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**THE EFFECTS OF bFGF TREATMENT IN THE AGED BRAIN 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

MICHAEL R ZEIGLER

Bachelor of Science, Liberty University, 2008

Major Director: Dong Sun, MD, Ph.D.

Associate Professor

Department of Neurosurgery

Virginia Commonwealth University

Richmond, Virginia

June 2010

Acknowledgements

I would first like to thank my adviser, Dr. Dong Sun, for her continuous support, expert guidance, and enduring patience during my time working in her laboratory. It was an honor and a privilege to be able to work alongside her and her staff while earning my degree. I would also like to thank my committee members, Dr. Robert Hamm and Dr. Raymond Colello, for their time and assistance. Through their valuable advice, they demonstrated the high level of scientific knowledge and genuine dedication of the VCU School of Medicine faculty and their desire to help students learn and appreciate scientific research.

I also have a deep appreciation for the other members of our laboratory. I would like to extend my thanks to Dr. Wendy Reid, our post-doc, whose help and encouragement was invaluable to me as a student new to the research community. I would like to thank research assistants Andrew Rolfe and Guoyan Gao, who trained me in the procedures necessary for my project and assisted me with the massive workload that came along with it. The incredible amount of assistance they provided, as well as the good conversations and friendships that developed, helped to pass the long hours of work and make my time that much more enjoyable.

In addition, I would like to acknowledge Dr. George Leichnetz, to whom I am indebted for his always helpful, always appropriate advice and for his supporting me every step of the way.

Lastly, I would like to thank my friends and family for their unending encouragement and support, especially my parents, Ray and Kelly Zeigler, who have always told me I could do it.

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List of Abbreviations

ABC	Avidin-biotin complex
aFGF	Acidic fibroblast growth factor
atm	Atmosphere
bFGF	Basic fibroblast growth factor
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
EGF	Epidermal growth factor
FG	Fluorogold
FPI	Fluid percussion injury
GCL	Granule cell layer

GZ	Granular zone
Hr	Hour
i.p.	Intraperitoneal
L-FPI	Lateral fluid percussion injury
MCAo	Middle cerebral artery occlusion
MWM	Morris water maze
NIA	National Institute of Aging
NIH	National Institute of Health
NOR	Novel object recognition
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

Abstract

THE EFFECTS OF bFGF TREATMENT IN THE AGED BRAIN FOLLOWING TRAUMATIC BRAIN INJURY

By Michael R. Zeigler, Master of Science

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science at Virginia Commonwealth University

Virginia Commonwealth University, 2010

Major Director: Dong Sun, MD, PhD., Associate Professor, Department of Neurosurgery

The mature mammalian brain continually generates new neurons in the subventricular zone and hippocampus throughout life. Adult neurogenesis in the hippocampus is associated with hippocampal-dependent learning and memory function. During aging, this endogenous neurogenic potential is reduced which is accompanied by decreased cognitive function seen in the aging population. We have previously found that the injured adult brain shows heightened levels of endogenous neurogenesis and this response is associated with innate cognitive recovery. We have also found that basic fibroblast growth factor (bFGF), a potent neurotrophic polypeptide, can enhance injury-induced hippocampal neurogenesis and improve cognitive recovery following TBI. In this study, we administered bFGF into the lateral ventricle of aged rats following TBI and assessed the effect of bFGF treatment on hippocampal neurogenesis and

cognitive recovery in aged animals. Specifically, male Fisher-344 rats at the age of 20 months received intraventricular infusion of bFGF for 7 days through osmotic mini-pump immediately following a moderate lateral fluid percussion injury. To label cell proliferation, animals received daily single i.p. BrdU injections for 6 days beginning 48 hr after injury. One group of animals was perfused at 1 wk after injury to assess cell proliferation. Another group of animals was first assessed for cognitive performance using the Morris water maze (MWM) at 21-25 days post-injury, then sacrificed at 4 weeks after injury to examine differentiation of newly generated cells. Brain sections were sliced and immunostained for BrdU, early neuronal marker doublecortin (DCX) and other cell type specific markers. Results showed that at 1 week post-injury, injured-aged animals infused with either vehicle or bFGF had a significantly higher number of cell proliferation in the dentate gyrus compared to sham animals. However, cell proliferation in the bFGF-infused animals was not significantly higher than vehicle-treated animals. Nevertheless, the number of DCX-labeled early stage neurons was significantly higher in the injured bFGF-treated animals than in vehicle-treated sham and injured animals. In MWM tests, unlike what we have observed in bFGF-treated younger animals, injured aged rats treated with bFGF did not show improved cognitive function. Furthermore, at 4 weeks post-injury, higher numbers of BrdU-labeled proliferative cells persisted in both injured groups, many of these cells labeled with glial and inflammatory cell markers. Collectively, the current data suggests that bFGF can enhance neurogenesis in the injured-aged hippocampus; however, this effect is not sufficient to improve functional recovery of aged rats following TBI due to the profound injury-induced inflammatory response.

Chapter 1 - Introduction and Background

Traumatic brain injury (TBI) is a major healthcare concern in the United States and around the world. As millions of Americans sustain a TBI each year, TBI is one of the leading causes of death and long-term disability in the U.S. One of the hallmarks of TBI-related disability is loss of cognitive function. The central nervous system (CNS) is especially vulnerable to injury due to its limited ability to repair or replenish neurons that have been damaged or lost. The cognitive deficits seen in TBI patients have been shown to result in large part from the loss of hippocampal neurons, and much research has been done to elucidate the biological mechanisms underlying these cognitive impairments in the hopes of developing an effective treatment. The presence of neural progenitor cells in the adult mammalian CNS suggests that the CNS retains a level of regenerative capacity throughout life. These cells persist in two regions of the CNS, including the subventricular zone (SVZ), which lines the lateral ventricles, and the dentate gyrus (DG) of the hippocampus. Although heightened during development, the ability of these neural stem/progenitor cells (NS/NPCs) to proliferate and differentiate into neurons and glia continues throughout adulthood. Additionally, research has shown that TBI significantly increases the proliferation of NS/NPC's (Sun et al., 2005; Chirumamilla et al., 2002). More specifically, the level of TBI-induced cell proliferation in the neurogenic regions is higher in the younger brain and decreases with age, which correlates with clinical findings that juvenile patients show greater cognitive improvement than older patients

following TBI (Kuhn, 1996; Eiben et al., 1984). Furthermore, the number of newly generated cells that assume a neuronal maturational fate decreases with age, which suggests a link between neurogenesis and cognitive recovery (Sun et al., 2005; Sun et al., 2007). This crucial link is indicative of an endogenous repair mechanism within the brain. Studies examining the underlying biological basis of this innate repair mechanism have found elevated levels of growth factor expression following TBI (Mattson and Scheff, 1994; Oyesiku et al. 1999). One of these growth factors, basic fibroblast growth factor (bFGF), has been shown to stimulate the proliferation of NS/NPC's *in vitro* and *in vivo* (Shetty et al., 2005; Vicario-Abejon, 2004 ; Ray et al., 1993 ; Caday et al., 1990). These findings strongly suggest that bFGF plays a role in mediating the TBI-induced proliferative response. Additionally, a recent study in our lab has succeeded in using bFGF treatment to enhance cell proliferation and neurogenesis and improve cognitive recovery following TBI in adult rats (Sun et al., 2009), and an association between integration of newly generated hippocampal neurons into existing neuronal circuitry and cognitive recovery has also been established (Sun et al., 2005). These studies collectively demonstrated that bFGF treatment improves cognitive recovery in adult rats following TBI.

Taken together, we postulate that if the robust TBI-induced cell proliferation observed in juveniles could be mimicked in their older counterparts by treatment with bFGF, this could potentially enhance neurogenesis and aid the elderly to achieve significant improvements in cognitive recovery. More specifically, it was hypothesized for this thesis that bFGF treatment following TBI would significantly improve cognitive recovery in aged rats. To test this hypothesis, this study set out to assess the effects of bFGF treatment on hippocampal neurogenesis in the aged brain and on cognitive recovery following TBI. In order to determine the degree of cell proliferation and the survival of these cells, proliferating cells were labeled

with BrdU from days 2-7 post-injury. BrdU-positive cells were quantified using stereological methods in the DG at 7 and 28 days post-injury in injured vehicle-treated and bFGF-treated animals as well as sham animals. In order to assess the effect of bFGF treatment on neurogenesis in the aged animals, young neurons were labeled with DCX and quantified using stereological methods at 7 days post-injury. To assess the maturational fate of cells generated following TBI, sections from animals sacrificed at 4 weeks post-injury were processed for immunofluorescent double-labeling of BrdU with cell-type specific markers for mature neurons (NeuN), astrocytes (GFAP), and infiltrating macrophages and microglia (ED1), and were examined using confocal microscopy. To assess the effect of bFGF on cognitive recovery of aged rats following injury, a novel object recognition test was performed on 3, 7, 14, and 28 days post-injury, and the Morris water maze was performed on post-injury days 21-25 with a probe trial 24 hrs later.

As the purpose of this study was to evaluate bFGF as a potential treatment in the aged animal following TBI, this thesis will first focus on the epidemiology and pathology of TBI, followed by a brief review of cell proliferation and neurogenesis in the aged brain and the effects of bFGF treatment on these processes.

Epidemiology and pathology of TBI

Traumatic brain injury continues to be a serious global health concern and is one of the leading causes of disability and death in the United States (Langlois et al., 2006). Each year approximately 1.5 million Americans suffer from TBI, with the most common causes involving violence or assault, motor vehicle accidents, falls, or sports-related injury (Langlois et al., 2006; Thurman et al., 1999). Of these known cases of TBI, 1.1 million are treated in Emergency

Departments, 235,000 require hospitalization, and 50,000 result in death. An additional 200,000 TBI patients are treated in outpatient care settings, such as primary care offices, and many more are unaccounted for, which include those who are treated in domestic and overseas U.S. military installations, those not seeking medical care, and those who are misdiagnosed (Langlois et al., 2006). Those at the highest risk for TBI include people from ages 15-24 years and those above the age of 64 years, with males showing an incidence rate 2 times higher than females and a mortality rate 3.3 times higher (Thurman et al., 1999). According to the CDC and the U.S. Dept. of Health and Human Services, advances in critical care have resulted in fewer fatalities and a greater chance of survival for those that have sustained severe TBI. However, 80,000-90,000 Americans experience the onset of long-term debilitating loss of function resulting from TBI annually, which has contributed to the growing population of those living with TBI-related disability in the U.S., which is currently estimated to be 5.3 million, or about 2% of the U.S. population (CDC, 1999). The cost of medical care, rehabilitation, and loss of productivity incurred by TBI is estimated to be an astounding \$60 billion every year, which represents a significant financial burden on the U.S. economy, affected victims, and their families (Langlois et al., 2006; Thurman et al., 1999). Even more burdensome for TBI patients and their loved ones is the often lifelong struggle they face to regain cognitive and physical functioning.

The pathology associated with TBI is widely described as a biphasic process involving a primary insult which is mechanical in nature and a secondary insult consisting of damaging physiological effects which can last from a few hours to a few weeks following injury (Dutton and McCunn, 2003; Davis, 2000). Primary brain insult may be focal, resulting from a direct blow or penetrating force to the head, or diffuse, resulting from rotational motion of the head. More commonly, TBI manifests as a combination of both focal and diffuse injury, causing direct

tissue damage, more global diffuse axonal injury, and, ultimately, a variety of neurobehavioral, motor, and cognitive deficits depending upon severity and location (Davis, 2000). The more delayed secondary insult which follows is characterized by excitotoxicity, changes in cerebral blood flow, local and systemic inflammation, alterations in oxygen delivery and metabolism, and both ischemic and apoptotic death of cells in the CNS. The inflammatory response to injury is beneficial in that it is necessary for healing and repair; however, the reaction is exaggerated and is harmful to many cells in the CNS (Dutton and McCunn, 2003).

The hippocampus is exceptionally vulnerable to secondary insult and has been linked to learning and memory deficits, which are the hallmarks of brain injury. The excitotoxic cascades which are a part of secondary insult result in the damage or loss of many neurons in the CA1 and CA3 subfields of the hippocampus to a greater extent than surrounding structures in the brain (Smith et al., 1991). The cumulative loss of neurons in the hippocampus along with alterations in the excitability and synaptic connections of remaining hippocampal neurons leads to the variety of cognitive deficits seen in TBI patients (Witgen et al., 2005). TBI-related disabilities range from cognitive impairments resulting from mild to moderate brain injury to a persistent vegetative state resulting from severe brain injury (McArthur et al., 2004; Hilton, 1994). The growing number of TBI-related disabilities in the U.S. is having an ever-increasing impact on the economy and on society in general. Many persons who have sustained a TBI cannot return to work for a considerable amount of time and often are unable to work indefinitely (van der Naalt et al., 1999). In addition to affecting TBI patients' ability to work, cognitive deficits may also limit their ability to interact socially, which further prevents them from becoming productive members of society and drastically affects their quality of life (Thurman et al., 1999; Hilton, 1994).

Hippocampal-dependent learning and memory deficits are consistently the most enduring and devastating effects of TBI; however, most TBI patients demonstrate a slow but significant improvement in cognitive function which may take weeks or even months to achieve (McArthur et al., 2004; Schmidt et al., 1999). Although spontaneous cognitive recovery occurs following TBI, age-related differences exist in the extent to which this recovery is seen. Clinical studies have shown that younger TBI patients display a greater degree of cognitive improvement and recovery of functional independence in comparison to their older counterparts (Eiben et al., 1984). In TBI patients, old age has been associated with significantly poorer cognitive performance immediately following TBI and poorer long-term cognitive and functional outcomes after maximum spontaneous recovery (Senathi-Raja et al., 2010). In order to explore these age-related differences in functional recovery and to more fully understand the pathology of TBI, experimental animal models have been developed and utilized in the research of TBI with the ultimate goal of developing an effective treatment for this debilitating condition.

Experimental TBI

The most widely used *in vivo* TBI models are CCI (Controlled Cortical Impact) and FPI (Fluid Percussion Injury), which have been effective in producing the TBI-induced pathological sequelae and subsequent cognitive deficits seen clinically (Kline et al., 2002; Hamm et al., 1996; Gorman et al., 1993). In particular, L-FPI (Lateral Fluid Percussion Injury) produces a combination of focal and diffuse injury and consistently induces TBI-related cognitive dysfunction, which has led to L-FPI being used extensively as a valid reproducible model of TBI to investigate the pathology of TBI and evaluate potential treatment paradigms designed to improve functional outcome following TBI (Thompson et al., 2005). Many studies have employed L-FPI in rats to characterize age-related differences in functional outcome after brain

insult. Similar to what is seen clinically, rats experience an increase in neurological deficits and mortality rate with age following TBI (Hamm et al., 1991). It has also been shown that juvenile rats significantly outperformed their aged counterparts on hippocampal-dependent learning and memory functions as assessed by the Morris water maze test on days 11-15 following L-FPI (Hamm et al., 1996).

Although a great deal of research has been devoted to investigating the neurobiological basis of cognitive recovery from brain trauma, the molecular mechanisms underlying these observed age-related differences in functional outcome following TBI have not been fully elucidated. However, the spontaneous cognitive recovery that is seen in humans and animals following TBI, limited as it may be, suggests that the CNS has an inherent potential to regenerate and repair itself following TBI. One potential mechanism of this endogenous repair response in the CNS is thought to involve the proliferation of neural progenitor cells located in specific germinal zones of the brain and their subsequent differentiation into neurons and glia. A thorough look at these neural progenitor cells and their role in repair and cognition is necessary.

Neurogenesis in the mature CNS

The scientific community had long held that no new neurons could be generated in the CNS. However, as early as 1965, investigators demonstrated that the SVZ (subventricular zone), adjacent to the ependymal layer of the lateral ventricles, and the DG (dentate gyrus) of the hippocampus continuously generate new cells throughout life in the mature mammalian brain, and that these cells have the capacity to differentiate into neurons or glia (Altman and Das, 1965; Lois and Alvarez-Buylla, 1993). Reserves of multipotent neural stem/progenitor cells (NS/NPCs) are localized at the SVZ and DG, and endogenous cell proliferation in the mature

mammalian CNS is limited to these regions (Peterson, 2002). The SVZ contains the largest population of dividing NPC's, the progeny of which migrate into the olfactory bulb (Peterson, 2002; Temple and Alvarez-Buylla, 1999). In the DG of the mature mammalian brain, NPC's are generated in the subgranular zone (SGZ), which is at the border between the hilus and granule cell layer (Kuhn et al., 1996). These NS/NPCs and their daughter cells migrate laterally to the granule cell layer where they become mature functioning granular neurons (Parent, 2003). Ordinarily, a large percentage of these newly generated granule cells undergo apoptosis (Cameron and McKay, 2001). However, the majority of newly generated cells that persist in the DG for an extended period of time become mature granule neurons (Sun et al., 2007). Those new granule cells which survive extend their axonal projections to their appropriate targets in the CA3 subfield of the hippocampus and integrate into the existing neuronal circuitry as mature, functional granule neurons (Hastings and Gould, 1999; Markakis and Gage, 1999). Furthermore, studies have confirmed that these new neurons display passive membrane properties, generate action potentials, and form functional synaptic inputs, which are characteristics of mature dentate granule neurons (van Praag et al., 2002).

Hippocampal neurogenesis is enhanced following TBI

Although more robust in younger animals, the adult mammalian CNS maintains certain degree of NS/NPC proliferation and neurogenesis throughout life, and these processes are significantly enhanced in response to TBI (Sun et al., 2005; Gould and Gross, 2002; Temple and Alvarez-Buylla, 1999). As these post-TBI changes are closely correlated to spontaneous cognitive recovery, recent studies have attempted to shed more light on the mechanisms of this endogenous repair response.

Injury to the brain is accompanied by a robust cell proliferative response in the SVZ, DG, and at the site of injury, which suggests that the CNS retains an innate regenerative and reparative capacity which may account for cognitive recovery observed following TBI (Chirumamilla et al., 2002). As an illustration, enhanced proliferation of granule cell precursors in the SGZ of the DG can be directly stimulated by the death of mature hippocampal granule neurons (Gould and Tanapat, 1997). A previous study in our lab has shown that TBI generated a nearly threefold increase in cell proliferation in the SGZ in juvenile and adult rats as compared to uninjured age-matched sham controls, with TBI-induced cell proliferation reaching a peak at 2 days post-injury before tapering off and returning to basal levels by 14 days post-injury (Fig. 1.1; Sun et al., 2005). Our lab later demonstrated that out of this large pool of newly generated cells, many new cells persist in the DG for an extended period of time (Fig. 1.2; Sun et al., 2007). There is evidence that these newly generated cells are involved in the endogenous repair response and subsequent cognitive recovery. The loss of these injury-induced proliferating cells, which can be caused experimentally by irradiation, resulted in significantly diminished cognitive recovery in adult rats following TBI as assessed by MWM testing (Tada et al., 2000). This suggests a role for these new cells in neurogenesis and cognitive recovery following TBI.

