The Effect of Minocycline Treatment on Cell Proliferation and Neurogenesis in the Hippocampus in Young and Aged Brains Following Traumatic Brain Injury

Ashley Harvin
Virginia Commonwealth University

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THE EFFECT OF MINOCYCLINE TREATMENT ON CELL PROLIFERATION AND NEUROGENESIS IN THE HIPPOCAMPUS IN YOUNG AND AGED BRAINS FOLLOWING TRAUMATIC BRAIN INJURY

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

by

ASHLEY L. HARVIN
Bachelor of Science, University of Virginia, 2010

Major Director: Dong Sun, MD, PhD.
Associate Professor

Department of Neurosurgery

Virginia Commonwealth University Richmond, VA
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<tbody>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>Atm</td>
<td>Atmosphere</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>CCI</td>
<td>Controlled cortical impact</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DAB</td>
<td>5,5-diaminobenzidine</td>
</tr>
<tr>
<td>DCX</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>FG</td>
<td>Fluorogold</td>
</tr>
<tr>
<td>FPI</td>
<td>Fluid percussion injury</td>
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<tr>
<td>GCL</td>
<td>Granule cell layer</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GZ</td>
<td>Granular zone</td>
</tr>
<tr>
<td>Hr</td>
<td>Hour</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>Iba1</td>
<td>Ionized calcium binding adaptor molecule 1</td>
</tr>
<tr>
<td>ICP</td>
<td>Intracranial pressure</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-13</td>
<td>Interleukin-13</td>
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<tr>
<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LFPI</td>
<td>Lateral fluid percussion injury</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<tr>
<td>NPC</td>
<td>Neural progenitor cell</td>
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<tr>
<td>NSC</td>
<td>Neural stem cell</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SE</td>
<td>Status epilepticus</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error margin</td>
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<tr>
<td>SGZ</td>
<td>Subgranular zone</td>
</tr>
<tr>
<td>SSC</td>
<td>Saline sodium citrate</td>
</tr>
<tr>
<td>SVZ</td>
<td>Subventricular zone</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
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</table>
THE EFFECT OF MINOCYCLINE TREATMENT ON CELL PROLIFERATION AND NEUROGENESIS IN THE HIPPOCAMPUS IN YOUNG AND AGED BRAINS FOLLOWING TRAUMATIC BRAIN INJURY

Ashley L. Harvin

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

Virginia Commonwealth University, 2012
Advisor: Dong Sun, MD, PhD., Department of Neurosurgery

Following traumatic brain injury, there is an enhanced cell proliferative and neurogenic response in the young adult hippocampus, which may be associated with innate cognitive recovery. However, in the aged brain, an increased level of inflammatory cell responses was observed following injury concomitant to decreased hippocampal neurogenesis and cognitive recovery in the aging population. This suggests that excessive inflammation produced in the injured aging brain has a detrimental effect on neurogenesis and cognitive function. In this study, we examined the effect of anti-inflammatory treatment with minocycline on cell proliferation and generation of new neurons in the dentate gyrus (DG) of the hippocampus in both young and aged rats. Fisher 344 rats aged at 3 months and 20 months were subjected to a moderate lateral fluid percussion injury (LFPI) or cortical impact injury (CCI). Minocycline was administered intraperitoneally starting either at 30 minutes or 4 hours
post-injury, thereafter twice daily for 2 days. BrdU was injected at 2 days post-injury to label injury-induced proliferating cells. To examine the effect of minocycline on cell proliferation, generation of new neurons, and inflammatory cell response at the acute stage post-injury, the rats were perfused 3 days post-injury. Brain sections were immunostained for BrdU and early neuronal marker doublecortin (DCX). The results show that short-term anti-inflammatory treatment with minocycline reduces the cell proliferative response, presumably inflammatory cell responses, in young and aged rats following LFPI and CCI injury, and enhances generation of new neurons in the hippocampus in both young and aged rats following LFPI and in aged rats following CCI injury. Therapies that enhance hippocampal neurogenesis may also have potential to improve cognitive recovery following TBI.
Traumatic brain injury (TBI) is a serious disorder that can result in severe behavioral and cognitive deficits. TBI is the leading cause of morbidity and mortality after trauma and affects 3 out of every 1,000 Americans each year (Dutton and McCunn, 2003). Post-traumatic cerebral inflammation, a secondary consequence of TBI, is characterized by glial activation, leukocyte recruitment, and upregulation and secretion of cytokines and chemokines (Ziebell and Morganti-Kossmann, 2010; Cederberg and Siesjö, 2009). As the primary mediators of the inflammatory response to CNS trauma, microglia play a vital role in the injured brain. While short-term microglial activation is necessary to remove dying and damaged neurons and promote tissue repair, chronic microglial activation is considered to be the most damaging response of microglia to injury. Associations between damaged neurons and uncontrolled, hyperactivated microglia can result in a self-propagating positive feed-forward loop that leads to dysregulated and prolonged microglial activation and neuronal cell death, thus driving the chronic progression of neurodegeneration.

The hippocampus is particularly vulnerable to insults (Dash et al., 2001), and brain trauma is often characterized by learning, memory, and cognitive deficits (Witgen et al., 2005). However, these deficits may spontaneously recover over time, suggesting that there are innate repair and regeneration mechanisms that exist within the brain. Previous studies from our laboratory and others have shown a significant increase in cell proliferation in the hippocampal dentate gyrus (DG) in both injured juvenile and adult rats after lateral fluid percussion injury and controlled cortical impact injury (Sun et al., 2005; Rice et al., 2003; Chirumamilla et al., 2002; Dash et al., 2001). These injury-
induced newly proliferating cells are capable of differentiating into neurons and integrating into the existing hippocampal circuitry, and thus may contribute to the potential of innate cognitive recovery following TBI (Sun et al., 2007).

It is well known that aged patients recover to a lesser extent than younger patients following TBI, but the mechanisms underlying this age-related disparity are not fully understood (Hamm et al, 1992; Sun et al, 2005). Although new neurons are continually generated throughout adulthood, multiple studies support that there is an 80% decline in neural stem cell proliferation and neurogenesis in the normal aged brain (Seki and Arai, 1995; Olariu et al., 2007; Galvan and Jin, 2007; Kuhn et al., 1996; Rao et al., 2006). Additional evidence indicates that in the aged brain, microglia when activated exhibit an amplified inflammatory response (Cekic and Stein, 2010; Loane and Byrnes, 2010). Our laboratory has observed that the injury-induced proliferative response in the aged brain predominately generates microglial cells, and that newly generated neurons fail to survive in this environment for an extended period of time (unpublished data from the Sun laboratory). These findings led us to hypothesize that enhanced levels of inflammation in the injured aged brain may contribute to reduced neurogenesis and cognitive function, and that hippocampal neurogenesis in response to injury can be restored by inhibiting the inflammatory response in the aged brain. To test this hypothesis, we administered minocycline treatments to young adult and aged rats to assess whether anti-inflammatory treatment could enhance hippocampal neurogenesis after injury, particularly in the aged brain where microglia exhibit an amplified response.
EPIDEMIOLOGY OF TBI

Traumatic brain injury (TBI) is a major global health concern and has affected approximately 57 million people worldwide, resulting in approximately 10 million deaths and hospitalizations each year. An estimated 1.5 million Americans sustain a TBI annually, making it a leading cause of death and life-long disability in the United States (McArthur et al., 2006). These injuries result in 1.1 million emergency department visits, 230,000 hospitalizations in which patients survive, and 80,000 people that experience long-term disability (Thurman et al., 1999a). The CDC estimates that TBI is responsible for one-third of all injury-related deaths, with approximately 50,000 Americans dying each year following TBI (McArthur et al., 2006; Langlois et al., 2006). The populations at the highest risk are adolescents, young adults, and the elderly. The incidence of TBI is twice as high among males as it is among females, and adults aged 75 years and older have the highest incidence of hospitalizations following TBI, mainly due to falls (Langlois et al., 2006). Motor vehicle crashes, falls, and violence are the most common causes of TBI (Thurman et al., 1999b; McArthur et al., 2006). Blasts are the leading cause of military-associated TBI. Other major causes of TBI include sports and recreational activities (Langlois et al., 2006).

Many people who survive TBI are faced with permanent neurologic problems and disabilities including cognitive, behavioral, emotional, and sensory and motor impairments (Hilton, 1994; Langlois et al., 2006). Even mild TBI can result in long-term cognitive deficits that may interfere with an individual’s ability to carry out daily tasks or return to work (Langlois et al., 2006). A person’s career and social and family relationships may be profoundly and permanently altered following TBI. Cognitive
functional deficits may cause the loss of communication skills and memory, organizational and problem-solving skills, decreased attention span, and impaired concentration (Thurman et al., 1999c). Emotional instability, personality changes, agitation and aggressiveness, and impulsiveness may be a part of the injury-induced behavioral sequelae typical of TBI (Hilton, 1994). In elderly persons, there is a 1.5 times increased risk of depression and a 2.3 to 4.5 times increased risk of Alzheimer’s disease depending on injury severity, making complications due to TBI especially difficult for this age group (Langlois et al., 2006).

**EXPERIMENTAL TBI**

TBI can result in both focal and diffuse injury to brain tissue. The mechanisms of injury include impact, rotational and inertial effects, and the combination of impact with rotation. The type and severity of injury depends on the amount of energy transferred to the head, the duration of injury, and the site of impact (McArthur et al., 2006). Focal injuries are those that are caused by a blow to the head at the site of impact. It is estimated that two-thirds of deaths from brain injury are due to focal injuries. Diffuse injuries include those that result in severely dysfunctional and vegetative states as well as mild concussive brain injuries. Severe diffuse injuries are the primary cause of persistent neurological disability (Davis, 2000). The profile of injury typically seen in human TBI represents a combination of focal and diffuse injury (McAllister, 2011).

In the laboratory setting, various experimental animal models are used to study the effects of TBI (McArthur et al., 2006). In rodents, fluid percussion injury (FPI) is the most widely used TBI model as it produces many of the pathologies observed in human
TBI, such as neuronal loss, gliosis, and perturbations in metabolic and ionic homeostasis (Witgen et al., 2005; Dixon et al., 1987). With this model, injury levels can be graded, where lower magnitudes of injury produce an injury similar to a concussion, with brief neurological and physiological disruptions in the absence of remarkable structural damage, and a higher magnitude of injury produces greater neurological deficits, death, and widespread structural damage (Dixon et al., 1987). The controlled cortical impact (CCI) model is often used to study focal brain injuries. The CCI model uses a pneumatically driven impactor to induce focal TBI and causes significant cortical tissue loss at the site of impact as well as hippocampal neuronal cell loss (Dixon et al., 1991).

**PATHOPHYSIOLOGY OF TBI**

Although they are fundamentally different, focal and diffuse injuries share common mechanisms in the pathophysiology of TBI (McAllister, 2011). Primary injury results from immediate disruption of brain tissues following mechanical impact. Primary injuries include focal injuries, diffuse injuries, penetrating injuries, and fractures (Davis, 2000). As a result, complex cellular and molecular cascades constitute the secondary injury processes that are initiated and progress over a duration of hours to days after the primary injury. A secondary injury cascade follows and causes the additional pathophysiology in the brain, significantly contributing to poor prognosis following TBI (Ray et al., 2002). These secondary responses includes changes in cerebral blood flow, local and systemic inflammation, oxygen free radical generation, ischemia, edema, increased intracranial pressure (ICP), apoptosis of neural cells, and excitotoxicity.
The inflammatory cascade that is induced via the secondary response can be both beneficial or detrimental, as it is a necessary part of healing yet harmful if exaggerated and uncontrolled (Dutton and McCunn, 2003). Repair responses may also occur following TBI in an attempt to promote functional recovery and regeneration of damaged neurons (Ray et al., 2002).

