NEUROPROTECTION THERAPY FOR TRAUMATIC BRAIN INJURY

A Thesis

by

DANIEL K JONES

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Chair of Committee,	D. Samba Reddy
Committee Members,	Leonide Gerard Toussaint
	Jane Welsh
Program Director,	Warren Zimmer

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ABSTRACT

The main goal of this project was to test the effectiveness of a novel combination neuroprotection therapy for traumatic brain injury (TBI). TBI affects millions of people worldwide every year. Neuroinflammation, excitotoxicity and neuronal death as well as related mechanisms contribute to the development of acute and complex neurological deficits, including post-traumatic seizures and cognitive dysfunction. Neuroprotection approaches targeting acute and chronic phases of TBI are needed to limit the damage and prevent post-TBI dysfunction. A variety of neuroprotection approaches such as statins, progesterone (P), cyclosporine A and anti-inflammatory agents have been tested that either target neurons or non-neuronal cells in animal models of TBI. Herein we evaluated the neuroprotective potential of the neurosteroid Ganaxolone (GX) in a mouse TBI model. GX is a synthetic neurosteroid related to allopregnanolone that has sedative, anxiolytic, and anticonvulsant effects. To our knowledge GX has not been used as a neuroprotective agent for TBI. We utilized a controlled cortical impact (CCI) model, which simulates aspects of concussions, brain contusions, and hemorrhages seen in human TBI. Our pilot studies showed the feasibility of TBI-induced chronic epilepsy model in mice. Ganaxolone treatment had positive outcomes on motor function and additional promising disease-modifying or protective potential to reduce epileptic seizures. This pilot study will be advanced further in a larger cohort to confirm Ganaxolone's ability to reduce or prevent PTE.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

BACKGROUND AND INTRODUCTION

1.1 Traumatic Brain Injury

Traumatic brain injury (TBI) is a major cause of mortality and morbidity, particularly at the two ends of the age spectrum, with large direct and indirect costs to society. In the United States (US) it has been estimated that more than 1.7 million individuals suffered a TBI occurrence annually, whether through an isolated injury or in congruence with another injury (Faul *et al.*, 2010), and the annual burden of TBI has been estimated at close to US $76.5 billion (Finkelstein *et al.*, 2006), though these numbers markedly underestimate the incidences and costs. In US Center for Disease Control and Prevention data (Faul *et al.*, 2010) sports-related injuries are not listed among the top categories, but some have estimated the incidence of such head injuries at 1.6–3.8 million per year (Langlois *et al.*, 2006). Globally, the incidence of TBI is also increasing, particularly in developing countries (Maas *et al.*, 2008). In Europe, it is estimated to cost health care an annually 33 billion euros for TBI, while among those suffering, a large portion of the individuals will have physical disabilities that will lead to a burden on the health care system. Perhaps more importantly though, is the functional impairments that remain, adversely affecting the patient's quality of life, and exacerbating the burden of TBI on society (Weaver at al. 2012, Dardiotis *et al.* 2012; 2010). Moreover, TBI is also thought to lead to outcomes of physical inactivity such as obesity, diabetes, and heart disease (Michael *et al.* 2010; Gallek *et al.*, 2011).

TBI is a highly complex disorder that comprises varying degrees of contusion, DAI, hemorrhage and hypoxia (Saatman *et al.*, 2008, 1983). TBI can be organized or identified on the basis of the mechanism of injury (Graham *et al.* 2009), and with these classifications, we can group TBI injuries into characteristics of mechanical force (acceleration, amplitude, duration, and velocity) determining if the injury is static or dynamic. In terms of rating severity of TBI, the 15-point Glasgow Coma Scale (GCS) is most commonly used. This rating scales measures three vital parameters: eye-opening response to stimuli, verbal, and motor (Marion, 2000). To demonstrate this rating given an example, we see in mild TBI (mTBI), a GCS score greater than or equal to thirteen, while in moderate TBI we see a nine to twelve score, and in severe TBI, a score 8 or less. Complicating the use of any scoring system, it should also not be assumed that the same injury severity will have the same distribution of damage within the brain and the type of damage whether that is gray or white matter (Saatman *et al.* 2008). Regardless of severity and pathological differences between severities though, collectively, these effects induce biochemical and metabolic changes that lead to progressive tissue damage and associated cell death (McIntosh, *et al.*, 2008). The vital point here is that, even though we can see the same injury severity amongst individuals, it should not be assumed that they are both following the same pathological and clinical endophenotypes.

TBI can result in the development of complex neurological deficits caused through primary and secondary injury mechanisms. Primary injury events encompass the

mechanical damage that occur at the time of trauma to neurons, axons, glia and blood vessels as a result of shearing, tearing or stretching (Saatman *et al.*; Adams *et al.*, 2008; 1983). In addition, secondary injury evolves over minutes to days and even months after the initial traumatic insult and results from delayed biochemical, metabolic and cellular changes that are initiated by the primary event.

This neurological disease is a significant and life-altering health issue that affects many service members and veterans during wars and following. It is often referred to the signature wound of the Iraq and Afghanistan wars predominantly occurring through a sudden traumatic or head related injury that disrupts the normal functions of the brain (Tanielian, 2008). The most common types of causations within the military include: explosive devices, falls, and vehicle or motorcycle accidents. During the Iraq war, several thousand military service members have sustained a TBI, 69% of those as a result of a blast (Warden, 2006). There is an increased rate for sustaining a TBI injury in active duty and reserve service members in comparison to civilians. These results are due to multiple factors that include the specific demographics of the military, e.g. in general, young men that are between the ages of 18 to 24 are at a greater risk of TBI. It is routine within the military to see many training activities and operational employments that can be physically demanding and potentially dangerous, even with stringent safety measures in place. Military service members are constantly deployed to high-risk areas where they could potentially experience blast exposures from

improvised explosive devices (IEDs), suicide bombers, mortar rounds, land mines and rocket-propelled grenades.

An analysis of data that was collected from March 2004-September 2004 from 115 patients from the Navy-Marine Corps that were identified with TBI showed that IEDs were the most common mechanism of injury, and were responsible for 52% of TBI cases overall. In addition, the analysis showed that intracranial injuries, specifically concussions, were the most common within diagnosis, and 51% of the patient group had only an intracranial injury with no accompanying open wound in the head or head fractures. It was also found that 115 patients analyzed, roughly 63% of the patients were wearing helmets at the time of Injury (Clark *et al.*, 2007). This augmented rate of TBI and blast-related concussion events that result from combat operations will lead to a direct impact on the; health, safety, overall well-being of individual service members, affected families of service members, and fellow comrades. The high impacts of TBI are shared within each branch of the service and throughout both the Department of Defense (DOD and the Department of Veterans Affairs (VA) health care systems. Specifically within the VA, TBI has become a large focus, leading to the acknowledgement of the necessities for additional resources to provide health care and vocational retraining for individuals that suffer from a TBI diagnosis, while they transition from active duty.

TBI is not limited to the realm of military combat situations but is also seen in sports. The marked difference between combat-related concussion and sports-related concussions is the mechanism of injury. Among the vast amount of people that suffer from TBIs each year in the United States, approximately 10% of those are due to sports and recreational activities (Gilchrist *et al.*, 2001; 2009). In sport-related concussion, the most common causations of a TBI injury are through head-to-head impacts in helmeted athletes. In the United States alone it is estimated that 300,000 sports-related concussions annually are due to high impact head-to-head collisions (Gessel et al, 2007). Sports are second only to motor vehicle accidents as one of the leading causes of civilian concussions within individuals between the ages of 15-24. Concussions are far more predominant in football (47.1% of the 1,936 concussions that occurred in high school athletes during the 2008-2010 school years) in comparison to any other sport, with the next highest impact TBI sport among young men being hockey and lacrosse (Marar *et al.* 2010). As the numbers of participants in youth sports continue to grow, the incidence of brain injuries proportionally rise as well (Gilchrist *et al.*, 2009). In congruence with these reports, we see an increased awareness for the potential short- and long-term sequelae of sports players that suffer from brain related injuries, such as the increased chance for re-injury, cognitive deficiencies, early onset of Alzheimer's, chronic traumatic encephalopathy, and second impact syndrome (CDC 1997; Cantu *et al.* 1995; Collins *et al.* 1999; Lovell *et al.*, 2003; Omalu *et al.* 2010). The related clinical signs and symptoms that follow an mTBI (mild Traumatic Brain Injury) in athletes can

range from subtle mood changes to obvious loss of consciousness, the onset of which can be immediate, or potentially several minutes after injury (Kelly Rosenberg, 1997).

According to the American Academy of Neurology (AAN), the identifying signs of mTBI are thought to be amnesia, behavior or personality changes, confabulation, delayed verbal and motor responses, disequilibrium, disorientation, emotional liability, loss of consciousness, slurred/incoherent speech, or a vacant stare. Symptoms of mTBI may include blurry/double vision, confusion, dizziness, excessive drowsiness, sleep difficulties, hazy feelings, foggy or groggy, headache, inability to focus or concentrate nausea, vomiting, and photo- or phonophobia (AAN 1997). It is vitally important that clinicians consider their evaluations of athletes as a unique population subset, simply because the sporting world has a culture and mental perspective that predicates the pushing of athletes beyond their perceived physical and mental abilities on and off the field, where even the slightest injury can be compounded if left untreated or undiagnosed. In response to increased TBI injuries amongst sports players, the Federal and State governments, in congruence with multiple sports governing officials and companies have implemented rule and policy changes that are specifically designed to increase the protection of athletes and to standardize medical care.