The function of hippocampal neurogenesis

The function of adult neurogenesis is not fully understood. However, substantial evidences have shown a correlation between hippocampal neurogenesis and hippocampal-dependent learning and memory. It has been shown that physical activity stimulates a notable increase in neuronal generation with subsequent enhancements in spatial learning and long-term potentiation (van Praag et al., 1999). In contrast, diminished hippocampal neurogenesis, which can be replicated by administration of an anti-mitotic drug or irradiation, has been associated

with poor performance on trace eyeblink classical conditioning which is a hippocampus-dependent task (Shors et al., 2004). Also, mouse strains with genetically low levels of endogenous neurogenesis have been observed to perform poorly on hippocampal-dependent learning tasks when compared to those with a higher level of baseline neurogenesis (Kempermann et al., 1997; Kempermann et al., 1998). Moreover, through studies examining hippocampal neurogenesis in various strains of inbred mice and evaluation with MWM testing, adult neurogenesis has been found to be involved in specific aspects of hippocampal function, such as the acquisition of new information (Kempermann and Gage, 2002). Collectively, these studies show compelling evidence supporting the role of hippocampal neurogenesis in cognitive function.

Although the connection between cognitive function and neurogenesis in the adult hippocampus is apparent, the underlying mechanisms contributing to innate recovery following brain injury are largely unknown. Previous studies revealing the association of hippocampal neurogenesis to normal cognitive function in the uninjured brain have led to speculation that neurogenesis may contribute to cognitive recovery following TBI. As previously outlined, there is a marked increase in cell proliferation in the DG of the hippocampus seen following TBI (Chirumamilla et al, 2002; Sun et al., 2005; Dash et al. 2001). The majority of TBI-induced newly generated cells in the adult DG that survive for an extended period of time assume a neuronal fate (Sun et al., 2007), and some of these surviving neurons have been reported to extend axonal projections to the target CA3 region as early as 14 days post-TBI (Emery et al., 2005). Furthermore, the integration of these new cells into the existing hippocampal circuitry has been demonstrated to coincide with the time course of cognitive recovery as assessed by MWM (Fig. 1.5; Sun et al., 2006; Sun et al., 2007). Despite the ability of the mature CNS to

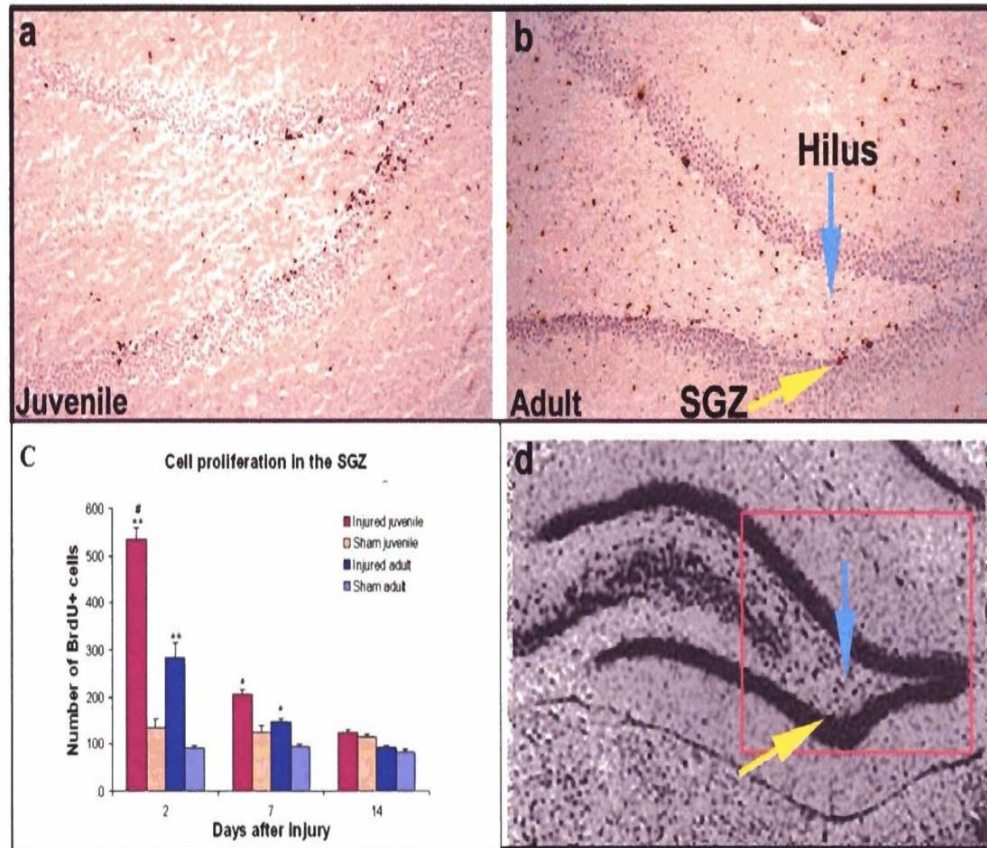


Figure 1.1: Cell proliferation in the ipsilateral dentate gyrus in juvenile and adult rats

following TBI. Coronal sections taken from injured juvenile (a) and adult (b) rats 2 days post-injury showing an elevated level of cell proliferation as labeled with BrdU in the DG of injured juvenile and adult rats. Arrows indicated BrdU-positive cells are clustered and concentrated in the subgranular zone (SGZ) of the DG. (c) Quantification of BrdU-positive cells in the SGZ. Graph shows that cell proliferation was significantly enhanced in injured juvenile and adult rats at both 2 and 7 days post injury as compared to age-matched sham controls (* $p < 0.05$, ** $p < 0.01$). The total number of proliferating cells in injured juveniles was significantly higher than injured adult rats at 2 days post injury (* $p < 0.05$, $n = 4/\text{group}$). (d) Nissl stained sections cross the hippocampal dentate gyrus with red box showing the corresponding area in (a) and (b) (Sun et al., 2005). In addition, our lab has demonstrated that new granule neurons generated following TBI can establish the correct anatomical connections to the CA3 region (Fig. 1.3). To further illustrate that these new neurons integrate into the existing neuronal circuitry, using synaptic vesicle marker synaptophysin we have shown that newly generated granule neurons form synapses with existing hippocampal neurons (Fig. 1.4; Sun et al., 2007).

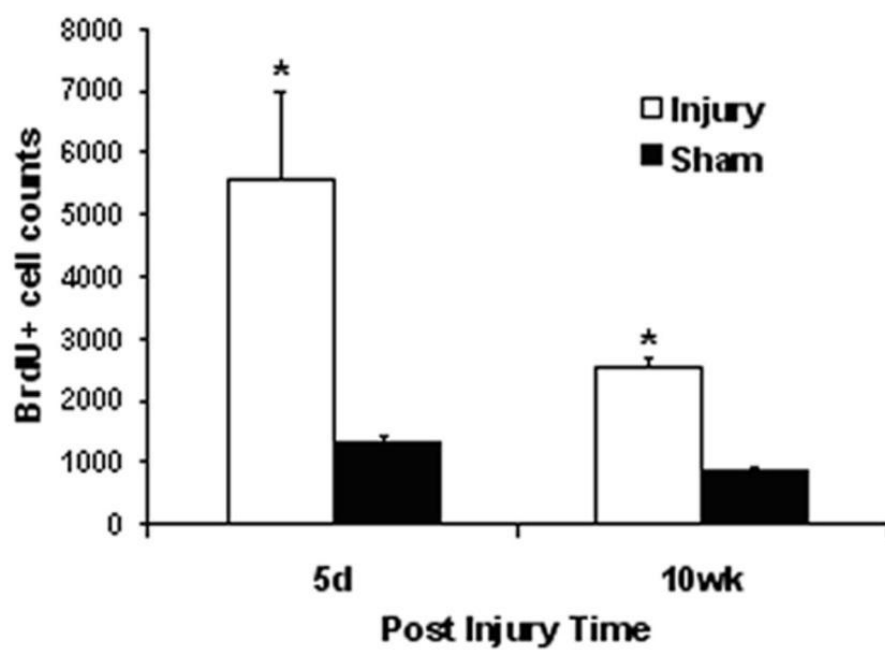


Figure 1.2: The number of BrdU-positive cells in the ipsilateral dentate gyrus in adult rats at 5 days and 10 weeks post-injury. Moderate lateral fluid percussion injury induced a 4-fold increase in the number of BrdU+ cells in the ipsilateral GCL as compared to sham animals at 5 days post-injury (* $p < 0.05$). The total number of BrdU+ cells decreased in both injured and sham animals over time. At 10 weeks post-injury, the overall number of BrdU+ cells remained 3-fold higher in injured animals compared to sham animals (* $p < 0.01$)(Sun et al., 2007).

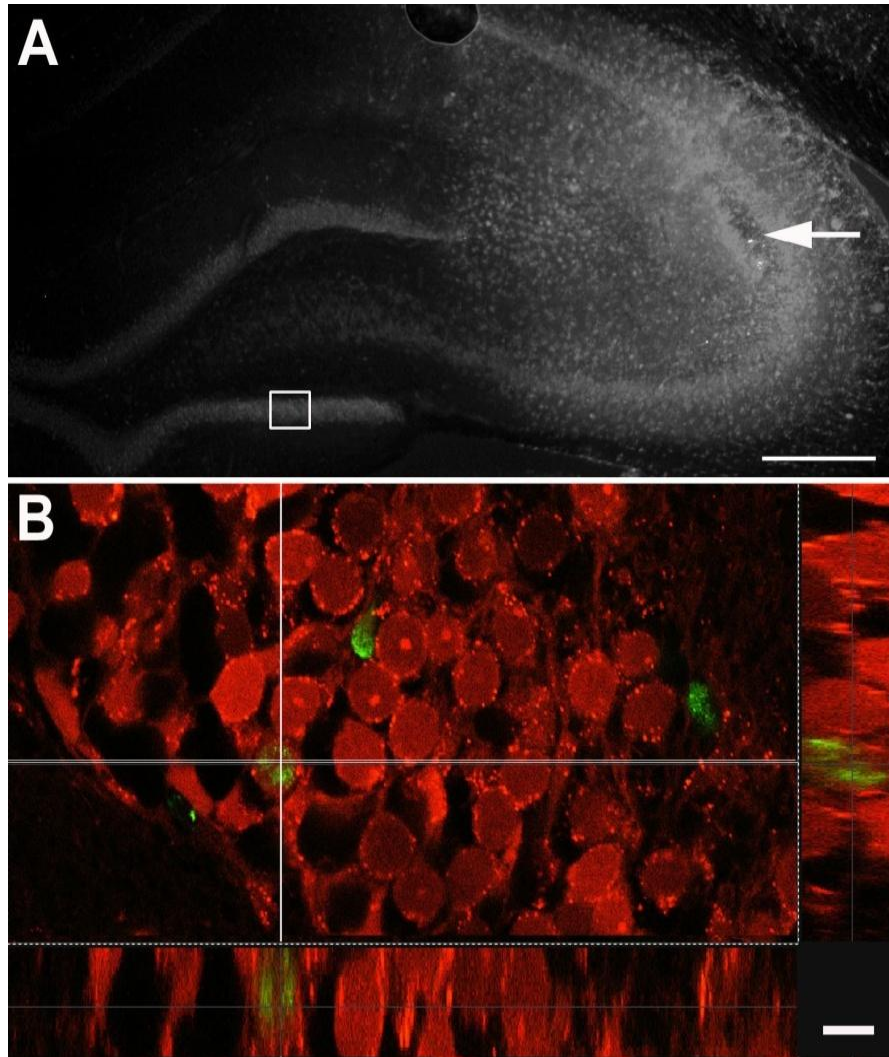


Figure 1.3: Newly generated neurons project axons to the CA3 region of the hippocampus.

a) Coronal section through the hippocampus of an injured adult rat which had received a fluorogold injection in the CA3 region at 8 weeks post-TBI with arrow indicating the injection epicenter. **b)** Confocal micrograph of the boxed area in panel (a) showing colocalization of BrdU (green) and FG (red), confirming that this newly generated cell had projected its axon to the CA3 region. Scale bars: (a): 250 μm ; (b): 20 μm . (Sun et al., 2007).

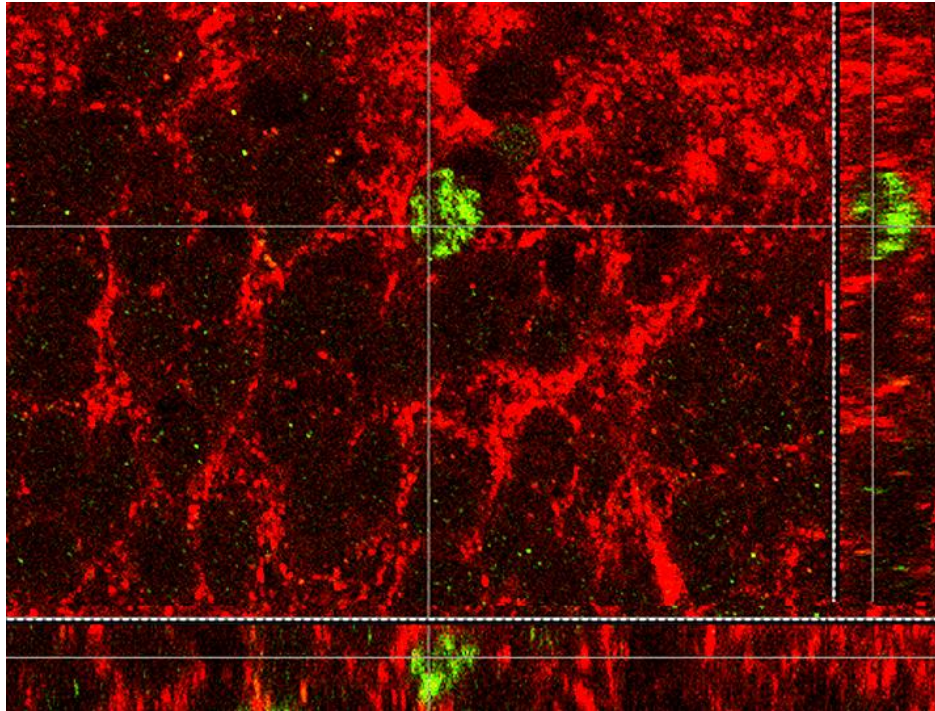


Figure 1.4: Newly generated granule neurons are retrogradely labeled with FG (fluorogold) and express synaptophysin. Confocal micrograph throughout the z -axis showing a newly generated BrdU⁺ cell (green) that is retrogradely incorporated with FG (red) after 10 weeks. This same BrdU⁺ cell is surrounded by a latticework of synaptophysin staining (blue). Scale bar: 30 μ m. (Sun et al., 2007).

replenish lost or damaged neurons throughout life, this regenerative capacity has been shown to decrease with age (Kuhn et al., 1996), which has led to increased investigation into the underlying causes of these age-related differences.

TBI and the aging brain

It has long been accepted that juvenile mammals recover to a greater extent than adults following brain trauma. Past clinical studies have found that children exhibit greater cognitive recovery and are less dependent on assistance than young adults following brain injury (Eiben et al., 1984). Experimental studies have shown that 3 month old injured rats performed significantly better than their 20 month old counterparts on hippocampal-dependent memory tasks such as the Morris water maze, which correlates with age-related differences in cognitive recovery seen in a clinical setting (Fig. 1.6; Hamm et al., 1992). The underlying biological basis for these age-related differences in functional recovery is not fully understood. However, a decrease has been observed in the proliferative and neurogenic capacity of the mammalian CNS with age, which may contribute to the significantly limited cognitive recovery seen in older TBI patients (Kuhn et al., 1996). Past studies in our lab have demonstrated that the juvenile brain is capable of generating a much more robust level of cell proliferation in response to TBI than the adult brain (Fig. 1.7; Sun et al., 2005). In addition to the more pronounced proliferative response seen in the juvenile brain, a greater percentage of newly generated cells differentiate into neurons in juveniles following brain insult as compared to injured adults (Sun et al., 2005). Another study pointed out a correlation between decreased survival of newly proliferated cells and a decline in learning and memory function (Wati et al., 2006). Overall, it is likely that the significantly enhanced levels of cell proliferation and neurogenesis seen in the juvenile brain contribute to its increased level of recovery above that of aged animals following TBI. Not only

has decreased hippocampal neurogenesis in the aged brain been associated with memory and learning deficits, but also has been tied to degenerative structural changes that occur naturally with aging (Driscoll et al., 2006). There is a progressive decline in CNS structure and function with age which is characterized by decreased plasticity, decreased cortical volume, decreased synaptic density, white matter degeneration, and glial cell reactivity, along with many other metabolic and micro-environmental changes; however, the hippocampus experiences relatively few structural changes as the brain ages and retains the capacity to generate new, functional neurons throughout life (Brazel and Rao, 2004). The observed decrease in neurogenesis with age has been shown in mice to be caused in some measure by a substantial loss of neural precursor cells in the SVZ, as well as a reduction in expression of important transcription factors and neurogenic factors (Ahlenius et al., 2009; Rao et al., 2006). Other studies have shown exacerbated oxidative damage, diminished antioxidant capacity, and a significantly increased loss of tissue in aged rats compared to young adult rats following TBI (Shao et al., 2006). Additionally, there is an exacerbated inflammatory response involving the prolonged activation of microglia and astrocytes in the aged hippocampus following TBI, which may contribute to the poorer cognitive outcomes seen in the elderly (Sandhir et al., 2008). As a whole, these changes may contribute to an overall impaired ability to respond to the various pathological sequelae and recover from functional deficits induced by brain insult.

The senescent CNS still retains the capacity for neurogenesis and plasticity, albeit not to the level seen in the juvenile brain. However, the aging brain presents an area of research which has the potential to benefit an ever-growing and increasingly disadvantaged population, and many studies hold some therapeutic promise. Although there is a steady decrease in endogenous neurogenesis with age, this happens primarily through a decrease in new cell generation without

any observable changes in the proportion of cells assuming a neuronal fate or survival of certain cell types (Olariu et al., 2007). In a rat model, the observed decrease in neurogenesis with age has been attributed in part to a decrease in available neural precursor cells, and a level of neurogenesis similar to that of a younger adult can be restored in the aged brain by addition of neural precursor cells to the dentate gyrus (Olariu et al., 2007). It has also been observed that some level of neurogenesis can be restored in the aged brain by removing glucocorticoids, which have been shown to inhibit neurogenesis in the dentate gyrus (Gould et al., 1992; Olariu et al., 2007; Kempermann et al., 1998). Experience-induced neurogenesis has been demonstrated in the aging mouse dentate gyrus which was stimulated through an enriched environment (Kempermann et al., 1998). Exercise-induced hippocampal neurogenesis has also been displayed in the aged mouse and was associated with a significant reduction in some of the negative morphological and behavioral consequences of aging (van Praag et al., 2005). While the age-related differences seen in neurogenesis and functional outcome following TBI are not fully understood, and no effective cure has been developed, it is widely suspected that there is an underlying neurogenic basis for these differences which is mediated by growth factor expression (Sun et al., 2001).