INFLAMMATORY RESPONSE TO TBI

Inflammation is a stereotypical reaction to tissue damage and has been extensively reported in both experimental and clinical TBI. Cerebral inflammation in response to trauma is a complex and multifactorial process characterized by glial activation, leukocyte recruitment, and upregulation and secretion of cytokines and chemokines (Ziebell and Morganti-Kossmann, 2010; Cederberg and Siesjö, 2009). Post-traumatic inflammation had previously been thought to be deleterious for the injured brain. However, recent studies suggest that inflammation has both beneficial and detrimental roles, but the mechanisms underlying this dichotomy are largely unknown (Ziebell and Morganti-Kossmann, 2010; Cederberg and Siesjö, 2009). In order to achieve the benefits, the inflammatory response must be controlled and persist for a defined period of time. However, sustained, excessive, or inappropriate inflammation is the cause of numerous neuropathologies, including multiple sclerosis, Alzheimer’s disease, and Parkinson’s disease (Ziebell and Morganti-Kossmann, 2010; Lucas et al., 2006). Since the post-traumatic inflammatory response occurs after the initial insult and partially parallels the development of secondary tissue damage, there has been much
focus on the possibility of inhibiting particular components of inflammation to reduce secondary damage (Cederberg and Siesjö, 2009).

The inflammatory cascade induced by brain trauma is mediated by the release of pro- and anti-inflammatory cytokines, which are rapidly upregulated in response to pathological or stressful situations yet barely detectable in healthy tissue (Ziebell and Morganti-Kossmann, 2010; Lucas et al., 2006). The role of neuroinflammation in the pathophysiology of TBI is controversial due to the dual role of cytokines in various neuropathologies, as they can contribute to tissue repair as well as tissue destruction (Morganti-Kossmann et al., 2001). Pro-inflammatory cytokines include interleukin (IL)-1α and IL-1β, tumor necrosis factor (TNF)-α, interferon-γ, and IL-6, and are thought to initiate inflammation via upregulation of adhesion molecules, release of chemokines, activation of inflammatory cells, and production of nitric oxide (NO) and reactive oxygen species (ROS) (Lucas et al., 2006). In contrast, anti-inflammatory cytokines, such as IL-10, IL-4, IL-13, and transforming growth factor (TGF)-β, function to downregulate and counteract inflammatory and cytotoxic events (Cederberg and Siesjö, 2009). Chemokines, or chemotactic factors, have the ability to attract inflammatory cells to the injured site (Morganti-Kossmann et al., 2001).

Post-traumatic edema formation is a secondary consequence of TBI and results in the breakdown of the blood-brain barrier (BBB) and subsequent cytotoxic events and vascular leakage (Ray et al., 2002; Lenzlinger et al., 2001). The impairment of the BBB in the acute post-traumatic period allows the entry of circulating neutrophils, monocytes, and lymphocytes to the site of injury (Ziebell and Morganti-Kossmann, 2010; Lucas et
al., 2006). The disruption of the BBB and subsequent accumulation of blood-borne immune cells within the parenchyma has been observed in a variety of experimental TBI models as well as in human TBI (Ziebell and Morganti-Kossmann, 2010; Lenzlinger et al., 2001). These activated cells release an array of substances that are highly toxic to cells including prostaglandins, free radicals, and pro-inflammatory cytokines, which then induce the expression of chemokines and cell adhesion molecules and mobilize immune and glial cells to the injured site (Ziebell and Morganti-Kossmann, 2010; Cederberg and Siesjö, 2009). Eventually, production of anti-inflammatory mediators suppresses humoral and cellular immune activation (Ziebell and Morganti-Kossmann, 2010). With time, repair mechanisms seal the sites of BBB leakage. However, a period of time exists in which the endothelium in the damaged region remains permeable to small molecules, which results in sustainment of an altered homeostasis of the brain parenchyma and continued impairment of neuronal function. This prolonged permeability allows for a window of opportunity in which drugs can reach the region of injured brain tissue when they would otherwise be excluded from the CNS (Ziebell and Morganti-Kossmann, 2010).

Neuronal dysfunction or death after injury or disease ultimately results in the loss of CNS functions (Raghupathi, 2004). As the primary responders to CNS insults, glial cells may play a vital role in the determination of clinical outcome (Sofroniew, 2005). The activation of microglia is one of the central inflammatory responses to brain injury. Microglia represent 10-20% of the total cell population in the adult CNS and function as the primary mediators of the brain’s immune response to infection, injury, and disease (Loane and Byrnes, 2010). Resident microglia in the CNS have an innate inflammatory
capacity and play several roles in the injured brain (Cederberg and Siesjö, 2009; Ziebell and Morganti-Kossmann, 2010). Whether the microglial response to injury is beneficial or detrimental is under debate, with strong evidence of a dual role and differential activation (Loane and Byrnes, 2010; Hailer, 2008). However, it is important to note that microglial responses vary depending on the severity of injury and injury model (Loane and Byrnes, 2010).

The chief functions of microglia are to constantly survey their microenvironment and detect any noxious agents or injurious processes. They clear toxic substances and cellular debris after various kinds of tissue destruction by phagocytosis in order to maintain cellular homeostasis in the CNS (Loane and Byrnes, 2010; Hailer, 2008). In the CNS, resident microglia react to neuronal injury and BBB disruption caused by the initial insult within minutes and can become chronically activated (Ramlackhansingh et al., 2011). Chronic microglial activation persists weeks and months after the initial brain injury (Loane and Byrnes, 2010). Microglia undergo morphological and behavioral changes in response to injury. When activated, microglia contract their processes and take on an ameboid morphology similar to that of peripheral macrophages, and then proliferate and migrate toward the injured tissue (Loane and Byrnes, 2010; Hailer, 2008). With this transformation, microglia become indistinguishable from blood-borne macrophages and function as antigen-presenting cells (Ramlackhansingh et al., 2011). Activated microglia secrete cytokines and trophic factors that can exert protective or harmful effects on neighboring cells (Lucas et al., 2006).

The view that microglia have a detrimental effect in the CNS following TBI is supported by their release of vast numbers of cytotoxic chemicals and their association
with neuronal cell death (Loane and Byrnes, 2010; Hailer, 2008). Numerous studies have reported that microglia release pro-inflammatory mediators, including pro-inflammatory cytokines, chemokines, NO, and superoxide free radicals that produce ROS and reactive nitrogen species (RNS) after brain trauma. These neurotoxic substances contribute to neuronal dysfunction and cell death following injury (Hailer, 2008). Although short-term microglial activation is not thought to be detrimental, chronic microglial activation is considered to be the most damaging response of microglia to injury. Associations between damaged neurons and uncontrolled, hyperactivated microglia can result in a self-propagating positive feed-forward loop that leads to dysregulated and prolonged microglial activation and neuronal cell death, thus driving the chronic progression of neurodegeneration (Loane and Byrnes, 2010). In contrast, short-term microglial activation may play a beneficial role in response to injury. Since microglia phagocytose cellular debris and maintain cellular homeostasis, they function to protect and preserve healthy tissue. Additionally, in response to injury microglia release neuroprotective agents that promote neuronal survival or regeneration such as anti-inflammatory cytokines and neurotrophic factors, including nerve growth factor (NGF), TGF-β, IL-10, and IL-1 receptor antagonist (IL-1ra) (Hailer, 2008).

Astrocytes, the predominant cell type in the human brain, may also influence neuronal survival and functional outcome after TBI (Laird et al., 2008). In the uninjured brain, astrocytes carry out several duties that are essential for neuronal function and survival, including homeostatic maintenance of extracellular ionic environment and pH, clearance of extracellular glutamate, production of pro- and anti-inflammatory cytokines and chemokines, production of growth factors, and free radical release and scavenging
(Myer et al., 2006; Sofroniew, 2005). Astrocytes become “reactive” in response to all 
CNS insults (Sofroniew, 2005). It is not known how reactive astrocytes affect the 
progression of the injury response or overall outcome after TBI (Myer et al., 2006). As 
with microglia, both beneficial and detrimental effects have been associated with 
reactive astrocytes. Recent studies suggest that astrocytes may limit brain injury via 
their role in regulation of homeostasis. However, other work indicates that reactive 
astrocytes may contribute to increased neuroinflammation, the development of cerebral 
edema, and increased ICP, thereby exacerbating secondary injury following TBI (Laird 
et al., 2008). For example, a protective role may be provided by free radical scavenging 
or neurotrophin release, while a harmful effect may be caused by free radical release or 
pro-inflammatory cytokine production (Myer et al., 2006).

With edema formation and swelling, activation of resident immune cells, and 
cerebral infiltration by immunocompetent cells, the post-traumatic brain shows the 
hallmark signs of inflammation (Lenzlinger et al., 2001). The previous belief that 
inflammation in TBI exacerbates cell death has been challenged by numerous reports 
demonstrating that inhibition of the inflammatory response can actually increase cell 
death and secondary tissue damage. It now appears that the most crucial element of 
anti-inflammatory intervention is the timing of its administration. While pro-inflammatory 
cytokines such as IL-1, IL-6, and TNF-α can all induce secondary tissue damage initially 
after TBI, they may well do the opposite at later stages. Although no clear mechanism 
for this has been presented, studies from non-CNS tissue damage indicate that the 
initial inflammatory response induces anti-inflammatory and wound healing factors such 
as IL-4, TGF-β, and IL-10 at later stages (Cederberg and Siesjö, 2009).
The scientific community had previously thought that recovery from TBI was severely limited due to the inability of the adult brain to replenish damaged neurons (Ming and Song, 2005). However, several studies in the last decade indicate that the mammalian CNS does have the potential to replace damaged neurons via neural stem/progenitor cell (NSC/NPC) proliferation (Chirumamilla et al., 2002; Rice et al., 2003). These stem and progenitor cells are present in both the developing mammalian nervous system and in the adult nervous system (Song et al., 2002). NSC/NPCs produce neurons and glial cells well into childhood and maintain cell replacement and addition throughout adulthood, albeit to a lesser extent (Emery et al., 2005; Dash et al., 2001; Jin et al., 2001). Glial progenitor cells are abundant and allow for continuous production of glia throughout the brain (Carpentier and Palmer, 2009). However, production of new neurons is restricted to two regions in the adult brain: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Lois and Alvarez-Buylla, 1993). NSC/NPCs in the SVZ migrate down the rostral migratory pathway and replace olfactory neurons in the olfactory bulb (Rice et al., 2003). In the SGZ, these cells migrate into the hippocampal dentate gyrus (DG) and differentiate into granular cell layer (GCL) neurons that are involved in networks that modulate mood as well as short-term learning and memory (Carpentier and Palmer, 2009).

In the normal hippocampus, new neurons are continually generated and migrate laterally from the hilus and SGZ into the GCL and eventually assume the nuclear and cytoplasmic morphology of surrounding granule neurons (Emery et al., 2005; Cameron...
et al., 1993). Although many of these cells undergo apoptosis, those that survive can integrate into the hippocampal circuitry by extending their axonal projections into the CA3 region, the normal granule cell target area (Sun et al., 2007; Cameron and McKay, 2001). It has been reported that newly generated neurons in the SGZ and mature granule neurons share the same neurophysiological characteristics, even when they are born after an ischemic insult (van Praag et al., 2002; Song et al., 2002).

The hippocampus, a region responsible for memory and learning, is particularly vulnerable to insults (Dash et al., 2001). Learning, memory, and cognitive deficits are the hallmarks of brain trauma (Witgen et al., 2005). These deficits are observed early on as a result of mild to moderate TBI, but spontaneously recover with time (Schmidt et al., 1999). This limited capacity of the brain to restore itself naturally after injury suggests that innate repair and regeneration mechanisms exist within the brain (Sun et al., 2005). Aside from activation of microglia and astrocytes and the proliferation of cells at the site of injury, the adult CNS has an additional response to injury that may have functional significance and serve as an endogenous source for repair of the injured brain (Chirumamilla et al., 2002; Rice et al., 2003).

