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DIFFERENTIAL GLIAL CELL RESPONSES IN THE DENTATE GYRUS IN YOUNG ADULT AND AGED BRAINS FOLLOWING TRAUMATIC BRAIN INJURY

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DIFFERENTIAL GLIAL CELL RESPONSES IN THE DENTATE GYRUS IN YOUNG ADULT 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

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List of Abbreviations

ABC   Avidin-biotin complex

Atm   Atmosphere

BrdU   5-bromo-2-deoxyuridine

CCI   Controlled cortical impact

CDC   Center for Disease Control

CNS   Central nervous system

CSF   Cerebrospinal fluid

DAB   5, 5-diaminobenzidine

DCX   Doublecortin

DG   Dentate gyrus

DNA   Deoxyribonucleic acid

FG   Fluorogold

FPI   Fluid percussion injury

GCL   Granule cell layer

GFAP   Glial fibrillary acidic protein

GZ   Granular zone

Hr   Hour

IBA1   Ionized calcium binding adaptor molecule 1

i.p.   Intraperitoneal

L-FPI   Lateral fluid percussion injury

NPC	Neural progenitor cell
NSC	Neural stem cell
PBS	Phosphate buffer saline
PNS	Peripheral nervous system
Sec	Second
SEM	Standard error margin
SGZ	Subgranular zone
SSC	Saline sodium citrate
SVZ	Subventricular zone
TBI	Traumatic brain injury

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Abstract

DIFFERENTIAL GLIAL CELL RESPONSES IN THE DENTATE GYRUS IN YOUNG ADULT AND AGED BRAINS FOLLOWING TRAUMATIC BRAIN INJURY

By Christopher H. Shin

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

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Advisor: Dong Sun, MD, PhD., Department of Neurosurgery

Traumatic brain injury (TBI) affects 3 out of every 1000 Americans each year, and is the leading cause of morbidity and mortality after trauma, accounting for as many as 56,000 deaths per year (Dutton and McCunn, 2003). The Centers for Disease and Control and Prevention found that TBI most commonly occurs in adolescents and young adults aged from 15 to 24 years and in the elderly (75 years and older). Following injury, the secondary injury begins almost immediately after the primary injury and is the result of a number of cascades where once activated, exacerbate the already altered homeostasis of the injured brain. Brain trauma leads to complex secondary injury responses that trigger many cellular and molecular pathways, especially inflammation. The cerebral inflammation that occurs after TBI has been described through the processes of glial activation followed by leukocyte recruitment, and upreglation and secretion of cytokines and chemokines. With aging there is a decrease in the production of anti-inflammatory cytokines along with increasing amounts of pro-inflammatory cytokines by

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peripheral blood monoculear cells, microglia, and astrocytes. Studies have shown that inflammation has a strong negative effect on neurogenesis in the adult brain due to the impact of the pro-inflammatory cytokines that are released following the acute injury or disease. In this study, we first examined the differences in glial cells responses in young adult brain and aged brain following a moderate lateral fluid percussion injury and the correlation of glial cell activation with hippocampal neurogenesis. We then examined the effect of anti-inflammation treatment on glial cell response in the young and aged brain. The levels of astrocytic and microglial responses in the DG of the hippocampus following injury at 3, 7 or 28 days post-injury were measured using densitometry image analysis on GFAP or Iba1 immunofluorescent labeled brain tissue sections. We found that injury increased both astrocyte and microglial activation and proliferation in both young and aged brain. The young injured animals exhibited greater levels of GFAP while the aged injured animals exhibited greater levels of Iba1 expression at all three time points. We also found that short time anti-inflammatory treatment with minocycline decreased levels of Iba1 expression while increased levels of GFAP expression in both young and aged brain following injury. Our data suggests that there are differences in glial response in the injured young and aged brain that may contribute to the differences in the regenerative and recovery potential in the two age groups following injury.

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Chapter 1 - Introduction:

Traumatic brain injury continues to be a serious global health concern and is gaining more attention as new studies present the complexity of the secondary injuries that follow the initial primarily insult. A major secondary cascade that truly attests to the complexity of treating TBI is the inflammatory response that stems from the activation of resident microglia and resident astrocytes. These activated microglia release a gradient of pro-inflammatory and anti-inflammatory cytokines that may vary depending on the type of injury that is induced. Most consistently, the gradient favors toward the pro-inflammatory cytokines being released at greater amounts and can cause a recruitment of peripheral neutrophils and monocytes that cross the ruptured blood-brain barrier to the site of injury. Once these neutrophils and monocytes are activated as well, a potentially dangerous feed-forward loop may occur causing a continuous induction of inflammation. TBI in the aged population has been shown to cause an even greater detrimental effect due to the state of the brain during the course of aging. Aged brain in humans and animal studies show that the brain possesses higher densities of resident microglia, though they are not always in an activated state. Aged brains have seen a greater damaging effect following TBI with larger lesion sizes, increased neuronal degeneration, an upregulated inflammatory response, and decreased injury induced neurogenesis.

The central nervous system, once thought not possible in producing new neurons, has shown the ability of constantly generating new cells throughout life in the neurogenic regions, ie. the subventricular zone and the subgranular zone in the hippocampus. Studies have shown that these newly generated neurons integrate into existing neural circuitry and become fully functional neurons. Studies have also found that adult neurogenesis can be affected by many factors. Among them, neural inflammation has a negative effect on neurogenesis especially in

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the aging brain. We observed previously that TBI enhanced neurogenesis in young adult animals; however, in the aged brain, this injury-enhanced neurogenic effect was not observed (Sun et al., 2005). This project is set to investigate the differences of glial cells responses and its association to neurogenesis particularly in the hippocampus in young and aged animals following TBI. We hypothesize that the aged brain has a greater inflammatory response following TBI which presents a greater negative effect on neurogenesis.

Epidemiology of TBI

Traumatic brain injury (TBI) is a critical health care concern affecting over 57 million people around world, especially 1.4 million people in the United States alone each year. (Langlois et al., 2006). Among the 1.4 million Americans that suffer TBI yearly, 1.1 million visit the emergency department, 235,000 are hospitalized, 50,000 become deceased, and males being twice as likely than females to experience a TBI (Langlois et al., 2006). According to recent studies conducted by the Centers for disease control and prevention, TBI accounts for roughly 30.5% of all injury related deaths in the United States. Falls, motor vehicle crashes, struck by or against events, and assaults or physical retaliation are reported as the leading causes of TBI each year (Langlois et al., 2006). Sports injuries, primarily concussions, are also a major cause of TBI with nearly 1.6 million to 3.8 million sports-related TBI occurring yearly (Langlois et al., 2006). Blast injuries have developed in becoming the leading cause of TBI for military soldiers in active duty (Scott et al., 2005).

The most common age distribution of the patients experiencing a TBI has shown a bi-modal trend with the first peak occurring in young adults (late teens through twenties) mostly

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due to motor vehicle crashes, and the second peak occurring among the elderly population primarily due to falls (Rutland-Brown et al., 2006; Sendroy-Terrill et al., 2010). A patient that has suffered a TBI may develop long-term and potential lifelong physical, cognitive, behavioral, and emotional consequences depending on the severity of the TBI. Recently, approximately 5.3 million Americans were reported to be living with a long-term or lifelong disability due to TBI (Thurman et al. 1999). Thus, these patients who suffer long-term problems stemming from TBI also have difficulties in being at social events, performing daily activities, and maintaining stability at work (Langlois et al., 2006). It has also been reported that injuries from TBI contribute to 15.7% of injury-related productivity loss and is fourteen times greater than compared to similar central nervous system injuries such as spinal cord injury (Finkelstein et al., 2006). Each year $60 billion is spent annually with the money supporting the needs for patients' medical care, rehabilitation, and loss of productivity (Thurman et al., 1999, Langlois et al., 2006). TBI is truly a health problem whose disabling nature takes a dangerous toll on the patients' quality of life while representing an enormous financial burden to our country's economy.

Pathophysiology of TBI

Falls, motor vehicle collisions, and struck by or against interactions cause high-energy acceleration or deceleration of the brain within the cranium (Dutton and McCunn, 2003), and can lead to an elevation of ventricular intracranial pressure that can indicate poor outcome after TBI (Marshall et al., 1979; Ray et al., 2002). The effects of TBI can affect patient health on two levels beginning with the primary insult followed by a more gradual secondary injury beginning

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from minutes to hours after the primary insult and may even last months (Gentleman et al., 2004). The primary injury arises when an external impact is delivered to the head. The initial impact causes two different injury manifestations of TBI, focal injuries and diffuse injuries (Ray et al., 2002). Focal injuries such as skull fractures and hematoma (epidural and subdural) are caused by contact forces that prevent the head from moving after impact while diffuse injuries such as concussion stem from inertial forces providing an acceleration to the head either translationally or rotationally or both (Graham et al., 1995).

The secondary injury begins almost immediately after the primary injury and is the result of a number of cascades where once activated, exacerbate the already altered homeostasis of the injured brain.   Increased vascular permeability, altered ionic balance, oxidative stress, excitotoxic damage, inflammation, and mitochondrial dysfunction are the causes of edema, increased intracranial pressure, neuronal cell death, and ultimately neurologic impairment (Morganti-Kossmann et al., 2002). Understanding and targeting the secondary injury phase of TBI has been the major focus of research in order to prevent further tissue damage, stimulate neuronal repair and neurogenesis, and restoring cognitive functions. As the brain enters the stages of secondary injury, different mechanisms within the brain that alter cerebral blood flow, ion homeostasis, inducing excitotoxicity, exacerbating local and systemic inflammation, and apoptotic death of neuronal cells contribute to the ongoing effects post-injury (Ray et al., 2002). Molecularly, oxygen free radicals are generated due to either post-traumatic ischemia from altered cerebral blood flow (Kontos and Povlishock, 1986; Ikeda and Long, 1990; Traystman et al., 1991, Ray et al., 2002) or the increase of intracellular free Ca^{2+} concentrations (Kontos and Povlishock, 1986; Tymianski and Tator, 1996; Ray et al., 2002). Oxygen free radicals are a threat because they damage cell membrane by lipid peroxidation and oxidize intracellular

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proteins and nucleic acids. Secondly, glutamate and aspartate, known to be excitatory amino acids, are present in high concentrations in the extracellular space and cerebrospinal fluid after TBI (Faden et al., 1989; Palmer et al., 1993). Aspartate and glutamate cause excitotoxicity through its key role of serving as a ligand for NMDA receptors and AMPA receptors. It has been documented that the increased influx of Na^+ and Ca^{2+} ions and efflux of K^+ within the neuron leading to acute neuronal and glial swelling as well as delayed damage (Choi et al., 1987; Reynolds and Miller, 1988; Ray et al., 2002). Furthermore, the heightened influx of Ca^{2+} ions after TBI can leads to an over-activation of Ca^{2+}-dependant proteases including calpain. Calpain normally mediates cytoskeletal protein degradation and neurodegeneration. However, when overexpressed, leads to harmful neurofilament protein degradation and cell death (Banik et al., 1997).

TBI induced inflammation

During the secondary injury phase, local inflammation occurring in the brain following injury has been widely studied. Brain trauma leads to complex secondary injury responses that trigger many cellular and molecular pathways, especially inflammation (Raghupathi, 2004; Ziebell and Morganti-Kossmann, 2010). The cerebral inflammation that occurs after TBI has been described through the processes of glial activation followed by leukocyte recruitment, and upregulation and secretion of cytokines and chemokines (Morganti-Kossman et al., 2001). Studies throughout the literature show the dual nature of inflammation having both positive and negative roles for the injured brain. The bimodality of inflammation has been determined through comprehensive research and identification of the cytokines that are released in the brain.

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Inflammation provides clear benefits when it is controlled in a regulated manner for a defined period of time (Ziebell and Morganti-Kossman, 2010). However, when inflammation is chronic and excessive in the injured brain and it greatly contributes to many harmful processes (Correale and Villa, 2004).

A major change occurring after TBI is the impairment of the blood brain barrier (BBB). In the acute post-traumatic period, the leaky BBB allows for the infiltration of peripheral monocytes, neutrophils, and leukocytes to the injured region of the brain. These activated cells enter the brain parenchyma and release factors such as prostaglandins, free radicals, complement factors, anti-inflammatory and pro-inflammatory cytokines (Werner and Engelhard, 2007). The sites of leakage within the BBB become sealed by repair mechanisms; however, it is possible that during a brief period of time, it still remains permeable to small molecules (Tanno et al., 1992). This leads to a continuous altered state of homeostasis of the brain parenchyma and potentially affecting neuronal function (Ziebell and Morganti-Kossmann, 2010).

Resident microglia whose function is patrolling the CNS and removing cell debris, play a major role in the inflammatory process once activated after injury. They are constantly surveying the microenvironment within the brain for noxious agents and injurious processes (Nimmerjahn et al., 2005). The role of microglia has been under heavy scrutiny in determining whether its activated response is beneficial or harmful. Microglia represents approximately 10 – 20% of the total cell population in the adult human CNS (Spranger and Fontana, 1996). These cells belong to the mononuclear phagocyte lineage and are the primary mediators of the brain's innate immune response to disease, infection, and especially injury (Loane and Byrnes, 2010). In the literature, a variety of CNS injury models have been used to investigate the role of microglia in the inflammatory response. The microglial responses have shown varying results

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depending on the type of injury model and the severity of the injury (Loane and Byrnes, 2010). The activation of microglia following injury causes them to undergo morphological changes (**Fig 1.1**). Their processes contract and their cell bodies change to an ameboid morphology while they begin proliferating and migrating toward the site of injury (Vilhardt, 2005).

Chronic inflammation has been correlated to chronic microglial activation within the brain. Chronic activation of microglia is considered to be the most damaging to injured or diseased CNS because interactions occurring between damaged neurons and hyper-activated microglia can create a self propagating positive feedback loop leading to a potential prolonged microglial activation and neuronal cell death (Block and Hong, 2007; Gao and Hong, 2008). Despite the harmful responses that microglia inflict onto the injured the brain, they also are key players in beneficial responses as well. Microglia phagocytose cellular debris and maintain normal cellular homeostasis providing preservation and protection for the brain tissue. As the microglia are activated after injury, they have been shown to react within hours migrating toward the site of injury. Once at the destination of injury, their processes fuse and form an area of containment between healthy and injured tissues (Davalos et al., 2005). Following the progression of brain injury, the role of inflammation in the injured brain may shift from detrimental to beneficial with decreased production of pro-inflammatory cytokines and increased production of anti-inflammatory cytokines such as IL-10 and prostaglandin E2 (PGE2) (Ekdahl et al., 2009). A study from Bonde et al., 2006 showed that after status epilepticus induced inflammation, neurons that survived after the first month of deleterious microglial activation continued to survive for at least six months. Thus, it can be postulated that chronically activated microglia potentially have a neuroprotective role (Bonde et al., 2006).

I   II   III   IV
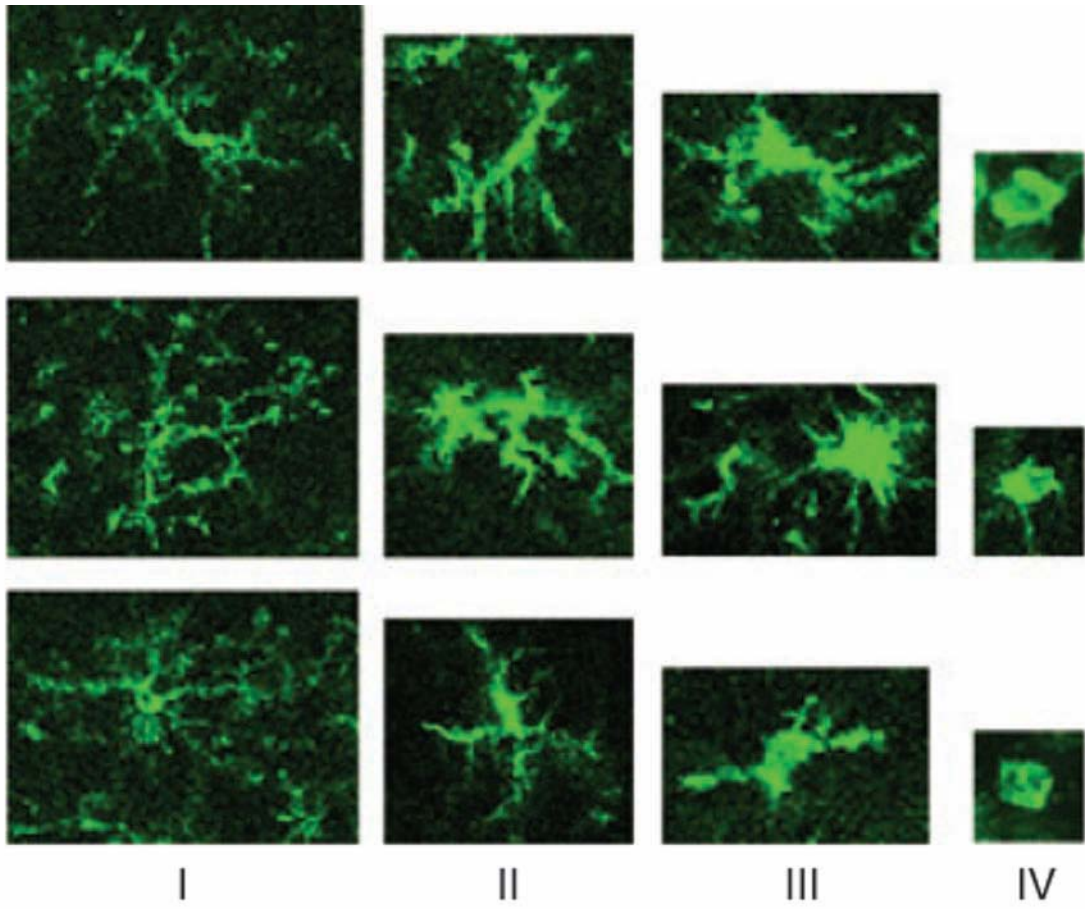
Figure 1.1: Characteristic morphologies of resting and activated microglia in the adult rat brain. Photographs of taken from Matthieu et al. (2010). (I) Stage I: Cells have rod-shaped cell bodies with fine, ramified processes and are defined as resting microglial cells. (II) Stage II: activated microglia cells have elongated cell bodies with long thick processes. (III) Stage III: activated microglia cells have small, thick processes, and a rounded morphology. (IV) Stage IV: activated microglia cells have a rounded shape with barely any process, vacuolated cytoplasm.

