Glial and apoptosis modulation following in a modified controlled cortical impact (CCI) model in juvenile male rats



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A Senior Comprehensive Project
Colleen Bodnar
April 3, 2017

I hereby recognize and pledge to fulfill my responsibilities as defined by the Honor Code and to maintain the integrity of myself and the college community.

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Pledge

Approved by the Senior Thesis Committee

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Jeff Cross

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Jeff Hollerman

Acknowledgements

To my Mom: You taught me what it means to be a strong woman. From your example, I have learned how to pursue my passion with no fear and for that I am forever grateful. Thank you for all your love and support throughout my life and encouraging me to go outside my comfort zone to make myself a better person.

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Abstract

Over 50,000 people in the United States die annually from traumatic brain injury (TBI) and even more people are diagnosed with a TBI of varying severities. The only current treatments for TBI are palliative in nature. Understanding how to prevent progressive post-injury symptoms holds significance for improving recovery from TBI. A major pathology that can be targeted through various avenues following TBI is increased inflammation leading to cell death. Microglia in the injured brain can take on a pro-inflammatory pathology which hinders axonal regeneration. Minocycline, a tetracycline antibiotic, has been found to decrease this pro-inflammatory pathology of microglia. Astrocytes also become activated after TBI and work to repair damage. However, they often become dysregulated which leads to dysfunction of neurons and neuronal circuits. Decreasing astroglial activation post injury through the administration of propentofylline (PPF) has been shown to promote repair in the brain. Damage to neurons following TBI also leads to cell death through apoptotic processes. By increasing bcl-2, an inhibitor of the proapoptotic protein, Bax, through administration of 4-phenyl 1-(4-phenylbutyl) piperidine maleate (4-PPBP), has been found to increase neuroprotection in ischemia models. As glia can be responsible or both neuroprotective and neurodegenerative mechanisms, there may be a small effective temporal window for treatment of TBI. The present study was an attempt to determine if modification of neurodegenerative processes at the level of glia and cell death processes could improve functional outcome and degeneration of neurons in the ipsilateral cortex following brain injury. To do this, a modified closed cortical impact (CCI) model was used to deliver a TBI to an anesthetized rat. Treatment with the glial and apoptosis modifiers propentofylline, minocycline, and PPBP occurred ten minutes after the induction of injury. All drug treatments significantly improved motor and spatial learning behaviors compared to untreated animals following CCI. Histological analysis revealed no significant group effects following drug administration of minocycline, PPF, or 4-PPBP.

Introduction

1. **Traumatic Brain Injury**

1.1 Prevalence of Traumatic Brain Injury: The Center for Disease Control and Prevention estimates that over 50,000 people die annually from traumatic brain injury (TBI) in the United States. This statistic places TBI among the leading causes of death in the United States, just below those who die from the flu and pneumonia (Center for Disease, 2014). Yet TBI is often overlooked as a significant area of research. Every year in the United States, around 2 million people are diagnosed with a TBI (Li et al., 2012; Tang-Schomer, et al., 2012; Turtzo et al., 2013) and over 60 million dollars are spent on care (Gatson et al., 2013). These numbers reflect only the individuals that seek treatment. Therefore, actual numbers of individuals who have suffered from brain injury could potentially be much higher. When examining the numbers worldwide, around 10 million individuals are diagnosed with a TBI annually (Hyder et al., 2007). The prevalence of TBI is an increasing and staggering statistic.

1.2 Mechanism of Injury: TBI broadly defined occurs because of a blow to the body or the head which then causes disruption and movement of the brain within the cranial cavity (Gardner & Yaffe, 2015). Movement of the brain within the cranial cavity can result in direct damage of neural tissue. Neurons have an inherit elastic capacity which protects the cells from damage. When the brain moves during normal activity, the neurons can adjust to the movements without sustaining damage. However, when the brain is impacted at a high intensity such as in a TBI, the elastic capacity of the neurons is exceeded and the neurons stretch to their breaking point (Johnson et al., 2013). The breaks along axons are responsible for various symptoms and pathology that occur after TBI.

1.3 Severities of TBI: The mildest form of brain injury (mild TBI) is most commonly referred to as a concussion (Carman et al., 2015). Concussions are commonly sports related (Stewart et al., 2012), with a conservative estimate of 3.8 million affected individuals annually in the United States alone (Covassin et al., 2013). Individuals who suffer from concussions do not normally succumb to their injuries and therefore are not included in the 50,000 that die from TBI annually. TBIs that do not fall

into the mild category are considered either moderate or severe. Clarification of TBI becomes more difficult as severity increases as there is no standard method of classification (Stein, 2015) and there are varying degrees of pathology in the clinical population. Typically, doctors employ scales to place TBI cases into either the moderate or severe category. One such scale is the Glasgow Coma Scale (GCS), which ranges from three to fifteen, with a score of three being the most severe TBI. For the GCS, the physician rates the patients' ability on three separate tasks including eye opening, verbal responses, and motor responses (Gill et al., 2005). Severe TBI victims receive a score of three to eight, while moderate TBIs produce a GCS score of nine to twelve (Stein, 2015). Besides scales, doctors also take into consideration the duration of loss of consciousness and the extent of amnesia following the injury to determine severity. These values are prone to subjectivity, making defining and treating, as well as studying TBI more difficult.

1.4 Symptoms Following TBI: The presence of symptoms differs greatly between TBI cases and can be influenced by the severity of injury, the mechanism of injury, and the history of the patient among other variables. TBI symptoms include cognitive, physical, emotional, and sleep disturbances ("Get the Facts", 2016). Cognitive deficits can entail changes in processing speed, attention, word finding, memory, concentration, and executive functioning (Kennedy et al., 2009). Physical symptoms include loss of consciousness, headache, vision disturbances, nausea and dizziness, sensitivity to noise and light, balance and motor issues, and overall fatigue. The emotional symptoms include irritability, sadness and depression, and anxiety. Sleep disturbances can include either increases or decreases in sleep time and quality ("Get the Facts", 2016). The degree and presence of these symptoms have the potential to influence recovery if they are not corrected in a timely manner. Symptoms can interfere with return to work, school, family, and community activities (Kennedy et al., 2009). Symptom profiles differ between patients and can change throughout the course of recovery. Understanding the cause of these diverse symptoms has thus become a focus for many TBI researchers.

1.5 Primary Pathology: Part of the difficulty in defining TBI comes from the fact that TBI does not result in a single pathology. In fact, many different pathologies are seen following TBI in different

combinations in each patient. Primary injuries result from the actual mechanical force on the brain (Karve et al., 2016) and ultimately lead to neuronal cell death (Raghupathi et al., 2000). Contusions, damage to blood vessels, hemorrhage, and axonal shearing are all included in primary injuries (Gaetz et al., 2004). These injuries can lead to secondary pathologies in the brain of a TBI patient.

1.6 Secondary Pathology: Because of their severity and potential implications, secondary injuries are the typical cause of death following TBI (Adams et al., 2010). These injuries can be seen from minutes to months following the primary injury depending on the individual case (Bramlett & Dietrich, 2007). Secondary injuries result from cascades of metabolic, cellular, and molecular events. These can manifest as glutamate excitotoxicity, disruption of cellular calcium homeostasis, increased free radical generation, increased lipid peroxidation, dysfunction of the mitochondria and cellular energy processes, inflammation, apoptosis, and diffuse axonal injury (Xiong et al., 2013). Combinations of secondary pathologies lead to both acute and chronic cell death (Bye et al., 2006) potentially leading to sustained functional impairment. Scientists have begun to focus on interfering with secondary injury pathologies to prevent symptoms from developing and to increase functional recovery following TBI.

1.6.1 Diffuse Axonal Injury: Diffuse axonal injury (DAI) is observed in all levels of TBI (Siedler et al., 2014; Tang-Schomer et al., 2012). DAI has been regarded as being specifically implicated in the mortality and morbidity seen following TBI (Smith et al., 2013). The forces exerted on the brain during TBI cause normally flexible axons to surpass their elastic capacity (Siedler et al., 2014; Tang-Schomer et al., 2014; Johnson et al., 2013). The damage that accumulates along these axons is characterized by swellings along the length of the axon and an accumulation of swollen axonal bulbs (Siedler et al., 2014). Progressive disruption of axonal transport competency within these varicosities can lead to disconnection of the neuron from its network and subsequent degeneration of the neurons (Johnson, Stewart, & Smith, 2013). Neuronal degeneration can be visualized

in post mortem tissue of experimental TBI animals through use of Fluorojade-C (FJ-C) which also labels activated astrocytes (Sato et al, 2001).

1.6.2 Immune Response: Another typical secondary pathology following TBI is inflammation. Inflammatory responses are upregulated following TBI (Vonder Haar et al., 2014) and begin with the activation of the innate immune system in the central nervous system which includes microglia, astrocytes, granulocytes, macrophages, and T-lymphocytes and can last for months (Lin & Wen, 2013; Vonder Haar et al., 2014). The inflammatory response following TBI is both beneficial and detrimental to the recovery of the brain (Vonder Haar et al., 2014). As there is this continuum of influence on recovery of inflammation following TBI, defining and intervening within the correct therapeutic window has been a difficult task.

1.7 Current Treatments: There are currently no effective treatments to prevent the damage due to the primary and secondary pathologies following TBI. Treatment strategies for individuals suffering from the effects of a TBI consist of palliative care (Haber et al., 2013). There has been failure of drugs in clinical trials aimed at neuroprotection in TBI despite promising results in pre-clinical trials (Stein, 2015). This disparity between pre-clinical and clinical trial success may account for the disparity in current research funding. According to NIH records, $401 million were spent on influenza and pneumonia research in 2016, with 55,000 deaths in the United States per year (NIH Categorical Spending Tables). Funding for research on TBI, which results in a comparable number of deaths, was limited to $99 million in 2016 (NIH Categorical Spending Tables). With an increasing prevalence of TBI in the media, more attention has been brought to this issue an increasing pressure to find an effective treatment strategy has also increased.

2. TBI and Neuroglia

2.1 Astrocytes: Glia are a group of non-neuronal cells in the central nervous system that are responsible for many functions within the brain including myelin formation, ion channel homeostasis and immune function. As the most abundant cells in the brain, astrocytes are responsible for a variety of

functions in both the normal and the injured brain. Astrocytic end-feet wrap around blood cells to comprise a major portion of the blood brain barrier (BBB) (Lungaard et al., 2013). Astrocytes maintain the ionic and pH balance in the brain through regulation of extracellular potassium, glutamate, and glucose. Other responsibilities of the astrocyte include regulating cerebral blood flow, production of lipoproteins, release of ATP, regulation of the sleep wake cycle, and facilitation of learning and memory (Lungaard et al., 2013). Astrocytes are connected through gap junctions and can regulate large and diffuse areas of the brain (Mayer et al., 2014). Astrocytes respond to changes in different homeostatic mechanisms and make constant adjustments.

2.1.1 Role in Brain Injury: Following TBI, there is a shift in the activation and morphology state of astrocytes in the human brain. This shift is termed reactive astrogliosis (Bondan et al., 2016) and active astrocytes are found in the lesioned cortex following TBI (Susarla et al., 2014). The activation of astrocytes is part of an attempt to recover function and protect from tissue damage (Pekny et al., 2014). However, astrocyte activation can be both beneficial and detrimental to recovery from injury (Mayer et al., 2013). After a TBI astrocytes will become hypertrophic, exhibiting an enlarged morphology causing swelling (Karve et al., 2015). Swelling due to astrocytes have been found early as four hours following TBI (Lin et al., 2013). Further, a glial scar will form around the lesion site following TBI (Mayer et al., 2014) which can become a seizure focus following injury. Astrocyte dysregulation can also be detrimental due to a loss of BBB function. The BBB becomes vulnerable to infiltration from peripheral immune cells triggering oxidative stress, inflammation, and cell death within the cortex (Lozano et al., 2015). Astrocyte function must be corrected for normal functioning and recovery to occur. However, since their actions are both beneficial and detrimental following TBI it has been difficult to determine when and how to intervene.

