axotomies and excitotoxicity.

Although none of these in vitro models can accurately encompass all aspects of a TBI and may not be as translatable to clinical settings as *in vivo* models, models of TBI can provide valuable information about secondary injury pathways.

1.5 Secondary Injury

Primary injuries in TBI include contusion, hematoma, shearing, and skull fracture (Coles, 2004; Park, Bell, and Baker, 2008; Xiong, Zhang, Mahmood, and Chopp, 2015) that occur as the result of the brain being displaced in the skull at the time of trauma (Prins, Greco, Alexander, and Giza, 2013). Primary injury is typically focal in nature (with some exceptions for rotational injuries) whereas secondary injury mechanisms exacerbate and expand upon the original injury (Borgens and Snyder, 2012).

Generally, secondary injury is defined as a "restricted set of intracellular biochemical processes that occur over time after cells are compromised" and includes various injuries such as edema, ischemia and hemorrhage (Borgens and Snyder, 2012). Brain tissue, as well as spinal cord tissue, is particularly susceptible to hemorrhage,

ischemia, and edema as the tissue is surrounded by inflexible bone. When an injury does occur, the pressure on the tissue increases, causing further damage following the original insult (Borgens and Snyder, 2012). A TBI can sever or rupture blood vessels and this restriction in/lack of blood flow (ischemia) deprives the tissue of oxygen and glucose which can lead to delayed cell death (Borgens and Snyder, 2012).

1.5.1 Edema

Edema, an increase in water content, can exacerbate ischemia if the swelling restricts blood flow, but more importantly, edema can lead to compression of the brainstem, which can cause death (Borgens and Snyder, 2012; Ho, Rojas, and Eisenberg, 2012; Xiong et al., 2015). Edema can be caused by various mechanisms but is usually classified as either cytotoxic, vasogenic, interstitial, or combined (Ho et al., 2012). Typically, edema following TBIs is listed under the combined category and can have multiple contributing factors (Ho et al., 2012).

Cytotoxic edema typically results from ischemia or excitotoxicity. This results in increased fluid in neurons, glia, axons and myelin, with gray matter being impacted first. If there is repeated insult, the blood brain barrier (BBB) may become compromised (Ho et al., 2012). Vasogenic edema occurs when the endothelial junctions in the BBB become compromised, which can happen from the BBB being physically disturbed. This type of edema can be corrected in the early stages if the BBB is reconstructed, however, if vasogenic edema becomes too advanced or occurs repeatedly, the resulting damage is not correctable and may cause or exacerbate ischemia or lead to cytotoxic edema (Ho et al., 2012). Interstitial edema varies from the previous types as the increased pressure is in the ventricles. This increase in pressure causes the lining of the ventricles to rupture which in

turn allows the cerebral spinal fluid (CSF) to move to extracellular space. This type of edema is most commonly caused by conditions such as tumors, meningitis, hydrocephalus, and subarachnoid hemorrhage (Ho et al., 2012). Interstitial edema is also open to intervention depending on the cause (e.g. removal of mass, insertion of catheter, etc.) (Ho et al., 2012).

Edema is one type of secondary injury that has been invested for therapeutic intervention. One of the factors that mediates vasogenic edema is bradykinin, (Borgens and Snyder, 2012). Bradykinin is an important peptide that plays a role in regulating blood pressure and inflammatory response by increasing vascular permeability (Golias, Charalabopoulos, Stagikas, Charalabopoulos, and Batistatou, 2007). Because of this, a bradykinin antagonist (Bradycor) went to clinical trials. Bradycor failed the clinical trial, but the trial was cut short due to an animal study that came out following the start of the clinical trial which aroused concerns about the safety of Bradycor (Narayan et al., 2002). In addition to vasogenic factors (such as bradykinin), there are also cytotoxic factors that contribute to edema and may provide other therapeutic targets. These cytotoxic factors include glutamate, arachidonic acid metabolites, and free radicals, etc. (Borgens and Snyder, 2012). While breakdown of the BBB can result in edema, it also allows for infiltration of immune cells from the periphery into the brain which can cause neuroinflammation, another major type of secondary injury (Schimmel, Acosta, and Lozano, 2017).

1.5.2 Inflammation

The inflammatory process is initiated shortly after trauma occurs. There is some debate over the usefulness of inflammation following TBI. In general, inflammation is complex set of interactions that are triggered by the introduction of trauma, infectious agents, and toxins, etc. (Nathan, 2002). TBIs (as well as ischemia) are categorized as sterile triggers of inflammation (as opposed to infectious triggers) (Rock, Latz, Ontiveros, and Kono, 2009). Neuroinflammation is complex as it can be both beneficial as well as damaging. Neuroinflammation is associated with the release of growth factors and proteolytic enzymes that aid in regeneration (Borgens and Snyder, 2012). Macrophages help clear debris (dead and damaged cells and myelin) that otherwise prevents regeneration (Borgens and Snyder, 2012). On the other hand, neuroinflammation can be detrimental as an overabundance of inflammatory cells can exacerbate edema, lead to more apoptosis which is mediated through cytokines and free radicals (Borgens and Snyder, 2012).

The inflammatory response following trauma includes a variety of cells including macrophages, neutrophils, astrocytes and microglia, and T cells (Corps, Roth, and McGavern, 2015). The TBI causes the release of alarmins, endogenous damage-associated molecular pattern molecules (DAMPs), which are interpreted as “danger” or “warning” signals (Bianchi, 2007; Corps et al., 2015). These alarmins, which include HMGB1, ATP, DNA, and interleukins, as well as many others, lead to the triggering of the inflammatory response (Corps et al., 2015). All cells contain ATP, but when it is released, it is believed to play a role in regulating a host of functions from cardiac functioning to vasodilation to neurotransmission (Bours, Swennen, Virgilio, Cronstein, and Dagnelie, 2006; Yegutkin, 2008). In particular, ATP release following trauma triggers immunological and

inflammatory responses through purinergic signaling (Bours, et al., 2006; Corps et al., 2015). ATP can get into the extracellular space either through controlled release from living cells (Junger, 2011) or can rush from cells that have been injured (Virgilio, 2005). Following trauma, ATP is believed to be released from cells surrounding the trauma area as well as from the cells damaged in the initial injury (Virgilio, 2005). In one study of spinal cord injury (SCI), the cells in the perilesion area released even more ATP than cells that were in the original area of trauma (Wang et al., 2004). Once it is released from damaged cells, ATP activates purinergic 2 (P2) receptors. However, as ATP is metabolized from ATP to ADP to AMP to adenosine, this process of hydrolysis ends the activation of the P2 receptors, and enables receptor desensitization allowing for quick and frequent signaling (Junger, 2011; Yegutkin, 2008). During this process, the sterile immune response tends to diminish as ATP is converted to adenosine (Corps et al., 2015). Astrocytes and microglia both express ectoenzymes, needed for the conversion of ATP to adenosine, giving astrocytes and microglia the ability to reduce ATP-mediated inflammatory responses (Corps et al., 2015). When neutrophils and T cells are activated, they too release ATP, which results in stronger activation stimuli for these immune cells (Junger, 2011).

Microglial activation is a key component to neuroinflammation (Tang and Le, 2016), however, the role of microglia activation following CNS injury is not fully understood and is somewhat controversial. Microglia arrive to the site of trauma within minutes and their role is dependent on purinergic signaling and astrocyte ATP-dependent ATP release (Corps et al., 2015). Microglia, considered to be the macrophages of the CNS (e.g. Perry and Teeling, 2013; Yin, Valin, Dixon, and Leavenworth, 2017), can transform into phagocytic cells in response to cell death and clear dead and damaged cell debris.

Depending on the area of damage, microglia may serve additional functions (Corps et al., 2015). For instance, in the event of damage to the glial limitans, microglia can insert themselves into the gaps left by dead and injured astrocytes and reinforce this barrier, preventing material leakage into the parenchyma of the brain (Corps et al., 2015). These data imply the role of microglia, at least acutely, are mainly beneficial following trauma, however, other data indicate the presence of microglia following trauma can be detrimental to the healing process. Microglia can be pushed into overactive states when in the presence of certain toxins, and damaged/dead neurons. Once that happens, the microglia begin to overexpress cytotoxic factors (Block, Zecca, and Hong, 2007). One proposed theory behind the dual functions of microglia (neuroprotective versus cytotoxic or anti-inflammatory versus pro-inflammatory) is that there are two distinct phenotypes of microglia/macrophages. Phenotype 1 (M1) is sometimes referred to as "pro-inflammatory" and produces superoxide, nitric oxide, reactive oxygen species (ROS) and pro-inflammatory cytokines including tumor necrosis factor-α (TNF- α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) (Kigerl et al., 2009; Tang and Le, 2016). M1 microglia are typically found at the sight of injury minutes following trauma and persist for an extended period of time. In comparison, phenotype 2 (M2) microglia show a smaller and more fleeting response to injury (Kigerl et al., 2009). M2 microglia promote and aid in phagocytosis of damaged cells and proteins, angiogenesis, repair of the extracellular matrix, tissue repair and neuron survival as well as combating the pro-inflammatory response of the M1 microglia (Kigerl et al., 2009; Tang and Le, 2016). This dichotomy may be an oversimplification, and some researchers oppose these designations quite strongly. Distinct M1and M2 phenotypes have only been shown in *in vitro* experiments

after exposing macrophages or microglia to specific cytokines (Jassam, Izzy, Whalen, McGavern, and El Khoury, 2017). Additionally, the actual environment for microglia and macrophages in the body following trauma is vastly more complicated than this *in vitro* model would suggest, limiting the clinical relevance of the M1 versus M2 phenotype hypothesis. In reality, as opposed to one or two cytokines, microglia following trauma are surrounded by various cytokines (some upregulated and some downregulated), adhesion molecules, the surrounding cells and matrix as well as the age of the cell (Jassam et al., 2017). Evidence indicates that macrophages isolated from TBI animals failed to fall into distinct phenotypes. In fact, these cells have unique activation states and possess both pro-inflammatory as well as healing attributes, further complicating the role of microglia following TBI (Kim, Nakamura, and Hsieh, 2016).

Similar to microglia, the role of neutrophils in the inflammatory response following TBI is complex and twofold. Neutrophils are typically believed to be pro-inflammatory, but they can also be beneficial for wound healing by aiding in phagocytosis, the release of metalloproteinase and the production of growth factors (Corps et al., 2015). Neutrophils can also play a role in breaking down the BBB and promoting neuronal death following trauma by releasing proteases, TNF-α and ROS (Corps et al., 2015). Neutrophils are also involved in the recruitment of monocytes, however the role of monocytes following TBI is not well understood (Corps et al., 2015). In mice, monocytes can be categorized as either classical, nonclassical or intermediate. Classical monocytes are believed to infiltrate the brain following trauma where they differentiate into macrophages, however the role of nonclassical monocytes is less understood. (Corps et al., 2015; Makinde, Cuda, Just, Perlman, and Schwulst, 2017). Briefly, while the global depletion of monocytes was

correlated with improved outcomes, evidence suggest that both classical and nonclassical monocytes both play a role in exacerbating damage as well as repairing damage following trauma (Makinde et al., 2017).

T cells are important for adaptive immunity, however their role following TBI is unknown (Corps et al., 2015). One study of T cell infiltration in mice following CCI showed that, while T cells were present in the first day following CCI, peak T cell infiltration did not occur until day seven following trauma (Clausen et al., 2009). Interestingly, the same lab two years previously found different results in rats, where peak infiltration of T cells was seen at 24 hours and slowly decreased through seven days post injury. Additionally, they found the presence of T cells in the subcortical white matter at 72 hours and at day 7 post injury in a subset of injured animals (Clausen, Lorant, Lewén, and Hillered, 2007). This indicates there may be differences in the pattern and time course of T cell infiltration across species (Clausen, 2009; Kelso and Gendelman, 2014), however these time points may only be applicable in males, as neither female mice nor rats were used. In mice, it has been shown that females have less immune cell infiltration into the brain post injury compared to males and this may be responsible for comparatively diminished neuroinflammatory response in females compared to males following CCI (Doran et al., 2018). Preclinical evidence suggests that endogenous hormones in female mice may offer a level of neuroprotection, improving outcome measures post TBI (Clevenger et al., 2018). Clinical studies have produced mixed findings as to whether TBI outcomes are different between men and women, with some studies showing better, equitable, or worse outcomes in women compared to men (reviewed in Clevenger et al., 2018).

A concern has also been raised that many studies of T cell infiltration rely on a marker that is not specific for T cells and labels phagocytes as well (Kelso and Gendelman, 2014). One study found no difference in severity or neurological impairment following closed head injury in wild-type mice versus *Rag1* knockout mice, which lack mature T cells and B cells, up to a week following injury, indicating T cells do not play a major role acutely following TBI (Weckbach et al., 2012). However, evidence from transplant studies potentially offer some support for role of T cells acutely following other types of trauma. A study utilizing a MPTP model of Parkinson's disease showed that regulatory T cells (T_{reg}) were able to attenuate the effects of T_H17 cells, a subset of helper T cells that promotes migration through the BBB that are implicated in autoimmune diseases of the CNS (Kebir et al., 2007; Reynolds et al., 2010). Whether this is directly translatable to TBI is unclear. Overall, the current understanding of the role of T cells following TBI is uncertain and disputed. As the roles of many cells, as well as neuroinflammation in general, have this dual persona of benefit and damage, attempting to alter the neuroinflammatory response following TBI is difficult and non-effective (Borgens and Snyder, 2012; Narayan, 2002; Stahel et al., 2000).

1.5.3 Excitotoxicity

Excitotoxicity refers to glutamate and other related excitatory amino acids destroying neurons (Olney, 1986). Glutamate is the most abundant excitatory amino acid in the CNS (Borgens and Snyder, 2012; Yi and Hazell, 2006) and is fairly ubiquitous throughout the brain in low (millimolar) concentrations, however, when neurons are exposed to an overabundance of glutamate, they die (Choi, 1992). Astrocytes play a critical role in maintaining homeostasis in the brain under normal conditions, and in particular play

a role in glutamate uptake (Kelso and Gendelman, 2014). Under normal circumstances, glutamate is encapsulated in vesicles in neurons and is released following the influx of calcium (Ca^{2+}) from depolarizing cells (Borgens and Snyder, 2012; Guerriero, Giza, and Rotenberg, 2015). Following this release, astrocytes collect up the remaining glutamate, it is converted to glutamine and returned back to the neurons for glutamate synthesis (Borgens and Snyder, 2012). In addition to benefitting signal transmission, the clearing of this extracellular glutamate is vital in preventing excitotoxic damage to the neurons (Floyd and Lyeth, 2007). Following a TBI, astrocytes become reactive and undergo a functional (increased expression of GFAP) and conformational change (increased cell size and elongated projections), but it is unclear if this process is beneficial or harmful (Kelso and Gendleman, 2014). Shortly following a TBI, there is a massive release of glutamate (Guerriero et al., 2015). Both human and rodent microdialysis studies have shown an increase in extracellular glutamate following TBIs (e.g. Bullock et al., 1998; Chamoun, Suki, Gopinath, Goodman, and Roberston, 2010; Faden, Demediuk, Panter, and Vink, 1989; Folkersma et al., 2011).

In humans with severe TBI, glutamate levels seem to peak within the first 24 hours post injury and slowly return to baseline, although there is variation between patients with some having low levels of glutamate throughout the entire studies, some peaking by 24 hours and returning to baseline, some having initially low levels but increasing over time, and other initially high and either remaining high or increasing over the course of the study (Chamoun et al., 2010). Patients that either maintained low levels of glutamate or peaked within 24 hours but returned to normal over the course of the study had better outcomes and lower mortality rates than patients with glutamate levels that either increased or

remained abnormally elevated over the course of the study (Chamoun et al., 2010). While this is interesting, given the heterogeneous nature of TBIs in the clinical population and the large trial inclusion window (up to 48 hours post TBI), these findings may not be easily generalizable. Microdialysis studies in rats found peak glutamate levels between 60 and 70 minutes following CCI injury (Folkersma et al., 2011), and around 90 minutes following FPI (Katayama, Becker, Tamura and Hovda, 1990).