Previous studies from our laboratory and others have shown a significant increase in cell proliferation in the hippocampal DG in both injured juvenile and adult rats after LFPI (Sun et al., 2005; Rice et al., 2003; Chirumamilla et al., 2002; Dash et al., 2001). In particular, a study in our laboratory demonstrated that TBI generated a four-fold increase in cell proliferation in the SGZ in injured juvenile rats and a three-fold increase in injured adult rats compared to age-matched shams, with TBI-induced proliferation reaching a peak at 2 days post-injury before returning to basal levels by 14
days post injury (Sun et al., 2005) (Figure 1-1, Sun, 2007). In the CCI model, increased proliferation has also been observed in the DG that peaks at 3 days post-injury and stays elevated at 60 days post-injury (Rice et al., 2003).

Several studies have been conducted to determine the composition of the proliferative response in the neurogenic region of the DG in the juvenile and adult brains. Results from our laboratory have shown that the percentage of newly generated cells that differentiate into neurons in the SGZ was approximately two times higher in juveniles than in adults at 7, 14, and 28 days post-injury. In contrast, significantly more cells express astrocytic marker in adults compared to juveniles post-injury (Sun et al., 2005). Our lab later examined the maturational fate of these newly generated cells 10 weeks after TBI and reported that the majority of the new cells that persisted in the DG for this period of time differentiated into mature neurons (Figure 1-2). All of these cells also exhibited positive immunostaining for calbindin, a protein present in all granule neurons in the DG. Additionally, some of these surviving granule neurons have been reported to extend axonal projections to the CA3 region as early as 14 days post-TBI (Emery et al., 2005). Furthermore, these cells establish synaptic connections with existing cells within the hippocampus, as determined with synaptophysin immunoflorescent staining (Figure 1-3; Sun et al., 2007).
Figure 1-1. Cell proliferation in the ipsilateral dentate gyrus in juvenile and adult rats following TBI. Coronal sections taken from injured juvenile (a) and adult (b) rats 2 days post-injury showing an elevated level of cell proliferation as labeled with BrdU in the DG of injured juvenile and adult rats. Arrows indicated BrdU-positive cells are clustered and concentrated in the subgranular zone (SGZ) of the DG. (c) Quantification of BrdU-positive cells in the SGZ. Graph shows that cell proliferation was significantly enhanced in injured juvenile and adult rats at both 2 and 7 days post injury as compared to age-matched sham controls (* p<0.05, ** p<0.01). The total number of proliferating cells in injured juveniles was significantly higher than injured adult rats at 2 days post injury (*p<0.05, n = 4/group). (d) Nissl stained sections cross the hippocampal dentate gyrus with red box showing the corresponding area in (a) and (b) (Sun et al., 2005). In addition, our lab has demonstrated that new granule neurons generated following TBI can establish the correct anatomical connections to the CA3 region (Fig. 1.3). To further illustrate that these new neurons integrate into the existing neuronal circuitry, using synaptic vesicle marker synaptophysin we have shown that newly generated granule neurons form synapses with existing hippocampal neurons (Fig. 1.4; Sun et al., 2007).
Figure 1-2. Newly generated cells display markers for mature granule cells. (a) Confocal micrograph showing newly generated BrdU+ cells within the granule cell layer (green) at ten weeks post injury. In (b) the same cells are labeled with the mature neuronal marker, NeuN (blue). (c) Granule neurons retrograde incorporated with FG (red). (d) Merged image of A, B and C. Arrow points to one of the newly generated cells labeled with BrdU, NeuN and FG while the arrow head points to a cell labeled with BrdU and NeuN only. Scale bar: 30μm. (e-h) Confocal photomicrograph showing newly generated cells express the granule cell marker, calbindin. The arrow indicates a BrdU-labeled cell (green, E) which expressed calbindin (blue, f) and was retrogradely incorporated with FG (red, G). (H) Merged image of E, F, and G. Scale bar: 30μm.
Figure 1-3. Newly generated granule neurons are retrogradely labeled with FG and express synaptophysin. Confocal micrograph throughout the z-axis showing (a) a newly generated BrdU+ cell (green) that is retrogradely incorporated with FG (red) at ten weeks after its generation. (b) The same BrdU-labeled cell is surrounded by a latticework of synaptophysin staining (blue). (c) Merged image of (a) and (b). Scale bar: 30µm.
FUNCTION OF HIPPOCAMPAL NEUROGENESIS

The function of newly generated neurons in the adult brain still remains unclear, but recent evidence indicates that hippocampal neurogenesis has a critical functional role in the normal adult brain, particularly in learning, long-term spatial memory, and mood (Rao et al., 2006). It has been demonstrated that the mammalian hippocampus regulates adult neurogenesis in an activity-dependent manner (Gould et al., 1999). Additionally, increasing evidence has shown that neurogenesis in the adult hippocampus may contribute to learning and memory functions (Sun et al., 2007). For example, it has been demonstrated that living in an enriched environment induces a robust increase in adult neurogenesis and enhanced survival of new neurons, and this neurogenesis was paralleled by improved performance on a hippocampal-dependent learning task. (Kempermann, 2002; van Praag, 1999). Evidence shows that physical activity also increases adult hippocampal neurogenesis and is associated with improved cognitive function and enhancement in spatial learning and long-term potentiation (van Praag, 1999). Shors et al (2001) demonstrated that performance in a hippocampal-dependent learning task was dependent on the replication of NSCs, indicating that adult hippocampal neurogenesis has a role in learning and memory (Shors et al., 2001). It appears that the association between neurogenesis in the adult hippocampus and learning may be bidirectional, as one study showed that training in hippocampal-dependent learning tasks enhanced the number of new granule cells and rescued new granule cells from death (Kempermann, 2002; Gould et al., 2000). In contrast, significant hippocampal cell loss after brain trauma has been associated with deficits in memory function that are directly related to injury severity (Smith et al., 1991). Additionally, diminished hippocampal neurogenesis, as observed following the
administration of anti-mitotic drugs, irradiation or genetic ablation, was associated with worse performance on hippocampus-dependent trace eyeblink conditioning (Shors et al., 2001), contextual fear conditioning (Shors et al., 2002) and long term spatial memory function tests (Rola et al., 2004; Jessberger et al., 2009). Collectively, these studies support a strong association between normal adult hippocampal neurogenesis and hippocampal function.

Despite the connection between neurogenesis and cognitive function, the underlying mechanisms contributing to endogenous repair and regeneration following TBI are not fully understood. In the injured brain, increased levels of proliferation of granule cell precursors may be initiated by the death of mature hippocampal granule neurons (Jin et al., 2001). Neuronal loss and dysfunction are thought to be the underlying causes of behavior and cognitive deficits observed following TBI. Both necrotic and apoptotic cell loss have been reported in the CA1, CA3, hilus, and dentate gyrus regions of the hippocampus. (Dash et al., 2001). However, in both clinical and experimental TBI studies, hippocampal-dependent learning and memory deficits often diminish over time after injury (Sun et al., 2007). Together, these observations suggest that innate mechanisms for repair and regeneration exist within the brain. The existence of NSC/NPCs in the adult hippocampal DG throughout life and their enhanced levels of proliferation in response to brain injury has led our lab to hypothesize that neurogenesis may contribute to cognitive recovery after TBI. Furthermore, our laboratory has observed that the time frame in which injury-induced newly generated cells differentiate into mature granule neurons and integrate into existing hippocampal circuitry is
coincident with the time course of spontaneous cognitive recovery as determined by Morris Water Maze data following injury (Figure 1-4, Sun et al., 2007).
Figure 1-4. Cognitive deficits following TBI recover over time. Graph comparing Morris water maze performance of injured rats to sham animals during trials at days 11-15, 26-30 or days 56-60 following injury. Injured animals displayed significant cognitive deficits, as characterized by a longer latency, at 11-15 days post injury when compared to sham animals (*p<0.05). These deficits persisted at 26-30 days (*p<0.05). At days 56-60, injured animals showed cognitive recovery with a shorter latency which was not significantly different to sham animals (Sun et al., 2007).
Age is one of the most reliable and important predictors of morbidity and mortality following TBI (Senathi-Raja et al., 2010). It is well known that juveniles exhibit greater functional recovery than adult patients following TBI. Himanen et al. (2006) found that higher age at injury (especially over 60 years of age) was a significant risk factor for cognitive decline, while younger age at injury was associated with cognitive improvement in a 30-year follow-up study (Himanen et al., 2006). After the age of 12, there is a progressive increase in mortality and long-term morbidity after injury. For example, elderly patients are twice as likely as younger patients to remain severely disabled or vegetative after moderate TBI (Hamm et al., 1992). The age-associated increases in morbidity and mortality following TBI can be observed in rodents. Hamm et al. (1992) has shown that aged rats (20 months) were more than twice as likely to die after fluid percussion TBI than young adult rats (3 months). It was also shown that when young adult and aged rats are subjected to injury of the same severity, the aged animals experience more severe impairment of both motor and cognitive function as assessed by beam-walking and MWM performance. These greater cognitive and motor deficits observed in aged rats are consistent with human clinical TBI (Hamm et al., 1992).

The exact mechanisms underlying this age-related increase in vulnerability to TBI are unknown. In the normal brain, there is a progressive decline in function with increasing age (Lazarov et al., 2010). The normal aging hippocampus undergoes many degenerative changes both morphologically and functionally that may contribute to memory and learning deficits associated with older populations (Kuhn et al., 1996;
Driscoll et al., 2006). Importantly, granule cells in the DG continue to be generated throughout adulthood, but there is an 80% decline in NSC proliferation and neurogenesis in the normal aged brain across several species (Seki and Arai, 1995; Olariu et al., 2007; Galvan and Jin, 2007; Kuhn et al., 1996; Rao et al., 2006). However, despite the reduced hippocampal neurogenesis associated with aging, the neurons that are newly generated appear functionally equivalent to those in the young brain, suggesting that neurogenesis in the aged brain is downregulated as opposed to aberrant (Lazarov et al., 2010).

Advancing age is accompanied by a decrease in the volume of the hippocampal formation thought to be due to shrinking neuronal size, reduced neuronal and synaptic density, decreased plasticity, and decreasing neurotransmitter levels (Senathi-Raja et al., 2010; Driscoll et al., 2006; Brazel and Rao, 2004). Additionally, it has been observed that aged rats with spatial memory impairments exhibit lower levels of dentate gyrus neurogenesis than aged rats with preserved spatial memory (Rao et al., 2006). Thus, reduced hippocampal neurogenesis appears to be related to both structural and functional deficits of the hippocampus associated with aging (Driscoll et al., 2006; Lazarov et al., 2010). Cellular changes in the aged brain such as increased oxidative stress and damage to DNA and proteins may also contribute to cognitive decline (Brazel and Rao, 2004).