Resident astrocytes are also important in contributing to the inflammatory process. Astrocytes are the predominant cell type within the neurovascular unit (neurons, astrocytes, blood vessels) having a 10:1 ratio compared to neurons in the human brain (Laird et al., 2008). Astrocytes have been shown to contribute to the formation of the blood brain barrier, regulate cerebral blood flow according to neuronal activity, provide metabolic substrates for neurons, and regulate oxidative balance in the brain (Abbot et al., 1992; Janzer and Raff, 1987; Takano et al., 2006; Pellerin and Magistretti, 2004; Wilson, 1997). Once astrocytes are activated following neurotrauma, they undergo a phenotypic change characterized by cellular hypertrophy and hyperplasia, cytoplasmic enlargement, elongated cytoplasmic processes, and increased expression of glial fibrillary acidic protein (GFAP) (Casteon, 1998; Baldwin and Scheff, 1996). Reactive astrocytes, like reactive microglia, have also been shown to exhibit a dual nature of inducing both potentially positive and harmful reactions following TBI.

Studies have shown that reactive astrocytes following TBI form a protective scar to contain brain injury, provide and improve niche for reparative responses, restore the integrity of the blood brain barrier, and aid in the establishment of an environment that is helpful for neurite outgrowth, synaptogenesis and synapse maturation through modulate levels of matrix metalloproteinase-3 released (Sofroniew, 2005; Bush et al., 1999; Myer et al., 2006; Reeves et al., 2002; Falo et al., 2006). However, reactive astrocytes also contribute greatly in exacerbating inflammation following TBI. First, astrocytes are known to regulate extracellular glutamate excitoxicity within the brain due to their expression of GLT-1 and GLAST receptors which serve as glutamate transporters (Pawlak et al., 2005). However, after injury, these receptors are downregulated following experimental TBI in rodents suggesting that this may lead to an increased inflammation and exacerbated excitotoxicity (Rao et al., 1998). Astrocytes also

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release and respond to pro-inflammatory cytokines such as TNF-α, Fas, and IL-1β and therefore, further amplifying the inflammatory response following TBI (Laird et al., 2010). The inflammatory cascade that ensues after TBI heavily relies on the concentration gradients between the amounts of released pro- and anti-inflammatory cytokines. TBI causes the upregulation and secretion of pro-inflammatory cytokines such as interleukins IL-1β, IL-6, IL-10 and tumor necrosis factor-α (TNF-α) (Ray et al., 2002). Cytokines are a group of proteins that act as chemical messengers between immune cells. The pro-inflammatory cytokines are believed to initiate inflammation. Generally, these cytokines are shown to be released after tissue damage and ischemia triggering the activation of resident microglia and astrocytes and upregulating the release of chemokines (Cederberg and Siesjo, 2010).

Looking more specifically into the roles of the pro-inflammatory cytokines, we learn that each of these cytokines plays a pivotal role in triggering a different portion of the inflammatory response. However, studies have also shown that these inflammatory cytokines may also have a dual role in helping the brain recover through inflammatory response. The anti-inflammatory cytokines such as IL-4, IL-10, IL-1Rα and TGF-β have been shown to counteract and regulate inflammatory reactions (Cederberg and Siesjo, 2010). The expressions of chemokines are induced leading to an upreglution of cell adhesion of molecules and mobilization of immune and glial cells to the injured site (Ziebell and Morganti-Kossmann, 2010).

Aging brain in response to TBI

It is established that juvenile and young adult mammals recover to a greater extent than aged adult following neurotrauma. Clinical studies examining cognitive recovery have showed

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that children exhibit greater cognitive recovery and are less-dependant on assistance than young adults following brain injury (Eiben et al., 1984). With increasing age, the older population show lower levels of recovery compared to their younger counterparts. For example, in experimental TBI animal studies, three month old injured rats performed significantly better than twenty month old aged rats on hippocampal-dependant memory tests, particularly the Morris Water Maze following injury (Hamm et al., 1992). Changes in the CNS in response to aging are similar to the changes that occur in other cells, and include increased oxidative stress, altered protein accumulation, nucleic acid damage, and dysfunction of energy homeostasis. The cellular changes during normal aging make neurons increasingly susceptible to excitotoxic damage through the impairment of ion pumps, dysregulation of Ca2+ homeostasis, and decreased mitochondrial function. Given that all these processes are involved in the evolution of the injury after traumatic insult, the process of aging itself could significantly increase vulnerability and impair the potential for recovery from TBI in aged individuals (Cekic and Stein, 2010). Previous studies done in our lab have shown that the younger brain is capable of generating more robust levels of cell proliferation in response to an injury compared to the aged brain, and in accordance, the younger brain's also showed greater levels of the newly generated neurons cells in the hippocampus (Rolfe et al., 2009) (**Fig 1.2**).

Aging is generally associated with the activation of inflammatory response due to the chronic antigenic stress on innate immunity experienced over a lifetime (Vasto et al., 2007). With aging there is a decrease in the production of anti-inflammatory cytokines along with increasing amounts of pro-inflammatory cytokines by peripheral blood monoculear cells,

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A — Cell Proliferation in the GCL at 2 Days Post Injury

B — Cell Proliferation in the Hilus at 2 Days Post Injury
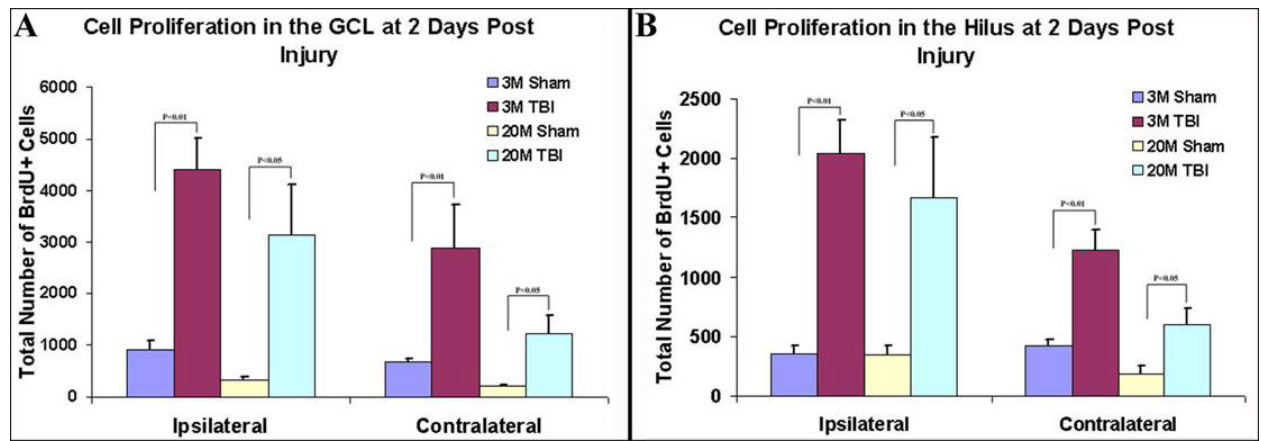
Figures 1.2: TBI induces cell proliferation in the DG in both young adult and aged brain.

Taken from previous studies in the Sun lab. Compared to shams, injured both young and aged animals had significantly increased cell proliferation in both the ipsilateral and contralateral side of the granule cell layer (GCL, A). Similarly, injured rats in both age groups had higher number of proliferating cells in both sides of the hilus region as compared to their age matched sham (B).

microglia and astrocytes. In the aged brain, microglia have been shown to take on a more activated phenotype in the resting state, and they have been shown exhibiting hypertrophic and shortened processes that confirm a more activated cellular morphology (Chung et al., 2010). Furthermore, studies have show that the aged brain has increased levels of IL-1β, IL-6 and TNF-α expression at resting baseline levels (Gelinas and McLaurin, 2005; Godbout and Johnson, 2004; Loane and Byrnes, 2010). Microglia in the aged brain compared to those of younger brains have been characterized to respond to injury more rapidly, produce more pro-inflammatory cytokines and responses, and proliferate more vigorously (Godbout et al., 2005; Conde and Streit, 2006).

Thus, aging creates a brain environment where microglial sensitivity to immune activation is increased leading to potential exacerbation of the secondary injury in older subjects and severe systemic immune responses (Cekic and Stein, 2010). Exaggerated inflammation can also interfere with neuroplasticity as the brain attempts to recover from injury along with memory consolidation and hippocampal neurogenesis (Barrientos et al., 2006; Vallieres et al., 2002). The aged brain gives an appearance of tissue that exists in a chronic state of inflammation that is associated with increased immune reactivity (Dilger and Johnson, 2008). It is well accepted that exacerbated microglial and astrocytic responses are involved in the enhanced susceptibility to injury and poor recovery from TBI among elderly patients (Galbraith, 1987; Pennings et al., 1993; Loane and Byrnes, 2010).

Neurogenesis in the hippocampus in the adult brain

It has been shown now through extensive research that neural stem cells give rise to the generation of neurons and glia throughout adult life. The generation of new neurons in the adult brain is mostly seen in two regions: the subventricular zone (SVZ) adjacent to the lateral ventricles where newly generated neuroblasts migrate to the olfactory bulb, and the subgranular zone (SGZ) of the hippocampal dentate gyrus where the newly generated neurons become dentate granular neurons involving in mood modulation, learning and memory function (Franklin and Ffrench-Constant, 2008; Ming and Song, 2005). The process of adult neurogenesis has been described through these steps: proliferation of stem/progenitor cells, migration to appropriate location, differentiation to region specific phenotypes, survival of immature neurons and development of functional synaptic connectivity counteracting disease symptoms (Ekdahl et al., 2009). Studies from our lab and others have shown that TBI and other CNS insults results in increased levels of cell proliferation in the neurogenic areas (Sun et al., 2005; Parent, 2003). Astrocytes have been shown to play a role in regulating almost every stage of the process of neurogenesis including self-renewal, fast specification of adult neural stem cells (NSCs), migration, differentiation and integration (Song et al. 2002; Barkho et al., 2006; Jiao and Chen, 2008; Mathieu et al., 2010). Although glial progenitor cells are seen to be abundant and mediate continued production of glia throughout the brain, the natural production of new neurons is restricted to only the SVZ and SGZ (Carpentier and Palmer, 2009).

However, despite the presence of new neurons being formed, it has been shown that only a few newborn cells are actually incorporated into the circuitry while majority of the population are presumed to die at the immature neuron stage through apoptosis, and the apoptotic debris is removed from the SGZ by microglia (Ma et al., 2009, Sierra et al. 2010). Following injury, we previously found that proximately 46% of newly generated cells persisted in the DG for an

extended period of time and became mature granule neurons (Sun et al., 2007). Furthermore, the newly formed neurons in the granule cell layer send axonal projections to the CA3 subfield of the hippocampus (Sun et al, 2007) and spineless dendrites to the molecular layer (Lazarov et al., 2010). The newly integrated neurons have been shown to display passive membrane properties, generating action potentials, and forming synaptic inputs (van Praag et al., 2002).

With aging, as with other organs in our body, the brain also exhibits a progressive decline in its regenerative abilities with increasing age leading to functional deterioration and poor recovery from injury and disease. Stem cell numbers have shown to decrease with age leading one to think that stem cell aging applies to the natural biology of aging (Sharpless and DePinho, 2007). Therefore, as NSCs are increasingly becoming acknowledged for their functional significance and potential for repairing the injured and diseased brain, dramatic declines in neurogenesis with age have also become an interesting topic of study (Lazarov et al., 2010). It has been observed that both the SVZ and SGZ exhibit age-related declines in the production of new neurons (**Fig 1.3 and 1.4**). Especially in the SGZ, the decreased counts of new cell proliferation as well as newly integrated neurons are correlated with a decline in hippocampal-dependant spatial memory (Bernal and Peterson, 2004).

However, despite the decline in the numbers of surviving and integrated new neurons, the neurons that did integrate themselves appear fully functional and equivalent to those seen in the young adult brains (Rao et al., 2005; Lazarov et al., 2010). Previous studies in our lab also showed that aged animals showed low levels of neurogenesis following injury compared to young adult animals (Fig 1.4).
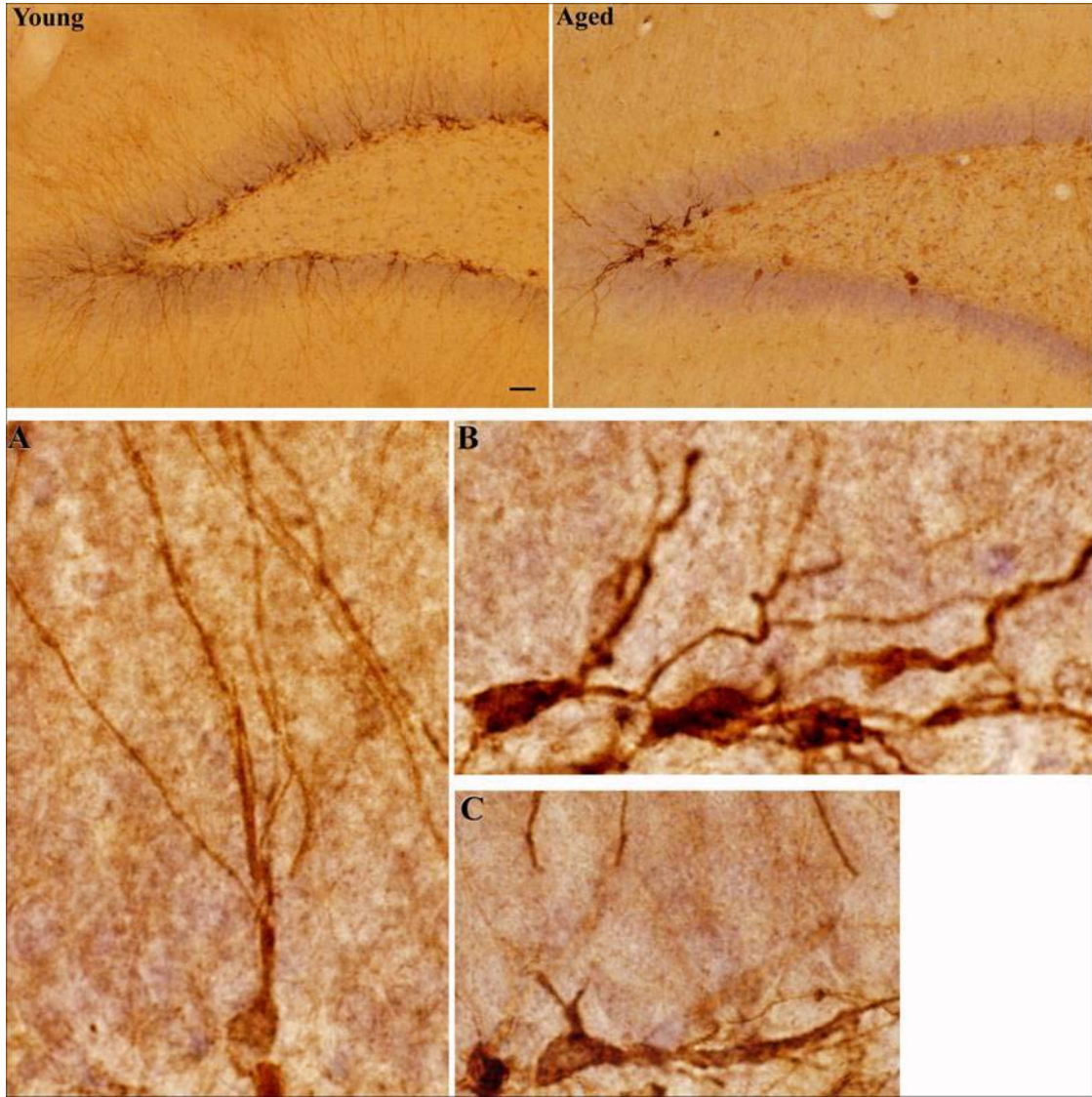
Figure 1.3: Generation of new neurons in the DG. Taken from previous studies in the Sun lab. <u>Upper panel</u>: in younger brain, densely DCX-labeled newly generated neurons are present throughout the GCL compared to a few DCX-labeled new neurons in the aged brain. Bar=100µm. <u>Lower panel</u>: three 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).

