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INHIBITION OF INJURY-INDUCED CELL PROLIFERATION IN THE DENTATE
GYRUS IMPAIRS COGNITIVE RECOVERY FOLLOWING TRAUMATIC BRAIN
INJURY

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

by

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List of Abbreviations

ABC	Avidin-biotin complex
ANOVA	Analysis of variance
AP	Anterior-posterior
Ara-C	Arabinofuranosyl Cytidine
Ara-CTP	1-beta-D-arabinofuranosylcytosine triphosphate
asf	average sampling fraction
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
CT	Contralateral
DAB	5, 5-diaminobenzidine
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
FG	Fluorogold
FPI	Fluid percussion injury
GCL	Granule cell layer ix

GFAP	Glial fibrillary acidic protein
Gw	Giga-watt
GZ	Granular zone
Hr	Hour
i.p.	Intraperitoneal
IACUC	Institution of Animal Care and Use Committee
ICP	Intracranial Pressure
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Ipsilateral
LFPI	Lateral fluid percussion injury
MAM	Methylazomethanol acetate
ML	Medial-lateral
MWM	Morris water maze
NIH	National Institutes of Health
NOR	Novel object recognition
NPC	Neural progenitor cell
NSC	Neural stem cell
NS/NPC	Neural stem/progenitor cell
PBS	Phosphate buffer saline
PNS	Peripheral nervous system
PSA-NCAM	Polysialylated neural cell adhesion molecule
Sec	Second

SEM	Standard error margin
SGZ	Subgranular zone
SSC	Saline sodium citrate
ssf	section sampling fraction
SVZ	Subventricular zone
TBI	Traumatic brain injury
Veh	Vehicle
VMN	Ventromedial Nucleus
WHO	World Health Organization

Abstract

INHIBITION OF INJURY-INDUCED CELL PROLIFERATION IN THE DENTATE GYRUS IMPAIRS COGNITIVE RECOVERY FOLLOWING TRAUMATIC BRAIN INJURY

By Teresa E. Daniels

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

Virginia Commonwealth University, 2012

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

Traumatic brain injury (TBI) induces a robust cellular proliferative response among neural stem/progenitor cells (NS/NPCs) in the dentate gyrus of the hippocampus. This proliferative effect is thought to contribute to the innate cognitive recovery observed following TBI. Inhibition of hippocampal neurogenesis impairs cognitive function. Furthermore, enhancement of injury-induced hippocampal neurogenesis via intraventricular administration of basic fibroblast growth factor (bFGF) improves cognitive function in animals following TBI. In this experiment, we investigated the direct association between injury-induced hippocampal neurogenesis and cognitive recovery utilizing an antimetabolic agent, arabinofuranosyl cytidine (Ara-C). In this study, adult rats received a moderate lateral fluid percussion injury (LFPI). Immediately following injury, Ara-C with or without bFGF was infused into the lateral ventricle via an osmotic mini-pump for 7 days. To label dividing cells animals received daily single injections of 5-bromo-2'-deoxyuridine (BrdU) at 2-7 days post-injury. To examine the

effect of Ara-C on cell proliferation, a group of animals was sacrificed at 1 week following injury. Brain sections were immunostained for BrdU and cell type specific markers, and the number of BrdU+ cells in the hippocampus was assessed by stereology. To examine the effect of inhibition of injury-induced cell proliferation on cognitive recovery, animals were assessed on Morris water maze tasks (MWM) either at 21 to 25 days or 56-60 days post-injury. We found that post-injury Ara-C treatment significantly reduces injury-induced cell proliferation in the DG and abolishes the innate cognitive recovery on MWM performance at 56-60 days post-injury. Additionally, Ara-C diminishes bFGF enhanced cell proliferation in the DG and cognitive recovery following TBI. These results support the causal relationship between injury-induced hippocampal neurogenesis and cognitive functional recovery. Our studies suggest that the post-TBI neurogenic response is an endogenous repair mechanism that contributes to the restoration of hippocampal function post-injury.

