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Post-TBI Hippocampal Neurogenesis in Different TBI Models

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

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Abbreviations

bFGF	Basic Fibroblast Growth Factor
BrdU	5-Bromo-2-deoxyuridine
CCI	Controlled Cortical Impact
CDC	Centers for Disease Control and Prevention
CNS	Central Nervous System
CT	Contralateral
DAB	3,3'-Diaminobenzadine-tetra-hydrochloride
DCX	Doublecortin
DG	Dentate Gyrus
ED	Emergency Department
EGF	Epidermal Growth Factor
FPI	Fluid Percussion Injury
GCL	Granular Cell Layer
GZ	Granular Zone
I.P.	Intraperitoneal
IP	Ipsilateral
LFPI	Lateral Fluid Percussion Injury
LSD	Fisher Least Significant Difference
LFPI	Lateral Fluid Percussion Injury
MWM	Morris Water Maze
NPC	Neural Progenitor Cells

NSC	Neural Stem Cells
PBS	Phosphate-Buffered Saline
SGZ	Subgranular Zone
SSC	Saline Sodium Citrate
SVZ	Subventricular Zone
TBI	Traumatic Brain Injury
TNF	Tumor Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

Abstract

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Traumatic brain injury (TBI) leads to short-term and long-term consequences that

can cause many different life-long disorders. Studies of TBI have generally focused on

the acute stage; however, it is now becoming important to investigate chronic responses

following TBI as clinical reports of dementia and cognitive impairments have been linked

to a history of TBI. Recent data have established that cognitive function is associated

with hippocampal neurogenesis. Chronic injury induced changes in the brain may affect

this endogenous process. Chronic responses following TBI include cell death pathways

and inflammatory responses that are persistent in the brain for months to years after

injury. In this study we investigate the chronic consequences of TBI on adult

neurogenesis and the possible involvement of chronic-inflammation in regulating adult

neurogenesis. We used two popular TBI animal models, Control Cortical Impact (CCI)

and Lateral Fluid Percussion Injury (LFPI) models, to examine focal and diffuse injury responses respectively. Adult rats received CCI, LFPI, or sham injury and were sacrificed at either 15 days or 3 months after injury to examine either subacute or chronic TBI-induced responses respectively. We found no change in levels of proliferation activity at both time points in both TBI models compared to sham animals. Using Doublecortin immunolabeling we found an enhanced generation of new neurons at 15 days after injury and by 3 months this activity was significantly reduced in both TBI models compared to sham animals. We also found persistent inflammation in the injured brains at both time points. Morphological assessment showed that LFPI model of TBI causes shrinkage of the ipsilateral hippocampus. Our results show that moderate TBI induced hippocampal neurogenesis in both models at the early time post-injury. However, at chronic stage, reduced hippocampal neurogenesis is observed in both models and this is accompanied by chronic inflammation. These results suggest that persistent inflammatory responses maybe detrimental to normal neurogenic activity, leading to cognitive impairment and neurodegeneration in long-term TBI survivors.

Chapter 1 - Introduction and Background

Traumatic Brain Injury (TBI) is a major cause of death and life-long disability worldwide. It is defined as a physical insult to the brain, which disrupts normal brain functions and can be caused by a bump, blow, or jolt to the head (Marr and Coronado, 2004). Various accidents such as car crashes, sports injuries, war related injuries, etc., could lead to TBI. Damaging factors of TBI can have acute and chronic consequences leading to many neurological disorders such as memory loss, behavioral dysfunction, epilepsy, dementia, and sleep disorders.

Various animal models have been developed to study TBI. These models work by replicating specific aspects of a TBI to study acute and/or chronic injury mechanisms. Using various TBI animal models, many studies have shown an injury-induced up-regulation in adult neurogenesis (Sun et al., 2005; Sun, 2016). These study have found an increase in proliferation of neural stem cells (NSC) and neural progenitor cells (NPC) in response to injury in the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus (DG) of the hippocampus (Dash et al., 2001; Rice et al., 2003). This neurogenic response after injury indicates that the brain has a limited ability to initiate mechanisms for repair and regeneration. Our lab has shown that injury-induced changes in hippocampal neurogenesis are related to innate cognitive recovery (Sun et al., 2015). Spontaneous cognitive recovery seen in many TBI patients may be a result of this endogenous neurogenic response.

Most TBI studies focus on acute and subacute changes after injury. However, there are growing number of clinical reports of long-term TBI patients that have developed neurodegenerative diseases and cognitive dysfunction (Mayeux et al., 1995; Castriotta et al., 2007). We hypothesize that neurodegeneration and cognitive dysfunction maybe a consequence of long-term injury-induced changes in adult neurogenesis. We suspect persistent secondary injury mechanisms of chronic-TBI such as neuroinflammation may be responsible for these neurogenic changes.

Studies have shown that chronic-neuroinflammation can have a detrimental impact on adult neurogenesis (Fuster-Matanzo et al., 2013). In this study we used two popular TBI animal models, controlled cortical impact (CCI) injury and lateral fluid percussion injury (LFPI) models, to explore changes in adult neurogenesis in the chronic-TBI stage and the association of these changes to neuroinflammation.

Epidemiology

 TBI affects millions of people worldwide every year. According to the Centers for Disease Control and Prevention (CDC), in the United States alone, 16% of all injury related emergency department (ED) visits are diagnosed as TBI. About 1.7 million people each year are affected by TBI in the United States. Many people that survive the injury develop life long disabilities. In the beginning of 2005 about 3.17 million people in the United States were living with long-term disability resulting from TBI (Zaloshnja et al., 2008). In 2010 the economic burden of TBI was about $76.5 billion, not including TBIs managed in non-hospital locations and at federal institutions (Finkelstein et al., 2006).

Highest rate of TBI related hospitalizations and deaths occur in adults over 75 years of age and 775,000 elderly adults develop life-long disabilities (Zaloshnja et al., 2008). With pre-existing medical conditions and slower rates of functional improvements, elderly adults are more likely to have longer hospital stays and higher risk of mortality (Mosenthal et al., 2004; Thompson et al., 2006).

The leading cause of TBI related ED visits is due to falls, and leading causes of TBI related deaths is motor vehicle accidents and suicides (Coronado et al., 2012). In 2010, TBI caused about 50,000 total deaths, not accounting for people that did not receive medical care or people diagnosed at federal facilities (Faul et al., 2010). According to the Department of Defense, about 5.6 million United States military members from 2000 – 2011 were diagnosed with TBI (CDC, 2015). Combat situations that result in mild TBI may lead to increased risk of post-traumatic stress disorder (Bryant et al., 2010) which can further increase risk for various post-concussive symptoms (Brenner et al., 2010).

Since 1980, hospitalization rate for mild TBI has significantly decreased due to improved safety and accident prevention programs whereas hospitalization rate for severe TBI has increased mainly due to improved availability and quality of medical treatments leading to greater survivability (Summers et al., 2009). This increase in survival rate leads to a greater number of people suffering from long-term disabilities after TBI. It is important to address these concerns of chronic-TBI by studying the mechanisms

regulating the spread of injury damages and developing treatments to reduce and/or reverse these damages.

Biomechanics and mechanisms of TBI

The cause of a TBI is a physical force that acts on the head resulting in structural damages and eventually causes damages to the brain at the cellular and biochemical levels. These cellular and biochemical changes then lead to cognitive and behavioral dysfunction. The underlying damages of a TBI can vary depending on the type and way injury is induced. The mechanical insults to the head can be characterized in three general categories: impact, impulse, and static. Impact occurs to the head with high magnitude and short duration of 50 ms or less causing deformation of the skull and brain tissue damage (Davis, 2000). Impulse occurs when there is a sudden change of head motion due to acceleration or deceleration of the body and does not involve deformation of the skull (Davis, 2000). Static involves long duration, greater than 200 ms of compressive force to the head causing skull deformation (Davis, 2000).

The injuries caused to the brain tissue can be focal or diffuse. Focal injury occurs when the injury is focused on a particular area of the brain caused by a direct blow to the head and involves contusion, brain laceration, and hemorrhage (Gennarelli, 1993). Diffuse injury usually involves widespread microscopic damages caused by sudden head movement and involves concussion and diffuse axonal injury (Gennarelli, 1993).

The magnitude and duration of the mechanical force determines the extent of brain injury along with accompanying damages such as brain hemorrhage and swelling (Xiong et al., 2013). The resulting immediate damages are referred to as the primary injury. Besides the skull fracture and brain tissue damage, primary injury can also cause the initiation of secondary injury mechanisms (Xiong et al., 2013). Secondary injury involves non-mechanical damages such as increased intracranial pressure, reduced cerebral blood flow, blood brain barrier damage, bleeding, edema, diffuse axonal injury, cell death, and neurovascular damage (Xiong et al., 2015). A complex interplay of cellular and biochemical mechanisms such as excitotoxicity, mitochondrial damage, changes in gene expression, inflammatory responses, and oxidative stress are involved in the spread of secondary injury (Xiong et al., 2013). However, the onset of secondary injury mechanisms can happen seconds to months after injury, providing a therapeutic window of opportunity for treatment against the spread of damages after TBI.

Pathology of TBI

Physical damages to the brain can lead to long-term cognitive impairments and significantly increase chances of developing neurological diseases (Koponen et al., 2002). Diffuse axonal injury serves as a clinical hallmark of human TBI and studies show that damage to white matter tracks correlate with declined cognitive function (Kraus et al., 2007). For example, using diffuse tensor imaging and neurophysiological testing, one study showed that TBI patients with greater white matter pathology had greater cognitive deficits (Kraus et al., 2007). Memory and attention are commonly impaired at all levels of TBI injury (Lovell and Franzen, 1994).

Cognitive deficits can have a devastating impact on social behavior and lifestyle, which also affects the lives of caregivers such as family and friends. Furthermore, behavioral changes in TBI patients may implicate the development of psychiatric disorders (Castriotta et al., 2007). Studies have reported that as many as half of TBI outpatients may be suffering from psychiatric disorders, and patients with pre-existing conditions have a higher likelihood of developing other disorders such as depression, anxiety, sleep disorders, and substance abuse (Koponen et al., 2002; Hibbard et al., 1998). Disruption of the normal sleep cycle, causing various sleep disorders such as obstructive sleep apnea, posttraumatic hypersomnia, and narcolepsy, has also been linked to post-TBI consequences in about half of TBI outpatients (Castriotta et al., 2007). TBI also increases chance of death from other diseases. In some studies, TBI patients were more likely to die from seizures, septicemia, pneumonia, digestive disorders, and circulatory disorders compared to patients without a TBI history (Harrison-Felix et al., 2006; Harrison-Felix et al., 2009; Shavelle et al., 2001).

Surviving TBI patients often suffer from long-term neurological disorders such as epilepsy and dementia. There is a rising concern about the relationship between TBI and dementia. For example, TBI patients who have the ApoR E4 allele, which has been implicated in Alzheimer's disease, are 10 times more likely to develop Alzheimer's disease than healthy people (Mayeux et al., 1995). Studies have reported that TBI induces up-regulation or activation of certain biochemical mechanisms that have also been found to play a role in these degenerative diseases (Uryu et al., 2007). Although the detailed mechanisms of these processes are unclear, secondary injury mechanisms such as damage

to the blood brain barrier, free oxygen radicals causing oxidative stress, and other biochemical changes may be responsible for the onset of degenerative processes in the brain inducing dementia and other chronic diseases (Lye and Shores, 2000; Zhang et al., 2000).

Adult Neurogenesis

In 1928 Ramon y Cajal proposed the idea that the brain loses the ability of growth and cellular regeneration after development (y Cajal, 1959). However, there is now overwhelming evidence indicating certain areas of the mammalian brain retain the ability to regenerate neural and glia cells. Evidence of adult neurogenesis in mammalian brain is largely found in rodent studies although it is also shown in many primate studies including humans (Rakic et al., 1985).

The most active neurogenic regions in the adult mammalian brain are restricted to the SVZ of the lateral ventricles and the subgranular zone (SGZ) of the DG in the hippocampus (Lois and Alvarez-Buylla, 1993; Altman and Das, 1965). Some studies have also reported evidence of adult neurogenesis in the neocortex, striatum, and hypothalamus although these findings are controversial (Cameron and Dayer, 2008; Gould, 2007).

The neurogenic process starts with the NSC proliferation, giving rise to more NSC and sometimes asymmetrically dividing into NPC, which are more determined to differentiate into either the neural or glial lineage (Gage, 2000). NSC division involves

both asymmetric and symmetric division. During asymmetric division, one NSC divides to produce one NSC and one NPC (Kriegstein and Alvarez-Buylla, 2009). Symmetric cell division can be proliferative, where one NSC divides into two identical NSCs, or differentative, where one NSC divides into two NPCs (Kriegstein and Alvarez-Buylla, 2009). NPC then migrate to their destinations where they can differentiate and have functional potential. In the SVZ, most NPC migrate to the olfactory bulb, via the rostral migratory stream, where they differentiate into olfactory interneurons (Gritti et al., 2002). In the DG, migration is much more restricted, however, NPC do travel a short distance from the SGZ to the granular cell layer, where they can differentiate into granular neurons (Kempermann and Gage, 2000; van Praag et al., 2002). In both regions, migration of the cells and extension of dendrites and axons is guided by extracellular matrix molecules (Bovetti et al., 2007) and chemoattractive and repulsive molecules similar to migratory signaling pathways of development (Wu et al., 1999).

In the DG, differentiation from NPC to mature functional granular neurons involves transition through several cell types. The proliferation stage of neurogenesis involves Type 1 and Type 2 progenitor cells. Type 1 cells, also called radial glial cells, divide slowly and asymmetrically into Type 2 cells, and Type 2 cells divide rapidly into more Type 2 cells or differentiate into neuroblasts (Bonaguidi et al., 2012). Neuroblasts then differentiate into immature neurons which, if allowed to survive, mature into granular neurons that extend their dendrites into the molecular layer and extend axons into the correct area of the CA3 region of the DG (**Figure 1.1**, Aimone et al., 2014; Hastings and Gould, 1999).

In both the SVZ and DG, pre-existing neurons are continuously replaced by newly generated neurons (Gage, 2000). In the DG, this amount of neuronal turnover varies in different regions such as the superficial granular cell layer, where 50% of the existing cells are replaced by late postnatal neurogenesis, and the deeper granular cell layer, where almost all pre-existing granular neurons are replaced (Imayoshi et al., 2008). The addition of these new neurons in the DG is significant enough to impact network function and has been shown to be important in normal cognitive function and behavior (Sun, 2016).

During the maturation period of immature neurons, when they are undergoing morphological and physiological changes, their survival and potential for integration into the existing network is modulated by experience and on-going activity of the animal (Tashiro et al., 2007; Kee et al., 2007). Studies inhibiting adult neurogenesis in DG of rodents show that although inhibition may not cause depletion of existing neuron populations, it does retard the formation and integration of new neurons into the existing population which causes defects in spatial memory learning tasks such as Morris Water Maze (MWM) (Imayoshi et al., 2008; Jessberger et al., 2009). This supports the idea that the role of neurogenesis in the DG is for modulation of existing neural networks for hippocampal-dependent learning and memory functions, important during postnatal cognitive development.

Additionally, many studies show that cognitive function is affected by the level of adult neurogenesis which can be modulated by environmental experience, activity, and drugs, further confirming that adult neurogenesis plays an important role in hippocampal-dependent learning and memory functions. Animals placed in enriched environment or given physical exercise show increased levels of granule cell proliferation and improved performance in spatial memory tasks such as MWM (Brown et al., 2003; Van et al., 1999). Similar results have been found with exogenous administration of growth factors such as Basic Fibroblast Growth Factor (bFGF) (Wagner et al., 1999; Sun et al., 2009).

Basket cell

type 1

type 2

Astrocyte

3 days

1 wk

2 wk

3 wk

>4 wk

to CA3

Proliferation

Differentiation

Survival
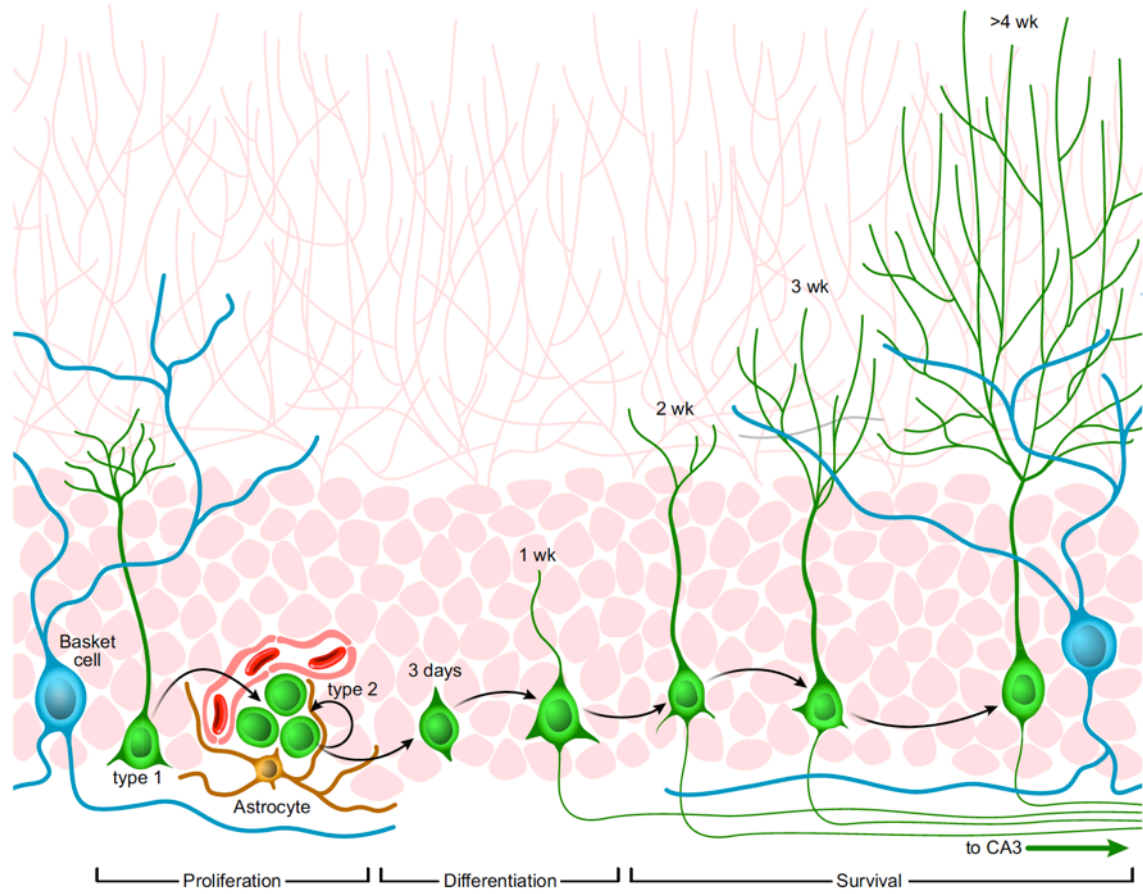
Figure 1.1. Adult neurogenic process in the dentate gyrus. Image extracted from

Aimone et al., 2014. Proliferation step involves Type 1 cells asymmetrically dividing into

neuroblasts by day three. By one week these neuroblasts become immature neurons,

which then enter a survival period to mature into integrated granular neurons extending

their dendrites into the molecular layer and axon into the CA3 region by 3-4 weeks.