Despite the increases in glutamate seen following TBI in microdialysis studies, studies utilizing magnetic resonance spectroscopy (MRS) show an overall decrease in glutamatergic levels following TBI, with an increase in glutamine (Guerriero et al., 2015). However, this discrepancy is believed to be due to sampling methods as microdialysis solely measures glutamate in extracellular space where as MRS studies can measure glutamate stored in vesicles as well as extracellular glutamate (Guerriero et al., 2015). This overabundance of glutamate in the synapse causes overstimulation of the glutamatergic receptors which trigger other harmful events that result in necrotic death, such as ion imbalance from extended depolarization, ATP depletion, and increases in intracellular levels of Ca^{2+} (Yin and Hazell, 2006). Taken together, these can cause edema, increase intracranial pressure, hernia, etc. and these complications can result in the death of the patient. (Yin and Hazell, 2006). Similarly, when glutamate is administered directly in the brain, initially neurons and dendrites swell (edema), followed by the shut down and deterioration of organelles, leading to the neuron becoming necrotic and finally being phagocytized (Choi, 1992).

Excess glutamate can be caused by various means. Glutamate can be released from cells following the formation of micropores in the membrane following trauma. Glutamate

is also able to permeate the brain following trauma-induced BBB disruption (Yi and Hazell, 2006). Some evidence suggests that specific subtypes of glutamate transporters are acutely down-regulated following trauma, though levels return to normal by 24 hours post injury (Yi and Hazell, 2006). In addition to this decrease in glutamate transporters, the normal Na+/K+ gradient is disturbed following TBI, diminishing the capacity of the glutamate transporters (Yi and Hazell, 2006). The overstimulation of cells caused by excessive glutamate causes a massive influx of calcium into the neuron (Weber, 2012). Excessive $Ca^{2+}$and the activation of Ca^{2+}-dependent enzymes from the over stimulation of glutamate receptors is believed to be a major contributor to excitotoxicity (Yi and Hazell, 2006). Proper CNS functioning is heavily dependent on Ca^{2+}signaling and homeostasis (Clapham, 1995; Weber, 2012). Specifically, Ca^{2+} is involved in neurotransmission (Wojda, Salinska, and Kuznicki, 2008), development, controlling gene expression, and synaptic plasticity (Wojda et al., 2008). In addition to its role in trauma, disrupted Ca^{2+} homeostasis is believed to be a component of various neurological and neurodegenerative disorders (Clapham, 1995; Wojda et al., 2008). Under normal conditions, intracellular levels of Ca^{2+} are maintained at approximately 100 nM whereas extracellular concentrations are maintained around the significantly higher concentration of 1-2 mM (Clapham, 1995; Weber, 2012). Following TBI, voltage gated calcium channels are activated and this, combined with Ca^{2+} leaking into cells through damaged membranes and the release of Ca^{2+} from intracellular stores, results in elevated intracellular Ca^{2+} levels (Weber, 2012; Wojda et al., 2008). This increase in intracellular Ca^{2+} activates various enzymes that trigger cascades that result in damage and death to the cells (Trump and Berezesky, 1995; Weber, 2012, Wojda et al., 2008). Excessive intracellular Ca^{2+} levels are also responsible for

activating calpains which can break down structural as well as enzymatic components of the cell (Wojda et al, 2008). One of the endoplasmic reticulum (ER)'s functions is to sequester intracellular calcium via Ca^{2+}-binding proteins (Clapham, 1995). The mitochondria can also store Ca^{2+}, but typically only when Ca^{2+} levels are pathologically high (Clapham, 1995). High levels of Ca^{2+} in the mitochondria can disrupt the transmembrane potential, which signals the release of cytochrome c, followed by apoptosis (Wojda et al., 2008).

Of the ionotropic glutamatergic receptors, N-methyl-D-aspartate (NMDA) receptors are believed to play the largest role in excitotoxicity as they have higher permeability to Ca^{2+} than the others (Yi and Hazell, 2006). Understandably, NMDA receptor antagonists have gone to clinical trials, but unfortunately none have yielded viable therapeutic options due to adverse side effects and small therapeutic windows for treatment (Narayan et al., 2002; Yi and Hazell, 2006). Blockage of the NMDA receptor outside this narrow therapeutic window can range from ineffective to damaging, as it can inhibit normal restorative processes (Narayan et al., 2002). In addition to the alteration in glutamatergic signaling, NMDA receptors themselves are effected following traumatic injury. One study found that, following CCI, protein levels (as measured by western blot) for the NR1, NR2A and NR2B were significantly decreased in the hippocampus compared to sham animals at six and 12 hours post injury, but these levels recovered by the 24-hour mark (Kumar, Zou, Yuan, Long, and Yang, 2002). Despite this, there was not a similar pattern found for mRNA expression of these subunits, indicating the change in NMDA subunit is not due to down regulated gene expression (Kumar et al., 2002). One possible explanation for the

discrepancy is that these subunits may be decreased due to enzymatic degradation from calpain (Kumar et al., 2002).

1.6 Medication Development

Primary injury can be prevented or minimized (e.g. use of helmets, enforcement of speed limits, educating public on dangers of alcohol abuse, etc.), but once a TBI has occurred, the primary injury is immediate and does not offer a therapeutic window for treatment (Park et al., 2008; McKee and Daneshvar, 2015). Current treatments for TBI patients focuses on preventing, minimizing, and/or correcting secondary injury responses (Park et al., 2008). Unfortunately, almost every medication that has made it to clinical trials has failed to consistently show efficacy (Xiong, Mahmood, and Chopp, 2009). The heterogeneous nature of TBIs may be responsible for the lack of effective treatments, despite promising pre-clinical data (McKee and Daneshvar, 2015; Morales et al., 2005; Schouten, 2007; Shultz et al., 2017). However, it is also important to consider that animal models of TBI may not be fully representative of the clinical TBI populations (Morales et al., 2005). A wide range of drugs have been tested in clinical trials including both competitive and non-competitive glutamate antagonists, steroids, free radical scavengers, growth factors, Ca^{2+} channel blockers, bradykinin antagonists, cannabinoid derivatives, and anti-convulsants, etc., and have shown inconsistent efficacy, no efficacy, or have even exacerbated the condition (Narayan et al., 2002; Xiong et al., 2015). While many of these treatments may truly fail to improve outcomes following TBIs, the design of most of the trials, a lack of standardization across trials, incorrect or inconsistent outcomes measures as well as a lack of solid preclinical data may have contributed to the failure of some of these compounds (Narayan et al., 2002).

1.7 Alcohol Use Disorder

Alcohol consumption is a risk factor for TBI as between 25% and 51% of patients with TBIs in the U.S. were intoxicated when the injury took place (Shandro et al., 2009). Alcohol use and Alcohol Use Disorder (AUD) continue to be major health care concerns in the United States. As of 2015, an estimated 15.1 million adults and over 600,000 adolescents (ages 12-17) in the U.S. had an AUD (National Survey on Drug Use Health, 2015). Alcohol related deaths are the third preventable cause of death in the U.S., with alcohol being implicated in 88,000 deaths annually (CDC, 2018), half of which are from binge drinking (Kanny, Naimi, Liu, Lu, and Brewer, 2018). Alcohol misuse costs the U.S. an estimated $249 billion annually (Sacks, Gonzales, Bouchery, Tomedi, and Brewer, 2015). The majority of this economic burden is related specifically to binge drinking (Sacks et al., 2015). While all age groups can engage in binge drinking, binge drinking is most commonly seen in individuals aged 18-34 years (Kanny et al., 2018). Binge drinking in young adulthood may be especially concerning as individuals that start drinking at an early age are at higher risk for developing AUD later in life (Higson, Heeren, and Winter, 2006).

1.8 Risk Factors for AUD

There are several risk factors for the development of AUD. While there is no single cause of AUD, as with many conditions, evidence supports the interaction of genetic and environmental factors (Dick, 2011; Hopfer, Crowley, and Hewitt, 2003). While the definition for AUD keeps evolving, one risk factor is displaying a risky pattern of drinking behavior (Oscar-Berman and Marinković, 2007). Patterns of risky drinking behaviors can involve episodes of binge drinking, chronic alcohol use, or both. Risky drinking in males

has been defined as more than fourteen drinks per week, or more than four drinks per setting, at least once a month. The definition for risky drinking behavior in women is similar to men, but at reduced quantities; specifically, ingestion of more than seven drinks per week or more than three drinks per day, at least once a month (Dawson, Grant, Stinson, and Zhou, 2005).

1.9 Ethanol Induced Damage

Effects of alcohol are variable based on the amount consumed, time since alcohol ingestion, pattern of use, as well as several individual factors, such as age, sex, genetics, family history of drinking, and socioeconomic background (Oscar-Berman and Marinković, 2007). These individual factors can help to explain why some individuals with AUD suffer severe, and sometimes permanent, impairments in motor, sensory and cognitive functions while others with AUD seem to lack any noticeable impairments (Oscar-Berman and Marinković, 2007). One hypothesis, the premature aging hypothesis, is that the damage induced by chronic ethanol consumption is comparable to the neuroanatomical changes seen in aging (reviewed in Oscar-Berman and Marinković, 2007). Generally, both chronic alcohol use and normal aging result in atrophy of the frontal lobes, enlargement of ventricles, as well as increased width of sulci (Oscar-Berman and Marinković, 2007; Sullivan and Pfefferbaum, 2013). Several imaging studies have shown additional abnormalities following chronic ethanol consumption including a reduction in volume of limbic structures (thalamus, mammillary bodies, anterior hippocampus), striatal structures (caudate and putamen), as well as the cerebellar hemispheres and the vermis in individuals with a history of chronic alcohol use (reviewed in Sullivan and Pfefferbaum, 2013). Not surprisingly, individuals with Wernicke-Korsakoff's Syndrome (WKS)

showed an even greater loss of volume than individuals with a history of chronic alcohol use without WKS (Sullivan and Pfefferbaum, 2009).

In addition to loss of volume in several important structures, studies utilizing diffusion tensor imaging (DTI) have shown individuals with a history of alcohol use show compromised integrity of the corpus callosum and of the centrum semiovale (Sullivan and Pfefferbaum, 2013). In fact, alcohol-induced damage to white matter structures is correlated with specific cognitive deficits seen in individuals with a history of chronic alcohol use. Specifically, higher diffusivity in the genu is correlated with decreased working memory and higher diffusivity in the splenium is correlated with impairments in a spatial reasoning task (Pfefferbaum, Adalsteinsson, and Sullivan, 2006). Mean diffusivity measures water diffusion in the brain tissue. Normally, cell barriers serve to restrict the diffusion of water, however, in many disease states as well as chronic alcohol use, these naturally occurring barriers can become compromised, resulting in higher diffusivity (Clark et al., 2011). In addition to corpus callosum white matter tracts, a studying using quantitative fiber tracking found that men with a history of chronic alcohol use show up to an 18% reduction in white matter tracts per volume between the midbrain and pons when compared to control men (Chanraud et al., 2009). This reduction is correlated with impaired performance on Trail-Making Test Part B, a task believed to measure cognitive flexibility (Kortte, Horner, and Windham, 2002; Chanraud et al., 2009). These changes in neuroanatomy following chronic alcohol use account for the neurological impairments seen in individuals with a history of alcohol use (Bernardin, Maheut-Bosser, and Paille, 2014; Chanraud et al., 2007). Although individuals with a history of chronic alcohol use show damage in various areas of the brain, the frontal lobes seem to be

especially vulnerable to ethanol induced damage and can show anatomical abnormalities (e.g. cerebral atrophy, and reduction in blood flow and glucose utilization) before cognitive and executive function becomes noticeably compromised (for review, see Moselhy, Georgiou, and Khan, 2001). Although some may not show noticeable cognitive deficit at least initially, other individuals with AUD can show significant impairment in executive functions. For example, one study showed that individuals meeting criteria for alcohol dependence (DSM-IV) showed impairments in the Trail Making Test Part B, the Wallace Adult Intelligence Scale-letter-number sequencing, and in the Wisconsin Card Sort Task. These tasks assess an individual's cognitive flexibility, working memory, and abstract thinking and ability to understand and alter responses based on feedback, respectively (Chanraud et al., 2007). However, they also found that these alcohol dependent individuals did not differ from control participants on the Letter Fluency Test, a measure of verbal fluency, or on The Stroop Color Word test, which assesses an individual's selective attention, ability to ignore interfering stimuli and the speed of cognitive processing (Chanraud et al., 2007). Another study also showed that heavier alcohol consumption was associated with poorer scores on the Dysexecutive Functioning Questionnaire, Trail Making Task (part A and B), the GoStop Task, and the Wisconsin Card Sort, but no difference in the Digit Span task or the Stroop Interference task (Houston et al., 2014).

In addition to these neuroanatomical deficits previously discussed, fMRI studies show that, even when individuals with a history of alcohol use perform a task without noticeable impairments from control participants, they display a different pattern of brain activity. In general, when given tasks, they use alternative pathways, more widespread areas, or both (reviewed in Sullivan and Pfefferbaum, 2013). These differing patterns of

activation support the inefficient processing hypothesis, which suggest that individuals with a history of chronic alcohol use have to activate and recruit more regions of their brain to perform a task the same as a control participant (Nixon, Tivis, Ceballos, Varner, and Rohrbaugh, 2002; Sullivan and Pfefferbaum, 2013).

A factor in the neurodegeneration caused in people with a history of alcohol dependence is the pattern of use followed by abstinence (Duka et al., 2004; Mello and Mendleson 1972). Additionally, the pattern of withdrawals leads to the neuroadaptations (Duka et al., 2004). Chronic use of alcohol causes increased expression of NMDA receptor as well as increasing the function of the NMDA receptor (Chandrasekar, 2013). Following chronic alcohol exposure, the glutamatergic system goes to a sensitized or hyperactive state and this can lead to some of the damage previously listed with this excitotoxic mechanism (Lovinger, 1993). Given that excitotoxicity is already a mechanism of secondary injury seen following traumatic brain injury (e.g. Bullock et al., Chamoun et al., 2010; Faden et al., 1989, Folkersma et al., 2011; Guerriero et al., 2015), it is possible that individuals with a history of this pattern of alcohol use followed with withdrawal may be sensitized to the effects of excitotoxicity.

1.10 Alcohol Use Prior to TBI

Alcohol is a major risk factor for injuries, including TBIs (Kolakowsky-Hayner et al., 1999; Shandro et al., 2009). While blood alcohol levels are not always collected for TBI patients, when these measures are taken, between 25% and 51% of TBI patients are intoxicated at the time of the injury (for reviews see Corrigan, 1995; Shandro et al., 2009). While this is quite a large range, studies examining blood alcohol concentration (BAC), have shown a bimodal distribution, with studies clustering around 35% and 50% of TBI

patients were intoxicated at the time of the injury (Corrigan, 1995). Part of the discrepancy could be due to the lack of testing for blood alcohol levels in all patients. There is evidence to suggest a bias in which patients are have their blood alcohol levels measured. Patients that are dead on the scene had their blood alcohol levels tested 93% of the time and patients that arrived at the hospital dead had their blood alcohol levels tested 99% of the time (Kraus, Morgenstern, Fife, Conroy, and Nourjah, 1989). However, among survivors of TBIs, they found a marked difference in prevalence of testing based on severity of injury. Patients suffering from severe TBIs (GCS of 8 or less) had their blood alcohol levels tested 61% of the time, patients with moderate TBIs (GCS 9-12) had their blood alcohol levels tested 54% of the time, but patients with mild TBIs (GCS 13-15) only had their blood alcohol levels tested 30% of the time (Kraus et al., 1989). The rates of intoxication reported for TBIs are also impacted by the BAC level used to define "intoxication" in these studies. Many studies examining the prevalence of alcohol intoxication at the time of TBI used a BAC of 0.1 or greater as their definition of intoxication (e.g. Gurney et al., 1992; Kraus et al., 1989; Lange, Iverson, and Franzen, 2007; Lin et al., 2014; Sparadeo and Gill, 1989). Many of these studies were prior to the federal initiative to lower the legal limit to 0.08 for illegal operation of a motor vehicle (National Highway Traffic Safety Administration, 2000) or use archival data from before this switch. However, impairments may be seen at much lower blood alcohol concentrations than 0.1 (National Highway Traffic Safety Administration, 2016; Shanin and Robertson, 2012), so the operational definitions of intoxication may muddy the results as well. Another potential confound is the time the blood is drawn for TBI patients in regards to time of injury (Shandro et al., 2009). While there are many factors impacting the absorption and metabolism of alcohol (for review see

Cederbaum, 2012), if blood alcohol levels are not taken at the time of the injury, readings will be artificially lowered, confounding the results of these studies.