TBI presents a significant public challenge in today's society, and approximately 1.7 million civilians in the U.S. each year suffer from some form of TBI that will at least require a hospital visit (Faul *et al.*, 2010). The fraction of severe TBI cases within the

general population-which is around 3% of total annual cases- is deemed to require the most intensive medical care and is more often than not complicated by additional injuries to other body regions. Moderate brain injury within the general population represents roughly 22% of the annual incidence and commonly renders victims with persistent deficits that will follow them the rest of their life. Mild TBI, though egregiously under-diagnosed due to the lack of response on the victim or surrounding affected friends or family, has the highest incidence rate in the general population-75% of total TBI injuries accrued- and may potentially be higher than reported in epidemiological studies, owing to the prior mentioned underreported cases in young populations (Laker, 2011). In total, the socioeconomic toll is daunting, and TBI remains the number one prevalent cause of death in adults aged less than 45 years old and is considered to be the highest cause of long-term disability (CDC, 2013). Additionally as the population ages, we are seeing an increased incidence in the elderly population, where TBI is now only second to cancer as a causation of death in individuals over the age of 65 (Coronado *et al.*, 2007). Thus with the growing awareness of TBI in civilian, sports and military populations, it is no longer considered a silent epidemic.

Neuroprotection approaches for both acute and chronic neurodegenerative disorders have historically been dominated by a neuronocentric view in which modification of neuronal-based injury mechanisms is the primary or even exclusive focus of the neuroprotective strategy. However, increasing evidence in the literature underscores the importance of viewing injury more broadly to include endothelial cells, astroglia,

microglia, oligodendroglia and precursor cells. More recent neuroprotection approaches have recognized this complex structure and interplay, emphasizing therapeutic strategies that promote the recovery and optimal functioning of non-neuronal cells in addition to inhibiting neuronal cell death pathways (Stoica *et al.*, 2009; Nimmo, 2009).

1.2 Epidemiology

TBI injuries can be classified as one of the singular causes of death seen in patients under the age of 25 and considered to be responsible for one third of all deaths involving a trauma experience (Kasmaei *et al.*, 2015). Therefore, the importance of collecting epidemiologic patterns and education between different populations is vitally needed for gaining knowledge into the epidemiology of this high-impact disease.

According to the CDC, an estimated 1.7 million documented cases of TBI are reported annually, with an unreported analysis suggesting upwards of 3.8 million (CDC, 2013). Direct and indirect cost associated with medical attention for TBI patients before and after injury totaled in the United States in the year 2000 roughly $76.5 billion (Finkelstein *et al.* 2006, Coronado, 2007). From 2001-2009, US physicians annually treated around 170,000 cases of mTBI in adolescents (0-19yo) (Gilchrist *et al.*, 2001-2009). During the same 10 year period, emergency departments visits for both recreational- and sports-related injuries (mTBIs) among children and young adults increased by a 60% annual rate (Gilchrist *et al.* 2001-2009) from 153,375, in 2001 to

248,418 in 2009 (Morbidity and Mortality Weekly Report, 2011). From 1997-2007 emergency department visits involving mTBI injuries from organized sports doubled in children (8-13yo), and tripled in young adults (14-19yo) (Mitka, 2010). A thorough analysis of these results show that 71% of all sports- and recreation-related TBI emergency department visits were in males between the ages of ten to nineteen (Gilchrist, *et al.*, 2001-2009). Furthermore, the activities that were predominantly linked with TBI-related emergency department visits were football and bicycling, and closely followed by soccer, basketball, and playground activities (Gilchrist *et al.*, 2001-2009).

In a recent cross sectional study, 1000 patients affected through some sort of TBI injury were profiled using a simple random sampling technique. Within the study, the variables that were examined included: outcome of patient, admission to ICU care, computed tomography scan results, surgery care, level of consciousness, accompanying injuries, hospitalization stay, demographics, season, and mechanism of injury. The results showed that within the 1000 patients that were affected by TBI, 81.8% were males with a mean age between 38.5±21.7 years old. In spring, the hospital referral rate of individuals within the study was 31.4%; more than any other season ($p<0.01$). The researchers used the Glasgow Coma Scale (lower scores correlated with deficiencies of normal functions), which measures Eye Opening (1-4), Verbal Response (1-5), and Motor Response (1-6) to measure consciousness within their test subjects. Their results showed 45.9% had some level of a “coma” measuring less than nine on

that scale. According to CT scans, 45.9% had subdural bleeding, 23.7% had epidural bleeding, and 23.3% patients suffered mortalities. These results suggest an increased frequency of TBI in male patients 20-60 years old (Kasmaei, 2015).

1.3 Pathophysiological Mechanisms

An injury considered to be a trademark of TBI is a DAI (Diffuse Axonal Injury) and commonly is initiated during the early phases of neuronal and parenchymal damage, as a direct result of varying traumatic external forces. DAI is a consequence of various rotational forces, and is vital to differentiate between cortical concussions and other differing hemorrhages that may be caused by linear acceleration/deceleration injuries (Goodman, 1994). Unfortunately, the primary injury may not always be treatable and thus can lead to fatal conditions. At a macroscopic level, damages may include shearing of white matter tracts, focal contusions, hematomas, and diffuse edema. At the cellular level, it is common to see premature sequelae of neurotrauma, such as: stearic conformational changes in proteins, leaky ion channels, and microporation of membranes. Another common result that is seen during a neurotraumatic event is the appearance of micro-hemorrhages, due to the tearing of blood vessels (Maas *et al.*, 2008). It is very clear that the most critical factor for diminishing damage resulting from a primary injury is through preventative measures.

A secondary delayed-phase may occur within hours, to days to weeks, and has been observed in TBI models to include: inflammatory cascade activation, edema, ischemia,

free radical effects, excitatory amino acids, ion release, and programmed cell death (Chestnut, Marshall, 1993). More often than not, one will see a disruption of axonal neurofilament organization occurring that results in an impairment of axonal transport that then lead to axonal swelling, Wallerian degeneration, and transection (Povlishock and Katz, 2005). Additional secondary injuries may be due to the release of excitatory neurotransmitters such as acetylcholine, glutamate and aspartate, and the generation of free radicals.

The pathophysiology of TBI that is involved in both focal and diffuse damage is multifaceted and heterogeneous, something that must be accounted for when designing neuroprotective treatments. Current and future studies should focus on elucidating why many neuroprotective therapies, to date have yet to yield results that improve the clinical outcome in this neurodegenerative disease. Two major neurodegenerative diseases that are commonly linked with the pathology of a traumatic brain event are Alzheimer's disease and Parkinson's disease.

1.4 TBI and Epileptogenesis

Epilepsy is a multifaceted diagnosis that is comparative to the complexity of the terminology. The ILAE (International League Against Epilepsy) definition defines epilepsy as a history of at least one ictal event, with an enduring alteration in the brain that elevates the possibilities of future seizures as well as associated neurobiological, cognitive, psychological, and social disturbances (Fisher *et al.*, 2005). As illustrated in

Figure 1, depending on the time of delay from TBI to the occurrence of the first seizures, post-TBI seizures are categorized into immediate (<24 h), early (1-7 days), or late seizures (> 1 week after TBI) (Annegers *et al.*, 1998). Therefore the association of TBI and a single unprovoked late seizure can be characterized as post-traumatic epilepsy (PTE) diagnosis.

PTE is responsible for 10-20% of symptomatic epilepsy in the general population and 5% of all epilepsy (Herman, 2002). Within civilian and military populations epidemiologic studies, the majority of which that were conducted before the relevant imaging era, the risk factors for PTE are inclusive to penetrating injuries, old age, injury severity (GSC<10), intracranial hemorrhage, bi-parietal or multiple contusions, frontal or temporal locations of lesion, >5 mm brain midline shift, duration of coma >24 h, loss of consciousness >24 h, prolonged length of post-traumatic amnesia, multiple intracranial procedures, and the occurrence of early post-traumatic seizures (Herman, 2000). In terms of methodology, the majority of the risk factors that have been identified are directly or indirectly comparative to the severity of brain injury, strengthening the idea that the risk for PTE rises with the severity of TBI (Pitkanen, 2014).

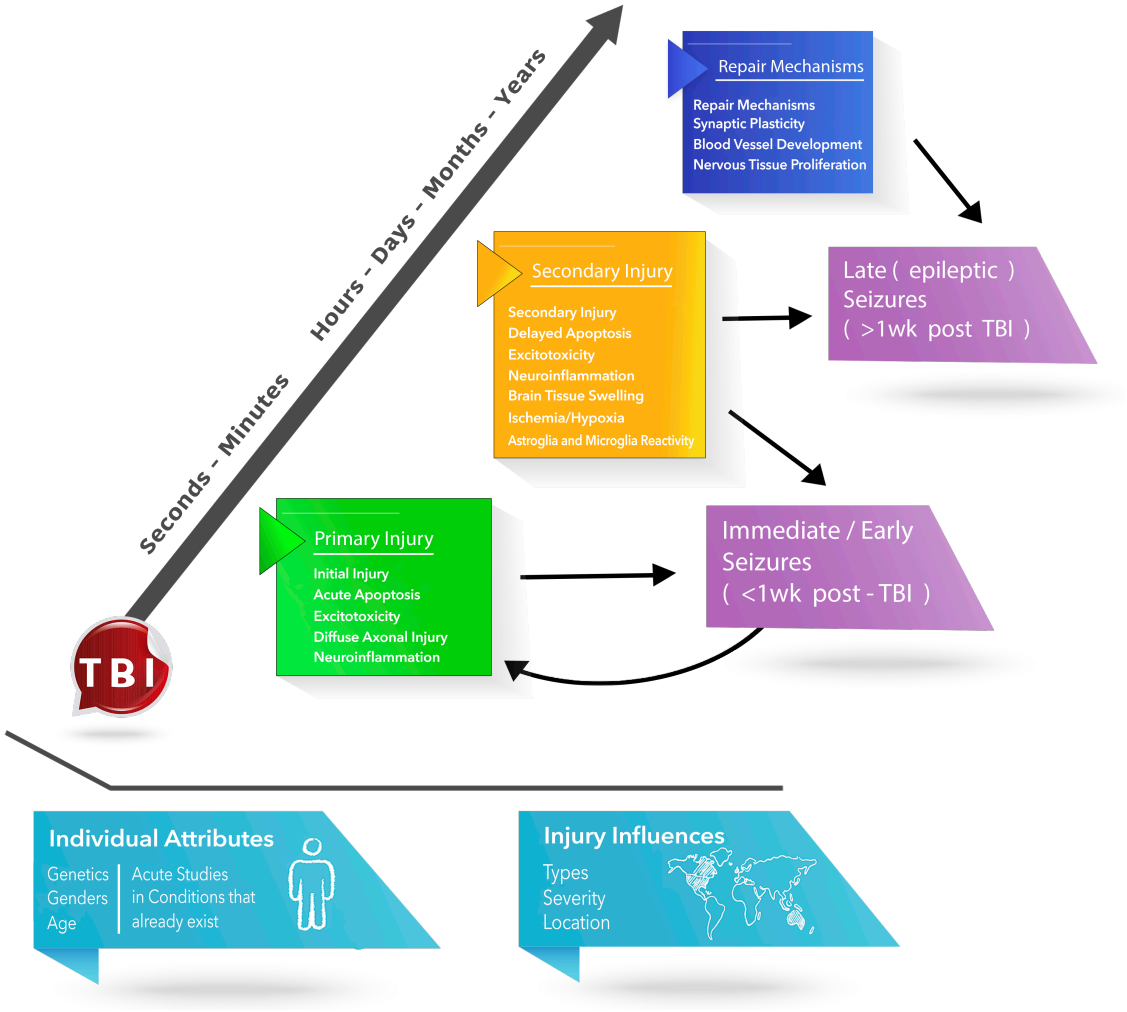

Figure 1. Schematic illustration adapted from Hunt *et al.* (2013) of TBI-Induced neuronal injury and epilepsy.