TBI Induced Neurogenesis

Modulation of adult neurogenesis has also been confirmed in TBI studies. This TBI stimulated endogenous neurogenesis is a natural response of the brain to promote repair (Sun, 2016). Although the exact mechanisms underlying the TBI-induced neurogenic response are unclear, studies have reported the up-regulation of growth factors such as Vascular Endothelial Growth Factor (VEGF), p75 NTR, bFGF, and Epidermal Growth Factor (EGF) following injury may regulate this process (Lee and Agoston, 2010; Catts et al., 2008; Sun et al., 2009; Sun et al., 2010). TBI animal models have shown increased proliferation of new cells in both the DG and SVZ (Dash et al., 2001; Rice et al., 2003). In the DG, cell proliferation peaks at 2 days post-injury thereafter gradually declining and reaches baseline level at 14 days post-injury (**Figure 1.2**, Sun et al., 2005). In terms of differentiation and survival of new neurons, studies have reported different results and these differences could be attributed to many different experimental factors such as different injury models or species used (Sun et al., 2007; Villasana et al., 2014). In the DG, almost half of all TBI-induced newly generated cells are able to survive to 10 weeks post-injury and most of these cells become mature neurons integrated into the functional hippocampal network, leading to cognitive recovery (**Figure 1.3**, Sun et al., 2007). In TBI animal studies, newly generated cells take about 14 days to fully mature and integrate into the functional network, which at that time animals begin to show cognitive recovery in MWM test, and by 60 days their performance is not significantly different from sham animals (Sun et al., 2007). This structural and functional recovery, via the neurogenic response of the brain following

injury, can be exogenously stimulated to promote regeneration and repair as a strategy for TBI treatment.

Current developments for TBI treatment include different methods of further stimulating neurogenesis following injury. Exogenous administration of growth factors such as bFGF and EGF has shown increased proliferation in the DG and SVZ compared to untreated animals after injury (Sun et al., 2009; Sun et al., 2010). Similar treatments of other growth factors such as Insulin-like growth factor 1, brain-derived neurotrophic factor, VEGF, and S100B in injured animals have shown increased generation of new neurons and improved cognitive and functional recovery compared to untreated animals (Carlson et al., 2014; Gao et al., 2009; Thau-Zuchman et al., 2010; Kleindienst et al., 2005). Pharmacological agents have also been developed to target post-TBI neurogenesis such as Cerebrolysin, 7,8-Dihydroxyflavone, LM11A-31, and carbamylated erythropoietin, showing increased neurogenesis with improved cognitive and functional recovery (Zhang et al., 2015; Chen et al., 2015; Shi et al., 2013; Xiong et al., 2011). Simple exercise such as running or putting animals in an enriched environment can also stimulate neurogenesis and improve recovery after injury (Gaulk et al., 2005; Piao et al., 2013).

A

BrdU cell counts in the SGZ

Legend:
- ■ Injured juvenile
- □ Sham juvenile
- ▨ Injured adult
- ▩ Sham adult

Y-axis: Number of BrdU+ cells

X-axis: Days after injury
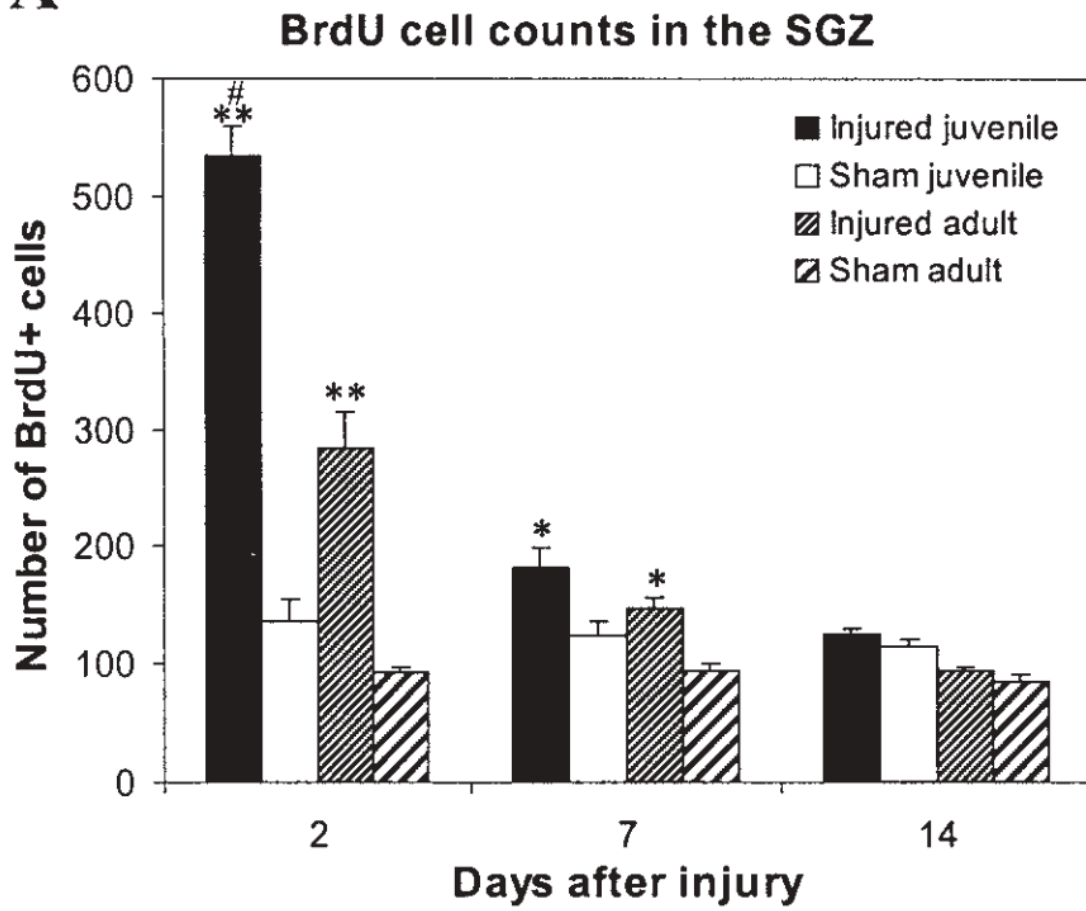
Figure 1.2. Proliferation timeline in the dentate gyrus. Results image extracted from Sun et al., 2005. The number of BrdU positive cells in the SGZ of the DG are shown at 2, 7, and 14 days after injury. Animals received I.P. injections of BrdU at 2, 7, or 14 days after injury and were sacrificed 24 hours after the last injection. Injured juvenile and injured adult groups show peak proliferation at 2 days post injury and no difference in proliferation compared to sham animals at 14 days. Also, injured juveniles had almost twice the proliferation level as that of the adults at 2 days post injury.
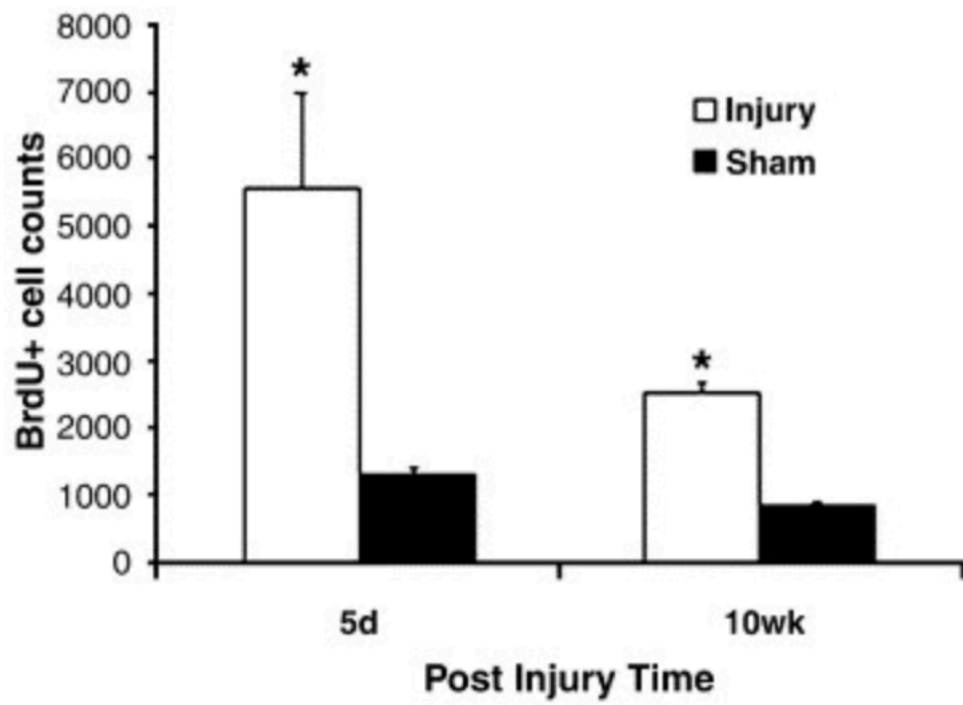
Figure 1.3. New cells survive long time after injury. Results image extracted from Sun et al., 2007. Animals received I.P. BrdU injections at 5 days post injury. Injured animals show 4-fold increase of the number of BrdU cells at 5 days post injury. At 10 weeks post injury the number of BrdU cells was 3-fold higher than sham animals indicating that a significant number of BrdU cells survive even at 10 weeks post injury.

Animal models of TBI

Our current understanding of TBI, mechanisms regulating damages, and development of potential treatments can be attributed to the use of animal models of TBI. To understand the heterogeneity of TBI, different animal models are in use that mimic specific aspects about the injury and resulting damages. These various models include fluid percussion injury (FPI), CCI, penetrating ballistic-like brain injury, weight-drop, Blast, and repeated mild TBI (Xiong et al., 2013). The most popular and widely used models are FPI and CCI.

Since TBI can be caused in various situations including car accidents, sports, and military grounds, these models are set up to replicate primary and secondary injuries to the brain. For example, the FPI models are designed to replicate focal cortical contusion with diffuse subcortical neuronal injury, without skull fracture (Xiong et al., 2013). The CCI model is designed to replicate mostly focal injury mechanisms (O'Connor et al., 2011). These models bypass the skull fracture, which allows for specific replication of injury aspects and control of injury parameters without randomized damages created by skull fragments.

Both the CCI and FPI models allow for control of the injury causing parameters. In the FPI model, injury severity can be controlled by the height of the pendulum, which hits the fluid tube exerting a fluid pressure pulse to the brain (Kabadi et al., 2010). The CCI model has an advantage over the FPI model in that it allows for more control of parameters such as time, velocity, and depth of impact (O'Connor et al., 2011).

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Nevertheless, both models have been successfully used to reproduce TBI and also to study the modulation of neurogenesis following injury.

TBI-induced Neuroinflammation

One of the several pathological conditions of TBI includes neuroinflammation. The complexity of many different molecular interactions and signaling pathways involved in neuroinflammation makes it difficult to elucidate the detailed mechanisms regulating this condition (Morganti-Kossmann et al., 2002). However, studies have reported that the immune system and central nervous system (CNS) resident glia cells are involved in inflammatory immune response after injury (Morganti-Kossmann et al., 2007). Although the CNS is considered to be unaffected by the immune system, the disruption of the blood brain barrier after injury causes an increased expression of endothelial adhesion molecules and disruption of endothelial tight junctions leading to increased migration of immune cells such as leukocytes from systemic circulation into the brain parenchyma via interaction of endothelial adhesion molecules with integrins on the surface of leukocytes (**Figure 1.4**, Ziebell and Morganti-Kossmann, 2010; Morganti-Kossmann et al., 2007). Tissue damage also triggers non-infection immune reactions that are activated by the release of damage associated molecular pattern molecules (DAMPs) from the injured tissue, which participate in the activation of the innate immune system (Matzinger et al., 1994; Manson et al., 2004). TBI-induced neuroinflammation, however, has been a controversial topic as studies suggest both beneficial and detrimental outcomes (Morganti-Kossmann et al., 2002).

Microgila, the resident immune cells of the CNS, are a major source of cytokine and chemokine release after injury. Conflicting evidence has been reported about the role of cytokines and chemokines, whether they promote repair functions or exacerbate the damages (Morganti-Kossmann et al., 2002). Enhanced tumor necrosis factor (TNF) levels in the brain after TBI has been associated with neurologic deficits, neuronal cell death, and blood brain dysfunction (Shohami et al., 1999). On the other hand, TNF knockout studies demonstrate that TNF may contribute to an early neurotoxic effect and a neuroprotective effect later in post-traumatic phase (Scherbel et al., 1999). TNF also triggers a rise in IL-6, which is a multifunctional factor that has anti-inflammatory effects, neurotrophic properties, induces nerve growth factor, promotes neuronal differentiation and survival, and counteracts excitotoxicity (Morganti-Kossmann et al., 2001). This suggests that there is a feedback loop balancing the release of pro- and anti-inflammatory cytokine release, further complicating the roles of these molecules (Morganti-Kossmann et al., 2002). IL-10, another anti-inflammatory cytokine, is well studied for its beneficial role in neuropathology and has also shown to diminish TNF synthesis (Morganti-Kossmann et al., 2000). However, a study in human TBI showed that high levels of IL-10 were associated with increased mortality (Bell et al., 1997). Other cytokines that have shown this dual nature include TGF-β, IFN-γ, and IL-18 (Fuster-Matanzo et al., 2013). The complicated roles of these molecules may be a result of a complex interplay of molecular signaling and a fundamental importance of timing and concentration of their expression.

Neuroinflammatory Effects on Neurogenesis

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As for the effects of neuroinflammation on adult neurogenesis, whether it is beneficial or detrimental, seems to depend on the magnitude and duration of the inflammation (Fuster-Matanzo et al., 2013). Microglia play a critical role in the regulation and balance of these effects. In the normal uninjured brain, microglia regulate the balance of neurogenesis by releasing factors that instruct proliferated cells to differentiate into neurons, survival of immature neurons, or phagocytosis of apoptotic new cells (Sierra et al., 2010; Walton et al., 2006). Microglia are involved in releasing both anti-inflammatory and pro-inflammatory molecules providing pro-neurogenic and anti-neurogenic effects respectively (Fuster-Matanzo et al., 2013). However, there is strong evidence suggesting that chronic-inflammation leads to decreased neurogenesis through the accumulation of cytotoxic substances such as oxidative stress molecules and pro-inflammatory cytokines (Choi et al., 2009). Over-activation of microglia disturbs the homeostatic balance of pro- and anti-inflammatory cytokines inducing cell death pathways and leading to neurodegenerative diseases such Alzheimer's and multiple sclerosis (Gao and Hong, 2008). Also, these apoptotic-mediating molecules are sustained long after TBI (Raghupathi et al., 2000). These effects have also been demonstrated in aging studies where aged mice have greater neurogenic deficits from neuroinflammation (Russo et al., 2011). Studies have shown that age is a critical factor that determines the level of endogenous neurogenesis as older animals show lower levels of neurogenesis (Sun et al., 2005).

Although the immune response is a way for the body to restore homeostasis, after TBI, a sustained immune activation can ultimately lead to the blockage of restoration and

repair functions that otherwise could lead to a better outcome (Corps et al., 2015). These studies suggest that despite the initial benefits of inflammation after TBI, chronic inflammation may be detrimental to adult neurogenesis.
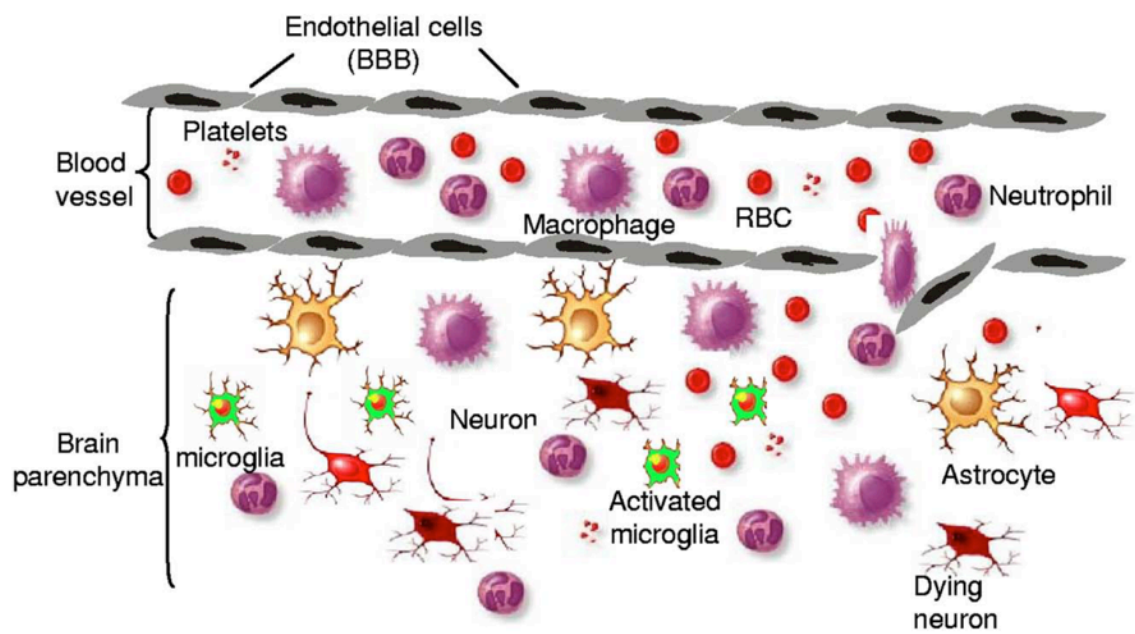
Figure 1.4. Immune system involvement in TBI. Image extracted from Ziebell and Morganti-Kossmann, 2010. Injury-induced blood brain barrier damage causes infiltration of leukocytes into the brain parenchyma via interaction of endothelial adhesion molecules with integrins on the surface of leukocytes, which can cause immune activation and inflammation in the brain.

Hypothesis

In this study, we examine the differences in the neurogenic response following TBI in the subacute and chronic TBI stage using the CCI and LFPI models. Post-TBI cognitive decline or dementia is a significant issue. As hippocampal neurogenesis plays an important role in cognitive function, we speculate that TBI may have a long lasting effect on hippocampal neurogenesis, which may be affecting cognitive function. Specifically, we propose that in the chronic TBI stage there is a decline of neurogenesis below the baseline level and this decline is due to either the exhaustion of the NSC pool, from over stimulation of symmetrical differentiative divisions during neurogenesis, or persistent neuroinflammation.

Because TBI is such a complex and complicated neurological disorder with impact on other neurological diseases, it is difficult to study TBI using one animal model. Since TBI encompasses a vast array of complex mechanisms and each type of animal model is used to study specific aspects of TBI, it is practical to use multiple different animal models when studying TBI and treatments. However, in terms of studying TBI-induced neurogenesis, there are no studies that compare the levels of injury-induced neurogenesis among different animal models. In this study we used two popular TBI animal models, CCI injury and LFPI models, to study focal and diffuse injuries responses respectively.

Chapter 2 - Materials and Methods

Experimental Animals

All animals used in this study were 3 months old male Sprague-Dawley rats that weighed around 300g and were purchased from Harlan Inc., Indiana. A total of 25 rats were included in this study. All animals were housed in pair at the Virginia Commonwealth University animal care facility and were given a 12 hour light/dark cycle at room temperature with adequate food and water *ad libitum*. Proper maintenance and animal care procedures were followed, which were approved by the Institution of Animal Care and Use Committee (IACUC) of Virginia Commonwealth University and the Guide for Care and Use of Laboratory Animals by the U.S. Department of Health and Human Services.

Experimental Setup

Animals were divided into two groups. Group one included 15 total rats and group two included 10 total rats. All animals received the same housing and care. Group one animals included four animals that received LFPI, seven animals that received CCI injury, and four sham animals. All group one animals were sacrificed 15 days after injury. Group two animals included four LFPI animals, four CCI injury animals, and two sham animals. All group two animals were given one BrdU injection 2 hours before sacrifice and were sacrificed 3 months after injury. The experimental set up is also described in **Table 2.1**.