Given the number of individuals that are intoxicated at the time of their injury, it is important to understand how alcohol impacts patient outcomes and mortality. First and foremost, it is vital to recognize that, regardless of the effects of alcohol on outcomes, intoxication is considered the predominant risk factor for sustaining a TBI (Kolakowsky-Hayner et al., 1999). There is no consensus on how alcohol consumption before injury or intoxication at the time of injury affects TBI prognosis. Several studies have found that alcohol intoxication at the time of injuries is associated with worse outcomes. Specifically, intoxication has been correlated with increased likelihood of severe computerized tomography (CT) scan lesions (Cunningham, Maio, Hill, and Zink, 2002) and more severely graded TBIs (Scheene et al., 2016), worse cognitive outcomes (Joseph et al., 2015; Mathias and Osborn, 2018), increased complications such as intubation, intracranial pressure monitoring, and surgical intervention (Gurney et al., 1992; Pandit et al., 2014; Shahin and Robertson, 2012), longer hospital stays, increased need for rehabilitation (Pandit et al., 2014), higher levels of disability (Mathias and Osborn, 2018) and increased mortality (Pandit et al., 2014; Ruff et al., 1990) compared to sober patients. Additionally, a study from India also found that TBI patients that used alcohol had TBIs that were more severe, had longer hospitalizations, and worse outcomes than patients that did not use alcohol (Gururaj, 2004). However, other studies have found there to be no difference between sober and intoxicated patient in terms of neuropsychological outcomes (e.g. Dikmen, Donovan, Løberg, Machamer, and Temkin, 1993; Lange et al., 2014; Lange, Iverson, and Franzen, 2008) no effect of intoxication on outcomes aside from very subtle

cognitive differences (Mathias and Osborn, 2018), and no effect on mortality in hospital, 90 days post injury, or one-year post injury (Shandro et al., 2009). Additionally, other studies have shown that patients who were intoxicated at the time of injury actually fared better than patients that were sober at the time of injury. Patients that were intoxicated at the time of injury have shown the same or better cognitive function (Lange et al., 2008), better outcomes two weeks following injury (Scheenen et al., 2016) and lower rates of mortality than sober TBI patients (Berry et al., 2011; Raj et al., 2015). A trend has also been shown that increasing BACs are correlated with improved survival, decreased likelihood of intensive care unit (ICU) admission and shorter ICU and hospital stays (Berry et al., 2011). Tien and colleagues (2006) conducted a retrospective study and found that individuals with moderate blood alcohol levels (operationally defined as a BAC between 0 and 230 mg/dL) had lower in-hospital mortality rates than individuals with a BAC of 0 at the time of injury. There was a non-statistically significant trend (p=.10) for patients with high BACs (BAC greater than 230 mg/dL) having a higher rate of in-hospital mortality than individuals with a BAC of 0 (Tien et al., 2006). There was also a trend for individuals with high BACs to die earlier in their hospital stays than individuals with a BAC of 0 (Tien et al., 2006).

While a few of these studies may seem to support the theory that acute alcohol intoxication may be neuroprotective at the time of TBI, the nature of these studies prevents conjectures about causality and some studies only looked at acute mortality or outcomes, which may not accurately represent the full picture. However, the speculation that low to moderate doses of alcohol at the time of injury may be neuroprotective via the inhibition of NMDA receptors and a subsequent dampening of the excitotoxicity following a TBI is

not without reason (Opreanu, Kuhn, and Basson, 2010). Preclinical trials of glutamate antagonists (e.g. Selfotel, Cerestat, CP 101-606) have shown efficacy in preclinical studies, but have not been shown effective in clinical trials (Narayan et al., 2002). There are many possible explanations for this discrepancy. Many of these trials suffered from design flaws, making it difficult to draw conclusions about the promise of the proposed mechanism of action (Narayan et al., 2002). However, one main difference between preclinical and clinical studies was the timeframe in which the drugs were administered. In preclinical trials, pharmaceutical intervention is typically given as a pretreatment or given soon after injury, but in clinical trials, medication is not able to be given until several hours after injury (Daiz-Arrastia et al., 2014; Mohamadpour, Whitney and Bergold, 2019; Narayan et al., 2002; Shohami and Biegon, 2014). Ideally, potential medications need to be effective at least 12 hours after injury (Mohamadpour et al., 2019). The gap between ideal and realistic therapeutic windows is not unique to glutamatergic agents and has plagued almost all medications (with various mechanisms of action) in TBI clinical trials, including Cyclosporine A (Sullivan, Sebastian, and Hall, 2011), progesterone (Skolnick et al., 2014; Wright et al., 2014), ziconotide and CDP-choline (Mohamadpour et al., 2019). In addition to the small therapeutic window for intervention, agents that inhibit glutamatergic signaling have additional complications.

Although there may be a benefit of NMDAr inhibition immediately following a TBI, the proposed benefit of alcohol in limiting excitotoxicity is subject to the same narrow therapeutic window NMDAr antagonists face (Shohami and Biegon, 2014). In fact, depending on the time since injury, it can be more beneficial to administer an NMDAr agonist (Biegon et al., 2004). After the initial, and fleeting, increase in glutatmatergic

activity following a TBI, there is a loss of NMDA receptors and those that remain are desensitized (e.g. Biegon et al., 2004; Grossman et al., 2003). In addition to not improving outcomes after TBIs, administration of NMDAr antagonist may even hinder the recovery process (Shohami and Biegon, 2014). Given the confusion surrounding the influence of alcohol on TBIs, the circumstances through which alcohol may improve or exacerbate TBI damage are important to understand.

The purpose of these studies is to characterize a model for studying the interaction of alcohol and TBIs in a hippocampal slice culture method. Using hippocampal slices allows understanding of the interaction of alcohol and TBIs and potential cellular mechanisms that may be beneficial in preventing or treating the secondary injury mechanisms. The purpose of experiment 1 is determine the effect of alcohol intoxication at the time of injury on cell death in hippocampal slice cultures. It is hypothesized that ethanol intoxication at the time of injury will result in more cell death compared to slices that are given a TBI in control media. However, the clinical data offers conflicting reports on the effects of alcohol intoxication and it is possible that ethanol intoxication at the time of injury may not have an effect or be neuroprotective compared to the TBI alone group. The purpose of experiment 2 is to determine how ethanol withdrawal at the time of injury will affect cell death in hippocampal slice cultures. It is hypothesized that ethanol withdrawal prior to a TBI will result in more cell death in the slices compared to the slices that receive a TBI in control media. The rationale is that the ethanol withdrawal will cause sensitization of the NMDA receptors at the time of the TBI and exacerbate the excitotoxicity seen following TBIs. The purpose of experiment 3 is to determine how a calpain inhibitor (MDL) prior to injury will affect cell death in hippocampal slice cultures. It is hypothesized that MDL will

reduce the amount of cell death in the group administered MDL prior to the TBI compared to the group that is administered the TBI in control media by inhibition of calpains.

CHAPTER 2. METHODS

2.1 Hippocampal Slice Culture

Eight-day old male and female Sprague Dawley pups (Harlan Laboratories; Indianapolis, IN) were humanely euthanized and their brains were harvested using aseptic technique. After removal, the brains were placed into cold dissecting media containing Minimum Essential Media (MEM; Invitrogen, Carlsbad, CA), (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*) (HEPES; Sigma, St. Louis, MO), streptomycin/penicillin (Invitrogen) and Amphotericin B solution (Sigma). The brains were cut midsagittally and the hippocampi removed. Any excess tissue was cleared from the hippocampi using a scalpel. Cleaned hippocampi were then chopped from front to back into slices 200 µm thick using a McIllwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Slices were then selected for culturing. Slices were only selected for culturing if they had all three of the major regions of the hippocampus intact (the CA1, the CA3, and the dentate gyrus (DG)). These slices were then plated onto biopore membrane inserts with three to four slices per membrane. Slices were plated in such a way to ensure enough space to prevent overlapping of the slices. Any excess media surrounding the slice was drawn up with a pipette to keep the slice from drowning. These inserts were then placed into six-well plates that contained approximately 1milliliter of culture media in the bottom. Slices were incubated for 5 days prior to any treatment to allow them to adhere to the membrane surface and to allow for damage from dissection to heal. Following the treatment(s), slices were imaged for cytotoxicity using Propidium Iodide (PI) stain. Slices were then fixed with a 10% formalin solution.

2.2 Experiment I: Ethanol Intoxication Prior to TBI

After 5-days in vitro, inserts containing the hippocampal slice cultures were transferred to new six-well plates. Cultures were randomly assigned to control or ethanol media (50mM, approximately equivalent to a blood alcohol level of .23). Plates with control media were placed into a Tupperware container with 50mL of distilled water and then placed into a Ziploc bag, filled with 5% CO_2 and 95% air, and then sealed. Slices treated with ethanol media were similarly placed in a Tupperware container with 50 mL of

50mM ethanol, placed in a Ziploc bag filled with 5% CO_2 and 95% air, and then sealed. Cultures were then placed in the incubator for an additional five days. At 10 days in vitro, all tissue was placed into new six-well plates with either control or ethanol media (50mM) media containing 7.48 µM PI. TBIs were administered to one-half of the cultures in the control media and in the ethanol media. Tissue receiving a TBI was placed under a stereoscope for easier identification of the CA1 region. TBI administration was done via a single cut into the CA1 region of the hippocampus with No. 15 blade scalpel. If the cut did not hit the CA1, if the cut hits the CA3 or dentate gyrus, or if multiple cuts were made into the tissue, that slice was not included in the analyses. Following the TBI procedure, tissue was placed in Tupperware containers with either 50mL of distilled water or 50mM ethanol, placed in Ziploc bags and filled with 5% CO_2 and 95% air, then sealed before the tissue was returned to the incubator. Tissue was imaged at 24, 48, and 72 hours post TBI. Following imaging at the 72-hour time point, tissue was fixed with a 10% formalin solution

2.3 Experiment II: Ethanol Withdrawal Prior to TBI

After 5-days in vitro, inserts containing the hippocampal slice cultures were transferred to new six-well plates. Cultures were randomly be assigned to control or ethanol media (50mM, approximately equivalent to a blood alcohol level of .23). Plates with control media were then placed into a Tupperware container with 50mL of distilled water and placed into a Ziploc bag, filled with 5% CO_2 and 95% air, and then sealed. Slices treated with ethanol media were similarly placed in a Tupperware container with 50 mL of 50mM ethanol, placed in a Ziploc bag filled with 5% CO_2 and 95% air, and then sealed. Cultures were then placed in the incubator for an additional five days. At 10 days in vitro, all tissue was placed into new six-well plates with control media containing 7.48 µM PI. Tissue was then returned to the incubator for an additional 24 hours to induce ethanol withdrawal. After 24-hour withdrawal, TBIs were administered to one-half of the cultures in the control media and in the ethanol media. Tissue that received a TBI was placed under

a stereoscope for easier identification of the CA1 region. TBI administration was done via a single cut into the CA1 region of the hippocampus with No. 15 blade scalpel. If the cut did not hit the CA1, if the cut hit the CA3 or dentate gyrus, or if multiple cuts were made into the tissue, that slice was not included in the analysis. Following the TBI procedure, tissue was placed in the incubator. Tissue was imaged at 24, 48, and 72 hours post TBI. Following imaging at the 72-hour time point, tissue was fixed with a 10% formalin solution.

2.4 Experiment III: Influence of Calpain Inhibition on TBI Induced Cell Death

After 5-days in vitro, inserts containing the hippocampal slice cultures were transferred to new six-well plates. Cultures were randomly assigned to control or TBIs. Plates were placed into a Tupperware container with 50mL of distilled water and then placed into a Ziploc bag, filled with 5% CO_2 and 95% air, and then sealed. Cultures were then placed in the incubator for an additional five days. At 10 days in vitro, all tissue was placed into new six-well plates with control media and returned to the incubator for 24 hours to account for lack of withdrawal time. Then, half of the tissue in the Control and half of the tissue in the TBI group were place in either control media containing 7.48 µM PI or media containing 7.48 µM PI and MDL-28170 (25 µM) and returned to the incubator for two hours. Following this two-hour incubation with MDL-28170, TBIs were administered. Tissue receiving a TBI was placed under a stereoscope for easier identification of the CA1 region. TBI administration was done via a single cut into the CA1 region of the hippocampus with No. 15 blade scalpel. If the cut did not hit the CA1, if the cut hit the CA3 or dentate gyrus, or if multiple cuts were made into the tissue, that slice was not included in the analysis. Following the TBI procedure, tissue was placed in the incubator. Tissue was imaged at 24, 48, and 72 hours post TBI. Following imaging at the 72-hour time point, tissue was fixed with a 10% formalin solution.

2.5 Staining of Cultures with Propidium Iodide

During final treatments of tissue, Propidium Iodide (PI) is added to culture media at 7.48µM concentration. PI is a highly polar fluorescent compound (Zimmer, Kristensen, Jakosen, and Noraberg, 2000) which used as a marker of cell membrane integrity and indirectly as a measure of cell death (Cummings, Willis, and Schnellmann, 2004) and correlates with other measures of cell death (Wilkins et al., 2006). As PI is a polar compound, it can only enter cells that have a compromised cell membrane (dead or dying cells), it then binds to the cell's nucleic acids (DNA) and gives off a fluorescent signal (Macklis and Madison, 1990). Cell cultures were then be imaged using a Leica DMIRB microscope (W. Nuhsbahm Inc.; McHenry, IL, USA) capable of detecting fluorescence (via mercury lamp) under a 5x objective lens. The microscope is connected to a computer with SPOT advanced software for Windows (version 4.0.2) through a SPOT 7.2 color mosaic camera (W. Nuhsburg). The optical density of these fluorescent images was then quantified using Image J software (National Institutes of Health, Bethesda, MD). For optical density, three regions of the hippocampus were assessed- the CA1, CA3, and the dentate gyrus. Prior to assessment of these areas of interest, a measure of background optical density was taken for each slice. This background measurement was subtracted from each measure of optical density for that slice prior to analysis. To account for potential variability, all optical density measurements were normalized to a percentage of control, using the formula (S-B)/C where S is the intensity of a region of a given slice, B is the background value from that image, and C is the mean fluorescent value for the control slices (see Mulholland et al., 2005). Image J software was also be used to quantify the area of trauma in the CA1 in slices that are subjected to TBI treatments. Following the final

imaging, the slices were preserved by fixing in a 10% formalin solution then stored at 4 degrees Celsius.

2.6 Statistical Analysis

Statistical analyses were conducted to determine the effect of treatment (e.g. Control, Ethanol, TBI, etc.) on severity of cell death in specific regions of the hippocampus. Cultures were also assessed for sex effects. Data were assessed in SPSS (version 24) using a Two Factor ANOVA (Treatment x Sex), with significance level set at $p \leq 0.05$ and, if appropriate, post hoc analyses were conducted using Fisher's Least Significant Difference (LSD).

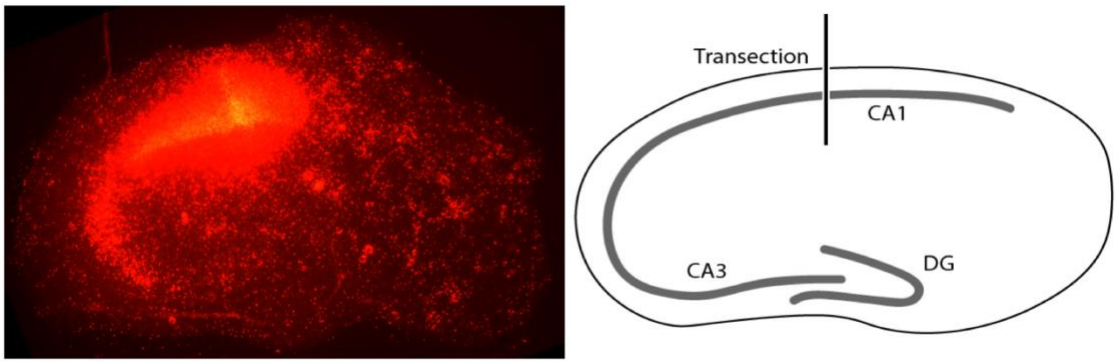


Figure 2.1 Transection Model. Left: Representative image of transection model of traumatic brain injury in hippocampal slice culture. A single cut was administered with a No. 15 scalpel near the midpoint of the CA1 of the hippocampus. Right: Simplified drawing of transection model of TBI for clarity.