The progression of an acquired epileptic disorder suggests an abnormal neuronal reorganization that happens over an extended period of time following a specific cerebral insult (Engel, 1989). Such morbidities include neurodegeneration,

neurogenesis, axonal sprouting, axonal injury, glial cell activation invasion of peripheral inflammatory cells, vascular damage and angiogenesis, alterations to the extracellular matrix, and changes in the molecular structure of cellular facets such as receptor-gated ion channels, and ligands. The majority of these alterations are normally discovered at the time when seizure threshold is diminished and devoid of spontaneous seizure occurrences. They will then proceed to develop beyond the time of the initial unprovoked seizure and can even be implicated long after epilepsy diagnosis (Pitkanen and Sutula, 2002). From the viewpoint of a neurobiologist, the starting point of epileptogenic network reorganization can be defined as the direct moment of insult such as a TBI. Conversely, it is difficult to define the endpoint of epileptogenesis, especially relative to the number of seizures.

In humans, the detailed neurobiology of epileptogenesis following various brain insults is poorly known. The data that is available would suggest that the majority of patients acquire epilepsy within 2 years after SE, TBI, or stroke, which would implicate some connections between etiologies (Bladin *et al.*, 2000). Roughly 10-20% of acquired epilepsy is caused by TBI with a 30-year overall incidence of epilepsy being 2.1% for mild, 4.2% for moderate, and 16.7% for severe injuries. TBI patients that suffer from a penetrating head injury such as a bullet wound, up to 53% develop epilepsy. In roughly 8-10% of cases, epilepsy develops within 2 years, though the elevated risk can reach up to 10 years post-TBI (Haltiner *et al.*, 1997).

1.5 Animal Model

In accordance with the heterogeneous nature of clinical circumstances in TBI, a number of animal model have evolved. Larger mammals are more interpretable to human physiology for research purposes, but rodents are mostly used in TBI research due to their low costs, small size and the ability to standardize resulting outcomes. More recent models of TBI have been geared toward targeting and expanding our knowledge of the detrimental, and complex molecular cascades that are due to the results of traumatic head injuries. Among the current models, four are commonly used in research: fluid percussion injury (FPI) (Dixon *et al.*, 1987), cortical impact injury (CCI) (Lightwall, 1988), weight drop-impact acceleration injury (Marmarou *et al.*, 1994), and blast injury (Cernak *et al.*, 1996). Within the study, we utilized the CCI model.

In the CCI model, a pneumatic or electromagnetic impact device drives a rigid impactor into the exposed intact dura, and replicates the cortical tissue loss, acute subdural hematoma, axonal injury, concussion, blood-brain barrier (BBB) dysfunction and also coma (Dixon 1991; Lighthall 1988; Smith *et al.*, 1995; Morales 2005; Lighthall *et al.*, 1990). This model has been used on ferret, rats, mice, swine, and monkeys, and is delivered to the intact dura in the form of a unilateral craniotomy. This technique is most often applied between the bregma and lambda and results in a deformation of the underlying cortex (Dixon *et al.*, 1991).

One of the positive attributes associated with the model in comparison with other TBI models is the enhanced control of mechanical factors, time, velocity and depth of impact. All these factors make it a more useful model than the FPI model for biomechanical studies of TBI (Mao *et al.* 2010; Hall *et al.*, 2005). An additional positive attribute of CCI, in comparison with other models that are gravity-driven devices, is the relative increased safety allowing for increasing cortical deformation and impact velocity, providing data in severe, but survivable injuries (Goodman *et al.*, 1994; Saatman *et al.*, 2006). With the elevation of cortical deformation and impact velocity, we see an increase in histopathological severity of CCI. This allows for the adjustment of the severity injury deemed proportionate for specific experimental requirements. Within this model, functional deficit such as cognitive impairments are measured using the Morris water maze test, and are strongly associated with both the depth of deformation and the velocity of the impact in mice and rat models (Fox *et al.*, 1998; Marklunc and Hillered, 2011). The model shows a deficit in emotional behavior that was also quantified in the forced swim test, elevated-plus maze, and pre-pulse inhibition of acoustic startle in mice (Kochanek *et al.*, 2002). In relation to injury severity, we see a strong association of elevated cognitive deficiencies that suggest the threshold for emotional changes post experimental TBI is low (Washington *et al.* 2012). Despite its cost and complexity, the swine CCI model has the ability to replicate injury that shares common pathologic features similar to human TBI. This large animal model presents us with the opportunity to gather physiological data post brain injury, in a

scenario that would mimic the intensive care unit and may further facilitate translation of animal data into clinical practice (Alessandri *et al.*, 2003).

1.6 Treatment Strategies

1.6.1 Antiepileptic Drugs

Seizures are a common post-TBI symptom that affects roughly 50% of patients up to 15 years following a penetrating injury. Post-traumatic injuries can be identified within weeks or as late as years, with incidence rates of 4%-25% and 9%-42%, respectively, in untreated patients (Klein *et al.*, 2008). According to the Brain Trauma Foundation (BTF) management guidelines, level I recommendations for anti-seizure prophylaxis do not exist, but instead level II recommendations suggest using anticonvulsants such as phenytoin and valproate to prevent early seizures. These anticonvulsants do not prevent late seizures. Additional anticonvulsants, such as phenobarbital and carbamazepine are generally not used due to adverse effects and pharmacodynamics profiles. Besides acute therapy, there seems to be a relative paucity in pharmacological options for epilepsy post-TBI. Though, recent preliminary trials have revealed some promising agents that are listed below.

1.6.2 Anti-Inflammatory Agents

Damage that is acquired through a primary injury is almost impossible to treat, which is why researchers and clinicians focus on secondary cell death when developing treatments for TBI. The delayed onset of damage that is caused by secondary events allows for a window of opportunity to treat TBI victims. This makes it extremely

convenient to target neuroinflammation for TBI therapy. It should be taken into consideration when developing treatments for neuroinflammation in TBI; inflammation can be both beneficial and detrimental. Prior studies have indicated that high concentration of anti-inflammatory agents can actually lead to worse outcomes.

Multiple stand-alone drugs have been implicated and used for the targeting and treatment of inflammation in TBI. Minocycline, a tetracycline derivative that holds anti-inflammatory properties, is one of these potential treatment drugs for TBI (Lampl *et al.*, 2007; Ma *et al.*, 2007). In studies using animal models, minocycline showed improvement in both tissue damage and inflammation that lead to improved outcomes. The drug decreases the secretion of proinflammatory cytokines and chemokines, that when taken together with other mediators in inflammation, can reduce nitric oxide through direct scavenging and inhibition of over-activation and proliferation of microglia cells (Jordan *et al.*, 2007; Sanchez Mejia *et al.*, 2001). The targeting of microglial cells is vital to linking together the reduction of cytokines IL-1beta, IL-6 and MMp-9, all of which mediate the proinflammatory response (Jordan *et al.*, 2007; Sanchez Mejia *et al.*, 2001).

An additional stand-alone drug that is being investigated is melatonin. Melatonin is a hormone from the pineal gland, and has shown to possess some neuroprotective properties (Campolo *et al.*, 2013). As a lipophilic enzyme, it can easily access cell membranes to carry out its function. Its anti-inflammatory properties are thought to

inhibit microglial activation and lower secretion of proinflammation cytokines that include IL-1beta and TNF-alpha (Wang *et al.*, 2013; Ding *et al.*, 2014) which are directly connected with increased inflammatory response. Successful trials have showed decreased brain edema and reduced cortical neural degeneration, suggesting improvements in cognitive deficits (Ding *et al.*, 2014).

G-CSF, a drug that has been approved by the US Food and Drug Administration for TBI treatment, is a cytokine that acts through receptor-mediated transport by recruiting endogenous stem cells, found in bone marrow into the peripheral blood. This allows for the stem cells to act as promoters for neuroprotection at the injury site (Acosta *et al.*, 2014). Experimental date has suggested that G-CSF can cross the BBB and bind to the G-CSF receptor on neurons and microglia leading to: inflammatory cytokine down-regulation, angiogenesis promotion, and antiapoptotic triggered pathways (Acosta *et al.*, 2014). Stem cells mobilized from bone marrow can additionally interact indirectly in the periphery through synthesizing and releasing trophic growth factors, chemokines, and cytokines that contribute to the protection of damage tissue and lead to improved brain repair (Acosta *et al.*, 2014).