There is evidence that growth factors may be involved in regulating various activities of neural precursor cells, particularly proliferation and differentiation (Kelly et al., 2005). The aged brain retains the capacity to respond to exogenous growth factors, as increased neurogenesis was exhibited in the hippocampus and the SVZ in 20 month old mice in response to bFGF (Jin et al., 2003). Administration of exogenous bFGF in aged rats resulted in improved functional outcomes and increased neurogenesis following middle cerebral artery occlusion (MCAO) (Won et al., 2006). When removed from their normal environment, neural precursor cells from adult

and aged SVZ respond similarly when challenged with growth factors *in vitro*, indicating a similar capacity for proliferation and differentiation (Ahlenius et al., 2009). We speculate that the TBI-enhanced proliferative response and subsequent differentiation of newly generated cells is mediated by endogenously expressed growth factors in the CNS. Therefore, it is thought that differences in growth factor expression may account for the observed age-related differences in cognitive recovery following TBI. A clearer understanding of the post-TBI micro-environment that most effectively promotes neurogenesis will allow this phenomenon to be exploited for therapeutic benefit in the aging brain.

bFGF as a potential treatment for TBI

In both the injured and uninjured brain, a number of observations have linked endogenous growth factor expression levels to cell proliferation and neuronal differentiation. For instance, previous *in vivo* studies have shown that expression of a number of growth factors is significantly elevated during development, which is a period of time during which the establishment of the numbers and types of cells is taking place (Caday et al., 1990; Lazar and Blum, 1992). Growth factors are produced by both neurons and non-neuronal cell types during brain development, and evidence suggests that they are responsible for the proliferation and differentiation of the various cell types in the CNS (Caday et al., 1990; Plata-Salaman, 1991). It has also been shown that various growth factors which are known to stimulate neurogenesis experience a significant decline in expression with increased age as compared to developmental expression levels (Cintra et al., 1994; Shetty et al., 2005), which corresponds well with the decrease in hippocampal neurogenesis seen during the natural aging process and suggests a causal relationship (Seki and Arai, 1995; Kuhn et al., 1996). Expression levels of mitogenic growth factors bFGF and EGF are significantly increased in the adult brain following TBI, and

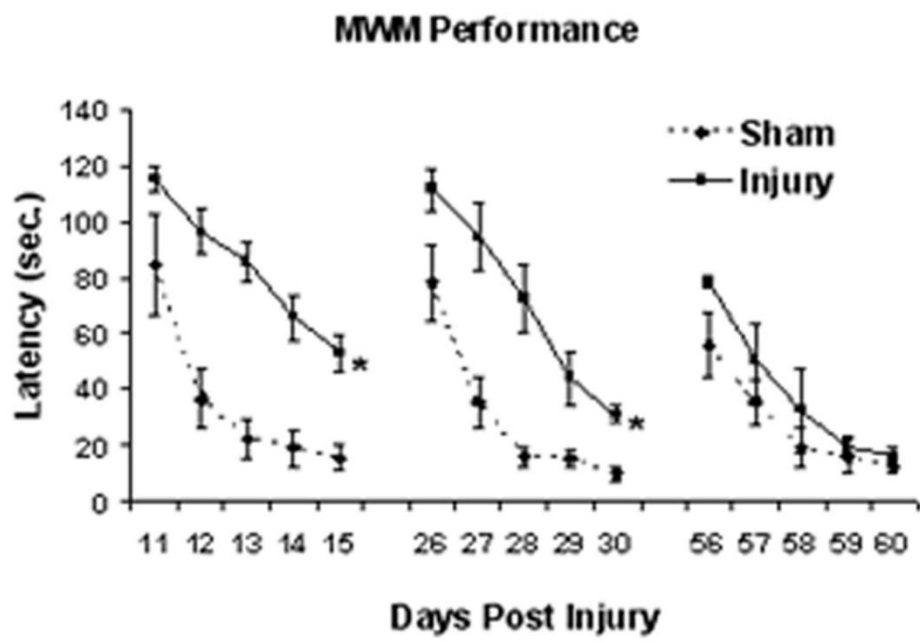


Figure 1.5: Cognitive deficits following TBI recover over time. Graph comparing Morris water maze performance of injured rats to sham animals during trials at days 11-15, 26-30, or days 56-60 following injury. Injured animals displayed significant cognitive deficits, as characterized by longer latency, at 11-15 days post-injury when compared to sham animals ($*p<0.05$). These deficits persisted at 26-30 days ($*p<0.05$). At days 56-60, injured animals showed cognitive recovery with a shorter latency which was not significantly different to sham animals. (Sun et al., 2007).

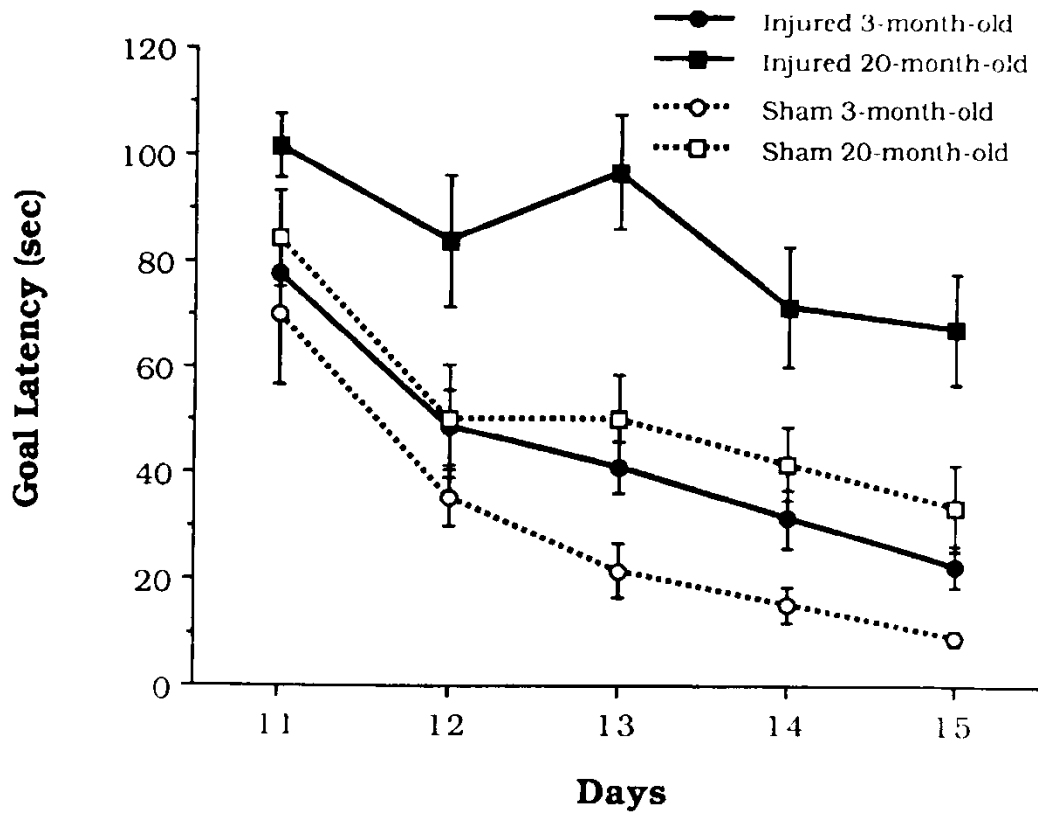


Figure 1.6: Younger animals exhibit a significantly greater degree of cognitive recovery following TBI than aged animals as assessed by the Morris water maze. Graph showing the mean (\pm SEM) of the latency of injured and sham-injured 3-month-old and 20-month-old rats to find the goal platform on days 11-15 after injury. Brain injury produced a deficit in performance in both age groups ($p < 0.0001$). However, injury resulted in a more severe impairment of function in the aged animals ($p < 0.05$). (Hamm et al., 1992).

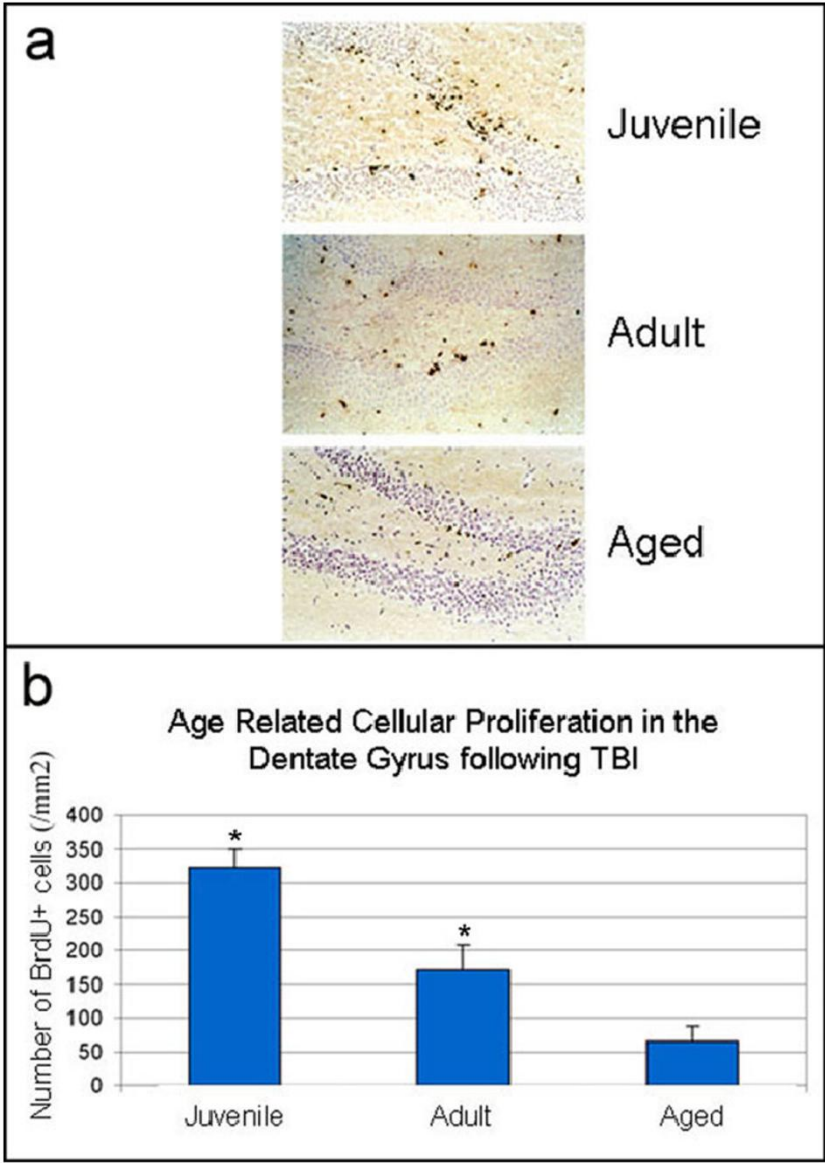


Figure 1.7: Cellular proliferation after TBI decreases with age. **a)** Coronal section of the of the DG in injured juvenile, adult, and aged rats stained for BrdU showing the decreased level of TBI-induced cell proliferation with age. **b)** Graph showing the decrease in total number of BrdU+ cells in the ipsilateral DG found with age ($*p<0.01$, $n = 4/\text{group}$). This graph represents a direct comparison of the injury-induced cell proliferative response between juvenile, adult, and aged rats, after subtracting out the number of proliferating cells in uninjured sham rats of the corresponding age groups. (unpublished data).

this elevated growth factor expression closely parallels the increase in cell proliferation seen following injury (Mattson and Scheff, 1994; Oyesiku et al. 1999). Taken together, these findings suggest a growth factor-mediated proliferative response to TBI.

Among these growth factors, bFGF has shown promise as a potential treatment for TBI related deficits. Fibroblast growth factors (FGF), namely aFGF and bFGF, are heparin-binding polypeptide signaling molecules that have an important role in nervous system development and maintenance and are also involved in angiogenesis and wound repair (Burgess and Maciag, 1989). FGFs elicit a broad spectrum of cellular responses by interacting with the polysaccharide portion of heparin sulfate proteoglycans (HSPG), a component of the extracellular matrix, and FGF receptors (FGFR1-5), which are receptor tyrosine kinases; FGFR activation induces tyrosine phosphorylation and recruitment of SHP2 which promotes sustained activation of the Ras to ERK pathway and leads to changes in gene transcription (Goldfarb, 2001). FGFs are present in high levels in the brain and have been shown *in vitro* to take action on various CNS and PNS cell types (Eckenstein et al., 1991). Moreover, studies have shown that bFGF is a potent mitogenic factor for neural precursor cells both *in vitro* and *in vivo*. Cultured hippocampal neural progenitor cells divide in response to bFGF *in vitro* (Ray et al., 1993; Vicario-Abejon, 2004). *In vivo* studies have demonstrated that bFGF expression levels are elevated during CNS development and diminish with maturity (Shetty et al., 2005; Caday et al., 1990). This decrease in bFGF expression is effectively reversed following various forms of brain injury (Kumon et al., 1993; Logan et al., 1992). Additionally, both subcutaneous and intraventricular deliveries of bFGF to normal adult animals enhance endogenous neural progenitor cell proliferation in the DG and the SVZ (Kuhn et al., 1997; Wagner et al., 1999). Furthermore, the injury-induced proliferative response is eliminated in bFGF null mice, but can

be restored by administration of exogenous bFGF (Yoshimura et al., 2001). Collectively, these studies implicate bFGF as an important regulatory factor in neurogenesis and brain repair.

Recent studies in our lab have explored the therapeutic potential of bFGF for brain repair in the rat FPI model. Consequently, exogenous administration of bFGF for 1 week post-injury was found to significantly enhance the TBI induced proliferative response in the SVZ and DG of adult rats (Fig. 1.8; Sun et al., 2009). In addition, post-TBI infusion of bFGF was also found to enhance endogenous neurogenesis above what is seen in untreated injured animals (Sun et al., 2009). Administration of bFGF immediately after injury not only enhanced TBI-induced cell proliferation and neurogenesis, but also attenuated the cognitive deficits associated with TBI in the adult brain as measured by MWM testing on days 21-25 post- injury (Fig. 1.9). Injured rats infused with bFGF displayed significant cognitive recovery as compared to their injured untreated counterparts (Sun et al., 2009). Having successfully demonstrated that intraventricular infusion of bFGF following injury has profound beneficial effects on the endogenous repair response and functional recovery in the adult rat brain, this study set out to use similar measures to determine if similar improvement in functional outcome can be achieved in the aged rat brain. We hypothesized that post-TBI treatment with bFGF can enhance hippocampal neurogenesis and improve cognitive recovery of injured aged animals. In this study, we will deliver bFGF through intraventricular infusion for 7 days. Hippocampal neurogenesis will be assessed with BrdU and neuronal markers. Cognitive function will be examined with novel object recognition and Morris water maze tests.

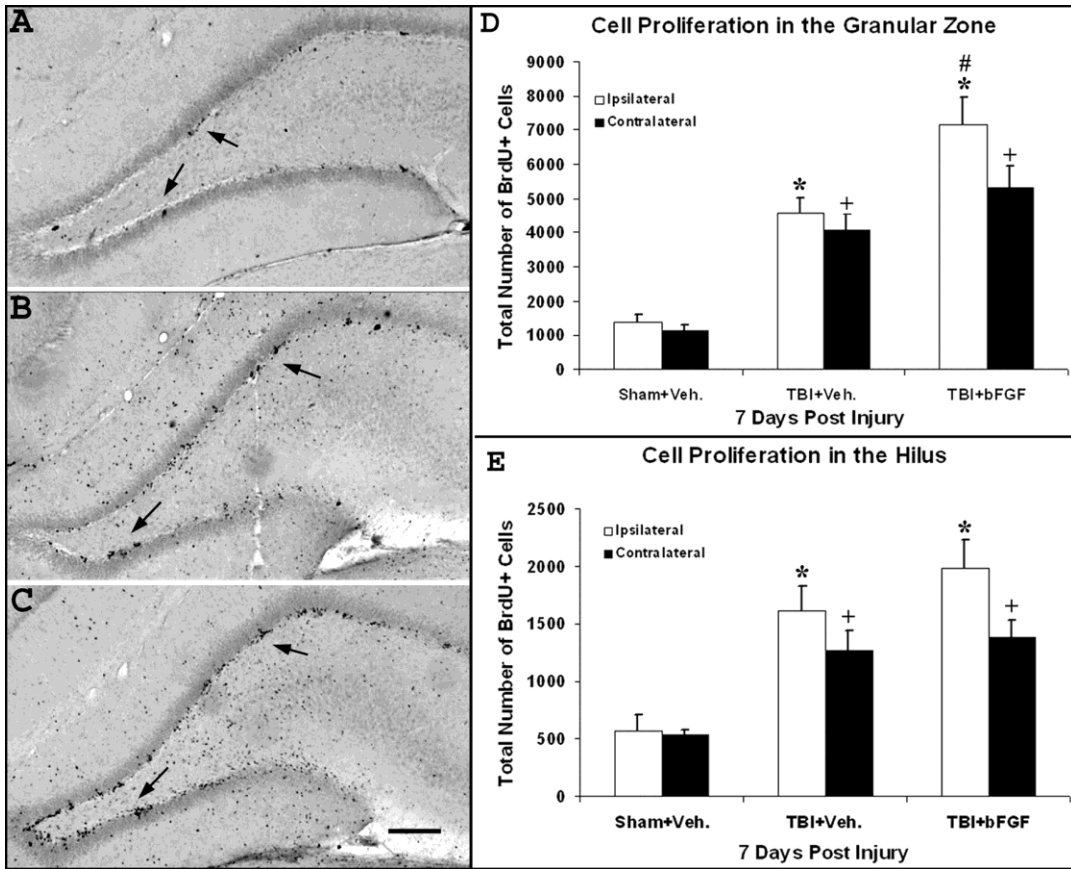
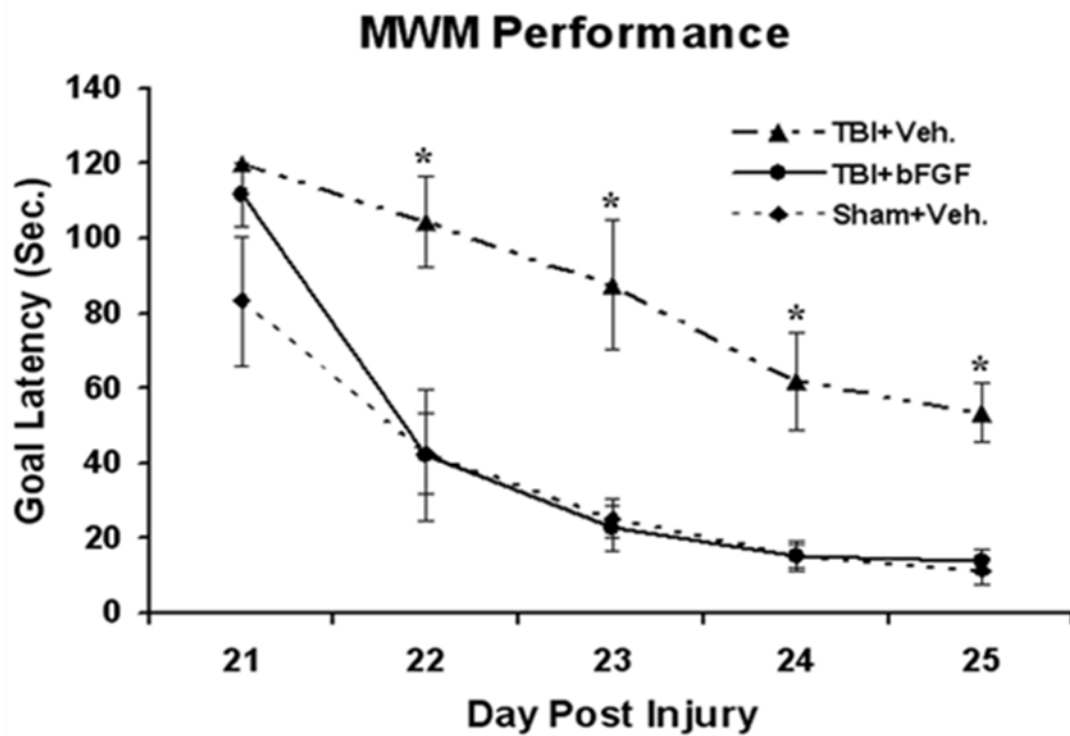


Figure 1.8: Intraventricular infusion of bFGF enhances cell proliferation in the DG.