Table 2.1. Experimental Setup

	Group 1	Group 2
Sham	4 Rats	2 Rats
LFPI	4 Rats	4 Rats
CCI	7 Rats	4 Rats
Sacrifice Time After Injury	15 Days	3 months
BrdU Administration		2 Hours before sacrifice
Immunostaining	DCX, Ki67, OX6, ED1	DCX, Ki67, BrdU, OX6, ED1
Hippocampus Size Comparison		Cresyl violet stained sections

Table 2.1. Experimental setup. Animals were divided between Group 1 and Group 2. Group 1 includes 4 shams, 4 LFPI, and 7 CCI rats, which were sacrificed 15 days after injury. Hippocampal sections of these rat brains were used for DCX, Ki67, OX6, and ED1 immunostaining. Group 2 included 2 shams, 4 LFPI, and 4 CCI rats, which received BrdU injections 2 hours before sacrifice and were sacrificed 3 months after injury. Hippocampal sections of these rat brains were used for DCX, Ki67, BrdU, OX6, and ED1 immunostaining as well as hippocampal size comparison.

Surgical procedures

Animals were randomly assigned to receive LFPI, CCI, or Sham injuries. Before surgery, all surgical tools and instruments were sterilized and aseptic procedures were followed during surgery. Animals were first put in a Plexiglas chamber with 5% isoflurane to be anesthetized. Once the animal was unconscious, the animal's head was shaved to remove excess fur. Then the animal's head was fixed in a stable position in a stereotaxic apparatus while the animal was intubated and kept anesthetized throughout surgery with 2.5% isoflurane in a gas mixture of 30% O_2 and 70% N_2. Betadine was applied to sterilize the head at the surgical site. Paralube ointment was applied to both eyes of the animal to prevent the eyes from drying. Using a scapula, a midline incision was made on the head and hemostats were used to retract the skin on both sides of the head to expose the skull. Connective tissue was removed by rubbing the skull with sterilized cotton tip applicators. A 4.0 mm craniotomy was made half way between the lambda and bregma sutures on the left parietal bone using a trephine and a Dremel drill fitted with a small dental drill bit. After cutting the bone, it was removed and any bone shards were removed from the craniotomy. All animals, including shams received the above surgical procedures. For LFPI and CCI injury animals, appropriate injuries were performed as described below. After surgery, the surgical incision was sutured in a simple continuous pattern using a sterilized stainless steel suture needle and a 5-0 polyamide surgical suture. Lidocaine hydrochloride jelly, which is a local anesthetic, and a triple antibiotic ointment were applied to the closed suture. Then, the isoflurane was turned off and intubation was removed. The animal was monitored until it regained normal breathing pattern. The animal's cage was lined with surgical drape and once the

animal righted, it was placed back into the cage and observed for three hours before returning to the animal housing facility.

Lateral Fluid Percussion Injury

A total of 8 animals received LFPI in this study. After the craniotomy, a Luer lock hub was cemented over the craniotomy using cyanoacrylic. Dental acrylic was used to seal the base around the hub and allowed to dry. To test the seal, the hub was filled with 0.9% saline solution, which also served as a medium for the injury pulse from the FPI device. Then, for 4 minutes, the isoflurane rate was reduced to 1%. During this time the FPI device (**Figure 2.1,** Thompson et al., 2005) was prepared by measuring the height of the pendulum that was needed to produce the target pressure pulse. This was done by calibrating the device by repeated test uses to produce pressure pulses around a target value of 2.0 atm. The pressure was measured by an oscilloscope (Tektronix) and a pressure transducer amplifier, which were connected to the FPI device. The isoflurane was turned off and the Luer lock hub on the animal was connected to the injury device and the injury was administered. Then the hub was disconnected from the injury device and the animal was returned to the surgical preparation area. The Luer lock hub and acrylic base were detached from the animal's head and the animal was placed on the surgical mat. The animal was observed until it righted and the righting time was recorded. Once the animal regained normal breathing, it was placed back on to the stereotaxic frame and was anesthetized with 5% isoflurane before the surgical site was sutured.

A.

B.
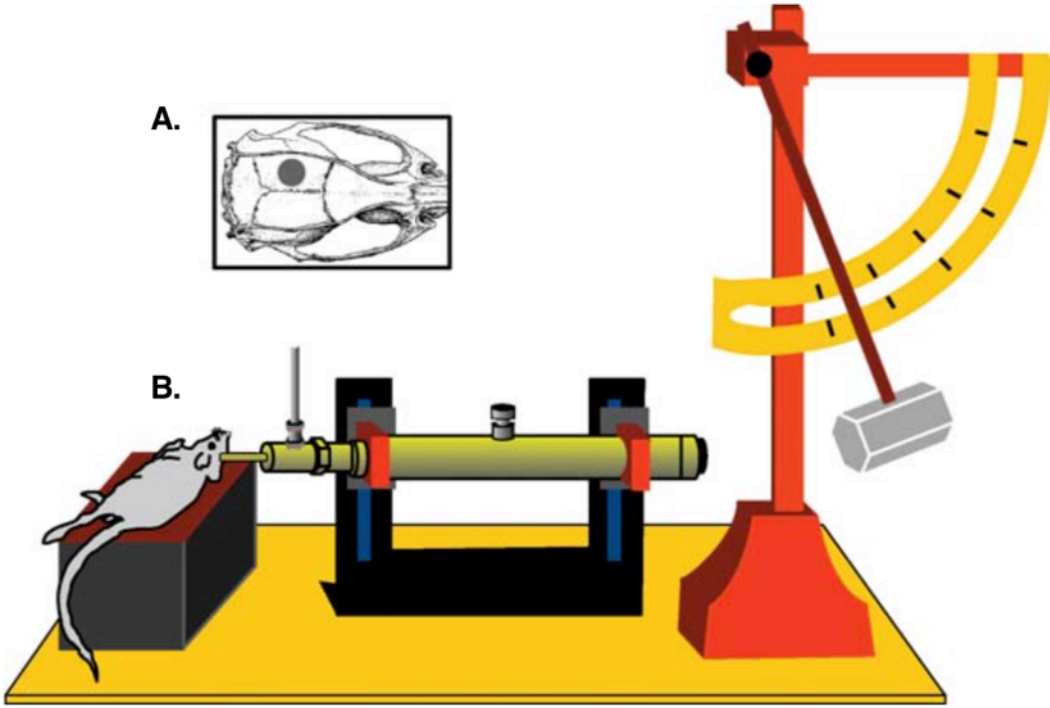
Figure 2.1. Lateral fluid percussion injury setup. (A) All injuries were made on the left hemisphere in the middle of bregma and lambda sutures as represented in this image. (B) This cartoon depicts the LFPI device used in this study and the position of the animal during injury.

Controlled Cortical Impact Injury

A total of 11 animals received CCI injury in this study. After the craniotomy, animals were kept anesthetized. A Leica Benchmark Stereotaxic Impactor was connected to the stereotaxic frame (**Figure 2.2,** Galgano et al., 2015). The 3 mm rigid impactor tip was positioned above the craniotomy and the zero point of impact was obtained by lowering the tip of the impactor until it touched the dura mater. The tip was driven by an electromagnetic piston and moved at 4 m/sec and remained on the tissue for 500 ms. A 2.5 mm depression in the tissue was set to cause a moderate level of focal injury. After the tip retracted, it was raised away from the animal. The animal was then sutured and monitored for normal breathing before returning to home cage.

A.

B.

Figure 2.2. CCI device. Photographs representing the (A) Controlled Cortical Impact injury device and (B) position of the animal during injury.
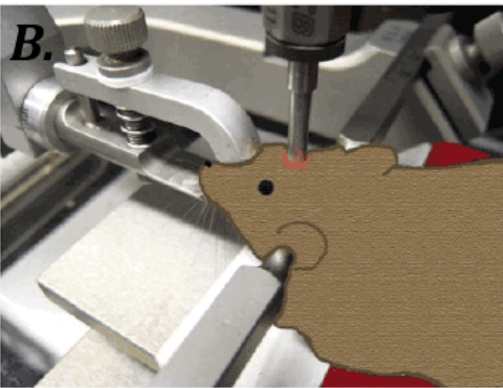
BrdU injections

BrdU (Sigma-Aldrich Co.), a thymidine analog that incorporates into DNA during cell division, is used to permanently label dividing cells. Group 2 animals received one dose (50 mg/kg of body weight) via intraperitoneal (I.P.) injection at 2 hours before sacrifice.

Sacrifice and Tissue Processing

Group one animals were sacrificed 15 days after injury while group two animals were sacrificed 3 months after injury. Each animal was placed in a Plexiglas chamber with 5% isoflurane and deeply anesthetized. The animal was taken out and a surgical gauze pad soaked in 100% isoflurane was placed on the animals nose to keep it anesthetized while it was transcardially perfused with 400 mL phosphate-buffered saline (PBS) and then with 400 mL of 4% paraformaldehyde in PBS fixative. The brains were removed and stored at 4°C in 4% paraformaldehyde in PBS fixative. After 48 hours, the brains were sliced into 60 μm coronal sections using a vibratome (Leica). The sections were stored in 24 well plates filled with 0.01% sodium azide in PBS and stored at 4°C. Brain sections were used as needed for different immunostaining protocols.

BrdU Immunostaining

Six sequential hippocampal brain sections from each brain of group two animals were selected for BrdU immunostaining. Sections were first washed in 1X PBS two times for five minutes each. Then they were incubated in a denatured solution with 50% formamide, 37.5% H_2O, and 12.5% 20X saline sodium citrate (SSC) buffer for one hour

at 65°C. Then the sections were rinsed in 2X SSC two times for 5 minutes each, and further denatured in 2N hydrochloric acid for 30 minutes at 37°C. Then they were rinsed with 1X PBS two times for 5 minute each, and placed in 3% hydrogen peroxide for one hour. The sections were then rinsed with 0.3% Triton X-100 in 1X PBS (washing buffer) for 10 minutes and placed in 5% normal horse serum in washing buffer (blocking buffer) overnight at 4°C. Then the sections were placed in the BrdU antibody solution for 48 hours at 4°C. The antibody solution was prepared with monoclonal mouse anti-BrdU antibody (Dako) diluted in blocking buffer at a 1 to 200 dilution. The sections were then allowed to come back to room temperature and rinsed in washing buffer three times for 10 minutes each, before placing them in blocking buffer for three hours at room temperature. Then the sections were placed in secondary antibody solution overnight at 4°C. This solution was prepared with the Biotin-conjugated anti-mouse IgG antibody (Vector) diluted in blocking buffer at a 1 to 200 dilution. The sections were then allowed to come back to room temperature and rinsed in washing buffer three times for 10 minutes each, before placing them in Avidin-biotin Complex reagent for two hours at room temperature. This solution was prepared 30 minutes before use from the Avidin-biotin Complex kit (Vector Labs, Burlingame, CA) and diluted in 1X PBS at a 1 to 200 dilution. Then the sections were rinsed in washing buffer three times for 10 minutes each, before placing them in 3,3' diaminobenzadine tetra-hydrochloride (DAB) (Sigma) solution. Once the sections were properly stained by the reaction, which was noted by visible stained cells under the microscope, the sections were rinsed with 1X PBS three times and mounted onto glass slides and allowed to air dry. Then they were

counterstained with 0.1% cresyl violet, dehydrated through gradient ethanol and cover slipped with Permount.

DCX, Ki67, OX6, ED1 Immunostaining

Sequential brain sections of all animals were processed for Doublecortin (DCX), Ki67, OX6, and ED1 immunostaining. Similar steps were taken as described in BrdU immunostaining, with the exception of denaturing procedure. After sections were rinsed with PBS, they were directly placed in 3% hydrogen peroxide solution for one hour, thereafter, BrdU immunostaining protocol was followed. For DCX immunostaining, the primary antibody solution was prepared with polyclonal goat anti-DCX (1:1000, Santa Cruz) and the secondary antibody solution was prepared with the Biotin-conjugated anti-goat IgG antibody (1:200, Vector). For Ki67 immunostaining, the primary antibody solution was prepared with rabbit anti-Ki67 (1:500, Abcam) and the secondary antibody solution was prepared with Biotin-conjugated anti-rabbit IgG antibody (1:200, Vector). For OX6 immunostaining, the primary antibody solution was prepared with mouse anti rat MHC Class II OX6 antibody (1:1000, AbD Serotec) and the secondary antibody solution was prepared with the Biotin-conjugated anti-mouse IgG antibody (1:200, Vector). For ED1 immunostaining, the primary antibody solution was prepared with mouse anti rat ED1 (1:1000, AbD Serotec) and the secondary antibody solution was prepared with the Biotin-conjugated anti-mouse IgG antibody (1:200, Vector).

Densitometry

The intensity of OX6 and ED1 staining was quantified by densitometry analysis using Image J program. Briefly, images of selected regions of OX6 or ED1 stained section was taken using the 4x objective on an inverted light microscope (1X71, Olympus) and Olympus controller program. Images were taken with automatic adjusted white balance, and exposure was set to produce the best image. Exposure was kept same for all sections of animal brains to be compared. ImageJ program was used to measure the optical density of the staining in a selected region of interest at a 0.621 pixels/um scale. The images were first converted to a RGB stack image. The threshold for detection was determined by selecting automatically according to the image exposure. For each brain, two sections were used and the average of measurements was taken. Measurements were recorded as percentage of selected area stained.

Stereology

All stained hippocampal sections were observed under an inverted light microscope (1X71, Olympus) microscope for quantifying the number of stained cells. For all cell counting, the Visiopharm program (Olympus) was used. First, a 4x objective was used to outline the region of interest. The stereological optical fractionator method was used to count the number of stained cells in the outlined region. For Ki67 and BrdU stained sections, the granular zone (GZ), which includes the SGZ and granular cell layer (GCL), were outlined. For DCX stained sections, cells were separately counted in the SGZ plus inner 1/3 GCL, middle 1/3 GCL, and outer 1/3 GCL. To derive the total DCX positive cell count of the GZ, cell counts of following three regions were added: the SGZ plus inner 1/3 GCL, middle 1/3 GCL, and inner 1/3 GCL. For Brdu, Ki67, and DCX

sections, stained cells were also counted in the hilus region. Cells outside of the optical

dissector counting frame were omitted. The dissector height (h) was set to 15 μm.

Measuring the focal plane at five different locations on each section and then taking the

average, the average thickness (t) of each brain section was calculated. These averages

were then averaged for the total five sections per brain. Total cell counts (n) were

estimated to be $n = \Sigma Q^- \cdot (t/h)(1/asf)(1/ssf)$, were ΣQ^- represents the number of counted

cells. *asf* is the average sampling fraction and because the entire region was counted in

these sections, it is set to one. *ssf* is the sample sectioning fraction and it is set to 0.125

because five sections were used from each brain, representing 1/8 of the total

hippocampus.

Hippocampal Size

To examine the injury-induced change of the hippocampus size, the ipsilateral and

contralateral hippocampal perimeters were measured using the Visiopharm program.

Cresyl violet stained sections from group two animal brains were observed under the

light microscope. The ipsilateral and contralateral hippocampi were separately outlined

using a 4X objective and perimeter of the outline was obtained. Due to inter-animal

variability of brain structures, we compared ipsilateral to contralateral hippocampi within

the same animal by taking a percent ratio of ipsilateral to contralateral hippocampal

perimeters. For each animal brain, 5 sections were used and their measurements were

averaged to calculate the hippocampal size percent change of each animal.

Statistical Analysis

We performed One-Way ANOVA using SPSS software to determine statistical significant difference of three injury groups, sham, CCI injury, and LFPI, in all experiments. To compare pairwise statistical significance of any two groups, we performed a post hoc Fisher Least Significant Difference (LSD) test. A p value of 0.05 or less was considered statistically significant.

Chapter 3 - Results

The purpose of this study was to evaluate the differences in TBI-induced neurogenic responses in subacute and chronic-TBI using different TBI animal models. Two popular TBI animal models, CCI and LFPI, were compared at two different injury time points, 15 days after injury and 3 months after injury. The degree of neurogenic difference was evaluated by comparing levels of proliferation, generation of new neurons, and neuroblast migration after injury in the DG.

Experiment 1. TBI-Induced Subacute and Chronic Proliferation

We used the cell proliferation markers Ki67 and BrdU to identify newly proliferating cells after injury in three groups: sham, CCI, and LFPI groups. Ki67 is a popular marker for proliferating cells because during interphase the Ki67 protein is located in the nucleus whereas during the mitotic cell phases it is relocated to the surface of the chromosomes where it can be detected by Ki67 antibodies (Scholzen and Gerdes, 2000). We also used BrdU, which is a thymidine analogue that incorporates into replicating DNA during the S phase of cell cycle, taking place of thymidine, and can later be detected by BrdU antibodies in cells that were proliferating during the time of BrdU incorporation (Kee et al., 2002).

Proliferative responses in Subacute-TBI

At 15 days after injury, we observed similar proliferative levels in all three groups (**Figure 3.1**) and stereological quantification demonstrated that the number of Ki67 positive cells between the three groups in the granular zone and hilus regions of the DG

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remained relatively similar (**Figure 3.2**). This was true for both ipsilateral and contralateral side. We used One-Way ANOVA to compare statistical significant difference of all three groups and found no significant difference in the ipsilateral GZ (p=0.325), ipsilateral hilus (p=0.465), contralateral GZ (p=0.826), and contralateral hilus (p=0.376) regions of the three groups.
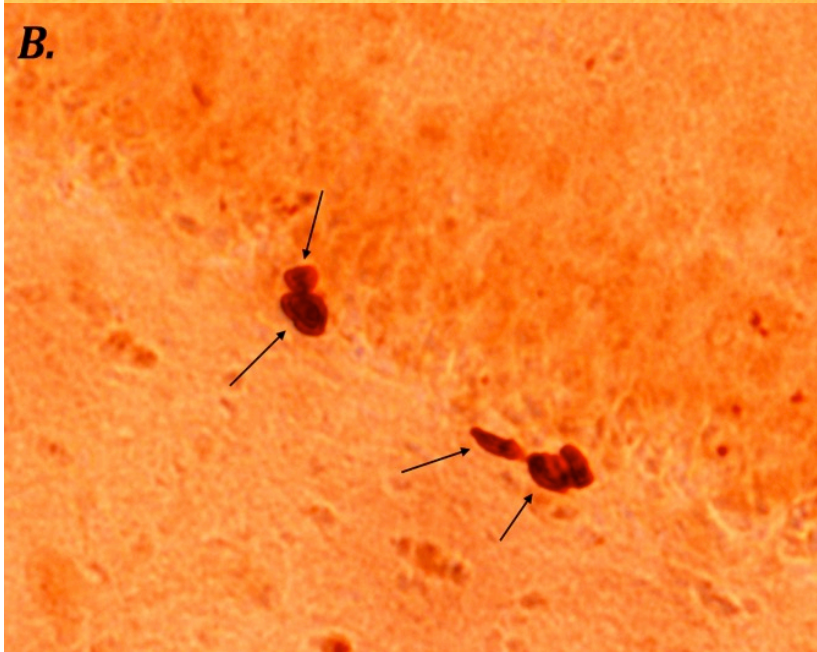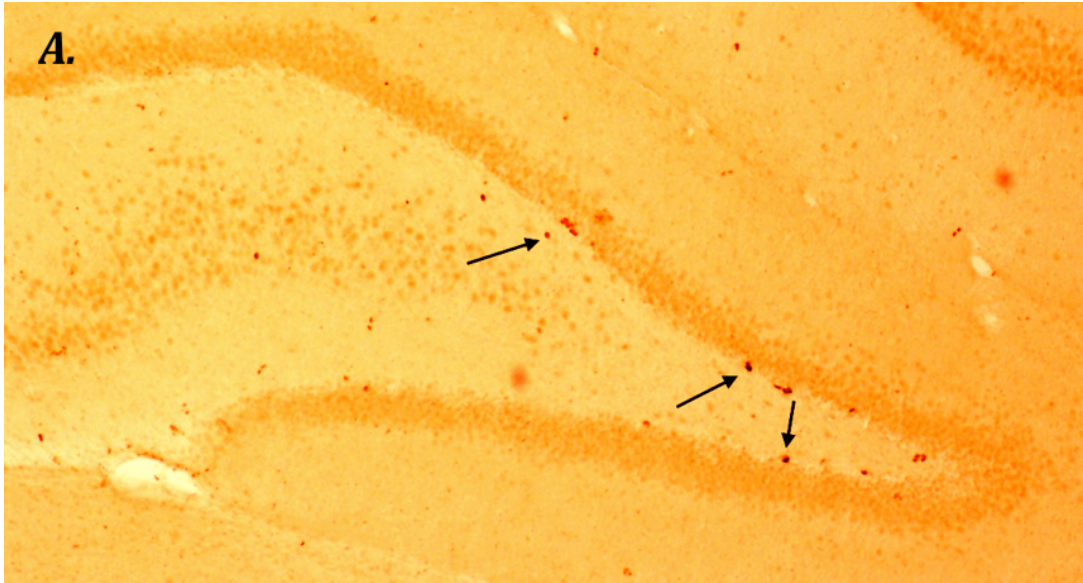
Figure 3.1. Cell proliferation at 15 days after injury. Images of a coronal section of Sprague Dawley rat brain showing Ki67 staining pattern of the ipsilateral dentate gyrus. The arrows indicate Ki67 positive cells. Using the (A) 4X objective the entire staining pattern of the dentate gyrus can be seen and using the (B) 40X objective the individual stained cells are identified. This staining pattern is consistent in all injury groups indicating similar levels of proliferation.