It has been suggested that the brain may use the same mechanisms for adaptation to aging that it does for recovery, and thus has a finite capacity for plasticity. It appears that collective effects of TBI and aging may have a synergistic adverse effect on cognitive outcome following injury, which may be in part due to the limited capacity of
the aged brain to compensate during initial recovery (Senathi-Raja et al., 2010). The aged brain demonstrates gene expression profiles representative of microglial activation and neuroinflammation. Additionally, there is evidence that microglia in aged animals exhibit an amplified inflammatory response when stimulated (Cekic and Stein, 2010; Loane and Byrnes, 2010). Our laboratory conducted a study to measure the glial response in injured young adult and aged brains at 28 days post-injury. The 20 month old injured rat had slightly higher levels of GFAP, and significantly higher levels of ED1 and Iba1 than the 3 month old injured rat, suggesting that the aged animals have an increased inflammatory response relative to the younger rats (Figure 1-5; unpublished data from Sun, 2011). The neurological dysfunction observed in aged animals may be in part due to this neuroinflammatory priming. We posit that enhanced microglial sensitivity to immune activation does not resolve in the environment within the aged brain, which may exacerbate secondary injury resulting in both a severe immune response as well as immune factor release from damaged cells within the brain (Cekic and Stein, 2010).
Figure 1-5. Level of glial response in the injured brain at 28 days post-injury. (a) and (b) In the ipsilateral GCL and hilus, the injured 20M old rat had slightly higher percentage of GFAP expression than the 3M old rat, but not statistically significant. (c) and (d) The injured 20M old had a significantly higher percentage of ED1 expression in the ipsilateral GCL and hilus regions than the 3M old counterpart. (e) and (f) The injured 20M old had significantly higher expression of Iba1 in both the GCL and hilus regions than the injured 3M old (Sun et al., unpublished data).
NEUROINFLAMMATION AND NEUROGENESIS

The inflammatory response to injury can produce both detrimental and beneficial conditions for neurogenesis, as it imposes varying effects on neural progenitor cell (NPC) proliferation, migration, differentiation, survival, and incorporation of newly born neurons into the existing hippocampal circuitry (Sierra et al., 2010; Mathieu et al., 2010). The profile of inflammatory mediators depends on the severity of inflammation, resulting in varying consequences on neurogenesis. CNS inflammation ranges from mild acute to uncontrolled chronic inflammation (Lucas et al., 2006; Whitney et al., 2009). During mild acute inflammation, inflammatory mediators tend to promote neurogenesis, but uncontrolled inflammation produces an environment that opposes neurogenesis (Sierra et al., 2010; Pluchino et al., 2008). Additionally, the pro- and anti-neurogenic effects of inflammation may also depend on the type and duration of microglial and/or astrocytic activation (Das and Basu, 2008). An understanding of the potential effects of inflammation on neurogenesis may potentially help identify therapeutic strategies for treatment of neuronal injury (Whitney et al., 2009).

Independent studies have shown that microglial cell activation induced by an LPS challenge in the brain is associated with a reduction in hippocampal neurogenesis (Jakubs et al., 2008; Ekdahl, 2012). Additionally, hippocampal neurogenesis can be restored by anti-inflammatory drug administration. These studies reported a negative association between the number of activated microglia and the number of newly generated neurons (Mathieu et al., 2010). Pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α are released by activated microglia and are thought to mediate the relationship between microglia and neurogenesis. Microglial activation may also
negatively regulate different steps of neurogenesis via release of prostaglandins (Ekdahl, 2012).

TNF-α is primarily released by activated microglia and plays a role in several neurodegenerative disorders. Based on the model and methods of injury, TNF-α can display both pro- and anti-neurogenic effects. Lipopolysaccharide (LPS)-activated microglia have been reported to inhibit neuronal differentiation via TNF-α expression. When a TNF-α inhibitor was administered, neuronal differentiation was partially restored, indicating that it has an anti-neurogenic effect when combined with LPS-conditioned media. Furthermore, other data suggests that TNF-α released from microglia may contribute to death of hippocampal NPCs after injury (Whitney et al., 2009). Activated microglia may also suppress neurogenesis via production of IL-6. One study reported that chronic production of IL-6 in a transgenic mouse model resulted in dramatically reduced hippocampal neurogenesis via reduction of NPC proliferation, survival, and differentiation (Whitney et al., 2009). Mathieu et al. studied the effects of chronic expression of IL-1β and observed a 50% reduction in the percentage of injury-induced proliferating cells that differentiated into neurons (Mathieu et al., 2010).

In contrast, the anti-inflammatory cascade is thought to promote neurogenesis, as administration of anti-inflammatory cytokine TGF-β has been reported to support neurogenesis (Ekdahl, 2012). However, in the aged brain, chronically activated microglia induce a prolonged and amplified neuroinflammatory response (Cekic and Stein, 2010). Ongoing inflammation in the CNS is associated with many neurodegenerative disorders, and is known to play a role in neuronal death observed in Alzheimer’s and Parkinson’s diseases (Ekdahl et al., 2003). Further, studies support
that persistent inflammation may contribute to the memory loss in these disorders by disrupting formation of new neurons (Das and Basu, 2008). The exaggerated neuroinflammation in the aged brain results in reduced plasticity during recovery, continued release of oxidative stressors, and enhanced production of inflammatory cytokines such as TNFα, IL-1β, and IL-6. In situations of chronic microglial activation, these cytokines disrupt long-term potentiation, memory consolidation, neurite outgrowth, and hippocampal neurogenesis (Cekic and Stein, 2010).

As discussed in the previous section, following TBI, there is a full range of inflammatory cell responses including infiltration of macrophages and activation of microglia and astrocytes accompanied with production of inflammatory cytokines. Our laboratory has recently observed that following TBI, the aged brain retains cell proliferative capacity; however, this injury-induced proliferative response predominantly generates microglial cells. Moreover, new neurons generated in the aged brain failed to survive for an extended period. These findings lead us to explore the hypotheses that heightened levels of injury-induced inflammation in the aged brain may play a pivotal role influencing neurogenesis and cognitive function, and that inhibition of this injury-induced inflammatory response may restore hippocampal neurogenic response and ultimately improve cognitive function of the injured aged animals.
MINOCYCLINE AS A POTENTIAL THERAPY

Inflammation is a key component in the pathological environment following TBI, and several components of this response have been identified as targets for therapeutic intervention (Helmy et al., 2011). Potential inflammatory targets have included microglial activation, leukocyte extravasation via adhesion molecules or matrix metalloproteinases (MMPs), IL-1, TNF-α, and NO, among several others (Lucas et al., 2006). Minocycline, a tetracycline antibiotic, has multiple targets and exhibits anti-inflammatory and neuroprotective properties that have been demonstrated in many models of brain injury (Hewlett and Corbett, 2006). Minocycline reduces cytokine expression via inhibition of microglial activation and inhibits MMP and the inducible form of nitric oxide synthase (iNOS) actions (Yrjanheikki, 1999; Lucas et al., 2006). It also downregulates caspase-1, which contributes to its anti-apoptotic properties. Minocycline has been used extensively in humans for treatment of both chronic and acute conditions, including acne, staphylococcal infections, and rheumatoid arthritis (Sanchez Mejia et al., 2001).

Minocycline is able to cross the BBB and inhibit microglial activation in neurodegenerative disease models (Ekdahl et al., 2003). Most studies that have reported neuroprotective effects of minocycline in rodents following brain injury have used doses of 10-90 mg/kg administered intraperitoneally. Minocycline reaches peak concentration in systemic circulation at 2.5 hours after intraperitoneal injection and has a half-life of 3 hours in rats (Elewa et al., 2006). In a study examining the effects of LPS-induced microglia in a status epilepticus (SE) model, administration of minocycline during a 5 week period after SE resulted in enhanced neurogenesis and a decrease in the microglial population (Ekdahl et al., 2009), but minocycline did not affect
neurogenesis in control animals (Ekdahl et al., 2003). Similarly, minocycline has been shown to reduce functional impairment resulting from cerebral focal ischemia, and this improvement is associated with decreased microglial activation and enhanced hippocampal neurogenesis in addition to an improved neural environment after chronic treatment (Liu et al., 2007). In another study, intraperitoneal administration of minocycline 12 hours before or 30 minutes after TBI resulted in improved neurological function compared to controls injected with saline (Sanchez Mejia et al., 2001).

Collectively, these studies suggest that modulation of microglia activation by minocycline can enhance hippocampal neurogenesis. In this study, we will explore the efficacy of anti-inflammatory treatment with minocycline as a potential therapy to restore hippocampal neurogenesis and cognitive function following TBI. We administered minocycline to both young adult and aged rats to begin to explore whether inflammation-mediated reduction of hippocampal neurogenesis can be reversed by inhibition of microglial activation, especially in the aged brain where microglia exhibit an amplified response.
Male Fisher 344 rats aged at 3 months and 20 months (NIA) were used for this study. The 3 month-old rats weighed approximately 300 grams and were housed in pairs. The 20 month-old rats weighed approximately 450 grams and were housed individually. Both groups were housed in a facility that maintained a temperature of 20-22 degrees Celsius with a 12-hour light/dark cycle and with food and water available ad libitum. Of the twenty-four young rats employed for this study, 16 were used to examine minocycline treatment on injury-induced cell proliferation and generation of new neurons at the early time post-injury. Of the seventeen aged rats employed for this study, 16 were used to study these effects. The remaining rats died immediately following injury or within the first week following injury. The mortality rate for 3 month-old rats was 10% following CCI injury and 43.8% following LFPI injury. The mortality rate for 20 month-old rats was 0% following CCI injury and 30.8% following LFPI injury. All protocols and animals used for this study complied with NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Seven aged and seven young rats were subjected to a moderate lateral fluid percussion injury (LFPI), and nine aged and nine young rats were subjected to a moderate controlled cortical impact (CCI) injury following standard protocols. All tools used for the surgical procedure were sterilized in an autoclave, and an aseptic environment was prepared for the surgical procedures. The rats undergoing FPI were
anesthetized with 5% isofluorane in a Plexiglas chamber, intubated, and ventilated with 2.5% isofluorane in a gas mixture (30% oxygen, 70% nitrogen). The rats undergoing CCI injury were anesthetized with 5% isofluorane in a Plexiglas chamber, and were ventilated through a nose cone with 2.5% isofluorane in a gas mixture (30% O₂, 70% N₂) throughout the surgery. After shaving the scalp, the animal was secured in a stereotaxic frame. To disinfect the site of incision, betadine was applied to the shaved region of the head. Puralube ointment was placed in the eyes to prevent dryness during the procedure. To expose the skull, an incision was made along the midline and the surrounding connective tissue was pulled back and clamped with hemostats. A 4.9 mm craniotomy was made on the left parietal bone midway between lambda and bregma using a trephine and a Dremel drill with a small drill bit. The bone chip over the craniotomy site was detached and any remaining bone fragments along the edges of the craniotomy were carefully removed.