Coronal sections of the ipsilateral DG were taken from the following animals at 7 days post-injury: **a)** sham with vehicle infusion; **b)** injured with vehicle infusion; and **c)** injured with bFGF infusion. Increased numbers of BrdU+ cells were observed in the injured animals with either vehicle or bFGF infusions compared to the sham (BrdU+ cell clusters indicated by arrows). BrdU+ cells in the DG were clustered and mainly located in the SGZ. Scale bar: 200 μ m. **d)** Quantification analysis of the degree of cell proliferation in the DG. Compared to shams, injured animals with vehicle or bFGF infusion had significantly more proliferating cells in the ipsilateral granular zone ($*p<0.05$) and the contralateral side ($+p<0.05$). Injured animals which received bFGF had a significantly higher number of BrdU+ cells in the ipsilateral granular zone compared to injured animals to injured animals with vehicle ($\#p<0.05$). **e)** Quantification analysis of the degree of cell proliferation in the hilus region. Compared to sham animals, proliferation cells in both the ipsi- and contralateral hilus were significantly higher in the injured animals with either vehicle or bFGF ($*/+p<0.05$). (Sun et al., 2009).



Sun et al., 2009

Figure: 1.9: Post-TBI infusion of bFGF improves cognitive recovery as assessed by Morris water maze testing. Graph compared MWM performance of injured rats infused with either bFGF or vehicle, to sham animals infused with vehicle alone. Injured rats infused with bFGF showed a significant improvement of cognitive recovery as compared to injured rats with vehicle ($*p < 0.01$, $n = 10$ in each group). This cognitive recovery, as characterized by shorter goal latency in the water maze performance, reached similar levels to that observed in sham animals through days 22-25 following injury. (Sun et al., 2009).

Chapter 2 - Materials and Methods

Subjects

All protocols and animals used for this study followed NIH guidelines and were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. A total of 47 male Fischer 344 rats (NIA, NIH) aged at 20 months weighing approximately 450g were used. These animals were housed in individual cages with food and water available *ad libitum*. The room where these animals were stored was maintained at a temperature of 20-22 degrees Celsius with a 12 hour light/dark cycle. Of the 47 rats employed for this study, 28 were used for data analysis.

Surgical preparation and procedure

Animals were subjected to a moderate lateral fluid percussive injury (L-FPI) following a standard protocol. All surgical tools were sterile and aseptic surgical procedures were carried out throughout the surgical processes. Each rat was anesthetized in a Plexiglas chamber with 5% isoflurane, intubated, and ventilated with 2.5% isoflurane in a gas mixture (30% O₂, 70% N₂). The top of the rat's head was shaved, and the rat was secured in a stereotaxic frame. The shaved region was cleansed and scrubbed with Betadine, and Puralube ointment was applied to the eyes. An incision was made sagittally along the midline to expose the skull, and the connective tissue and fascia were retracted using hemostats. A 4.9 mm craniotomy was made on the left parietal

bone half way between the lambda and bregma sutures using a trephine and a Dremel drill fitted with a small dental drill bit (Fig. 2.1). The resulting bone chip was removed, and a Luer lock fitting, or hub, was cemented to the skull with cyanoacrylic. After the integrity of the seal between the hub and the skull was confirmed, dental acrylic was applied liberally around the hub and allowed to dry. In preparation for injury, the male end of a spacing tube was then inserted into the hub and filled with 0.9% saline without introducing any air bubbles.

Lateral fluid percussion injury (L-FPI)

The injury model chosen for this study was L-FPI because it has been shown to reproduce similar pathophysiological effects observed in the clinic following TBI (McIntosh et al., 1989). When surgical preparation was complete, isoflurane anesthesia was switched off. Once the rat regained consciousness showing toe and tail reflexes, the intubation tube was disconnected, the rat was removed from the stereotaxic frame and connected to the pre-calibrated fluid percussion injury device through the Luer-Lock and the fitting tube, and a moderate fluid impulse (1.8 ± 1 atm) was administered (Fig. 2.2). Immediately after the injury, the spacer/hub assembly was removed from the rat's skull, and the rat was reconnected to ventilation and allowed to recover without isoflurane. During the recovery period, paw reflex, tail reflex, and righting times were recorded as a method of assessing injury severity. The righting time is defined as the amount of time that lapses from the point of injury until the rat returns spontaneously to an upright position from being placed in a supine position. Injured rats were also checked for spontaneous respiration. Ventilation was continued until an appropriate breathing response was regained. Isoflurane anesthesia at 2.5% was resumed in preparation for the placement of an infusion pump into the ventricle once the rat demonstrated a righting reflex. Sham animals were subjected to the same surgical procedure without any injury.

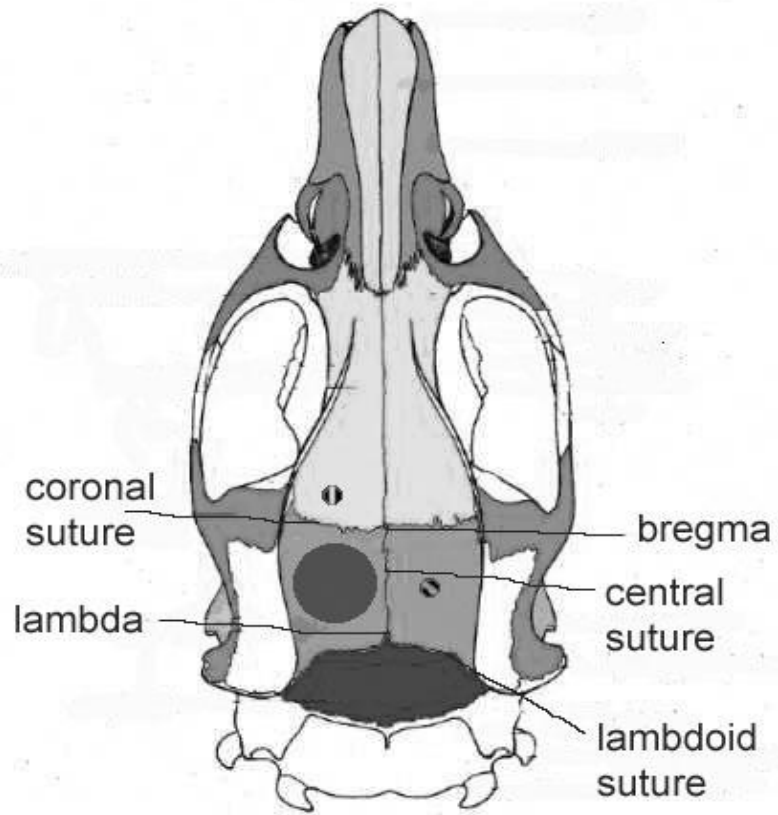


Figure 2.1: Craniotomy and injury site. A 4.9 mm craniotomy was made on the left parietal bone half way between the lambda and bregma sutures which served as the site where fluid percussion injury was delivered.

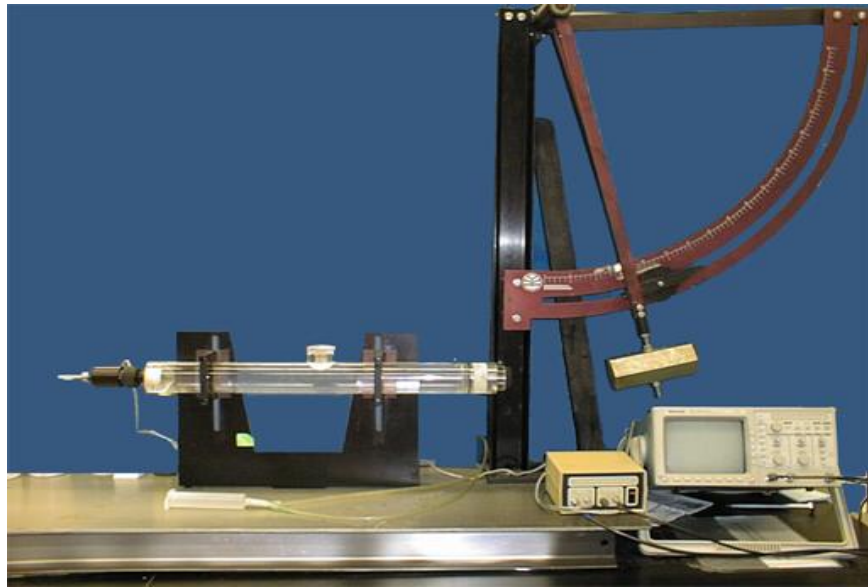


Figure 2.2: A photograph of a fluid percussion injury device.

Intracerebroventricular infusion

Thirty minutes after receiving the injury, when the rat was fully re-anesthetized, an Alzet brain infusion cannula connected to an Alzet brain mini-osmotic pump was stereotactically implanted into the posterior lateral ventricle ipsilateral to the injury site. The coordinates used for cannula placement were -0.8 mm along the anteroposterior axis and 1.4 mm laterally at a depth of 3.5 mm beneath the pial surface. Before the surgical incision was sutured, the mini-osmotic pump was placed subcutaneously on the back of the neck. A total of 10 rats receiving an injury were infused with recombinant human bFGF reconstituted in sterile artificial CSF (148 mM NaCl, 3 mM KCl, 1.4 mM CaCl₂, 0.8 mM MgCl₂, 1.5 mM Na₂HPO₄, 0.2 mM NaH₂PO₄, pH 7.4) containing 100 µg/ml bovine serum albumin and 10 µg/ml heparin for a final concentration of 33 µg/ml. A total of 10 rats receiving an injury and a total of 10 sham uninjured rats were infused with the vehicle solution. Solutions were delivered for 7 consecutive days at a flow rate of 0.5 µl/hr (approximately 400 ng/day for bFGF). After successful placement of the cannula and mini-osmotic pump, the surgical incision was closed using a sterile stainless steel suture needle and 5-0 polyamide surgical suture in a simple continuous pattern. After closure of the incision, triple antibiotic ointment and 2% lidocaine hydrochloride jelly were applied to the incision. Upon recovering from anesthesia and regaining mobility, the rat was returned to a clean cage with a surgical drape and warmed on top of a heating pad, and observed for 3 hours before being returned to the animal housing facility. During the following 7 days after surgery and pump placement, the body weight of each animal was recorded and indicators of general health, such as lethargy, porphyrin staining, wound healing, and weight loss were assessed and recorded on a scale of 0-4. At post-injury day 7, the infusion cannula and Alzet mini pump was removed from each animal 2 hours after the last BrdU injection. For rats that were sacrificed on

post-injury day 7, the pump was removed after transcardial perfusion. For rats that were sacrificed on day 28, removal of infusion pumps was performed under isoflurane anesthetic via a nose cone. After removal, each infusion cannula was examined for blockage and the remaining fluid in the mini-osmotic pump was also examined.

BrdU injections

To label dividing cells, all rats were given single daily intraperitoneal injections of 5-bromo-2-deoxyuridine (BrdU) at a dosage of 50 mg/kg for 6 consecutive days starting at 48 hours post-injury. One group of animals was sacrificed at post-injury day 7 for the purpose of studying the effects of exogenous bFGF administration on cell proliferation as compared to injured rats and non-injured sham rats infused with the vehicle. A second group of rats was permitted to survive until 4 weeks post-injury for the purpose of assessing newly generated neural precursor cell survival and determining the maturational fates of these cells. This particular group was also used to assess recovery of cognitive function with a novel object recognition test and the Morris water maze test.

Tissue processing

Animals were euthanized at either 7 or 28 days post-injury with deep isoflurane anesthesia, then transcardially perfused with 300-400 ml 1x phosphate buffer saline (PBS) followed by 300-400 ml 4% paraformaldehyde in PBS. The brains were then dissected out and post-fixed in 4% paraformaldehyde in PBS for 48 h at 4° C, after which the fixative was changed with PBS. A vibratome was used to cut the brains coronally into 60 µm sections throughout the rostro-caudal extent of the brain. Sections were collected into 24-well plates filled with PBS

plus 0.01% sodium azide and were stored at 4° C. Four sets of 30 sections were collected from each brain so multiple types of immunostaining could be performed.

Immunohistochemistry

From each brain, one set of 30 serial sections was processed for BrdU immunohistochemistry in order to assess the number of dividing cells labeled with BrdU. From the group of animals sacrificed at 7 days post-injury, one set of 30 serial sections from each brain was processed for neuronal marker doublecortin (DCX) immunohistochemistry in order to assess generation of new neurons. Parallel sections were selected from the brains of rats sacrificed at 28 days post-injury and double-labeled with BrdU and a cell type specific marker including NeuN (mature neuron), GFAP (astrocyte), or ED1 (infiltrating macrophages and activated microglia) for the purpose of determining the maturational fate of the newly-generated cells.

BrdU immunostaining

Sections were washed 2 times for 5 minutes with PBS, and DNA was denatured with 50% formamide for 60 min at 60 °C. After washing with 2x saline sodium citrate (SSC) 3 times for 5 min, the sections were incubated with 2N HCl for 30 min at 37 °C. Following the denaturing process, sections were washed with 1x PBS 3 times for 5 min and incubated at room temperature for 1 hr in 3% H₂O₂ in order to block endogenous peroxidase. After washing 3 times with 1x PBS for 5 minutes, sections were blocked overnight in 5% normal horse serum (in 1x PBS with 0.3% Triton) and then incubated with mouse anti-BrdU antibody (Dako, CA) for 48 hrs at 4 °C. For this step, the primary antibody was prepared at a ratio of 1:200 in 5% normal horse serum, and 300 µl were added to each well. After this 48 hr incubation, the sections were washed in 1x PBS with 0.3% Triton 3 times for 10 min in preparation for the addition of the

secondary antibody, which was biotinylated anti-mouse-IgG (Jackson Laboratories, ME). For this step, the secondary antibody was prepared at a ratio of 1:200 in 5% normal horse serum, and 300 µl were added to each well. After incubating the sections for 24 hrs in the secondary antibody, the sections were washed in 1x PBS with 0.3% Triton 3 times for 10 minutes and once with 1x PBS for 5 minutes. The avidin-biotin complex (ABC kit, Vector Laboratories) was prepared 30 minutes before use at a concentration of 1:200 avidin and 1:200 biotin combined with a peroxidase included in the ABC kit into 1x PBS. 300 µl of ABC was added to each well, and the sections were incubated at room temperature for 2 hrs. The sections were then washed in 1x PBS 3 times for 10 minutes. The sections were then briefly incubated in a chromogen 5, 5-diaminobenzidine (DAB) solution at room temperature. To make the DAB solution, 2 drops of phosphate buffer at pH 7.5, 4 drops of 3,3'-Diaminobenzidine, and 2 drops of hydrogen peroxide were added to each 5 ml of dH₂O using a Vector DAB kit. The reaction was observed under a microscope until the sections were stained adequately and then quenched by adding 1x PBS to the wells. The sections were washed with 1x PBS 3 times for 5 minutes and stored at 4° C before mounting. The sections were washed in dH₂O and mounted to glass microscope slides.

DCX immunostaining

The staining procedure is similar to BrdU immunostaining, with the only difference being that the DNA denaturing process with formamide and HCl was omitted. Briefly, sections were washed with PBS, and endogenous peroxidase was blocked in 3% H₂O₂. This was followed by washing 3 times in 1x PBS with 0.3% Triton for 10 minutes and overnight blocking in 5% normal horse serum. Sections were then incubated with a goat anti-DCX antibody (1:1000, Santa Cruz, CA) for 48 hrs at 4 °C in blocking buffer. After this, the sections were washed in 1x PBS with 0.3% Triton 3 times for 10 min followed by incubation with biotinylated anti-goat IgG

(1:200, Jackson Laboratories, ME) for 24 hrs at 4 °C in blocking buffer. After wash, sections were incubated with ABC complex at room temperature for 2 hrs. The sections were then washed with PBS, and briefly incubated in a DAB solution at room temperature. The reaction was observed under a microscope until the sections were stained adequately and then quenched by adding 1x PBS to the wells. Sections were washed with PBS and dH₂O before being mounted to glass microscope slides.

Counterstaining procedure

Mounted sections were placed in water, stained with 0.1% cresyl violet solution for 1 min, then washed in running tap water, and sequentially placed into increasing concentrations of ethanol followed by differentiation of stain in acidic alcohol. Sections were dehydrated in 100% ethanol twice 1 min each, followed by placement in Citrisolv twice 5 min each, and cover slipped using Permount.

Immunofluorescent double-labeling

For immunofluorescent double-labeling, sections were processed using the same procedure described above for BrdU immunostaining and incubated for 48 hrs at 4° C in a rat (1:200, Immunologicals Direct, Oxford, UK) or mouse anti-BrdU antibody along with one of the following cell type specific markers: rabbit anti-GFAP (1:1000, Dako) to label astrocytes; mouse anti-NeuN (1:500, Chemicon) to label mature neurons, and mouse anti- ED-1 (1:1000, Chemicon) to label macrophages and activated microglia. Secondary antibodies used were Alexa Fluor 488 anti-rat IgG, Alexa Fluor 488 anti-mouse IgG, or Alexa Fluor 568 anti-rabbit IgG (1:200, Molecular Probes) for 2 hours at room temperature. After washing with PBS and

water, sections were mounted on glass slides and cover slipped with Vectorshield mounting media (Vector Labs, CA).