A. Proliferation in GZ 15 Days Post Injury
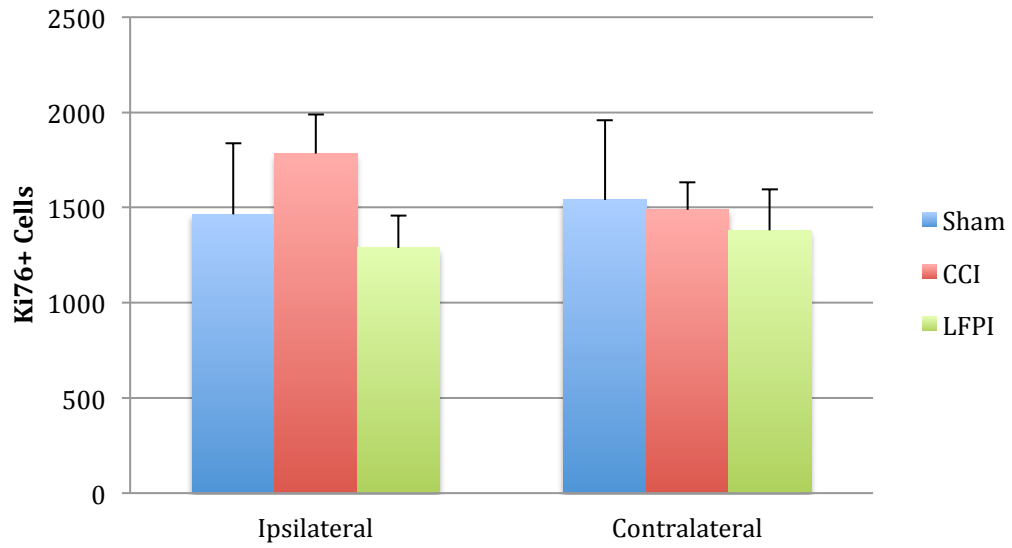
B. Proliferation in Hilus 15 Days Post Injury
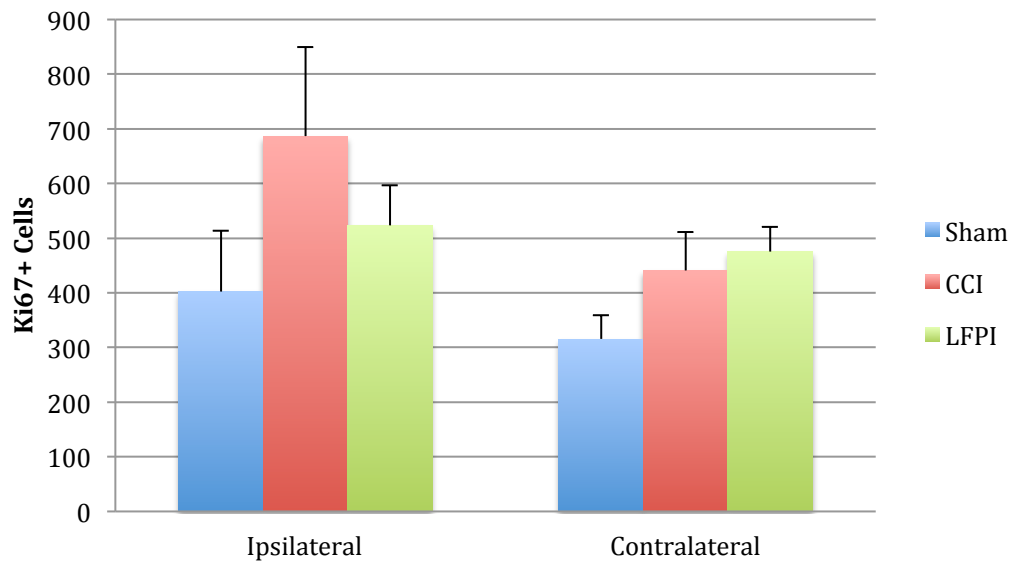
Figure 3.2. Quantification of Ki67 positive cells at 15 days after injury. The number of Ki67 positive cells in the dentate gyrus of ipsilateral and contralateral sides of the injury were quantified in the sham, CCI, and LFPI injury groups. At 15 days after injury, the level of proliferation in the (A) GZ was not significantly different in injured groups from sham animals. This was also true for proliferation levels in the (B) hilus region.

Proliferative responses in Chronic-TBI

To understand long-term effects of TBI on adult neurogenesis, we examined the level of proliferation at 3 months after injury. In our results, we did not observe a difference in the level of proliferation in injured animals compared to sham animals at 3 months after injury (**Figure 3.3**). Stereological quantification analysis using One-Way ANOVA demonstrated that there was no significant difference in the levels of Ki67 positive cells in the ipsilateral (IP) and contralateral (CT) dentate gyrus of injury and sham animals (IP: GZ *p=0.838,* hilus *p=0.605*; CT: GZ *p=0.885,* hilus *p=0.785*) (**Figure 3.4**). To further verify these findings, we used another cell proliferation marker, BrdU, to label proliferating cells, and also found no difference in proliferation in sham and injured groups. Stereological quantification analysis demonstrated that there was no significant difference in the levels of BrdU positive cells in the ipsilateral (IP) and contralateral (CT) dentate gyrus of injury and sham animals (IP: GZ *p=0.438*, hilus *p=0.143*; CT: GZ *p=0.627*, hilus *p=0.371*) (**Figure 3.5**).
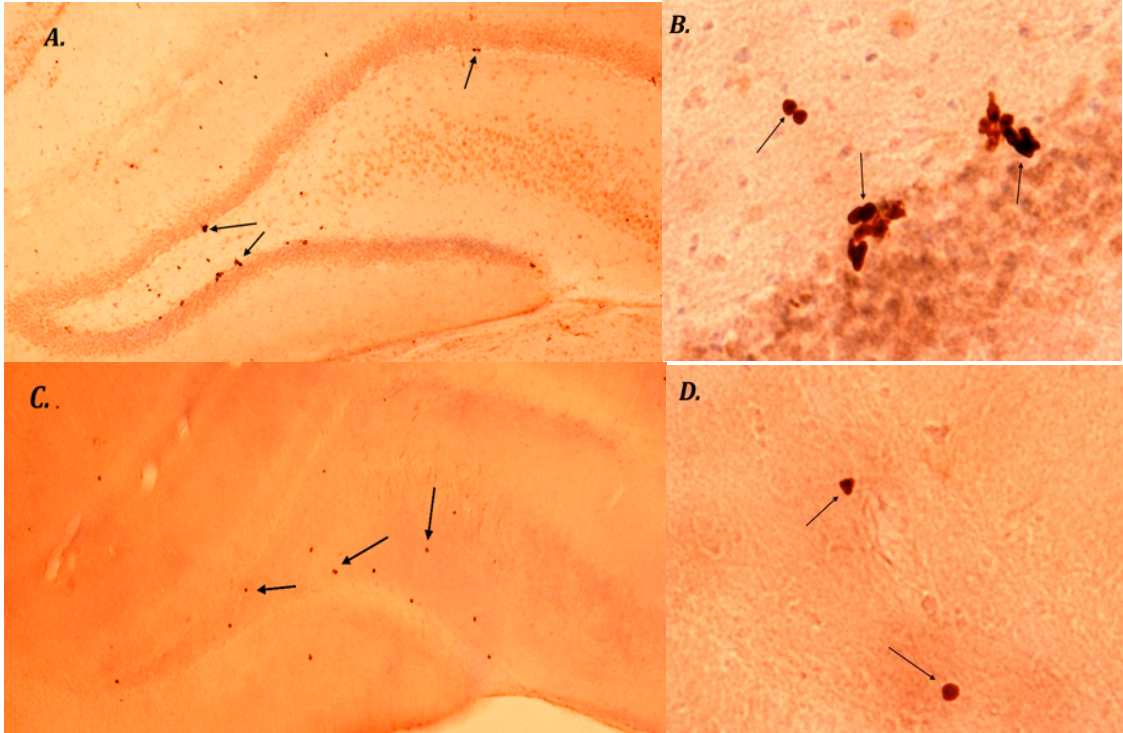
Figure 3.3. Cell proliferation at 3 months after injury. Images of coronal sections of Sprague Dawley rat brain showing Ki67 and BrdU staining patterns of the ipsilateral dentate gyrus. The arrows indicate stained positive cells. Using the (A) 4X objective, the Ki67 staining pattern of the entire dentate gyrus can be seen and using the (B) 40X objective the individual stained cells are identified. The observed Ki67 staining pattern is consistent in sham, CCI injury, and LFPI animals, indicating similar levels of proliferation. Immunolabeled images of BrdU staing using (C) 4X objective to show the entire dentate gyrus and (D) 40X objective to show individual stained cells also indicate consistent proliferation levels in sham, CCI injury, and LFPI animals.

A. Proliferation in GCL 3 Months Post Injury
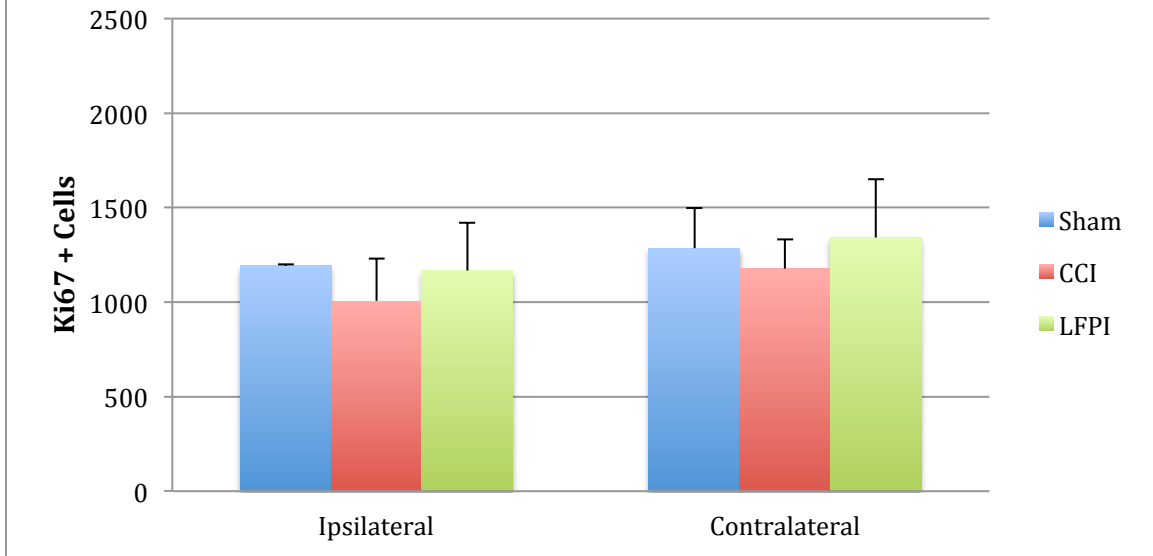
B. Proliferation in Hilus 3 Months Post Injury
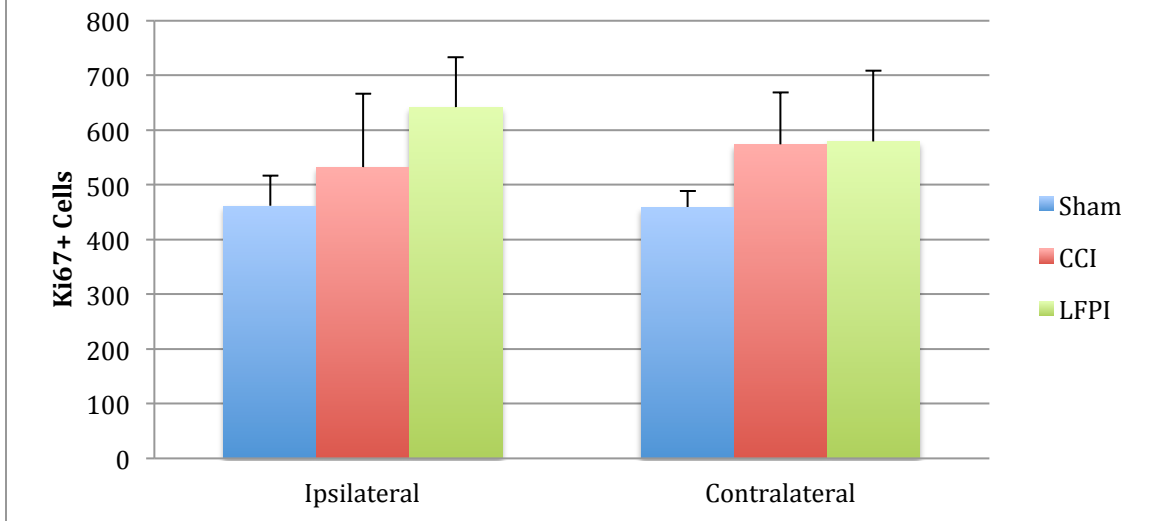
Figure 3.4. Quantification of Ki67 positive cells at 3 months after injury. The number of Ki67 positive cells in the dentate gyrus of ipsilateral and contralateral sides of the injury were quantified in the sham, CCI, and LFPI injury groups. At 3 months after injury, the level of proliferation in the (A) GZ was not significantly different in injured groups from sham animals. This was also true for proliferation levels in the (B) hilus region.

A. Cell Proliferation in GZ 3 Months Post Injury
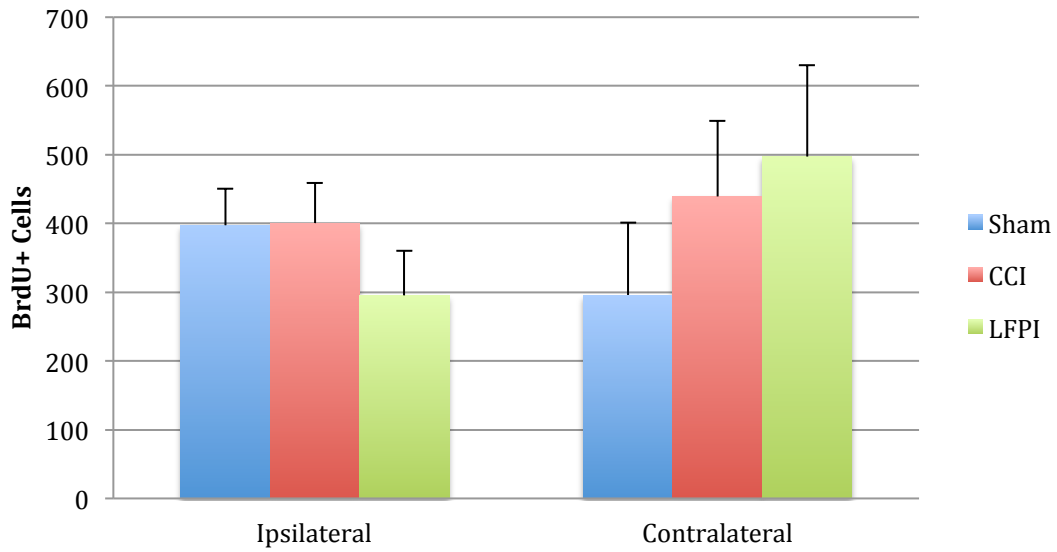
B. Cell Proliferation in Hilus 3 Months Post Injury

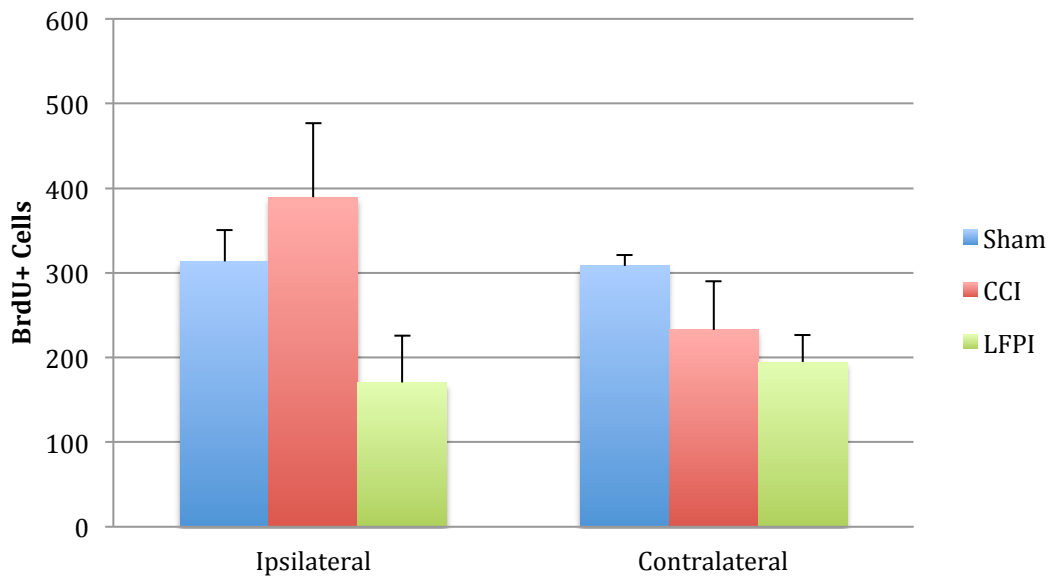**Figure 3.5. Quantification of BrdU positive cells at 3 months after injury.** The numbers of BrdU positive cells in the ipsilateral and contralateral dentate gyrus were quantified in the sham, CCI, and LFPI injury groups. At 3 months after injury, the level of proliferation in the (A) GZ was not significantly different in injured groups from sham animals. This was also true for proliferation levels in the (B) hilus region.

Experiment 2. TBI-Induced Generation of New Neurons

After proliferation, surviving cells in neurogenic regions differentiate into either neuronal or glial cell lineage. Cells that become determined for a neuronal lineage express certain biomarkers including DCX. To determine the impact of TBI on the generation of new neurons, we used the DCX antibody to label newly generated neurons. DCX is a microtubule binding protein expressed in immature neurons and migrating neuroblasts in neurogenic regions of the adult mammalian brain (Brown et al., 2003). We compared levels of newly generated neurons at 15 days after injury and 3 months after injury in the DG of animals that received sham, CCI, or LFPI injury.

Generation of New Neurons in Subacute-TBI

In our results, we observed an increase in the number of newly formed neurons at 15 days after injury in the ipsilateral dentate gyrus of the injured animals compared to sham animals (**Figure 3.6**). We used One-Way ANOVA to compare significant difference of all three groups and found statistically significant difference of the levels of DCX positive cells in the ipsilateral GCL (p=0.002) and ipsilateral hilus region (p=0.001) (**Figure 3.7**). We used a post hoc LSD test to determine pair wise significance of the group means, which revealed that although there was a significant increase of DCX positive cells in the ipsilateral GCL of injured animals compared to sham (sham vs. CCI, p=0.001; sham vs. LFPI, p=0.015), there was no significant difference between the CCI injury and LFPI groups (p=0.061). In the ipsilateral hilus region, a pairwise comparison of each injury group to sham showed significant increase of DCX positive cells (sham vs.