2.2 Microglia: Microglia also have several functions within the central nervous system (CNS). These cells are typically responsible for the regulation of immune function in the CNS (Hu et al., 2016). As

part of the immune response, microglia possess phagocytic and antigen presenting capacities to remove foreign molecules from the CNS (Karve et al., 2015). Microglia have a distinct morphology at rest consisting of a ramified structure. Activated microglia can be determined from their hypertrophic morphology (Karve et al., 2015). Microglia will polarize to two opposing functions when activated, termed M1 and M2. The M1 phenotype is the classical activation of microglia (Kobayashi et al., 2013). This phenotype is pro-inflammatory, promoting the immune response in the brain (Haber et al., 2013; Karve et al., 2015; Tang & Le, 2015). Increased expression of the M1 phenotype is harmful on a long-term basis and has been correlated with obesity, insulin resistance, and type 2 diabetes, all which have a chronic inflammatory component (Tang & Le, 2015). The M2 phenotype on the other hand is associated with anti-inflammatory markers (Karve et al., 2015). The M2 phenotype is neuroprotective, promoting repair in the CNS (Hu et al., 2016). The M1-M2 polarization possibilities can shift depending on the environment and injury to the system.

2.2.1 Role in Brain Injury: Normally the immune responses of the microglia in the CNS are controlled to maintain stable homeostasis. However, TBI has the potential to skew the response of the microglia to one end of the two extremes (Tang & Le, 2015). There an increase in M2 activated phenotype immediately after an injury promoting recovery (Karve et al., 2015). Then the polarization of microglia shift toward M1 with a delayed and prolonged response (Karve et al., 2015). It is this shift toward the M1 phenotype that has the potential to be detrimental to the recovery of the brain following TBI. The M1 phenotype dominates several days after TBI and can last for several weeks promoting inflammation and hindering regeneration (Hu et al., 2016; Lu et al., 2011; Nakayama et al., 2010, Homsi et al., 2010). The M2 to M1 shift creates an unsuitable environment for repair (Koh & DiPietro, 2011). The degree of injury to the brain can influence the response of microglia (Hu et al., 2016). Recent work has determined that activated microglia in the brain after TBI release microparticles that travel throughout that brain and carry pro-inflammatory cytokines (Kumar et al., 2017). Microparticles are a type of

extracellular vesicle that is derived from the plasma membrane in response to cell activation or cell death. The pro-inflammatory cytokines released from these microparticles are thus suggested as key mediators of the neuroinflammation seen following TBI (Kumar et al., 2017). In summary, following TBI, there is a shift in the microglia polarization toward the pro-inflammatory M1 phenotype and an increase in microparticles which has been correlated with decreased recovery.

3. **Cell Death Following TBI:** Following brain injury there is cell death in various cell types. Neuronal cell death has been linked to more focal injuries, while death to glial cells such as oligodendrocytes is correlated with diffuse injuries (Raghupathi et al., 2000). Neuronal cell loss occurs various regions within the cortex of TBI patients including the cortex, hippocampus (dentate gyrus), cerebellum, and thalamus (Clark et al., 1997; Raghupathi et al., 2000). Cell death, like astrocyte and microglia activation, can be both beneficial and detrimental. When this process becomes overactive following TBI, neurodegeneration can occur leading to further issues (Cory et al., 2003).

   3.1 Apoptosis: Apoptosis is typically a controlled process in the brain meant to play a role in the development and maintenance of the tissue (Clark et al., 1997; Raghupathi et al., 2000). In the fluid-percussion model of TBI in rodents, increases in apoptosis were found in the cortex and hippocampus of an injured rat as early as 12 hours after injury induction (Rink et al., 1995). These results have also been found in the controlled cortical impact (CCI) model of TBI (Zhou et al., 2012). Study of apoptotic mechanisms following brain injury have illustrated the ability to modulate this pathway.

      3.1.1 BCL-2: Apoptosis is regulated by different factors within the central nervous system. One such gene is *bcl-2,* a proto-oncogene (Clark et al., 1997). *Bcl-2* is part of a family of genes that have been shown to protect cells from a variety of different central nervous system insults (Cory et al., 2003). Bcl-2 inhibits the activation of Bax, a pro-apoptotic protein (Cory et al., 2003). Bax is responsible for damage to the mitochondria and endoplasmic reticulum which then activate caspases leading to the death of the cell. In *in vitro* cell cultures as well as TBI models, neuroprotection was found to be correlated with

preserved values of *bcl-2* expression (Clark et al., 1997; Yang et al., 2007). Cells that were identified as degenerating were found to lack bcl-2 expression in this same experiment. Therefore, it is important for the survival of neurons after TBI to preserve or induce the expression of *bcl-2* to prevent further cell death from apoptotic mechanisms.

3.2 Necrosis: Typical apoptosis in the brain following TBI is not the only mechanism responsible for cell death. A process known as necrosis has also been proposed to be active following TBI (Raghupathi et al., 2000). Necrosis is typically regarded as a pathological form of cell death while apoptosis is a programmed mechanism of cell death. Necrosis occurs in neurons following TBI (Portera-Cailliau et al., 1997). These two pathways result from activation of different receptors; activation of the *N*-methy-D-aspartate (NMDA) receptor may lead to necrosis and activation of non-NMDA receptors may lead to apoptosis (Portera-Cailliau et al., 1997). Preventing this pathological form of cell death after TBI may be a potential avenue for new treatments for the patients who have been victims of such injuries.

4. **Minocycline:** Minocycline (Figure 1) is a derivative of tetracycline, and has traditionally been used for its antibiotic properties (Wang et al., 2004; Vonder Haar et al., 2014). As a highly lipophilic compound able to cross the blood brain barrier, minocycline has also been used to suppress microglial activation in the central nervous system (Homsi et al., 2009). Minocycline has thus been used as a neuroprotectant in models of ischemic stroke (Arvin et al., 2002), Huntington's disease (Chen et al., 2000), amyotrophic lateral sclerosis (Zhu et al., 2002), TBI (Sanchez et al., 2001), multiple sclerosis (Popovic et al., 2002), and Parkinson's disease (Du et al., 2001). Minocycline has several potential pathways of interfering with pathology following brain injury. Minocycline increases *bcl-2* expression which prevents cell death through apoptotic pathways (Wang et al., 2004). Another major role of minocycline in neuroprotection is the inhibition of microglial activation, specifically preventing the M1 microglial phenotype. Treatment with minocycline has inhibited M1 polarization in both ALS and TBI models (Kobayashi et al., 2013, Homsi et al., 2009), increased motor

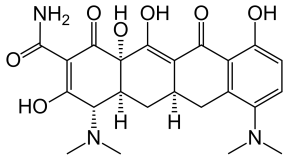

Figure 1: Chemical structure of minocycline, a tetracycline derivative, previously used for its antibiotic properties.

function after TBI (Vonder Haar et al., 2013), and decreased lesion size following TBI (Homsi et al., 2009; Vonder Haar et al., 2013). Further study on minocycline's effects following TBI in different models is important to furthering the study of the drug and implementation of the drug into clinical patients. Many of the previous studies with minocycline have used several doses over the course of the recovery. Very little study into the potential benefits of a single dose in TBI patients has been undertaken.

5. **Propentofylline (PPF):** Propentofylline (PPF) (Figure 2) is a synthetic derivative of xanthine (Buytaert-Hoefen et al., 2002). This drug has a rich history of over 30 years of use within a variety of disease states including vasodilation, anti-inflammation, anti-proliferation, neuroprotection, platelet aggregator inhibition, glial modulation, and glutamate inhibition along with others (Sweitzer & DeLeo, 2011). PPF has been found to negotiate these diseases through a variety of mechanisms. PPF interferes with adenosine availability and inhibits actions of cyclic AMP (Sweitzer & DeLeo, 2011). Increasing the amount of adenosine available for action effects several brain cell types and leads to neuroprotection (Rudolphi et al., 1997). Increasing adenosine availability leads to hyperpolarization of the neuron, which counteracts depolarization that typically results from different types of brain injury (Rudolphi et al., 1997). In animal tests, PPF administration has decreased lesion size following ischemia (Johnson et al., 1998), increased growth factors after contusion (Buytaert-Hoefen et al., 2002), and decreased astroglial activation and increased repair (Bondan et al., 2016). With propentofylline's diverse actions in the brain following many different types of brain injuries, it is expected that it will also have similar effects in TBI.

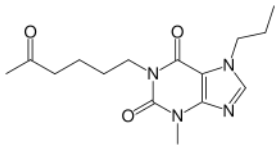

Figure 2: Chemical structure of propentofylline (PPF), a xanthine derivative used throughout history for a variety of effects.

6. **4-phenyl 1-(4-phenylbutyl) piperidine maleate:** The drug 4-phenyl 1-(4-phenylbutyl) piperidine maleate (4-PPBP) (Figure 3) has been used previously as a neuroprotective agent. 4-PPBP is a potent sigma-1 receptor agonist (Schetz et al., 2007). The sigma receptor family can be divided into two families based on their functions within the body, sigma-1 and sigma-2. Sigma-1 receptors interact with glutamate, dopamine, serotonin, norepinephrine, and cholinergic systems (Rousseaux et al., 2015).

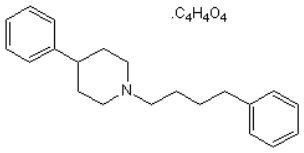

Figure 3: Chemical structure of 4-phenyl 1-(4-phenylbutyl) piperidine maleate (PPBP), a potent sigma-1 agonist.

The sigma-1 receptor is found everywhere in the body, specifically on the cell membrane, the endoplasmic reticulum of neurons, and in oligodendrocytes. As a potent sigma-1 receptor agonist, PPBP has been used in different models to increase neuroprotection. Administration of 4-PPBP has decreased neurotoxicity (Harukuni et al., 1998), decreased pro-inflammatory M1 markers (Harukuni et al., 1998), decrease infarction size (Takahashi et al., 1995; Schetz et al., 2007; Goyagi et al., 2001), and decrease loss of *bcl-2* mRNA (Yang et al., 2007). In summary, 4-PPBP can be used to both decrease the amount of apoptotic cell death through regulation of bcl-2 and acting as a sigma-1 receptor agonist to modulate excitotoxic pathways following TBI.

7. **Present Study:** TBI is becoming increasingly prevalent with various mechanisms of injury. Considering the only option that doctors currently have to treat victims of TBI is palliative in nature, it is important to understand if there is a potential to prevent or minimize symptoms before they occur. Understanding the involvement of glia in the brain following TBI is a promising avenue. As glia are responsible or both neuroprotective and neurodegenerative mechanisms, there is a small effective temporal window for treatment. The present study was an attempt to determine if modification of neurodegenerative processes at the level of glia can improve functional and histological outcomes the ipsilateral cortex following TBI. To do this, a modified closed cortical impact (CCI) model was be used to deliver a TBI to an anesthetized rat. Treatment with glia and apoptosis modifiers propentofylline, minocycline, and 4-PPBP occured ten minutes after the induction of injury. We hypothesized that improved functional recovery and histological outcomes are modulated by glial activation and prevention of cell death. We expected that administration of the three drugs would improve functional recovery compared to a group with TBI but no drug treatment. We further expected that drug treatment would cause decreases in lesion size and cell death compared to no drug treatment.

Methods

1. **Animals and Husbandry:** Twenty-nine adolescent (2-4 months) male Sprague-Dawley rats (200-500g) were used. Animals were group housed, no more than four animals per cage. Following induction of brain injury, the animals were individually housed for the duration of the experiment to reduce infection. The animals were kept on a 12-hour light/dark cycle and fed and given water *ad libitum*.
2. **Induction of Brain Injury:** The novel model here was created to model the closed cortical injury model of head injury in the rat. Protocol for brain injury was adapted from (Dixon et al., 1991). Rats were anesthetized with ketamine (70ug)/ xylazine (6ug) mixture (.1ml/100 grams). The animal was then placed into the stereotaxic device (Figure 4) and a sterile incision made down the midline of the scalp. The periosteum was moved away from the incision to expose the midline suture, bregma, and lambda (Figure 5). Once the skull was free of blood, a 3-mm craniotomy was created over the right cortex between bregma and lambda with a drill (Figure 6). A carbon-dioxide powered air pistol was then used to deliver a concentrated force to the exposed brain tissue. The pistol was held 5 cm over the craniotomy and fired (Figure 7). A brief hypo-ventilation can be seen following the injury. If this occurred, a 0.05 cc/kg of atropine was delivered to recover normal ventilation along with chest massages. The animals were then sutured and returned to an individual cage and given antibiotics (Terramycin in peanut butter) for three days.