Stereological quantification

Quantification of the number of BrdU-positive and DCX-positive cells was carried out in the hippocampal dentate gyrus (DG). BrdU or DCX stained sections were examined using the Olympus Image CAST program (Olympus, Denmark). Ten 60 μm thick sections spaced 240 μm apart at the level of the DG were selected from each brain and examined using unbiased stereological methods. Cells of interest in the ipsilateral and contralateral granule cell layer (GCL) including subgranular zone (SGZ) and hilus were counted throughout the entirety of each region. For this study, the granular zone and subgranular zone were counted together as the granule cell layer. This was carried out by outlining the region of interest under 4x objective, then counting the cells within the region of interest under 60x oil immersion objective focusing through the thickness of the section. Cells in the upper and lower most focal planes were ignored. Average section thicknesses were obtained by measuring the depth of each section from one focal plane to another in 5 different randomly selected locations in the DG. Since counting frames were not used, and the entire region of interest was counted for each section rather than random samples, the average sampling fraction (asf) was equal to 1. The disector height ($h=15\mu\text{m}$) was known relative to the section thickness (t). With these parameters in place, the total number of cells counted was estimated as $N = (\sum Q)(t/h)(1/asf)(1/ssf)$, where ssf was the section-sampling fraction ($=0.25$), and $\sum Q$ was the raw number of cells counted.

Confocal microscopy

Immunofluorescent double-labeled sections were examined using a confocal microscope (Leica TCS SP2) in order to quantify the percentage of BrdU-positive cells that have differentiated to neurons, astrocytes, or microglia. The entire granule cell layer of each section was examined through its entire thickness, and every BrdU-positive cell was scrutinized to assess the co-localization of BrdU with cell type specific markers. Each BrdU-positive cell in the granular zone was manually viewed in its full z dimension, and only those cells for which the BrdU-positive nucleus was unambiguously associated with a given cell type specific marker was considered to be co-labeled. The percentage of co-labeled cells was calculated as the number of BrdU+/NeuN+, BrdU+/GFAP+, or BrdU+/ED1+ cells against the total number of BrdU-positive cells.

Novel object recognition test

To assess cognitive function, animals that were sacrificed at 4 weeks post-injury were evaluated with a novel object recognition test (NOR) at 3, 7, 14, and 28 days post-injury. The object recognition chamber used for this study was a 4 ft. x 4 ft. x 2 ft. box with a wooden bottom painted white and Plexiglas walls lined with opaque white paper externally. The chamber was divided into 4 quadrants. Objects were fastened with Velcro at the center of diagonally opposed quadrants, and the same quadrants were utilized throughout the entire study for consistency. Many different objects which varied in size, shape, color, and material were used for testing. Each animal being tested was individually habituated to the new environment of the chamber for 30 minutes without any objects present 24 hours before testing. For the first part of the NOR test, each rat was placed into the chamber alone with 2 identical sample objects for 5 minutes, then removed from the chamber for a period of 30 seconds while one of the

objects was replaced with a new object different from the sample. After the 30 second delay, the rat was placed back into the chamber for 5 minutes and allowed to explore the objects. For the second part of the NOR test, the rat was placed in the chamber for 5 minutes with 2 identical sample objects different from the objects used in previous tests. After a 4 hr. delay, the rat was placed back into the chamber for 5 min. with one of these sample objects and a novel object. All testing was performed in a dimly lit room to minimize any visual cues external to the chamber. The chamber and all objects were sprayed with 75% EtOH in H₂O and wiped clean between animals, allowing enough time for the EtOH to evaporate. All testing was recorded to VHS with a video camera mounted above the chamber, and tapes were later viewed to document the amount of time each animal spent exploring each individual object during the sample and testing phases. The same procedure was used on each day of testing, and no objects were reused for the same animal on a later test.

Morris water maze: Latency and probe trial

The Morris water maze performance (MWM) has been established as a valid method of assessing hippocampal-dependent cognitive function in rats (Brandeis et al. 1989, Hamm 2001). A total of 16 rats were assessed for cognitive function using the MWM on post-injury days 21-25 and a probe trial on post-injury day 26. A large circular tank 180 cm in diameter and 45 cm high was used for testing. The tank was filled to a depth of 30 cm with water which was maintained at 25-28° C. White latex-based paint was mixed into the water for opacity and a goal platform was concealed under the water 45 cm from the edge of the tank in the center of the southeast quadrant (Fig. 2.3). The testing was performed in a room with numerous external visual cues that remained constant throughout testing. Each rat was given 4 trials per day for 5 consecutive days. Each rat was placed on the goal platform for 30 seconds on the first day before any trials

were run in order to be habituated to the goal location. Rats were placed into the maze facing the wall at one of 4 starting points (N, S, E, or W) for each trial and each possible starting point was used once per day in a randomized fashion. Once the goal was reached, rats were allowed to stay on the hidden platform for 30 seconds. Rats were given a maximum of 120 seconds to find the platform and were placed on the platform for 30 seconds after this time. Rats were placed in an incubator between trials. Latency and path length was recorded using a video tracking system (Videomex, Columbus Instruments, Columbus, OH). In order to rule out any motor deficits that could potentially affect performance, swim speeds were calculated from each trial and compared between rats. Each rat was tested with a single probe trial the day following the final latency trial. For this test, the platform was removed from the tank and each rat was placed into the water facing the wall from a random starting point (N, S, E, or W). Rats were allowed to swim for 60 seconds then removed from the tank. The Videomex video tracking system was used to determine the amount of time each rat spent swimming in the goal quadrant and average proximity to the goal location.

Statistical analysis

All statistical analysis of data was done using SPSS software. A one-way ANOVA was used to determine any statistically significant differences in cell quantification and probe trial data, and post-hoc Student *t*-test was used to determine differences within groups, with *p* value less than 0.05 being considered statistically significant. MWM data was analyzed using a repeated measure ANOVA with the exception of swim speed, which was analyzed using a one-way ANOVA. All data are presented as mean \pm SEM.

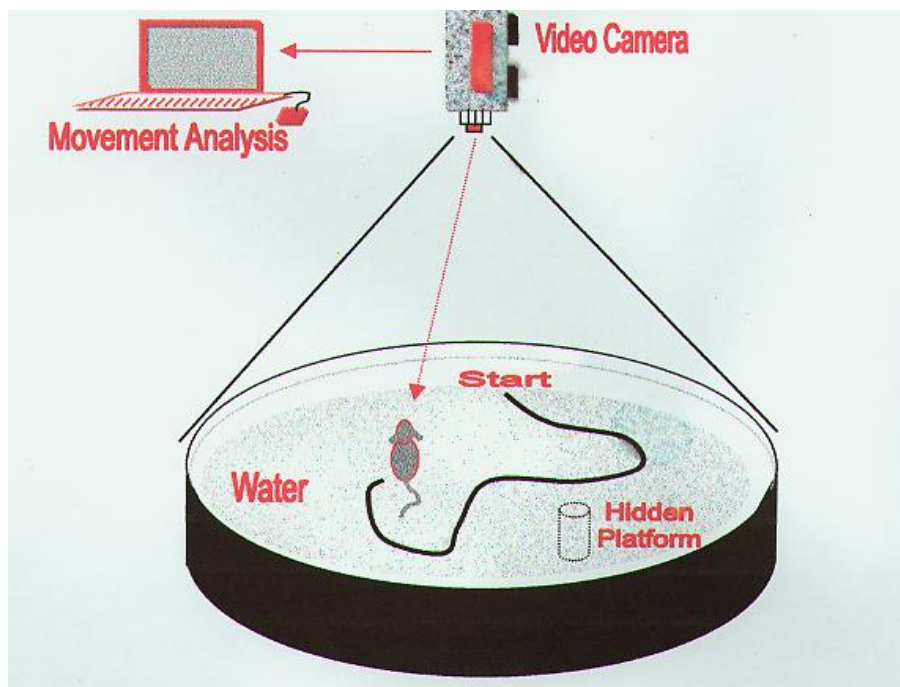


Figure 2.3: Morris water maze. An illustration the Morris water maze apparatus. A rat is placed in a tub of water and is allowed to search for a hidden platform while a camera connected to a computer records the movements of the rat and the length of time it takes for the rat to reach the goal.

Chapter 3 – Results

Based on previous studies done in our lab, intraventricular infusion of bFGF enhances the neurogenic response in the SVZ and in the hippocampal dentate gyrus, and improves cognitive recovery in adult rats following TBI. The aim of this study was to determine if treatment with bFGF following injury similarly improves neurogenesis and cognitive recovery in aged rats. In this study, a total of 47 Fisher 344 rats at the age of 20 months were used. Of the 47 rats, 17 died either immediately following TBI or within the first week following TBI. The calculated mortality rate for rats used in this study was 36.2%, which is similar to what was reported before (Hamm et al., 1991).

Righting response

The post-injury righting response was used to assess whether a similar level of injury was received by all animals injured in this study. The amount of time required for the return of the righting reflex, or duration of righting response suppression, is related to neuromotor deficits and is a valid indicator for determining injury severity (Morehead et al. 1994, Hamm 2001).

Righting times were recorded for each injured animal and compared between those animals that received bFGF infusion and those that received vehicle infusion. The mean (\pm SEM) duration of righting response suppression following injury was 15.89 ± 1.34 min for the vehicle-infused animals and 12.92 ± 1.31 min for bFGF-infused animals. A *t*-test was performed on this data

which demonstrated no significant difference between the righting times of bFGF- versus vehicle-treated rats ($p=0.14$), supporting the conclusion that a similar level of injury severity was received by both groups.

Effect of bFGF treatment on cell proliferation in the hippocampal dentate gyrus

In order to assess whether the injury-induced cell proliferation response in the DG of aged animals could be further augmented through exogenous bFGF administration, these animals were subjected to a moderate L-FPI followed by a 7-day infusion of recombinant bFGF into the lateral ventricle ipsilateral to the site of injury. Animals received daily single BrdU injections from 2 days post-injury for 6 days to label proliferating cells. These animals were then sacrificed at 7 days post-injury and processed for BrdU immunohistochemistry.

In the sham animals, a few BrdU-labeled proliferating cells were observed in the SGZ of the DG (Fig. 3.1). In the injured animal, a massive amount of BrdU-labeled cells was found in the ipsilateral DG in the granule cell layer (including SGZ) and the hilus region, as well as the outer molecular layers in animals either bFGF or vehicle-treated as compared to sham animals (Fig.3.1). Stereological quantification of BrdU-positive cells in selected subregions within the DG showed that the total number of proliferating cells was significantly enhanced in the ipsilateral GCL (SGZ included) and hilus of injured animals receiving vehicle or bFGF infusion compared to sham (Fig. 3.2a & b, $p<0.05$). In injured animals, the number of BrdU-positive cells in the ipsilateral GCL was higher in the bFGF-treated group than vehicle-treated animals, but did not reach statistical significance ($p=0.36$, Fig. 3.2b). No significant difference was found in the total number of BrdU-positive cells in the contralateral GCL and hilus regions (Fig. 3.2a and b).

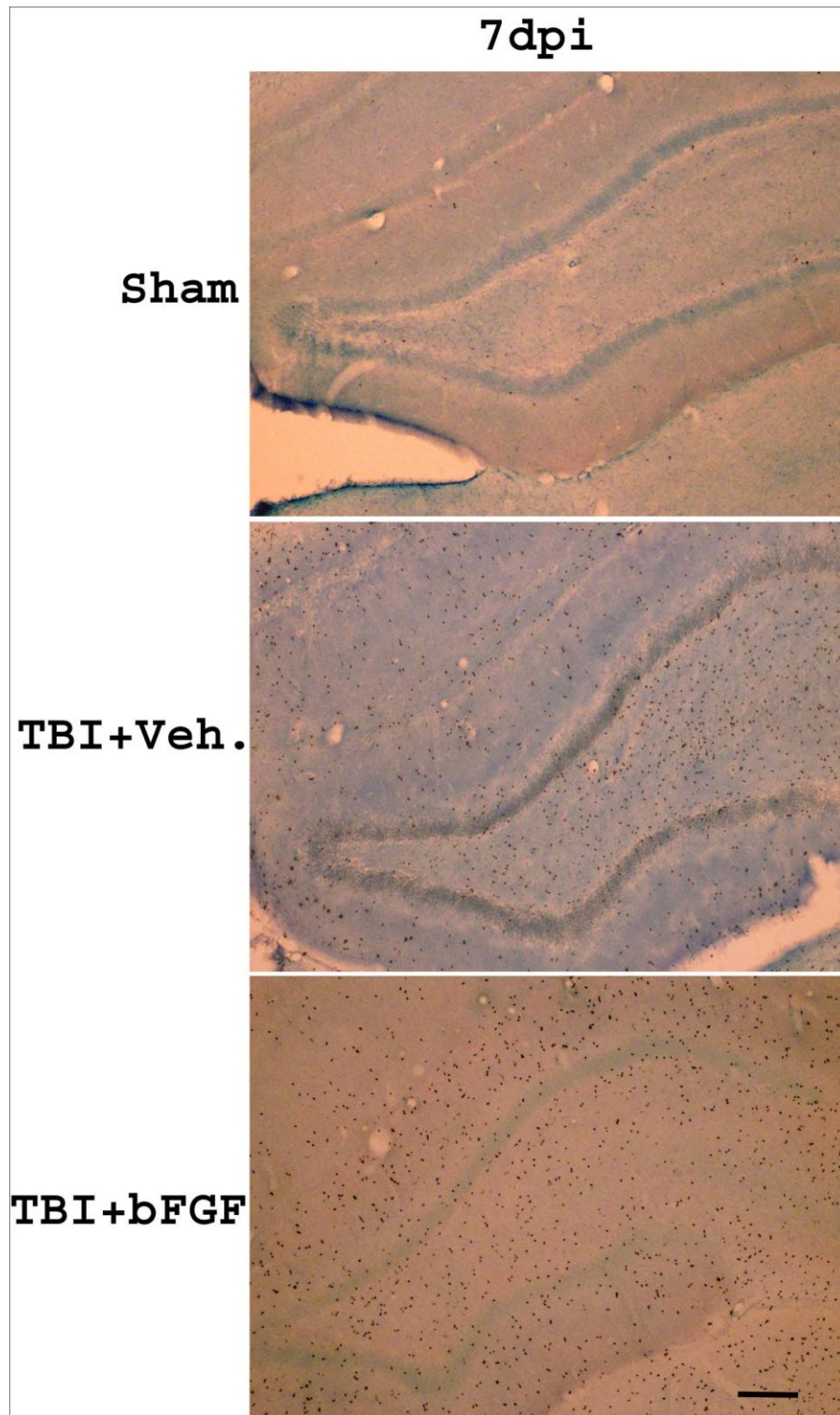


Figure 3.1. Cell proliferation at 7 days post-injury. Micrographs taken from BrdU stained coronal sections in the ipsilateral DG from sham, injured with vehicle or bFGF treated animals show that, **a)** in the sham animal, a few BrdU-positive cells were located mostly in the SGZ of the DG; **b)** in the injured vehicle-treated animal, a massive amount of BrdU-labeled cells in the ipsilateral DG in the GCL (including SGZ) and the hilus region, as well as the outer molecular layers; **c)** similar pattern of BrdU staining as shown in (b) was observed in injured bFGF-treated animals. Brown dots indicated BrdU-labeling. Bar scale = 500 μ m.

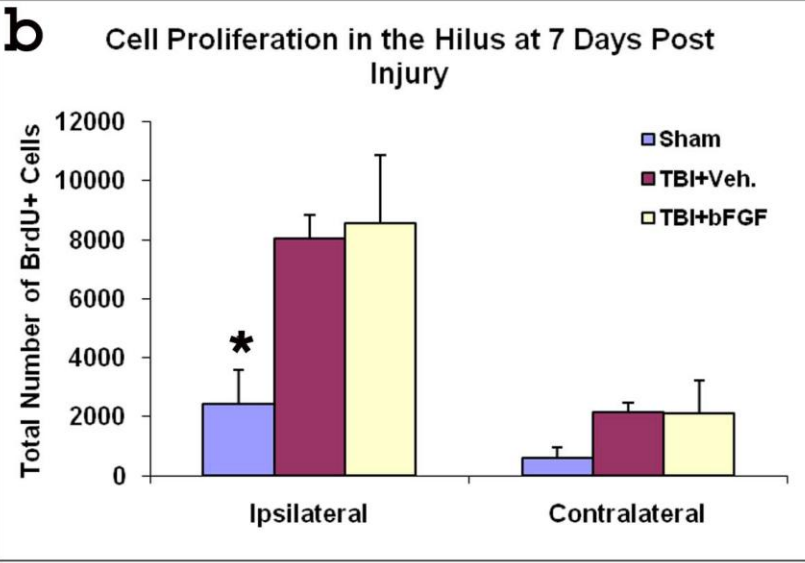
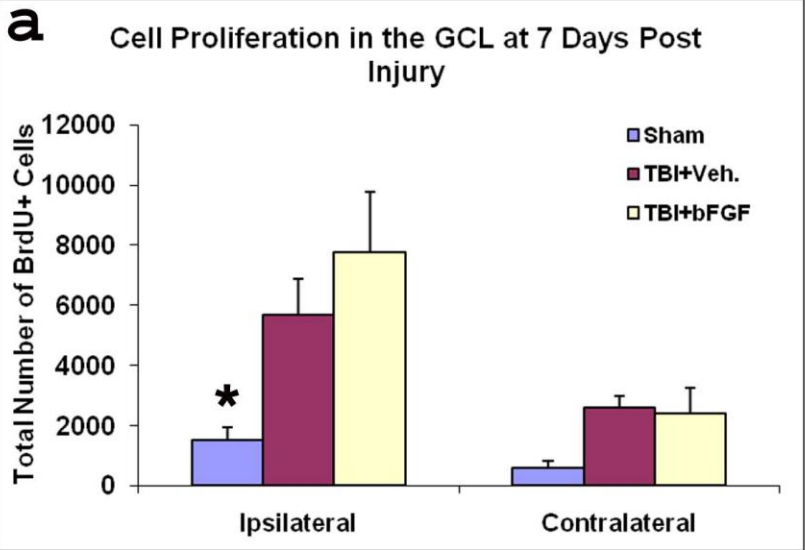


Figure 3.2: Quantitative analysis of the degree of cell proliferation in the granule cell layer and hilus in the DG at 7 days post-injury. **a)** In the GCL (SGZ included), compared to sham animals, injured animals treated with either vehicle or bFGF had a significant increase in the total number of BrdU-positive cells in the ipsilateral GCL ($F_{2,9}=4.34$, $*p<0.05$) but not in the contralateral GCL. Among injured animals, the total number of BrdU-positive cells in the ipsilateral GCL in the bFGF-treated group was higher than vehicle-treated group, but did not reach statistical significance ($p=0.36$). **b)** In the hilus region, injured animals had a significantly higher number of BrdU-positive cells than sham animals in the ipsilateral side ($F_{2,9}=4.29$, $*p<0.05$), but not in the contralateral side. Among injured animals, no significant difference in the number of BrdU-positive cells was found between groups ($p=0.81$).