TBI is highly implicated in long-term neurodegenerative diseases and is linked to the risk factors in the development of Alzheimer's disease (AD). A hallmark sign of AD or similar neurodegenerative diseases are the presence of Beta amyloid (AB) peptide deposits. These deposits increase following a TBI in both animal models and patients

who have suffered head traumas (Ekici *et al.*, 2014). Lithium has the ability to inhibit GSK-3 activity through alleviating beta amyloid deposits (Yu *et al.*, 2012). A study in mice treated with lithium following a TBI resulted in significant reductions of beta amyloid deposits through B-secretase inhibition. Lithium is thought to block Beta-secretase 1 (BACE1) expression, upregulated in humans and rats following head damage, resulting in a reduction of AB load. Partially, this leads to the contributory effects of improved spatial learning/memory and improved hippocampal preservation, determined by enhanced neurobehavioral testing with TBI and sham groups (Blasko *et al.*, 2004; Loane *et al.*, 2009; Uryu *et al.*, 2007; Yu *et al.*, 2012).

HDAC Inhibitors. The regulation of histone deacetylase (HDAC) inhibition is also thought to be neuroprotective in nature. Its ability to restore histone acetylation levels and correct transcriptional deficits involving multiple models of brain disorders has been a main focus in TBI rodent models (Chuang *et al.*, 2009; Dash *et al.*, 2009; Shein *et al.*, 2009; Zhang *et al.*, 2008). A recent study in 2013 by Fengshan and colleagues demonstrated the effects of pooled sub-therapeutic valproate (VPA)-an additional mood stabilizing drug- and lithium in a mouse model of TBI. Both lithium and VPA maintained the BBB through the inhibition of HDAC and TBI-induced MMP-9 overexpression (Yu *et al.*, 2013; Wang *et al.*, 2012; Chiu *et al.*, 2013). Combined sub-effective doses of lithium and VPA resulted in a significant decline of lesion volume while simultaneously preserving the BBB integrity in three-day post-TBI. This was not seen in treatment groups when lithium and VPA were used alone. Lithium alone did

show enhancements in fine motor neurobehavior at days 14 and 21, but combined treatments had more profound effects with significantly less Fluoro-Jade B (FJB)- a dye utilized for the labeling of degenerating neurons-positive cells at days 7,14, and 21 (Yu *et al.*, 2013). Both drugs at the sub-therapeutic levels resulted in, fewer side effects, improved tolerance to long term use, and multiple signal pathway targeting, owing to the diverse pathophysiology of TBI (Chiu *et al.*, 2013).

1.6.3 Neurosteroids

Neurosteroids are steroids synthesized in the brain. There is limited literature regarding neurosteroids in TBI. VanLandingham group tested the effect of a neurosteroid in model of CCI (VanLandignham *et al.*, 2007). CCI was performed on the animals and administration of allopregnanolone (ALLO) was administered (8 mg/kg) using an I.P. dose at 1 h and then a subcutaneous dose at 6 h and followed every 24hr post injury. Brains were collected at 48 h, used for mRNA analysis, and at 24 and 72 h post-injury for protein analysis. Animals treated with ALLO showed significantly lower CD55 protein expression when compared with controls. This gene encodes a glycoprotein in the regulation of the complement cascade. Through the binding of this encoded protein to other complement proteins, there is acceleration in their decay. This decay leads to a disruption of the cascade and prevent damage to the host cells. There was significantly lower expression of the CD55 gene in vehicle-treated injured brains, compared to ALLO treatments samples. Post-injury administration of the neurosteroid ALLO increases CD55 gene expression at 48 h in comparison to the vehicle-treated TBI and shams. The

treatment maintained protein expression at 24 and 72 h at the level of the uninjured controls, and when compared to the vehicle-treated injured brains, there was a significant decline in CD55 protein abundance. ALLO specifically has been documented to reduce edema, inflammation and lipid peroxidation in multiple models of brain injury, and now can be implicated in the control of expressed inflammatory mediators through the CD55-regulated mechanism (VanLandignham *et al.*, 2007).

Ganaxolone (GX, 3α-hydroxy-3β-methyl-5α-prengan-20-one) is the 3β-methylated analog of ALLO. As its analog compound, GX is a positive allosteric modulator of $GABA_A$-benzodiazepine receptor-chloride ionophore complex (Carter *et al.*, 1997; Nohria and Giller, 2007). GX is a CNS-selective $GABA_A$ modulator that has well-characterized targets in the brain, and anxiolytic and anticonvulsant effects. The compositions of the $GABA_A$/benzodiazepine receptor complexes are altered under stress-induced situations, resulting in the lack of response to hallmark benzodiazepine ligands. Additionally this receptor complex is involved in the dysfunctional productions of behaviors proceeding a stress or traumatic event are well published in both preclinical and clinical studies. Studies that involved anxiety disorders and depression in humans showed postmortem alterations in $GABA_A$ receptor binding and receptor subunit composition, as well as in GABA synthesis and transport (Vaiva *et al.*, 2004; Geuze *et al.*, 2008). This synthetic neurosteroid exhibited effectiveness in a large spectrum of animal models of epilepsy (Gasior *et al.*, 2000; Reddy and Rogawski 2000 a,b). Powerful anti-seizure effects of GX in the amygdala-kindled model in mice support the use of this substance in the

treatment of temporal lobe epilepsy, and according to Reddy and Rogawski (2009), GX treatment (7 mg/kg, sc) significantly reduced the frequency of spontaneous seizures in rats. Clinical studies of GX have shown to be well tolerated in adults and children. In early phase II studies, GX showed to be beneficial in adult patients with partial-onset seizures and in epileptic children with a history of infantile spasms. These results raise the possibilities that GX may provide a specific treatment for TBI in humans.

GX is the sole neurosteroid-like agent that has been utilized in human clinical trials for the treatment of epilepsy (Nohria *et al.*, 20010). Over the past decade, GX has been used in varying clinical trials to assess the efficacy and safety in the treatment of epilepsy (Laxer *et al.*, 2000) In a recent randomized, double-blind controlled study a comparison of GX (500 mg t.i.d.) to placebo in 147 adults with partial onset seizures who were refractory to conventional antiepileptic drugs. The primary endpoint, mean weekly seizure frequency, and the percent change from baseline in weekly seizure frequency were significantly improved in the GX group. More than 900 subjects have received GX in human clinical trials; where common treatment-related adverse events are dizziness and fatigue, but discontinuation rates have generally been similar to that of placebo. In complete, GX appears to be an efficacious, well-tolerated and safe treatment for partial seizures and would be an effective treatment for TBI.

CHAPTER II

AIMS AND OBJECTIVES

2.1 Neuroprotection Effects of GX

The main goal of this project is to test the effectiveness of a novel combination neuroprotection therapy for traumatic brain injury (TBI). TBI affects millions of people worldwide every year. In this study, we evaluated the neuroprotective potential of the neurosteroid Ganaxolone (GX) in a mouse TBI model. GX is a synthetic neurosteroid related to allopregnanolone that has sedative, anxiolytic, and anticonvulsant effects. To our knowledge GX has not been used as a neuroprotective agent for TBI. We utilized a controlled cortical impact (CCI) model, which simulates aspects of concussions, brain contusions, and hemorrhages seen in human TBI. The outcomes were analyzed based on several primary protection indices such as extent of neuronal injury, neurodegeneration, neuroinflammation, post-traumatic seizures, and behavioral/cognitive dysfunction.

CHAPTER III

MATERIALS AND METHODS

3.1 Animals

C57BL/6 adult male mice (8 to 12 weeks of age; weight 24-38g) were housed four to a cage with free access to food and water. The mice were housed in an environmentally controlled animal facility under a 12-h light/dark cycle. The animals were cared for strictly in compliance with the guidelines outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). All animal procedures were approved by our university's Institutional Animal Care and Use Committee.

3.2 Controlled Cortical Impact Model of TBI

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). They were positioned in the stereotaxic frame. Once the head was restrained, an approximate 5-mm craniectomy was performed using a portable drill over the left parietal temporal cortex and the bone flap was removed. Mice were subjected to CCI using the Hatteras Pinpoint Instrument (PCI3000 Precision Cortical Impactor™) with the actuator directly mounted on the stereotaxic instrument (Illustrated in Fig.2). The impactor 3.5 mm tip accelerated down to the 2.00 mm distance, reaching a preset velocity of 4.5m/s, and the applied electromagnetic force remained there for the dwell time of 100 ms, and then retracted automatically. The contact sensor indicated the exact point of contact for reproducible results. These CCI

parameters lead to an injury that is considered to be severe. A rounded two-channel electrode (model MS333/1; Plastics One, Roanoke, VA) was stereotaxically implanted in the right hippocampus (2.9 mm posterior, 3.0 mm lateral, and 3.0 mm below dura) (Franklin and Paxinos, 1997) and anchored with dental acrylic to three jeweler's screws placed in the skull. Before the dental cement was applied, a small plastic slip was placed over the impact zone to prevent the dental acrylic from contacting the brain. Following surgery, the animal was allowed to recover for a period of 7 to 10 days.

The experimental design and protocol is illustrated in Fig.3. Sham operated mice underwent the same surgical procedures (craniotomy) with the exception of the traumatic impact. For the first 2h post-CCI, mice were closely monitored in their cages. Histopathological and functional outcomes were compared between the untreated mice and treated mice at 24 h, 72 h, 7 d, 14 d, 30 d, 60 d, 90 d, and 120 d after trauma (2-4 mice per time per experimental group). Sham animals were tested at 0 h 24 h 72 h 7 d and 14 d.