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Generation of new neurons in the GCL at 3 days following TBI
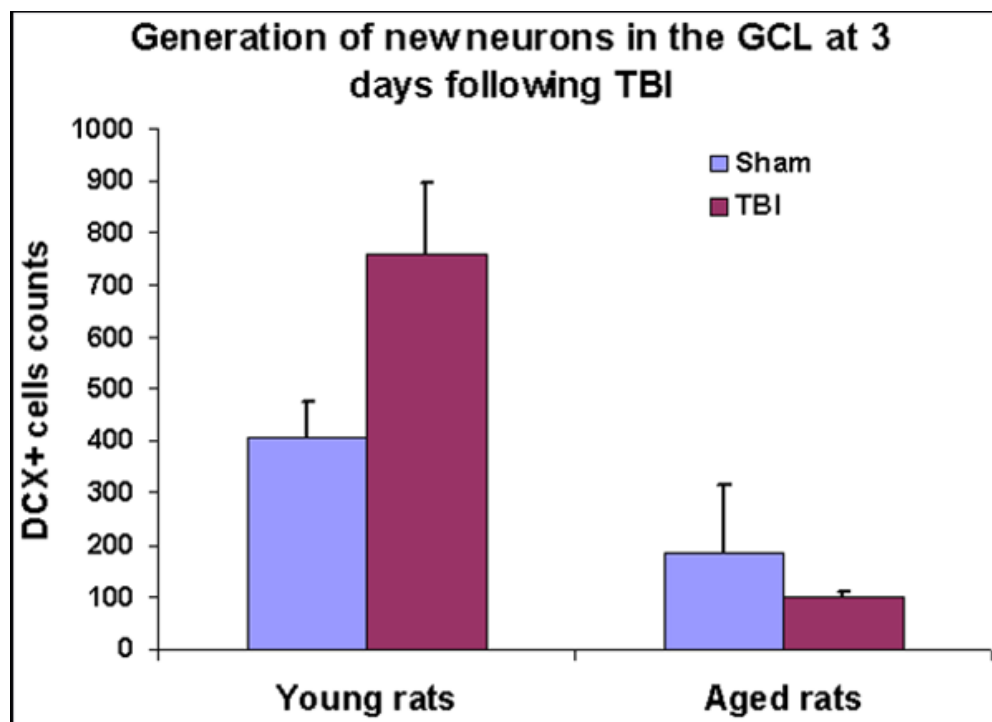
Figure 1.4: Quantification analysis of the number of newly generated neurons in the DG following injury. Taken from previous studies in the Sun lab. DCX+ cells which have horizontally oriented dendrites and mixed dendrites in the GCL representing newly generated neurons were quantified using stereological method. In young animals, injured brain have almost double amount of DCX+ neurons compared to sham animals. In aged brain, injured rats do not have increased number of newly generated DCX+ neurons compared to age matched sham control.

Glial activation and especially microglia activation is a trademark of inflammation in the CNS. Astrocytes and microglia that become activated following disease or injury produce a host of immune mediators. Inflammation is shown to affect neurogenesis in both the SGZ and the SVZ (Pluchino et al., 2008). Many studies have shown that the immune reaction is able to perturb adult NSCs activity and fate through the released signaling molecules such as the complex cascade of both pro-inflammatory and anti-inflammatory cytokines and chemokines (Carpentier and Palmer, 2009). NSCs in the adult brain are influenced by a broad range of inflammatory signaling molecules through their expression of receptors and ligands related to the innate and adaptive immune responses such as toll-like receptors, cytokine and chemokine receptors, major histocompatibility complex I (MHC I), and cell adhesion molecules (Covacu et al., 2009; Johansson et al., 2008; Tran et al., 2007; Carpentier and Palmer, 2009). For example, the pro-inflammatory cytokine IL-6 that is specifically expressed in lipopolysaccaride (LPS) induced microglia, was shown to play a role in the impairment of hippocampal neurogenesis (Monje et al., 2003). Interestingly, Song et al., 2002 showed that despite the negative and positive effects on neurogenesis from activated astrocytes, neural progenitor cells (NPC) co cultured with astrocytes induced a two-fold increase in NPC proliferation and a ten-fold increase in neuronal differentiation of adult rat NPC's.

Studies have shown that inflammation has a strong negative effect on neurogenesis in the adult brain due to the impact of the pro-inflammatory cytokines that are released following the acute injury or disease. The role of pro-inflammatory cytokines produced by activated microglia and astrocytes from the acute innate inflammatory response such as IL-6, IFN-γ, IL-1β, and

TNF-α play a pivotal role in suppressing neurogenesis. IL-6 compromises hippocampal neurogenesis by directing the differentiation of NPCs to astrocytes through the activation of the Janus kinase/signal transducer and activator of transcription and MAP kinase pathways (Nakanishi et al., 2007; Whitney et al., 2009). TNF-α decreases neurogenesis by shifting the differentiation of NPCs to astrocytes and also by inducing neuronal cell death (Peng et al., 2008). IL-1β negatively affects neurogenesis by inducing apoptosis of NPCs through the stress-activated protein kinase/JNK pathway (Wang et al., 2007). Lastly Ben-Hur et al., 2003 showed detrimental effects of IFN-γ on the survival and proliferation of neural stem cells and neural progenitor cells.

Inflammation not only affects neurogenesis in the generation and survival of new neurons, it also influences other aspects of newly generated neurons. It has been shown that inflammatory responses guide the migration of new neurons toward ischemic damage through the chemokine stromal cell-derived factor-1α (SDF-1α) and its receptor CXC4 (Imitola et al., 2004). In an LPS induced neural inflammatory response, Jakubs et al., (2008) found that inflammation not only affects the number of surviving new neurons that integrate themselves in the SGZ, but also alters the function of survived new neurons as neurons generated during the period of inflammation are morphologically normal yet displaying an accentuated inhibitory responses in immature neurons and excitatory responses in mature neurons.

Minocycline as a possible treatment for TBI

Following injury or disease, a number of studies have reported the anti-inflammatory effects of minocycline. Minocycline is a tetracycline derivative antibiotic that has been used

extensively for its effective non-steroidal anti-inflammatory and anti-apoptotic properties (Kim and Suh, 2009). It has been suggested that minocycline's effectiveness is largely due to its superior penetration of the blood-brain barrier (Saivin and Hovin, 1988; Yrjanheikki et al., 1998). Molecularly, minocycline interferes with bacterial protein synthesis by binding to the 30S ribosomal subunit, inhibiting messenger RNA-transfer RNA interaction and protein translation (Elewa et al., 2006).

The effects of minocycline's anti-inflammatory properties have been demonstrated throughout the literature in both acute and chronic brain injury (Elewa et al., 2006). Minocycline influences neutrophils, monocytes, microglia and neurons during inflammation by inhibiting neutrophil migration, degranulation and the formation of oxygen free radicals (Golub et al., 1991). Despite the fact that the mechanisms of action for minocycline treatments have not been fully understood, the beneficial effects stem from the drug's ability to decrease or inhibit microglia activation (Yrjanheikki et al., 1998). In an epilepsy induced animal model, minocycline treatment decreased the number of activated microglia in the brain while increasing the number of newborn neurons (Ekdahl et al., 2003).

The anti-apoptotic effect of minocycline has also made the drug a good candidate as a hopeful avenue for NSC restoration and neurogenesis. Studies have showed that minocycline prevents apoptosis by inhibiting the release of cytochrome c from mitchondria (Zhu et al., 2002), and up-regulation of anti-apoptic protein bcl-2 mRNA levels in neurons (Matsukawa et al., 2009). The effect of minocycline in the injured brain has been reported in several studies. For example, Mejia et al., (2001) showed that following TBI, administration of minocycline reduced the levels of caspase-1 (known as IL-1β converting enzyme), decreased post-traumatic lesion size, and increased levels of cognitive recovery. In a focal TBI model, it was reported that

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minocycline decreased the density of activated microglia and lesion size without significance effect in cognitive function or apoptosis (Bye et al., 2007). In combination with N-acetyl-cysteine, heightened cognitive restoration, decreased myelin lost and inhibited the production of IL-1β was observed following a cortical impact injury (Abdel Baki et al., 2010).

Minocycline also has been shown in studies to inhibit matrix metalloproteinases (MMPs). MMPs are proteins that are involved in degenerating extracellular proteins and extracellular matrix components in the brain. In rat arthritis and inflammatory encephalitis models, specific MMP2 and 9 were shown exhibiting reduced levels (Elewa et al., 2006). In summary, the literature supports the postulation that minocycline is able to influence multiple cascades of the secondary injury responses that occur following TBI.

Chapter 2 - Materials and Methods

Subjects

The two groups of animals used for this study were male, Fischer 344 rats (NIA, NIH) aged at 3 months and 20 months weighing approximately at 300 grams and 450 grams respectively. The 3 month-old animals were housed in pairs and the 20 month-old animals were housed individually with food and water supplied *ad libitum*. Both groups were housed at the animal facility in a room that was kept at a temperature of 20 – 22 degrees Celsius following a 12-hour light/dark cycle. All procedures performed during the study complied with NIH guidelines and approved by the Institution of Animal Care and Use Committee of Virginia Commonwealth University.

For the first aim, a total of 9 young animals and 6 aged animals were used for the sham GFAP densitometry data analysis along while 9 young animals and 9 aged animals were used for the sham IBA1 densitometry data analysis. For data analysis of densitometry analysis at different time points following injury observing both GFAP and IBA1 optical intensity: a total of 4 young animals and 4 aged animals were used for 3 dpi time point; 2 young animals and 5 aged animals were used for 7 dpi time point; lastly, 4 young animals and 4 aged were used for the 28 dpi time point.

For the second aim, a total of 2 young rats and 2 aged rats were used for the densitometry analysis for measuring both GFAP and IBA1 optical density following minocycline treatment.

Surgical preparation and procedure

Following a standard protocol, a moderate lateral fluid percussion injury (FPI) was performed to all animals used in the study. All surgical tools were sterilized through an autoclave procedure, and surgeries were performed in an aseptic setting. Each rat from both age groups (3 months and 20 months) endured the same surgical procedure under the same conditions. Adult rats were anesthetized in a plexiglass chamber with 5% isofluorane, intubated and ventilated with 2.5% isofluorane in a gas mixture (30% Oxygen, 70% nitrogen). The animals were then secured in a stereotaxic frame. The top of their heads were cleaned and scrubbed with Betadine to sterilize the site of incision, and Puralube ointment was placed over their eyes to prevent dryness. A midline incision was made to expose the skull, and the surrounding connective tissues on both sides were retracted using hemostats. Using a fitted trephine and a Dremel drill with a small drill bit, a 4.9 mm craniotomy was made on the left parietal bone midway between the lambda and bregma sutures (**Fig 2.1**). The resulting bone chip was then removed carefully, ensuring that the dura underneath was not breached. Afterwards, a modified Luer lock fitting was cemented onto skull over the craniotomy with an acrylic mixture. Once the mixture was completely dry, the hub was filled to brim with saline solution in order to check for leakage and to provide a continuous medium for the lateral fluid percussion injury device.

Lateral fluid percussion injury and recovery

Once surgery had been completed and the Luer lock hub stabilized, the animals were then taken off anesthesia for approximately 5 minutes or until consciousness was regained by checking tail and toe reflexes. During this time, the fluid percussion injury device was calibrated by repeated measurements of pressure every 30 seconds for 5 minutes, ensuring that the device

had reached consistency in delivering the target pressure of 1.8 ± 0.05 atm (**Fig 2.2**). The intubation tube was then disconnected, and the animals were transferred from the stereotaxic frame to the fluid percussion injury device. Once the animals were connected to the injury device through the Luer lock-fitting hub, a moderate fluid impulse (1.8 ± 0.05 atm) was administered. Immediately following the injury, the animals were taken back to the aseptic surgery bench in order to remove the Luer lock hub.   They were then laid on a supine position with a few pads of gauze covering the open incision, and reconnected to the intubation tube without isofluorane for ventilation. As the animals recovered from the injury, their tail and toe reflexes were periodically checked and recorded at the moment of first recovery response. The righting time, the moment after injury when the animal would begin returning to an upright position, was also recorded as the last measure of recovery. The animals were then placed under anesthesia again with 2.5% Isofluorane as the wound was sealed using a sterile stainless steel suture needle and 5-0 polyamide surgical suture in a simple continuous pattern. For post-surgery treatment, the wound was covered with triple antibiotic ointment and 2% lidocaine hydrochloride jelly. To retain body heat and provide comfort, the animals were then taken back to their cages that had been placed on heating pads with a surgical drape providing interior bedding. After 3 hours of post surgery observation and maintenance, the rats were taken back to the animal housing facility. Sham animals underwent the identical surgical procedures but were not given the injury pulse.

BrdU injections

5-Bromo-2'-deoxyuridine (BrdU, Sigma), a synthetic nucleoside that is analogous to thymidine within the DNA, was administered to the animals in order to label newly dividing cells after injury. Both age groups, the young adult (3 months) and aged (20 months) animals were divided into three groups for BrdU injection. For the three-day post injury group, all rats received a total of four intra-peritoneal (i.p.) injections of BrdU at 50 mg/kg (dissolved in sterile saline) every two hours at forty-eight hours after injury (2 days post injury). The seven-day post injury and twenty-eight day post injury groups also began receiving injections forty-eight hours
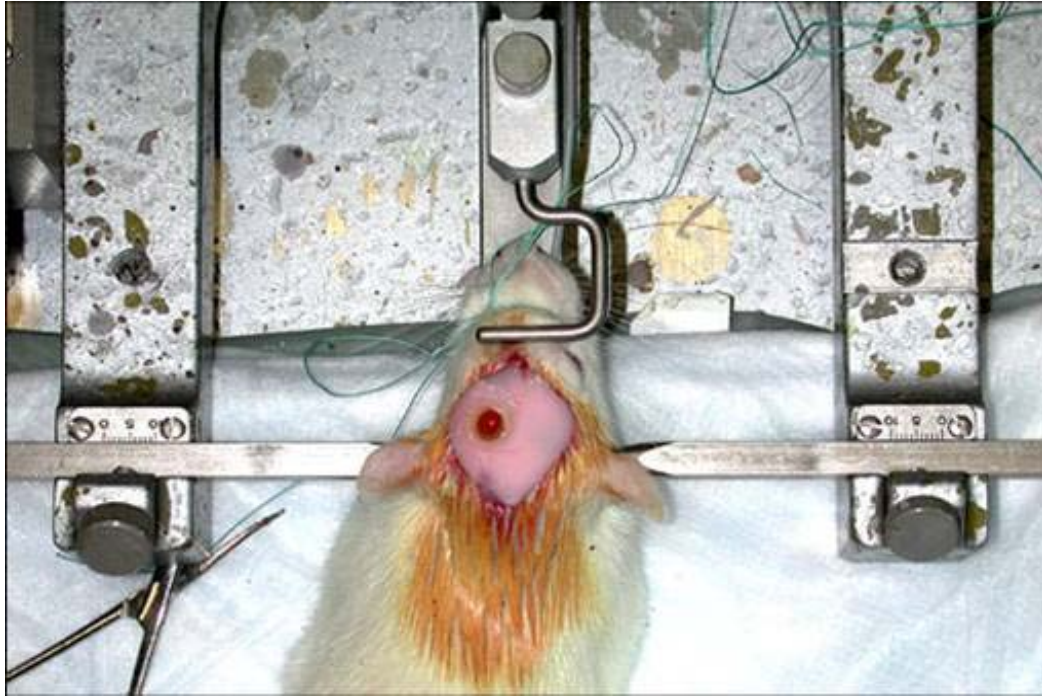
Figure 2.1: Craniotomy and injury site. A 4.9 mm craniotomy was made on the left parietal bone half way between the lambda and bregma sutures which served as the site where fluid percussion injury was delivered.
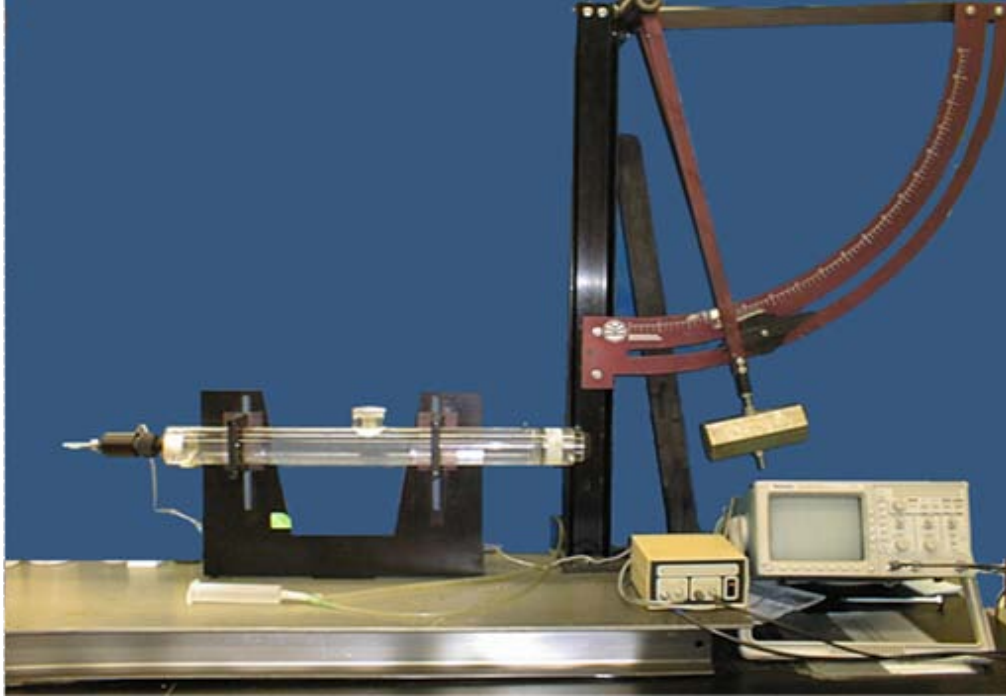
Figure 2.2: A photograph of a fluid percussion injury device.