Effect of bFGF treatment on generation of new neurons

To assess whether bFGF can augment generation of new neurons in the aged brain following injury, every 4th section from animals sacrificed at 7 days post-injury were stained for early neuronal marker DCX. Examination of DCX-positive cells in the aged rat DG revealed that DCX-labeled neuronal cell bodies were primarily localized in the SGZ (Fig. 3.3a). Various types of dendrite morphologies were observed, including neurons with vertically oriented dendrites, horizontally oriented dendrites, or a mixture of the two (Fig. 3.3b). DCX-labeled cell bodies were quantified using stereological methods and each cell was classified according to its dendrite morphology as vertical, horizontal, or mixed. Stereological quantification of DCX-positive cells in the ipsilateral and contralateral GCL (SGZ included) showed a significantly enhanced level in the number of DCX+ cells in both ipsi- and contralateral GCL in the bFGF-infused animals following injury as compared to injured vehicle-infused animals and sham animals (Fig.3.3a, $p < 0.01$). There were no significant differences in the number of DCX+ cells in the GCL in vehicle-infused injured animals and sham animals both ipsilaterally and contralaterally (Fig. 3.4a). In the hilus region, the number of DCX+ cells was higher in the ipsilateral side in the injured animals with vehicle or bFGF treatment than in sham animals, but did not reach statistical significance (Fig 3.4b, $F_{2,9}=1.36$, $p=0.30$). No significant differences were seen in the ipsilateral hilus among the two injured groups (Fig. 3.4b). In the contralateral hilus, the injured bFGF-treated group had a higher number of DCX+ cells than the vehicle treated or sham groups, but did not reach statistical significance (Fig.3.4b $F_{2,9}=3.08$, $p=0.10$). These data indicate that post-TBI administration of bFGF enhances generation of new neurons in the ipsilateral and contralateral granule cell layer in injured aged rats.

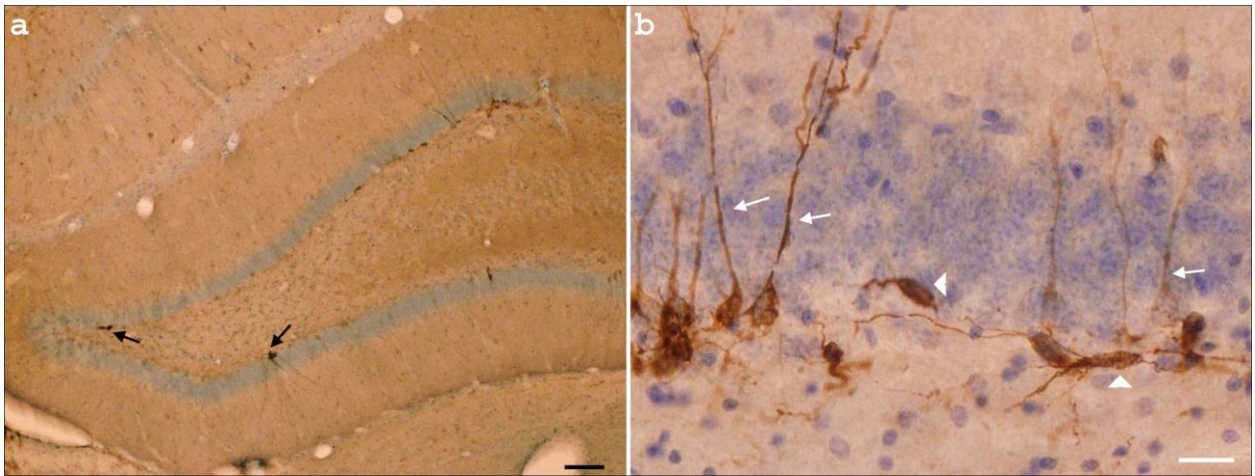


Figure 3.3: Generation of newly-generated neurons as labeled with DCX in the dentate gyrus in aged rats at 7 days post- injury. **a)** Photograph shows a few DCX-positive cells in the aging DG clustered primarily in the SGZ as indicated by arrows. **b)** DCX-stained neurons with vertically oriented dendrites (arrows), horizontally oriented dendrites (arrow head), or a mixture of the two were observed.

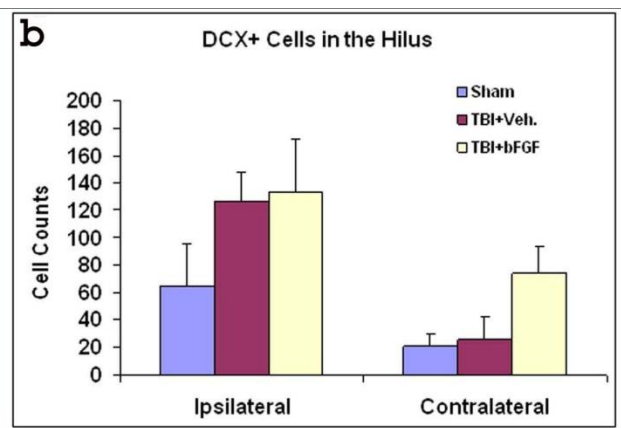
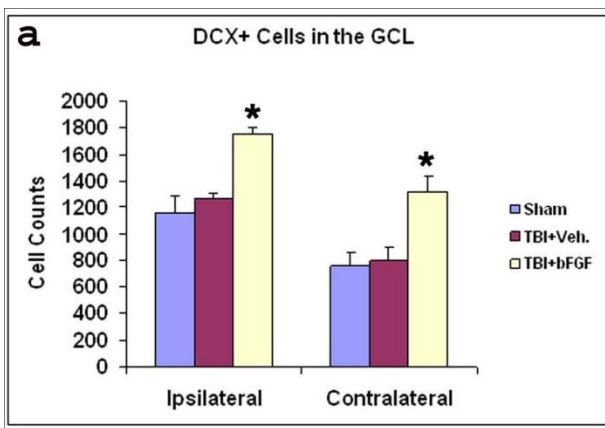


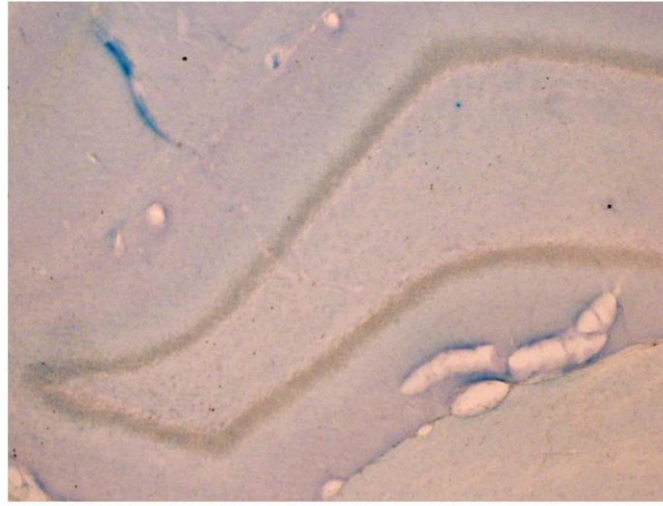
Figure 3.4: Quantitative analysis of the total number of newly-generated cells as labeled with DCX in the dentate gyrus at 7 days post-injury. a) In the GCL, injured animals treated with bFGF had a significantly higher number of DCX-labeled new neurons in both the ipsilateral and contralateral GCL (ipsilateral GCL: $F_{2,9}=18.32$, $*p<0.01$; contralateral GCL: $F_{2,9}=8.90$, $*p<0.01$). There were no significant differences in the number of DCX+ cells between injured vehicle-infused animals and sham animals both ipsilaterally and contralaterally. b) In the hilus region, injured animals treated with either vehicle or bFGF had a higher number of DCX-positive cells in the ipsilateral side compared to sham, but was not significant ($p=0.30$); no difference was found among the injured groups. In the contralateral hilus, the injured bFGF-treated group appeared to have a higher number of DCX-positive cells than injured vehicle-treated or sham groups, but no significant difference was found ($p=0.10$).

Effect of bFGF on survival of newly generated cells

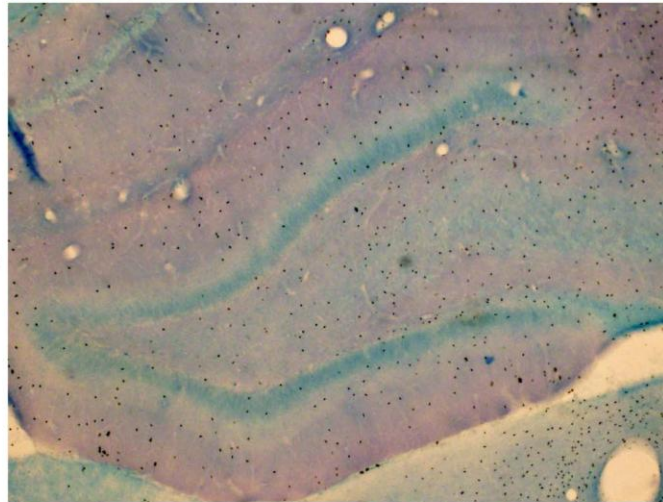
In order to assess whether bFGF treatment can enhance long term survival of the newly-generated cells, every 4th brain section from animals sacrificed at 4 weeks post-injury were processed for BrdU immunohistochemistry and the number of BrdU-positive cells in the DG was quantified. At 4 weeks post-TBI, a few sparse BrdU-labeled proliferating cells were observed in the DG in sham animals (Fig. 3.5). In the injured animal, a large amount of BrdU-labeled cells was observed in the ipsilateral DG in the GCL (with SGZ) and the hilus region, as well as the outer molecular layers in injured animals either bFGF or vehicle-treated as compared to sham animals (Fig.3.5). Stereological assessment was done in a similar manner as described for the 7 days post-injury assessment. The total number of BrdU-positive cells counted in the ipsilateral GCL (with SGZ) and hilus was significantly higher in injured animals treated with either bFGF or vehicle compared to sham animals (3.. 6a and e; GZ, $p<0.01$; hilus, $p<0.05$). In the ipsilateral GCL (with SGZ) and hilus in bFGF-infused animals, the number of BrdU-positive cells was higher than vehicle-treated animals; however, the difference was not statistically significant (Fig. 3.6a and b; GCL, $p=0.06$; hilus, $p=0.09$). In the contralateral GCL (with SGZ), in comparison to sham animals, the total number of BrdU-labeled cells was significantly higher in vehicle-treated injured animals (Fig. 3.6a, $p<0.001$), and higher in bFGF-treated injured animals, although not reaching statistical significance (Fig. 3.6a, $p=0.06$). No significant differences in the number of BrdU-positive cells were found between groups in the contralateral hilus (Fig. 3.6b). These data suggest that many of the cells generated following TBI persist for an extended period of time, and treatment with bFGF following injury does not significantly affect the survival of these cells.

28dpi

sham



TBI+Veh.



TBI+bFGF

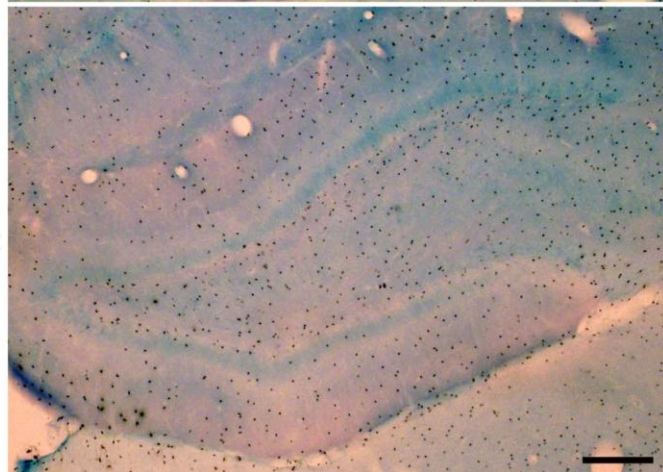


Figure 3.5: Survival of newly-generated cells in the DG at 28 days post-injury. Micrographs taken from BrdU stained coronal sections in the ipsilateral DG from **a)** sham, **b)** injured with vehicle and **c)** bFGF treated animals. **a)** In the sham animal, there were very few BrdU-positive cells in the DG. **b-c)** Many BrdU-labeled cells persisted in the ipsilateral DG in the injured vehicle-treated (**b)** and bFGF treated (**c)** animals. Brown dots indicated BrdU-labeling. Bar scale = 500 μ m.

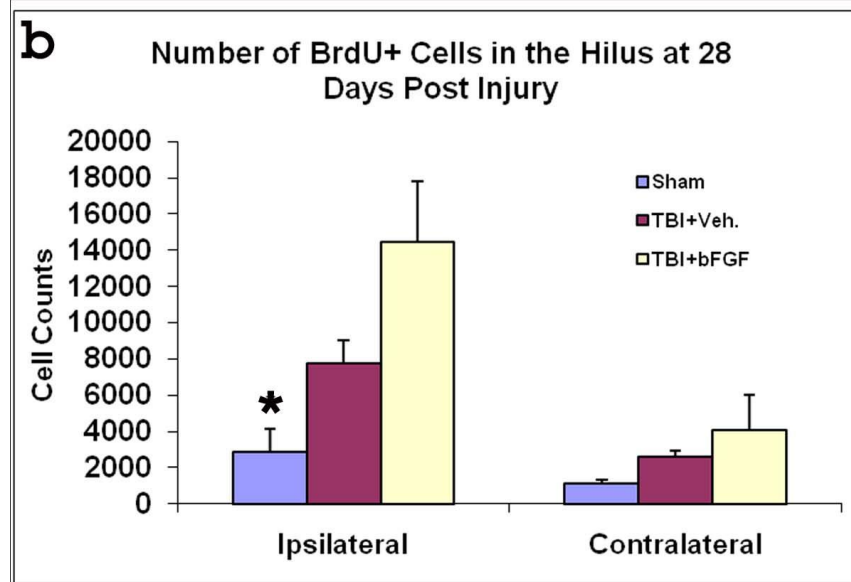
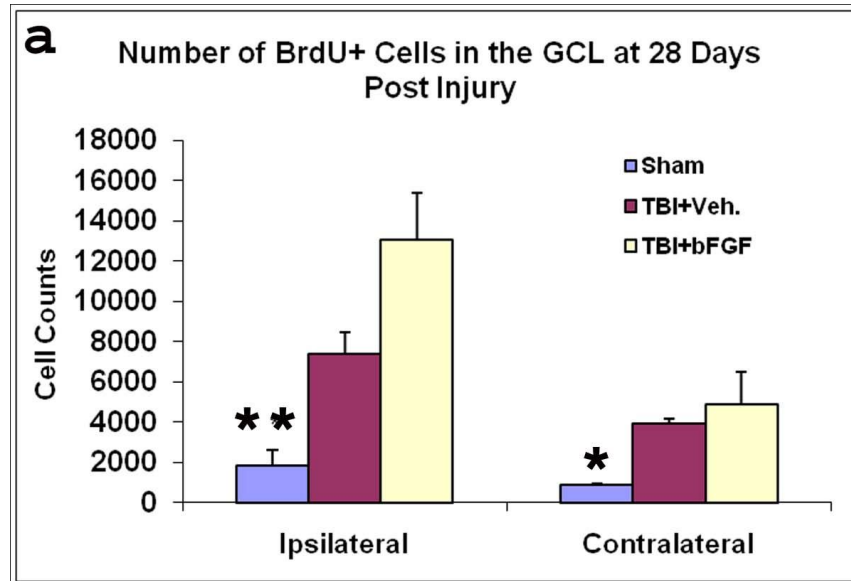


Figure 3.6: Quantitative analysis of surviving newly-generated cells as labeled by BrdU in the DG at 28 days post-injury. **a)** In the GCL, a significantly higher number of BrdU-positive cells were found in the injured animals treated with vehicle or bFGF compared to sham in both the ipsilateral and contralateral sides ($F_{2,7}=12.80$, $**p<0.01$; $F_{2,7}=5.91$, $*p<0.05$). **b)** In the hilus, injured animals treated with vehicle or bFGF had significantly more BrdU-positive cells in the ipsilateral side than sham ($F_{2,7}=7.56$, $*p<0.05$). No difference was found in the contralateral hilus ($F_{2,7}=1.75$, $p=0.24$).

Differentiation of newly generated cells

To assess the maturational fate of cells generated following TBI, sections from animals sacrificed at 4 weeks post-injury were processed for immunofluorescent double-labeling for BrdU combined with NeuN, GFAP, or ED1, which are cell type specific markers for mature neurons, astrocytes, or infiltrating macrophages and microglia, respectively. Immunofluorescent stained sections were examined using confocal microscopy. Some BrdU-positive cells in the GZ were co-labeled with NeuN in all three groups (Fig. 3.7a-c). BrdU and GFAP co-labeling were also found in all three groups (Fig. 3.7d-f). For BrdU-ED1 double staining, no co-localization of these two markers was found in the GZ in sham animals. However, there were many BrdU-ED1 co-labeled cells in the GZ in injured animals with vehicle or bFGF treatment.

Cells which were unambiguously co-labeled with BrdU and each of the aforementioned cell type specific markers were quantified and used to calculate their respective percentages against the total number of BrdU-labeled cells in the GZ. Preliminary data showed that in sham animals roughly 45% of BrdU-positive cells in the GZ are co-labeled with NeuN (Fig. 3.8). In injured animals, a lower percentage of BrdU-positive cells in the GZ are co-labeled with NeuN compared to sham animals. Of the BrdU-positive cells observed in the GZ of injured animals, about 15% were co-labeled with NeuN in vehicle-treated animals, and about 25% were co-labeled with NeuN in bFGF-treated animals (Fig. 3.8). Of the total number of BrdU-positive cells counted in the GZ in sham animals, approximately 25% were co-labeled with GFAP (Fig. 3.8). In vehicle-treated injured animals, nearly 40% of BrdU-positive cells in the GZ were co-labeled with GFAP, and in bFGF-treated injured animals, nearly 30% of BrdU-positive cells in the GZ were co-labeled with GFAP (Fig. 3.8). Whereas no BrdU-positive cells in the GZ in sham animals were co-labeled with ED1, approximately 30% of BrdU-positive cells in the GZ of

vehicle-treated injured animals were co-labeled with ED1, and approximately 35% of BrdU-positive cells in the GZ of bFGF-treated injured animals were co-labeled with ED1 (Fig. 3.8).

This preliminary evaluation of post-TBI cell differentiation in the aged brain suggests that a smaller percentage of the newly-generated cells which survived for 4 weeks had become mature neurons in injured animals regardless of treatment compared to sham animals. The percentage of astrocytic differentiation was similar in sham animals and injured animals. Strikingly, in contrast to what was seen in sham animals where no BrdU/ED1 co-localization was found, in injured animals either treated with bFGF or vehicle, many of the BrdU+ cells were ED1+ infiltrating macrophages or activated microglia. Overall, these preliminary data showed a trend of a decreased neuronal survival and apparent inflammatory response in the DG in the injured aged brain, and bFGF treatment did not show any protective or detrimental effect in this regard.