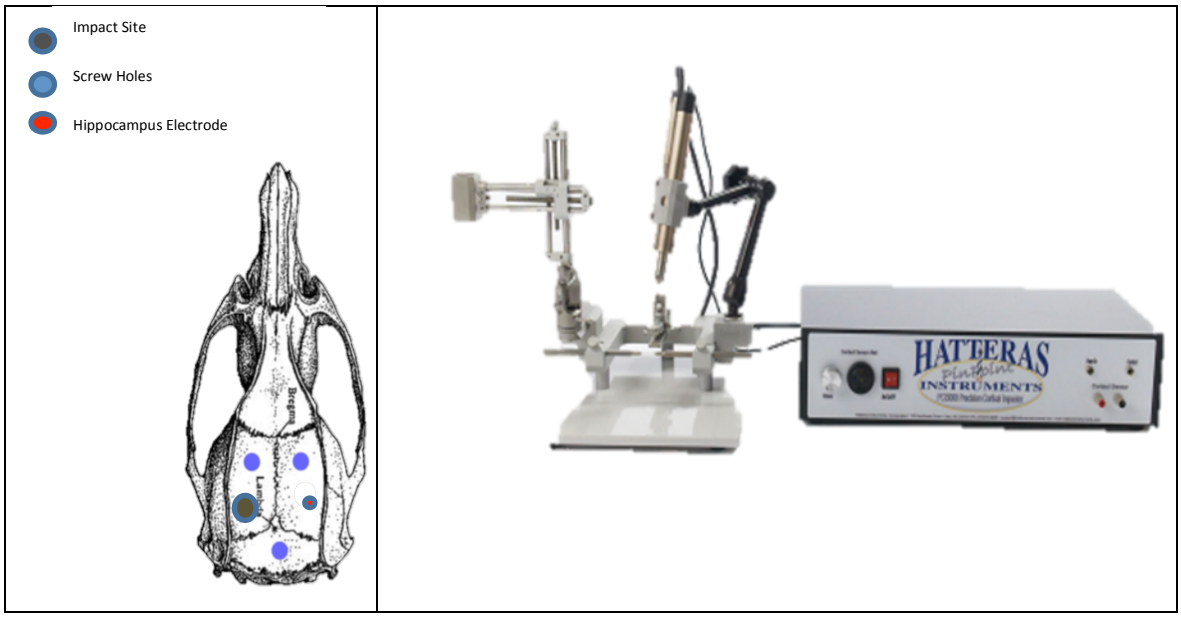

Figure 2. CCI model of TBI in mice. Hatteras Pin-Point Instrument that was used to perform TBI and the stereotaxic position of TBI impact site, anchor screws and hippocampal electrode implant.

Study Design:

Group 1: Sham group (N=10)

Group 2: CCI Control (N=10)

Group 3: CCI + GX Treatment (N=10)

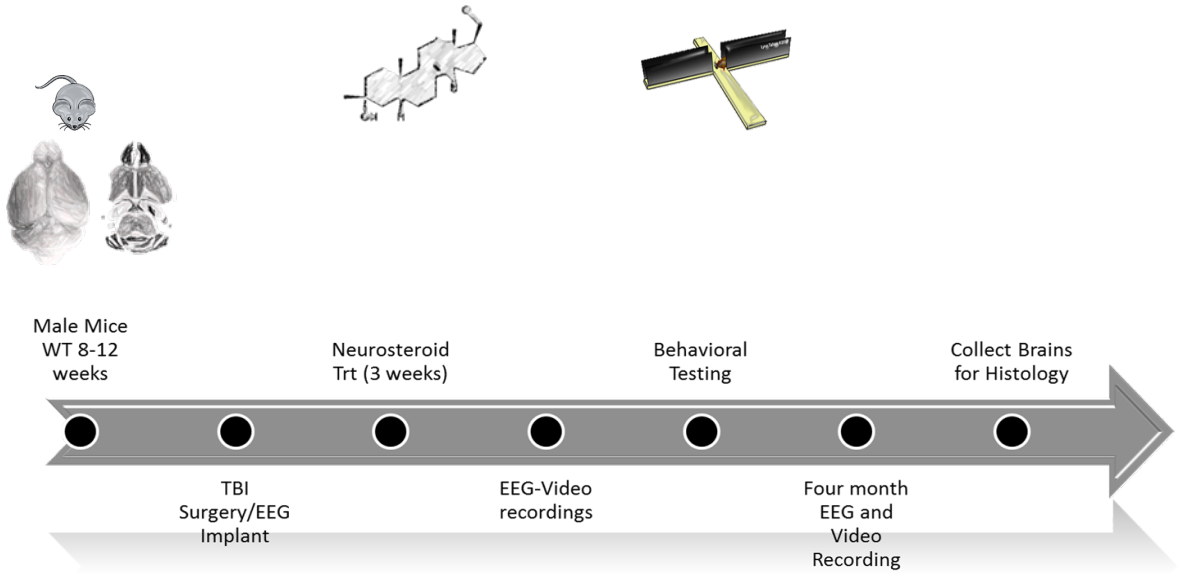

Figure 3. Overall protocol for TBI model and epilepsy studies.

3.3 Brain Perfusions and Histology

Mice were anesthetized with ketamine-xylaxine mixture and transcardially perfused with 4% formaldehyde solution (Fisher Scientific) in sodium phosphate buffer (PB, pH 7.4). Following this, the the brain was carefully removed from the skull of each animal and post-fixed in 4% formaldehyde for 16 h at 4°C. The brain tissues were next treated with PB (for 24 h), 10, 20 and 30% sucrose solution in PB for 72 h each respectively and then rapidly frozen with O.C.T compound on dry ice (Rao *et al.*, 2006; Kuruba *et al.*, 2011). Serial sections (30 µm thick) were cut coronally through the forebrain containing the amygdala and the hippocampus, approximately from bregma −0.24 to −7.44 mm

(Paxinos and Watson, 2007). The sections were collected serially in 24-well plates filled with PB. Every 10th section through the entire mouse hippocampus was then selected from all animals. Sections were taken at 300 μm intervals and processed for neuronal nuclei antigen (NeuN, total neurons), parvalbumin (PV, interneurons) and Fluoro-jade (FJB, degenerating neurons)-immunoreactivity.

The stereology system consists of an Olympus BX53 microscope (Olympus, Tokyo, Japan) fixed with a DP73 cooled digital color camera (Model: DP73-1-51, Olympus, Tokyo, Japan) or ORCA-R2 digital CCD camera (Hamamatsu, Hamamatsu City, Japan) for immunofluorescence images (Figure 1). A motorized stage (Model: H101ANNI, Prior Scientific, Rockland, MA, USA) controlled by universal microscope automation controller with encoder (Model: 500-H31XYZEF, ProScan III, Prior Scientific, Rockland, MA, USA) and Proscan III joystick (Model: P-PS3J100, Prior Scientific, Rockland, MA, USA). The BX53 microscope is fixed with a 1.25× objective (PLAPON1.25×, numerical aperture (NA) = 0.04, working distance (WD) = 5.1 mm, Olympus), 10× objective (UPLSAPO10X2, NA = 0.4, WD = 3.1 mm, Olympus), 20× objective (UPLSAPO20XNA = 0.75, WD = 0.65 mm, Olympus) and 60× oil immersion objective (UPLSAPO60XO, NA = 1.35, WD = 0.15 mm, Olympus). Olympus immersion oil type-F (IMMOIL-F30CC, Olympus) was used with the 60× oil immersion objective. Appropriate cleaning supplies are kept readily available for use such as lens cleaning solution, lens paper, and 99% ethanol for cleaning the slides. The stereology software used in this experiment is newCAST (Version: VIS4.6.1.630, Visiopharm, Denmark). A calibration slide with a

standard Visiopharm calibration grid embedded on the glass sheet in the center is included with the newCAST VIS software.

Super images of the entire slide containing hippocampal sections (three slices in each slide) were acquired by stereology newCAST software (Visiopharm, Denmark) with a 1.25× objective on the Olympus BX53 microscope. Before cell counting, the hippocampus was delineated into a single region of interest, which contained the dentate gyrus, dentate hilus, and cornu ammonis regions I-III. Neuronal density was performed on the hemisphere of the brain, which had received the craniotomy and TBI incident. Tissue volume was calculated using an application in the NewCast software that combined Cavalieri's theory and vertical sectioning. To produce an accurate volume estimate using the application, at least 200 points are required to be counted within the region of interest. Crosshairs in groups of 3X3 were overlayed onto the computer image of the slide and appeared as a guide for the volume calculation. Volumes for slides were calculated using the 10X objective lens. After the volume is found for the hippocampus, cell density calculations are performed using a separate application in the newcast software.

Because of the nature of TBI, the stereology procedures were performed on the hippocampus as a whole, rather than divided up into 5 regions. In many cases with this experiment, the TBI was chronic/severe and sections of tissue were missing and/or too damaged to perform stereology. Because of this, volumes between control and TBI

animals were compared to find how much tissue was lost due to injury—and cell density counts were performed in the same manner.

3.4 Motor Function

We used two different motor function tests: (1) Rota-rod test and (2) inverted screen test.

3.4.1 Rota-Rod Test

The ability of a rodent to maintain balance and keep pace with a rotating rod has been used in varying degrees of success in the past decade to assess motor function. The intent of this test is to subject the mouse to walking on a rotating rod of fixed diameter at a maintained speed over a predetermined period of time unto the animal can no longer maintain its position. The rota-rod apparatus used in this study consisted of a central drive rod connected to a stepper motor (IITC Life Science Instrument) that was divided into five separate testing stations. The speed at which the rod rotated can be accelerated up from 0 rmp to over 40 rpm over a set time period.

Mice that suffered from a traumatic injury were tested on the rota-rod 24 hours following the surgical procedure. The apparatus was set at a specific acceleration of 5 rpm over 60 sec. This was maintained as a good standard for young adult mice, although it should be noted that juvenile and older animals performed poorly during this task. Five mice were placed on the rota-rod, one per testing station, and then the timer and rotating rod was initiated. Our model came equipped with a timer that began

when the protocols were set and stopped when the animal fell to the floor of the apparatus, as was detected through a weight sensor. The mouse is required to walk continually in a stationary position. The latency to fall from the rotating rod was determined and taken as a measure of motor function.

Although locomotor testing has its skeptics, changes in any behavior can elucidate critical information in the diagnoses of neurological deficits. Brain injury models employed by many researchers can produce subtle or often pronounced alterations in motor behavior. However, these animal models are not devoid of drawbacks, which include profound changes in motor function that can muddle the interpretations of the behavioral results. Therefore, it is critical for neuroscientist to be conscious of and to characterize these changes carefully. The following examines several methods for assessing motor and exploratory functions in the TBI mouse model.

Locomotor and exploratory behavior may additionally be skewed by several other factors such as time of day (mice are generally nocturnal animals and are significantly more active during dark periods); anxiety (animals may or may not be more active dependent on what situation they are exposed to); state of wakefulness or arousal; motivation; age; general health; and genetic strain. All of these factors require detailed experimental designs and it is therefore prudent to control for these and maintain consistency from the outset. However, natural variations in activity and stress levels

more often than not exist between mice of the same strain despite controlling for all the factors. Thus, group-sizes that will balance out the statistical analysis are required.