CCI, p<0.001; sham vs. LFPI, p=0.03) and CCI injury animals had significantly more

DCX positive cells than LFPI animals (p=0.005).
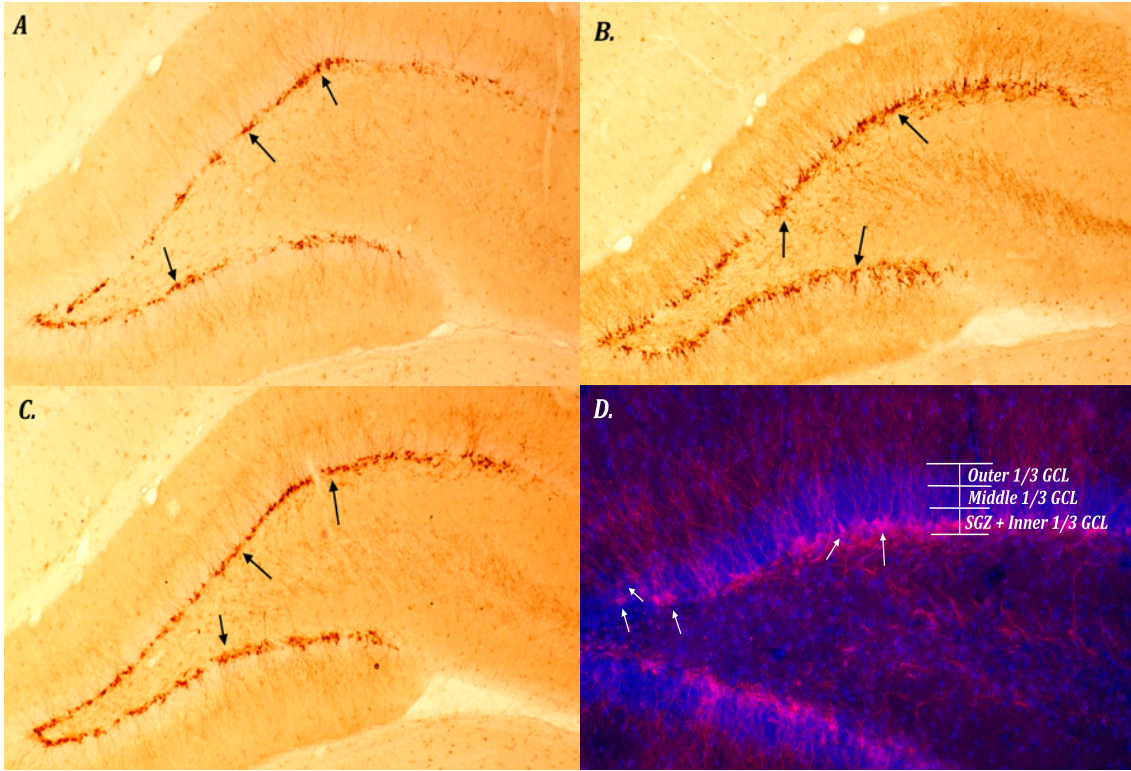
Figure 3.6. Generation of new neurons at 15 days after injury. Images of a coronal section of Sprague Dawley rat brain showing DCX staining pattern of the ipsilateral dentate gyrus. The arrows indicate DCX positive cells. In (A) sham animals, there is less observable DCX staining compared to (B) CCI injury and (C) LFPI. To determine neuronal migration, (D) the GZ was divided into three regions, the SGZ and 1/3 of the inner GCL, middle 1/3 of the GCL, and outer 1/3 of the GCL as shown.

A. Generation of New Neurons in GZ 15 Days Post Injury
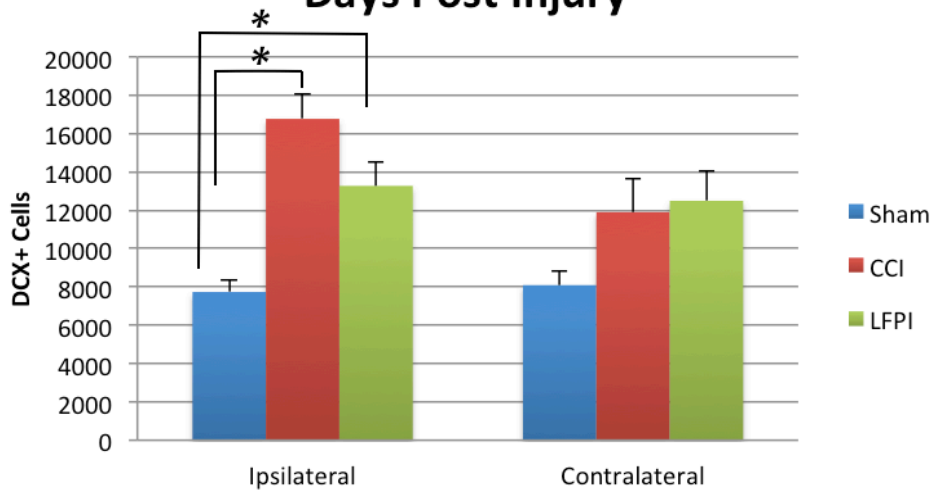
B. Generation of New Neurons in Hilus 15 Days Post Injury
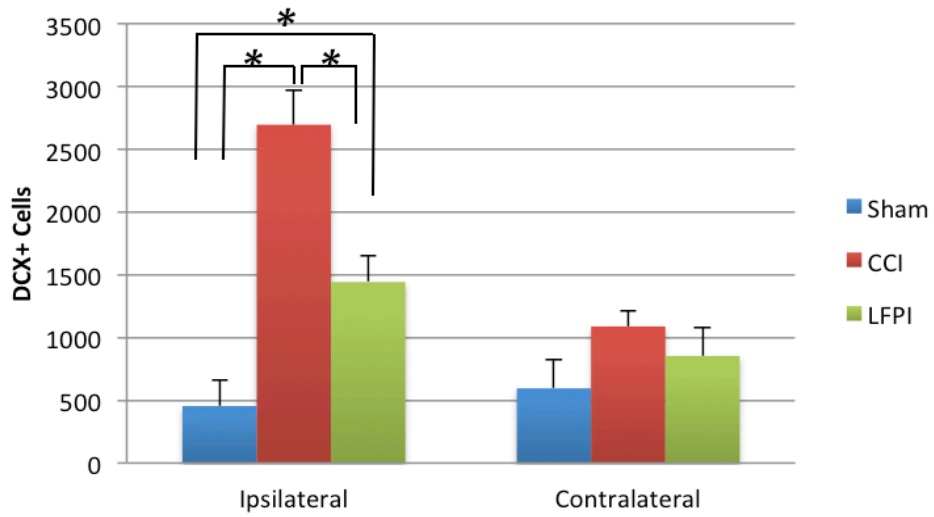
Figure 3.7. Quantification of DCX positive cells at 15 days after injury. In the (A)

ipsilateral GZ, both types of injuries resulted in significantly higher level of DCX

positive cells indicating more generation of new neurons compared to sham animals

(sham vs. CCI, p=0.001; sham vs. LFPI, p=0.015). In the (B) ipsilateral hilus region,

both types of injuries resulted in significantly higher level of DCX positive cells

compared to sham animals (sham vs. CCI, p<0.001; sham vs. LFPI, p=0.03), and the CCI

injury had a significantly higher number DCX positive cells compared to LFPI (p=0.005)

Differences in neuronal migration was determined by using the Visiopharm program to trace and divide the GZ into 3 regions, the SGZ plus inner 1/3 GCL, middle 1/3 of the GCL, and outer 1/3 of the GCL, and count the number of DCX positive cells in each region (**Figure 3.6**). Stereological quantification demonstrated that both types of injuries induced an increased number of DCX positive cells in the ipsilateral SGZ plus inner 1/3 GCL, but only CCI injury induced an increase of DCX positive cells in the ipsilateral middle 1/3 of the GCL, and ipsilateral outer 1/3 of the GCL. Using a post hoc LSD test to compare pairwise significance, we found that in the SGZ plus inner 1/3 GCL, each type of injury compared to sham had a statistically significant increase in DCX positive cells (sham vs. CCI, $p=0.005$; sham vs. LFPI, $p=0.021$), but there was no significant difference between CCI and LFPI groups ($p=0.418$) (**Figure 3.8**). In the ipsilateral middle 1/3 GCL, we found that CCI injury produced a statistically significant increase in DCX positive cells compared to sham and LFPI (sham vs. CCI, $p<0.001$; CCI vs. LFPI, $p<0.001$), but there was no significant difference between sham and LFPI groups ($p=0.52$) (**Figure 3.8**). In the ipsilateral outer 1/3 GCL, we found the same pattern of increase in DCX positive cells where CCI injury produced a statistically significant increase compared to sham and LFPI (sham vs. CCI, $p=0.034$; CCI vs. LFPI, $p=0.021$), but there was no significant difference between sham and LFPI groups ($p=0.948$) (**Figure 3.8**).

A. Generation of New Neurons in SGZ + Inner 1/3 GCL 15 Days Post Injury
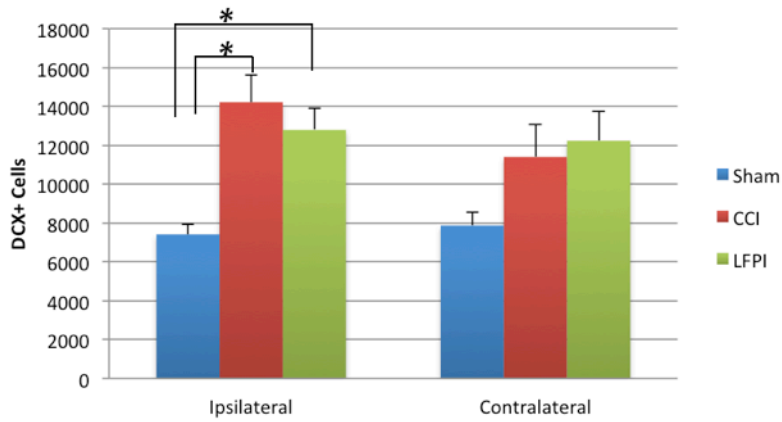
B. Generation of New Neurons in Middle 1/3 GCL 15 Days Post Injury
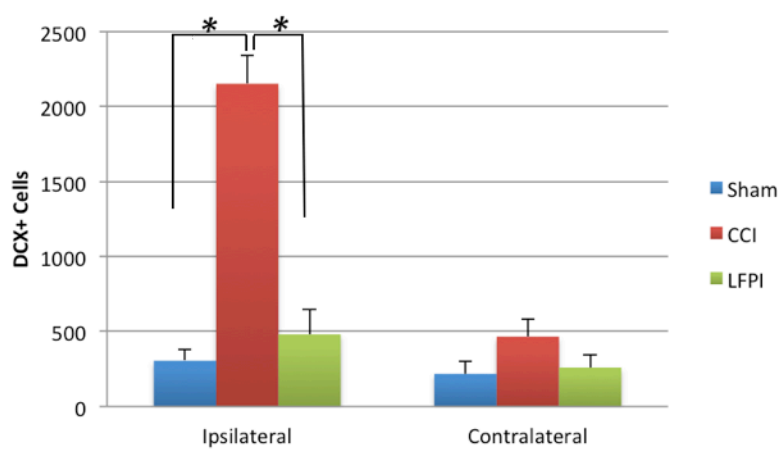
C. Generation of New Neurons in Outer 1/3 GCL 15 Days Post Injury
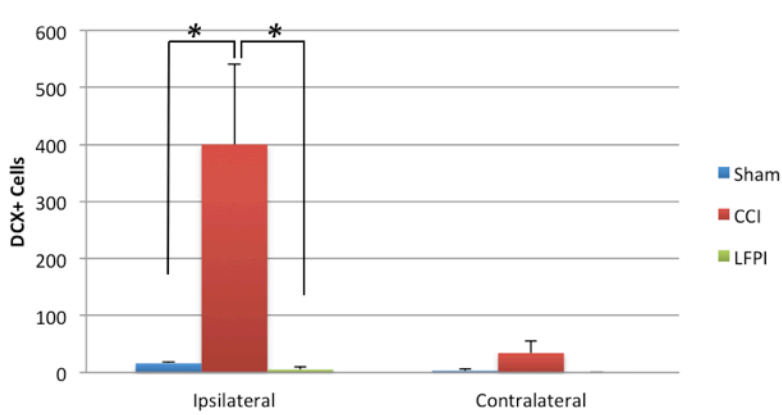
Figure 3.8. Migration of neuroblasts at 15 days after injury. In sham and injured animals, majority of the DCX positive cells of the GZ were located in the (A) SGZ plus inner 1/3 GCL, with a significantly higher number of DCX positive cells in injured animals compared to sham (sham vs. CCI, p=0.005; sham vs. LFPI, p=0.021) and no significant difference between CCI injury and LFPI groups (p=0.418). In the (B) middle 1/3 GCL, CCI injury resulted in a significantly higher number of DCX positive cells compared to sham and LFPI (sham vs. CCI, p<0.001; sham vs. LFPI, p=0.52; CCI vs. LFPI, p<0.001). Also in the (C) outer 1/3 GCL, CCI injury resulted in a significantly higher number of DCX positive cells compared to sham and LFPI (sham vs. CCI, p=0.034; sham vs. LFPI, p=0.948; CCI vs. LFPI, p=0.021).

Generation of New Neurons in Chronic-TBI

To further evaluate long-term effects of TBI on adult neurogenesis, we examined the generation of new neurons at 3 months after injury by DCX immunolabeling. In our results, we observed a decrease in the number of newly formed neurons at 3 months after injury in the ipsilateral and contralateral DG of the injured animals compared to sham animals (**Figure 3.9**). Stereological quantification analysis demonstrated that injured animals and sham animals had statistically different levels of DCX positive cells in the ipsilateral GZ (p=0.002), contralateral GZ (p=0.016), and ipsilateral hilus region (p=0.001) (**Figure 3.10**). Although we saw an observable difference between the three groups in the contralateral hilus region, the data was not statistically different. A pairwise comparison using post hoc LSD between two groups reveled that there was a significant decrease of DCX positive cells in the ipsilateral and contralateral GZ of injured animals compared to sham (IP: sham vs. CCI *p=0.001*, sham vs. LFPI *p=0.002*; CT: sham vs. CCI *p=0.009*, sham vs. LFPI *p=0.013*), however, there was no significant difference between the CCI injury and LFPI groups (IP: p=0.781; CT: p=0.823) (**Figure 3.10**). In the ipsilateral hilus region, a pairwise comparison of each injury group to sham revealed a statistically significant decrease of DCX positive cells in animals that received LFPI (sham vs. LFPI, p=0.006) but not in animals that received CCI injury (sham vs. CCI, p=0.118) (**Figure 3.10**). There was also no statistical difference between the two types of injuries (CCI vs. LFPI, p=0.097).
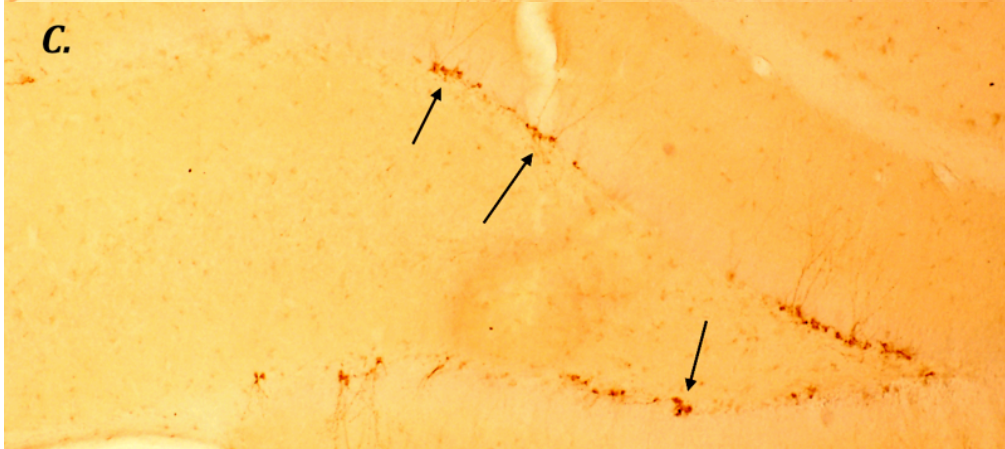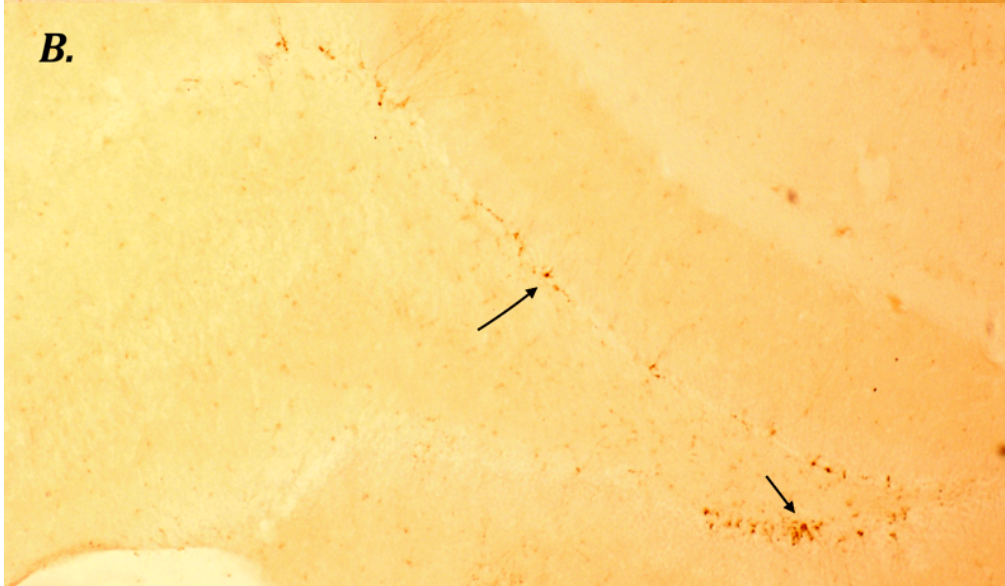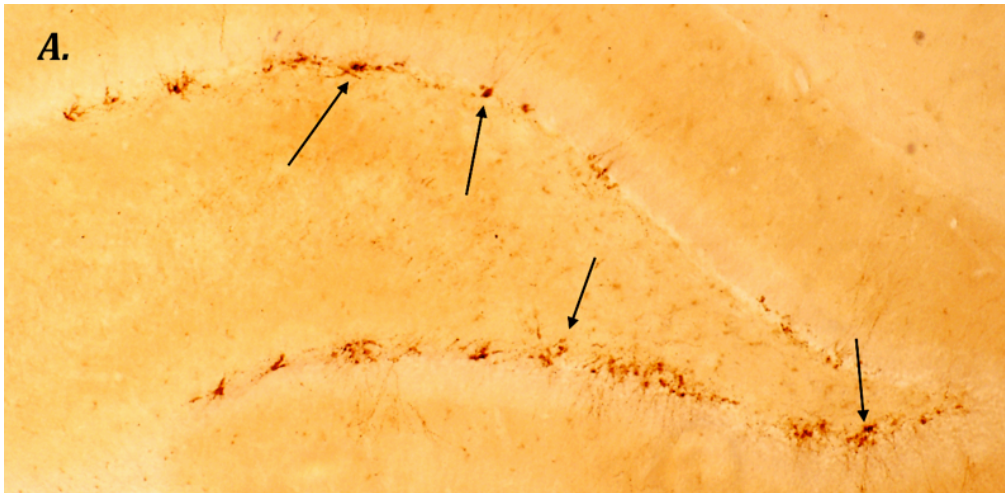
Figure 3.9. Generation of new neurons at 3 months after injury. Images of a coronal section of Sprague Dawley rat brain showing DCX staining pattern of the ipsilateral dentate gyrus. The arrows indicate DCX positive cells. In (A) sham animals, there are more observable DCX stained cells compared to (B) CCI injury and (C) LFPI.