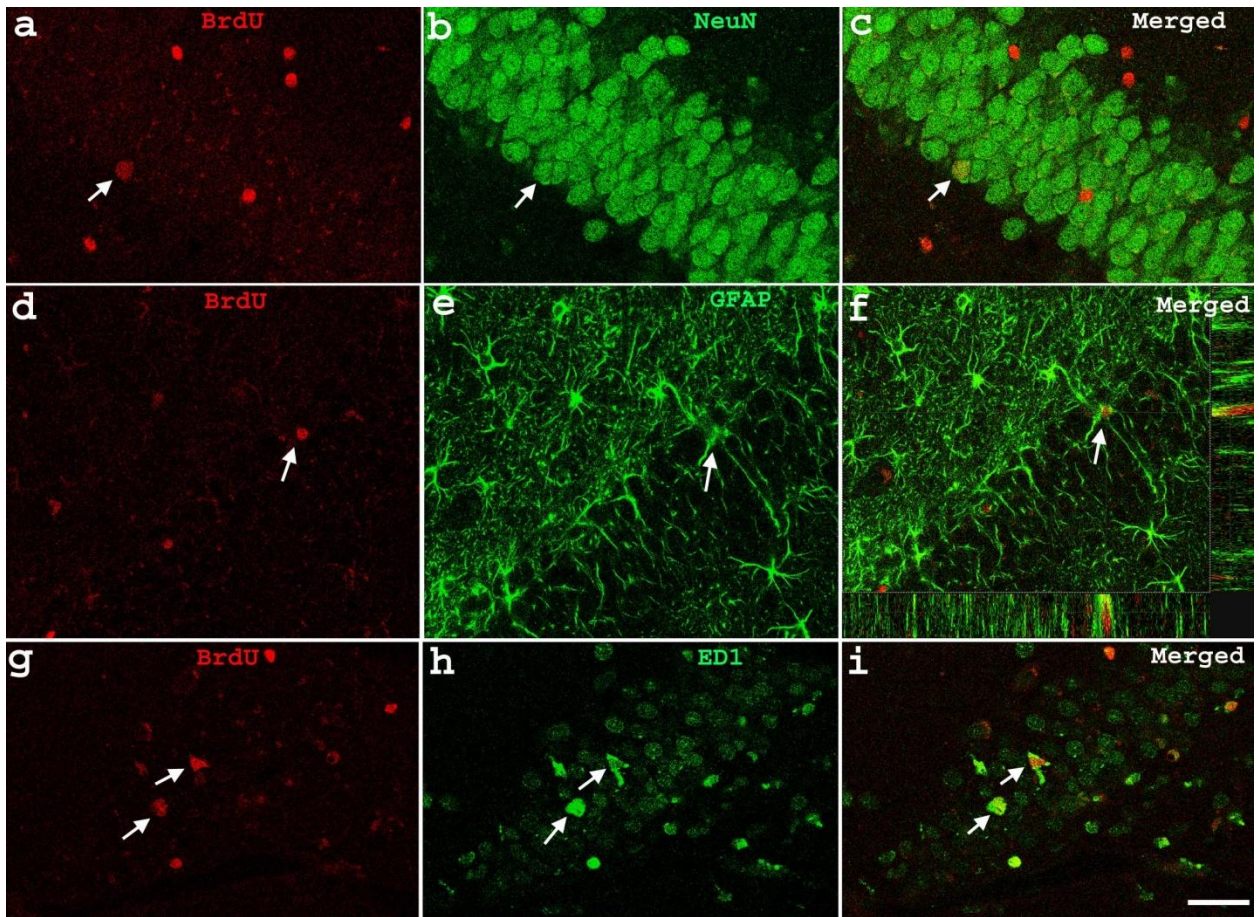


Figure 3.7: Differentiation of newly-generated cells at 28 days post-injury. **a-c)** Neuronal differentiation. Confocal microscopic images showing a newly generated BrdU-labeled cell as indicated by arrow co-labeled with NeuN indicating that this cell differentiated into a mature neuron. Note several BrdU-positive cells were not co-labeled. **d-f)** Astrocytic differentiation. The arrow indicated a BrdU-labeled new cell with cytoplasm and processes staining of GFAP. This co-localization is best viewed through the z axis. **g-i)** Co-labeling with ED1. Arrows pointed to two BrdU-positive cells co-labeled with ED1 in the GCL. Bar scale = 100 μ m

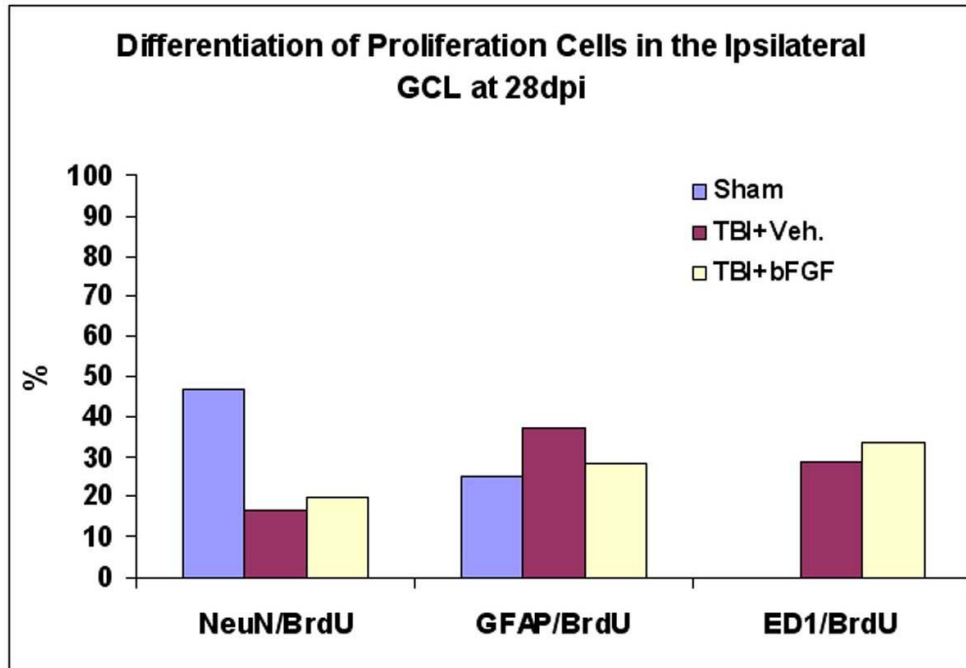


Figure 3.8: The percentage of neuronal or glial differentiation rate of the newly-generated cells in the GCL at 4 weeks post-injury. Cells in the GCL which were co-labeled with BrdU and NeuN, GFAP, or ED1 were calculated as a percent of co-labeling of each marker against the total number of BrdU-positive cells. A smaller percentage of the newly generated cells which survived for 4 weeks had become mature neurons in injured animals regardless of treatment compared to sham animals. The percentage of cells which differentiated into astrocytes was similar in sham animals and injured animals. No BrdU/ED1 co-localization was found in sham animals; however, in injured animals either treated with bFGF or vehicle, many of the BrdU+ cells were ED1+ infiltrating macrophages or activated microglia.

Cognitive Function: Novel object recognition

This study employed the novel object recognition test as one measure of cognitive recovery following TBI. Rats were tested on post-injury days 3, 7, 14, and 28 for their ability to recognize a novel object 30 seconds and 4 hrs after being exposed to sample objects for 5 min. Each rat's ability to recognize a novel object was measured by examining the amount of time spent exploring each object and the number of encounters with each object. In general, the rats did not respond well to the test and demonstrated considerable lethargy throughout the course of testing. Based on the data obtained, sham animals and injured animals alike spent very little time exploring the sample and novel objects in all phases of testing, often not encountering any of the objects in multiple trials throughout the time course of the test. Furthermore, statistical analysis using a split-plot analysis of variance (repeated measures ANOVA) showed no significant Group effect, Day effect, or Group x Day interaction for exploration time or object encounters between time points or among the animal groups.

Cognitive Function: Morris water maze latency and probe trial

Past studies in our lab have shown that moderate L-FPI induces cognitive deficits which recover over time and that the time course of this spontaneous cognitive recovery corresponds with the timeframe in which new granule neurons generated post-TBI integrate into existing hippocampal neuronal circuitry (Sun et al. 2007). Furthermore, post-TBI infusion of bFGF was shown to enhance TBI-induced neurogenesis in the DG as well as promote cognitive recovery in adult rats as assessed by the Morris water maze test (Sun et al. 2009).

In this study, the MWM followed by a probe trial was used to examine whether post-TBI infusion of bFGF improves cognitive function in aged rats as seen in younger rats. MWM tests

were performed daily from 21-25 days post-injury, and the probe trial was run the following day after MWM testing was completed (24 hrs later). Latency to reach the goal platform was used as a measure of cognitive function. The mean latency (*s*) to reach the goal platform for each group is presented in Fig. 3.9 (\pm SEM). The data were analyzed using a split-plot analysis of variance (repeated measures ANOVA, Group x Day). The results of the ANOVA did not reveal any significant differences in Group effect ($F_{2,13}=0.73, p=0.50$) or Day x Group interaction ($F_{2,13}=0.86, p=0.56$). The results of the ANOVA did reveal a significance in Day effect ($F_{2,13}=21.00, p<0.001$). Overall, goal latency was significantly shorter in all groups by the fifth day of MWM testing; however, no significant differences in goal latency between groups were found.

For the probe trial, the platform was removed from the pool and each rat was allowed to swim for 1 min while the average proximity (*cm*) to the location of the goal and time spent in the goal quadrant (*s*) were recorded. Based on the data obtained from the probe trial, bFGF-treated injured animals appeared to spend less time in the goal quadrant than vehicle-treated injured animals or sham animals (Fig. 3.10a); however, no statistical difference was found upon analysis ($F_{2,13}=3.54, p=0.06$). The average proximity to goal location for each group is presented in Fig. 3.10b. No significant differences in goal proximity were found between the groups ($F_{2,13}=1.28, p=0.31$). No significant differences were observed in swim speed between groups, demonstrating that motor deficits did not contribute to any potential differences in the recorded latencies ($F_{2,13}=2.79, p=0.10$).

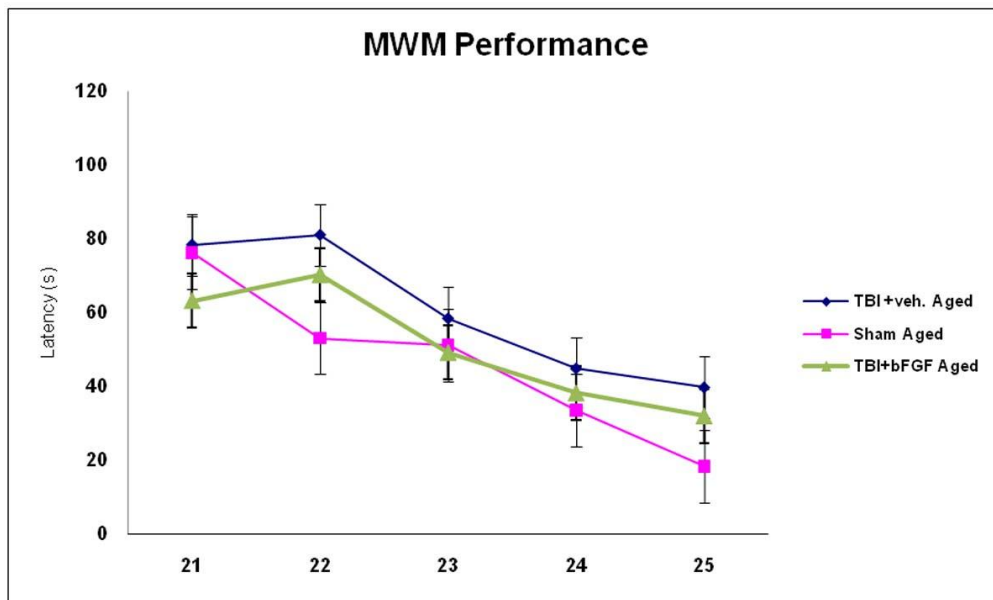


Figure 3.9: Hippocampal-dependent learning tests using Morris water maze performance.

All animals were tested with the MWM at 21-25 days post-injury. Latency to reach the goal platform was used as a measure for cognitive function. Injured-vehicle treated animals spent a slightly longer amount of time learning to locate the platform than bFGF treated or sham animals, but no significant differences were found between groups ($F_{2,13}=0.73, p=0.50$). All groups showed significant improvement in goal latency by the fifth day of testing ($F_{2,13}=21.00, p<0.001$).

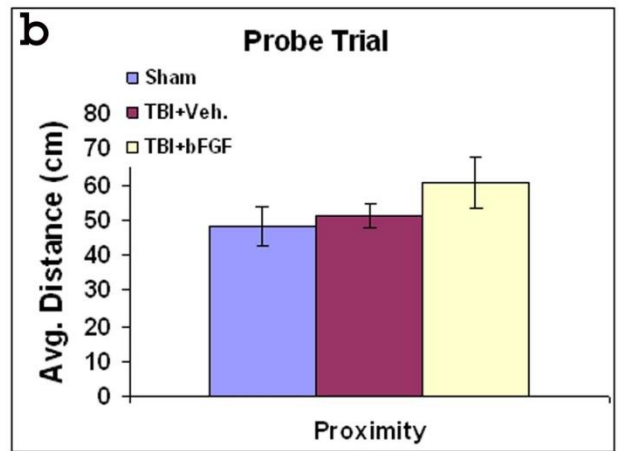
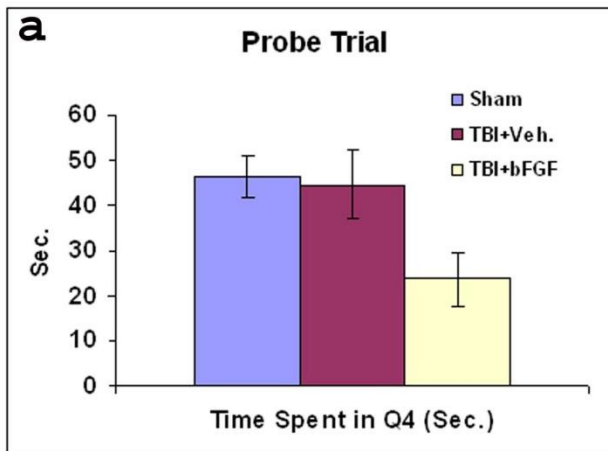


Figure 3.10: Memory retention tests with Probe trial. a) The time spent in the goal quadrant, and b) the average proximity to the goal location. Injured animals infused with bFGF appeared to spend less time in the goal quadrant than vehicle-treated injured animals or sham animals, but no significant difference was found ($F_{2,13}=3.54, p=0.06$). No significant differences in goal proximity were found between the groups ($F_{2,13}=1.28, p=0.31$).

Chapter 4 – Discussion

Traumatic brain injury is a major healthcare concern as it is a debilitating condition for which an adequate cure has not yet been developed. One of the foremost challenges faced in the development of an effective treatment for TBI is the limited capacity of the mature CNS to regenerate and repair itself after it has been damaged. Although many of those suffering from TBI achieve functional recovery to a certain extent, clinical and laboratory research has shown convincingly that old age leads to poorer outcomes following TBI (Eiben et al., 1984; Hamm et al., 1991; Senathi-Raja et al., 2010). Following the discovery of the existence of neural stem/progenitor cells in the mature mammalian CNS, recent progress in this research area has evidenced that neural stem/progenitor cells acting as an innate repair mechanism could contribute to the recovery and regeneration of the injured brain (Sun, 2005; Gould and Gross, 2002; Chirumamilla, 2002; Temple and Alvarez-Buylla, 1999). However, thus far, studies have also shown that this reparative capacity is limited, especially in the aging population (Kuhn et al., 1996). The spontaneous cognitive recovery observed following injury, as well as normal hippocampal-dependent learning and memory function, has been correlated to the generation of new neurons in the dentate gyrus in the mature mammalian CNS (Sun et al., 2007; Kempermann and Gage, 2002). As the brain ages, the degree of endogenous neurogenesis in the hippocampus decreases, which corresponds with a diminished ability for the aged brain to achieve cognitive recovery in the event of brain insult (Kuhn et al., 1996). It is thought that these age-related

changes in neurogenesis may be due, in part, to changes in trophic support for newly generated cells, as decreased levels of mitogenic growth factor expression have been observed in the aged brain (Mudo et al., 2009). Because growth factors play an essential role in regulating neural stem/progenitor cells, growth factors could be a potential therapy for TBI in the particularly vulnerable aged population through enhancing endogenous repair mechanisms.

Previous studies have shown that several growth factors that drive the proliferation and differentiation of cells in the CNS during development continue to be expressed in declining levels as the CNS matures (Cintra et al., 1994; Shetty et al., 2005). Expression levels of EGF and bFGF in particular experience a sharp decline as the brain ages, but are increased significantly following TBI (Mattson and Scheff, 1994; Oyesiku et al. 1999). These growth factors have been shown *in vitro* and *in vivo* to be important regulators of neural stem and progenitor cell proliferation, differentiation, and survival (Caday et al., 1990; Plata-Salaman, 1991). Subsequently, recent studies in our lab have attempted to evaluate EGF and bFGF as potential treatments for TBI. Exogenous administration of EGF following TBI in adult rats was found to enhance TBI-induced cell proliferation in the SVZ and hippocampus and to aid in cognitive recovery, while having no effect on neurogenesis (Sun, 2010). Treatment of adult rats with bFGF infusion was shown to further augment the TBI-induced proliferative response and significantly enhance neurogenesis and cognitive recovery following injury (Sun, 2009). In light of the positive neurogenic effects of bFGF in adult rats, the current study sought to evaluate the effectiveness of exogenous bFGF administration as a treatment for TBI in aged rats. Therefore, we hypothesized for this study that bFGF infusion following injury would enhance neurogenesis and cognitive recovery in older subjects as seen in their younger counterparts.

Results summary

In this study, aged rats were subjected to moderate L-FPI followed by infusion of either bFGF or a vehicle solution into the lateral ventricle for a period of 7 days post-injury. Evaluation of newly-generated cells at 7 days post-injury with BrdU-labeling revealed that TBI substantially enhances cell proliferation in the ipsilateral hippocampal DG in the aged brain. At 7 days post-injury, bFGF infusion slightly further enhanced the TBI-induced proliferative response in the ipsilateral GCL. Though the effect of bFGF in augmenting TBI-induced cell proliferation was not statistically significant, the level of neurogenesis at 7 days post-injury was significantly increased in bFGF-treated animals in both the ipsi- and contralateral dentate gyrus as assessed by DCX staining. Injured vehicle-treated animals showed no difference in the number of newly generated DCX+ neurons in the ipsi- or contralateral GCL compared to sham animals. In the hilus region, where neuronal cell loss is often observed following TBI, injured animals in both the vehicle and bFGF treated groups had enhanced cell proliferation, and bFGF showed no further enhancement.