Figure 4: Experimental animal placed in stereotaxic device, with incision area shaved and prepared for surgery.



Figure 5: Midline incision with bregma, lambda, and midline suture exposed after the periosteum had been moved from the surgery site.



Figure 6: A 3-mm craniotomy performed using a drill between bregma and lambda exposing the intact dura matter through which an experimental TBI will be performed.

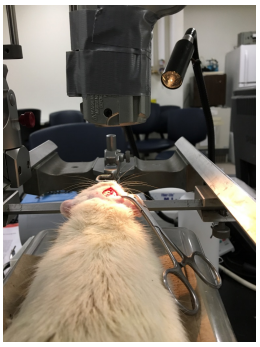

Figure 7: CO2 powered pistol held 7 cm over the center of the craniotomy. Pistol was fired and then the animal was suture and returned to an individual cage.

3. Behavioral Tests

3.1 Beam Walk: The animal was placed in the middle of a beam that measured 108 cm high, 127 cm long, and 3.8 cm wide. Latency to reach either end of the beam was recorded (Figure 8). Reaching the end was considered when the head crossed over the final inch of the beam. A maximum latency of 90 seconds was given for animals that failed to reach the end of the beam. Daily assessment consisted of three consecutive trials from which the mean was taken. The animal rested for 30 seconds in the hands of the experimenter between trials. Baseline was the latency recorded immediately prior to surgery. Post injury induction, the animals was tested for 5 consecutive days starting 24 hours after surgery. Averages per day were collected.



Figure 8: Beam walk behavioral test with animal near the end. Animals are placed in the center of the beam and latency to reach either end of the platform is recorded. Three trials per day were completed for five days of which averages were taken.

3.2 Beam Balance Test: The animal was placed on a wooden beam (23 cm high, 70.5 cm long, and 2cm wide) (Figure 9). The quality of the balance was graded per the following scale originally outlined by Beaumont et al. (1999): 1, balances with steady posture; 2, grasps beam, but steady; 3, grasps beam with lateral/medial balancing movements, generally unsteady; 4, falls in 10-60 seconds; 5, falls in 0-10 seconds; 6, no attempt to balance at all. Daily assessment consisted of 3 trials from which the mean score was taken. Baseline was measured immediately prior to surgery and post injury measures were taken starting at 24 hours after injury and continued for five consecutive days of which averages were taken.



Figure 9: Beam balance behavioral test with animal exhibiting a rating of one, balancing without grasping the beam or swaying from side to side.

3.3 Labyrinth Water Maze: The maze was 94x94x41 (cm; W x L X D) and consisted of left and right turns to reach a platform that stood out of the surface of the water level. The water was kept at room temperature (20 degrees C) and at a depth of 24 cm. The platform (20.5 cm height) was kept at a fixed point at the end of the maze. Latency to reach the end of the maze from the starting position was recorded (Figure 10). Errors were measured as a half of a body length in the wrong direction through the maze. Being removed from the room temperature water was a positive reinforcer to the animal to learn the maze as the body temperature for a rat is higher than room temperature (35-37 degrees C). Daily assessment consisted of three consecutive trials of which the average was taken. Testing continued for five consecutive days beginning 10 days after the surgery to ensure that the wounds were healed before being placed into water.



Figure 10: Labyrinth Water Maze with start and finish positions labeled. Animals were placed at the start point and required to learn the path through eh maze to the finish position while latencies were recorded.

4. **Histological Preparation:** Following cessation of all behavioral testing, the animal was perfused with formalin solution. Briefly, this is accomplished by delivering a fatal dose of sodium pentobarbital solution (Euthasol). The animal was then perfused with 10% formal saline solution and the brain was removed from the skull and stored at least overnight in the same mixture. The brain was then blocked into two coronal sections at the midline of the lesion. One half of the lesion was then sectioned with alternating coronal sections being used for Nissl and Fluorojade-C. For sectioning the tissue was frozen to -21° C, and serially sectioned on a cryostat. Cover slipping medium was used following staining procedures (DPX for fluoro-jade C staining, and paramount for Nissl). Slides were then allowed to fully dry and analyzed on a Nikon Eclipse E600 fluorescent microscope. Images were collected and analyzed using the Leica Application Suite Program.

4.1 Nissl Stain: The cresyl fast violet, Nissl stain will be used to determine changed in cell counts following both TBI and treatment with each drug. Cresyl violet staining solution will be mixed by combining 2.5 grams of cresyl fast violet, 1.7 ml of glacial acetic acid, and 500 ml of distilled H2O. Solution B will be prepared by combining 300 ml of 70% ethanol with 3 ml of glacial acetic acid. Glass mounted sections

will be first rinsed in distilled water for 2 minutes. This is followed by a four-minute immersion in the cresyl violet stain and then a one-minute rinse in distilled H2O. The sections are then placed into solution B for 2 minutes followed by successive rinses in 95% ETOH (1 minute), 100% ETOH (1 minute), and xylene (for at least 5 minutes). Sections will then be dried and cover slipped with paramount mounting medium. The slides will be analyzed under light microscopy. For histology analysis, lesion perimeters were collected using the Leica Application Suite. Lesion perimeter was drawn by hand around the lesion as indicated by a ring of dark stained neurons after Nissl staining on an image taken at 4x magnification and recorded in μm. Three lesion perimeters were taken per animal and then averaged. Cell counts were on cells in the CA1 region of the hippocampus on the ipsilateral side. Three sections per animal were analyzed. A region of interest was drawn on around CA1 and was saved in the Leica Application Suite program. Images were then collected (3 per animal) at 10x magnification. Once images were collected, all cells were counted by hand using the Leica object count tool. Cell counts were performed in the hippocampus due to the function of the hippocampus in spatial learning and new memory formation (Langston et al., 2010). The CA1 region of the hippocampus has been specifically implicated in temporal encoding of spatial memories (Langston et al., 2010) which is important to the successful completion of the labyrinth water maze.

4.2 Fluoro-jade C Staining. Histology methods were adapted from Gao & Chen (2011). Fully dried slides containing sections of tissue are first immersed in a 1% sodium hydroxide 80% alcohol mixture for five minutes. This is followed by two rinses, the first for two minutes in 70% alcohol and two minutes in distilled water. The slides are then moved to a 0.06% potassium permanganate solution for 10 minutes, and rinsed in distilled water for 2 minutes. A staining solution of 0.0004% of fluoro-jade C was prepared in 0.1% acetic acid vehicle and the slides are immersed in this solution for fifteen minutes. The slides are then washed in three distilled water washes for one minute each. The slides are then briefly drained, allowed to dry fully and then immersed in xylene for at least one minute before cover slipping with DPX and being sealed.

5. **Drug Administration:** TBI control and sham will receive no injections (n=7). Single doses of protentofylline, minocycline, and 4-PPBP was administered to the drug treatment groups. The propentofylline (Sigma Aldrich) group (n=5) received a single 10 mg/kg dose (Buytaert-Hoefen et al., 2002). Minocycline (Sigma Aldrich) (n=5) were administered at a dose of 25 mg/kg (Meuwissen et al., 2013). Finally, 4-PPBP (Tocris) (n=5) was delivered at a dose of 1 mg per animal. Each drug was delivered 10 minutes following induction of head injury. This is because there is an effective window that was found to be between five minutes and an hour (Haber et al., 2013). Further, when emergency medical services are called, 75% of calls respond in ten minutes (Meyer et al., 2008).
6. **Statistical Analysis:** Statistical analyses were run to determine if there are any statistical differences between both the sham and the experimental group, and the treatment and non-treatment groups. Repeated measures analysis of variance (ANOVA) were performed to determine what these differences were in the beam walk, beam balance, and labyrinth water maze. A one-way ANOVA was performed for lesion perimeter and cell counts. Following ANOVA, the Tukey's HSD post-hoc test was performed to determine differences between adjacent cells in the experimental design.

Results

1. Behavioral Tests:

1.1 Beam Walk: The 4-PPBP group had significant side effects following drug administration including blood in the urine as well as difficulty regulating blood pressure. One animal in this group was unable to care for itself following TBI and was excluded from behavioral analysis. Beam walk test was used to determine motor abilities for the first five days following a modified CCI in rats. A two-way ANOVA determined that there was a significant main interaction between days and group ($F(20,95) = 6.228$, $p < .001$). The sham group latencies were significantly lower than the TBI control group ($p < .001$) across all post-injury testing days. All drug treatment groups, minocycline, PPF, and 4-PPBP, had post-injury latencies in the beam walk significantly lower than the TBI control group ($p < .05$) while there were no significant differences between drug treatment and sham groups ($p > .05$) (Figure 11). Following a Tukey's HSD test there were no significant differences in average beam walk latencies across groups pre-injury ($p > .05$). When analyzing the effect of day, all injured animals regardless of drug treatment condition had increased latencies compared to pre-injury levels on the first testing day post-injury, excluding sham (Figure 12). The TBI group had significantly higher latencies on days one through four of the post-injury testing days compared to sham and all treatment groups (Figure 12).

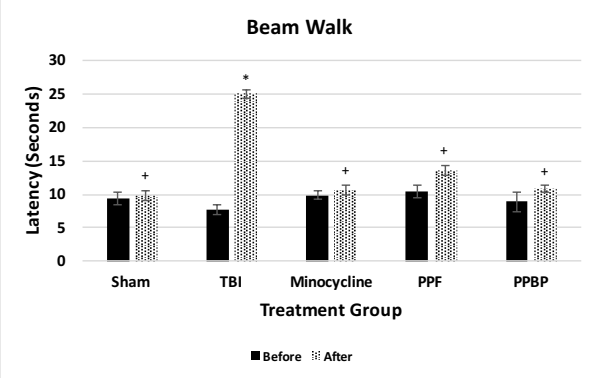

Figure 11: Beam walk average latencies are significantly increased following TBI compared to sham animals and increased latencies were recovered with minocycline, PPF, and 4-PPBP treatment. There were no statistical differences in pre-injury latencies in any of the experimental groups. The TBI treatment group had significantly higher latencies after injury. Both minocycline and PPBP did not have significant increases after injury. The PPF treatment group on average had a significant increase in latency following injury compared to pre-injury baseline. Error bars represent +/- one SEM. (*p<.05 compared to sham; +p<.05 compared to TBI)

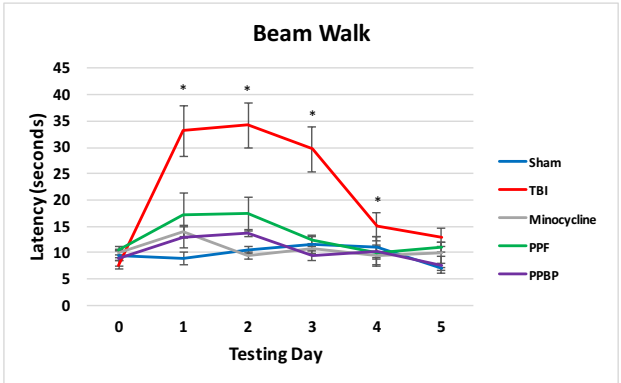

Figure 12: TBI caused sustained increases in beam walk latencies throughout the testing window compared to sham, minocycline, PPF, and 4-PPBP groups. Pre-injury averages (0) showed to significant differences across all experimental groups. None of the drug treatment groups showed significant increases in latencies on any one day across the five-day testing period. The TBI group had significant increases in beam walk latencies following TBI on days 1-4 and did not return to baseline values by day 5 but this increase was not significant. Error bars represent +/- one SEM. (*p<.05 compared to sham)

1.2 Beam Balance: A two-way ANOVA indicated a significant main interaction of day and group assignment in the beam balance behavioral measure ($F(20,95) = 7.507$, $p < .001$). Pre-injury ratings on beam balance showed no significant differences across all groups (Figure 13) and were statistically significantly lower than post injury day 1 ($p < .005$) across all groups. Average sham group performance was significantly lower than TBI group ($p < .005$). The TBI group had higher ratings on beam balance on days two and three (Figure 14). By days four and five, there were no significant differences in beam balance performance across all groups in accordance to previous results from Beaumont et al. (1999). All three drug treatments resulted in decreases beam balance ratings that were statistically significant compared to the TBI control group ($p < .005$).