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after injury. All animals in these two time groups were given single intra-peritoneal injections of 50 mg/kg per body weight daily for six days from day 2 to day 7 post injury. For clarification, the twenty-eight day post injury group did not receive any further BrdU injections after the seventh day post injury.

A second group of animals, consisting of both age groups of three months and twenty month old rats, were sacrificed at the three days post injury time point also followed the same procedure in receiving BrdU injections. This group however, was subjected to an anti-inflammatory treatment.

Minocycline injections

Minocycline (Sigma) is a widely used synthetic tetracycline antibiotic with known anti-inflammatory and anti-apoptic properties in several models for brain injury such as stroke and traumatic brain injury (Elewa et al., 2006). This treatment was given to a group of 3 months and 20 months animals and sacrificed at three -day post injury (N=2 for each age group). The animals first received 45 mg/kg of minocycline (dissolved in sterile saline, neutral pH maintained) thirty minutes after the initial fluid percussion injury. The same dosage (45 mg/kg) was administered again nine to twelve hours following the first injection on the same day of surgery. On the first and second day post injury, the animals were injected twice daily with 22.5 mg/kg of minocycline at nine to twelve hours between. The protocol was consistent for both the young adult (3 month) and aged (20 month) rats. Animals received BrdU injection at 48 hr after injury following the protocol described above and were sacrificed at three days post injury.

Tissue Processing

All animals, young adult and aged, were euthanized at either three days, seven days or twenty-eight days post injury. These rats were deeply anesthetized with isofluorane until their breathing had completely ceased, and then transcardially perfused with 400 ml of 1x Phosphate buffer solution (PBS) followed by 400 ml of 4% paraformaldehyde + PBS solution. Afterwards, the brains of each animal was dissected out of the skull and further fixed in 4% paraformaldehyde + PBS solution for at least forty-eight hours. These brains were stored at 4°C allowing for complete preservation of the freshly dissected tissue.

After 48hr fixation in 4% paraformaldehyde, the brain was stored in PBS plus 0.01% sodium azide before slicing. On the day of making coronal sections of the brains, the 4% paraformaldehyde + PBS solution was changed to PBS. For tissue slicing, a vibratome was then used to make 60 μm coronal sections beginning from the rostral end of the brain, through the hippocampus were sliced with a vibratome (Leica). The sections were collected into twenty-four well plates filled with a PBS + 0.01% sodium azide solution, and were stored in a 4°C refrigerator. A total of four groups consisting of thirty coronal sections were collected, filling a total of five twenty-four well plates in order to have enough tissue available when conducting different staining protocols.

Immunohistochemistry

For different markers, the number of sections stained varied. For fluorescent immunostaining for GFAP (marker for astrocytes) or IBA1 (marker for microglia), two coronal sections from a rostral and caudal section of the hippocampus 960um in between were stained.

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Sections were washed with PBS and then incubated with 3% hydrogen peroxide for 1 hr at room temperature to block endogenous peroxidase. The brain sections were rinsed with a 1x PBS + 0.3% Triton solution for ten minutes and were blocked overnight at 4°C in blocking buffer solution (5% normal horse serum + 1x PBS with 0.3% Triton).

The next day, the tissue sections were treated with the primary antibody solution. The primary antibody solutions were rabbit anti-GFAP (1:1000, Dako, CA) or rabbit anti-Iba1 (1:1000, Wako) in blocking buffer solution as mentioned previously. 300 µl of the primary antibody solution was placed in each well. The sections were incubated at 4°C for forty-eight hours on shaker.

Once the sections were brought back to room temperature after being incubated with the primary antibody solution, they were rinsed with 1x PBS with 0.3% triton solution for ten minutes and repeated two more times. The sections were then blocked for three hours in blocking buffer solution. Afterwards, the sections were given 300 µl of secondary antibody solution and incubated at 4°C overnight. The secondary antibody solution was prepared with a 1:200 dilution of Alex Fluor 488 anti-rabbit IgG (Invitrogen) in blocking buffer solution.

Upon the completing the incubation with the secondary antibody solution, the sections were brought back to room temperature, washed with 1x PBS for ten minutes three times, rinsed with distilled water (dH$_2$0) and mount the finished tissue sections onto glass microscope slides coverslipped with Vestorshield (Vector Lab).

Immunofluorescent double-label staining

The selected coronal sections were rinsed twice with PBS for five minutes, and then denatured in a 50% formamide solution at 65°C for one hour. Sections were then rinsed with 2x

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saline sodium citrate (SSC) for five minutes at room temperature. The sections were further denatured in 2N hydrochloric acid (HCL) for thirty minutes at 37°C. Following the completion of the denaturation process, the sections were rinsed twice with PBS for five minutes each wash. Once the PBS washing was complete, the tissue sections were treated with 3% hydrogen peroxide for one hour at room temperature to block endogenous hydrogen peroxidase. The primary antibodies used were anti-mouse BrdU combined with anti-rabbit GFAP (1:200 for BrdU, 1:1000 for GFAP; Dako, CA) or anti-mouse BrdU with anti-rabbit Iba1 (1:200 for BrdU, 1:1000 for Iba1, Wako). The secondary antibody solution was prepared with a 1:200 dilution of an Alexa Fluor 488 anti-mouse IgG along with a 1:200 dilution of an Alexa Fluro 568 anti-rabbit IgG in blocking buffer solution. After incubation with the primary and secondary antibodies, a 4', 6-diamidino-2-phenylindole (DAPI) solution (1:1000 in PBS) was then added to the tissue sections for a ten-minute incubation for nuclei staining. Lastly, the sections were washed with 1x PBS three times and then each section was quickly rinsed with distilled water before they were mounted onto glass slides and coverslipped with Vectorshield (Vector Labs, CA) and sealed with nail polish.

Densitometry Image Analysis

This procedure was used to measure the fluorescent optical intensity of GFAP and Iba1 staining. This procedure allowed us to compare the fluorescent optical intensity between the three-month and twenty-month age groups as well as the different time points post injury of a particular cell specific marker.

The fluorescent antibody tagged tissue sections were examined under a 4x objective lens on an Olympus fluorescent microscope. The sections were then scanned under a 488 nm or 568 nm wavelength light measurements in order to visualize the specific glial cells that had been labeled with GFAP or Iba1. To set the exposure time, sections from sham animals for each time point were used as reference points. This was an important step in the protocol because the tissue samples that were used for this experiment were not all stained for the specific cell markers at the same time. Some samples that were examined for this quantification study had previously been stained and photographed from past experiments. Therefore, it was imperative in finding the closest reference point so that the different age groups at different time points post injury could be compared. Once the exposure time of the sham groups was set, the ipsilateral dentate gyrus region in the hippocampus was photographed for all the tissue sections.

The photo files were then transferred to the computer program IPLAB, a densitometry program that was used to measure the amount of fluorescent optical intensity of glial cells marked with a specific fluorescent antibody within a region of interest. The segmentation feature of this program was the main tool for measuring fluorescent intensity. This feature was designed to mark and overlap the areas of fluorescence detected. Therefore, the settings placed on this feature also had to be controlled in order to prevent from over or under coverage of fluorescence. This was also done by adjusting the minimum and maximum sensitivity of coverage to the sham animal groups within each time point post injury. Once the segmentation level was determined, the region of interest was then traced over the photo of the dentate gyrus. The two regions of interest analyzed were the granule cell layer and the hilus regions. Stringent settings were also placed so that no overlapping fluorescent signals between both regions of interest would occur. This was important in confirming that the regions of interest drawn were

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providing clear boundaries for analysis. The photos were also measured at grayscale in order to place all tissue sections on an equal optic background.

Once the numerical data was obtained from each section measured, it was copied to an excel spreadsheet. Since the staining of the tissue sections was conducted over different time points, setting consistent standards for the sham animal groups first allowed us to use the raw data of fluorescent optical intensity measured from the regions of interest. Another important part of this procedure was that a young adult and aged sham animal group was included for each time point post injury. This was necessary in order to ensure that we were able to measure a change in fluorescent optical intensity from sham animal groups to injured animal groups. This allowed us the ability to also compare between the young and aged groups because once the sham young levels were set first, these settings were then held consistent throughout each batch of tissue sections for their respective time point, allowing us to look at the normalized changes in GFAP or IBA1 expression at each time point compared to their sham groups.

Confocal microscopy

This procedure was used to quantify the number of immunofluorescent double labeled cells that were marked with either GFAP and BrdU or IBA1 and BrdU. The granule cell layer regions of the tissue sections were examined on a Leica confocal microscope (TCS SP2 model) under a 40x oil immersion lens. The entire span of the granule cell layer on the ipsilateral side of the injury was examined by looking through its entire thickness in order to examine every BrdU-positively labeled cell. Only BrdU-specific fluorescently labeled cells with an unambiguous nucleus was deemed a positive cell. Double labeling with either Iba1 was seen through the

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multiple channels that were set to co-visualize the different light intensities of each fluorescent antibody. For quantification, the total numbers of BrdU-positive cells were compared to the number of cells that were positive for BrdU and either Iba1 or GFAP.

Data analysis

All statistical analysis of data was done using SPSS software. A univariate ANOVA was used to determine any statistically significant differences in densitometry analysis and cell quantification data. Post-hoc student t-tests (only two-tail t-tests were considered) were used to determine differences within groups within groups when cross comparing between the young and the aged animal groups, with p-values less than 0.05 being considered statistically significant.

Chapter 3 - Results

In preliminary studies conducted in our lab, we found that the aged brain exhibited increased expression of inflammatory cell markers such as Iba-1, ED1, and GFAP in the dentate gyrus of the hippocampus following TBI. We have also found that the aged brain shows decreased neurogenic capacity in the hippocampus following injury. Based on these observations, this study sought to establish the connection between the heightened levels of inflammatory cell responses and decreased levels of neurogenesis in the injured aged hippocampus by comparing the degree of glial cell responses and neurogenic cell response following injury between aged brains and younger brains. The first aim is to examine the differences of glial cell responses in the aged and younger brains. The second aim is to assess whether an anti-inflammation treatment with minocycline will down regulate the injury-induced inflammatory cell response and upregulate neurogenesis in the aged and young brains. For the first aim, brain tissue samples from twenty-three 20 month-old Fisher 344 rats and twenty-three 3 month-old Fisher 344 rats were used. For Aim2, a total of six Fisher 344 rats at 20 months of age along with six Fisher 344 rats at 3 months of age were used.

Morphological changes of astrocytes and microglia following TBI

Following injury, astrocytes and microglia transformed morphologically and functionally from a resting, inactivated state to a more robust, activated state. The morphological differences between resident microglia and astrocytes compared to their activated counterparts were observed within the dentate gyrus of the hippocampus. Compared to the resident microglia

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mostly seen in sham animals, activated microglia showed an increased size of cell body and shorter projections as a result of TBI (**Fig 3.1 a-b**). Compared to inactive astrocytes, activated astrocytes also showed increased density and size of the cell bodies while displaying an increase in the length and thickness of their projections following TBI (**Fig 3.1 c-d**). These morphological changes were consistent in the observed granule cell layer and hilus regions within the hippocampus.

Levels of GFAP expression at different time points in the dentate gyrus following TBI

GFAP, an intermediate filament protein expressed by astrocytes, is used as a marker for astrocyte activation. The level of GFAP expression was quantified using densitometry analysis. GFAP expression in sham young and aged animals was used as the standard for comparison for their respective injured groups. The levels of GFAP expression at different time points after injury in the injured animals were measured in granule cell layer (including the SGZ) and the hilus regions in the dentate gyrus of the hippocampus. The granule cell layer and the hilus regions were analyzed separately.

We found that the sham young animals displayed greater GFAP expression in both the granule cell layer and hilus regions compared to the aged sham animals (**Fig 3.2**). However, the differences between the young and aged animal groups in both regions were not statistically significant (**Fig 3.2**).

Following TBI, in the young injured animals, GFAP expression was increased at 3 and 7 days post injury (dpi) while showing decreased levels at 28 dpi time point post-TBI in both the GCL and hilus regions in the dentate gyrus (**Fig. 3.3**). GFAP expression was greatest at 7 days post injury in both the GCL and hilus regions (**Fig 3.3 C**). Statistical analysis revealed that in the

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GCL region, compared to the sham group, the optical intensity levels measured at 3 dpi and 7dpi were significantly greater (Fig 3.5 a, p = 0.02 and p = 0.003 respectively) while the 28 dpi time point did not show a significant difference (Fig 3.4 a). When comparing the optical intensity within the young injured group at different time points, GFAP expression at 7 days post injury between 28 days post injury reached a statistic significance (p = 0.019) (Fig 3.4 a). Statistical analysis in the hilus region showed that GFAP expression in the injured animals was significantly higher in the 7dpi and 28 dpi time points compared to sham group (Fig 3.4 b, p = 0.033 and p = 0.014 respectively) whereas the 3 dpi time point compared to the sham group did not reach significance. Optical intensity measured between the different time points following injury did not show statistical significance.

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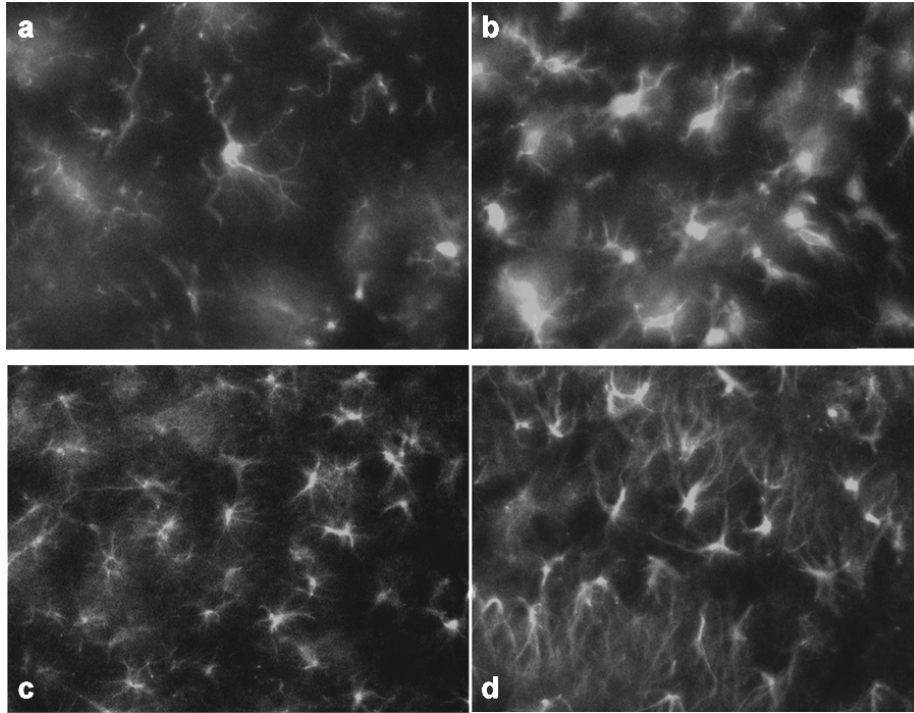
Figure 3.1: Morphological changes of microglia and astrocytes following injury.