LATERAL FLUID PERCUSSION INJURY (L-FPI)

Once the craniotomy site was cleaned, a Luer lock fitting was placed over the craniotomy and cemented in place with a dental acrylic mixture. When the acrylic had dried, the fitting was filled with saline solution in order to ensure that it was completely sealed and to provide a continuous medium though which the injury would be applied. At this time, the rat was removed from isofluorane anesthesia until it regained consciousness as assessed by demonstration of paw and tail reflexes. The fluid percussion injury device (Figure 2-1) was calibrated by performing pressure measurements every 30 seconds until the animal regained consciousness in order to ensure that the device would produce an injury level within the target pressure range of
1.77 ± 0.05 atm. The intubation tube was disconnected, the rat was removed from the stereotaxic frame, and was then transferred and connected to the injury device via the Luer lock fitting. A moderate fluid impulse of 1.77 ± 0.05 atm was applied. Once the injury was administered, the animal was taken back to the surgery bench where the fitting was removed from the skull. The rat was reconnected to ventilation without isofluorane and placed in a supine position with gauze underneath the open incision. Paw and tail reflexes and righting time were recorded during the recovery period in order to assess the injury severity and verify that all animals received a comparable injury. The rat’s righting time is the amount of time that passed from the point of injury until the rat spontaneously returns to an upright position. Once this occurred, the animals were again placed under anesthesia with 2.5% isofluorane and the incision was sealed using 5-0 surgical suture with a sterile stainless steel suture needle. A triple antibiotic ointment and 2% lidocaine hydrochloride jelly were applied to the sutured region. A surgical drape was placed inside the cage and the rat was transferred back to the cage, which was situated on a heating pad to aid the recovery process.
**Figure 2-1. Fluid percussion injury device.** A photograph of the injury device used to deliver lateral fluid percussion injury (LFPI) to young and aged rats.
CONTROLLED CORTICAL IMPACT (CCI) INJURY

Once the craniotomy site was cleaned, the rat was removed from isofluorane anesthesia and transferred to the CCI injury device (Figure 2-2). To prepare the injury device, the 3 mm steel impactor tip was carefully adjusted to a location perpendicular to the surface of the brain just over the craniotomy so that the dura would not be pierced upon delivery of the injury. Once the rat demonstrated a tail reflex, a moderate injury corresponding to a 2.3 mm depression of the dura was delivered with a velocity of 4 m/s and a dwell time of 0.05 seconds. After the injury was delivered, the incision was sutured and the animal was allowed to recover following the same procedure as described for the FPI injury. The time to regain paw and tail reflexes and righting time were recorded as previously described, and once normal breathing was observed the animal was transferred to its cage with a surgical drape placed inside and with the cage over a heating pad. Sham animals underwent the same surgical procedure without injury delivery.
Figure 2-2. **Controlled cortical impact injury device.** A photograph of the injury device used to deliver controlled cortical impact injury (CCI) to young and aged rats.
Minocycline is a tetracycline antibiotic that exhibits anti-inflammatory and anti-apoptotic properties that have been demonstrated in many models of brain injury (Hewlett and Corbett, 2006). Minocycline (Sigma) was administered intraperitoneally (i.p.) to the 3 month-old and 20 month-old rats that underwent FPI and CCI injuries. These animals were divided into two different minocycline treatment groups, group 1 and group 2 (Table 2-1). Of the seven aged rats subjected to FPI, four were assigned to group 1 and three were assigned to group 2. Of the seven young rats subjected to FPI, three were assigned to group 1 and four were assigned to group 2. Nine young and nine aged rats received CCI injuries. In both age groups, three animals were vehicles, three were assigned to group 1, and three were assigned to group 2.

Minocycline was dissolved in sterile saline at a 15 mg/ml concentration before boiling point with neutral pH maintained. In treatment group 1, animals received a first i.p. injection of minocycline at the dose of 45 mg/kg at 30 minutes post-TBI, followed by a second dose of 45 mg/kg at 8 hours post-TBI. In treatment group 2, animals received the first 45 mg/kg dose at 4 hours post-TBI, followed by a second dose at 8 hours post-TBI. On the first and second days post-injury, both groups received minocycline i.p. injections twice daily at a dose of 22.5 mg/kg with 12 hours in between. Vehicle treatment animals received equal volume of sterile saline injection.

To label cell proliferation, 5-bromo-2'-deoxyuridine (BrdU, Sigma), a thymidine analog that can be incorporated into the newly synthesized DNA of replicating cells, was used. All animals received 4 doses of i.p. BrdU injection (50mg/kg, two hrs in between
each injection) at two days post-injury. All animals in the study were sacrificed at 3
days following TBI (24 hrs after the last BrdU/minocycline injection).
Table 2-1. Animal treatment groups in minocycline study. Table showing the number of animals in each group in the minocycline study based on injury model, age, and treatment group.
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TISSUE PROCESSING

Animals were sacrificed at 3 days post-injury. After deep anesthetization with isofluorane until complete cessation of breathing, the rats were transcardially perfused with 400 ml of 1x phosphate buffer solution (PBS) followed by 400 ml of 4% paraformaldehyde in PBS. The skulls were then opened and the brains were removed and post-fixed in 4% paraformaldehyde in PBS for a minimum of 48 hours at 4°C.

After 48 hours of post-fixation, the brains were placed in PBS. A Leica vibratome was used to slice each brain into 60 µm thick coronal sections throughout the rostro-caudal extent of the brain. Four groups of 30 serial coronal sections were collected from each brain, and the sliced tissue sections were collected into five 24-well plates filled with PBS + 0.01% sodium azide and were stored in a refrigerator kept at 4°C until needed for staining procedures.

IMMUNOHISTOCHEMISTRY

One set of 30 sections from each brain used in the study was stained for BrdU immunohistochemistry to determine the number of newly proliferating cells in the DG after injury. Another set of 30 sections from every brain used in the study was processed for doublecortin (DCX), a microtubule-associated protein expressed by immature neurons, to assess the number of newly generated neurons after injury.

BRDU IMMUNOSTAINING

Selected sections were first washed twice with PBS for 5 minutes and then denatured in 50% formamide for 1 hour at 65°C. The sections were washed with 2x saline sodium citrate (SSC) 2 times for 5 minutes, and then 2N HCl was placed on the
sections for 30 minutes at 37°C. The sections were rinsed with PBS 2 times for 5 minutes, and quenched with 3% hydrogen peroxide for 1 hour on a shaker. The sections were rinsed with PBS + 0.3% Triton100 for 10 minutes on the shaker before adding a blocking buffer consisting of 5% normal horse serum in PBS + 0.3% Triton. The sections were blocked overnight at 4°C on a shaker, and then incubated with the primary antibody solution for 48 hours at 4°C on a shaker. The primary antibody solution consisted of mouse anti-BrdU antibody (Dako, CA) at a 1:200 dilution in the blocking buffer, and 300 µl was added to each well. Following 48 hour incubation, the sections were brought back to room temperature and rinsed in PBS + 0.3% Triton100 for 10 minutes 3 times. The sections were then blocked in the blocking buffer solution for 3 hours at room temperature before they were incubated with the secondary antibody solution at 4°C overnight on a shaker. The secondary antibody solution consisted of biotinylated anti-mouse IgG (Jackson Laboratories, ME) at a 1:200 dilution in blocking buffer, and 300 µl was added to each well. After overnight incubation, the sections were brought back to room temperature and washed with 1x PBS for 10 minutes 3 times. The avidin-biotin complex (ABC kit, Vector Laboratories) was prepared 30 minutes before use at a concentration of 1:200 avidin and 1:200 biotin in 1x PBS. The sections were incubated with ABC for 2 hours at room temperature on a shaker, and then washed with 1x PBS for 10 minutes 3 times. The liquid DAB solution was prepared using a Vector DAB kit by adding 2 drops of phosphate buffer at a pH of 7.5, 4 drops of 3,3’- Diaminobenzidine, and 2 drops of hydrogen peroxide to each 5 ml of nanopure water required to incubate each section with 300 µl of DAB. The sections were incubated with the DAB solution and the reaction was monitored under a microscope until the sections
were adequately stained, and then quenched with 1x PBS to stop the reaction. The sections were washed with 1x PBS for 5 minutes 3 times and stored in a refrigerator at 4°C. The sections were washed in distilled water and mounted to glass microscope slides, with 6 sequential sections per slide.

**DCX IMMUNOSTAINING**

The sections were washed with 1x PBS for 5 minutes 2 times and then quenched with 3% hydrogen peroxide 1 hour. They were then washed with PBS + 0.3% Triton100 for 5 minutes 3 times before incubation with the blocking buffer solution (5% normal horse serum in 1x PBS + 0.3%Triton100) for 3 hours at room temperature on a shaker. The sections were then incubated with a goat anti-rat DCX antibody (Santa Cruz, CA) solution at a dilution of 1:1000 in blocking buffer for 48 hours at 4°C. After incubation, the sections were washed with 1x PBS + 0.3% Triton100 for 10 minutes 3 times. Next, they were blocked in blocking buffer for at least 3 hours at room temperature prior to overnight incubation with biotinylated anti-goat IgG (Jackson Laboratories, ME) at a dilution of 1:200 in blocking buffer at 4°C. The sections were then washed with 1x PBS + 0.3% Triton100 for 10 minutes 3 times and incubated with ABC for 2 hours at room temperature. They were then washed with PBS and incubated with DAB at room temperature, and the reaction was observed under a microscope. When adequately stained, the sections were quenched with PBS and washed 3 times with PBS. They were rinsed with distilled water and mounted on glass microscope slides, with 6 sequential sections per slide.
COUNTERSTAINING PROCEDURE

Sections mounted on the glass slides were placed in distilled water and then stained with 0.1% cresyl violet solution for 1 minute. They were washed in running tap water 3 times, or until the water ran clear, and then placed back in distilled water. The sections were then placed in ethanol in increasing concentrations (two 75% solutions, one 95% solution) for approximately 1 minute each. They were placed in an acid alcohol solution for approximately 30 seconds or until the counterstain color was appropriate, and then dehydrated in 2 containers filled with 100% ethanol for 1 minute each. They were placed in Citrisolv twice for 5 minutes each, and cover slipped with Permount.

STEREOLOGICAL QUANTIFICATION

The BrdU- and DCX-positive cells present in the DG were quantified using the Olympic Image CAST program (Olympus, Denmark). Of the 30 coronal sections obtained from each brain for each staining technique, 10 sections spanning the DG (-2.56 mm to -5 mm of the bregma) were selected for examination using unbiased stereological methods. Positively labeled cells in the ipsilateral hilus, subgranular zone (SGZ), and granule cell layer (GCL) were counted in each of these sections. For the BrdU quantification, the SGZ and GCL were counted together as the GCL. For the DCX quantification, the SGZ and the hilus were counted together as the hilus. Under 4x objective, the GCL and hilus were outlined and the cells within each region of interest were counted under 40x objective focusing through the thickness of each section. In the DCX stereological procedure, all neurons were categorized based on the vertical, horizontal, or mixed orientation of their dendrites. The average thickness of the DG of each brain was determined by measuring the depth of each section from one focal
plane to another in 5 randomly selected locations in the DG, and then averaging the thickness of all 10 sections to calculate the average thickness (t) of each brain. The entire region of interest in each section was examined for stereological quantification instead of random samples, so the average sampling fraction (asf) was equal to 1. The dissector height (h) was set to 15 µm. Using these parameters, the total number of cells in each DG was estimated as \( N=(\Sigma Q)(t/h)(1/asf)(1/ssf) \), where ssf was the section-sampling fraction (ssf = 0.25), and \( \Sigma Q \) was the raw number of cells counted.

STATISTICAL ANALYSIS

Analysis of the righting times for animals following surgery was performed using post-hoc student t-tests (only one-tailed t-tests were considered) to determine any statistically significant differences within groups. Statistical analysis of cell proliferation and neurogenesis data was performed using one-way ANOVA followed by post-hoc LSD tests to determine differences in BrdU and DCX quantification data within treatment groups. P-values less than 0.05 were considered statistically significant. No two-way ANOVA analysis was performed.
Numerous studies have suggested that inflammation plays a critical role in modulating neurogenesis, and that the increased inflammatory response in the aged brain may contribute to decreased neurogenesis and poorer cognitive recovery following TBI. This study aimed to determine the effects of anti-inflammatory treatment with minocycline on cell proliferation and neurogenesis in 3-month old young rats and 20-month old aged rats in the acute post-traumatic period. Two different minocycline treatment groups were employed, with one group receiving a first minocycline dose at 30 minutes post-TBI (treatment group 1) and the other group receiving a first dose at 4 hours post-TBI (treatment group 2). Additionally, the effects of minocycline administered in these two time courses in young and aged rats were examined in two most widely used TBI models, namely, LFPI and CCI injury models. Micrographs showing the location of the LFPI and CCI injuries relative to the hippocampus are shown in Figure 3-1. Fisher 344 rats were used for this study because it is the only strain that the aged rats are available. A 3-month old Fisher 344 rat represents a fully mature, young adult, and a 20-month old Fisher 344 rat represents a late middle-aged animal that is comparable to a human approximately 50 years old (Hamm et al., 1992).