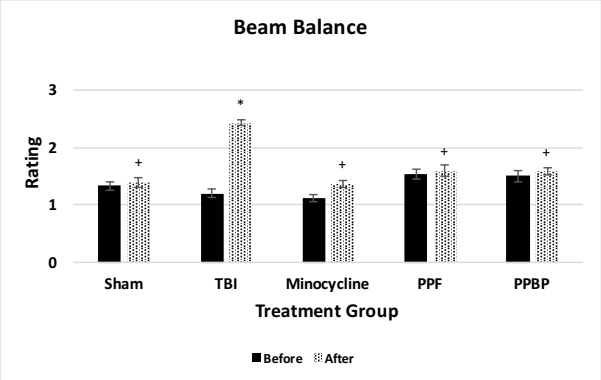

Figure 13: Average beam balance ratings were significantly higher following TBI with no drug treatment but not following drug treatment. Ratings were given as follows 1, balances with steady posture; 2, grasps beam, but steady; 3, grasps beam with lateral/medial balancing movements, generally unsteady; 4, falls in 10-60 seconds; 5, falls in 0-10 seconds; 6, no attempt to balance at all. No groups showed significant differences in beam balance rating at baseline. Following injury, only the TBI group had a significant increase in beam balance rating indicating a decrease in performance. Error bars represent +/- one SEM. (*p<.05 compared to sham; +p<.05 compared to TBI)

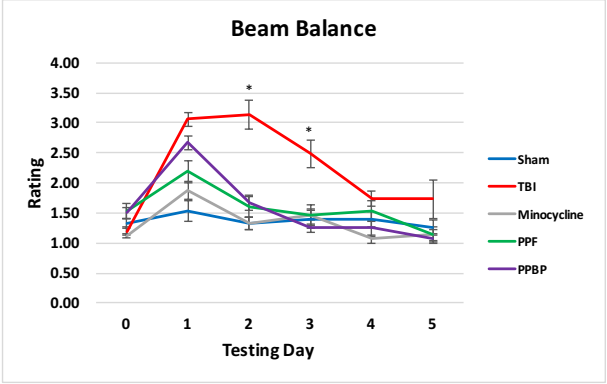

Figure 14: TBI causes decreased performance on beam balance across testing days. Ratings were given as follows 1, balances with steady posture; 2, grasps beam, but steady; 3, grasps beam with lateral/medial balancing movements, generally unsteady; 4, falls in 10-60 seconds; 5, falls in 0-10 seconds; 6, no attempt to balance at all. No differences between group were seen on pre-injury averages (testing day 0). All groups experienced decreases in performance as evidenced by increased ratings after injury (day 1). The TBI group had significantly higher ratings on days 2 and 3 than all other experimental groups. By day 4 and 5 these increases in the TBI group began to return to sham levels. Error bars represent +/- one SEM. (*p<.05 compared to sham)

1.3 Labyrinth Water Maze: To determine TBI effects on spatial learning, a labyrinth water maze test was run 10 days after injury. A two-way ANOVA determined that there was a significant group by day interaction ($F(20,65) = 2.049$, $p = .016$). All groups showed increases in performance as evidenced by decreases in latencies throughout the five-day testing window (Figure 15). The TBI group had significantly longer average latencies compared to sham groups (Figure 16). No drug treatment significantly decreased water maze latencies to sham levels on average (Figure 16). However, when analyzing differences across each individual testing day, the TBI group had significantly longer latencies than sham group on testing days two through five

(p<.05), as well as all drug treatment groups (p<.05). A secondary measure of performance on the labyrinth water maze task was the number of errors committed during each trial. Average number of errors for the TBI group were significantly higher than the sham group on days three through five (p<.05) (Figure 17). All drug treatment groups had significantly lower error averages compared to the TBI group but also had significantly higher averages than the sham group (p<.05) (Figure 18).

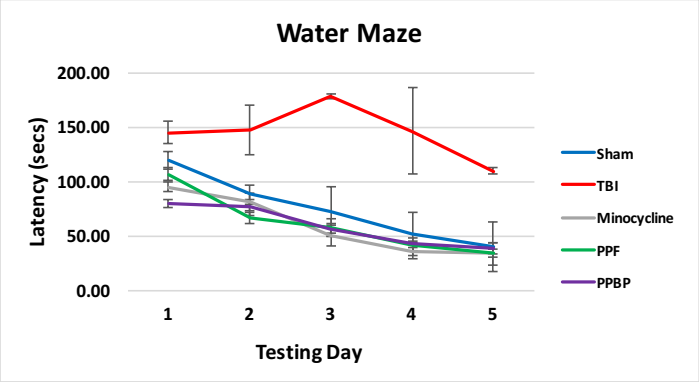

Figure 15: Animals with a TBI have sustained increases in labyrinth water maze latencies across post-injury testing days. Sham and all drug treatment groups (minocycline, PPF, and PPBP) had high initial latencies that improved throughout the five-day testing window. The TBI group had no improvement in performance. On testing days two through five, the TBI group had significantly longer latencies in the water maze compared to all other groups. There were no statistically significant differences between the sham group and all drug treatment groups. Error bars represent +/- one SEM. (*p<.05 compared to sham)

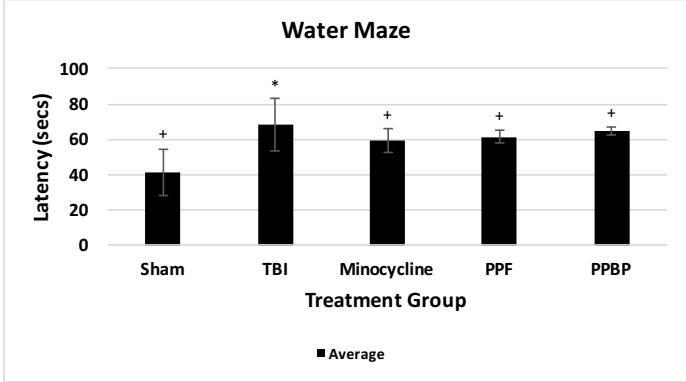

Figure 16: Minocycline, PPF, and 4-PPBP treatment reduces labyrinth water maze latencies following TBI. Looking at the overall averages across all five post-injury latencies there were not any statistically significant differences between any of the groups expect the TBI group. The TBI group has a longer average latency. Differences between groups can be seen throughout the testing days and not in overall averages. Error bars represent +/- one SEM. (*p<.05 compared to sham; +p<.05 compared to TBI)

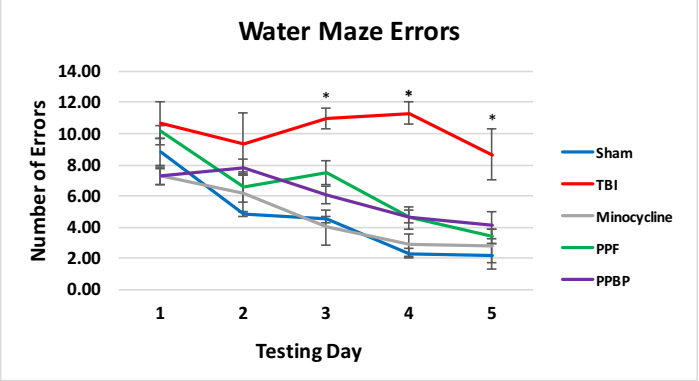

Figure 17. Minocycline, PPF, and 4-PPBP attenuate labyrinth water maze errors compared to TBI animals. Average number of errors for the sham, minocycline, PPF, and PPBP groups decreased throughout the testing window indicating learning. The TBI group had higher levels of errors and had significantly higher error averages on testing days three through five. Error bars represent +/- one SEM. (*p<.05 compared to sham)

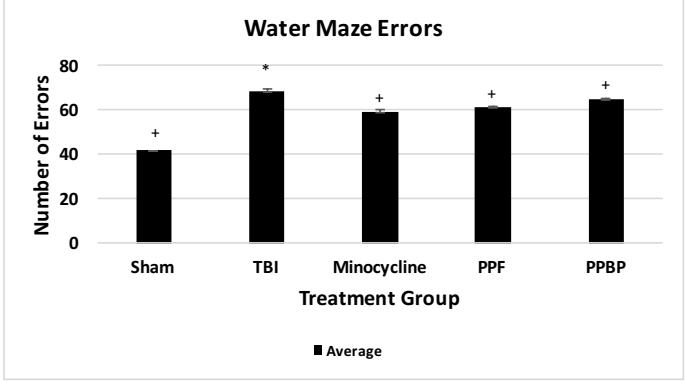

Figure 18: Total water maze errors show increase errors following TBI with improvement following drug treatment. Following injury, the TBI group had significantly higher error averages than the sham group. All drug treatment groups had significantly higher averages of errors than the sham group but significantly lower than the TBI group. Error bars represent +/- one SEM. (*p<.05 compared to sham; +p<.05 compared to TBI)

2. **Histology:** After perfusion of all experimental animals, lesion sizes were observed across the groups. Sham animals did have a small visible lesion on the surface of the brain which measured on average 3 mm (Figure 19-A). With variation within each group, there were sizable lesions in the TBI group (Figure 19 – B), the minocycline treated animals (Figure 19 – C), the propentofylline treated animals (Figure 19 – D), and the animals treated with 4-PPBP (Figure 19 – E).

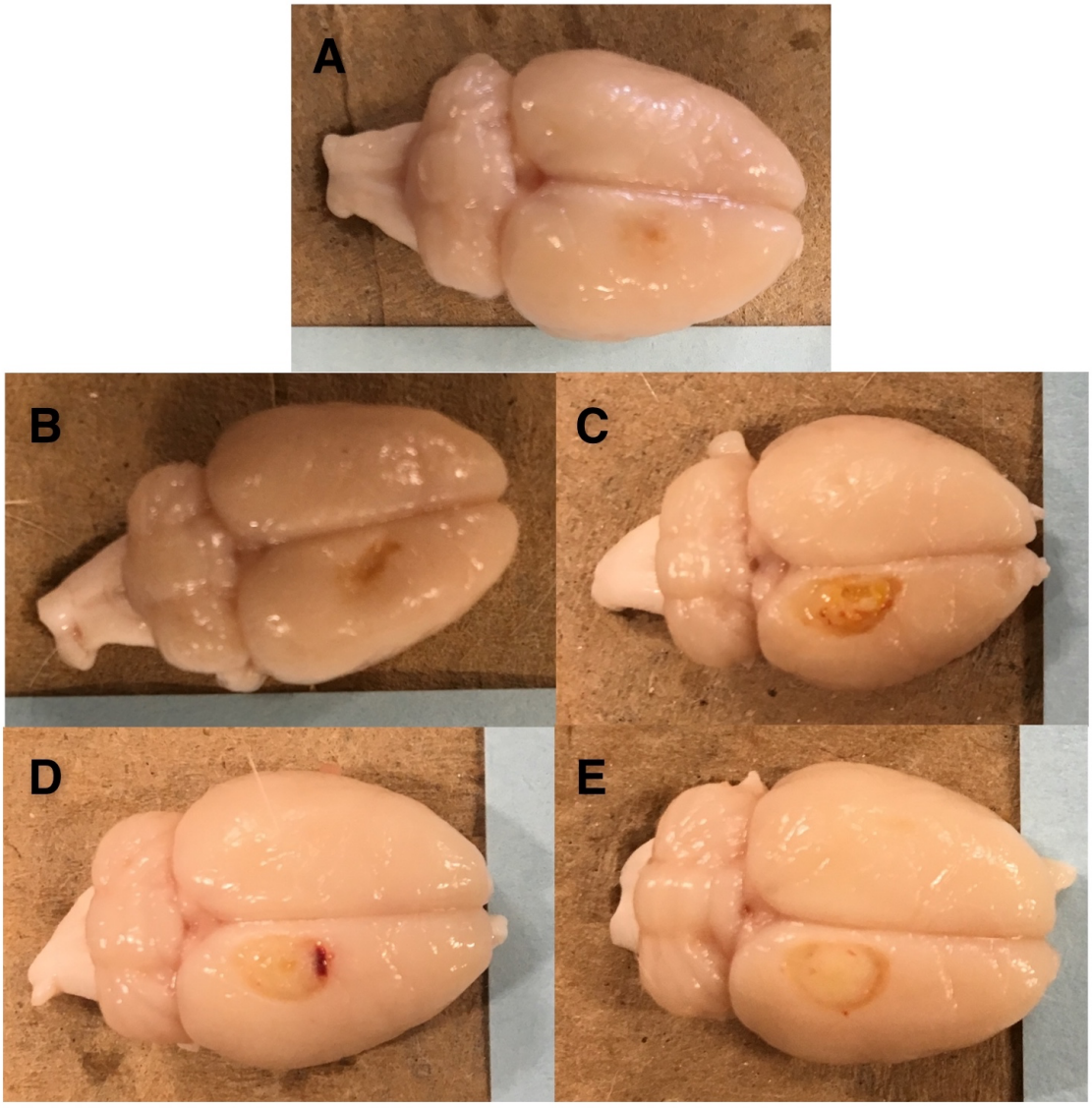

Figure 19. TBI created lesions on the injured cortex regardless of treatment condition compared to sham animals. Animals were overdosed with a sodium pentobarbital solution and perfused with formal saline 15 days after modified CCI injury. Brains collected from sham (A) animals had smaller visible lesions than TBI (B), Minocycline (C), Propentofylline (D), and 4-PPBP (E) treatment groups, which had larger and deeper visible lesions.