CHAPTER 3. RESULTS

3.1 Experiment I: Ethanol Intoxication Prior to TBI

3.1.1 Effect of Treatment

3.1.1.1 24 Hour

There was a main effect of treatment in the CA1 region at 24 hours, $F(3,170) = 32.63$, $p < .001$ (Figure 3.1.1). Both the TBI ($M = 206.99$, $SE = 10.54$) and EtOH+TBI ($M = 194.84$, $SE = 10.41$) groups showed more cell death compared to slices in the control condition ($M = 100.00$, $SE = 9.72$), $p < .001$. However, there was no difference in cell death between slices in the EtOH treated group ($M = 102.02$, $SE = 9.824$) and slices in the control condition ($M = 100.00$, $SE = 9.72$), $p = .884$. There was no difference in cell death between slices that were kept in ethanol prior to the TBI (EtOH+TBI) compared with slices that were kept in control media prior to the TBI, $p = .413$. There was no effect of treatment on the CA3 (Figure 3.1.2) or the DG (Figure 3.1.3) regions of the hippocampal slices at the 24-hour time point, $F(3,170) = 0.93$, $p = .427$ and $F(3,170) = 0.716$, $p = .544$, respectively.

3.1.1.2 48 Hour

There was an effect of treatment in the CA1 region at the 48-hour time point, $F(3,170) = 29.554$, $p < .001$ (Figure 3.2.1). Both the TBI ($M = 175.63$, $SE = 7.79$) and EtOH+TBI ($M = 166.12$, $SE = 7.70$) groups showed more cell death compared to slices in the control condition ($M = 100.00$, $SE = 7.19$), $p < .001$. There was no difference between slices in the EtOH groups ($M = 101.24$, $SE = 7.26$) and slices in the control condition ($M = 100.00$, $SE = 7.19$), $p = .903$. There was no difference in cell death between slices in the

EtOH+TBI group and the TBI group, $p = .386$. There was no effect of treatment in the CA3 (Figure 3.2.2) or DG (Figure 3.2.3) regions, $F(3, 170) = 1.50$, $p = .217$ and $F(3,170) = 0.95$, $p = .418$.

3.1.1.3 72 Hour

There was an effect of treatment in the CA1 region at the 72-hour time point, $F(3,170) = 21.64$, $p < .001$ (Figure 3.3.1). Both the TBI ($M = 161.10$, $SE = 6.80$) and EtOH+TBI ($M = 147.15$, $SE = 6.71$) groups had more cell death compared with slices in the control condition ($M = 100.00$, $SE = 6.27$), $p < .001$. There was no difference between slices in the EtOH group ($M = 104.84$, $SE = 6.34$) and slices in the control group ($M = 100.00$, $SE = 6.27$), $p = .588$. There was no difference between slices in the TBI condition and slices in the EtOH+TBI condition, $p = .146$. There was no effect of treatment in the CA3 (Figure 3.3.2) or DG (Figure 3.3.3) regions of the hippocampal slices, $F(3,170) = 0.81$, $p = .490$ and $F(3,170) = 0.35$, $p = .791$, respectively.

3.1.2 Effect of Sex

3.1.2.1 24 Hour

There was not a sex difference in the CA1, $F(1,170) = 0.19$, $p = .661$ (Figure 3.1.1), the CA3, $F(1,170) = 1.30$, $p = .255$ (Figure 3.1.2), or the DG, $F(1,170) = 0.45$, $p = .504$ (Figure 3.1.3).

3.1.2.2 48 Hour

There was not a sex difference in the CA1, $F(1,170) = 2.55$, $p = .112$ (Figure 3.2.1). However, there was a sex difference in the CA3 (Figure 3.2.2) and DG (Figure 3.2.3) regions of the hippocampus, $F(1,170) = 4.07$, $p = .045$ and $F(1,170) = 3.95$, $p = .049$, respectively. In the CA3, female slices ($M = 112.87$, $SE = 6.53$) had more cell death on average compared to male slices ($M = 94.25$, $SE = 6.52$). The same pattern was seen in the DG, with the female slices ($M = 114.67$, SE 6.00) had more cell death on average compared with the male slices ($M = 97.84$, $SE = 5.99$).

3.1.2.3 72 Hour

There was not a sex difference in the CA1, $F(1,170) = 0.08$, $p = .774$ (Figure 3.3.1), the CA3, $F(1,170) = 0.82$, $p = .366$ (Figure 3.3.2), or the DG, $F(1,170) = 0.15$, $p = .699$ (Figure 3.3.3).

3.2 Experiment II: Ethanol Withdrawal Prior to TBI

3.2.1 Effect of Treatment

3.2.1.1 24 Hour

There was an effect of treatment in the CA1 at the 24-hour time point, $F(3,338) = 95.07$, $p < .001$ (Figure 3.4.1). The EWD ($M = 132.12$, $SE = 7.42$), TBI ($M = 236.57$, $SE = 7.77$), and EWD+TBI ($M = 252.47$, $SE = 7.77$) all had more cell death compared with the control group ($M = 100.00$, $SE = 7.86$), $p = .003$, $p < .001$, and $p < .001$, respectively. There was no difference between slices in the TBI condition and slices in the EWD+TBI condition, $p = .149$. Similarly, in the CA3, there was an effect of treatment, $F(3,338) =$

4.46, $p = .004$ (Figure 3.4.2). The EWD ($M = 128.50$, $SE = 7.19$), TBI ($M = 129.76$, $SE = 7.52$), and EWD+TBI ($M = 135.78$, $SE = 7.52$) all had more cell death compared with the control group ($M = 100.00$, $SE = 7.61$), $p = .007$, $p = .006$, and $p = .001$, respectively. There was no difference between slices in the TBI and EWD+TBI groups, $p = .571$. In the DG, the same pattern was seen. There was a main effect of treatment, $F(3,338) = 4.42$, $p = .005$ (Figure 3.4.3). The EWD ($M = 127.75$, $SE = 7.87$), TBI ($M = 135.39$, $SE = 8.23$), and EWD+TBI ($M = 138.12$, $SE = 8.23$) all had more cell death compared with the control group ($M = 100.00$, $SE = 8.33$), $p = .016$, $p = .003$, and $p = .001$, respectively. There was no difference between the TBI and the EWD+TBI conditions, $p = .815$.

3.2.1.2 48 Hour

There was an effect of treatment in the CA1 at the 48-hour time point, $F(3,338) = 49.28$, $p < .001$ (Figure 3.5.1). The EWD ($M = 129.75$, $SE = 6.462$), TBI ($M = 196.17$, $SE = 6.77$), and EWD+TBI ($M = 192.61$, $SE = 6.76$) groups all had more cell death compared with the slices in the control group ($M = 100.00$, $SE = 6.84$), $p = .002$, $p < .001$, and $p < .001$, respectively. There was no difference between the TBI and EWD+TBI group, $p = .710$. Similarly, in the CA3, there was an effect of treatment, $F(3,338) = 4.60$, $p = .004$ (Figure 3.5.2). The EWD ($M = 135.89$, $SE = 9.162$), TBI ($M = 139.90$, $SE = 9.59$), and EWD+TBI ($M = 145.95$, $SE = 9.59$) groups all had more cell death compared with the slices in the control group ($M = 100.00$, $SE = 9.70$), $p = .008$, $p = .004$, and $p = .001$, respectively. There was no effect of treatment in the DG at the 48-hour time point, $F(3,338) = 2.23$, $p = .084$ (Figure 3.5.3).

3.2.1.3 72 Hour

The was an effect of treatment in the CA1 at 72 hours, $F(3,338) = 15.92$, $p < .001$ (Figure 3.6.1). Both the TBI ($M = 129.51$, $SE = 4.97$) and EWD+TBI ($M = 144.60$, $SE = 4.97$) had more cell death than the control condition ($M = 100.00$, $SE = 5.03$), $p < .001$. There was no difference between the EWD group ($M = 110.61$, $SE = 4.75$) compared with the control group ($M = 100.00$, $SE = 5.03$), $p = .126$. Additionally, there was significantly more cell death in the EWD+TBI group compared with the TBI group, $p = .032$. In the CA3, there was an effect of treatment, $F(3,338) = 2.92$, $p = .034$ (Figure 3.6.2). The EWD+TBI group ($M = 126.01$, $SE = 8.11$) had more cell death than the control condition ($M = 100.00$, $SE = 8.21$), $p = .025$ and more cell death than the TBI alone condition, $p = .025$. Neither the EWD condition ($M = 121.50$, $SE = 7.76$) or the TBI condition ($M = 100.12$, $SE = 8.12$) differed from the control condition ($M = 100.00$, $SE = 8.21$), $p = .058$ and $p = .992$, respectively. In the DG, there was an effect of treatment, $F(3,338) = 6.12$, $p < .001$ (Figure 3.6.3). Both the EWD ($M = 135.61$, $SE = 7.44$) and EWD+TBI ($M = 126.66$, $SE = 7.78$) conditions had more cell death compared to the control condition ($M = 100.00$, $SE = 7.88$), $p = .001$ and $p = .017$, respectively. The TBI group ($M = 97.87$, $SE = 7.78$) did not differ from the control condition ($M = 100.00$, $SE = 7.88$), $p = .848$. Additionally, the EWD+TBI condition had more cell death compared to the TBI alone condition, $p = .009$.

3.2.2 Effect of Sex

3.2.2.1 24 Hour

There was an effect of sex in the CA1 (Figure 3.4.1) and CA3 (Figure 3.4.2) regions of the hippocampus, $F(1,338) = 7.88$, $p = .005$ and $F(1,338) = 5.57$, $p = .019$, respectively.

In the CA1, the female slices (M = 191.10, SE = 5.48) showed more cell death on average compared with the male slices (M = 169.47, SE = 5.42). Similarly, in the CA3, the female slices (M = 132.31, SE = 5.31) had more cell death than the male slices (M = 114.70, SE = 5.25). In the DG, there was no effect of sex, $F(1,338) = 2.37$, $p = .124$ (Figure 3.4.3).

3.2.2.2 48 Hour

There was an effect of sex in the CA1 region at the 48-hour time point, $F(1,338) = 6.95$, $p = .009$ (Figure 3.5.1). The female slices (M = 163.48, SE = 4.77) had more cell death on average compared with male slices (M = 145.79, SE = 4.72). There was no effect of sex in the CA3 (Figure 3.5.2) or DG (Figure 3.5.3) regions at the 48-hour time point, $F(1,338) = 0.102$, $p = .749$ and $F(1,338) = 0.18$, $p = .674$.

3.2.2.3 72 Hour

There was no effect of sex at 72 hours in the CA1, $F(1,338) = 2.96$, $p = .086$ (Figure 3.6.1), CA3, $F(1,338) = 0.02$, $p = .890$ (Figure 3.6.2), or the DG, $F(1,338) = 2.05$, $p = .153$ (Figure 3.6.3).

3.3 Experiment III: Influence of Calpain Inhibition on TBI Induced Cell Death

3.3.1 Effect of Treatment

3.3.1.1 24 Hours

There was an effect of treatment in the CA1 at 24 hours, $F(3,181) = 45.28$, $p < .001$ (Figure 3.7.1). Both the TBI (M = 202.12, SE = 8.14) and MDL+TBI (M = 184.87, SE = 8.34) conditions had more cell death than the control condition (M = 100.00, SE = 8.24), $p < .001$. The MDL group (M = 97.51, SE = 8.14) was not different from the control

condition (M = 100.00, SE = 8.24), p = .830. There was no difference between the TBI and MDL+TBI conditions, p = .141. In the CA3, there was an effect of treatment, $F(3,181)$ = 2.78, p = .043 (Figure 3.7.2). The TBI group (M = 129.76, SE = 8.26) had more cell death than the control condition (M = 100.00, SE = 8.36), p = .012. There was no difference between either the MDL (M = 106.68, SE = 8.26) or MDL+TBI (M = 123.12, SE = 8.46) compared to the control condition (M = 100.00, SE = 8.36), p = .571 and p = .054, respectively. There was no effect of treatment in the DG at 24 hours, $F(3,181)$ = 2.63, p = .052 (Figure 3.7.3).

3.3.1.2 48 Hours

There was an effect of treatment in the CA1 at 48 hours, $F(3,181)$ = 37.81, p < .001 (Figure 3.8.1). Both the TBI (M = 173.30, SE = 6.72) and MDL+TBI (M = 166.52, SE = 6.88) conditions had more cell death compared to the control condition (M = 100.00, SE = 6.79), p < .001. There was no difference between the MDL condition (M = 96.12, SE = 6.72) and the control condition (M = 100.00, SE = 6.79), p = .685. There was no difference between the TBI and MDL+TBI groups, p = .481. In the CA3, there was an effect of treatment, $F(3,181)$ = 3.12, p = .028 (Figure 3.8.2). The TBI group (M = 128.80, SE = 7.65) had more cell death than the control condition (M = 100.00, SE = 7.73), p = .009. Neither the MDL (M = 104.00, SE = 7.65) or the MDL+TBI group (M = 120.32, SE = 7.83) differed from the control condition (M = 100.00, SE = 7.73), p = .713 and p = .067, respectively. There was no difference between the TBI and MDL+TBI groups, p = .440. There was an effect of treatment in the DG at 48 hours, $F(3,181)$ = 3.17, p = .026 (Figure 3.8.3). The TBI condition (M = 129.30, SE = 6.69) had more cell death than the control condition (M = 100.00, SE = 6.76), p = .002. Neither the MDL (M = 115.02, SE = 6.69) or

MDL+TBI (M = 115.95, SE =6.85) differed from the control condition (M = 100.00, SE = 6.76), p =.116 and p = .099, respectively. There was no difference between the TBI and MDL+TBI groups, p = .165.

3.3.1.3 72 Hours

There was an effect of treatment in the CA1 at 72 hours, $F(3,181) = 34.03$, $p < .001$ (Figure 3.9.1). Both the TBI (M = 169.50, SE = 6.41) and MDL+TBI (M = 172.42, SE = 6.57) groups had more cell death than the control condition (M = 100.00, SE = 6.49), $p <$ .001. There was no difference between the MDL (M = 112.25, SE = 6.41) and control conditions (M = 100.00, SE = 6.49), p = .181. There was no difference between the TBI group and the MDL+TBI group, p = .750. In the CA3, there was an effect of treatment, $F(3,181) = 5.09$, p = .002 (Figure 3.9.2). The MDL (M = 125.57, SE = 7.30), TBI (M = 127.20, SE = 7.30), and the MDL+TBI (M = 139.91, SE = 7.48) groups all had more cell death than the control group (M = 100.00, SE = 7.39), p = .015, p =.010, and $p < .001$, respectively. There was no difference between the TBI and the MDL+TBI groups, p = .225. In the DG, there was an effect of treatment, $F(3,181) = 9.014$, $p < .001$ (Figure 3.9.3). The MDL (M = 134.61, SE = 5.81), TBI (M = 137.36, SE = 5.81), and the MDL+TBI (M = 133.21, SE = 5.95) groups all had more cell death than the control group (M = 100.00, SE = 5.88), $p < .001$. There was no difference between the TBI and MDL+TBI conditions, p = .618.

3.3.2 Effect of Sex

3.3.2.1 24 Hours

There was no effect of sex at 24 hours in the CA1, $F(1,181) = 2.68$, $p = .103$ (Figure 3.7.1), the CA3, $F(1,181) = 0.58$ (Figure 3.7.2), $p = .446$, or in the DG, $F(1,181) = 1.97$, $p = .162$ (Figure 3.7.3).

3.3.2.2 48 Hours

There was no effect of sex in the CA1 at 48 hours, $F(1,181) = 1.94$, $p = .165$ (Figure 3.8.1) or in the CA3, $F(1,181) = 2.91$, $p = .090$ (Figure 3.8.2). There was an effect of sex in the DG, $F(1,181) = 11.28$, $p = .001$ (Figure 3.8.3). Female slices ($M = 103.74$, $SE = 4.68$) had less cell death compared to male slices ($M = 126.39$, $SE = 4.86$).

3.3.2.3 72 Hours

There was no effect of sex in the CA1, $F(1,181) = 1.81$, $p = .180$ (Figure 3.9.1) or in the CA3, $F(1,181) = 1.60$, $p = .208$ (Figure 3.9.2) at 72 hours. There was an effect of sex in the DG at 72 hours, $F(1,181) = 4.74$, $p = .031$ (Figure 3.9.3). Male slices ($M = 132.68$, $SE = 4.23$) had more cell death than female slices ($M = 119.91$, $SE = 4.07$).