3.4.2 Screen Test

For all tests, the mice were brought to the experimental room 5-20 minutes before testing to ensure arousal. Mice were returned to the home cage after each motor test to permit recovery of muscle strength. Evaluation for motor impairment was carried out using an inverted screen test that determines an animal's ability to support its own body weight by grasping a grid (Coughenour *et al.*, 1977). Mice (n=14/group) were placed on a horizontal metal grid (consisting of 1.5-mm-diameter rods situated 1 cm apart), which was then inverted and suspended roughly 45 cm above their home cage. The latency that the mouse remained on the grid was measured. Animals that fell from the grid within 10s were scored as positive for motor impairment.

Falling between 1-10 sec = 1 Falling between 11-15 sec =2 Falling between 26-60 sec + 3 Falling after 60 sec = 4.

3.5 Elevated-Plus Maze

The elevated plus maze test is one of the most widely used tests for measuring anxiety-like behavior. The test is validated on the natural aversion response of mice for open and elevated areas, and additionally on their natural spontaneous exploratory behavior in novel environments. The apparatus consists of open arms and closed arms, crossed in the middle perpendicularly to each other, and a center area. Mice are allowed access

to all of the arms and are permitted to move freely between them. The number of entries into the open arms and the time spent in the open arms are used as indices of open space-induced anxiety in mice.

The apparatus used for the elevated plus maze test is comprised of two open arms (25x5x0.5 cm) across from each other and perpendicular to two closed arms (25x5x16 cm) with a center platform (5x5x0.5 cm). The open arms have no walls, whereas the closed arms have high (16 cm) walls to enclose the arms. The entirety of the apparatus is 50 cm above the floor and is placed in an empty circular tank (100 cm diameter, 35 cm tall; and is normally used for the Morris water maze task) to protect the mice that fall or attempt to escape during the experiment. The apparatus is made of black glossy plexi-glass.

Mice were housed with a 12-h light/dark cycle. Behavioral testing was performed between 9:00 AM and 6:00 PM. All the experimental mice were transferred to the behavior testing room 30 min prior to beginning the first trial. The behavior testing rooms is soundproof and the illumination level is maintained at an appropriate lux. A mouse is placed in the center area of the maze with its head directed toward a closed arm. The number of entries (an entry is defined as the center of mass of the mouse entering an arm) into each arm and the time spent in each of the arms are recorded and serve as indices for measuring anxiety-like behavior.

Mice were permitted to move uninhibited about the maze for 5 min. Each mouse received three trials-one before surgery, one 7 days post-surgery, and the final one 14 days post-surgery. The number of entries into each arm, the time spent in each arm, and the percentage of entries into the open arms were calculated. Following each trial, all arms and the center area was cleaned with 70% isopropyl alcohol. This served to eradicate odor and served to prevent a bias based on olfactory cues, thus allowing for the conduction of tests under controlled conditions.

Infrequently (in less than 1% of mice tested), mice run to the edge of the open arms and fall off. When this occurred, the mouse was rapidly picked up and placed back onto the open arms of the maze. This behavior was recorded on the data sheet and taken into consideration when the behavioral data was analyzed. Behavioral data from an animal that does this would usually be excluded from analysis, however due to the nature of our study the animal's data was still used. If freezing were to occur due to a noise or movement, a note was taken on the data sheet and considered an anomaly if it was an extended period of time (more than 100s on the open arm), and was deemed an exclusion criteria. However, the animal was then tested as a later date to be commensurate with other animals in its cohort.

3.6 Assessment of Cerebral Edema

Brain water content, a sensitive measure of cerebral edema, was quantified using the wet-dry method (Dempsey *et al.*, 2000; Hewett *et al.*, 2006). At 24h and 72h post-

injury, time points associated with significant edema formation following experimental TBI (Kiening *et al.*, 2002), brain water content was estimated in a 3 mm coronal tissue section of the ipsilateral and contralateral cortex regions of the brain centered on the impact site. Whole, fresh brain was weighed using weigh-paper, dried for 72 hours at 57 degrees Celsius in a Thermo Scientific oven and reweighed to obtain a dry weight. Brain water content was defined as % of water calculated as (wet weight-dry weight)/ (wet weight) x 100. Additional equations used were Water content = (wet weight – dry weight)/dry weight and %Tissue swelling = 100*(final wet weight – initial wet weight)/initial wet weight.

3.7 Neurological Motor Function

The composite neuroscore test was assessed at 24h, 72h, 7d, 14d, 21d, 30d, 60d, 90d, and 120d post-injury using seven 4-point tests (maximal composite neuroscore = 28). Animals were scored on a 4 (normal) to 0 (severely impaired) integral scale for left and right forelimb flexion, left and right lateral pulsion, left and right hind limb flexion and the aptitude to stand on an inclined angle board in the horizontal and vertical positions.

3.8 EEG Analysis

For continuous EEG recordings, two surface electrodes (cortex and cerebellum) and a depth electrode in the hippocampus was implanted (4mm AP. 2.3mm ML. 3.4mm deep of DV) in the mice directly following the TBI event. Mice were then allowed to recover for 7-10 days. Once this recovery period was over the animals were placed into single

house cages, connected to an apparatus to record EEG data via the hippocampus electrode. The electrographic activity of the TBI-induced seizures was defined as the appearance of large amplitude repetitive discharges (>0.5 Hz with at least double the amplitude of the baseline).This activity was acquired from the hippocampal electrode using pClamp 10 software 2.0KHZ sample rate, with Digidata 1322A interface (Molecular Devices) through a Grass CP511 preamplifier (Astro-Med). This system (up to sixteen channels) allowed simultaneous monitoring and recording of animal behavior with high resolution video (1920x1080 pixels) and electrographic activity in awake, freely behaving mice, that have free access to water and food. Behavioral seizures (Video EEG recordings) were rated according to Racine's scale (Racine, 1972) as modified for the mouse: stage 0, no response or behavioral arrest; stage 1, chewing or facial twitches; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, falling. At the end of the study, mice were anesthetized and perfused transcardially with paraformaldehyde for Nissl staining to verify the electrode placement, and TIMM's stain to measure mossy fiber formation.

CHAPTER IV

RESULTS

CCI injury to the lateral cortex causes focal brain injury is highly associated with a number of contusion injuries that include intra-parenchymal petechial hemorrhages. Such hemorrhages are followed closely in incidence by epidural and subdural hematomas (see Fig.4A). Our histological analysis showed large lesions in the cortical gray matter, as well as axonal injury in the adjacent white matter, corpus callosum, and capsula interna (Fig.4B). In addition, there were areas of degenerations in the cortex, hippocampus and thalamus. Such anatomical changes are associated with a spectrum of cognitive and motor deficits (Thompson *et al.*, 2005). Recently Hunt *et al.* showed that CCI-induced TBI in the lateral cortex would trigger the development of PTE in CD1 mice. Behavioral seizures were seen in 20% of animals with mild injury and in 36% with severe injury 42-71 days post-injury. Our data also showed increased occurrences of electrographic spontaneous seizures after lateral CCI injury in C57BL/6 mice.

4.1 TBI-Induced Brain Injury (Histology)

Nissl staining was performed on an 8-12 week old C57BL/6 male mouse brain that had received a severe TBI using the CCI model (Fig.1B). A visible cavity can be seen extending into the hippocampus layer. This severe TBI event showed amplified distortions and cranial swelling post-impact, where the hippocampal and cortical

regions on the ipsilateral side are completely gone. We used this CCI model to determine the effects of severe TBI through the measurements of tissue deformation, neuronal death, and histopathological changes.

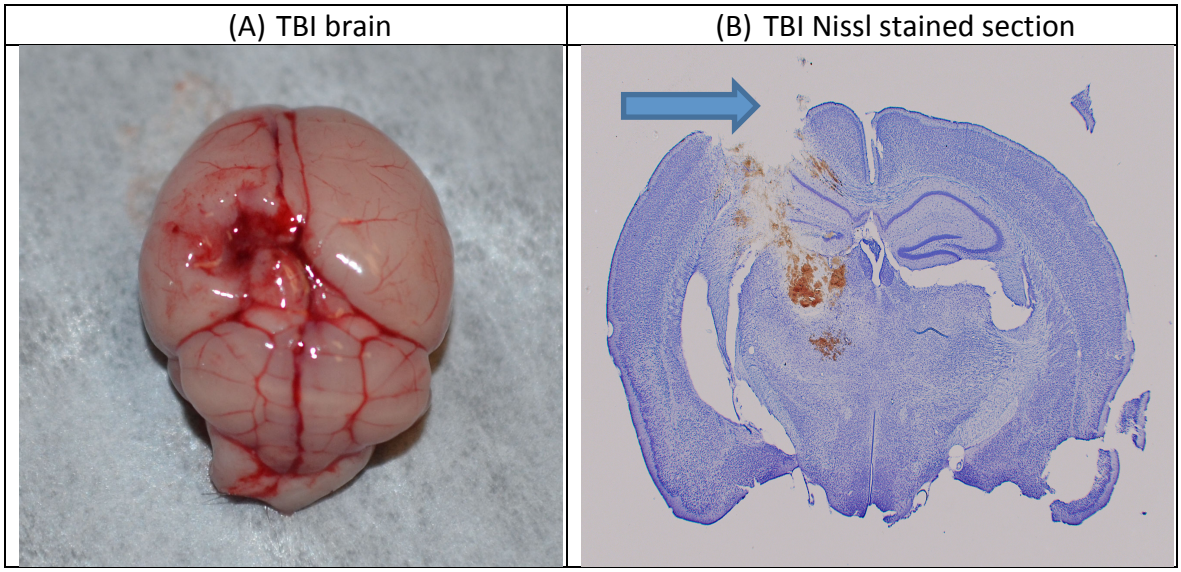

Figure 4. TBI impacted brain. (A) Sample CCI mouse brain representative of TBI injury to the ipsilateral cortical region. (B) Nissl staining of a severe TBI in a mouse brain section.