To assess whether anti-inflammation treatment with minocycline affects injury-induced cell proliferation in the DG after LFPI and CCI, animals were injected with BrdU four times 2 hours apart at 2 days post injury and sacrificed 3 days post injury. Ten sections from each brain were stained for BrdU to label newly proliferating cells. These cells are typically observed in the hilus or SGZ, which are the proliferating zones of the hippocampal DG, and fewer newly proliferating cells are observed in the GCL. For this
reason, BrdU-positive cells in the GCL and SGZ were taken together as the newly proliferating cells in the GZ, and were counted separately from BrdU-positive cells in the hilus. To assess whether anti-inflammation treatment with minocycline can enhance hippocampal neurogenesis in the young and aged brains following LFPI and CCI injury, ten sections from each brain of animals sacrificed 3 days post-injury were stained for early neuronal marker DCX. In all animals, DCX-positive cells were primarily localized in the SGZ of the DG, with some additional DCX-positive cells located in the GCL. In this study, DCX-positive neurons in the GCL, SGZ, and hilus were all counted together as newly generated neurons in the DG. Newly generated DCX-positive neurons were classified according to three types of dendrite morphologies: vertically oriented dendrites (Figure 3-2a), horizontally oriented dendrites (Figure 3-2b), and mixed dendrites (Figure 3-2c). Neurons with horizontal and mixed dendrites were taken to be newly generated cells after injury, while those with vertical dendrites were taken to be neurons that were generated prior to injury. Expression of DCX in new neurons lasts about 2 weeks.
Figure 3-1. Location of LFPI and CCI injury. Micrographs taken from a) 3M sham animal, b) 20M sham animal, c) 3M vehicle-treated animal following CCI injury, d) 20M vehicle-treated animal following CCI injury, e) 3M vehicle-treated animal following LFPI injury, and f) 20M vehicle-treated animal following LFPI injury. These images show the location of the injuries relative to the hippocampal region.
Figure 3-2. Characterization of DCX+ newly generated neurons. Three types of DCX+ cells are observed in the GCL: a) cell with vertically orientated soma and dendrites, b) cells with horizontally orientated soma and dendrites, c) cell with both horizontal and vertical dendrites (mixed dendrites). Confocal micrograph shows BrdU/DCX double-labeled cells which are newly generated DCX+ neurons at 2 days post-injury do not have vertical dendrites (Sun, unpublished data).
STUDY 1: EFFECT OF MINOCYCLINE TREATMENT IN LFPI MODEL

MORTALITY RATE AND RIGHTING TIME FOLLOWING LFPI

To assess the effect of minocycline on cell proliferation and neurogenesis after LFPI a total of 14 young rats and 13 aged rats were used. The mortality rate in young rats was 43.8% and in aged rats was 30.8%. These values are similar to those that have been reported in other studies for young adult rats, but lower than those typically reported for aged rats (Reid et al., 2010; Hamm et al., 1991). Due to the high sensitivity of Fisher 344 rats to LFPI injury, the target injury level was slightly lowered in order to increase survival rates during the study. The righting response, which is the time required for an injured animal placed on its back to spontaneously return to an upright position, was recorded immediately after injury as a measure of injury severity (Hamm, 2001). The mean (±SEM) righting time for young rats after LFPI was 12 ± 2.08 minutes for minocycline-treated animals and 10.13 ± 1.14 minutes for vehicle-treated animals. Analysis of this data with a t-test indicated that there was no significant difference between the righting times of the vehicles compared to the rats treated with minocycline, indicating that a similar severity of injury was received by both groups (p = 0.95). The mean (±SEM) righting time for aged rats after LFPI was 8.89 ± 0.93 minutes for minocycline-treated animals and 13.19 ± 2.17 minutes for vehicle-treated animals. This difference was also not statistically significant, indicating that all animals received a comparable injury (p = 0.07).
THE EFFECT OF MINOCYCLINE ON CELL PROLIFERATION IN YOUNG ADULT AND AGED RATS FOLLOWING LFPI

In 3 month-old sham animals, very few BrdU-positive cells were observed in the DG, but the injured vehicle animals showed a greatly enhanced number of BrdU-positive cells (Figure 3-3). Animals treated with minocycline both 30 minutes and 4 hours post-TBI showed a reduction in the number of BrdU-positive cells in the GZ following injury compared to vehicles. Stereological quantification of BrdU-positive proliferating cells in the GZ and hilus showed that the vehicle-treated animals had significantly more BrdU-positive cells compared to shams (p < 0.05 in GZ and hilus; Figure 3-4). There were no significant differences in the number of BrdU-positive cells in the GZ or hilus of animals treated with minocycline 30 minutes post-TBI or 4 hours post-TBI compared to vehicle-treated animals. However, animals treated with minocycline in either treatment group tended to show a reduced level of cell proliferation in the GZ and hilus compared to vehicles (Figure 3-4). There was no significant difference in the number of BrdU-positive cells observed in the GZ or hilus between treatment groups, but those treated 4 hours post-TBI tended to show a slightly greater reduction of cell proliferation following LFPI (Figure 3-4). This data suggests that administration of minocycline in either treatment group tends to reduce the injury-induced cell proliferative response in the DG in young rats after LFPI.

In the 20-month old rats, similar to young adult rats, very few BrdU-positive cells were observed in the DG. Following injury, there was a marked increase in BrdU-positive cells in the vehicle-treated animals (Figure 3-3). Compared to injured vehicle-treated animals, animals treated with minocycline starting at 30 minutes or 4 hours post-TBI had a lower number of BrdU-positive cells. Stereological quantification of BrdU-
positive cells in the ipsilateral DG showed a significantly higher number of cell proliferation in the GZ and hilus of vehicle-treated animals compared to shams (p < 0.05 in GZ and hilus, Figure 3-5). Following minocycline treatment, the number of BrdU-positive cells was decreased in the GZ in both treatment groups particularly in the 4 hr treatment group. The number of BrdU-positive cells in the hilus was not significantly affected (Figure 3-5). There was no significant difference in the number of newly proliferating BrdU-positive cells in the GZ or hilus between the two treatment groups. This data suggests that minocycline treatments administered 30 minutes or 4 hours post-TBI tend to reduce cell proliferation after LFPI in aged rats. The decrease in cell proliferation is more notable and trending toward significance in animals treated 4 hours post-TBI.
Figure 3-3. Newly generated cells in the ipsilateral DG at 3 days post-injury in young and aged rats following LFPI. Micrographs taken from BrdU-stained coronal sections in the ipsilateral DG from a) 3M sham animals, b) 3M injured vehicle-treated animals, c) 20M sham animals, d) 20M injured vehicle-treated animals. a) In the 3-month old sham, very few BrdU+ cells were observed in the DG. b) 3-month old injured vehicle animals showed a greatly enhanced number of BrdU+ cells in the GZ and hilus. c) Very few BrdU+ cells were observed in the DG of the 20-month old sham animal, but the injured vehicle showed many more BrdU+ cells in the GZ and hilus (d).
a. BrdU+ Cells in the GZ in the 3M Old Animals Following LFPI

b. BrdU+ Cells in the Hilus in the 3M Old Animals Following LFPI
Figure 3-4. Stereological quantification of cell proliferation in the granular zone and hilus of young rats at 3 days post injury after LFPI. a) In the ipsilateral GZ (GCL and SGZ), vehicle-treated injured animals had significantly more BrdU+ cells compared to shams (*p<0.05). Animals treated with minocycline 30 minutes and 4 hours post-TBI showed a reduction in the number of BrdU+ cells compared to vehicle-treated animals, but did not reach statistical significance. b) In the ipsilateral hilus, vehicle-treated animals showed a significant enhancement in the number of BrdU+ cells compared to shams (*p<0.05). Neither treatment group showed a significant difference in the number of BrdU+ newly proliferating cells after injury compared to vehicles, but the animals treated 4 hours post-TBI showed a reduction in the injury-induced cell proliferative response. There was no significant difference in the number of BrdU-positive cells between treatment groups among injured animals in the GZ or hilus.
a. BrdU+ Cells in the GZ in the Aged Animals Following LFPI

b. BrdU+ Cells in the Hilus in the Aged Animals Following LFPI
Figure 3-5. Stereological quantification of cell proliferation in the granular zone and hilus of aged rats at 3 days post-injury after LFPI. a) In the ipsilateral GZ (GCL and SGZ), vehicle-treated animals showed significantly enhanced cell proliferation compared to shams (*p<0.05). Animals treated with minocycline 30 minutes post-TBI and 4 hours post-TBI showed a reduced number of BrdU+ cells, which almost reached significance in the 4-hour treatment group. b) In the ipsilateral hilus, vehicle-treated animals had a significantly greater number of BrdU+ cells compared to shams (*p<0.05). Animals treated with minocycline 30 minutes post-TBI did not have a significantly different number of BrdU+ cells after injury compared to vehicles, but animals treated 4 hours post-TBI showed a reduction in the injury-induced proliferative response that almost reached significance.
To examine the effect of minocycline on neurogenesis, DCX staining was used to quantify the number of newly generated neurons. New neurons are constantly generated in the DG and new neurons express DCX for 2 weeks after generation. In our study, animals were sacrificed at 3 days post-injury, and at this time injury-induced new neurons were still at the early stage and lacking typical dendrites. DCX+ cells that have vertical dendrites are likely new neurons born before injury, in contrast, DCX+ cells that have not identifiable dendrites or with horizontal and mixed dendrites are likely new neurons born after or around the time of TBI (Figure 3-6).

In 3 month-old rats, stereological quantification showed that the number of DCX-positive cells bearing horizontal or mixed dendrites was significantly increased in the ipsilateral DG after LFPI in animals treated with minocycline at 30 minutes or 4 hours post-TBI ($p < 0.01$; Figure 3-7a). There were no significant differences in the numbers of DCX-positive cells in the DG between the two treatment groups or in the number of DCX-positive cells in vehicle-treated animals compared to sham animals. A decrease in the number of DCX-positive cells with vertical dendrites was observed in injured animals with or without minocycline treatment but was not statistically significant (Figure 3-7a). These data indicate that post-TBI administration of minocycline results in a significantly increased number of injury-induced new neurons in the DG of young rats after LPFI in both treatment time points.

In the 20 month-old rats, stereological quantification of DCX-positive cells in the ipsilateral DG showed a significantly higher number of DCX-positive cells with horizontal
and mixed dendrites in rats treated with minocycline starting at 30 minutes post-LFPI compared to vehicles and shams (p < 0.01). Animals treated with minocycline 4 hours post-TBI did not demonstrate an increased number of DCX-positive cells in the DG following LFPI, and had significantly less DCX-positive cells with horizontal and mixed dendrites than rats treated with minocycline 30 minutes post-TBI (p < 0.05, Figure 3-7b). Quantification of the number of DCX+ cells with vertical dendrites revealed a significant reduction in animals with minocycline treatment in both groups compared to vehicles or sham animals (p < 0.01, Figure 3-7b).
Figure 3-6. Newly generated DCX+ neurons in the DG at 3 days post-injury in young and aged rats following LFPI. Micrographs taken from DCX-stained coronal sections in the ipsilateral DG from LFPI injured animals at 3 or 20 months showing many DCX+ cells in the 3 month brain compared to aged brain.
a) DCX+ Cells in the DG in the 3M Old Animals Following LFPI

b) DCX+ Cells in the DG in the Aged Animals Following LFPI
Figure 3-7. Quantitative analysis of the number of newly generated DCX+ neurons in 3 and 20 month old rats in the ipsilateral DG at 3 days following LFPI. a) In the 3 month old animal, a significantly higher number of DCX+ new neurons with horizontal or mixed dendrites was observed in animals treated with minocycline either starting at 30 minutes or 4 hours post-TBI compared to vehicles and shams (**p<0.01). No significant difference in the number of DCX+ neurons was found between the two treatment groups. A lower number of DCX+ cells with vertical dendrites were observed in all injury groups compared to sham, but did not reach statistical significance. b) In the 20 month old animals, a significantly higher number of DCX+ cells with horizontal or mixed dendrites was observed in animals treated with minocycline starting at 30 minutes post-TBI compared to vehicles and shams (**p<0.01) and compared to animals treated with minocycline starting at 4 hours post-TBI (*p<0.05). Additionally, a reduced number of DCX+ cells with vertical dendrites was observed in animals in both minocycline treated groups compared to vehicles and shams (**p<0.01).
STUDY 2: EFFECT OF MINOCYCLINE TREATMENT IN CCI MODEL

MORTALITY RATE AND RIGHTING TIME FOLLOWING CCI INJURY

To assess the effect of minocycline on cell proliferation and neurogenesis after CCI, a total of twelve young rats and twelve aged rats were used. The mortality rate in young rats was 10% and in aged rats was 0%. The righting response was recorded immediately after injury as a measure of injury severity (Hamm, 2001). The mean (±SEM) righting time for young rats after CCI was 4.29 ± 0.60 minutes for minocycline-treated animals and 3.38 ± 0.71 minutes for vehicle-treated animals, which were not statistically different (p = 0.59). The mean (±SEM) righting time for aged rats after CCI was 5.63 ± 0.53 minutes for minocycline-treated animals and 4.25 ± 0.25 minutes for vehicle-treated animals. This difference was also not statistically significant, indicating that all animals received a similar severity of injury (p = 0.13).