A. Generation of New Neurons in GZ 3 Months Post Injury
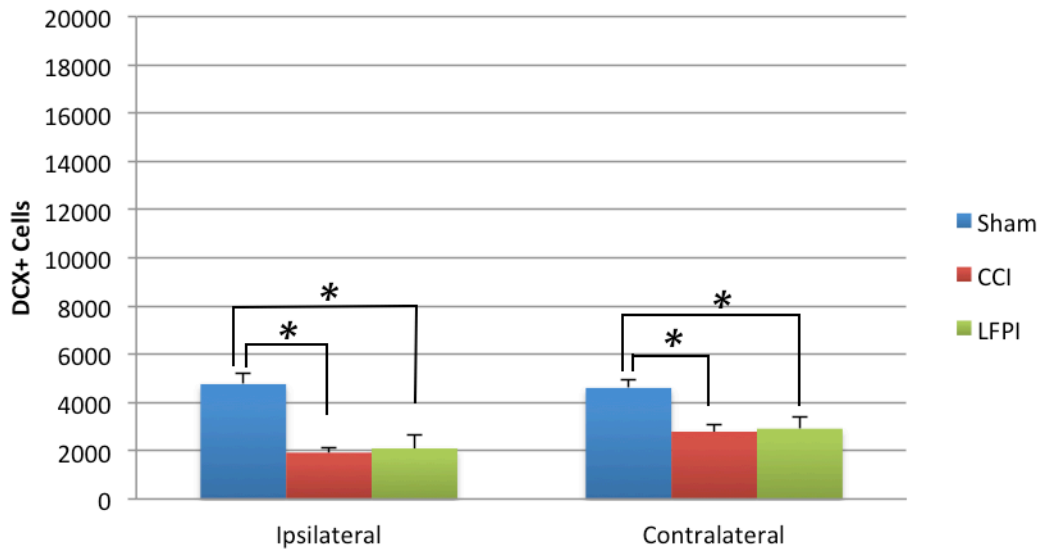
B. Generation of New Neurons in Hilus 3 Months Post Injury
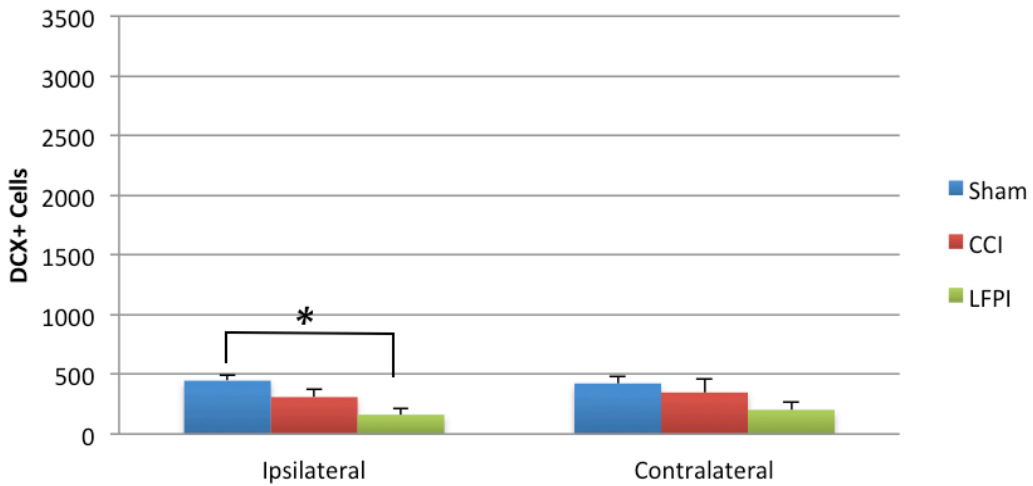
Figure 3.10. Quantification of DCX positive cells at 3 months after injury. In the (A)

ipsilateral and contralateral GZ, both types of injuries resulted in significantly lower

levels of DCX positive cells indicating less generation of new neurons compared to sham

animals (IP: sham vs. CCI *p=0.001*, sham vs. LFPI *p=0.002*; CT: sham vs. CCI *p=0.009*,

sham vs. LFPI *p=0.013*). In the (B) ipsilateral hilus region, animals that received LFPI

resulted in significantly lower level of DCX positive cells compared to sham animals

(sham vs. LFPI, p=0.006).

Differences in neuronal migration were determined by dividing the GZ into 3 regions as previously described (**Figure 3.6**). Stereological quantification demonstrated that both types of injury groups had reduced number of DCX positive cells in the ipsilateral and contralateral SGZ plus inner 1/3 GCL and middle 1/3 GCL compared to sham. Using a post hoc LSD pairwise comparison, in the ipsilateral and contralateral SGZ plus inner 1/3 GCL, we found that each type of injury compared to sham had a statistically significant decrease in DCX positive cells (IP: sham vs. CCI *p=0.001*, sham vs. LFPI, p=0.002; CT: sham vs. CCI *p=0.011*, sham vs. LFPI, p=0.018), but there was no significant difference between CCI and LFPI groups (IP: CCI vs. LFPI *p=0.676*; CT: CCI vs. LFPI *p=0.782*) (**Figure 3.11**). In the ipsilateral and contralateral middle 1/3 GCL, we also found that each type of injury compared to sham had a statistically significant decrease in DCX positive cells (IP: sham vs. CCI *p=0.011*, sham vs. LFPI, p=0.001; CT: sham vs. CCI *p=0.026*, sham vs. LFPI, p=0.008), but there was no significant difference between CCI and LFPI groups (IP: CCI vs. LFPI *p=0.1*; CT: CCI vs. LFPI *p=0.5*) (**Figure 3.11**). In the ipsilateral and contralateral outer 1/3 GCL, we did not find a statistical difference in DCX positive cells in the three groups (IP: p=0.878; CT: p=0.996) (**Figure 3.11**).

A. Generation of New Neurons in SGZ + Inner 1/3 GCL 3 Months Post Injury
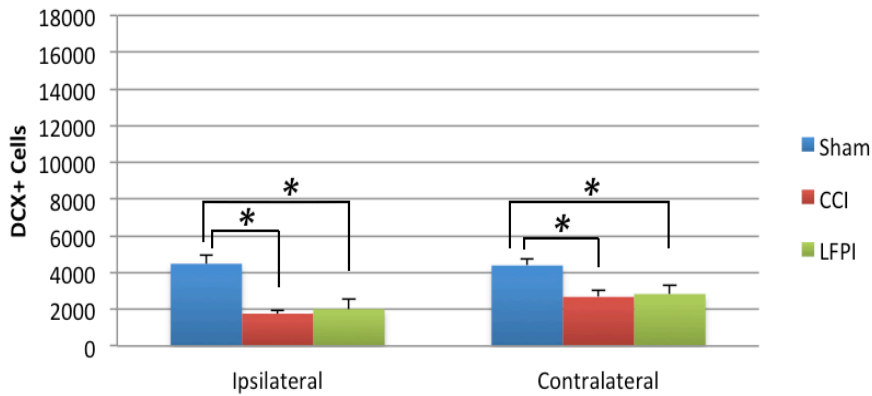
B. Generation of New Neurons in Middle 1/3 GCL 3 Months Post Injury
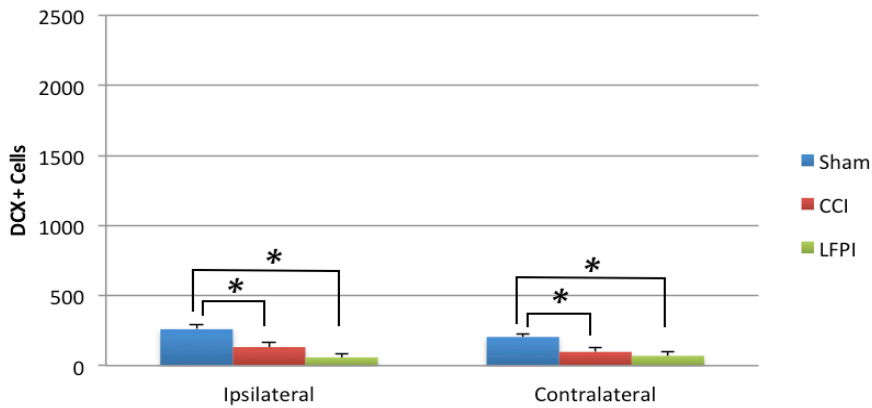
C. Generation of New Neurons in Outer 1/3 GCL 3 Months Post Injury
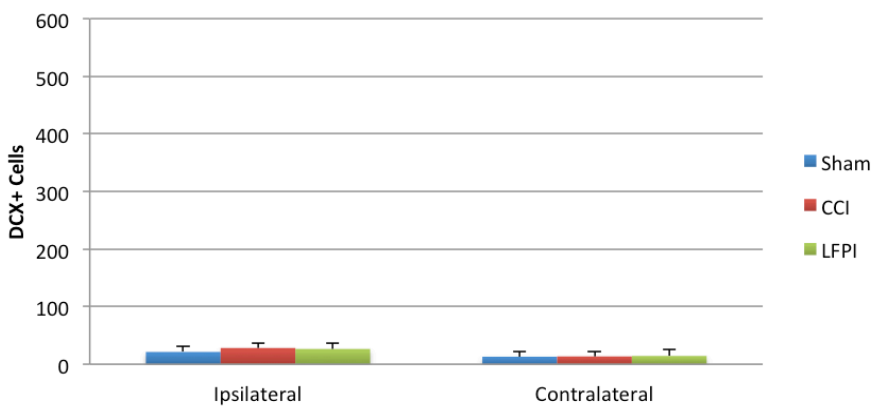
Figure 3.11. Migration of neuroblasts at 3 months after injury in the dentate gyrus.

In sham and injured animals, majority of the DCX positive cells of the GZ were located in the (A) SGZ plus inner 1/3 GCL, with a significantly higher number of DCX positive cells in Sham animals compared to injured animals (IP: sham vs. CCI *p=0.001*, sham vs. LFPI, p=0.002; CT: sham vs. CCI *p=0.011*, sham vs. LFPI, p=0.018). In the (B) middle 1/3 GCL, Sham animals had a significantly higher number of DCX positive cells compared to injured animals (IP: sham vs. CCI *p=0.011*, sham vs. LFPI, p=0.001; CT: sham vs. CCI *p=0.026*, sham vs. LFPI, p=0.008). In the (C) outer 1/3 GCL, there was no difference in the number of DCX positive cells between any group.

Experiment 3. Factors Which Are Associated to Post-TBI Neurogenesis

From experiments 1 and 2, our results indicate that TBI does not have an effect on the level of proliferation in the subacute and chronic-TBI stage. However, we did find that TBI does reduce the level of new neuron generation in the chronic stage, which is opposite of that in the subacute TBI stage. To investigate mechanisms that maybe associated to this change in neurogenesis in the chronic TBI stage, we examined neuroinflammatory responses of subacute and chronic TBI. We used two TBI animal models, CCI injury and LFPI, to study focal and diffuse injury effects at 15 days after injury and 3 months after injury. Inflammatory response was examined by markers for microglial activation in the brain. We used ED1 antibody to label ED1 proteins expressed in activated microglia that become phagocytic in the rat CNS during an inflammatory response (Damoiseaux et al., 1994). We also used the OX6 (anti-MHC class II) antibody to identify immunoreactive cells in the CNS. We checked for inflammation in the cortex, dentate gyrus, and thalamus.

Inflammation in subacute-TBI

At 15 days after injury, sham animals showed only scant amount of inflammation in the brain as detected by ED1 staining in the thalamus and OX6 staining in the DG. However, using both inflammatory markers, we show that injured animals had a significant inflammatory response in the ipsilateral side of injury in the cortex, dentate gyrus, and thalamus (**Figure 3.12**).

ED1 staining in the cortex revealed that CCI injured animals had a staining optical density of 10% +/- 1.8%, LFPI animals had a staining optical density of 14.5% +/- 11%, and both injury groups were not significantly different from each other (p=0.531) (**Figure 3.13**). In the dentate gyrus, CCI injured animals had a staining optical density of 4.7% +/- 3.3%, LFPI animals had a staining optical density of 0.57% +/- 0.28%, and both injury groups were significantly different from each other (p=0.002) (**Figure 3.13**). In the thalamus, sham animals had a staining optical density of 0.2% +/- 0.03%, CCI injured animals had a staining optical density of 2.7% +/- 1.8%, LFPI animals had a staining optical density of 9.3% +/- 1.17%. Both injury groups had significantly more inflammation in the thalamus compared to sham (sham vs. CCI, p<0.038; sham vs. LFPI, p<0.001) and both injury groups were significantly different from each other (p<0.001) (**Figure 3.13**). Both injury groups produced different inflammatory responses in the ipsilateral dentate gyrus and thalamus, with CCI injured animals having a more ED1 staining in the dentate gyrus and LFPI animals having more ED1 staining in the thalamus.

Similar trends were found with OX6 staining. In the cortex, CCI injured animals had a staining optical density of 2.3% +/- 0.98%, LFPI animals had a staining optical density of 12.5% +/- 9.9%, and both injury groups were significantly different from each other (p=0.016) (**Figure 3.14**). In the dentate gyrus, sham animals had a staining optical density of 0.29% +/- 0.29%, CCI injured animals had a staining optical density of 5.3% +/- 2.4%, LFPI animals had a staining optical density of 1.8% +/- 1.57%. Although both injury groups showed a greater inflammatory response in the DG compared to sham, only

CCI injury group had a statistically significant increase (sham vs. CCI, p=0.004; sham vs. LFPI, p=0.325). Also, both injury groups were significantly different from each other (p=0.017) (**Figure 3.14**). In the thalamus, CCI injured animals had a staining optical density of 11.3% +/- 2.76%, LFPI animals had a staining optical density of 26% +/- 1.76%, and both injury groups were significantly different from each other (p<0.001) (**Figure 3.14**). Both injury groups produced different inflammatory responses in the ipsilateral cortex, dentate gyrus, and thalamus, with CCI injured animals having a more OX6 staining in the dentate gyrus, and LFPI animals having more OX6 staining in the cortex and thalamus.

	Cortex	Dentate Gyrus	Thalamus

ED1

CCI

LFPI

OX6

CCI
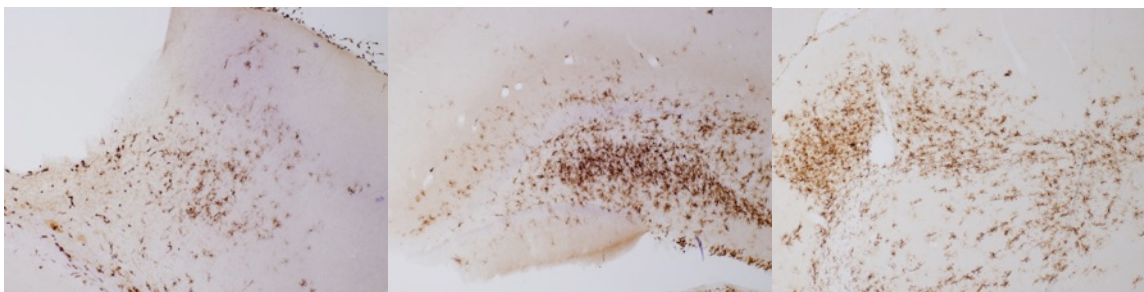
LFPI
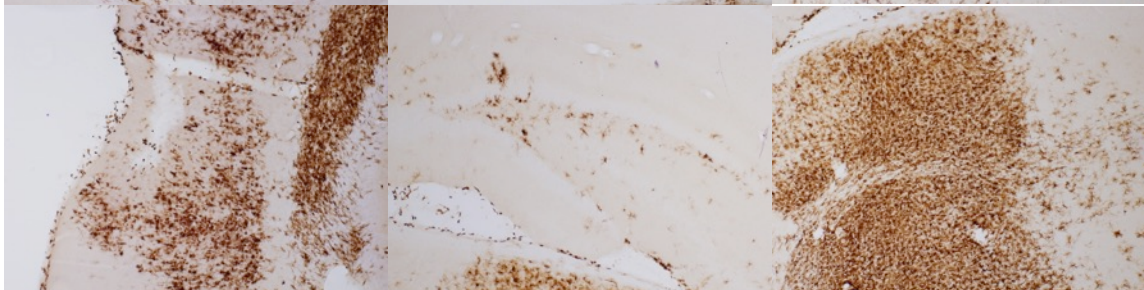
Figure 3.12. ED1 and OX6 Staining Patterns In the Injured Brain. The top panel of images shows ED1 inflammatory marker expression in the cortex, dentate gyrus, and thalamus brain regions after CCI injury or LFPI. The bottom panel of images shows OX6 inflammatory marker expression in the cortex, dentate gyrus, and thalamus brain regions after CCI injury or LFPI. Both inflammatory markers show inflammation in the injured brains. There is a noticeable difference of inflammatory responses between CCI injury and LFPI, with CCI injury producing more inflammation in the dentate gyrus, and LFPI producing more inflammation in the thalamus.

A. ED1 Staining Intensity in Cortex 15 Days Post Injury
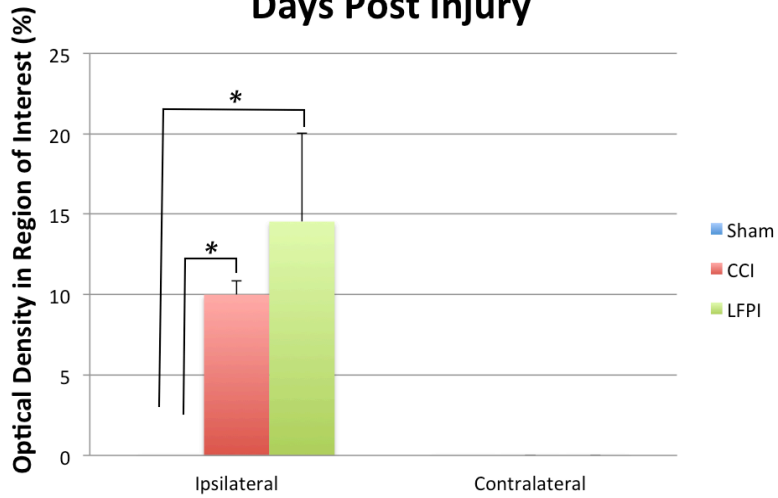
B. ED1 Staining Intensity in Dentate Gyrus 15 Days Post Injury
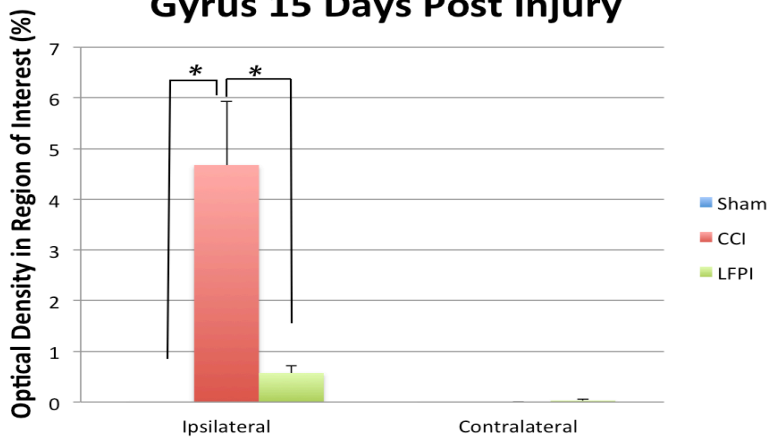
C. ED1 Staining Intensity in Thalamus 15 Days Post Injury
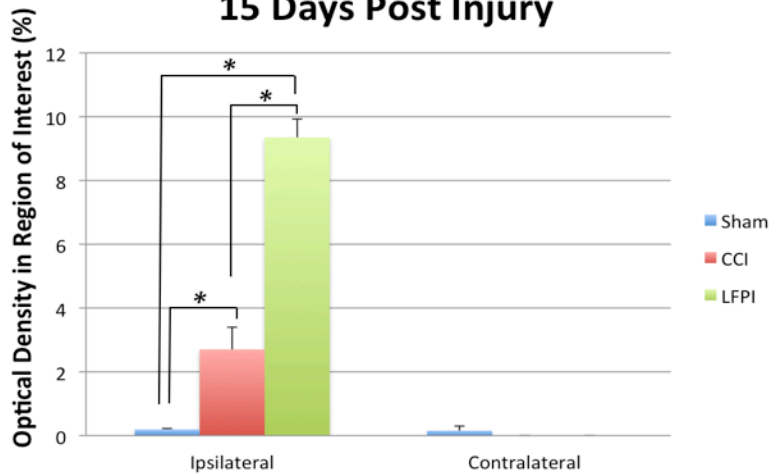
Figure 3.13. ED1 staining optical density at 15 days after injury. Optical density of ED1 staining was used to measure level of inflammation in the (A) cortex, (B) dentate gyrus, and (C) thalamus in sham and injured animals. Sham animals did not show ED1 staining in the cortex and DG and had scant inflammation in the thalamus. Animals of both types of injuries showed inflammation in all ipsilateral structures. CCI injury animals had significantly more inflammation in the dentate gyrus compared to LFPI animals (p=0.002). LFPI animals had significantly more inflammation in the thalamus compared to CCI injury animals (p<0.001).