4.2 Effect of GX on TBI-Induced Rota-Rod Motor Impairment

We examined the motor ability of the mice of all three groups (Sham, Control, and GX treated) with the rota-rod task. As shown in Figure 5, all of the mice in the Sham group were able to stay for the maximum time (60s) on the stationary rod in the pre-sessions.

The majority of the Control mice were unable to stay on the stationary rod. The GX mice mirrored the Sham mice. The effect of Sham surgery, Control mice and GX treated mice on time spent on the rotating-rod is shown below in Fig.5. Control mice showed no significant improvement over the course of the 14d period, while the GX mice exhibited a significantly increased performance over the 14d-testing period. Because of these pronounced deficits, it would be unwise to conduct cognitive experiments with a significant motor component (e.g., Morris water maze) during this time period. It can be concluded that mice treated with GX showed improved functional motor function on the Rota-rod test.

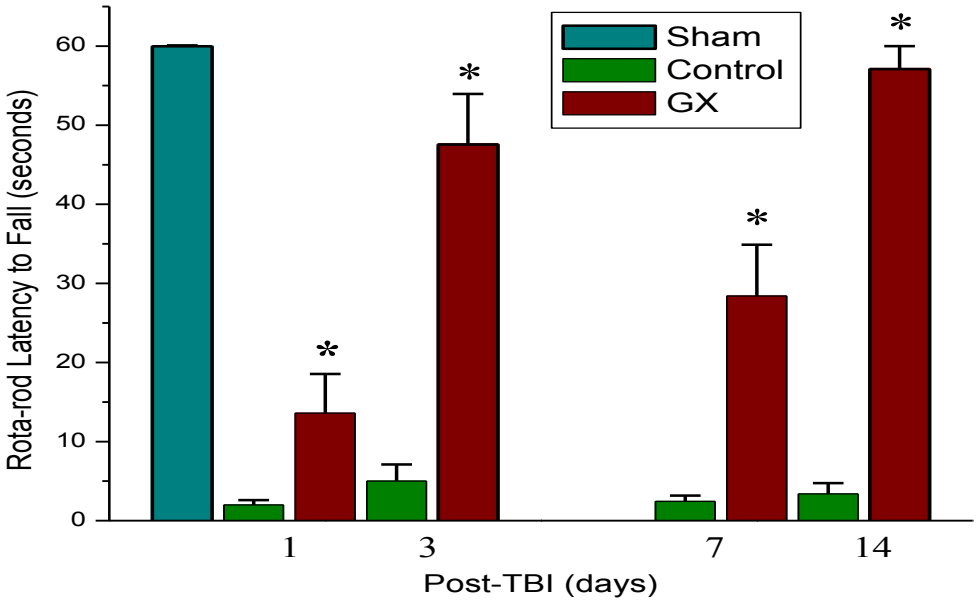

Figure 5. Effects of GX on the Rota-Rod apparatus used to measure the latency to fall in seconds.

4.3 Effect of GX on TBI-Induced Screen Test Motor Impairment

The screen test was used to measure grip strength in controlled versus GX treated mice. Our data was collected at 1d, 3d, 7d, and 14d, although preliminary, showed that mice treated with GX exhibited increased latency to fall over a period of time (Fig.6). Thus it can be concluded that mice treated with GX remained longer on the grid when compared to control mice. Additionally when compared to Sham mice, the GX treated mice were almost completely normalized, showing little neurological deficits in grip strength over a period of time.

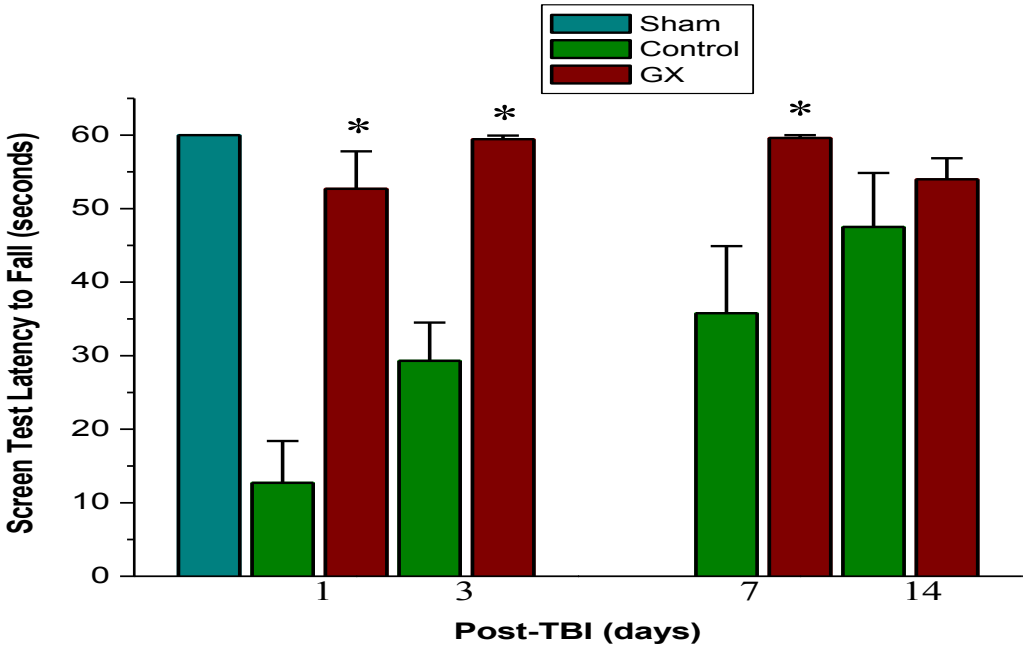

Figure 6. Effects of GX on the Screen Test used to measure the latency to fall in seconds.

4.4 Effect of GX on TBI-Induced Sensory Motor Functional Impairment

The composite neuroscore data assessed neurological deficits in our Control mice but showed no significant deficit in mice that were treated with GX (Fig.7). The average was taken of the scores and a significant difference was found between the treated group and the control animals. Additionally, the neuroscore analysis suggests normalization between the Sham animal scores and the scores of the GX treated group. We can

conclude that animals treated with GX showed improvements in neurological scores when compared to Control animals.

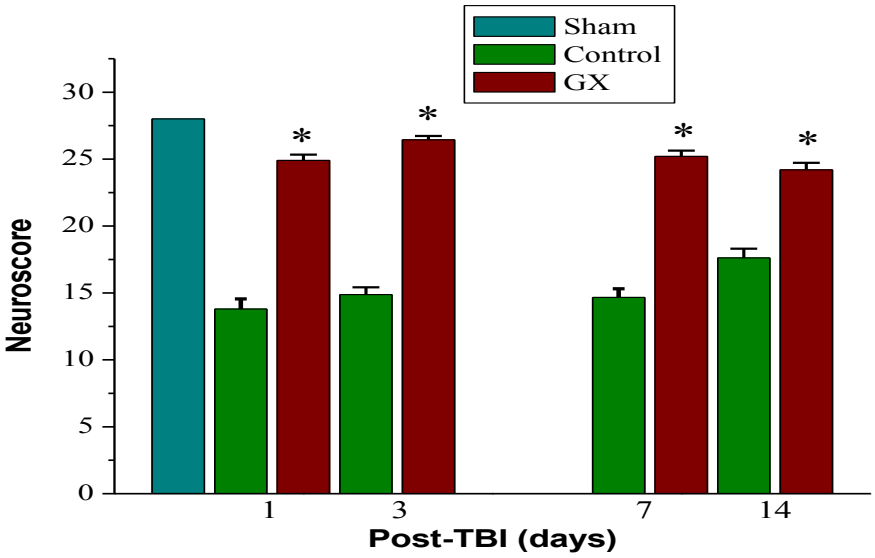

Figure 7. Effects of GX on Neuroscore data used to assess neurological deficits

4.5 Effect of GX on TBI-Induced Brain Edema

Brain edema has been a TBI target for decades now and is identified and continuously monitored in TBI patients through imaging, clinical exams, and intracranial pressure monitoring. Cerebral edema causations are cytotoxicity, and vasogenic- resulting from a disruption in the blood-brain barrier (BBB). As shown in Tables 1-3, our results indicates that animals treated with GX and Sham animals had significantly lower edema in both the percent Brain water content and the water content (g/g dry weight), while the non-treated animals had significantly increase percent changes in both the Brain water content and the dry weight comparisons. Additionally, our data showed a significant difference between the percentage of brain swelling in Sham and injured animals, but no significant difference between Sham and GX treated animals (Table 1-3). These results suggest that tissue swelling was reduced in GX treated animals and by all indications had less tissue swelling than Sham animals.

Table 1. Brain edema analysis. Measurements of % brain water content and % change between groups.

Model CCI	% Brain water content				
	Non-Injured (Sham)	Injured	Injured (GX Treated)	% Change Injured	% Change Injured GX
Contralateral	38%	76.69%	60%	39%	22%
Ipsilateral	45%	76.07%	56%	31%	11%
Contralateral	53%	76.8%	60%	24%	7%
Ipsilateral	56%	77.9%	58%	22%	2%

Table 2. Brain edema analysis. Measurement of the water content for the g/g dry weight and the % change between groups.

Model CCI	Water content (g/g dry weight)				
	Non-Injured (Sham)	Injured	Injured (GX Treated	% Change Injured	% Change Injured GX
Contralateral	0.83	3.17	1.26	282%	51.8%
Ipsilateral	1.13	3.29	1.5	191%	32.7%
Contralateral	1.28	3.3	1.39	158%	8.9%
Ipsilateral	0.615	3.53	1.52	474%	147%

Table 3. Brain edema analysis. Measurement of the brain swelling % change between groups.