Photographs taken from sections stained with Iba1 or GFAP in the ipsilateral DG. (a) Resting microglia displays a smaller cell body, long and thin processes. (b) Activated microglia possesses a larger cell body and short, sturdy processes. (c) Resting astrocytes display a smaller cell body and short star-like processes; (d) Activated astrocytes display bigger cells bodies and longer, thicker processes.

GFAP expression in the sham rats

■ 3M GCL
□ 20M GCL
□ 3M hilus
■ 20M hilus
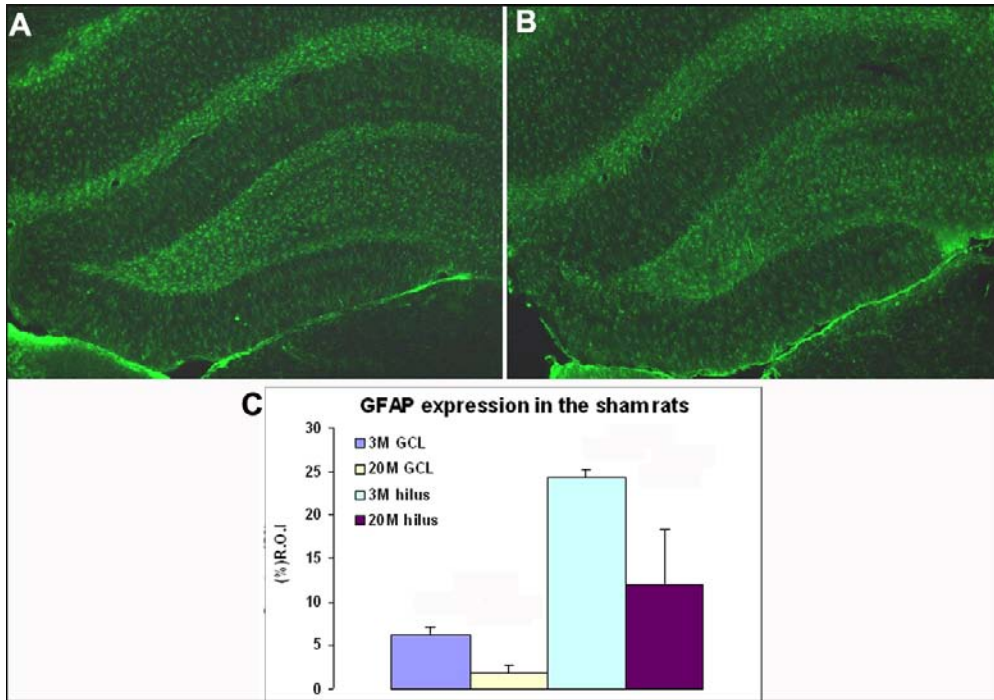
Figure 3.2: Comparison of baseline GFAP expression in sham young and aged rats with quantitative analysis of the degree of GFAP fluorescent optical intensity in both the GCL and hilus regions in the DG. Photographs taken from GFAP stained coronal sections in the ipsilateral DG from sham young (A) and aged animals (B). (C) Densitometry analysis showed that in the GCL (SGZ included), compared to the sham aged animals, sham young animals had a greater level of baseline GFAP signaling intensity but the difference was not significant ($F_{1, 13}$ = 2.718, p = 0.123). The hilus region of the sham young animals also had a greater baseline GFAP optical intensity levels when compared to the sham aged animals but the difference was not significant ($F_{1, 13}$ = 3.258, p = 0.094). Error bars represent the standard error mean.

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Figure 3.3: Astrocytic response following TBI in the young adult rat brain at 3, 7 and 28 days post injury. Photographs taken from GFAP stained coronal sections in the ipsilateral DG of the hippocampus in sham animal, 3, 7 and 28 days post injury. Graph represents densitometry analysis of GFAP staining intensity at different time points. In the GCL, measured GFAP optical intensity levels were significantly greater at the 3 dpi (*$p < 0.05$) and 7 dpi time points post-injury (**$p < 0.01$) compared to sham animal groups. The differences of GFAP optical intensity compared between the 7 dpi and 28 dpi time points showed a significant difference (*$p < 0.05$). In the hilus region, GFAP optical intensity levels was significantly greater compared to sham young animal groups at the 7dpi and 28 dpi time point post-injury (* $p < 0.05$). Differences between the GFAP intensity levels within the different time points did not show significance.

In the aged injured animals, GFAP expression was increased at 3 and 7 days post injury while showing decreased levels at 28 dpi time point post-TBI in the GCL while the hilus region in the showed a gradual increasing trend (**Fig. 3.4**). GFAP expression was greatest at 3 days post injury in the GCL region and at 28 days post injury in the hilus region (**Fig 3.4**). Statistical analysis revealed that in the GCL region, compared to the sham group, the optical intensity levels measured at 3 dpi and 7dpi were significantly greater (**Fig 3.4**, $p = 0.002$ and $p = 0.002$ respectively) while the 28 dpi time point did not show a significant difference. When comparing the optical intensity within the aged injured group at different time points, GFAP expression at 3 days post injury between 28 days post injury reached a statistic significance (**Fig 3.4**, $p = 0.044$). Statistical analysis in the hilus region showed that GFAP expression in the injured aged animals was significantly higher in the 7dpi and 28 dpi time points compared to sham group (**Fig 3.4**, $p = 0.038$ and $p = 0.019$ respectively) whereas the 3 dpi time point compared to the sham group did not reach significance. Optical intensity measured between the different time points following injury did not show statistical significance.

Aged Sham | Aged 3dpi

Aged 7dpi | Aged 28dpi

GFAP expression in the GCL in the 20M old rats
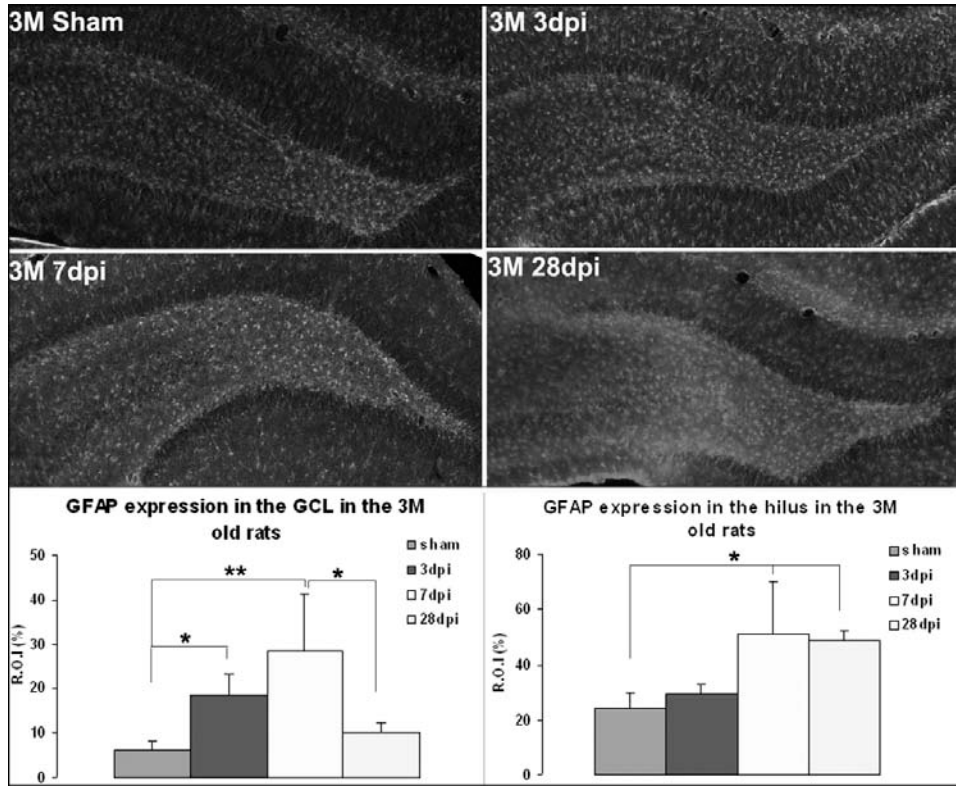
GFAP expression in the hilus in the 20M old rats
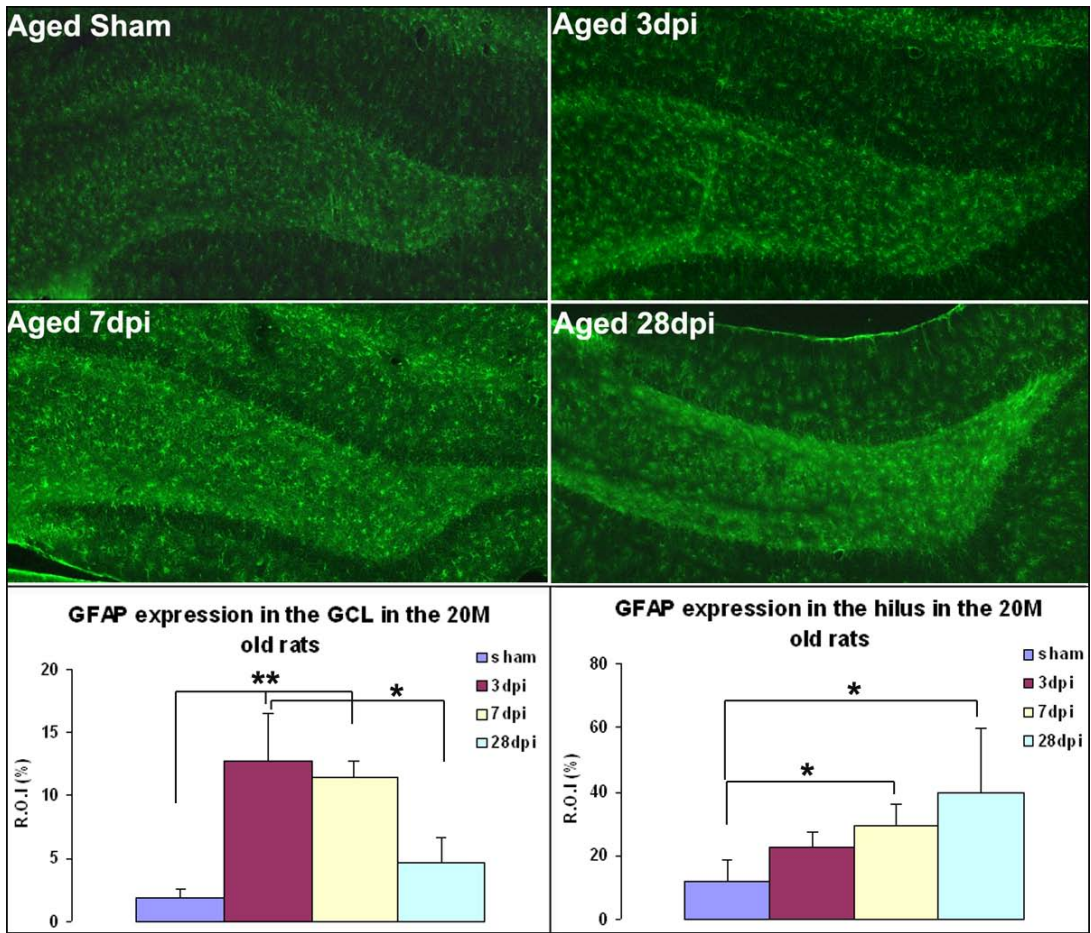
Figure 3.4: Astrocytic response following gTBI in the aged brain at 3, 7, and 28 day post injury. Photographs taken from GFAP stained coronal sections in the ipsilateral DG of the hippocampus in sham animal, 3, 7 and 28 days post injury. Graph represents densitometry analysis of GFAP staining intensity at different time points. In the GCL, measured GFAP optical intensity levels were significantly greater at the 3 dpi and 7 dpi time point post-injury compared to sham animal groups (** $p < 0.01$). The differences of GFAP optical intensity compared between the 3 dpi and 28 dpi time point reached significance (*$p < 0.05$). In the hilus region, GFAP optical intensity levels were also significantly greater compared to sham aged animal groups at 7 dpi and 28 dpi time points post-injury (* $p < 0.05$). Differences between the GFAP intensity levels within the different time points in the injured groups did not show significance.

Cross comparison of astrocyte activation between young and aged injured animal groups

Compared to the aged groups, the young adult animal groups displayed greater levels of GFAP optical intensity in sham animal and in injured groups in all three post injury time points in both the GCL and hilus regions. However, two tail t-test analyses revealed that the differences between two groups were no statistically significant in both GCL and hilus regions (**Fig 3.5**). Secondly, we looked at the normalized data in order to examine the changes in optical intensity compared to sham groups at each time point. Using our sham groups as a baseline 100%, the respective young and aged animals were then examined by looking at their difference from the 100% frame of reference. Compared to the young groups, the aged animal groups displayed greater levels of GFAP optical intensity changes at all three post injury time points in both the GCL and hilus regions. Again, at each time point the optical intensity measured was compared to sham levels. However, two tail t-test analyses revealed that the differences between two groups were no statistically significant in both GCL and hilus regions (**Fig 3.6**).

GFAP expression in the GCL in the 3M and 20M old rats
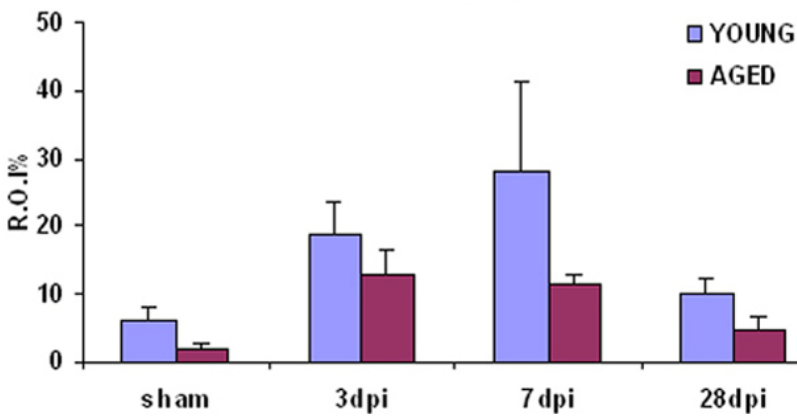
GFAP expression in the hilus in the 3M and 20M old rats

Figure 3.5: Comparison GFAP expression between aged and young animal groups in both the GCL and hilus regions. In the GCL and hilus regions, measured GFAP optical intensity levels displayed that younger animals both sham and injured at all three time points were greater than the aged animals. However, two-tail t-test analysis showed no statistical significance between the groups at each time point.

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Changes in GFAP expression relative to Sham in the GCL for 3M and 20M old rats

Changes in GFAP expression relative to sham in the Hilus for 3M and 20M old rats

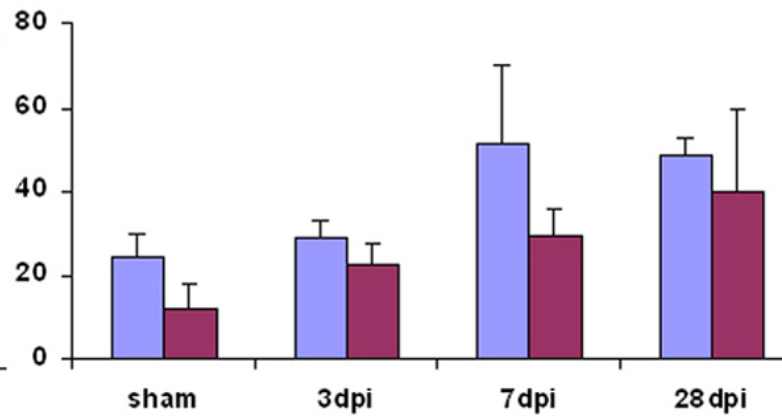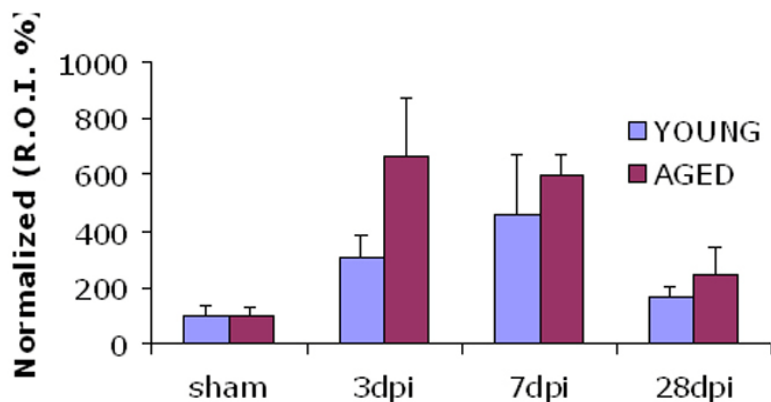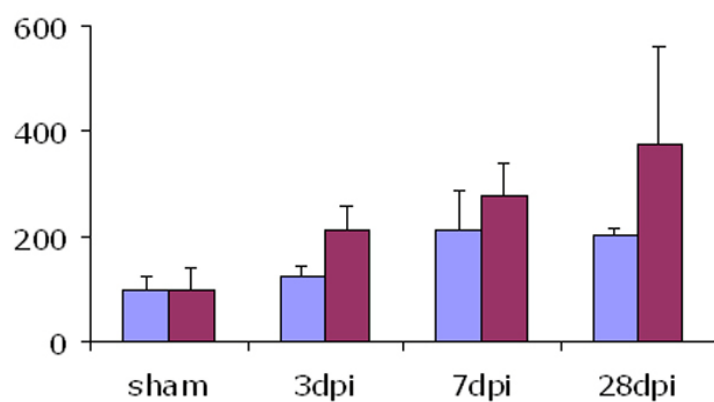**Figure 3.6: Normalized comparison of GFAP expression between aged and young animal groups in both the GCL and hilus regions.**   In the GCL and hilus regions, the normalized GFAP optical intensity levels displayed that younger animals at all three time points showed less changes relative to sham groups than the aged animals. However, two-tail t-test analysis showed no statistical significance between the groups at each time point.