A. OX6 Staining Intensity in Cortex 15 Days Post Injury
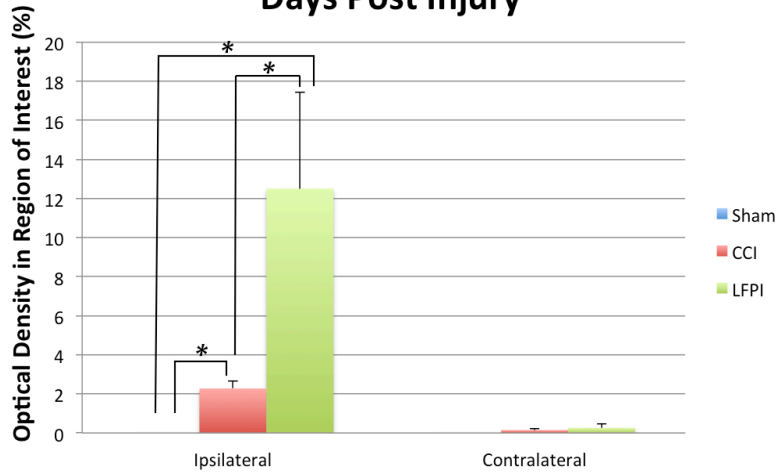
B. OX6 Staining Intensity in Dentate Gyrus 15 Days Post Injury

C. OX6 Staining Intensity in Thalamus 15 Days Post Injury
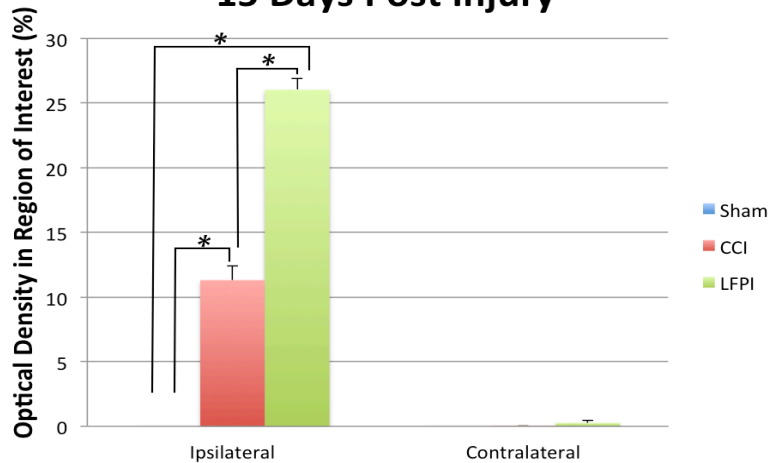
Figure 3.14. OX6 staining optical density at 15 days after injury. Optical density of OX6 staining was used to measure level of inflammation in the (A) cortex, (B) dentate gyrus, and (C) thalamus in sham and injured animals. Sham animals did not show OX6 staining in the cortex and thalamus and had scant inflammation in the DG. Animals of both types of injuries showed inflammation in all ipsilateral structures. CCI injury animals had significantly more inflammation in the dentate gyrus compared to LFPI animals ($p=0.017$). LFPI animals had significantly more inflammation in the cortex ($p=0.016$) and thalamus ($p<0.001$) compared to CCI injury animals.

Inflammation in Chronic-TBI

At 3 months after injury, using both inflammatory markers we assessed inflammatory responses in sham and injured animals. Injured animals showed more inflammation compared to sham animals. In the cortex, sham animals did not show ED1 staining. In the ipsilateral cortex, CCI injured animals had a staining optical density of 4.55% +/- 1.73%, LFPI animals had a staining optical density of 6.38% +/- 4.88%, and both injury groups were not significantly different from each other (p=0.507) (**Figure 3.15**). In the ipsilateral dentate gyrus, sham animals had a staining optical density of 0.16% +/- 0.03%, CCI injured animals had a staining optical density of 1.25% +/- 0.13%, and LFPI animals had a staining optical density of 0.56% +/- 0.1%. Both injury groups had significantly more inflammation in the dentate gyrus compared to sham (sham vs. CCI, p<0.001; sham vs. LFPI, p=0.004) and CCI injury group had significantly more inflammation compared to LFPI group (CCI vs. LFPI, p<0.001). Although ED1 staining was detected in the contralateral dentate gyrus in sham and injured groups, One-Way Anova comparison of the three groups showed no significant difference (p=0.604) (**Figure 3.15**). In the ipsilateral thalamus, sham animals had a staining optical density of 0.42% +/- 0.07%, CCI injured animals had a staining optical density of 1.22% +/- 0.22%, and LFPI animals had a staining optical density of 3.94% +/- 2.19%. LFPI group had significantly more inflammation in the thalamus compared to sham and CCI injury group (sham vs. LFPI, p=0.026; CCI vs. LFPI, p=0.032;), and CCI injury group was not significantly different from sham (p=0.542). Although ED1 staining was detected in the contralateral thalamus in sham and injured groups, One-Way ANOVA comparison of the three groups showed no significant difference (p=0.739) (**Figure 3.15**).

We also checked for inflammation in these brain regions with OX6 marker at 3 months after injury (**Figure 3.16**). In the cortex, sham animals did not show OX6 staining. In the ipsilateral cortex, CCI injured animals had a staining optical density of 3.08% +/- 1.61%, LFPI animals had a staining optical density of 3.7% +/- 3.5 %, and both injury groups were not significantly different from each other (p=0.76). In the contralateral cortex, OX6 staining was detected with both injury groups having significantly more inflammation compared to sham (sham vs. CCI, p=0.035; sham vs. LFPI, p=0.001) and LFPI group had significantly more inflammation compared to CCI injury group (CCI vs. LFPI, p=0.002). In the dentate gyrus, sham and injured animals showed OX6 staining, however, using One-Way ANOVA to compare all three groups, we found no significant difference in ipsilateral and contralateral sides (IP: p=0.686; CT: p=0.819). In the ipsilateral thalamus, sham animals had a staining optical density of 0.22% +/- 0.19%, CCI injured animals had a staining optical density of 0.2% +/- 0.08%, and LFPI animals had a staining optical density of 1.24% +/- 0.31%. LFPI group had significantly more inflammation in the ipsilateral thalamus compared to sham and CCI injury group (sham vs. LFPI, p<0.001; CCI vs. LFPI, p<0.001;), and CCI injury group was not significantly different from sham (p=0.936).

A. ED1 Staining Intensity in Cortex 3 Months Post Injury

B. ED1 Staining Intensity in Dentate Gyrus 3 Months Post Injury

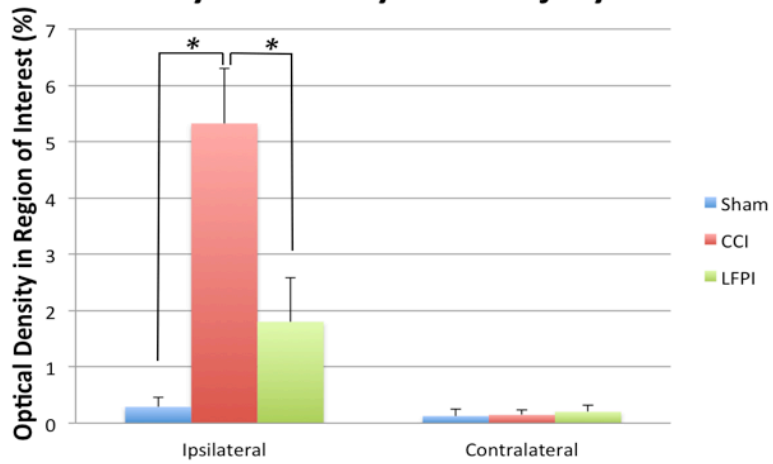**C.** ED1 Staining Intensity in Thalamus 3 Months Post Injury
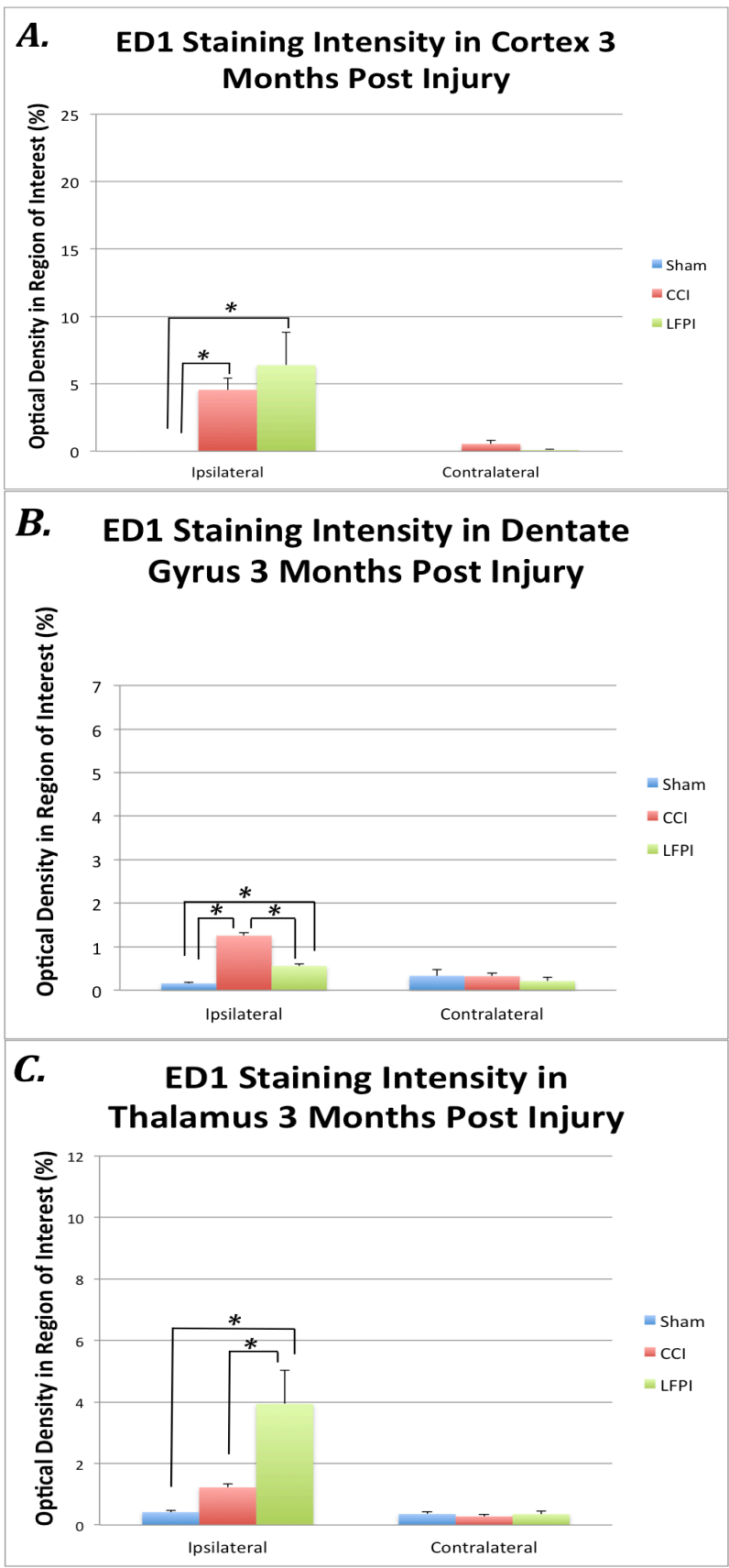
Figure 3.15. ED1 staining optical density at 3 months after injury. Optical density of ED1 staining was used to measure level of inflammation in the (A) cortex, (B) dentate gyrus, and (C) thalamus in sham and injured animals. Injured animals showed significantly more inflammation in ipsilateral cortex, dentate gyrus, and thalamus, compared to sham animals. CCI injury animals had significantly more inflammation in the dentate gyrus compared to LFPI animals (p<0.001). LFPI animals had significantly more inflammation in the thalamus compared to CCI injury animals (p=0.032).

A. OX6 Staining Intensity in Cortex 3 Months Post Injury
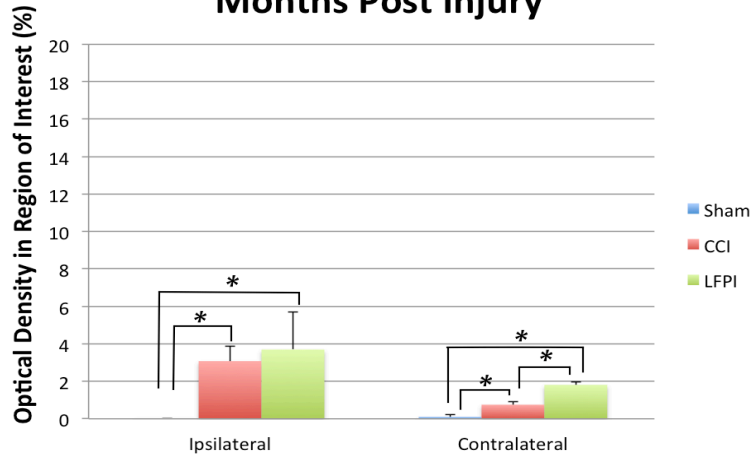
B. OX6 Staining Intensity in Dentate Gyrus 3 Months Post Injury
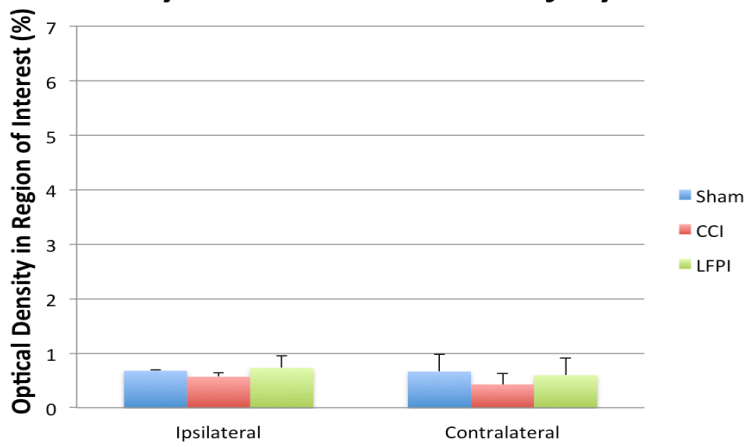
C. OX6 Staining Intensity in Thalamus 3 Months Post Injury
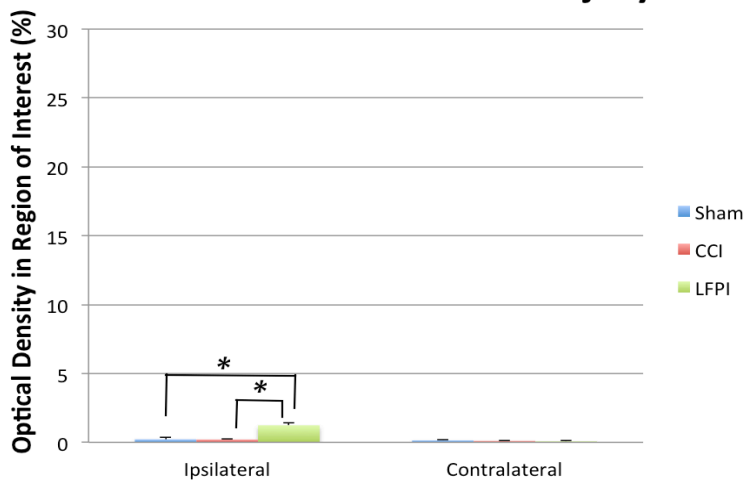
Figure 3.16. OX6 staining optical density at 3 months after injury. Optical density of OX6 staining was used to measure level of inflammation in the (A) cortex, (B) dentate gyrus, and (C) thalamus in sham and injured animals. Injured animals showed significantly more inflammation in the ipsilateral and contralateral cortex, and only LFPI group showed significantly more inflammation in ipsilateral thalamus, compared to sham animals.

Experiment 4. Morphological Changes of the Hippocampus in Chronic-TBI.

Apart from cellular changes that happen after TBI, morphological changes of overall brain structures such as the hippocampus have also been evaluated to assess injury characteristics. The purpose of this experiment was to evaluate TBI-induced morphological changes of the hippocampus in chronic-TBI stage using different TBI animal models. The CCI and LFPI models were used to compare changes of the hippocampal size after injury. The parameters of ipsilateral and contralateral hippocampi were measured in injured and sham animal groups. Percent change was calculated by taking a percentage of the ratio of ipsilateral to contralateral parameters.

In our results we found that only animals that received LFPI show reduction of hippocampal size at 3 months after injury (**Figure 3.17**). These animals had a 12% +/- 7.64% reduction of ipsilateral hippocampal size (**Figure 3.18**). Animals that received CCI injury and sham animals did not show changes in hippocampal size. Using One-Way ANOVA to compare change in all groups, we found that there is a statistically significant change (p=0.047). A post hoc LSD pairwise comparison, reveled that animals that received LFPI had statistically reduced ipsilateral hippocampal size compared to sham and CCI (Sham vs. LFPI, p=0.047; CCI vs. LFPI, P=0.027). Sham animals and CCI injury animals were not statistically different (p=0.894).