THE EFFECT OF MINOCYCLINE ON CELL PROLIFERATION IN YOUNG ADULT AND AGED RATS FOLLOWING CCI INJURY

In the 3-month old rats, very few BrdU-positive cells were observed in the ipsilateral DG of the sham animals. Following CCI injury, more BrdU-positive cells were found in the vehicle-treated animals following CCI compared sham or injured with minocycline treatment either starting at 30 minutes or 4 hours post-TBI (Figure 3-8). Stereological quantification showed that the total number of BrdU+ cells was not significantly different among sham, CCI-vehicle or CCI-minocycline groups. There were no significant differences in the number of BrdU-positive cells in the ipsilateral GZ or hilus between the two minocycline treatment groups (Figure 3-9).
In the aged rats, very few BrdU-positive cells were observed in the ipsilateral DG of the sham animals. Following injury a large number of BrdU-positive cells was observed in the GZ and hilus in vehicle-treated animals compared to shams after CCI injury ($p < 0.05$ in GZ, $p < 0.01$ in hilus; Figure 3-10 and Figure 3-11). There was no significant difference in the number of BrdU-positive cells in the GZ and hilus of animals treated with minocycline at 30 minutes post-TBI compared to vehicles. Similarly, while the animals treated with minocycline 4 hours post-TBI showed a decreased number of BrdU-positive cells in the GZ and hilus compared to vehicles, this difference did not reach statistical significance (Figure 3-10 and Figure 3-11). There was no significant difference in the number of BrdU+ cells in the GZ and the hilus of animals between treatment groups. These data indicate that minocycline treatment tends to reduce the injury-induced proliferative response in aged rats following CCI injury, but this reduction in BrdU-positive cells in treated groups does not reach statistical significance.
Figure 3-8. Newly generated cells in the DG at 3 days post-injury in young rats following CCI injury. Micrographs taken from BrdU-stained coronal sections in the ipsilateral DG from a) sham animals, b) injured vehicle-treated animals, c) animals treated with minocycline 30 minutes post-TBI, and d) animals treated with minocycline 4 hours post-TBI. (a) very few BrdU+ cells were observed in the sham. (b) CCI injury resulted in a greatly enhanced number of BrdU+ cells in the DG, but minocycline treatment 30 minutes post-TBI (c) and 4 hours post- TBI (d) caused a reduction in the injury-induced cell proliferative response.
a  BrdU+ Cells in the GZ in the 3M Old Animals Following CCI

b  BrdU+ Cells in the Hilus in the 3M Old Animals Following CCI
Figure 3-9. Stereological quantification of cell proliferation in the granular zone and hilus of young rats at 3 days post-injury after CCI. a) In the ipsilateral GZ, a higher number of BrdU+ cells was observed in the vehicle-treated animals compared to shams, but did not reach significance. Animals treated with minocycline both 30 minutes and 4 hours post-TBI showed a reduction in the injury-induced proliferative response, but this also did not reach significance. b) In the ipsilateral hilus, vehicle-treated animals had a greater number BrdU+ cells compared to shams, but were not statistically significant. Animals in both minocycline treatment groups showed a decreased number of BrdU+ cells compared to vehicles, but did not reach statistical significance. There was no significant difference in the number of BrdU+ cells between treatment groups.
Figure 3-10. Newly generated cells in the DG at 3 days post-injury in aged rats following CCI Injury. Micrographs taken from BrdU-stained coronal sections in the ipsilateral DG from a) sham animals, b) injured vehicle-treated animals, c) animals treated with minocycline 30 minutes post-TBI, and d) animals treated with minocycline 4 hours post-TBI. (a) In the sham animal, there were very few BrdU+ cells in the ipsilateral DG. (b) Much more BrdU+ cells were observed in the DG of the vehicle-treated animal after CCI injury. (c-d) Less BrdU+ cells were observed in minocycline treated animals.
a. BrdU+ Cells in the GZ in the Aged Animals Following CCI

b. BrdU+ Cells in the Hilus in the Aged Animals Following CCI
Figure 3-11. Stereological quantification of cell proliferation in the granular zone and hilus of aged rats at 3 days post-injury after CCI. (a) In the ipsilateral GZ, injured animals with vehicle or minocycline at 30 min had a significantly higher number of BrdU+ cells compared to shams (*p<0.05). Animals treated with minocycline starting at 4 hours post-TBI showed a reduced number of BrdU+ cells compared to vehicles, no statistical significance was reached compared to vehicles after CCI. (b) In the ipsilateral hilus, vehicle-treated animals showed a significantly enhanced number of BrdU+ cells compared to shams (**p<0.01). Animals treated with minocycline 4 hours post-TBI had a lower number of newly proliferating BrdU+ cells compared to vehicles, but there were no significant differences in the number of BrdU+ cells in either treatment group compared to vehicles. There were no significant differences in the number of BrdU+ cells in the GZ or hilus between two treatment groups.
In the 3 month-old animals, following CCI injury, the number of DCX-positive cells with horizontal and mixed dendrites was significantly higher in vehicle or minocycline treated groups when compared to shams (p < 0.01, Figure 3-12a). The number of DCX+ cells with vertical dendrites was slightly lower in the injured groups but was not statistically significant.

In the aged rats, a higher number of DCX-positive cells with horizontal and mixed dendrites was found in rats treated with minocycline at 30 minutes or 4 hours after CCI injury compared to vehicles, but this enhanced neurogenesis was not significant in either treatment group compared to vehicles (Figure 3-12b). However, injured animals treated with minocycline had significantly more DCX+ cells compared to shams (p < 0.01 in CCI-mino at 30 min; p < 0.05 in CCI-mino at 4 hr). There was a significant decrease in the number of DCX+ cells with vertical dendrites in the injured animals with vehicles or minocycline treatment as compared to shams (p < 0.05). No significant difference was found in the number of DCX+ cells with vertical dendrites among injured groups.
Figure 3-12. Quantitative analysis of the number of newly generated neurons in the ipsilateral DG of young and aged rats at 3 days following CCI. 

a) In the ipsilateral DG of young adult rats, animals treated with vehicle or minocycline starting either at 30 minutes or 4 hours post-TBI showed a significantly higher number of DCX+ cells compared to shams (**p<0.01). No significant difference was found in the number of DCX+ cells with vertical dendrites among all groups.

b) In the ipsilateral DG of aged rats, injured animals treated with vehicle or minocycline had a higher number of DCX+ cells with horizontal and mixed dendrites compared to shams, and this increase was statistically significant in animals treated 30 minutes post-TBI (**p<0.01) and in animals treated 4 hours post-TBI (*p<0.05). A significantly lower number of DCX+ cells with vertical dendrites was found in all injured animals compared to shams (*p<0.05), but not among injured groups.
RESULTS SUMMARY

In this study, young adult and aged rats were subjected to mild to moderate LFPI or CCI injury followed by a short-term minocycline treatment. The animals that received minocycline treatment were randomly assigned to one of two treatment groups. Animals in treatment group 1 received a first 45 mg/kg dose of minocycline at 30 minutes post-TBI, then a second 45 mg/kg dose 8 hours post-TBI. Animals in treatment group 2 received a first 45 mg/kg minocycline dose at 4 hours post-TBI, followed by a second 45 mg/kg dose at 8 hours post-TBI. Both groups received two 22.5 mg/kg minocycline treatments 12 hours apart on days 1 and 2 post-injury. The animals were sacrificed at 3 days post-injury to assess the effects of minocycline on cell proliferation and neurogenesis in the DG of the hippocampus using BrdU and DCX staining.

Following LFPI, both 3 month and aged animals show increased cell proliferation in the ipsilateral GZ and hilus regions compared to sham animals. Minocycline treatment starting at 30 min or 4 hr post injury decreased the injury-induced cell proliferation in both age groups, although this reduction did not reach statistical significance likely due to the big standard errors within the group. Minocycline treatment increased the number of newly generated neurons as characterized by horizontal or mixed dendrites with DCX staining in both young and aged animals. This treatment effect was significant when minocycline was given at both time points in young adult animals and at 30 min post-injury in aged animals. Minocycline treatment did not increase the number of DCX+ cells with vertical dendrites in both age groups, but rather decreased the number of this type of cells in the aged groups.
Following a mild to moderate CCI injury, all injured animals both young and aged with minocycline treatment had a reduced number of BrdU+ cells in the ipsilateral GZ and hilus region, but the reduction did not reach statistical significance similar to the LFPI model. In this model, minocycline treatment slightly increased generation of new neurons with DCX+ horizontal and mixed dendrites in the aged but not young rats.

**TBI INDUCES AN INCREASED PROLIFERATIVE RESPONSE IN THE DG IN BOTH YOUNG AND AGED RATS**

It has been reported that NSC/NPCs are present in the adult mammalian brain and are stimulated to rapidly proliferate in response to brain insults in what appears to be an attempt of the brain to heal itself naturally after injury (Rice et al., 2003). In this study, in the LFPI injury model, young and aged rats had increased cell proliferation in response to injury in animals with vehicle treatment compared to shams. This response is consistent with studies reported in our lab and others which have shown that injury-induced cell proliferation in the adult brain peaks at 2 days post-injury in the DG (Sun et al., 2005; Dash et al., 2001; Chirumamilla et al., 2002; Rice et al., 2003). Although NSC/NPCs are present throughout adulthood, studies have confirmed a major reduction in the number of these cells in the aged brain. This reduction was thought to contribute to the declined cognitive function observed in the elderly population (Dash et al., 2001; Artegaiani and Calegari, 2012). In our current study, we found that the aged brain showed a significantly increased number of proliferating cells in the DG following either LFPI or CCI injury as compared to their age-matched shams. This suggests that the aged brain retains proliferative capacity in the DG. Following CCI injury, in the young animals the number of BrdU+ proliferation cells was not significantly higher than sham
animals in the GZ and hilus, which is contrary to what has been reported before (Dast et. al., 2002). This is likely due to the lower animal numbers included.

MINOCYCLINE REDUCES THE INJURY-INDUCED PROLIFERATIVE RESPONSE

Studies from our lab have shown that in the hippocampus in adult animals, many injury-induced proliferative cells are astrocytes and microglia (Chirumamilla et al., 2002). Neuronal differentiation of newly generated cells was only observed in the neurogenic region of the SGZ (Dash et al., 2001). In the aged brain, there is significantly less neural progenitor proliferation, neuronal differentiation, and survival of new neurons compared to the younger brain, and newly proliferating cells in the aged animals differentiate predominantly into glial cells compared to young adult animals (Lee et al., 2012).