Levels of microglial marker Iba1 expression at different time points in the dentate gyrus following TBI

Microglia is described as the resident macrophages in the brain parenchyma, and they comprise approximately 15% of the total cells within the brain (Mathieu et al., 2010). Once microglia are activated following a traumatic insult or disease, they release a series of inflammatory cytokines into the brain. They are the major players that contribute to the inflammatory response due to their release of pro-inflammatory cytokines which cause a positive feedback loop creating a potentially dangerous state for the brain that is exacerbated especially in the aged population.

To assess the level of injury-induced microglia activation, we used Iba1 as a marker to label microglia. Iba1 is a calcium binding protein expressed by resting and activated microglia. The Iba1 staining intensity in the GCL and hilus of ipsialteral DG was quantified using densitometry analysis. Similar to GFAP quantification, Iba1 staining in the sham brain was used as the standard for comparison. In sham both young and aged animal, Iba1-labeled microglia are predominant at resting state with small irregular nuclear and fine slender processes. Densitometry analysis revealing that the baseline microglia Iba1 expression was higher in the aged sham compared to the sham young animal group in both the GCL and the hilus regions (**Fig. 3.7**). ANOVA analysis showed that the differences of Iba1 optical intensity between the sham aged and sham young were not statistically significant in the GCL region (**Fig 3.7**, $F_{1, 16} = 2.812$, $p = 0.113$). In the hilus region, statistical analysis showed that the sham aged animal group exhibited a significantly greater levels of IBA1 measurements compared to the sham young group (**Fig 3.7**, $F_{1, 16} = 4.538$, $p = 0.049$).

Baseline Iba1 expression
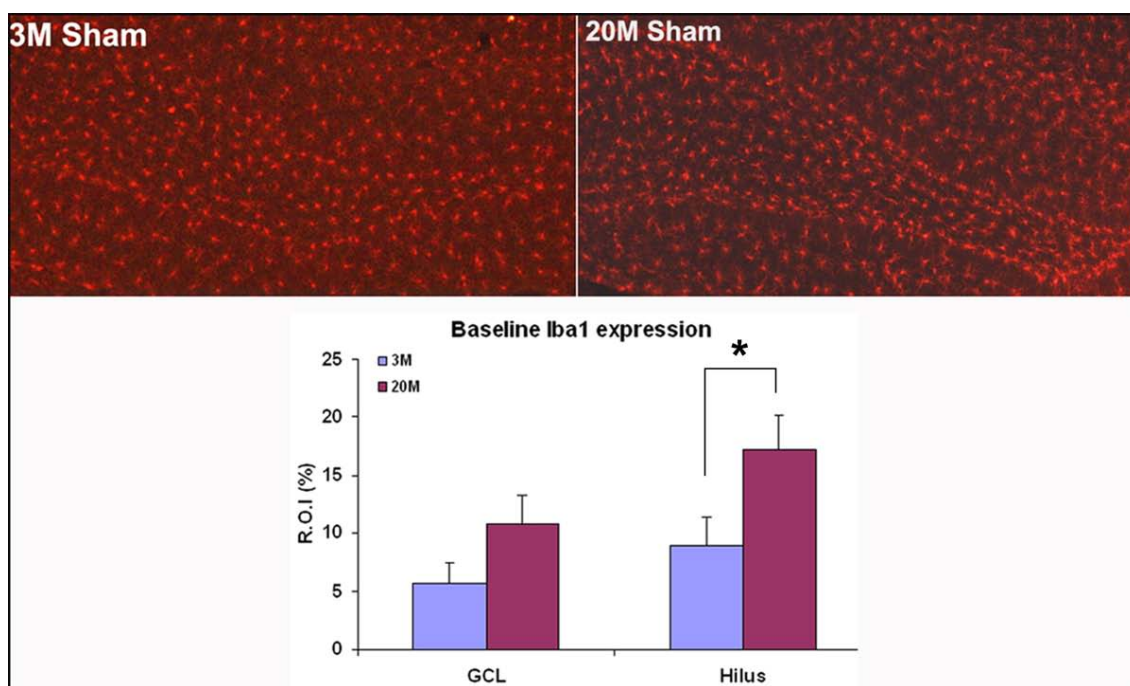
Figure 3.7: Comparison of baseline microglial Iba1 expression in sham young and aged rats with quantitative analysis in both the GCL and hilus regions in the DG. Photographs taken from Iba1 stained coronal sections in the ipsilateral DG from sham young and aged animals. Densitometry analysis revealed that in the GCL (SGZ included), compared to the sham young animals, sham aged animals had a greater level of baseline Iba1 expression but the difference was not significant ($F_{1, 16} = 2.812$, $p = 0.113$). In the hilus region, the sham aged animals also had a greater baseline Iba1 optical intensity levels when compared to the sham young animals and the difference was significance (* $p < 0.05$)

Following injury, in the young injured animals, Iba1 expression was increased at 3 dpi and 28 dpi while showing decreased levels at 7 dpi in the GCL region. In the hilus region, IBA1 showed an increasing trend in optical intensity levels measured at each successive time point (**Fig. 3.8**). Iba1 expression was greatest at 28 days post injury in both the GCL and hilus regions (**Fig 3.8**). Statistical analysis revealed that in the GCL region, the optical intensity levels measured at 3 dpi and 28 dpi were significantly greater compared to the sham young group (**Fig 3.8**, $p = 0.001$ and $p = 0.003$ respectively) while the 7 dpi time point did not show a significant difference ($p = 0.132$). When comparing the optical intensity within the injured groups at different time points, the differences were not statistically significant. In the hilus region, Iba1 expression in the injured animals was significantly higher at the 3 dpi, 7dpi and 28 dpi time points compared to sham group (**Fig 3.8**, $p = 0.003$, $p = 0.014$ and $p = 0.001$ respectively). Optical intensity measured between the different time points following injury in the hilus region did not show statistical significance.

Iba1 expression in the GCL in the 3M old rats

Iba1 expression in the hilus in the 3M old rats

Figure 3.8: Microglial cell response following TBI in the young adult brain at 3, 7, and 28 day post injury. Photographs taken from Iba1 stained coronal sections in the ipsilateral DG of the hippocampus in sham animal, 3, 7 and 28 days post injury. Graph represents densitometry analysis of Iba1 staining intensity at different time points. In the GCL, measured Iba1 optical intensity levels were significantly greater at the 3 dpi and 28 dpi time points post-injury compared to sham animal groups (**$p < 0.01$). In the hilus region, Iba1 optical intensity levels at all three time points post injury were significantly greater compared to sham animal groups (**$p < 0.01$).

In the injured aged animals, Iba1 expression was increased at 3 dpi and 28 dpi while showing decreased levels at 7 dpi in the GCL and the hilus region had a gradual increasing trend (**Fig. 3.9**). Iba1 expression was greatest at 28 days post injury in both the GCL and hilus regions (**Fig 3.9**). Statistical analysis revealed that in the GCL region, compared to the sham group, the optical intensity levels measured at 3 dpi and 28 dpi were significantly greater (**Fig 3.9**, p = 0.009 and p = 0.009 respectively) while the 7 dpi time point did not show a significant difference. When comparing the optical intensity within the aged injured group at different time points, Iba1 expression did not reach statistic significance. Statistical analysis in the hilus region showed that Iba1 expression in the injured aged animals was significantly higher in the 7dpi and 28 dpi compared to sham group (**Fig 3.9**, p = 0.017 and p = 0.002 respectively) whereas the 3 dpi did not reach significance when compared to the sham group. Optical intensity measured between the different time points following injury did not show statistical significance.

Cross comparison of microglial activation between young and aged injured animal groups

The aged injured animal group displayed greater levels of Iba1 optical intensity in sham and injured groups at all three time points in the GCL and hilus regions. Two tail t-tests were applied to assess the levels of significance between the levels of Iba1 optical intensity from the injured aged and injured young animal groups. There were no significant differences of Iba1 optical intensity at each post injury time point as well as the sham groups in both the GCL and hilus regions (**Fig 3.10**). Secondly, looking at the normalized comparison, the aged injured animal group displayed lower levels of Iba1 optical intensity in compared to sham groups at all three time points in the GCL and hilus regions. Two tail t-tests were applied to assess the levels of significance between the levels of Iba1 optical intensity from the injured aged and injured

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young animal groups. Only at 3 dpi time point, a significant difference of Iba1 optical intensity change was found (**Fig 3.11,** p = 0.012).
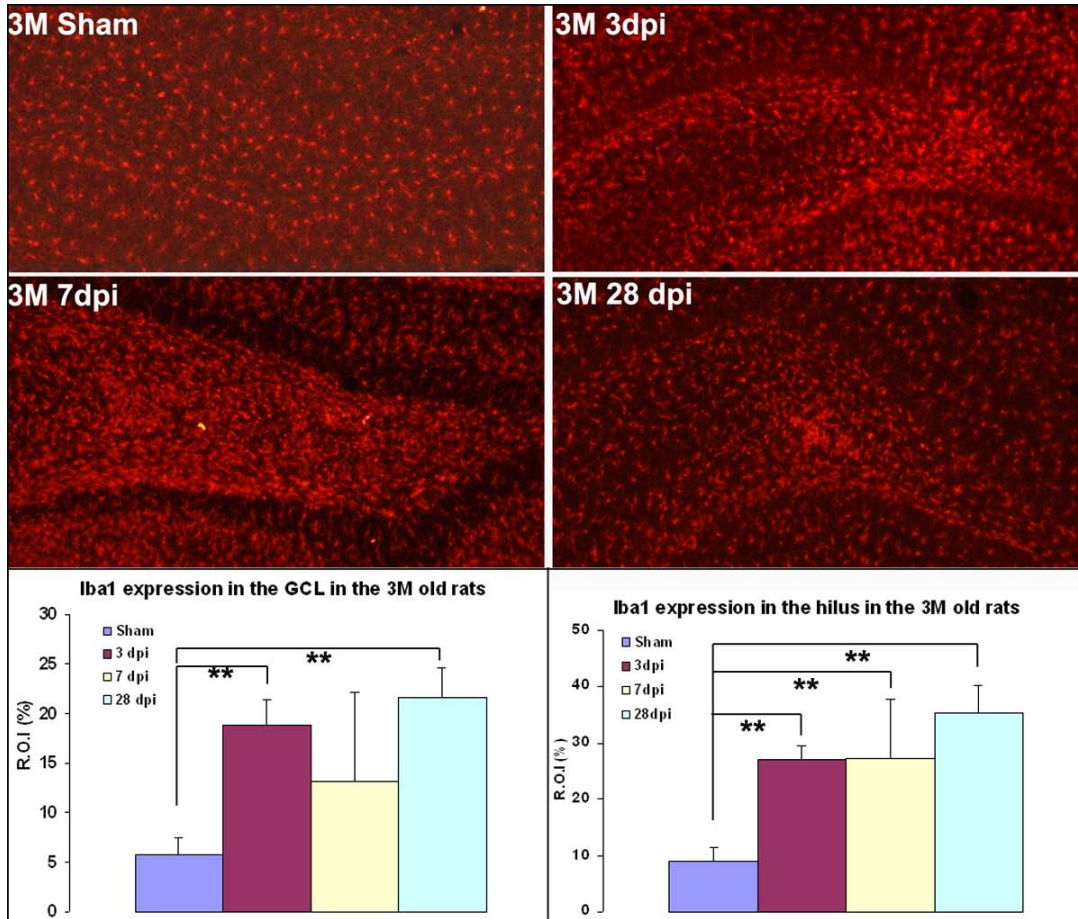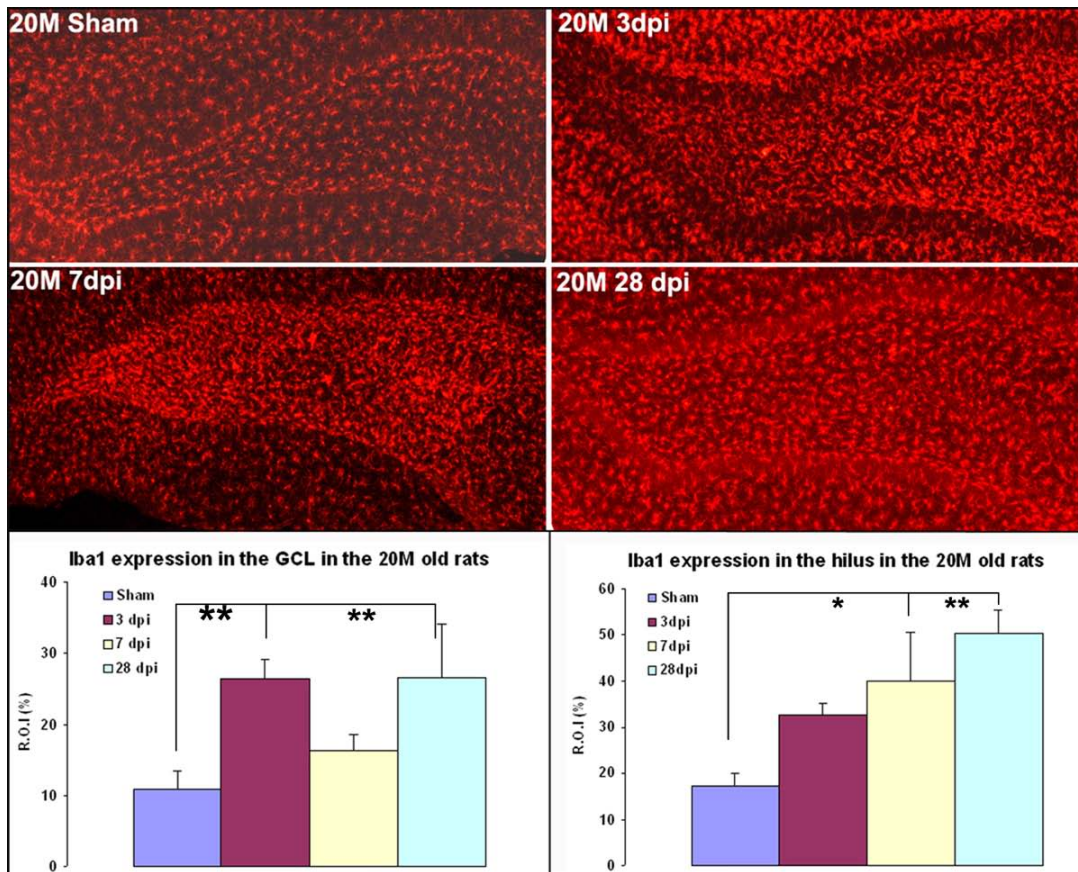
Figure 3.9: Microglial cell response following TBI in the aged brains at 3, 7, and 28 day post injury. Photographs taken from Iba1 stained coronal sections in the ipsilateral DG of the hippocampus in sham animal, 3, 7 and 28 days post injury. Graphs represent densitometry analysis of Iba1 staining intensity at different time points. In the GCL, measured Iba1 optical intensity levels were significantly greater at the 3 dpi and 28 dpi compared to sham animal groups (**$p < 0.01$). In the hilus region, Iba1 optical intensity levels were also significantly greater at the 7 dpi and 28 dpi compared to sham aged animal groups (*$p < 0.05$ and **$p < 0.01$). There were no significant differences within the GCL and hilus regions between the different injury time points following TBI.