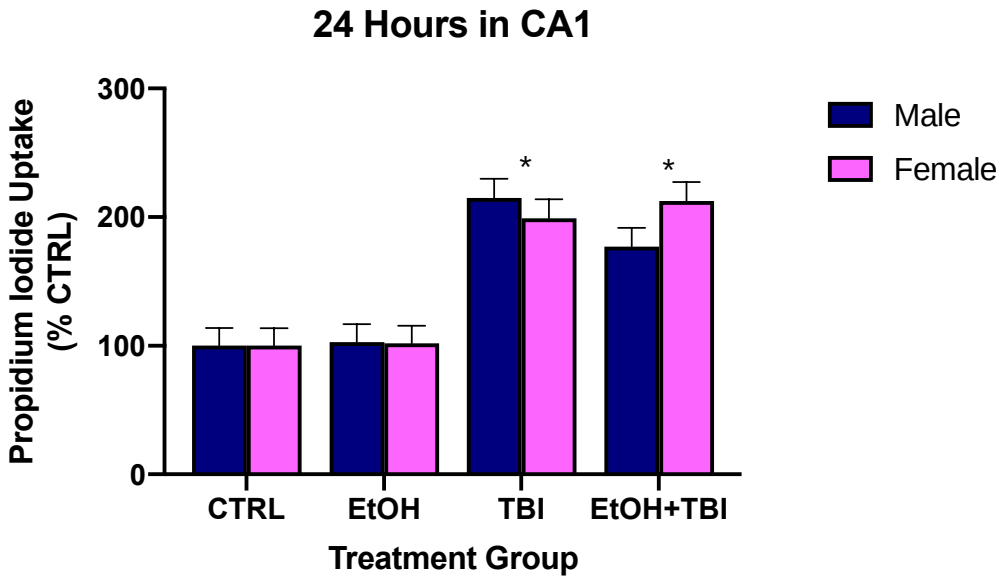


Figure 3.1.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 24 hours following treatment. There was no effect of EtOH treatment (five days of 50mM ethanol) compared to slices in the CTRL condition. Both the TBI and EtOH+TBI groups showed an increase uptake of PI in the CA1 at 24 hours, compared to the CTRL group, $p < .001$. There was no effect of sex, $p = .661$. Error bars represent standard error.

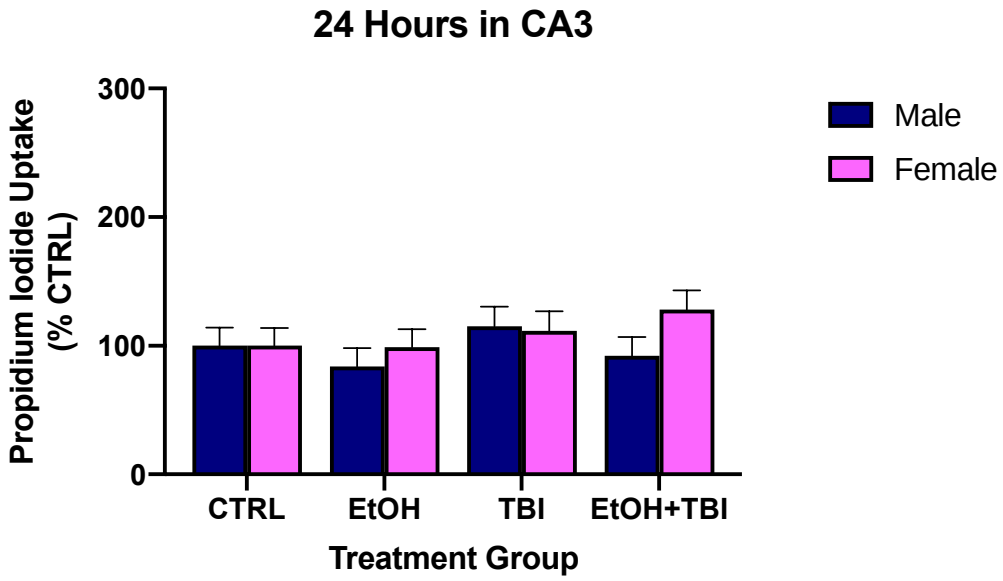


Figure 3.1.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 24 hours following treatment. There was no effect of treatment, p = .427 or an effect of sex, p = .255. Error bars represent standard error.

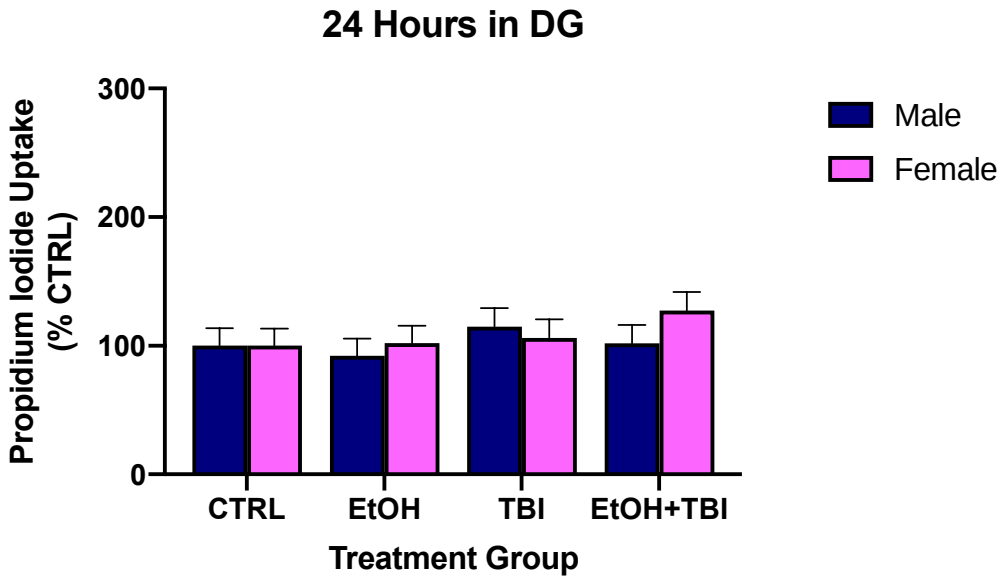


Figure 3.1.3: Effects of treatment on propidium iodide (PI) uptake in the DG region of hippocampal slices, 24 hours following treatment. There was no effect of treatment, p = .544 or an effect of sex, p = .504. Error bars represent standard error.

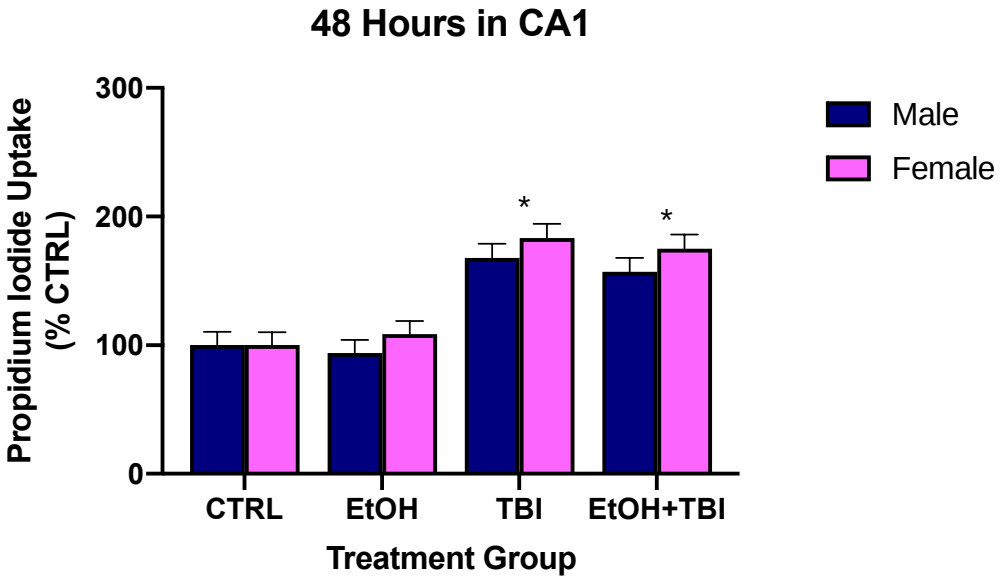


Figure 3.2.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 48 hours following treatment. There was no effect of EtOH treatment (five days of 50mM ethanol) compared to slices in the CTRL condition. Both the TBI and EtOH+TBI groups showed an increase uptake of PI in the CA1 at 48 hours, compared to the CTRL group, $p < .001$. There was no effect of sex, $p = .112$.

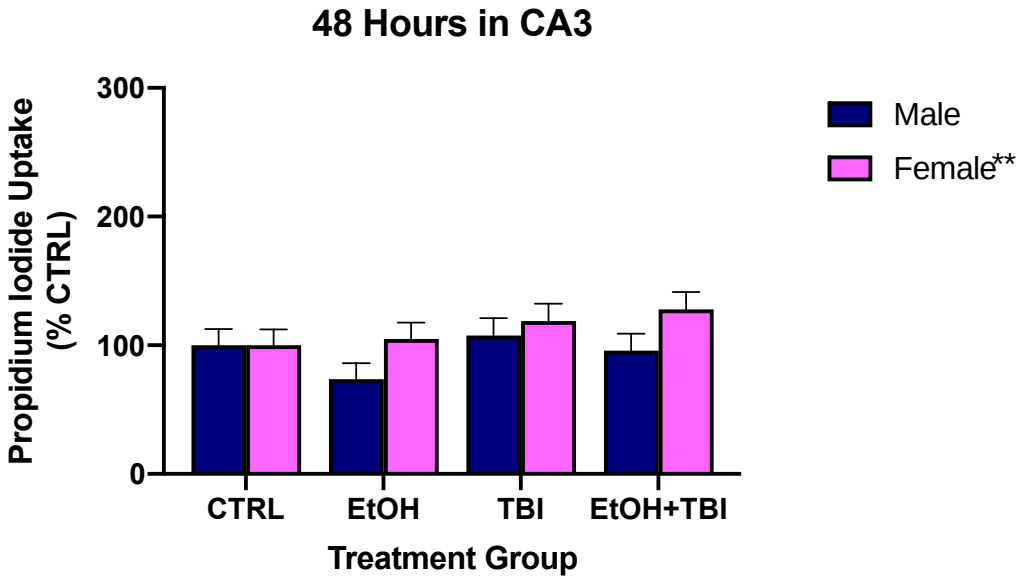


Figure 3.2.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 48 hours following treatment. There was no effect of treatment, p = .22. There was an effect of sex (**), p = .045. At 48 hours, the CA3 region of female slices took up more PI than the CA3 region of male slices. Error bars represent standard error.

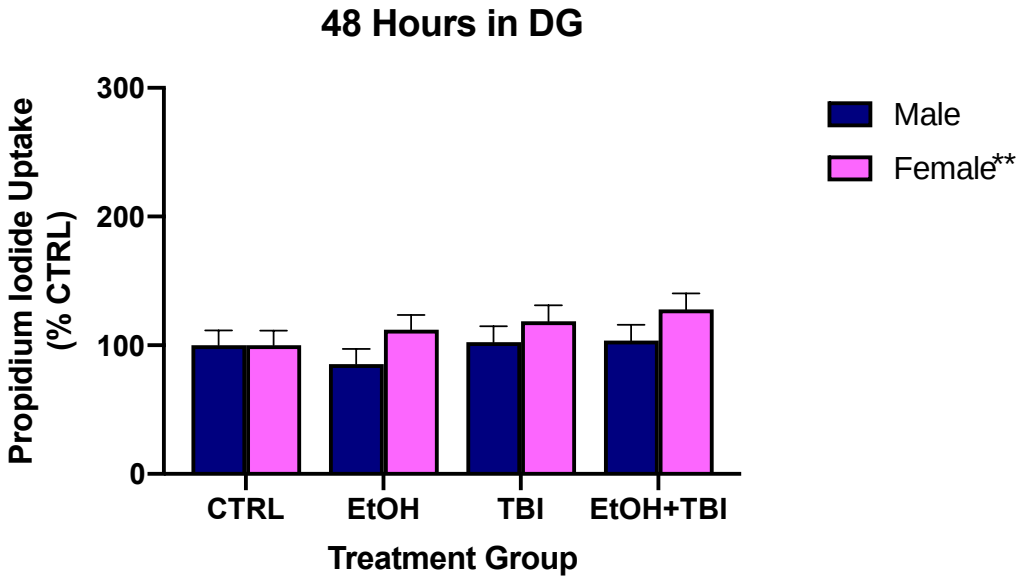


Figure 3.2.3: Effects of treatment on propidium iodide (PI) uptake in the DG region of hippocampal slices, 48 hours following treatment. There was no effect of treatment, p = .418. There was an effect of sex (**), p = .049. At 48 hours, the DG region of female slices took up more PI than the DG region of male slices. Error bars represent standard error.

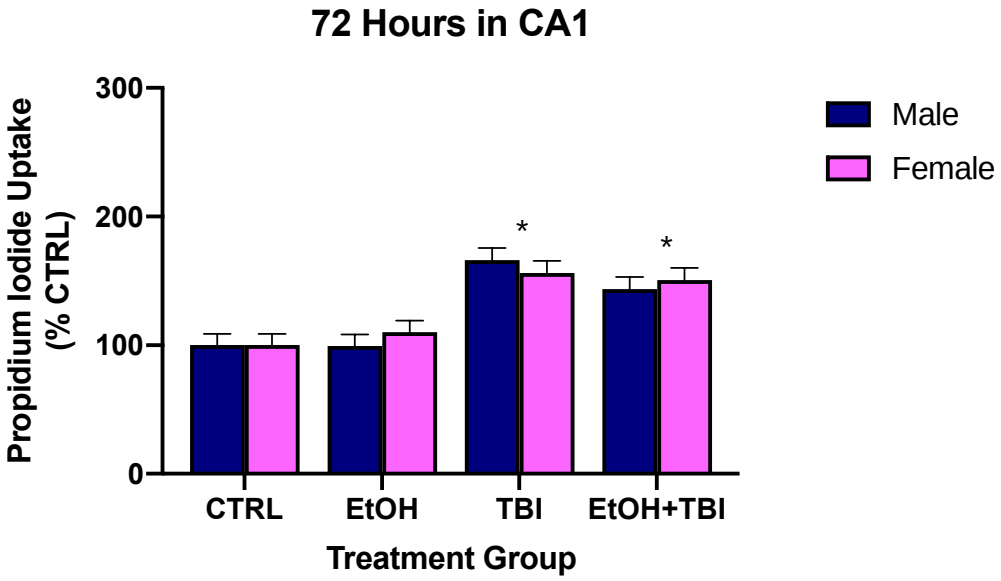


Figure 3.3.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 72 hours following treatment. There was no effect of EtOH treatment (five days of 50mM ethanol) compared to slices in the CTRL condition. Both the TBI and EtOH+TBI groups showed an increase uptake of PI in the CA1 at 72 hours, compared to the CTRL group, $p < .001$. There was no effect of sex, $p = .774$. Error bars represent standard error.

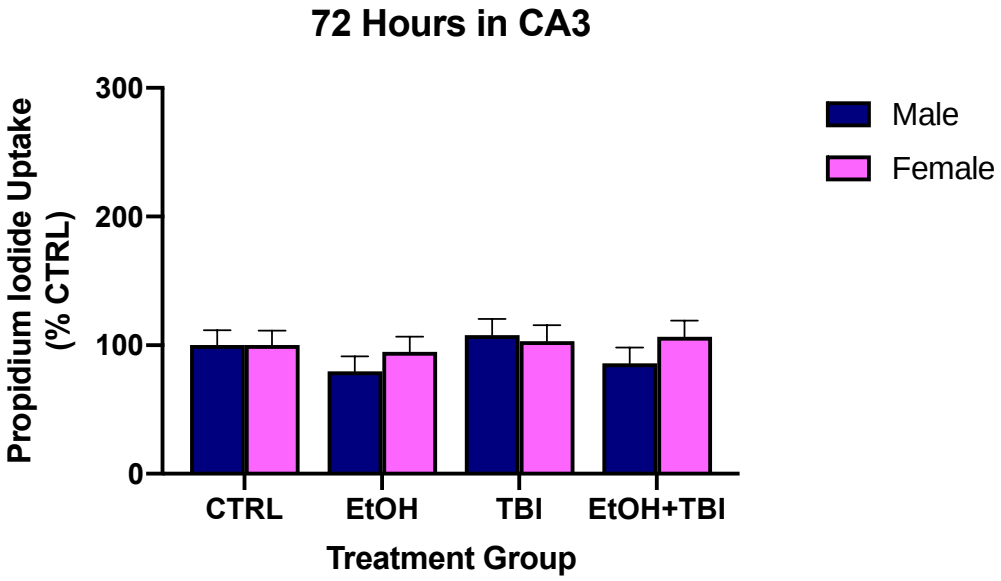


Figure 3.3.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 48 hours following treatment. There was no effect of treatment, p = .490 or an effect of sex, p = .366.