Inflammation is a strong inhibitor of neurogenesis and is heightened in the aged hippocampus. The concentration of activated microglia and secretion of pro-inflammatory cytokines in the brain increases with age. It has been reported that activated microglia from aged mice exhibit increased secretion of pro-inflammatory cytokines TNF-α and IL-6 compared to young mice, which is consistent with other studies suggesting that the aged brain exhibits an amplified inflammatory response when stimulated (Lee et al., 2012). Recently, our lab has observed that the aged brain retains cell proliferative capacity following TBI, but this injury-induced proliferative response predominantly generates microglial cells. In addition, we found that new neurons generated in the aged brain failed to survive for an extended period of time. We speculate that the amplified injury-induced inflammation in the aged brain may play a
critical role in modulating neurogenesis and cognitive function, and that inhibition of this injury-induced inflammatory response may restore the hippocampal neurogenic response and ultimately improve cognitive function of the injured aged animals.

In this study, minocycline, a widely used tetracycline antibiotic, was used to inhibit the injury-induced inflammatory response. Minocycline has anti-inflammatory effects inhibiting neutrophils, monocytes, and microglial cells (Elewa et al., 2006). In a stroke model, minocycline was reported to decrease the microglial response in the DG, preserve stroke-induced newly born hippocampal neurons, and reduce functional impairment (Liu et al., 2007). Minocycline was also reported to protect neurons in an acute toxin model of Parkinson’s disease where neuronal damage was predominantly caused by inflammation. Administration of minocycline in an immune-inflammatory encephalitis model resulted in decreased TNF-α release and enhanced secretion of anti-inflammatory cytokine IL-10. Additionally, the anti-inflammatory effect of minocycline have been observed in humans, as minocycline has been used to treat rheumatoid arthritis and multiple sclerosis (Elewa et al., 2006). Collectively, these studies suggest that minocycline has potential to modulate the inflammatory cascades induced by TBI and as a result enhance neurogenesis following injury, especially in aged patients where the inflammatory response is amplified.

In this study, both young and aged rats subjected to LFPI and CCI showed a reduced injury-induced proliferative response after minocycline treatment starting at 30 minutes or 4 hours post-TBI. Since the proliferative response in young adult rats, and especially aged rats, is predominately either microglia or astrocytes, these data are consistent with other studies that have reported reduced microglial activation after
minocycline treatment. While there were no statistically significant differences in the number of BrdU-positive cells in the ipsilateral DG between minocycline treatment groups in each age group and injury model, the reduction in the proliferative response to injury appeared to be more marked in the animals treated with minocycline at 4 hours post-TBI.

**MINOCYCLINE ENHANCES NEUROGENESIS IN YOUNG AND AGED ANIMALS FOLLOWING LFPI**

The aged brain exhibits lower levels of neurogenesis than its younger counterpart, which may contribute to learning and memory impairments associated with aging. However, neurogenesis in the aged brain can be increased or even restored to levels observed in the adult brain by several factors, including an enriched environment and infusion of growth factors. Additionally, despite the age-associated decline in hippocampal neurogenesis, studies suggest that age-related causes of reduced neurogenesis do not affect the morphological properties of newly generated neurons, which is important from a therapeutic perspective (Ahlenius et al., 2009).

The role of neuroinflammation in modulating neurogenesis is not fully understood. It appears that microglia in the normal brain are necessary to preserve neurogenesis and memory function, but microglia activated after brain insult impedes neurogenesis. It has been reported that LPS-induced inflammation resulted in an 85% reduction in the number of newly generated neurons during the inflammatory induction in young adult rats. Additionally, systematic administration of LPS was found to increase microglial activation and reduce neurogenesis, and this effect could be prevented by anti-inflammatory treatment with nonsteroidal anti-inflammatory drug (NSAID).
indomethacin. Microglia-mediated inflammation can regulate neurogenesis in two major ways. They have the potential to be neurotoxic when they are removing dysfunctional or damaged cells by producing reactive oxygen species and inflammatory cytokines (Gemma et al., 2010). Microglia are the major producers of the key innate cytokines IL-1β and TNF-α which are pro-inflammatory cytokines and capable of inhibiting NSCs from differentiating into neurons (Lee et al., 2012). We speculated that inhibition of microglia-mediated inflammation with minocycline would enhance neurogenesis, especially in aged rats where microglial activation is amplified.

In this study, both young adult and aged rats subjected to LFPI and treated with minocycline exhibited a reduction in the number of proliferating cells compared to the injured vehicle group. Additionally, minocycline-treated young and aged rats showed an increased number of new neurons as demonstrated by DCX+ cells bearing horizontal or mixed dendrites. This is likely due to the inhibition of injury-induced microglia proliferation and subsequent reduced inflammation. In our study, in young animals, minocycline given beginning at 30 min or 4 hr after injury equally reduces cell proliferation and increases DCX+ cells following LFPI injury but not CCI injury, whereas in the aged animals minocycline treatment starting at 30 min post-injury has a more positive effect in increasing the number of young DCX+ neurons. This suggests that there are model and age-related differences in the injury-induced cell proliferative and neurogenic responses. While inflammatory cell responses vary depending on injury model (Loane and Byrnes, 2010), similarities and differences in microglial responses and temporal cytokine profiles in the LFPI and CCI model have not been well documented. These results also suggest varying pharmacokinetics of minocycline.
treatment by age or other factors that affect stem/progenitor cell function, neuronal differentiation, or survival after injury (Gemma et al., 2010).

We speculated that aged animals would exhibit higher levels of neurogenesis after injury with minocycline treatment due to the enhanced inflammation observed in the aged brain relative to the younger brain. However, young adult animals also showed a reduced cell proliferative response and enhanced neurogenesis following LFPI injury, suggesting that minocycline may influence neurogenesis via another mechanism. In addition to their anti-inflammatory and anti-oxidant properties, tetracyclines such as minocycline have also been shown to have anti-tumor properties. Minocycline inhibits cell proliferation by altering the cell cycle to arrest cells in the G₀-G₁ phase (Pourgholami et al., 2012). It is possible that by suppressing cell proliferation in this way, minocycline may drive early differentiation of cells into a neuronal phenotype in younger animals.

Furthermore, we found that both young and aged injured animals with or without minocycline treatment have a lower number of DCX+ cells with vertical dendrites compared to their age-matched shams following both LFPI and CCI injury. Most DCX+ neurons bearing vertical dendrites are new neurons born before injury and they are vulnerable to TBI. This injury-induced reduction indicated the loss of these new neurons due to the injury. Importantly, the decreased number of DCX+ cells with vertical dendrites in injured animals was significant in aged animals but not young animals after both LFPI and CCI injury. These results are consistent with other studies that have reported increased apoptotic cell death in the hippocampus of aged rats compared to young adult rats following brain injury (Itoh, 2012). Further, infarct development progresses at an accelerated rate in aged rats compared to young rats, which is linked
to apoptosis and early neuronal death as well as neuroinflammation. The rapid infarct progression and cell death in the aged brain following TBI is at least in part due to free radicals and oxidative stress, which also increases with age (Itoh, 2012; Popa-Wagner et al., 2011). The increased vulnerability of brain tissue to TBI with age is consistent with the neuronal loss observed in vehicle-treated aged animals.

Minocycline-treated animals were expected to have less neuronal loss than vehicle animals due to the neuroprotective properties commonly attributed to minocycline, yet this effect was not observed. Recent studies have shown that minocycline has variable and even contradictory effects based on the species and model of neurological disorder. For example, minocycline has demonstrated neuroprotective effects in rodent models of ischemic injury and in global and focal cerebral ischemia (Diguet, 2004). In contrast, other studies have reported that minocycline had no effect on cell apoptosis in a mouse model of closed head injury (Bye, 2006) and had deleterious effects in Parkinson’s, Huntington’s, and hypoxic-ischemic brain injury models (Diguet, 2004). Although the neuroprotective properties of minocycline are likely associated with its anti-inflammatory properties and inhibition of caspase pathways, further exploration of the mechanisms of minocycline in different injury models would be useful in understanding its varying effects (Diguet, 2004).

Initially in this study, the effect of minocycline treatment on cell proliferation and neurogenesis was to be observed in the LFPI model only, as previous studies in our laboratory have been conducted using this model. However, Fisher 344 rats are highly sensitive to LFPI, and have been reported to exhibit higher mortality rates, more frequent seizure attacks, higher ICP, and more cell death than Sprague-Dawley rats of
the same age and subjected to the same injury level (Reid, 2010). Due to the high mortality rates observed early in the study, we slightly lowered the target injury level to increase survival rates following injury, especially in the aged rats that are particularly susceptible to LFPI. This adjustment in LFPI injury severity is responsible for the low mortality rates in the aged rats and the greater variation in LFPI injury results. The CCI injury model was included in this study because Fisher 344 rats are better able to tolerate the focal injury, as demonstrated by the almost complete lack of mortality following injury in both young and aged rats. For this reason, the CCI injury was more consistent among rats and produced more consistent results.

**FUTURE OF MINOCYCLINE AS A POTENTIAL THERAPY**

In this study, both young and aged rats showed a reduced injury-induced cell proliferative response after treatment with minocycline in both LFPI and CCI injury models. Furthermore, this reduction of injury-induced cell proliferation was concomitant with enhanced hippocampal neurogenesis in both young and aged rats following injury. While not all of these relationships showed statistical significance, the animals in the groups that did not reach significance showed a high degree of variability in stereological cell counts, which may have contributed to the lack of statistical significance in these groups. However, these groups showed a trend towards significance.

There were no statistically significant differences between minocycline treatment groups in either cell proliferation or generation of new neurons in young or aged rats after CCI or in young rats after LFPI, but aged rats treated with minocycline at 30
minutes post-LFPI showed significantly enhanced neurogenesis compared to those treated at 4 hours post-LFPI. The animals treated 4 hours post-TBI tended to show a more marked reduction of the injury-induced cell proliferative response than the animals treated 30 minutes post-TBI. The more effective minocycline treatment to enhance neurogenesis differed among age and injury model groups. However, enhanced neurogenesis was observed in all groups that showed a significant reduction of the injury-induced proliferative response. These varying results may be due to differing minocycline pharmacokinetics in rats of different ages or in response to different types of injuries. Due to the big variations, addition of more animal numbers to each group may reduce the observed variability in stereological counts and help identify the most effective minocycline treatment for the groups in this study. Importantly, this study identified the efficacy of minocycline treatment to reduce the number of injury-induced proliferating cells. Further study to confirm this reduced cell population is indeed microglia cells is needed. Overall, the data from this study shows that minocycline treatment can enhance hippocampal neurogenesis in both LFPI and CCI injury models concomitant to reduced injury-induced cell proliferation. These data strongly support that microglia-mediated inflammation is detrimental to injury-induced neurogenesis, and that inhibition of this inflammatory response may result in improved cognitive recovery in patients. Future studies should also investigate the long-term effect of minocycline on cell survival, neuronal differentiation, and inflammation.


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VITA

Ashley Harvin was born in Poquoson, Virginia on January 12, 1988. She graduated from Poquoson High School in 2006. After high school, she attended the University of Virginia in Charlottesville, Virginia where she earned a Bachelor of Science in Chemical Engineering in May of 2010. She attended Virginia Commonwealth University in Richmond, Virginia in August of 2010 to complete a Master of Science in Anatomy and Neurobiology. In August of 2012, she will be matriculating into the M.D. program at the VCU School of Medicine as a member of the Class of 2016.