Model CCI	Brain Swelling % Change
Non-Injured Sham	0.19%
	0.05%
Injured	0.203%
	0.23%
Injured (GX Treated)	0.18%
	0.038%

4.6 Effect of GX on TBI-Induced Plus-Maze Anxiety Behavior

In this study, we tested CCI Control injured mice and CCI injured GX treated mice in the elevated-plus maze 7d and 14d post-injury, which is described in detail above, to investigate the anti-anxiety effects of TBI. The results we have obtained using the elevated plus maze to determine the effects TBI are robust and replicable. As shown in Figure 8, we found that following a traumatic brain event, a significantly increased time was spent on the open arms of the plus maze of the Control mice when compared to GX and Sham treated mice. Thus, we can conclude that a traumatic event in Control mice caused a dis-inhibition behavior in the elevated plus maze test, which was not seen in the GX and Sham groups.

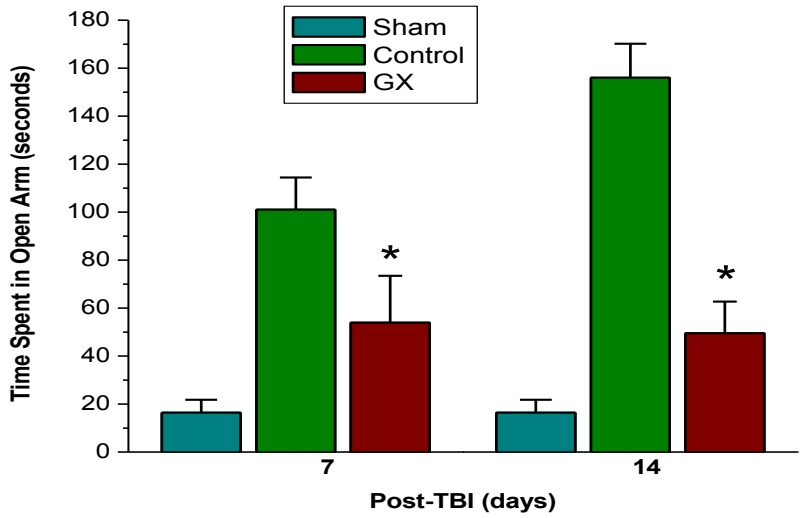

Figure 8. Effects of GX on TBI-Induced plus maze anxiety measuring time spent on the open arm in seconds.

4.7 TBI-Induced Epilepsy Development in a Control CCI Group

One of the major parts of epilepsy in regards to the pathophysiology is epileptogenesis, where a normal brain can become progressively epileptic due to injury factors. In this study, it was our goal to generate epileptogenic mice following a traumatic Injury event. The progression of spontaneous seizures is illustrated in Fig.9. Figure 10 shows two sample spontaneous recurring seizure (SRS) EEG events in two of our Control mice and a bar chart outlining the average number of seizures per day for 120d period in our control group. The first emergent (SRS) in this control group was seen at day 21 (Fig.9).

This was an unexpected result, in that the majority of the literature hadn't shown any type of seizure activity until around day 60. What was expected was the increase in seizure number per day and the clustering of the events. Our results show a relatively steady increase in the number of seizures per day in the control group, adhering to the published data, that once a brain suffers a seizure, the epileptogenesis susceptibility increases after every event.

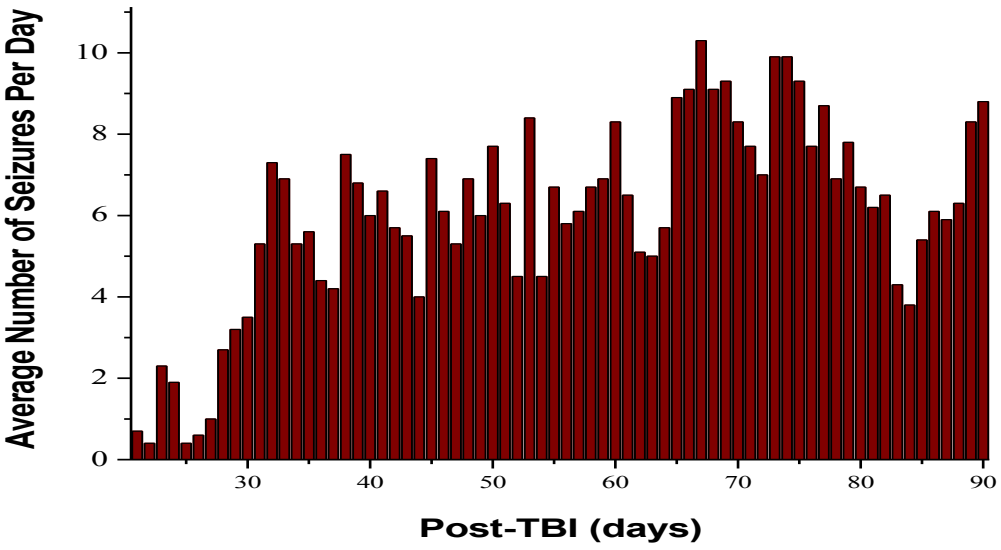

Figure 9. Development of epilepsy with spontaneous seizures in post-TBI control mice.

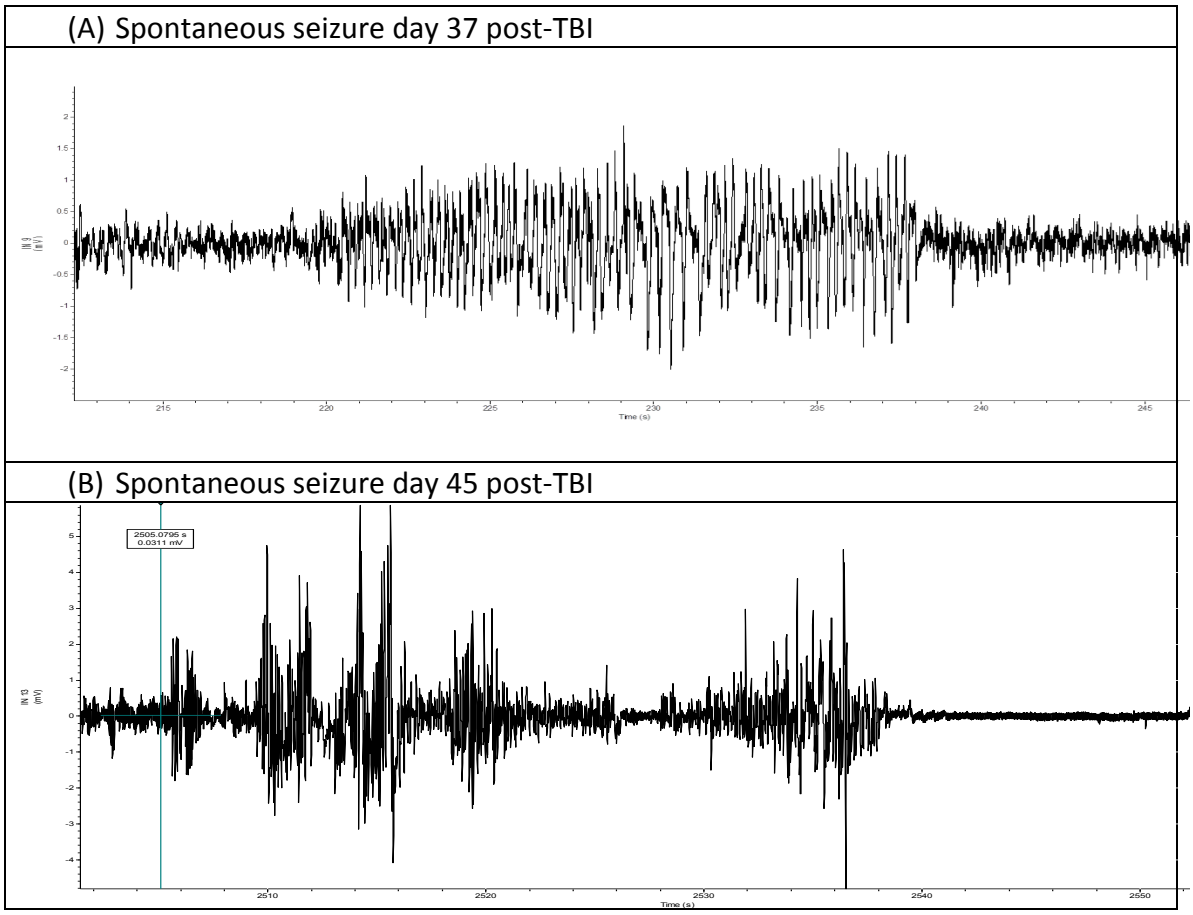

Figure 10. (A) & (B) Representative EEG traces of spontaneous seizures at various intervals after TBI in a mouse model.

CHAPTER V

CONCLUSIONS

This study was the first effort to adapt the CCI model of epileptogenesis to the AED testing of the neurosteroid GX, and it is separated from previous pre-clinical AED studies in several respects. First, the anti-epileptic effect of GX has never been evaluated in TBI patients or animal models. Second, continuoouse seizure monitoring by EEG and video recordings, has previously not been reported in TBI CCI injury models using GX treatment. Finally, the treatment itself is novel: the GX neurosteroid is resistant to tolerance mechanisms such as conversion to a 3-keto form. This model system yielded another fortuitous result- we were able to identify both convulsive seizures which were rare but lasted minutes, and non-convulsive seizures which were frequent but lasted tens of seconds. To our knowledge, this study is the first study to identify both seizure types in the TBI mouse model.

Our study established a mouse TBI model system capable of providing anatomic histopathological, electroencephalographic, and behavioral outcomes as a means to test therapies for this disease. Specifically, herein, we tested the use of GX neurosteroid after TBI and derived meaningful data on its usefulness. Our experimental studies to this point have indicated a potential for GX in improving the behavioral and functional neurological deficits observed following TBI. GX treated animals had significantly reduced brain tissue swelling, improved motor function when tested on the rota-rod, improved neurological deficits in grip strength over a period of time, a lack of dis-

inhibition effects, and improved Neuroscores. It was noted that animals within the GX edema group had slightly less tissue swelling than Sham animals. It was our assessment that animals in the Sham group were still subjected to a craniectomy, and thus was susceptible to tissue swelling as well. In conclusion our results demonstrate that GX has potent positive effects on the behavior associated deficits and edema in the CCI mouse model and considering the variety of potential mechanisms, GX may prove to be a valuable treatment for ameliorating post-traumatic epilepsy following a traumatic event.

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