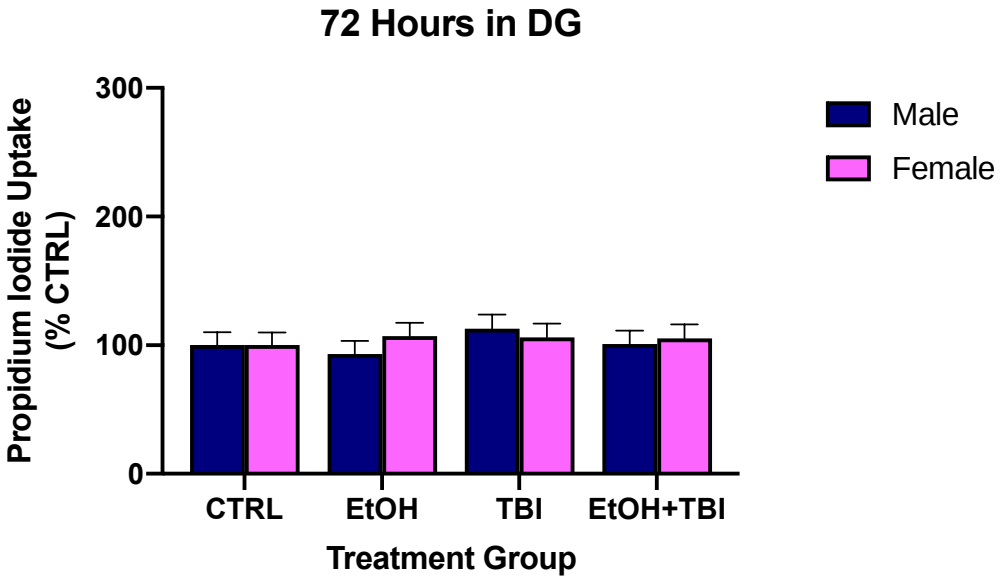


Figure 3.3.3: Effects of treatment on propidium iodide (PI) uptake in the DG region of hippocampal slices, 72 hours following treatment. There was no effect of treatment, p = .791 or an effect of sex, p = .699. Error bars represent standard error.

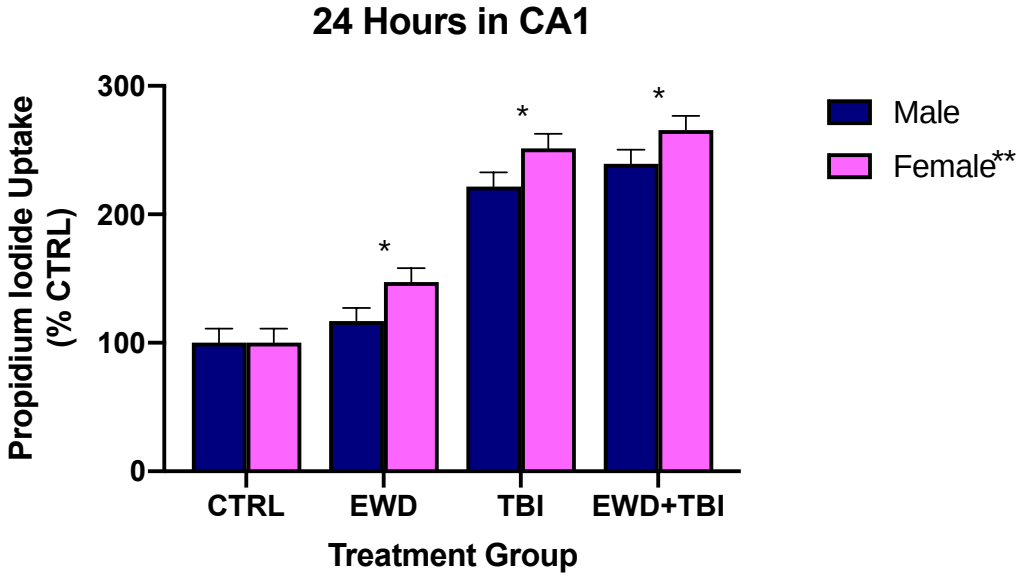


Figure 3.4.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 24 hours following treatment. All treatment conditions resulted in more uptake of PI compared to the CTRL group. EWD vs. CTRL, $p = .003$; TBI vs. CTRL, $p < .001$; and EWD+TBI vs. CTRL, $p < .001$. There was an effect of sex, $p = .005$. At 24 hours, the CA1 region of female slices took up more PI than the CA1 region of male slices. Error bars represent standard error.

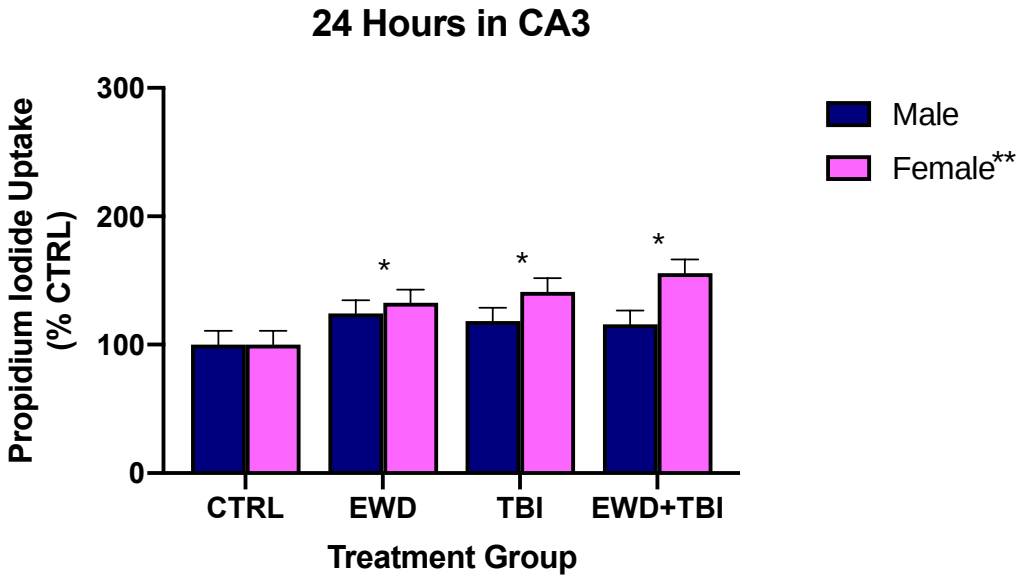


Figure 3.4.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 24 hours following treatment. All treatment conditions resulted in more uptake of PI compared to the CTRL group. EWD vs. CTRL, $p = .007$; TBI vs. CTRL, $p = .006$; and EWD+TBI vs. CTRL, $p < .001$. There was an effect of sex, $p = .019$. At 24 hours, the CA3 region of female slices took up more PI than the CA1 region of male slices. Error bars represent standard error.

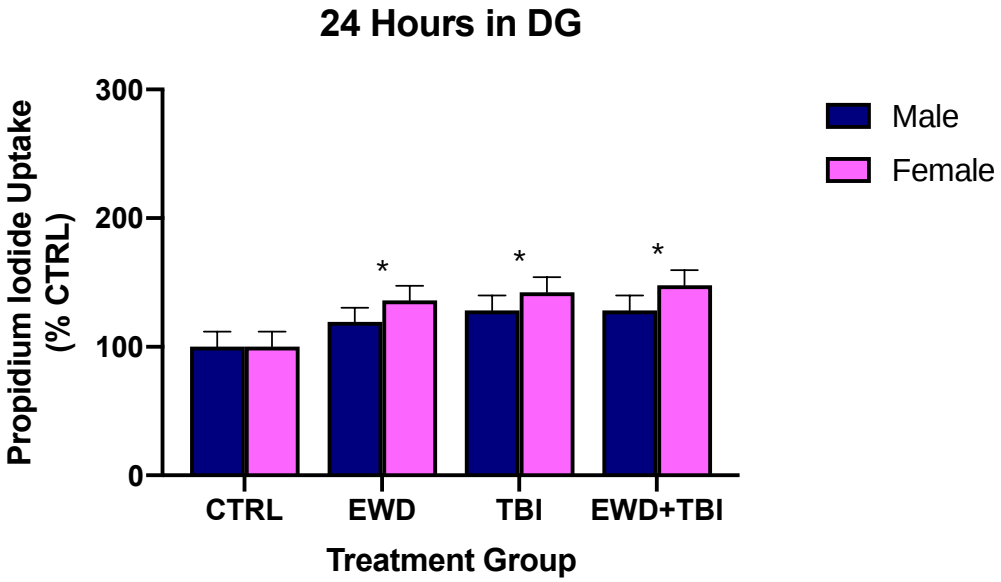


Figure 3.4.3: Effects of treatment on propidium iodide (PI) uptake in the DG region of hippocampal slices, 24 hours following treatment. All treatment conditions resulted in more uptake of PI compared to the CTRL group. EWD vs. CTRL, $p = .016$; TBI vs. CTRL, $p = .003$; and EWD+TBI vs. CTRL, $p = .001$. There was no effect of sex, $p = .124$. Error bars represent standard error.

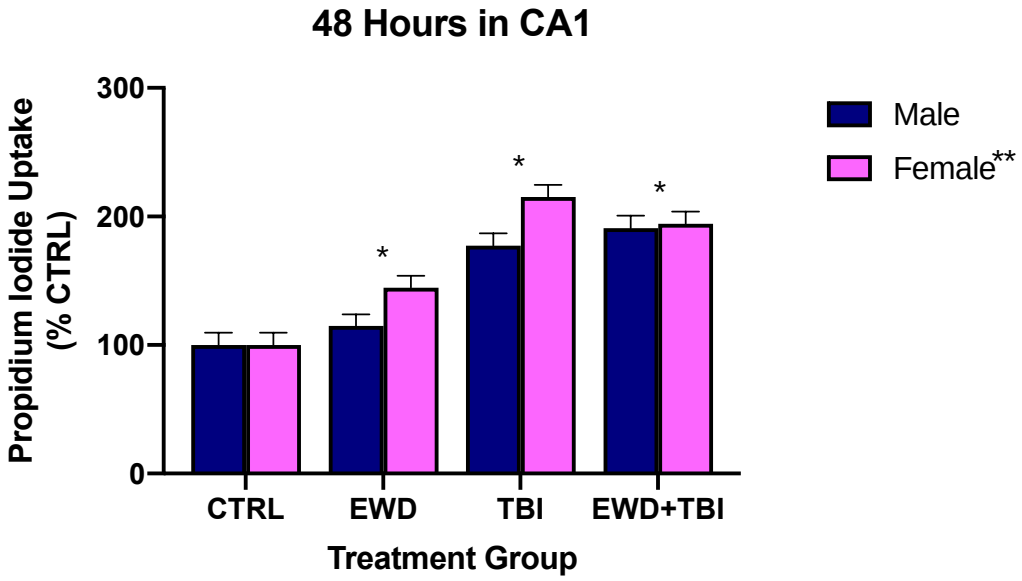


Figure 3.5.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 48 hours following treatment. All treatment conditions resulted in more uptake of PI compared to the CTRL group. EWD vs. CTRL, $p = .002$; TBI vs. CTRL, $p < .001$; and EWD+TBI vs. CTRL, $p < .001$. There was an effect of sex, $p = .009$. At 48 hours, the CA1 region of female slices took up more PI than the CA1 region of male slices. Error bars represent standard error.

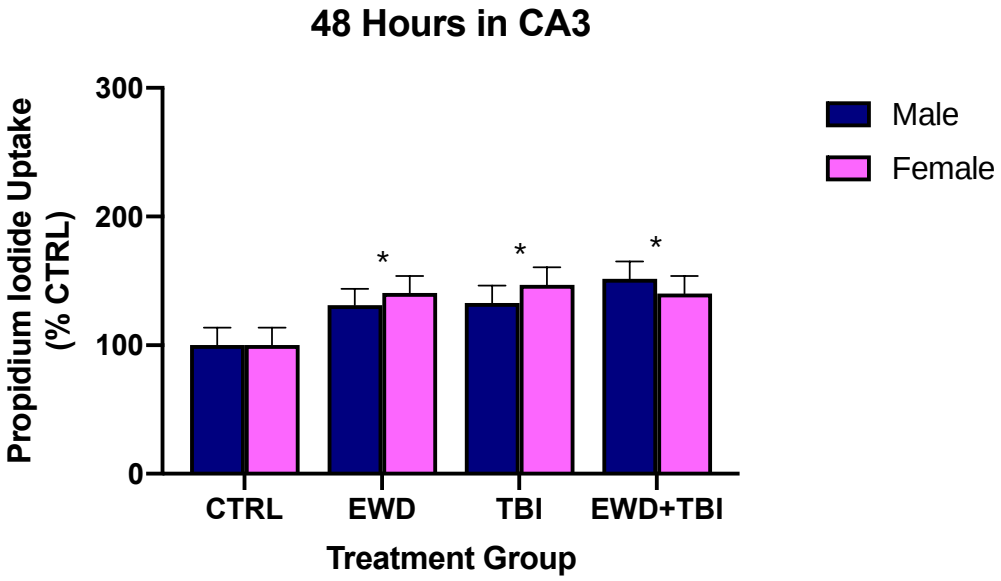


Figure 3.5.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 48 hours following treatment. All treatment conditions resulted in more uptake of PI compared to the CTRL group. EWD vs. CTRL, $p = .008$; TBI vs. CTRL, $p = .004$; and EWD+TBI vs. CTRL, $p = .001$. There was no effect of sex, $p = .749$. Error bars represent standard error.

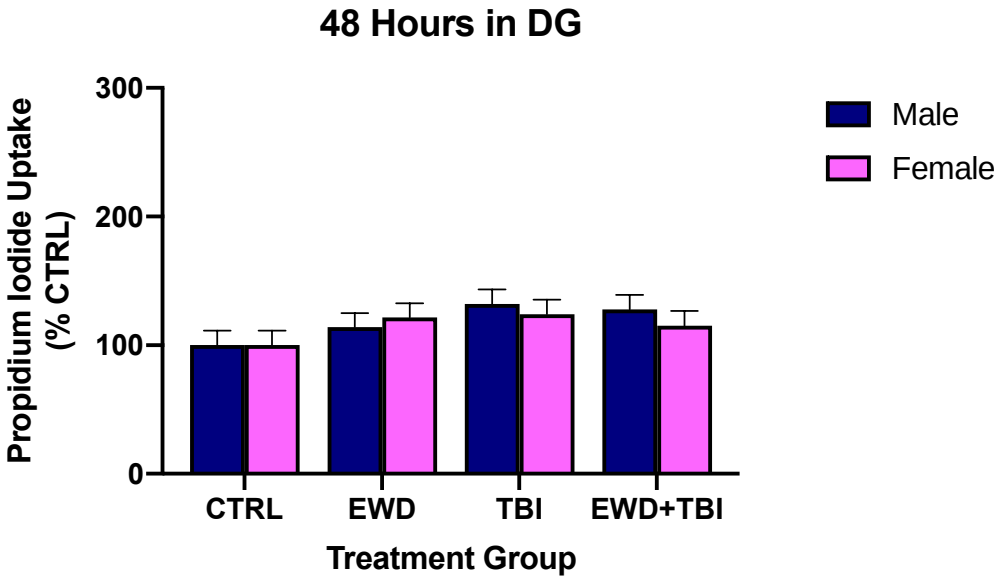


Figure 3.5.3: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 48 hours following treatment. There was no effect of treatment, p = .084 or an effect of sex, *p* = .674. Error bars represent standard error.