Ipsilateral　　　　　　　　　　**Contralateral**

Sham
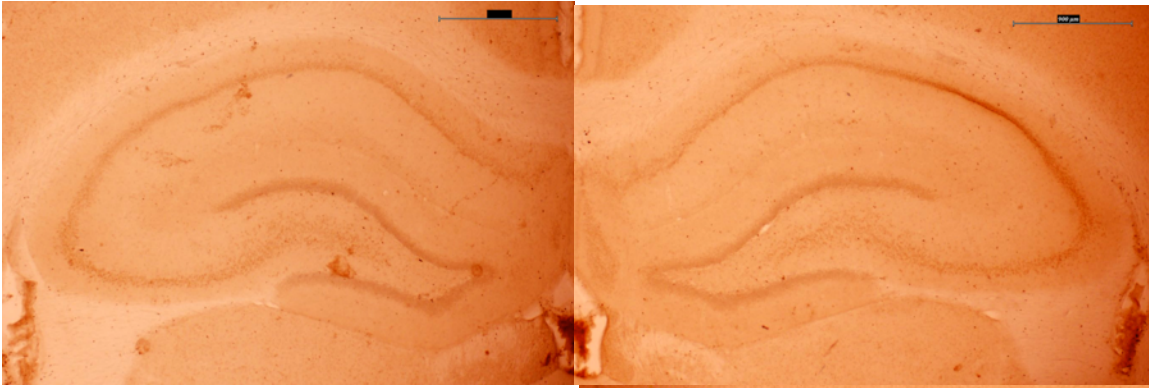
CCI
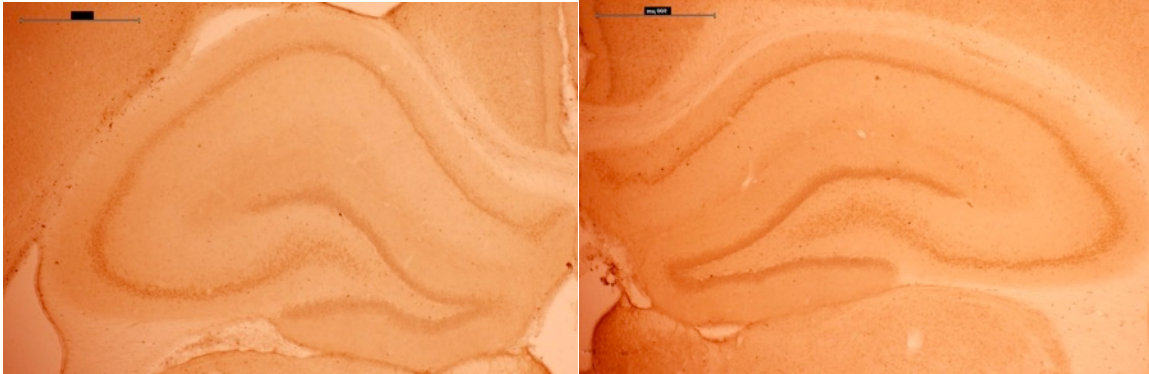
LFPI
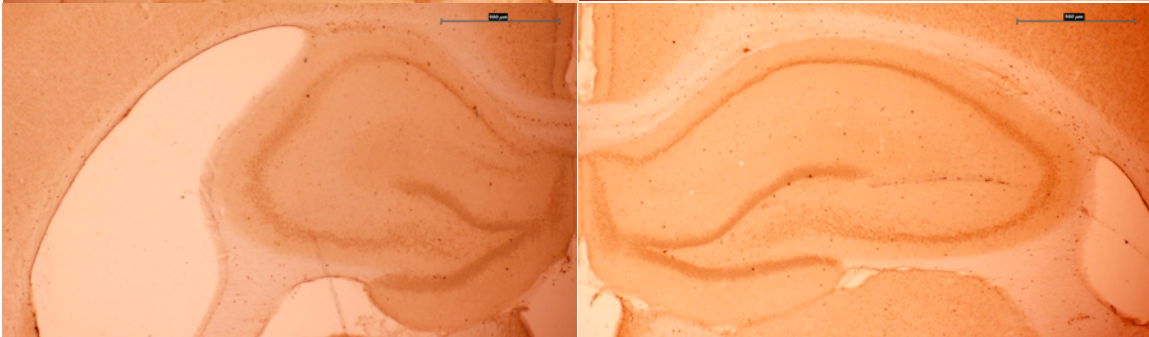
Figure 3.17. TBI-induced hippocampal size change at 3 months after injury. We observed the ipsilateral and contralateral hippocampal size differences of sham, CCI injury, and LFPI animal groups (scale bar= 900 μm). In sham animals, ipsilateral and contralateral hippocampal sides were similar in size. In CCI injury animals, ipsilateral hippocampus showed changes in shape but not in size compared to contralateral hippocampus. In LFPI animals, we found observable reduction in ipsilateral hippocampus size compared to contralateral hippocampus.

Comparison of Hippocampal Size at 3 Months Post Injury
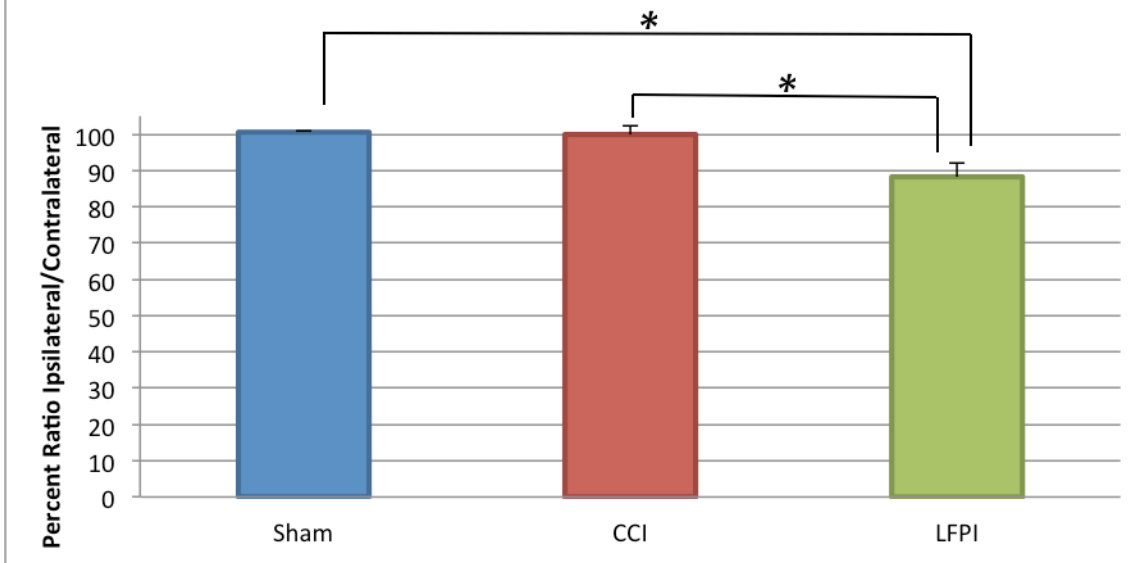
Figure 3.18. Quantification of hippocampal size change at 3 months after injury.

Percentage of the ratio of ipsilateral to contralateral hippocampus perimeter reveals the injury-induced change in ipsilateral hippocampal size. CCI injury animals and sham animals do not have a change in ipsilateral hippocampus size. Only animals that received LFPI show a reduction of ipsilateral hippocampal size with an average percent ratio of 88% +/- 7.64%, which is significantly different from sham animals (p=0.047) and CCI injury animals (p=0.027).

Chapter 4 - Discussion

TBI poses significant short and long-term challenges to millions of people around the world (Zaloshnja et al., 2008). According to the CDC, majority of these patients are either younger than 24 years old or over 65 years old. Clinical evaluations have shown that TBI patients are more likely to develop many other neurological complications over time than people without a TBI history (Koponen et al., 2002). Recently much attention has been drawn to the relationship between TBI and dementia, as TBI patients commonly develop neurodegenerative diseases later in life such as Alzheimer's and CTE (Smith et al., 2013). Over the past few decades we have learned a great deal about the mechanisms that regulate TBI, however, a central question still remains unanswered; what is causing these long-term TBI deficits?

One possibility could be that there is a change in adult neurogenesis in the chronic TBI stage. In the past few decades we have discovered evidence of lifelong adult neurogenesis, mainly in the SVZ and DG of the mammalian brain (Sun, 2016). Our lab and many others have demonstrated that adult neurogenesis can be stimulated and one of these stimuli is TBI (Sun et al., 2005; Gao and Chen, 2013). Following TBI, the brain initiates endogenous neurogenic responses in an attempt to repair the physical damages. Some studies suggest that TBI induced neurogenesis in the DG contributes to the short-term spontaneous cognitive recovery seen in clinical patients (Sun et al., 2015; León-Carrión and Machuca-Murga, 2001). Because the hippocampus is linked to cognitive function and memory consolidation, TBI induced cellular changes in this structure may explain cognitive and behavioral changes seen in TBI patients (Deng et al., 2009).

However, the neurogenic stimulus following injury is not continuous and does not fully repair the damages. Majority of these studies have focused on acute and subacute neurogenic changes following TBI. For this reason, we investigated the neurogenic changes from subacute to chronic-TBI stage, and the mechanisms that may regulate long-term neurogenic changes.

Summary of Results

To accurately study neurogenic responses after TBI, we used two different TBI animal models. Previous studies have reported conflicting results of TBI-induced neurogenic responses, particularly regarding cell differentiation and generation of new neurons (Sun et al., 2005; Kleindienst et al., 2005; Kernie et al., 2001; Rice et al., 2003; Gao and Chen, 2013). Some of these differences may be due to the use of different TBI animal models. Because TBI is a complex process involving both focal and diffuse injury, we used CCI and LFPI models to induce focal and diffuse injuries respectively, and bring attention to any differences that can be attributed to using different animal models. Brain tissue was collected 15 days after injury to evaluate subacute TBI, and 3 months after injury to evaluate chronic TBI.

In the subacute stage, TBI had no effect on the rate of proliferation in the GZ and hilus region. However, DCX immunolabeling showed that TBI induced an increase in the number of immature neurons in the ipsilateral side of injury in the GZ and hilus region. Further analysis showed that CCI injury resulted in more immature neurons in the hilus

than LFPI injury. Analysis of neural migration in the GZ revealed that TBI does cause more lateral migration but this is specific to focal injury induced by the CCI model.

In the chronic stage of TBI, proliferation was no different after injury compared to sham, similar to what we found in the subacute stage. Interestingly however, we found a significant reduction in the generation of new neurons at 3 months after injury in the ipsilateral and contralateral granular zone. In the hilus region a significant reduction was seen only in the diffuse injury model in the ipsilateral side of injury. Neuronal migration in the GZ was also reduced in chronic-TBI stage; however, this was only seen in the middle 1/3 of the GCL.

To investigate the mechanisms that may regulate the activity of generating new neurons in chronic-TBI, we examined neuroinflammation in the cortex, DG, and thalamus at both subacute and chronic-TBI stage. In the subacute-TBI stage, inflammation was seen only in the ipsilateral hemisphere of injured animals while sham animals show no inflammatory responses. Also, CCI injury model induces a significantly higher inflammatory response in the DG, while LFPI model induces a higher inflammatory response in the thalamus, which is indicative of focal injury caused by CCI model and diffuse injuries caused by LFPI model. In the chronic stage of TBI, we found a similar trend in inflammatory response. We found low levels of inflammation in the DG and thalamus of sham animals and significantly higher inflammatory responses in injured animals in cortex, DG, and thalamus. These inflammatory results indicate that TBI-induced inflammation is persistent long after TBI injury.

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Further assessment of TBI induced changes in chronic stage involved morphological analysis of the hippocampus. Our results indicate a changed morphology of the hippocampus after injury that can be seen at 3 months after injury. CCI injury model produced an observed change in shape of the ipsilateral hippocampus while LFPI model caused shrinkage of the ipsilateral hippocampus.

Subacute-TBI neurogenic response

The TBI-induced neurogenic response involves all aspects of neurogenesis including proliferation, differentiation, survival and integration (Sun, 2016). Our lab and others have shown that TBI-induced proliferative activity peaks at 2 days post injury and gradually returns to baseline by 14 days post injury (Sun et al., 2005; Gao and Chen, 2013). In this study, at 15 days after injury, we found baseline proliferation levels in injured animals, consistent with previous reports. However, the ability of TBI to stimulate an endogenous neurogenic response in the brain has been controversial. Most studies agree that TBI induces an up-regulation in proliferation in the DG and SVZ (Sun et al., 2005; Gao and Chen, 2013). However, discrepancies arise in discussion about the fate of these newly proliferated cells. Some studies report that increased proliferation in the neurogenic regions results in several fold increase of the number of new neurons that can be seen as long as 60 days post injury (Sun et al., 2005; Kleindienst et al., 2005; Kernie et al., 2001; Richardson et al., 2007). Other studies have found that TBI does not change the production of new neurons (Rice et al., 2003; Gao and Chen, 2013) or may even have a reductive effect on neuronal production (Rola et al., 2006). These

discrepancies may be due to many factors including different injury models used, assessment at different post injury time points, quantification methods, and/or human error. In our results, we found that at 15 days after injury using either CCI or LFPI model, TBI causes a significant increase in the number of immature neurons in the ipsilateral DG of the adult rat brain. This is in disagreement with another study that used similar approach, looking at the number of DCX positive cells after injury, to assess TBI-induced generation of immature neurons, in which they report a significant decrease (Rola et al., 2006). The difference in results may be due to different animals used as their study used mice. Also, cell counting methods were different as they only examined the number of DCX positive cells in the SGZ, excluding a major portion of cells that may have migrated into the inner 1/3 GCL after injury. Other studies that have shown a TBI-induced increase in the generation of new neurons, have not directly analyzed cell counts of immature neurons after injury.

Chronic-TBI Neurogenic Response

TBI-induced proliferation involves a robust increase in cell divisions of NSC into more NSCs and NPCs (Sun et al., 2005; Bonaguidi et al., 2012). We hypothesized that the acute-TBI neurogenic stimulus would exhaust the NSC pool through rapid symmetrical differentative cell divisions, where one NSC divides into two NPCs, resulting in reduced proliferative activity over time. Our proliferation results using both pulse BrdU paradigm and Ki67 staining at 3 months after injury however, did not match our expectations, as we did not find a reduction of proliferative activity in chronic TBI, indicating that the NSC pool may not be exhausted over time. Our results are inconsistent

with a previous study that reported reduction of proliferating cells 2 month after injury (Acosta et al., 2013). Although they used similar TBI model and immunohistochemical staining procedures, their cell counting method involved only examining the number of Ki67 positive cells in the SGZ, whereas we included proliferating cells of the entire granular zone.

To fully understand chronic-TBI impact on neurogenesis, we also looked at the generation of new neurons at 3 months after injury. Although proliferative activity is not impacted by TBI in the chronic stage, our results show significant reduction in the number of DCX positive cells, suggesting that TBI causes a long-term reduction in the ability to generate new neurons in the DG. Studies have suggested that chronic neuroinflammation may negatively regulate adult neurogenesis (Gao and Hong, 2008). In a rodent TBI study, sustained neuroinflammation was detected more than 8 weeks post-injury and showed hippocampal cell loss and down-regulation of proliferation in the SGZ of the hippocampus when compared to sham animals (Acosta et al., 2013). Considering that TBI-stimulated hippocampal proliferation returns to baseline levels by 14 days post-injury, at 8 weeks post-injury the baseline neurogenic stimulus may not be strong enough to overcome the negative effects of chronic-neuroinflammation, which could result in decreased neurogenesis below baseline levels in the chronic-TBI stage. One study shows that increasing the stimulation of neurogenesis through environmental enrichment and exercise rescues the neurogenic suppression via lipopolysaccharide-induced inflammatory conditions (Wu et al., 2007). This suppression of neurogenic activity via inflammatory responses would also explain many of the long-term pathological

conditions of chronic-TBI that are reported after injury such as neurodegenerative diseases, psychiatric disorders, and sleep disorders. These findings indicate that TBI-induced inflammatory responses may be active at 3 months post-injury, affecting the normal neurogenic activity.

TBI-induced Inflammation Affects Long-term Neurogenesis

Concurrent with stimulated neurogenic activity that induces benefits following TBI, studies show that other TBI induced mechanisms are also activated that are detrimental to recovery and normal function such as progressive cell death and inflammation in the brain (Bramlett and Dietrich, 2015). Especially in the hippocampus, these mechanisms can be responsible for some of the cognitive deficits, sensory and motor dysfunction, and neuropathology such as seizures seen in TBI patients (Fujimoto et al., 2004; Gupta and Gupta, 2005). Studies have found significant cell death in the DG after acute-TBI, especially of immature neurons (Gao et al., 2008). Inflammatory responses after injury have been demonstrated to play a role in the regulation of hippocampal cell death and reduced neurogenic activity through activation of microglia that release inflammatory mediators such as IL-1, IL-6, TNF-α, reaction oxygen species etc. (Monje et al., 2003; Ekdahl et al., 2003). These pro-inflammatory mediators are detrimental for survival of newly generated neurons (Ekdahl et al., 2003). For example, studies have shown that the number of activated microglia and the levels of pro-inflammatory cytokines, particularly IL-6 and TNF-α, produced by microglia are correlated with decreased survival of new hippocampal neurons, and inhibition of microglia activation with anti-inflammatory drug treatment increase the number of newly

formed neurons (Monje et al., 2003; Ekdahl et al., 2003). It is likely that the persistent

inflammation following TBI, especially after the TBI-induced acute neurogenic stimulus

is returned to baseline, curtail the neuronal differentiation or survival of newly generated

cells. Future study using combination of BrdU with cell type specific markers is needed

to answer this question. In this study we found inflammatory responses during subacute-

TBI, and this inflammation is persistent at 3 months after injury. In a previous study,

TBI-induced inflammatory responses have been detected in the striatum, thalamus, and

cerebral peduncle at 2 months after injury in conjunction with reduced levels of immature

neurons in the SGZ (Acosta et al., 2013). Our study adds to these findings showing that

inflammatory responses are extended to 3 months post injury and also detected in the DG

and cortex regions, concomitant with reduction of immature neurons in the DG.

Hippocampal morphological changes following TBI

Apart from cellular and biochemical changes, chronic assessment of TBI also

involves morphological changes. Temporal studies of histopathological changes after TBI

in rats demonstrate progressive cell death mechanisms in the cerebral cortex and

hippocampus over one year after injury (Smith et al., 1997). These cell death mechanisms

have also been linked to neurodegenerative changes. Clinical studies show changes in

white matter tracts and progressive atrophic changes in TBI patients at one year after

injury (Williams et al., 2001; MacKenzie et al., 2002). The hippocampus is particularly

vulnerable to TBI as evident from morphological changes seen in the temporal lobe,

specifically hippocampal atrophy (Ariza et al., 2006). In the present study we found TBI-

induced morphological changes of the hippocampus; particularly in the LFPI model at 3

100

months post-injury we found shrinkage of the hippocampus. This is consistent with previous findings showing reduced hippocampal volume at 2 months following LFPI in rats (Bramlett et al., 1997). Studies suggest that these morphological changes may contribute to cognitive and behavioral changes seen in many TBI patients (Jorge et al., 2007). Atrophy of the hippocampus may also be a contributing factor to the chronic changes of adult neurogenesis that we found in this study.

Conclusion and Future Direction

TBI induces both acute and chronic neurological consequences that can lead to pathological conditions such as cognitive deficits, neurodegenerative diseases, psychiatric disorders, and seizures among many other problems (Koponen et al., 2002; Castriotta et al., 2007; Uryu et al., 2007). The complex interplay of mechanisms that regulate these acute and chronic conditions is not completely understood. The present study in combination with previous studies shows involvement of a neurogenic response to TBI, in an endogenous effort to initiate brain repair mechanisms (Sun, 2016; Sun et al., 2005). This endogenous neurogenic response is stronger than the normal baseline adult neurogenic activity, however, it is not continuous (Sun et al., 2005). Once this TBI-induced neurogenic response returns to baseline levels, other persistent TBI induced mechanisms may affect normal adult neurogenic activity. In the present study we show the involvement of inflammatory mechanisms that may regulate normal neurogenic activity in chronic-TBI. Our study shows persistent inflammation concurrent with reduced generation of immature neurons in chronic TBI stage.

Further investigation is required to understand the true nature of this interaction between chronic inflammation and reduced neurogenic activity. It is also necessary to elucidate the phenotype of microglial activation in chronic-TBI. Studies have shown that specific microglial phenotypes have specialized functions, such as the M1 phenotype that has pro-inflammatory properties and the M2 phenotype that has anti-inflammatory properties (Hsieh et al., 2013). Distinguishing the phenotype of microglia activation will determine the nature of the inflammatory response in chronic stage of TBI.

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Vita

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