2.1 Nissl: Lesion morphology was observed by Nissl staining across all groups. At the edge of the lesion in every group, there were an increase in the darkly labeled cells compared to the contralateral hemisphere. All groups had dark stained cells around the lesion area including the sham group but to a smaller extent (Figure 20). One animal in the sham group had a larger lesion than the one presented in Figure 20 (A-B). The TBI group had two animals with lesions reaching to the edge of the corpus callosum (Figure 20, C-D), and two animals with lesions that were smaller but contained very dark stained cells. The TBI group most often had lesions that with no cells remaining in the impact zone. The minocycline group had two animals with lesions that reached to a depth at the corpus callosum with most lesions that had similar morphology to the representative image in Figure 20 (E-F). The PPF group had two animals with lesions that reached to the depth of the corpus callosum (Figure 20, G-H). The 4-PPBP group had variable sized lesions with 3 with lesions most like the TBI group missing most of the cells in the lesion area (Figure 20, I-J).

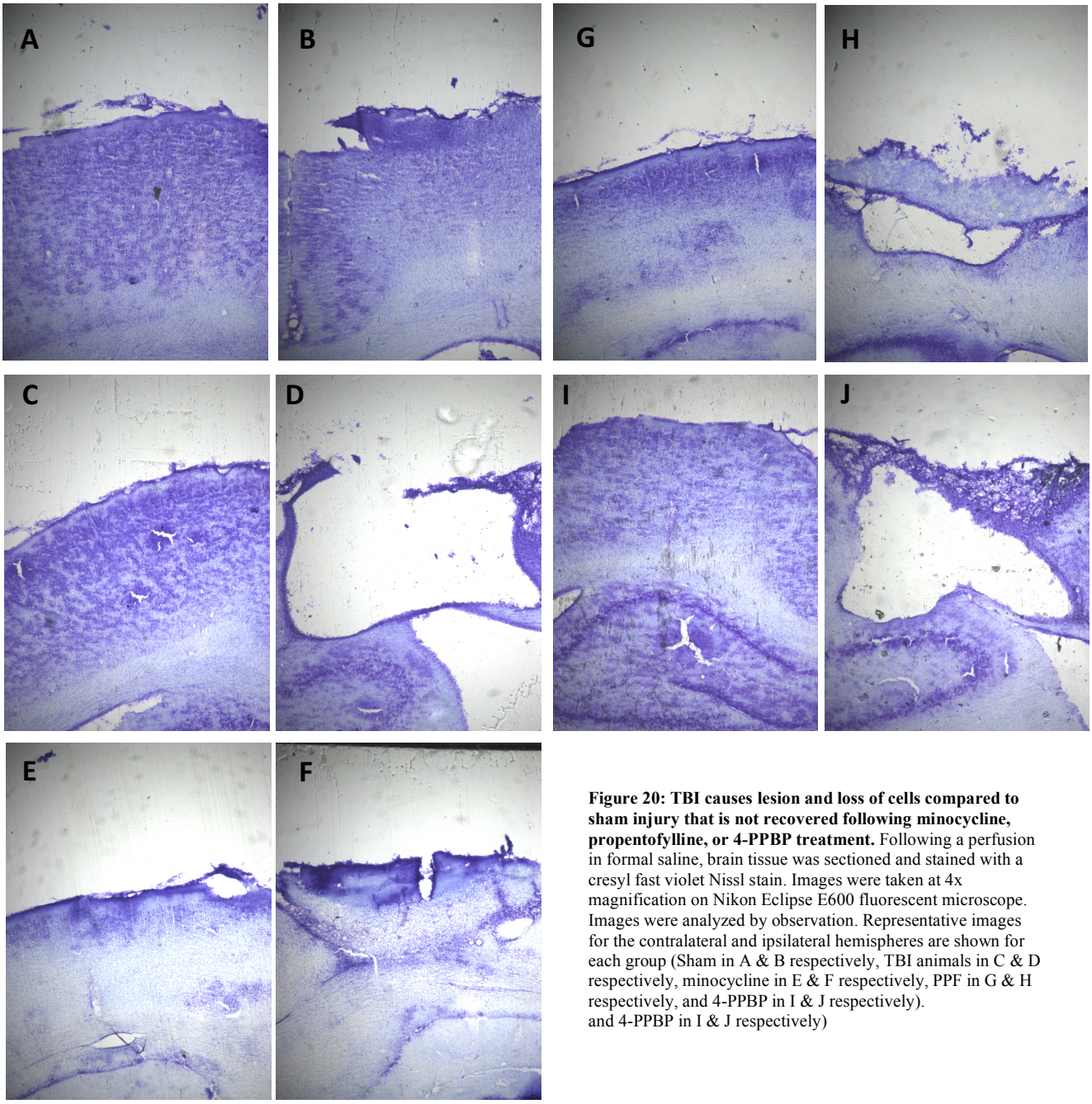

Figure 20: TBI causes lesion and loss of cells compared to sham injury that is not recovered following minocycline, propentofylline, or 4-PPBP treatment. Following a perfusion in formal saline, brain tissue was sectioned and stained with a cresyl fast violet Nissl stain. Images were taken at 4x magnification on Nikon Eclipse E600 fluorescent microscope. Images were analyzed by observation. Representative images for the contralateral and ipsilateral hemispheres are shown for each group (Sham in A & B respectively, TBI animals in C & D respectively, minocycline in E & F respectively, PPF in G & H respectively, and 4-PPBP in I & J respectively).
and 4-PPBP in I & J respectively)

2.1.1 Lesion Perimeter: Lesion perimeter was quantitatively measured across each group (Figure 21). Following a one-way ANOVA, there was no significant difference observed in lesion perimeter between groups ($F(4, 24) = 2.109$, $p = .111$). A Tukey's HSD test revealed that lesion size was larger in the TBI group ($p = .088$) than the sham group while not statistically significant. The TBI group had larger lesions than the minocycline treatment group ($p = .316$), slightly larger lesions than the PPF treatment group ($p = .569$) and no difference with the 4-PPBP treatment group ($p = .930$).

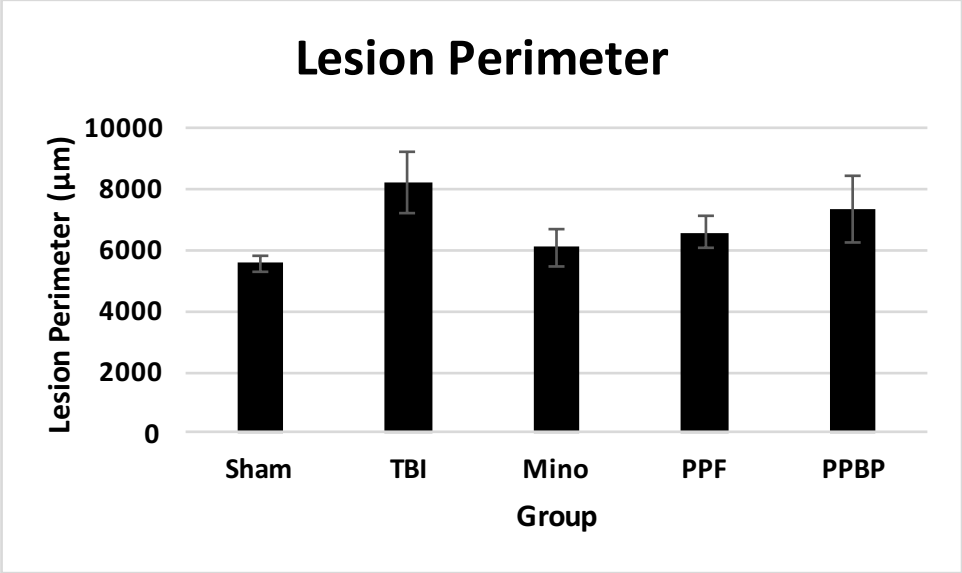

Figure 21. Average lesion perimeters for each group following Nissl staining. Nissl stained tissue was used to determine lesion perimeter as the cells in the lesion are darkly stained. Perimeter was drawn by hand and measured through computer algorithms on the microscope software. Measurements were recorded in micrometers (µm). Sham and TBI (n=7). Mino, PPF, and PPBP (n=5). Error bars represent standard error of the mean.

2.1.2 Cell Count: For further analysis, cell counts were performed in the CA1 region of the hippocampus. After one-way ANOVA, there were no significant between group differences ($F(4,20) = 0.375$, $p = .824$) (Figure 22). However, when looking at the individual between group comparisons, the sham group had cell counts that were lower than the TBI group but not significantly ($p = .332$). The sham group also had cell counts that were lower than the PPF group but not significantly ($p = .382$). There were no significant differences between the TBI group and any of the drug treatments.

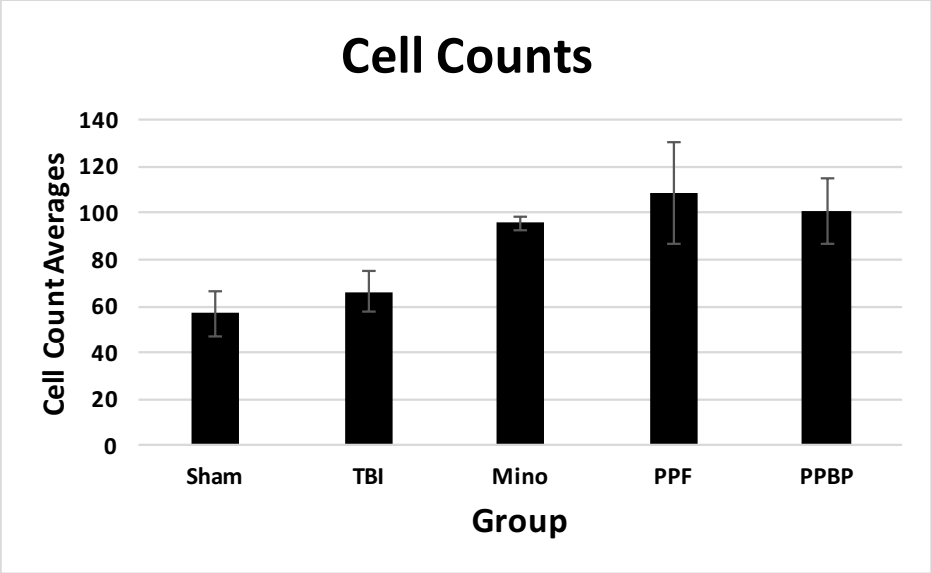

Figure 22: Average cell counts for each experimental group following Nissl staining after experimental TBI. Cell counts were collected by a region of interest in the CA1 of the hippocampus. Pictures to be analyzed were collected at 10x magnification. Cells were counted by hand in the region of interest. Sham and TBI (n=7). Mino, PPF, and PPBP (n=5). Error bars represent standard error of the mean.