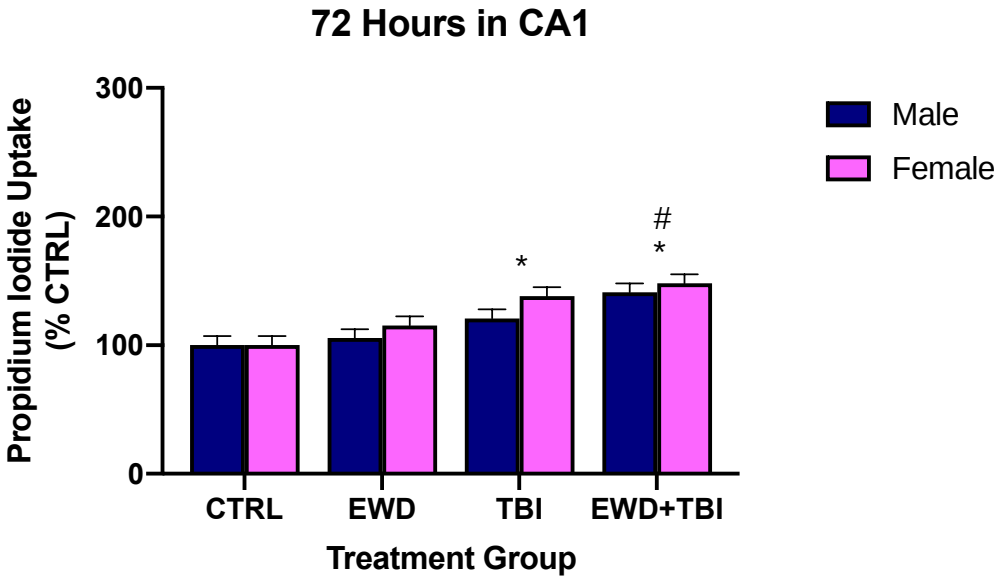


Figure 3.6.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 72 hours following treatment. There was no effect of EWD treatment (five days of 50mM ethanol followed by 24 hours of control media) compared to slices in the CTRL condition. Both the TBI and EWD+TBI groups showed an increase uptake of PI in the CA1 at 72 hours, compared to the CTRL group, $p < .001$. There was an increase in PI uptake in the EWD+TBI condition compared with the TBI group (#), $p = .032$. There was no effect of sex, $p = .086$. Error bars represent standard error.

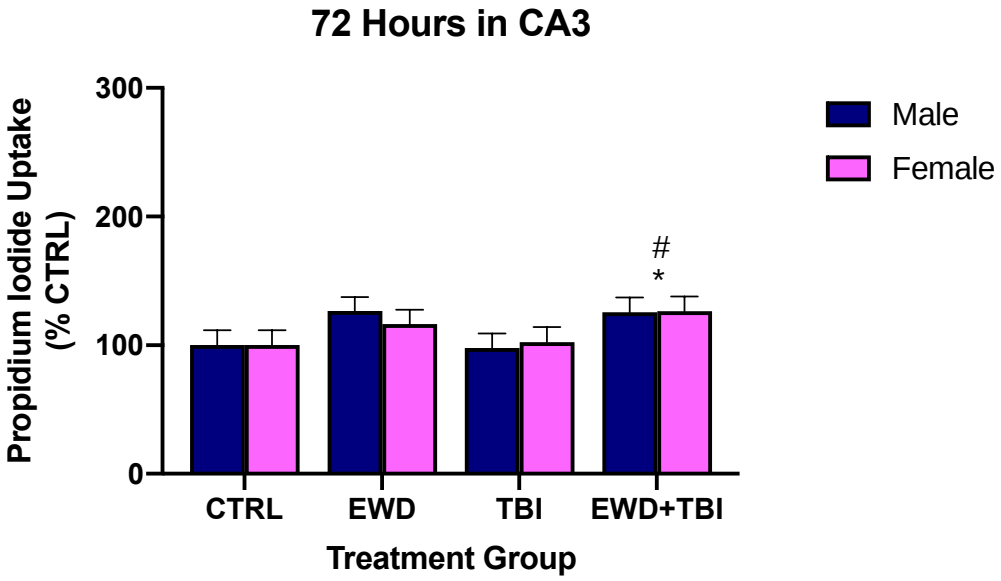


Figure 3.6.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 72 hours following treatment. There was no effect of EWD treatment (five days of 50mM ethanol followed by 24 hours of control media) compared to slices in the CTRL condition or an effect of TBI treatment compared to the CTRL condition. The EWD+TBI group showed an increase uptake of PI in the CA3 at 72 hours, compared to the CTRL group, p = .025. There was an increase in PI uptake in the EWD+TBI condition compared with the TBI group (#), p = .025. There was no effect of sex, p = .890. Error bars represent standard error.

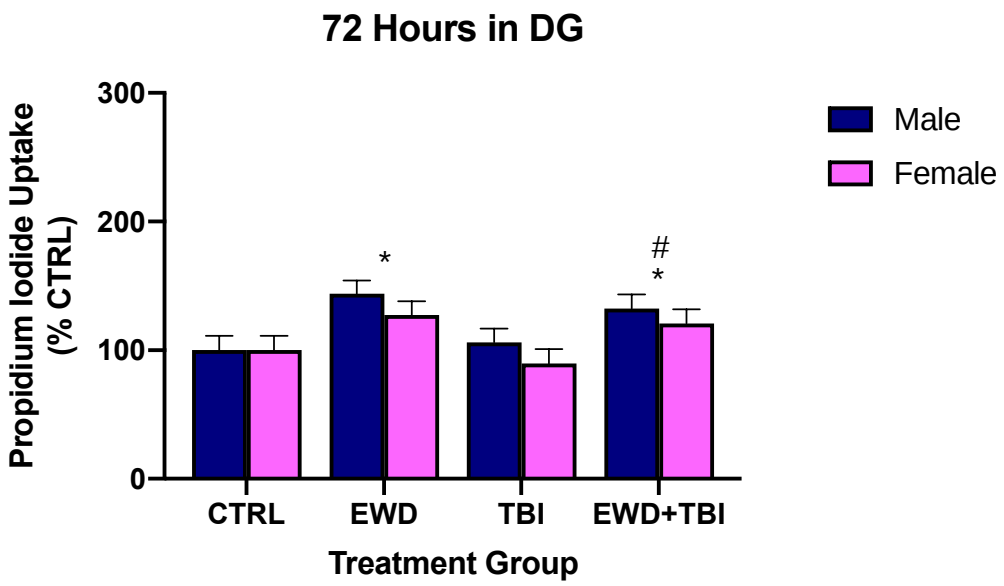


Figure 3.6.3: Effects of treatment on propidium iodide (PI) uptake in the DG region of hippocampal slices, 72 hours following treatment. There was an increased uptake of PI in the EWD and EWD+TBI groups compared with the CTRL group, $p = .001$ and $p = .017$, respectively. There was no effect of the TBI treatment compared to the CTRL condition. The EWD+TBI group showed an increase uptake of PI in the DG at 72 hours, compared to the CTRL group, $p = .025$. There was an increase in PI uptake in the EWD+TBI condition compared with the TBI group (#), $p = .009$. There was no effect of sex, $p = .153$. Error bars represent standard error.

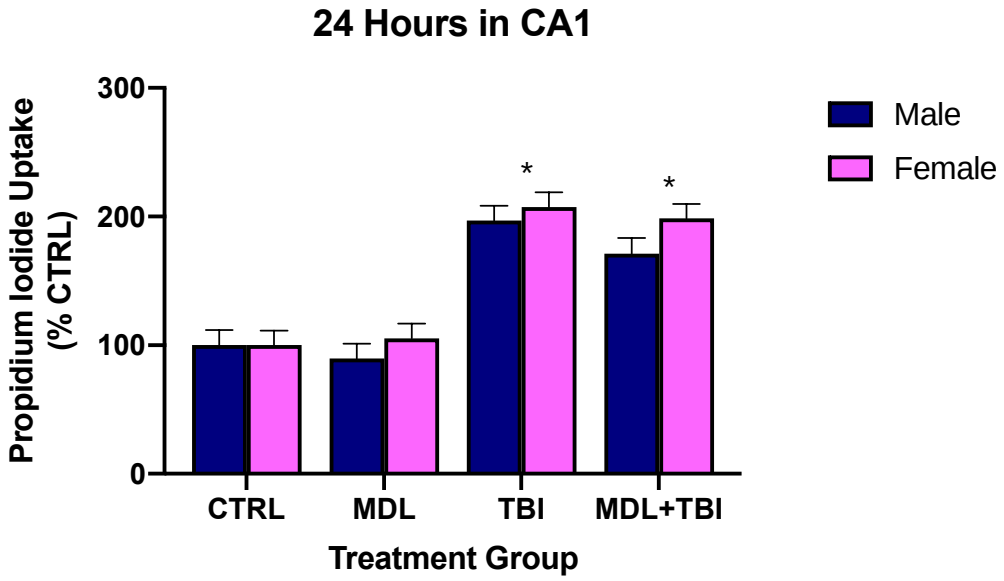


Figure 3.7.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 24 hours following treatment. There was no effect of MDL treatment compared to slices in the CTRL condition. Both the TBI and MDL+TBI groups showed an increase uptake of PI in the CA1 at 24 hours, compared to the CTRL group, $p < .001$. There was no effect of sex, $p = .103$. Error bars represent standard error.

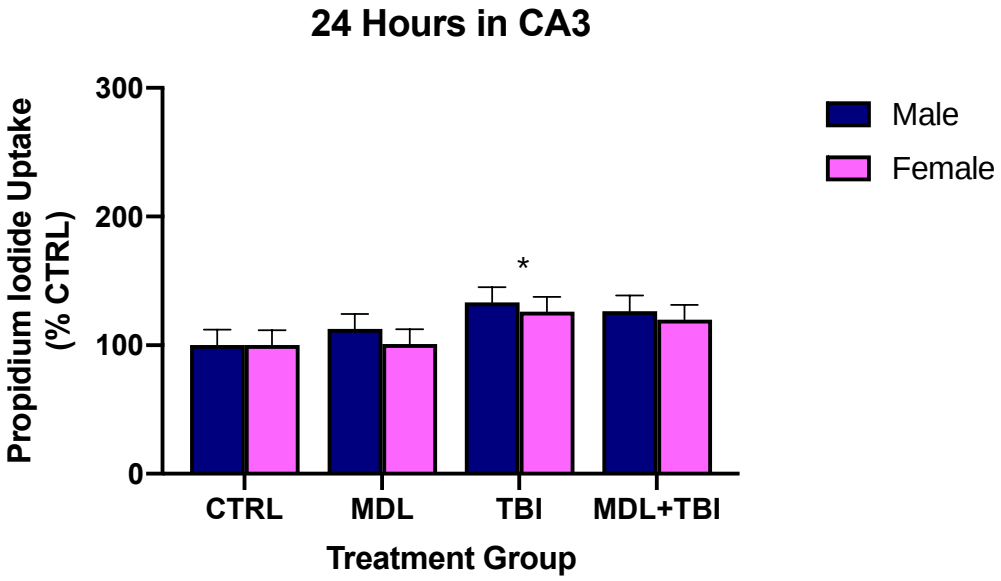


Figure 3.7.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 24 hours following treatment. There was no effect of MDL treatment or the MDL+TBI treatment compared to slices in the CTRL condition. The TBI group showed an increase uptake of PI in the CA3 at 24 hours, compared to the CTRL group, $p = .012$. There was no effect of sex, $p = .446$. Error bars represent standard error.

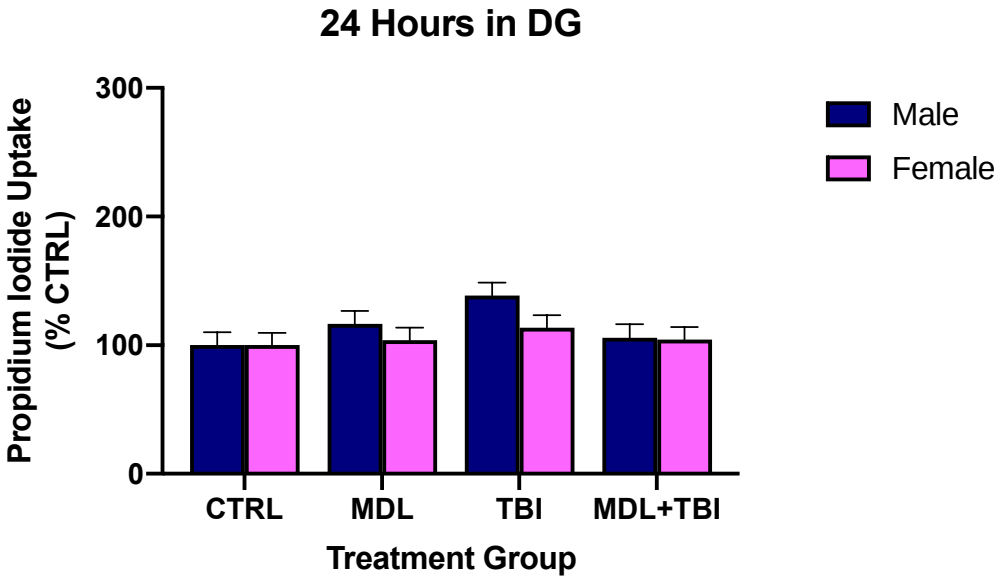
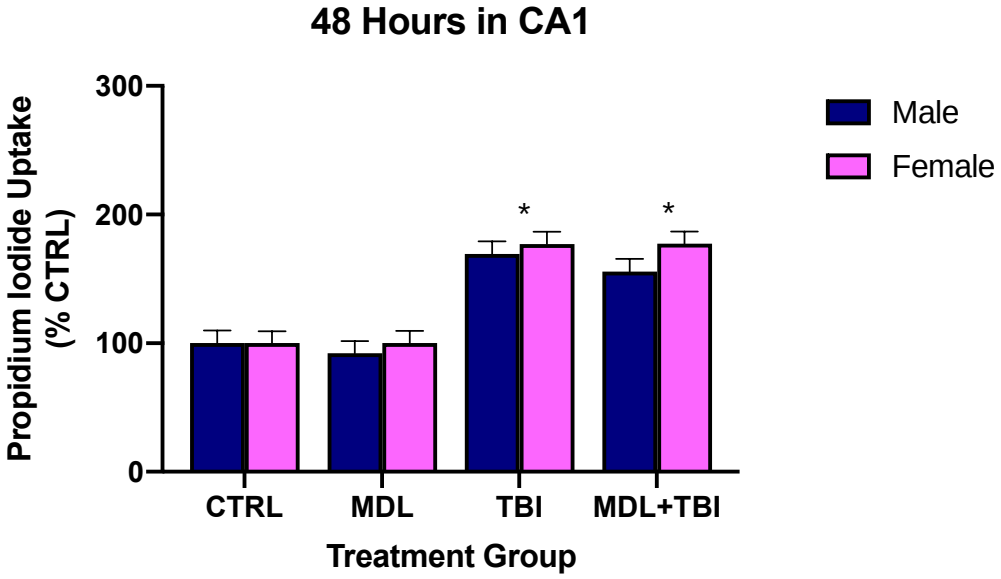


Figure 3.7.3: Effects of treatment on propidium iodide (PI) uptake in the DG region of hippocampal slices, 24 hours following treatment. There was no effect of treatment or an effect of sex, $p = .052$ and $p = .162$. Error bars represent standard error.



3.8.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 48 hours following treatment. There was no effect of MDL treatment compared to slices in the CTRL condition. Both the TBI and MDL+TBI groups showed an increase uptake of PI in the CA1 at 48 hours, compared to the CTRL group, $p < .001$. There was no effect of sex, $p = .165$. Error bars represent standard error.

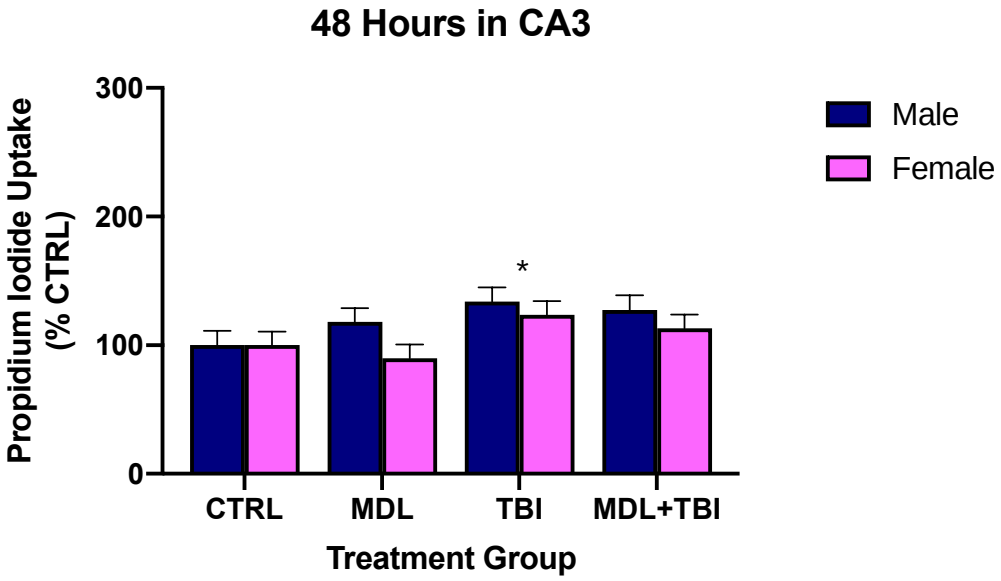


Figure 3.8.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 48 hours following treatment. There was no effect of MDL treatment or the MDL+TBI treatment compared to slices in the CTRL condition. The TBI group showed an increase uptake of PI in the CA3 at 48 hours, compared to the CTRL group, $p = .009$. There was no effect of sex, $p = .090$. Error bars represent standard error.