To examine the survival of newly-generated cells, brain sections taken from animals which survived for 28 days post-injury were processed for BrdU immunohistochemistry, and BrdU-positive cells were quantified. A significantly higher number of BrdU-labeled cells were observed in the ipsi- and contralateral GCL as well as the ipsilateral hilus in injured animals compared to shams. A slightly higher number of BrdU-positive cells were counted in the ipsilateral GCL and hilus in bFGF-treated injured animals compared to vehicle treated animals, though this trend was not statistically significant.

In order to assess the maturational fate of the newly generated cells which persisted in the GCL at 4 weeks post-injury, brain sections were double-labeled with BrdU and cell-type specific markers including NeuN, GFAP, and ED1. By examining the co-labeling of BrdU with NeuN, it was determined that a larger percentage of newly generated cells appeared to differentiate into neurons in sham animals than injured animals either with bFGF or vehicle infusion. As detected by co-labeling of BrdU with GFAP, the percentage of astrocytic differentiation of newly generated cells was similar in sham animals and injured animals regardless of treatment. Remarkably, in contrast to what was found in sham animals where no BrdU+ cells were co-labeled with ED1, injured animals treated with either bFGF or vehicle had a large number of BrdU-positive cells which were ED1-positive.

To assess whether bFGF infusion can improve cognitive functional recovery of aged rats following TBI, cognitive functions were tested with a novel object recognition test (NOR) and Morris water maze test. NOR was tested on days 3, 7, 14 and 28 following TBI. No differences or trends were observed in the amount of time all injured animals or sham animals spent exploring the objects or in the number of times encountering the objects in either the 30 sec trials or the 4 hr trials. Morris water maze tests were performed on 21-25 days post-injury for goal latency tests and followed by a probe trial 24 hrs later after the last latency test. Sham and both groups of injured animals demonstrated significant improvement in learning over the course of the 5 days of latency trials, and there was no significant difference between the groups. For the probe trial, no significant differences were found between groups in the analysis of time spent in the goal quadrant or in the proximity analysis.

TBI induces a proliferative response in the dentate gyrus of the hippocampus

We found in this study that TBI produced a significant proliferative response in the DG of the hippocampus in both vehicle- and bFGF-treated injured aged rats. The injury-induced proliferative response seen in this study is consistent with our previous research concerning endogenous repair mechanisms in the brain following TBI as well as reports from other labs (Sun et al., 2007; Chirumamilla et al., 2002; Dash et al., 2001). Previous work done in our lab has shown that cell proliferation following TBI peaks at 2 days post-injury in juvenile and adult rats (Sun et al., 2005). Other data from our lab has suggested that cell proliferation in the GCL and hilus of aged rats at 2 days post-injury is increased to levels not significantly different than what is seen in adult rats. The ability of the aged brain to mount such a response following injury is indicative of a possible mechanism for repair, although this regenerative potential is limited. Although an increase in newly generated cells was anticipated in the aged brain, how this proliferative response directly compares to what is seen in juvenile or adult rats is outside the scope of this study. Very few studies have directly examined trauma-induced cell proliferation in the aged brain, and further investigation into this area is necessary to understand the role of these newly generated cells in the injured senescent CNS.

Post-TBI infusion of bFGF does not significantly further enhance TBI-induced cell proliferation but does enhance neurogenesis in the aged hippocampus

In addition to the proliferative response observed following TBI, exogenous administration of bFGF may have the potential to augment this TBI-induced cell proliferation and neurogenesis in the injured aged brain. Previously we found that in 3 month old rats treatment with bFGF significantly further enhanced injury-induced cell proliferation in the GCL

and hilus (Sun et al., 2009), which led us to hypothesize that a similar effect would be expected in the aged rats. Although an increase in the number of newly-generated cells was observed in the ipsilateral GCL of bFGF-infused animals at 7 days post-injury compared to vehicle-treated animals, this study did not establish that this effect was significant. One of the explanations for this result is that there was a markedly high variance in the data. This variance may have been caused by human error, such as variations in injury severity, BrdU staining, or stereological quantification. To reduce the variance, higher animal numbers are needed. It must also be taken into account that FGFR expression decreases with age and the ability of NS/NPCs to respond to growth factor signaling is altered in the senescent brain. The juvenile hippocampus displays robust expression of FGFRs early in development which declines substantially with age (Brazel and Rao, 2004). The lower number of FGFRs present in the hippocampus may partially explain the observed difference in the proliferative response to bFGF in aged animals compared to younger animals. Nevertheless, using DCX as a marker for generation of new neurons, we did find that bFGF-infusion produced an elevated level of neurogenesis in the aged DG following injury, which is consistent with what is seen in adult rats from our previous bFGF study (Sun et al., 2009).

It is well known that bFGF is involved in regulation of neurogenesis in the normal and injured brain (Mudo et al., 2009; Yoshimura et al., 2003; Wagner et al., 1999). Basic FGF not only acts as a potent mitogen, but also plays a role in guiding differentiation, especially in the CNS. Treatment with bFGF may promote differentiation of newly generated cells into a neuronal phenotype. The precise role of bFGF in neurogenesis is not completely understood and demands further investigation; however, previous studies have shown that there is an endogenous bFGF-mediated increase in neurogenesis in the adult DG following injury, and that

bFGF treatment enhances this endogenous neurogenic response (Parent, 2003; Tureyen et al., 2005; Wagner et al., 1999). Though we observed enhanced cell proliferation in the aged brain following injury, interestingly, we did not observe increased neurogenesis in vehicle-treated animals, which suggests that the neurogenic capacity is significantly impeded in the aged CNS. This is in accordance with a previous study which demonstrated that the ability of the DG to further amplify neurogenesis in the event of brain insult may be lost as early as middle age (Hattiangady et al., 2008). The effect of bFGF to restore the neurogenic capacity in the aged brain after TBI suggests its potential therapeutic importance.

bFGF has no significant effect on the survival of newly generated cells in the aged hippocampus

We have found a large number of newly proliferated cells persisting in the DG in injured animals 4 weeks post-injury regardless of the treatment received. Our previous studies have shown that cell proliferation in the hippocampus reaches its peak at 2 days following injury and gradually returns to sham levels by 2 weeks post-injury, with the majority of new cells being generated by 1 week post-injury (Sun et al., 2005). As animals in this study were injected with BrdU from days 2-7 post-TBI, only newly proliferated cells from this period of time were labeled and counted. Our results suggested that many of these cells survived for an extended period of time; however, we were unable to determine the percentages of newly generated cells that survived or degenerated as the brain recovered from injury. The observed levels of newly born cells which survived for 4 weeks after TBI were surprisingly high, and seemed to be inconsistent with previously published data. In the normal adult rodent hippocampus, newly generated cells are likely to degenerate within approximately 1-2 weeks of their formation (Cameron and McKay, 2001; Dayer et al., 2003). Various degrees of new cell survival have been found in uninjured mice of different ages. Survival rates after 4 weeks varied from 25% in

2 month old mice, to 43% in 6 month old mice, and to 61% in 18 month old mice (Kempermann et al., 1998). In injured adult rats, 46% of newly generated cells persisted in the DG for 10 weeks post-injury, indicating an overall decrease in the number of surviving cells over time (Sun et al., 2007). However, long-term survival of injury-induced proliferating cells has not been thoroughly examined in the aged hippocampus, and further evaluation is needed for clarification of this phenomenon. Concerning the number of persistent cells that were found in the aged hippocampus in this current study, it must be taken into account that cells which incorporated BrdU during mitosis may continue to divide and produce daughter cells which are also BrdU-positive, although it is plausible that the BrdU staining in these cells would be diluted and would not stain as strongly. Cells undergoing apoptosis may also incorporate BrdU, which may have contributed to Type I error in cell quantification. An assay for apoptotic markers would be helpful to add in future studies. Our current methods of stereological quantification enabled us to accurately count BrdU-positive cells, but did not allow us to distinguish between cells which were generated during the period of BrdU-labeling, their progeny which may have been generated after this period, or cells undergoing apoptosis. Notwithstanding, these cells were counted towards the total number of newly generated cells surviving at 4-weeks post-injury.

Concerning the effect of bFGF-treatment on newly generated cell survival, we found that many BrdU-labeled cells persisted in the DG of the injured aged brain for an extended period of time regardless of treatment. The number of BrdU+ cells that was present in the DG at 4 weeks was higher in the bFGF-treated animals than in vehicle-treated animals to the extent that the difference between the 2 groups approached significance. Nevertheless, it is inconclusive whether bFGF affects cell survival in the injured aged animal. It is unknown whether the observed levels of new cells surviving for an extended period of time have a beneficial effect on

the aged brain following injury. These new cells might be involved in an attempt to maintain or restore normal function or could be a result of TBI-induced inflammation.

TBI induces a profound inflammatory response in the aged brain which is independent of bFGF treatment and which may affect the survival of new neurons

Based on the preliminary evaluation of the maturational fate of newly-generated cells at 4 weeks post-TBI, the percentage of neuronal differentiation is decreased in injured aged animals compared to sham animals. Although neurogenesis is decreased in the CNS of normal aged animals compared to juvenile or adult animals, a similar percentage of neuronal differentiation is found in all age groups (Ahlenius et al., 2009; Rao et al., 2006). However, in injured rats, a much lower percentage of newly generated cells assume a neuronal fate in adult rats as compared to juvenile rats, which suggests that the capacity for neuronal differentiation in response to injury decreases with age (Sun et al., 2005). In adult rats, the percentage of newly generated cells in the DG that differentiate into neurons in injured animals is similar to what is seen in sham animals (Sun et al., 2009). The findings of this current study suggest that the injured aged brain is limited in its capacity to generate new neurons compared to the injured younger brain.

Neuronal differentiation appeared to be decreased in injured animals at 28 days post-injury regardless of treatment, which is inconsistent with the increased neurogenesis observed at 7 days post-injury in bFGF treated animals. Treatment with bFGF was observed to enhance neurogenesis at 1 week post-injury, but this effect was not evident after 4 weeks, which suggests that these newly generated neurons did not survive. These new neurons may have died from lack of proper trophic support or may have undergone apoptosis as a result of inflammatory cytokines produced following injury. Basic FGF was seen to have a beneficial effect in promoting a

neuronal fate for newly generated cells, but this effect was not sustained. Future studies should include an assay for apoptotic markers along with cell type specific markers in order to more thoroughly evaluate the survival of different cell types and to determine what happens to newly generated neurons following bFGF treatment in the aged brain.

While neuronal differentiation appeared to be decreased in the injured aged brain, astrocytic differentiation was unaffected by injury. A similar level of newly generated cells differentiated into astrocytes in sham animals and injured animals regardless of treatment. This is consistent with what is found in adult rats (Sun et al., 2009). It is important to note that the percentage of astrocytic differentiation substantially increases with age. At 2 and 4 weeks post-injury, the percentage of new cells in the DG which differentiated into astrocytes was significantly increased in injured adult rats compared to injured juvenile rats (Sun et al. 2005). Furthermore, studies in aged mouse have found that an exacerbated astrocyte and microglial response to TBI might contribute to worse cognitive outcomes in the elderly following injury (Palermo et al., 2008). In this study, we have found that a large percentage of newly generated cells in injured animals at 4 weeks post-injury were ED1-positive infiltrating macrophages and activated microglia. This contrasted remarkably to what was found in sham animals, where no new cells were ED1-positive. It has been demonstrated previously that aging is associated with an increased glial response that may increase the susceptibility of the aged brain to injury, which was shown by a stronger and more persistent increase in ED1 expression in aged rats compared to younger rats following direct insult to the hippocampus (Zhu et al., 2003). Taken together, these observations suggest that TBI produces a profound inflammatory response in the aged brain that may affect the generation and survival of new neurons as well as functional recovery.

bFGF has no effect on cognitive recovery in aged rats following TBI

Despite the demonstrated positive effect of bFGF-treatment on hippocampal neurogenesis following TBI in the aged rat, no effect on cognitive recovery was observed as assessed by a novel object recognition test and the Morris water maze test. It is well established that old age is associated with poor cognitive outcomes following TBI (Senathi-Raja et al., 2010; Sandhir et al., 2008; Hamm et al., 1992; Eiben et al., 1984). The susceptibility of the aged brain to injury-induced functional deficits and the aged brain's limited regenerative capacity present a significant challenge to researchers in developing an effective treatment. It was shown in a previous study that uninjured aged rats performed significantly worse in the Morris water maze than uninjured adult rats, and injured aged rats performed significantly worse than injured adult rats (Hamm et al., 1992). In effect, normal aged animals exhibit decreased cognitive function in comparison to normal adult animals, and injury generally has a more devastating impact on the aged brain. This study hypothesized that exogenous administration of bFGF in the aged brain following TBI would enhance cognitive recovery, as we previously found that adult rats treated with bFGF displayed significantly improved cognitive recovery following injury as assessed by the Morris water maze (Sun et al., 2009). However, in the current study, we did not observe significant differences in cognitive deficits in injured animals regardless of treatment compared to sham. We have also observed no measurable improvement in cognitive function in bFGF-treated as compared to vehicle-treated groups. This could be a result of our utilization of Fisher 344 rats for this study as opposed to Sprague-Dawley rats due to the limited availability of aged rats. A recent study done in our lab has found that Fisher 344 rats are particularly susceptible to TBI with a higher mortality rate and a higher incidence of acute seizure activities, but surprisingly with less cognitive deficits when compared to Sprague-Dawley rats at similar injury

levels following FPI (Reid, 2010). The respective strain differences in response to FPI and the fact that the injury severity was lower in the current study due to the high mortality rate might explain why injured animals regardless of treatment did not show significant deficits in cognitive function. For this study, an injury level of 1.8 ± 1 atm, which is considered a mild to moderate injury, was used because the mortality rate increases severely above injury levels of 1.9 atm for Fisher 344 rats. Albeit, a more severe injury level may be necessary to induce appropriate cognitive deficits, the sensitivity of this particular rat strain makes achieving a higher level of injury more difficult and costly. Assessing cognitive measures at an earlier time may be beneficial due to the possibility that any cognitive deficits that exist would be more pronounced and, therefore, may be more detectable. For future studies, more sensitive measures of cognitive function should be evaluated and utilized to assess cognitive recovery in aged rats. It should also be considered that uninjured rats in this older age group display visibly poorer baseline motor and cognitive function than their younger counterparts, which could affect the extent to which investigators can induce potentially reversible deficits. For this study, it is also noted that the animal number included in the current data is relatively low for behavior analysis. Increasing the animal number is necessary for a definitive conclusion. Based on our current data, one might suggest that bFGF treatment does not have any effect in improving cognitive function in injured aged rats, which is unlike what we have observed in younger animals. The increased neurogenesis that was seen in response to bFGF treatment 1 week following TBI may not have had any effect on cognitive recovery considering that no evidence of increased neurogenesis was found after 4 weeks. If these new neurons did not survive and integrate into the existing neuronal circuitry in the hippocampus, they would not be able to contribute to improved cognitive function. The prominent inflammatory response observed in the aged brain following

TBI may contribute to this deficiency in cognitive recovery as the limited neurogenic effect of bFGF treatment could not compensate for such a detrimental inflammatory response.

The future of bFGF as a treatment for TBI

Although the results of this study did not reveal any significant effect of bFGF on cognitive recovery in aged rats following TBI, this novel research is of significant clinical relevance with respect to the therapeutic implications of bFGF. This study merely provides a preliminary look at the effects of bFGF in the injured aged brain and further study is needed. The important role of bFGF in endogenous repair in the CNS is undeniable, as bFGF has both neurogenic and neurotrophic effects.

Basic FGF is a well-known mitogen for both neuronal and non-neuronal cells, displaying multifunctional and pleiotropic actions both *in vitro* and *in vivo*. For example, it has been shown that bFGF provides crucial extracellular signals which regulate the proliferation and fate determination of neural stem and progenitor cells during CNS development (Calof, 1995). *In vitro* studies have demonstrated that bFGF provides mitogenic signals for neuroblasts and glial cells and is involved in directing differentiation of these cells, as well as promoting proliferation and survival of cultured neural stem cells (Gritti et al., 1996; Palmer et al., 1999; Qian et al., 1997). In addition to its trophic effects on neuronal cells, bFGF stimulates a proliferative response in astrocytes and oligodendrocytes as well (Bogler et al., 1990; Fressinaud et al., 1993; Hagoood et al., 2006). *In vivo* studies have shown that intraventricular or subcutaneous administration of bFGF increases cell proliferation in the SVZ of normal animals (Kuhn et al., 1997; Wagner et al., 1999). Administration of bFGF in normal aged animals restored neurogenesis in the hippocampus and the SVZ to levels seen in younger animals (Jin et al., 2003;

Rai et al., 2007). These findings taken together demonstrate that signals provided by bFGF are essential for the proliferation, differentiation, and survival of CNS cells. The role of bFGF in neurogenesis has also been well established. The anatomical distribution of bFGF and its receptor FGFR1 in the neurogenic regions of the adult brain, including the SVZ and DG, is suggestive of bFGF's role in endogenous neurogenesis in the mature mammalian CNS (Gonzalez et al., 1995; Weickert et al., 2005). Furthermore, it was shown in bFGF-null mice that bFGF is necessary and sufficient to induce cell proliferation and differentiation of NPCs in the hippocampus; bFGF knock-out mice were also shown to have a diminished level neurogenesis in the DG following seizure or TBI, which was reversed by exogenous treatment with bFGF (Yoshimura et al., 2001; Yoshimura et al., 2003). The beneficial effect of bFGF on neurogenesis and cognitive recovery in younger adult rats further demonstrates the therapeutic potential of this vital growth factor (Sun et al., 2009). Besides neural progenitor cell proliferation and neurogenesis, other processes have been shown to be influenced by bFGF and represent additional mechanisms by which bFGF may aid in cognitive recovery. When given systematically, bFGF may improve synaptic plasticity (Schuman, 1999) and modulate axonal branching and arborization (Ramirez et al., 1999). *In vitro*, bFGF was shown to promote synaptogenesis (Li et al., 2002) and axonal branching and growth in cultured rat hippocampal neurons (aoyagi et al., 1994; Patel and McNamara, 1995).

Evidence of the beneficial effects of bFGF in the mature CNS is rapidly accumulating, and the work being done in our lab has continued to contribute immensely to the available knowledge in this area, especially with regard to TBI research. This current study sought to evaluate the therapeutic potential of bFGF in the aged brain using a similar paradigm to what was previously used by our lab in adult subjects. We concluded that bFGF has the ability to

enhance neurogenesis in the injured aged hippocampus, but this effect was not sufficient to improve functional recovery of aged rats following TBI due to the profound injury-induced inflammatory response.

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Vita

Michael Ray Zeigler was born in Fort Walton Beach, Florida, on May 17, 1983, as a native and citizen of the United States of America. He graduated from Bethel Christian School in Hampton, Virginia, as the Valedictorian of the Class of 2001. He is an alumnus of Liberty University where he graduated *cum laude* receiving a Bachelor of Science in May of 2008. He attended VCU in Richmond, Virginia, later that year to complete a post-baccalaureate Pre-medical Certificate and decided to continue his graduate education in the Department of Anatomy and Neurobiology. In August, 2010, he will be matriculating to the MD program at the VCU School of Medicine as member of the Class of 2014.