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IBA1 expression relative to sham in the GCL for 3M and 20M old rats
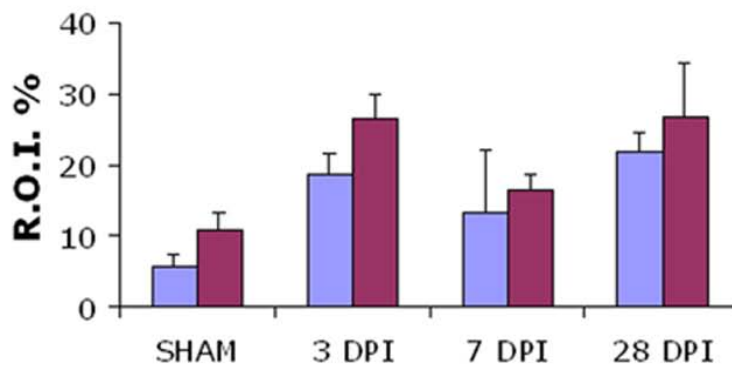
IBA1 expression relative to sham in the hilus for 3M and 20M old rats
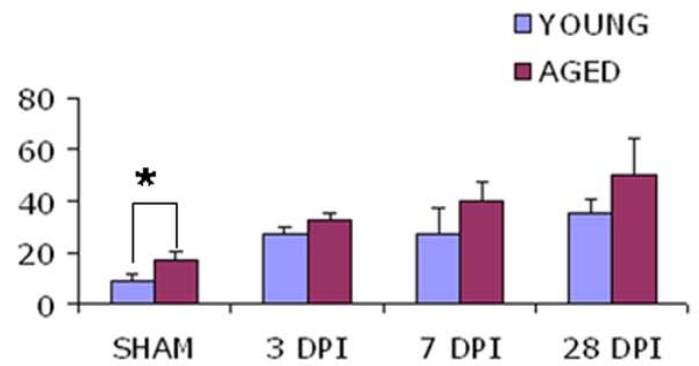
Figure 3.10: Comparison of Iba1 expression between injured aged and young animal groups at 3, 7, and 28 dpi in both the GCL and hilus regions. In the GCL and hilus regions, measured Iba1 optical intensity levels displayed that injured aged animals were greater than injured young animals at all time points. However, two-tail t-test analysis showed no significance between the injured young and aged groups at each time point.

Changed in IBA1 expression relative to sham in the GCL for 3M and 20M old rats
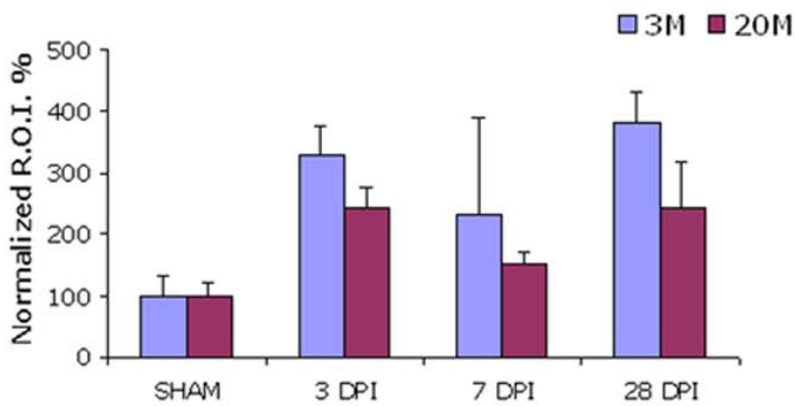
■ 3M ■ 20M

Changes in IBA1 expression relative to sham in the hilus for 3M and 20M old rats

■ 3M ■ 20M
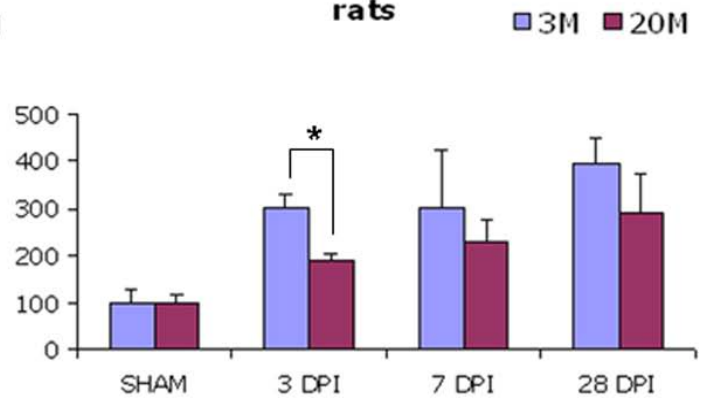
Figure 3.11: Normalized comparison of Iba1 expression between injured aged and young animal groups at 3, 7, and 28 dpi in both the GCL and hilus regions. In the GCL and hilus regions, measured Iba1 optical intensity levels displayed that injured aged animals were greater than injured young animals at all time points. A two-tail t-test analysis showed significance between the injured young and aged groups at each time point at the 3 dpi time point (*p < 0.05).

Percentage of inflammatory cells among injury-induced newly generated cells in the DG in both young and aged animal groups

In previous studies done in our lab, we found that TBI enhances cell proliferation in the injured DG in both young and aged brains (**Fig.1.2**). In order to determine whether inflammatory cells count for the injury-induced proliferating cell population, coronal brain sections from animals sacrificed at either sham, 3 days or 28 days post-injury in both young and aged animals were processed for immunofluorescent double-labeling for mitotic marker BrdU and microglia marker Iba1. Immunofluorescent stained sections were examined using a Leica confocal microscope. BrdU and Iba1 double-labeled cells in the GCL of the ipsilateral DG were quantified. At 3 days post injury, sham young animals displayed a 3.85% co-labeling of BrdU and Iba1. In the injured young animals, around 87.21% BrdU/Iba1 double-labeling was observed. In comparison with the younger counterparts, aged sham animals at 3 days post-injury had 10.21% BrdU/Iba1 double-labeling. Aged injured animals had 84.10% BrdU/ Iba1 co-labeled cells.

At 28 days post injury, young sham animals displayed 5.95% of BrdU positive cells that were co-labeled with Iba1, and injured young animals with 38.7% co-labeling of BrdU and Iba1. In the aged counterparts, sham animals displayed 55.2% of BrdU positive cells that were co-labeled with IBA1, while aged injured animals maintained a high percentage of BrdU and Iba1 double labeling at 81.7% (**Fig.3.12**).

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DAPI + BrdU   IBA1 + BrdU   Overlay

GCL (Granule Cell Layer)

A

3 DPI

IBA1/BrdU

GCL (Granule Cell Layer)

B

28 DPI

IBA1/BrdU

Legend: Young Sham, Young Injured, Adult Sham, Adult Injured
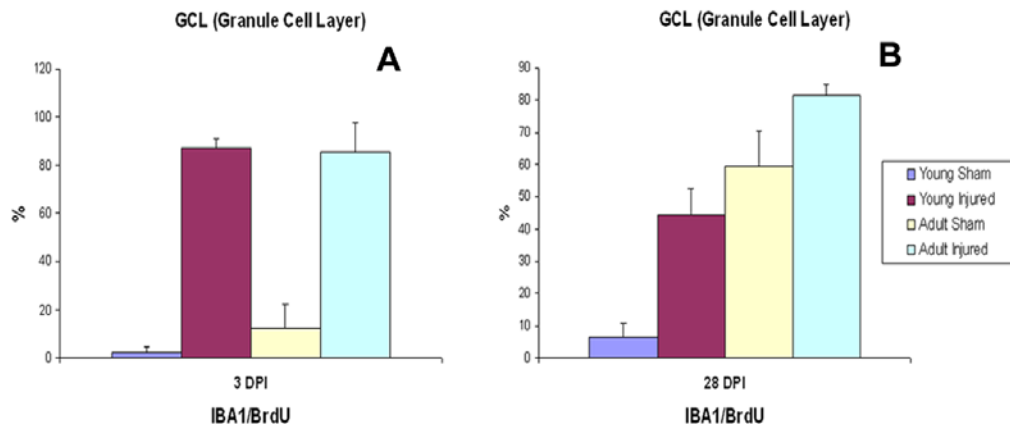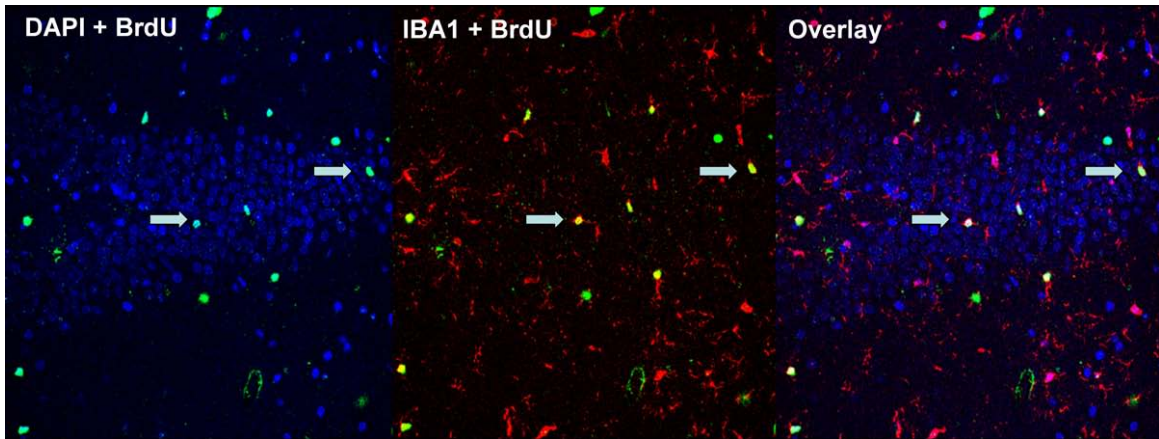
Figure 3.12: Differentiation of newly-generated cells into microglia. Confocal images showing A) newly generated BrdU-labeled cells in the GCL indicated by arrow; B) microglia identified by fluorescent Iba1 immunostaining indicated by arrow; and C) co-labeling of newly generated BrdU positive cell with Iba1. Graphs show the percentage of BrdU/Iba1 double-labeled cells in the GCL at 3 and 28 days post-injury in both young and aged brains. Cells in the GCL which were co-labeled with BrdU and Iba1 were calculated as a percent of co-labeling of against the total number of BrdU-positive cells.

Effect of anti-inflammation treatment with minocycline on glial cell responses in the hippocampal dentate gyrus at 3 days post-injury

Minocycline is a tetracycline derivative antibiotic which has anti-inflammatory properties. In order to assess whether minocycline's anti-inflammatory abilities can significantly decrease inflammatory cell responses in the injured brain, young and aged animals were subjected to a moderate L-FPI followed by a short minocycline treatment. Animals were sacrificed at 3 days post-injury and processed for immunofluorescent labeling of IBA1 or GFAP. The staining intensity of IBA1 and GFAP was quantified using densitometry image analysis.

For GFAP staining, the young injured animals treated with minocycline showed a significant increase in GFAP optical intensity especially in the GCL region compared to sham and injured non-treated animals (**Fig 3.13**, $p = 0.001$ and $p = 0.035$ respectively). In the hilus region, the young injured animal treated with minocycline also showed an increase in GFAP optical intensity compared to sham and injured non-treated animals. However the statistical analysis showed no significance (**Fig 3.13**). The effects of minocycline treatment on the GFAP optical intensity following TBI in the injured aged animal counterparts also showed an increase in both the GCL and hilus regions. In the GCL region, statistical analysis showed a significant increase in treatment animals compared with the sham animal group ($p = 0.018$) but no significance between the injured aged group and the aged treatment animals (**Fig 3.13**). In the hilus region, there were no statistical significant increases between groups (**Fig 3.13**). However, the treatment group compared to the sham group almost reached a significant increase ($p = 0.058$).

For microglia IBA1 staining, the young injured animals had a decrease in IBA1 optical intensity following minocycline treatment in both the GCL and hilus regions (**Fig 3.14**). In the GCL and hilus regions, there were no statistical significances between groups. However, in the hilus region of the injured young animal groups, the decreased levels of IBA1 optical intensity in the young treatment group compared to non-treatment injury group almost reached significance ($p = 0.085$). In the aged injured animal groups, IBA1 staining intensity was decreased in the injured brain following minocycline treatment was seen in both the GCL and hilus regions (**Fig 3.14**). In the GCL region, the aged treatment group compared to the non-treated injured aged group almost reached significance ($p = 0.074$). In the hilus region, despite the decreased levels of IBA1 optical intensity, there was no significance when compared to either the sham or non-treated injured aged animal groups.

A GFAP expression in the GCL in the 3M old rats at 3dpi

B GFAP expression in the GCL in the 20M old rats at 3dpi

C GFAP expression in the hilus in the 3M old rats at 3dpi

D GFAP expression in the hilus in the 20M old rats at 3dpi
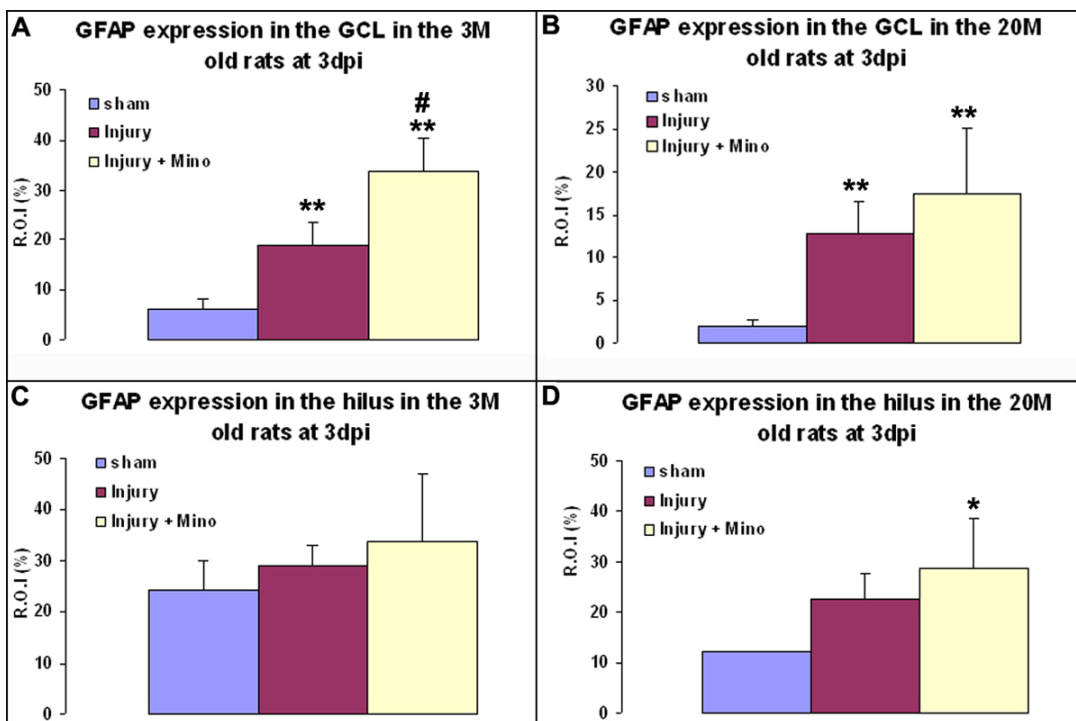
Figure 3.13: Quantitative analysis of astrocyte GFAP levels in the DG of injured young and aged animals at 3 days post-injury. A) In the young adult groups, significance in GFAP labeling was seen when comparing injured young animal groups to sham group (**$p = 0.035$), between the sham young animal groups and injured minocycline treated group (**$p = 0.014$), and between the treated young group to sham young groups in the GCL region (#$p = 0.001$). (B) In the aged GCL, significance in GFAP labeling was seen when comparing sham aged animal group to the treatment aged group (**$p = 0.010$), between the sham aged animal groups and injured (**$p = 0.018$). C) In the hilus region for the young animal group, though increased GFAP optical intensity levels were seen, there was no statistical significance between group comparisons. D) In the hilus region for the aged animal group, though increased GFAP optical intensity levels were seen, there was no statistical significance between group comparisons. Comparison between the sham aged animals and the treatment aged groups almost reached significance (*$p = 0.058$).