2.1.3 Hippocampal Morphology: Hippocampal morphology was compared between each treatment group and representative images are shown in Figure 23. There was a mix of the portion of the hippocampus that was available for analysis in each group. Most samples were from the medial portion of the hippocampus while four samples were more dorsal or ventral hippocampal sections. These sections were not included in the analysis as it is more difficult to differentiate between the different regions of the hippocampus at those locations. No visible differences in the ipsilateral and contralateral hippocampi were observed for the sham group (n=7) (Figure 23, A-B). The TBI group had deformed structure and darker cells of the ipsilateral hippocampus that was not seen in the contralateral hemisphere (Figure 23, C-D). This similar pattern was seen all the drug treatment groups with shape changes and darker cells (Figure 23, E-J).

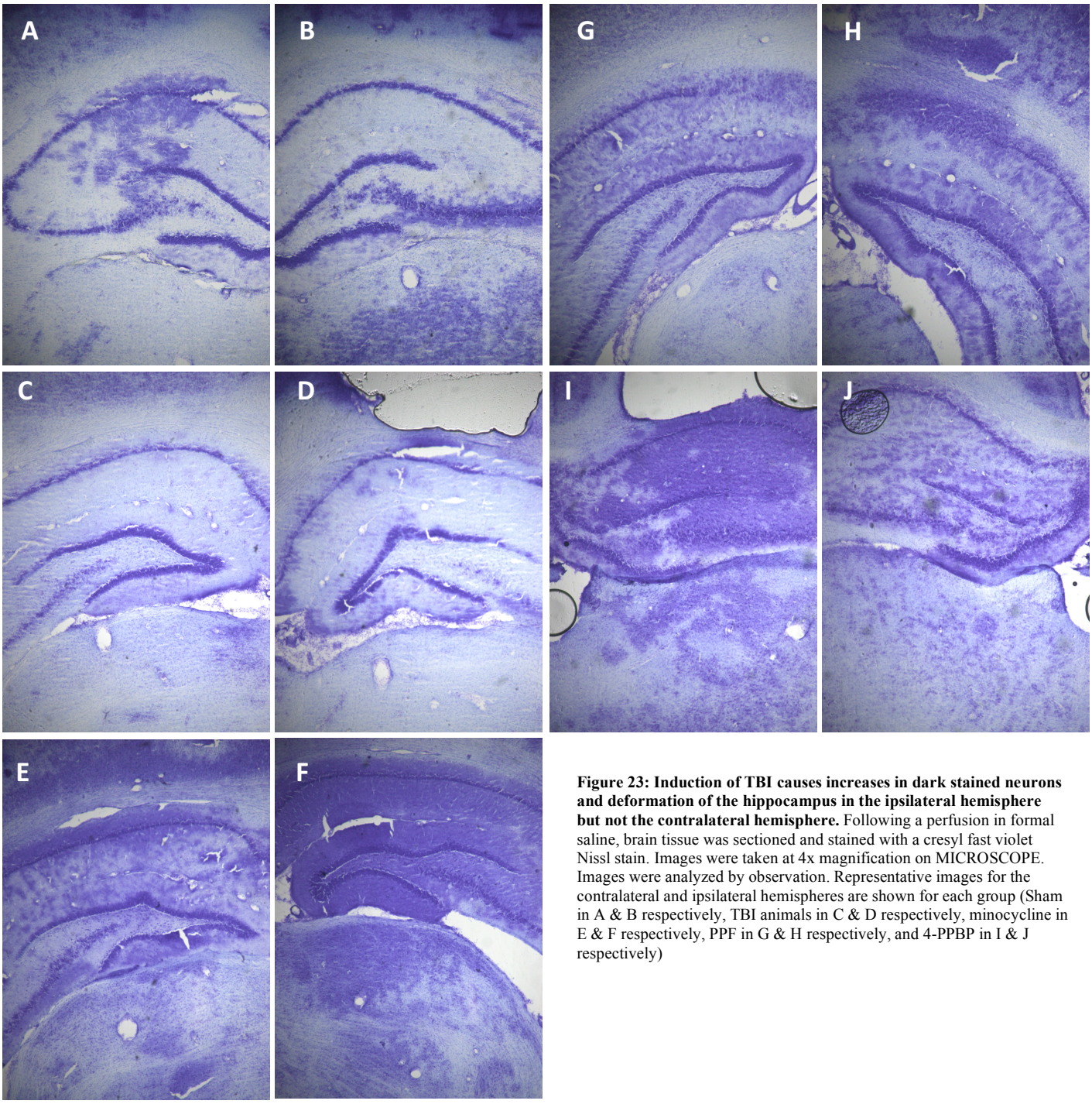

Figure 23: Induction of TBI causes increases in dark stained neurons and deformation of the hippocampus in the ipsilateral hemisphere but not the contralateral hemisphere. Following a perfusion in formal saline, brain tissue was sectioned and stained with a cresyl fast violet Nissl stain. Images were taken at 4x magnification on MICROSCOPE. Images were analyzed by observation. Representative images for the contralateral and ipsilateral hemispheres are shown for each group (Sham in A & B respectively, TBI animals in C & D respectively, minocycline in E & F respectively, PPF in G & H respectively, and 4-PPBP in I & J respectively)

2.2 Fluorojade-C: To label degenerating neurons, FJ-C has been used in following TBI. Staining patterns were observed on the ipsilateral and contralateral hemispheres. The contralateral hemispheres in the sham, TBI, minocycline, propentofylline, and 4-PPBP groups contained no visible differences (Figure 24 – A, C, E, G, I respectively). The sham group had small lesions with small amounts of visible staining around the perimeter of the lesion (Figure 24, B). The TBI group had larger lesions compared to the sham group which typically had extensive tissue loss as evident by holes in the tissue under the lesion (Figure 24, D). There was increased fluorescently labeled neurons around the lesion site as well. When comparing the TBI group to the all drug treatment groups, the staining patterns were similar with no significant differences across any of the treatments (Figure 24 - F, H, J). In the contralateral hemisphere of all groups little fluorescently labeled cells were found (Figure 24, A-sham, C-TBI, E-mino, G-PPF, I-PPBP).

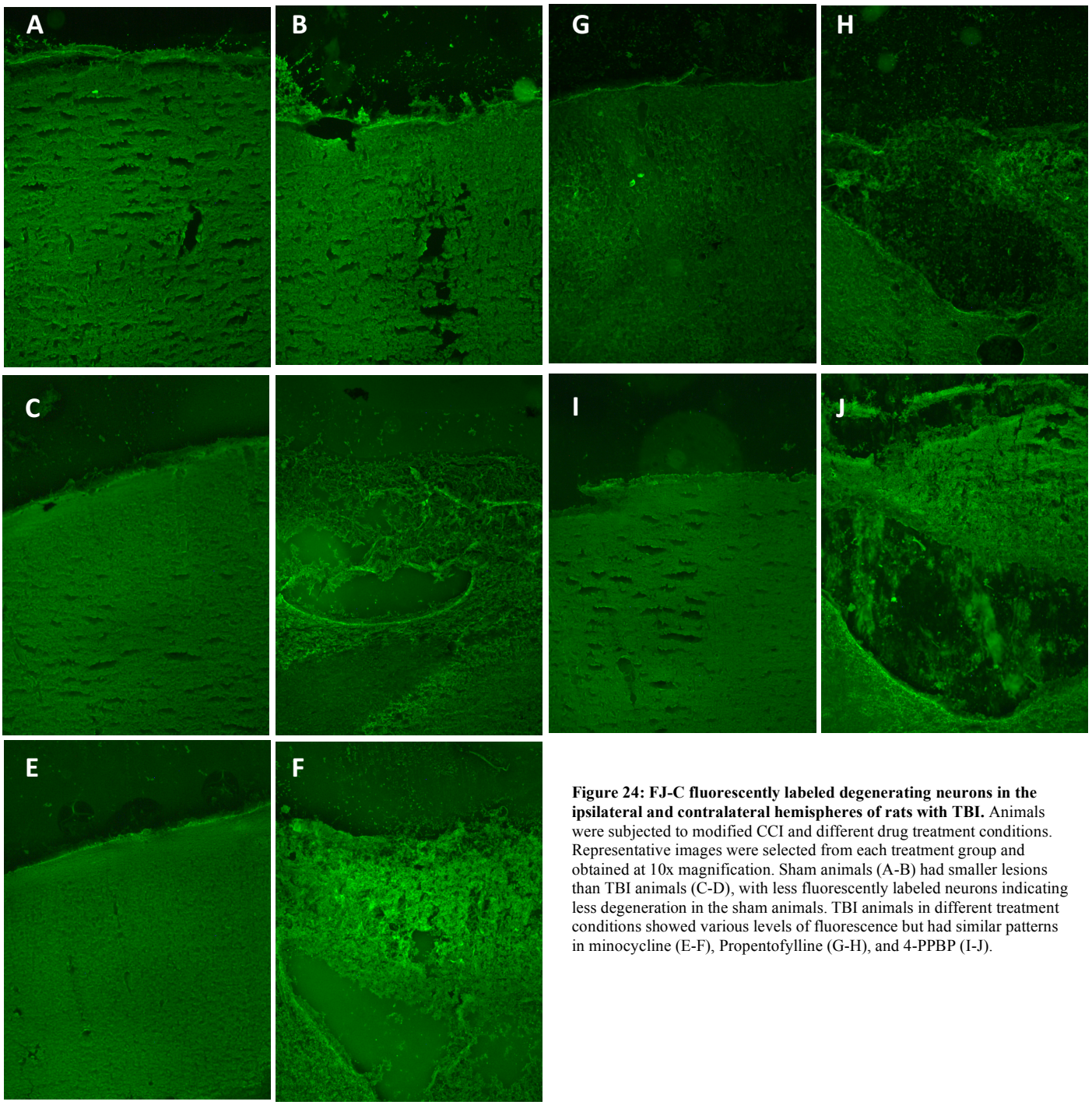

Figure 24: FJ-C fluorescently labeled degenerating neurons in the ipsilateral and contralateral hemispheres of rats with TBI. Animals were subjected to modified CCI and different drug treatment conditions. Representative images were selected from each treatment group and obtained at 10x magnification. Sham animals (A-B) had smaller lesions than TBI animals (C-D), with less fluorescently labeled neurons indicating less degeneration in the sham animals. TBI animals in different treatment conditions showed various levels of fluorescence but had similar patterns in minocycline (E-F), Propentofylline (G-H), and 4-PPBP (I-J).

2.2.1 Astrocytic Labeling: Fluorojade-C staining also revealed the presence of astrocytes which is consistent with previous work where astrocyte labeling was determined to indicate potential damage (Ehara & Ueda, 2009). The activation was strongest around and within the ventricles. In the sham group, there was astrocytes present in the third ventricle (Figure 25, A), a pattern that was carried throughout all other groups. Astrocytes were also present in the lateral ventricles of all groups (Figure 25, D & I). In the minocycline treated animals, a secondary infarct was found under the primary lesion that showed similar staining patterns with labeled neurons and astrocytes (Figure 25, C). Two animals in the propentofylline treatment group had secondary lesions on the dorsal surface of the ipsilateral hemisphere (Figure 25, E). Typically, astrocytes were found in both the contralateral and ipsilateral ventricles but were brighter on the ipsilateral side (Figure 25, G-contralateral, H-ipsilateral). With the FJ-C stain, it was also possible to visualize the choroid plexus within the ventricles (Figure 25, F).