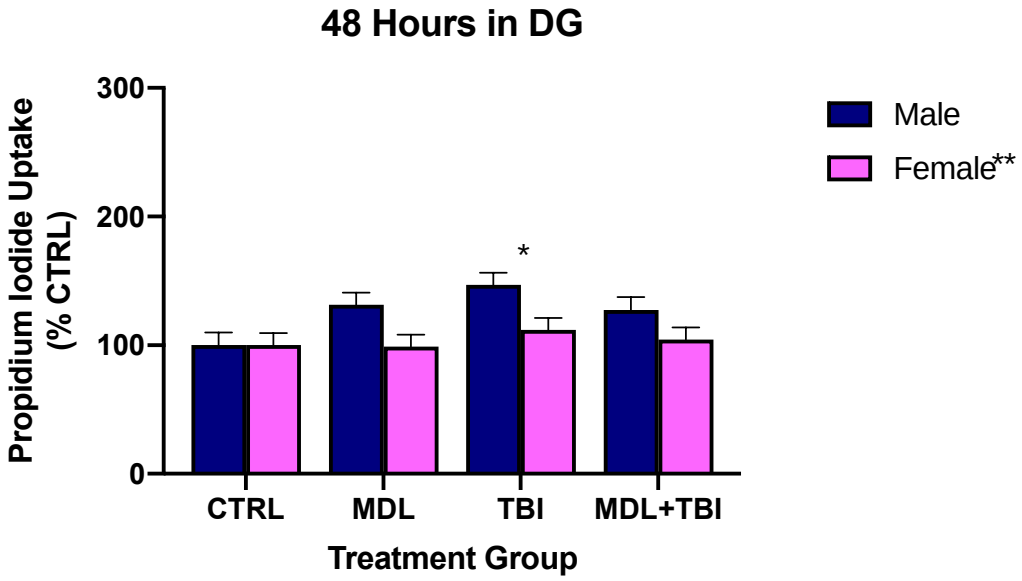


Figure 3.8.3: Effects of treatment on propidium iodide (PI) uptake in the DG region of hippocampal slices, 48 hours following treatment. There was no effect of MDL treatment or the MDL+TBI treatment compared to slices in the CTRL condition. The TBI group showed an increase uptake of PI in the DG at 48 hours, compared to the CTRL group, p = .002. There was an effect of sex (**), p = .001. At 48 hours, the DG region of male slices took up more PI than the DG region of female slices. Error bars represent standard error.

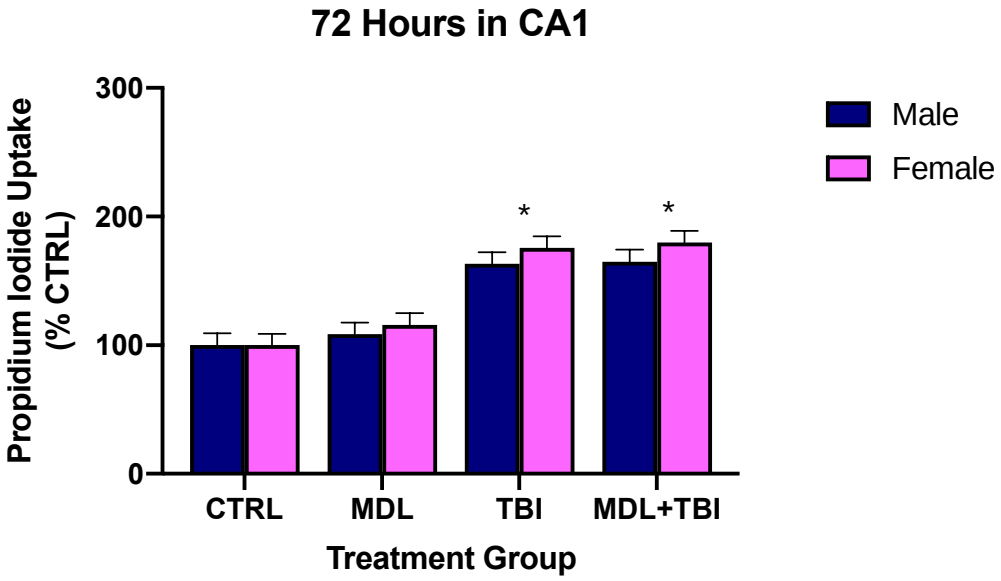


Figure 3.9.1: Effects of treatment on propidium iodide (PI) uptake in the CA1 region of hippocampal slices, 72 hours following treatment. There was no effect of MDL treatment compared to slices in the CTRL condition. Both the TBI and MDL+TBI groups showed an increase uptake of PI in the CA1 at 48 hours, compared to the CTRL group, $p < .001$. There was no effect of sex, $p = .180$. Error bars represent standard error.

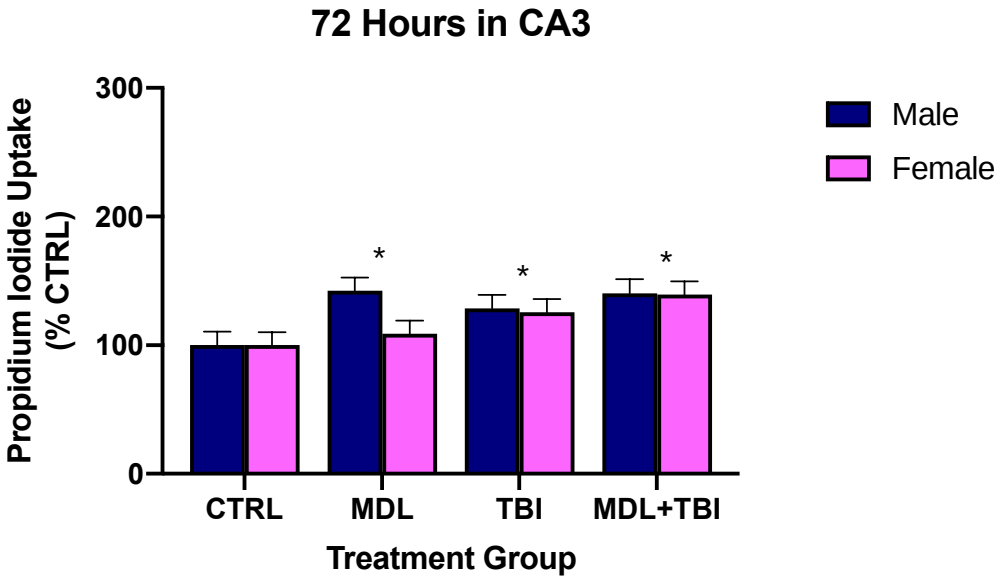


Figure 3.9.2: Effects of treatment on propidium iodide (PI) uptake in the CA3 region of hippocampal slices, 72 hours following treatment. All treatment conditions resulted in more uptake of PI compared to the CTRL group. MDL vs. CTRL, $p = .015$; TBI vs. CTRL, $p = .010$; and MDL+TBI vs. CTRL, $p < .001$. There was no effect of sex, $p = .208$. Error bars represent standard error.

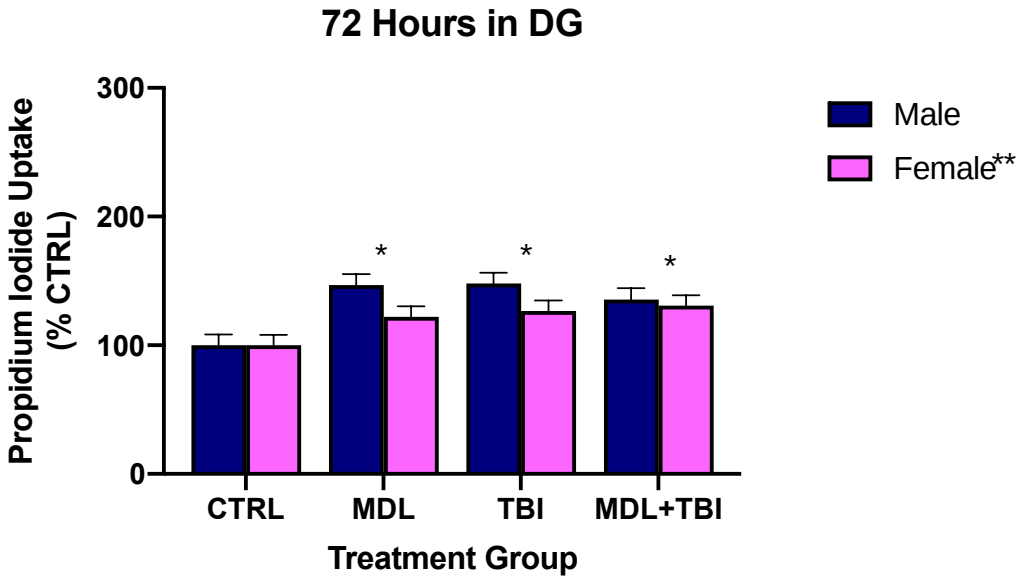


Figure 3.9.3: Effects of treatment on propidium iodide (PI) uptake in the DG region of hippocampal slices, 72 hours following treatment. All treatment conditions resulted in more uptake of PI compared to the CTRL group. MDL vs. CTRL, $p < .001$; TBI vs. CTRL, $p < .001$; and MDL+TBI vs. CTRL, $p < .001$. There was an effect of sex (**), $p = .031$. At 72 hours, the DG region of male slices took up more PI than the DG region of female slices. Error bars represent standard error.

CHAPTER 4. DISCUSSION

In experiment 1, unsurprisingly, the application of a transection (cut) to the CA1 in both the TBI alone and the EtOH+TBI resulted in increased propidium iodide (PI) uptake in the CA1, indicative of increased cell death. This was seen at each time point the slices were imaged. There was not an effect of the EtOH treatment on PI uptake in the CA1, but this is consistent with (Reynolds, Berry, Sharrett-Field, and Prendergast, 2015). Also the effect of the TBI treatment was limited to the area of injury (CA1), with no effect of treatment in either the CA3 or the DG. While it is not surprising that the most pronounced effect would be seen at the site of injury, it is unexpected that there was no effect of treatment seen in either the CA3 or the DG, as animal models of TBI show a tendency of injuries to spread after the initial insult due to secondary injury processes (Hawryluk and Manley, 2015). However, a possible explanation for this unexpected finding is that, although an effort was made to image at different time points to capture the progression of secondary injury mechanisms, the time points may have been too long after the initial insult to capture injury expansion. Different secondary injury mechanisms can occur minutes to days after the initial insult (e.g. Liu, Yin, Zhang, and Qian, 2014; Nakajima et al., 2010), so future investigations may wish to image more closely following the initial injury, as image at more frequent intervals. At the 48-hour mark, there was some indication that female slices had more cell death than male slices, but only in the CA3 and DG. Given that there was not an effect of treatment in these regions, caution should be used in the interpretation of this finding. Even in the CA1, where there was an effect of treatment and both TBI and EtOH+TBI showed an increase in cell death, there was no difference between

the slices that were intoxicated at the time of injury (EtOH+TBI) and slices in the control media at the time of injury. This finding adds to the confusing and conflicting previous results regarding alcohol intoxication at the time of TBIs (Berry et al., 2011; Cunningham et al., 2002; Dikmen et al., 1993; Gurney et al., 1992; Gururaj, 2004; Joseph et al., 2015; Lange et al., 2008; Lange et al., 2014; Mathias and Osborn, 2018; Pandit et al., 2014; Raj et al., 2015; Ruff et al., 1990; Scheene et al., 2016; Shahin and Robertson, 2012; Shandro et al., 2009; Tien et al., 2006). However, an additional limitation of this model is that the TBI mechanism used in this study was quite severe and focal in nature, which may have resulted in a ceiling effect. Efforts should be made to replicate these experiments using different, and less severe, models of TBI to determine if a potential ceiling effect may have influenced the findings. Although the values changed between time points, due in part to the fluorescent marker, in this experiment, overall the same pattern of effects were seen at 24, 48, and 72 hours following injury (aside from the unexpected sex effect in the CA3 and DG at 48 hours), which may indicate these time points may not be the most beneficial for capturing the dynamic nature of TBIs, as modeled in this experiment.

In experiment 2, in the CA1, CA3, and DG at 24 hours, all three treatment groups (EWD, TBI, and EWD+TBI) had more PI uptake (cell death) than the slices in the control condition. Unlike in experiment 1 where the damage was limited to the area of injury (CA1), in this experiment, increased cell death is seen in the other regions of the hippocampus. At 24 hours in the CA1, CA3, and at 48 hours in the CA1, there was an effect of sex. At these time points in these regions, female slices had more PI uptake n average compared to male slices. This result was not hypothesized, but was not necessarily surprising. Clinical data is mixed with some studies finding no difference in TBI outcomes

between men and women however, women experiencing worse outcomes following a TBI than men, and women experiencing better outcomes than men (for review, see Gupte, Brooks, Vukas, Pierce, and Harris, 2019). One concern in TBI research is that female participants are not always included or included in sufficient numbers (Gupte et al., 2019), which may be in part due to the increased likelihood of men obtaining TBIs compared to women (Faul et al., 2010). Interestingly, at the 72-hour time point, there is some indication that slices in the EWD+TBI group have more cell death than slices in the TBI alone group. In the CA1, while both the TBI and EWD+TBI have more cell death than the CTRL group, the EWD+TBI group has significantly more cell death than the TBI alone group. In the CA3, the only treatment group that has more PI uptake than the CTRL group is the EWD+TBI group, and it has more cell death than the TBI alone group, which was not different from CTRL slices. In the DG, only the EWD and EWD+TBI groups had more cell death than the CTRL group-the TBI alone group did not. Additionally, the EWD+TBI group had more cell death than the TBI alone group. This finding was anticipated, as it was hypothesized that EWD would sensitize NMDA receptors, exacerbating the excitotoxicity seen following the initial injury.

In experiment 3, in the CA1 at 24, 48, and 72 hours, both the TBI and MDL+TBI groups had more cell death than the CTRL group. Interestingly, in the CA3 at 24 and 48 hours as well as the DG at 48 hours there was more cell death in the TBI group compared to the CTRL group, but the MDL+TBI group did not. While there was no statistical difference between the TBI and MDL+TBI groups, this difference at the non-injured areas should be investigated further to see if this finding was an anomaly, or if there may be a true therapeutic effect of MDL in these regions. Also, at 72 hours in the CA3 and DG, in

addition to both the TBI and MDL+TBI groups having more cell death than the CTRL group, the MDL group has also become toxic and had more cell death than the CTRL group. Given the nonspecific mechanism MDL is targeting, it is possible that it can cause toxicity in its own right.

The purpose of these experiments was to characterize a model of TBI in OHSC and to study the effect of alcohol intoxication and withdrawal prior to TBI on cell death as well as to investigate a potential therapeutic to elucidate a secondary mechanism of injury in a controlled manner to further disease mechanisms prior to experiments in live animals. There were several limitations to these experiments. For one, the mechanism of injury used is not a common one in clinical TBI populations and was potentially too severe to determine the effects of alcohol intoxication and withdrawal prior to TBI. Another limitation was that imaging time points were chosen to try and capture the progressive nature of TBIs, but may have been too late following the injury. Another limitation was that the drug administered is not specific and can cause dysfunction in healthy cells. Additionally, given that different litters of pups can give very different results, it is important future studies use multiple litters to ensure the effects are consistent. Another limitation of this study was that only one mechanism of secondary injury was looked at- excitotoxicity. By also looking at another secondary injury mechanism, such as various inflammatory markers, a more complete picture can be made. In that same vein, by using a fluorescent marker such at NeuN that will specifically show live neurons, a more complete picture can be formed, instead of just looking at PI uptake, which will stain all types of dead cells.

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