A Iba1 expression in the GCL in the 3M old rats at 3 days post injury

B Iba1 expression in the GCL in the 20M old rats at 3 days post injury

C Iba1 expression in the hilus in the 3M old rats at 3 days post injury

D Iba1 expression in the hilus in the 20M old rats at 3 days post injury
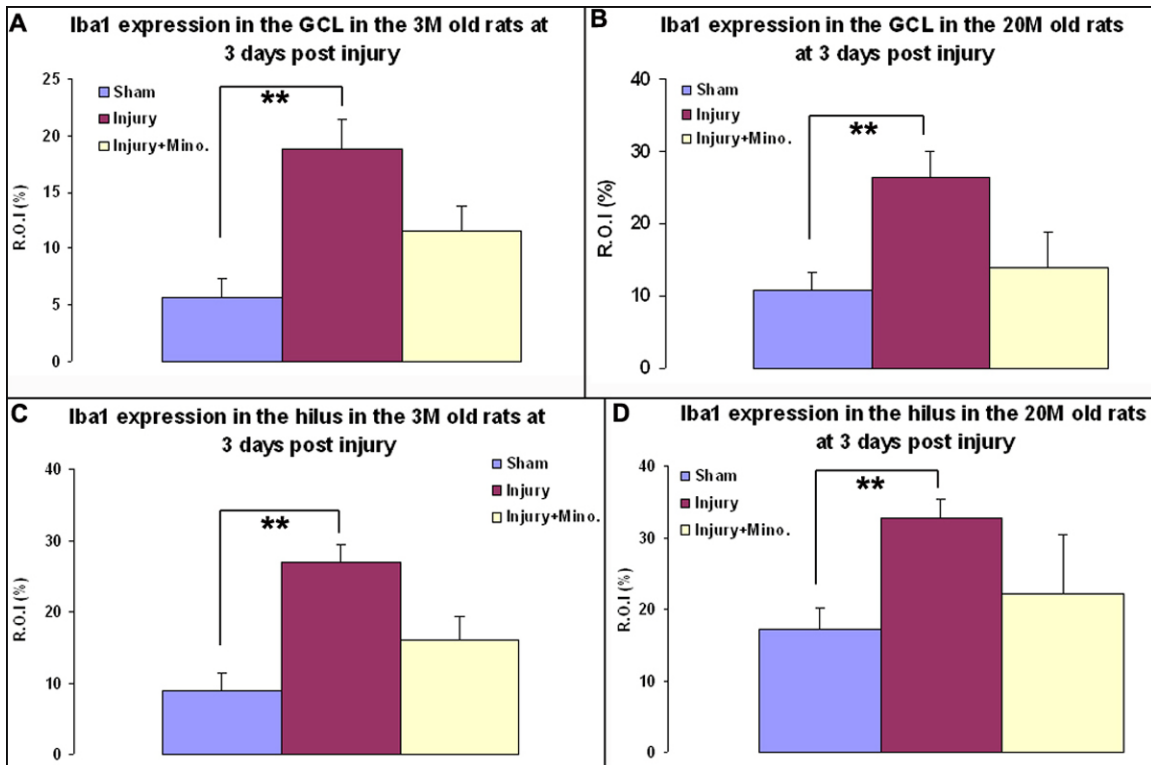
Figure 3.14: Quantitative analysis of microglial IBA1 expression levels in the DG of injured young and aged animals at 3 days post-injury. A) Significance in IBA1 labeling was seen when comparing the sham young animal groups and injured (**p = 0.001) (B) In the aged GCL, significance in IBA1 labeling was seen when comparing sham aged animal group to the injured aged group (**p = 0.004). C) In the hilus region for the young animal group, though decreased IBA1 optical intensity levels were seen following treatment, there was statistical significance between sham young and injured young animals (**p = 0.001). D) In the hilus region for the aged animal group, though decreased IBA1 optical intensity levels were seen following treatment, there was statistical significance between sham aged and injured aged animals (**p = 0.011). Note in both young and aged brains in the GCL and hilus regions, IBA1 expression was decreased in the micocycline treated groups.

Chapter 4 - Discussion

Results Summary

In this study, we first utilized coronal brain sections containing the hippocampus from previous studies done in our lab to compare the TBI-induced glial responses in the dentate gyrus of the hippocampus between 3-month old young adult and 20-month aged brains. We have found that aged rats had lower level of baseline expression of astroglial marker GFAP and higher level of baseline expression of microglial marker Iba1 in comparison to the younger counterparts. Following injury, in the young injured animal groups, astrocyte GFAP staining was increased at 3, 7, and 28 days post injury, and was peaked at 7 days post injury in both GCL and hilus regions. The aged injured animal groups also showed increased GFAP staining following injury. The highest GFAP staining was observed at the 3 and 7 days post injury for the GCL and hilus regions, respectively. Following injury, microglial Iba1 expressed was increased and was seen at the greatest at the 28 days post injury in the GCL and hilus regions for both young and aged animals. In summary, moderate LFPI had a significant and similar effect on glial responses in dentate gyrus in both young and aged animals.

After examining the level of injury induced glial responses in the DG, we next assessed the degree of TBI-induced generation of new microglial cells using BrdU and Iba1 double-labeling. We found that at 3 days post injury, both injured young and aged animals had a high percentage of double labeling of BrdU and Iba1. At 28 dpi, the measured levels of BrdU and Iba1 double labeled cells decreased in the young injured animal group whereas the aged injured animal group maintained high levels of BrdU and Iba1 double labeling. This suggests that TBI

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not only induces microglia activation through morphological changes but also through increasing cell numbers and this is more pronounced in the aged brain.

Lastly, we assessed the effect of anti-inflammatory treatment with minocycline on glial cells responses in both young and aged brains. Short time minocycline treatment increased GFAP expression while decreased Iba1 expression in both young and aged animal groups in the GCL and hilus regions.

Sham aged rats have higher baseline level of microglial Iba1expression and lower level of GFAP expression compared to sham young animals

Aging is generally associated with the activation of inflammatory response due to the chronic antigenic stress on innate immunity experienced over a lifetime (Vasto et al., 2007). We found in this study that sham aged animals exhibited greater levels of Iba1 fluorescent optical intensity in both the GCL and in the hilus regions compared to the sham young adult animals. The greater levels of measured Iba1 were probably due to the increased size of resting state microglial and the higher numbers of resident microglial cells in the aged brain. Our findings were in agreement with observations from other published studies report that the aged brain shows a chronic state of inflammation that is associated with increased immune reactivity (Dilger and Johnson, 2008). Furthermore, in the aged brain, microglia have been shown taking on a more activated phenotype in the resting state, and they have been shown exhibiting hypertrophic and shortened processes that confirm a more activated cellular morphology (Chung et al., 2010).

In this study, we also found that sham young adult animals exhibited higher GFAP expression than the aged brain. Astrocytes are the predominant cell type within the

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neurovascular unit (neurons, astrocytes, blood vessels) having a 10:1 ratio compared to neurons in the human brain (Laird et al., 2008). In the uninjured CNS, astrocytes provide many supportive activities essential for neuronal function such as homeostatic maintenance of pH and the extracellular ionic environment, and providing metabolic substrates for neurons (Sofroniew, 2005). Therefore, the lower levels of GFAP optical intensity in the sham aged animals could potentially be postulated that aged animals may have less neuronal support. In contrast to our results, other studies have shown that there is an age-related increase in basal expression of GFAP mRNA and protein levels in rodents and humans (Yoshida et al., 1996). Also, age related increases in GFAP mRNA or protein in extracts of the whole hippocampus have been attributed to more GFAP molecules per cell (Major et al., 1997). The disparity between our results and others may due to the methods used. This study used densitometry analysis of anti-GFAP fluorescent antibodies as markers to quantify GFAP. Compared to other methods for protein analysis such as Western blotting, the densitometry image analysis we used is less accurate while providing spatial benefits. Human errors could happen during the immuno-fluorescent staining, photo taking as well as during imaging analysis. Furthermore, our study focused only on the GCL and hilus regions of the dentate gyrus in the hippocampus while other studies accounted for the entire hippocampus including the CA1 and CA3 regions as well as the dentate gyrus which may affect the measured levels of GFAP.

Microglia response in young adult and aged brains following TBI

Microglia are the primary intrinsic immune effecter cells of the central nervous system and are involved in most pathological processes of brain related to inflammation (Berman et al.,

1999). Our study revealed significant microglial activation through increasing in number and size in both young and aged brains following injury. We also showed that the aged animals had higher levels of optical intensity of IBA1 in sham baseline level and following injury at all three time pointes examined compared to the young animal groups, though the differences did not reach statistical significance. However, when the changes in IBA1 expression at each time point were compared between young and aged animal groups, significance was found at the 3 dpi time point.

Studies have shown that chronic activation of microglia is considered to be the most damaging to injured or diseased CNS because interactions occurring between damaged neurons and hyper-activated microglia can create a self propagating positive feedback loop leading to a potential prolonged microglial activation and neuronal cell death (Block and Hong, 2007; Gao and Hong, 2008). Furthermore, microglia in the aged brain compared to those of younger brains have been characterized to respond to injury more rapidly, produce more pro-inflammatory cytokines leading to more pronounced inflammatory responses, and proliferate more vigorously (Godbout et al., 2005; Conde and Streit, 2006). More specifically, the aged brain has increased levels of the pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α expression at resting baseline levels while greatly increasing in production following injury or insult to the CNS (Gelinas and McLaurin, 2005; Godbout and Johnson, 2004; Loane and Byrnes, 2010). Thus, aging creates a brain environment where microglial sensitivity to immune activation is increased leading to potential exacerbation of the secondary injury in older subjects and severe systemic immune responses (Cekic and Stein, 2010).

In our study, we found that Iba1 optical intensity was lower at 7 than 3 and 28 days post injury in both aged and young animal groups. This maybe due to a rapid regression of injury-

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activated microglial cells at this time points compared to at 3 days post injury, and the injury-induced newly generated microglia cells contributing to the second wave of high Iba1 expression at 28 days post injury. A study by Sandhir et al., (2008) quantified Iba1 mRNA levels following the induction of controlled cortical impact injury and found highest levels of Iba1 mRNA at the 3 dpi time point supporting our observation. The Sandhir study also found that the aged animal groups had greater Iba1 levels compared to young adult animal groups at 3, 7 and 28 days post injury similar to what we found. Through the normalization data, we postulate that since the aged brain already has a higher baseline of IBA1 optical intensity, changes in IBA1 expression following injury were decreased due to the aged brain's pro-inflammatory homeostatic state.

Astrocytic response in young adult and aged brains following injury

Astrocytes are the predominant cell type of the nervous system, and they contribute many important neuroregulatory functions including regulation of neuron communication, neurosecretion, metabolism and synaptic plasticity (Mahesh et al., 2006). The wide ranges of astrocyte functions contribute to uncertainty whether these cells exert beneficial or detrimental effects after CNS injuries (Sandhir et al., 2008). It has been seen that astrocytes respond to TBI by pronounced changes in gene expression, cellular hypertrophy and cell proliferation, all of which occur in a graduated fashion in relation to the severity of the injury (Myer et al., 2006). Beneficially, studies have shown that reactive astrocytes following TBI form a protective scar to contain brain injury, provide and improve niche for reparative responses, restore the integrity of the blood brain barrier, and aid in the establishment of an environment that is helpful for neurite outgrowth, synaptogenesis and synapse maturation through modulate levels of matrix

metalloproteinase-3 released (Sofroniew, 2005; Bush et al., 1999; Myer et al., 2006; Reeves et al., 2002; Falo et al., 2006). However, as astrocytes are known to regulate extracellular glutamate excitoxicity within the brain due to their expression of GLT-1 and GLAST receptors which serve as glutamate transporters (Pawlak et al., 2005); after injury, these receptors are downregulated following experimental TBI in rodents suggesting that this may lead to an increased inflammation and exacerbated excitotoxicity (Rao et al., 1998). Astrocytes also release and respond to pro-inflammatory cytokines such as TNF-α, Fas, and IL-1β and therefore, further amplifying the inflammatory response following TBI (Laird et al., 2010).

In a study done by Sandhir et al. (2008), following injury GFAP mRNA expression levels were increased and peaked at the 7 days post injury and the aged animal groups showed significantly higher GFAP mRNA levels compared to the young adult animal groups. Our study found similar trend of injury-enhanced GFAP expression at the protein level especially in the young adult brain. The differences we found that aged brain had lower level of GFAP expression compared to younger brains maybe due to the intensity of the injury, the TBI model differences, differences in quantification method, as well as disparities between mRNAs and proteins. Interestingly, when we looked at the normalized data that measured the changes in GFAP expression at each time point, we observed that aged animals exhibited greater changes of GFAP optical intensity relative to sham groups at each time point following injury. The function of astrocytes in the injured brain has been under intensive studies in the past. Previously, reactive astrocytes were considered detrimental. However, in recent years, the beneficial role of reactive astrocytes has been recognized. Studies have shown that astrocyte activation following injury plays important roles in stabilization of injured environment and contribute to recovery (Floyd and Lyeth, 2007.).   For example, transgenic mice with deletions for reactive astrocytes

actually responded worse to trauma and insult (Myer et al., 2006). In our study, the increased changes of GFAP expression in the aged brain may correlate to the worse functional recovery we previously observed in the injured aged group.

Inflammation reduces neurogenic potential and shows increased dividing microglia following injury in the aged brain

With aging, as with other organs in our body, the brain exhibits a progressive decline in its regenerative abilities leading to functional deterioration and poorer recovery from injury and disease. Inflammation is shown to affect neurogenesis in both the SGZ and the SVZ (Pluchino et al., 2008). Many studies have shown that the immune reaction is able to perturb adult NSCs activity and fate through the released signaling molecules such as the complex cascade of both pro-inflammatory and anti-inflammatory cytokines and chemokines (Carpentier and Palmer, 2009). Exaggerated inflammation can also interfere with neuroplasticity as the brain attempts to recover from injury along with memory consolidation and hippocampal neurogenesis (Barrientos et al., 2006; Vallieres et al., 2002). Studies have shown that inflammation has a strong negative effect on neurogenesis in the adult brain due to the impact of the pro-inflammatory cytokines that are released following the acute injury or disease. The role of pro-inflammatory cytokines produced by activated microgla from the acute innate inflammatory response such as IL-6, IFN-γ, IL-1β, and TNF-α play a pivtol role in suppressing neurogenesis.

We previously found that injury induces significant cell proliferation in the DG and many of these newly generated cells become neurons in the young adult brain contributing to the innate cognitive recovery following injury (Sun et al., 2005, 2007). In this study, we found that at 28 days post injury, around 80% of injury-induced new cells became microglial cells in the aged

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brain which was significantly higher than what was observed in the younger adult brain where 40% BrdU/Iba1 double-labeling was found. Conversely, there were much less new neurons generated in the injured aged brain compared to the younger brains. As we previously observed, the aged brain retains cell proliferative capacity but generates less new neurons following injury compared to younger brains. Results from the current study suggest that the endogenous regenerative potential is decreased in the aged brain. In BrdU/Iba1 double-labeling study, we found that many of the proliferation cells at 3 days post injury were labeled with IBA1 in both young and aged injured brains, this probably reflects the rapid proliferation of microglia following injury. We postulate that microglia may continue to proliferate in the aged brains at the 28 dpi time point because microglia and neurons have different precursor cells. Therefore, the BrdU labeling portion may reflect the mitotic properties of microglia.

The effect of minocycline treatment on the glial cell response following TBI

Following injury or disease, a number of studies have reported the anti-inflammatory effects of minocycline. It has been suggested that minocycline's effectiveness is largely due to its superior penetration of the blood-brain barrier (Saivin and Hovin, 1988; Yrjanheikki et al., 1998). In our study, we found that a short 3-day minocycline treatment increased the levels of GFAP expression and decrease Iba1 expression in both young and aged animal groups at 3 days post-injury showing a positive effect in the injured hippocampus.

Minocycline influences neutrophils, monocytes, microglia and neurons during inflammation by inhibiting neutrophil migration, degranulation and the formation of oxygen free radicals (Golub et al., 1991). Despite the fact that the mechanisms of action for minocycline

treatments have not been fully understood, the beneficial effects stem from the drug's ability in decreasing or inhibiting microglia activation and proliferation along with the down-regulation of inducible nitric oxide (iNOS) synthase transcription (Yrjanheikki et al., 1998).

In the current preliminary study, we found that the effect of minocycline was consistent with the reports from the literature on reducing microglial activation in both young and aged animal groups. It is unknown whether reduced microglia activation and increased astrocytic activation by minocyline treatment would encourage neurogenesis. Published studies have shown mixed results of minocycline on neurogenesis. A study conducted by Ekdahl et al. (2003) studied minocycline's effects on neurogenesis in a status epilepticus experiments and found that in the SE model, minocycline increased the numbers of new neurons in the SGZ/GCL but did not see an increase in the number of matured neurons or increased injury induced cell proliferation. Minocycline had a clear effect after TBI, but there was no difference in the trends observed between aged groups.

Conclusion

In summary, our studies have examined injury-induced astrocyte and microglia cell responses in the young adult and aged brain at different time points post TBI. We have found similar trend of glial cell response in the injured brain in both age groups, and found that the aged brain has an increased microglia response and a decreased astrocytic cell response compared to the younger brain. The differences in glial cell responses between the two age groups may correlate to the differences in their regenerative and recovery potential following injury.

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Vita

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