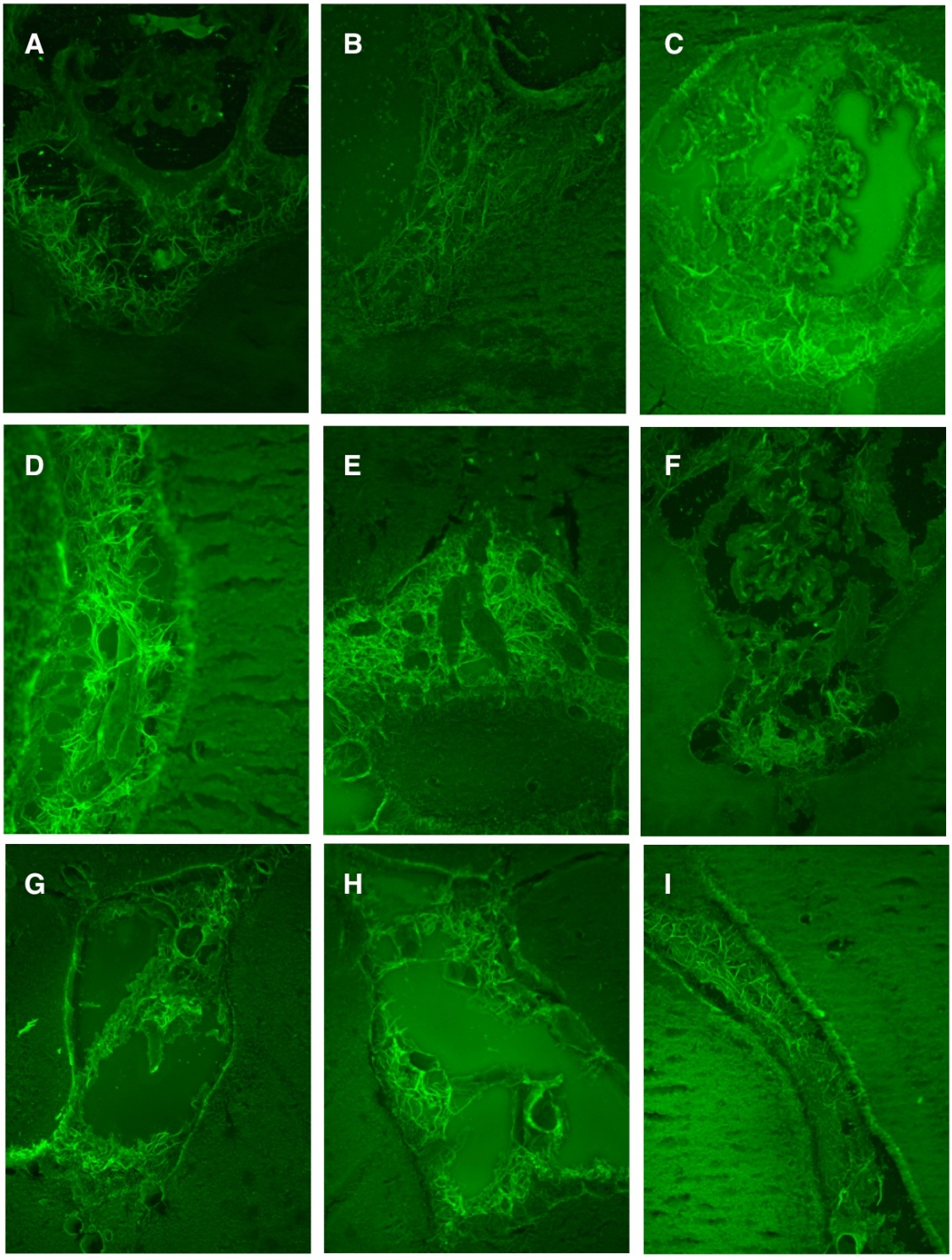

Figure 25: Different patterns of FJ-C staining in samples evident in every treatment group. Different patterns were evident in staining patterns in the different treatment groups. Representative images were selected from each treatment group and obtained at 10x magnification. Astrocytes were evident especially within the ventricles regardless of injury (A-ipsilateral, Sham animal). Astrocytes were particularly present around the lesion site (B- ipsilateral, TBI animal). Several animals had secondary infarct, some under the primarily lesion (C-ipsilateral, minocycline animal) and others on the dorsal surface of the ipsilateral hemisphere (E-PPF animal). Astrocytes were evident in the lateral ventricles of both the ipsilateral (D- minocycline animal, H-PPF animal) and contralateral hemispheres (G-PPF animal). The choroid plexus was evident with the FJ-C stain (F-4-PPBP animal) in all groups.

Discussion

The primary finding of the present study is that treatment with minocycline, PPF, or 4-PPBP following brain injury attenuated the behavioral deficits seen after TBI but was not able to correct histological measures. There were no significant behavioral differences between drug treatment groups and the sham group, indicating that drug treatment restored behavioral function. Our initial hypothesis that treatment with select drugs would be able to recover behavioral function as well as decrease lesion size was only partially supported. Behavioral improvements in all groups were consistent with previous work with brain injury indicating recovery of motor function following induction of experimental TBI in rats (Beaumont et al., 1999). Because TBI is a serious health concern not only in the United States but also around the world, recovery of behavioral function in an important goal of TBI research. However current treatments for TBI address symptoms, while failing to correct the underlying pathology (Haber et al., 2013) which was part of the goal of the study. Due to the potential actions of each individual treatment, further investigation is prompted to determine the mechanism by which these drugs improved behavioral outcomes following TBI.

Minocycline can interfere with both apoptotic and inflammatory pathways. As a chemical that can cross the blood brain barrier, minocycline directly interacts in the brain (Homsi et al., 2009). Consistent with previous work, treatment in this study improved functional recovery (Vonder Haar et al., 2013). However, minocycline has previously improved inflammation after TBI by shifting microglia toward the M2 phenotype as well as increasing *bcl-2* expression to decrease apoptosis (Homsi et al., 2009; Wang et al., 2004). These results that were not found in the present study. The potential discrepancy in these results may be because only a single dose was given in the current study while previous work has used multiple doses over the course of several days. While the functional improvements are consistent with this study, because of methodological weaknesses, it cannot be determined how minocycline facilitated these effects. Simple cell counts and degeneration of neurons as not able to explain the improvement in behavior. Future studies should attempt to measure microglial activation as well as *bcl-2* expression following minocycline treatment after experimental TBI to determine how minocycline mediates its effects. Further work should consider possible mechanisms by which minocycline specifically promotes functional recovery.

Previous work with PPF has determined that it has a variety of potential therapeutic benefits that would prove useful following brain injury. Inconsistent with previous work, no changes in astroglial activation were found following TBI (Bondon et al., 2016). However, the staining method used to visualize astrocytes, FJ-C has mixed support. Activated astrocytes and microglia may be able to uptake the stain, however it has not been determined what the morphology of the glial cells required for this to occur (Ehara & Ueda, 2009). The nonspecific nature by which FJ-C stains astrocytes may explain the non-significant findings in the current study. The improvement of functional recovery following PPF administration is consistent with previous work (Buytaert-Hoefen et al., 2002). However, the previous work with PPF have all used repeated doses following injury and our results suggest the potential for benefit with a single dose acutely following TBI. Future work with PPF administration following brain injury should characterize the morphology of microglia and astrocytes to determine if PPF mediated improved performance through glial interference.

The animals treated with 4-PPBP while they had significant improvements in behavior, had the largest lesion size of all drug treatments. The increased lesion size could be because of the side effects following drug treatment in this group. A methodological weakness of the present study was the inability to determine the effect of 4-PPBP administration on *bcl-2* levels. The high dose (1 mg) used in the present study had not been previously used. Previous work has used smaller doses (1 μmol/kg/hour) over longer periods of time and were able to see neuroprotective effects that were not replicated in the present study (Goyagi et al., 2001; Harukuni et al., 1998; Takahashi et al., 1996). As there were no cell count differences it is impossible to determine if 4-PPBP decreased cell death by the apoptotic processes by *bcl-2* expression (Clark et al., 1997). Future work with 4-PPBP following brain injury should consider lower single dose treatments following brain injury.

This study had several methodological weaknesses. First, there was a lack of naïve group. This would have been beneficial to determine if the consistency FJ-C staining patterns regardless of treatment group were due to artifact in the stain or due to the surgery itself. Also, there was no experimenter blinding during behavioral testing. This bias may explain why there were significant results in the behavioral measures, while there were no significant results in the histological measures. Further, there was no test to determine emotional changes that may have been caused by either the injury or the drug treatment. Emotionality and arousal influence the formation

of spatial memories (Wainwright, 2000). Finally, the method of TBI used in the present study has not be extensively characterized. It is thus subject to considerable heterogeneity of injury severity. This can be due to distance of the pistol from the brain surface as well as changes to the pressure of the CO2 canister. The heterogeneity of injury severity is reflected in large standard deviations in histology parameters.

There were several weaknesses with the histology methods. First, there was no ability to quantify the magnitude of fluorescence following FJ-C staining. The FJ-C stain has high background activity (Ehara & Ueda, 2009). Increasing the time that sections are pretreated with the potassium permanganate solution to ten minutes has been found to decrease background noise (Ehara & Ueda, 2009). While this time was used in this study, determining fluorescent staining patterns by eye is subject to human error and bias. Changes in the patterns following drug treatment may have been too small to detect by eye. Further, because of the non-specificity of the FJ-C stain, changes to the individual neurons due to DAI could not be analyzed.

Further, there was no analysis into the morphology of cells around the lesion. Dark stained neurons following Nissl staining have been previously found to have morphology consistent with apoptotic neurons (Kovesdi et al., 2014). Areas that stain dark after Nissl staining coincide with areas of subsequent neuronal death (Kovacs et al., 2014; Ooigawa et al., 2006). While the presence of dark stained neurons in this study is consistent with previous work with Nissl following TBI (Ooigawa et al., 2006), there was no morphological analysis to determine how these cells specifically changed. Understanding the morphology of the cells following injury would help to determine what kind of insult has occurred and what effect that may have on the functioning of the neurons.

Another weakness was that cell counts were only attempted in the CA1 region of the hippocampus. While CA1 has been found to be important in spatial encoding of information (Langston et al., 2010) other areas including the cortex, hippocampus (dentate gyrus, and the hilus), cerebellum, and thalamus have all shown cell loss following TBI (Clark et al., 1997; Cory et al., 2003; Raghupathi et al., 2000). Along with neuronal morphology, glial morphology was also not analyzed. To determine if drug intervention did influence glial activation, GFAP staining should be done to see if there is the expected increased activation following TBI (Susarla et al., 2014) and if this can be recovered with drug treatment. Finally, to determine if apoptosis was

decreased with the drug treatments, TUNEL staining should also be pursued to compare to previous work (Rink et al., 1995).

Future studies should attempt to fix these weaknesses and limitations. To do this, first, this study should be repeated in an accepted TBI model such as CCI or fluid percussion. This would give data that can be more easily compared to previous research as these models have been used extensively in research (Xiong et al., 2013). This may reduce the heterogeneity of the animals and improve statistical power. Further histological analyses should also be conducted. This can include FJ-C quantification, astrocyte and microglia labeling, cell (neuronal and glial) morphology, *bcl-2* expression levels, GFAP staining, TUNEL staining, as well as performing cell count in other areas. Future work should attempt to determine mechanism of action of these drugs specifically following TBI.

As this study was a first at attempting single dose administrations of these drugs, future research should work to determine drug dose effects. An important step in this process is determining dose responses of these drugs following TBI. Many of the present clinical trials have failed to incorporate this aspect into their study which may account for their failure in clinical trial (Stein, 2015). Dose responses should thus be performed in minocycline, PPF, and 4-PPBP. As the dose of 4-PPBP caused side effects, future studies should decrease the dose used. Future work should also combine both minocycline and propentofylline as they showed the most promise in this study. There is still much unknown about the actions of astrocytes and microglia following TBI, however, the benefits of both minocycline and propentofylline following TBI are promising for future therapeutic intervention.

While behavioral results significantly improved following drug treatment after experimental TBI, similar improvements were not seen in histological measures. This is because recovery on behavioral functions does not necessarily indicate recovery in other areas. A period of decreased functioning following TBI has been suggested to exist at a sub-clinical level that cannot be detected from behavioral tests (Beaumont et al., 1999). Another possible explanation for the significant results in the behavioral measures, but not histological, is that only certain animals developed secondary infarcts following the initial lesion and there was a large variation in the severity of

the injuries both within and between groups. The development of secondary injury can prevent a recovery (Beaumont et al., 1999) and mask significant results in the histology measures.

In conclusion, treatment with a single dose of minocycline, propentofylline, or 4-PPBP 10 minutes following experimental modified TBI could recover motor and spatial memory deficits by 15 days post-injury. No previous work has considered a comparison of these three drug treatments with only a single dose given at 10 minutes after brain injury. The use of a single dose can be helpful if implemented into first responder protocols for TBI. First responders arrive on average 10 minutes after the call (Meyer et al., 2008) which falls in the five minute to hour effective window of minocycline (Haber et al., 2013) and potentially other neuroprotective drugs after TBI. These treatments were not able to improve histological outcomes such as lesion size, cell count, astrocytic activation, or degenerating neurons.

Incidence of even a single brain injury has been linked to an increase in risk for development of neurodegenerative diseases such as Alzheimer's later in life (Fleminger, 2003). Alzheimer's disease accounts for even more deaths than TBI, with over 93,000 deaths per year (CDC, 2015). Individuals who survive TBI injuries may succumb to Alzheimer's disease later in life if effective treatment of the underlying pathologies of TBI are not found. By the year 2020, TBI is expected to be the leading cause of death and disability worldwide (Humphreys et al., 2013). Finding effective treatments for not only the symptoms but also the underlying causes such as microglia, astrocytes, and apoptosis is thus an imperative matter for health care.

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Supplementary Tables

S Table 1: **Group denotations.**

Group	Number
Sham	1
TBI	2
Minocycline	3
PPF	4
PPBP	5

S Table 2: **Beam Walk Averages for all experimental days. Latencies recorded in seconds and are the averages of three trials per day. Pre-injury days are denoted by negative numbers and post-injury days are denoted by positive numbers.**

Animal	Group	BW -3	BW -2	BW -1	BW 1	BW 2	BW 3	BW 4	BW 5
A	**3**	11.05	10.54	8.94	13.57	7.95	9.39	8.25	9.82
B	**3**	7.94	11.62	10.50	15.46	8.44	9.21	16.27	13.94
C	**3**	10.59	15.55	12.46	12.19	11.77	14.33	8.56	14.71
D	**3**	7.76	6.87	8.88	10.82	9.21	10.63	7.84	7.88
E	**3**	9.92	7.34	8.33	17.74	9.38	10.05	6.46	3.70
F	**2**	8.69	7.78	9.29	35.88	38.70	37.85	25.06	18.50
G	**2**	17.00	5.04	6.72	34.94	21.82	21.12	10.59	7.53
H	**2**	9.85	3.95	2.88	18.24	26.55	18.05	11.25	12.11
I	**2**	7.11	9.13	7.25	47.61	44.38	31.50	16.36	14.25
J	**2**	9.52	6.92	4.57	28.99	39.56	39.63	11.51	12.10
K	**4**	10.80	7.09	10.08	8.50	13.91	15.19	12.75	9.00
L	**4**	9.89	8.25	7.24	11.82	13.00	13.63	10.00	11.88
M	**4**	14.89	13.52	9.42	11.25	10.87	12.45	12.86	11.98
N	**4**	13.37	8.52	8.59	29.38	29.25	8.10	8.97	13.59
O	**4**	10.41	13.88	10.84	24.61	19.61	12.00	5.89	8.43
P	**1**	11.29	11.94	12.94	7.99	11.55	9.08	7.40	5.99
Q	**1**	7.54	6.50	7.26	10.27	10.82	10.60	13.35	6.40
R	**1**	10.15	6.78	7.92	7.84	11.54	9.66	7.35	6.77
S	**1**	13.35	7.81	12.00	13.11	10.48	13.23	17.37	7.80
T	**1**	11.13	9.06	5.48	5.70	8.23	15.99	9.95	7.62
U	**5**	7.36	5.65	10.15	18.98	14.46	6.45	3.96	2.67
V	**5**	14.41	5.86	8.62	90.00	16.90	12.15	27.11	15.41
W	**5**	5.96	6.35	5.70	9.49	11.79	10.18	9.23	10.22
X	**5**	24.53	8.90	7.67	10.16	14.86	9.12	18.74	9.66
Y	**5**	7.19	11.08	5.94	13.31	13.78	11.89	9.22	8.16

S Table 3: Average beam balance ratings following brain injury in all experimental animals. Ratings were as follows 1, balances with steady posture; 2, grasps beam, but steady; 3, grasps beam with lateral/medial balancing movements, generally unsteady; 4, falls in 10-60 seconds; 5, falls in 0-10 seconds; 6, no attempt to balance at all.

Animal	Group	BB -3	BB -2	BB -1	BB 1	BB 2	BB 3	BB 4	BB 5
A	**3**	1.00	1.33	1.00	1.67	1.00	1.33	1.00	1.00
B	**3**	1.00	1.00	1.00	2.00	1.33	1.67	1.00	1.00
C	**3**	1.33	1.00	1.33	1.67	1.67	1.33	1.33	1.67
D	**3**	1.00	1.50	1.00	2.33	1.33	1.67	1.00	1.00
E	**3**	1.33	1.00	1.00	1.67	1.33	1.33	1.00	1.00
F	**2**	1.33	1.00	1.00	3.00	3.00	2.33	1.67	1.33
G	**2**	1.33	1.33	1.00	2.70	2.33	1.70	1.30	1.00
H	**2**	1.00	1.33	1.67	3.30	3.67	3.00	2.00	2.33
I	**2**	1.33	1.67	1.00	3.00	3.67	2.70	1.70	1.33
J	**2**	1.00	1.00	1.00	3.30	3.00	2.70	2.00	2.67
K	**4**	1.33	1.33	1.00	2.33	1.00	1.33	1.33	1.00
L	**4**	1.67	1.00	1.33	1.67	2.00	2.00	2.00	1.33
M	**4**	1.67	1.33	1.67	2.33	1.67	1.00	1.67	1.00
N	**4**	2.00	2.00	1.67	2.67	1.67	1.67	1.67	1.00
O	**4**	2.00	1.67	1.33	2.00	1.67	1.33	1.00	1.33
P	**1**	1.00	1.67	1.00	1.67	1.67	2.00	1.67	1.33
Q	**1**	1.33	1.00	1.00	1.67	1.33	1.33	1.33	1.00
R	**1**	1.00	1.67	1.33	2.00	1.00	1.33	1.33	1.33
S	**1**	1.67	1.67	1.33	1.33	1.33	1.00	1.67	1.67
T	**1**	1.67	1.33	1.33	1.00	1.33	1.33	1.00	1.00
U	**5**	1.67	1.00	1.00	2.33	1.33	1.33	1.00	1.00
V	**5**	1.67	1.00	1.00	6.00	5.00	5.00	4.33	4.33
W	**5**	2.00	1.67	1.33	2.67	1.67	1.33	1.33	1.33
X	**5**	2.00	1.67	1.33	3.00	1.67	1.00	1.67	1.00
Y	**5**	2.00	1.33	1.00	2.67	2.00	1.33	1.00	1.00

S Table 4: Average labyrinth water maze latencies for all groups. Testing began 10 days after injury.

Animal	Group	WM 1	WM 2	WM 3	WM 4	WM 5
A	**3**	78.79	91.01	56.41	31.84	19.74
B	**3**	96.46	76.28	26.02	20.89	21.92
C	**3**	103.60	80.17	79.05	50.47	54.15
D	**3**	92.76	55.94	30.82	21.81	10.95
E	**3**	105.40	103.90	58.92	55.72	62.39
F	**2**	85.55	83.07	63.53	60.54	28.44
G	**2**	78.74	70.18	48.79	35.58	17.98
H	**2**	24.50	13.39	7.42	10.04	7.66
I	**2**	29.31	17.23	12.41	16.74	11.53
J	**2**	94.06	73.22	28.44	18.45	19.23
K	**4**	116.86	86.34	66.32	63.51	30.06
L	**4**	88.17	56.54	50.36	28.66	23.76
M	**4**	111.27	62.59	59.05	37.21	45.64
N	**4**	114.49	63.14	58.64	43.56	38.53
O	**4**	105.15	65.01	54.17	37.50	32.09
P	**1**	61.64	29.29	18.30	39.04	41.03
Q	**1**	66.69	46.29	26.84	13.29	8.41
R	**1**	37.48	34.43	22.84	19.50	9.52
S	**1**	39.23	25.96	22.97	12.41	12.12
T	**1**	51.76	24.39	13.90	18.48	16.67
U	**5**	78.27	89.14	64.15	45.70	30.27
V	**5**	111.59	106.08	85.28	70.52	75.76
W	**5**	70.53	55.38	51.53	39.91	56.60
X	**5**	89.82	87.32	54.70	50.49	39.93
Y	**5**	80.84	74.11	56.02	34.63	28.95
Z	**1**	127.73	79.80	50.22	32.51	17.46
AA	**1**	11.46	97.53	94.89	71.74	63.84
BB	**2**	134.96	124.38	180.03	106.55	106.57
CC	**2**	155.11	170.88	176.52	186.01	112.47

S Table 5: Average labyrinth water maze errors for the post-injury testing days. An error was recorded as half a body length in the wrong direction.

Animal	Group	WM 1	WM 2	WM 3	WM 4	WM 5
A	**3**	9.00	10.00	6.00	4.33	1.67
B	**3**	7.67	5.33	1.00	0.67	2.00
C	**3**	6.33	4.67	7.00	4.33	5.00
D	**3**	6.00	3.00	2.33	1.67	0.00
E	**3**	7.33	8.00	3.67	3.33	5.33
F	**2**	5.00	4.33	5.00	5.00	1.00
G	**2**	3.67	6.00	2.33	3.00	0.00
H	**2**	1.67	0.67	0.00	1.00	0.00
I	**2**	0.00	0.67	0.00	1.33	0.67
J	**2**	7.33	5.33	0.00	0.00	1.67
K	**4**	10.00	6.00	7.00	7.00	3.67
L	**4**	11.00	10.33	9.00	3.67	2.33
M	**4**	10.00	5.33	6.00	2.67	4.33
N	**4**	8.67	6.00	6.00	4.67	2.33
O	**4**	11.00	5.00	9.67	5.00	4.33
P	**1**	3.33	1.33	0.33	2.33	3.33
Q	**1**	3.67	3.00	0.67	0.33	0.00
R	**1**	2.33	2.33	3.67	2.67	0.33
S	**1**	3.00	0.67	0.00	0.67	0.00
T	**1**	2.67	0.67	1.33	1.00	0.67
U	**5**	9.00	9.00	7.67	5.00	3.00
V	**5**	10.60	12.33	9.00	9.00	6.33
W	**5**	7.33	6.33	5.67	3.67	7.00
X	**5**	6.00	8.00	4.67	5.67	3.67
Y	**5**	7.00	8.00	6.33	4.33	3.00
Z	**1**	9.67	5.00	4.67	2.67	1.33
AA	**1**	8.00	4.67	4.33	2.00	3.00
BB	**2**	9.33	11.33	10.33	6.33	10.33
CC	**2**	12.00	7.33	11.67	16.33	7.00

S Table 6: Average lesion perimeter taken from three representative sections from each animal.

Animal	Group	Perimeter
A	**3**	3848.442333
B	**3**	6687.094667
C	**3**	5751.508
D	**3**	6670.841
E	**3**	7407.331
F	**2**	6343.814
G	**2**	5348.544
H	**2**	7391.397
I	**2**	5670.975
J	**2**	11967.687
BB	**2**	9190.054
CC	**2**	11451.868
K	**4**	5629.03
L	**4**	5865.548
M	**4**	6380.701
N	**4**	6425.975
O	**4**	8514.975
P	**1**	5548.242
Q	**1**	6105.578
R	**1**	5743.24
S	**1**	5052.642
T	**1**	5333.323
Z	**1**	6510.728
AA	**1**	4537.052
U	**5**	5078.795
V	**5**	6211.178
W	**5**	6049.595
X	**5**	8323.522
Y	**5**	11010.858

S Table 7: Average cell counts for all animals. Averages were taken from 3 ROI images around the CA1 of the hippocampus. Some animals did not contain this region or could not be properly analyzed due to issues with the histology.

Animal	Group	Cell Count
A	**3**	106
B	**3**	95.67
C	**3**	96
D	**3**	91.33
E	**3**	90
F	**2**	90
G	**2**	103.67
H	**2**	81.67
I	**2**	N/A
J	**2**	122.67
BB	**2**	110
CC	**2**	143
K	**4**	87.67
L	**4**	N/A
M	**4**	106
N	**4**	70
O	**4**	170
P	**1**	99
Q	**1**	N/A
R	**1**	106.33
S	**1**	N/A
T	**1**	78.33
Z	**1**	64.33
AA	**1**	115
U	**5**	99
V	**5**	120
W	**5**	74.67
X	**5**	67.33
Y	**5**	142