Chapter 1 – Introduction and Background

Traumatic Brain Injury (TBI) is a major cause of death and disability worldwide and is a central topic of investigation in healthcare research. It is characterized by a broad spectrum of deficits, including cognitive and motor disabilities. The limited capacity of the central nervous system (CNS) to repair or regenerate neurons poses a significant obstacle to developing therapies to restore these functions. However, recent studies have revealed that the subventricular zone (SVZ) and the dentate gyrus of the hippocampus house neural stem/neural progenitor cells (NS/NPCs), which retain the ability to divide and develop into mature neurons and glia throughout adulthood. Further, these cells have been observed to substantially increase in proliferation following TBI. It is proposed that this proliferative response to injury may serve as an innate mechanism to restore cognitive function.

Damage to the hippocampus is responsible for the cognitive deficits associated with TBI. Thus, targeting injury-induced hippocampal neurogenesis may provide a promising avenue for potential therapies. This would especially serve older adults who have less capacity to achieve cognitive recovery than their younger counterparts. There is a significant body of evidence suggesting a relationship between post-injury hippocampal neurogenesis and cognitive recovery. Previous studies in this lab have shown that after injury, newly generated dentate granular neurons are able to anatomically integrate with existing neural networks and that this process is temporally correlated with cognitive recovery (Sun et al., 2007). Further, treatment with a growth factor post-TBI heightened

injury-induced neuronal proliferation and promoted cognitive recovery. For example, intraventricular infusion of bFGF, a known neuronal mitogen, both profoundly increased post-injury neuronal proliferation and enhanced cognitive recovery compared to vehicle-treated animals (Sun et al., 2009). While these studies strongly suggest a relationship between endogenous neurogenesis and cognitive recovery, the biological basis for the improved cognitive performance cannot be ascertained by these findings alone.

If increased neurogenesis resulting from the infusion of a growth factor indeed contributes to cognitive recovery post-injury, we postulated that infusion of a mitogen inhibitor post-TBI would abolish neuronal proliferation in the hippocampus and impair cognitive performance compared to vehicle-infused injured animals. We further proposed that infusion of both the growth factor and the mitogen inhibitor would suppress the cellular and behavioral benefits observed following bFGF infusion. To test our hypothesis that injury-induced neurogenesis directly contributes to innate cognitive recovery, we conducted a series of experiments investigating the effects of intraventricular infusion of Ara-C, a known mitotic inhibitor, on hippocampal neurogenesis and cognitive recovery following TBI in the presence or absence of bFGF.

Epidemiology of TBI

The World Health Organization (WHO) describes TBI as a “critical public health problem” that requires the attention of the international health care community (WHO, 2012). Recent data estimates that annually 1.7 million people in the U.S. and at least 10 million worldwide are afflicted with TBI severe enough to result in hospitalization or death (Coronado et al., 2011; Langlois et al., 2006). Nationally, 52,000 individuals die each year from TBI, 275,000 are hospitalized, and 1.365 million are treated and released

from an emergency department. TBI is a contributing factor to almost one-third of all injury related deaths in the U.S (Coronado et al., 2011). As a result of advancements in emergency room techniques and practices, an increasing number of TBI patients survive their injuries and live with long-term or lifelong physical and mental disabilities. In the US, approximately 80,000 people annually sustain a TBI that results in long-term disability (NIH, 2007). For example, TBI can result in neurological changes that affect memory, reasoning, sensation, language, emotion, and can cause epilepsy and chronic traumatic encephalopathy. It also increases risk for sleep disorders, Alzheimer's disease, Parkinson's disease and other brain disorders (Masel & DeWitt, 2010). It is estimated that 5.3 million Americans are living with long-term or lifelong disability as a result of TBI, but considering that not all individuals seek treatment in a hospital or an outpatient care facility, this figure is greatly reduced compared to the actual number (Langlois et al., 2006).

The costs accrued by patients and society as a result of TBI are staggering. In the U.S. alone, the estimated annual cost of medical bills and lost production is \$60 billion. Moreover, the effects on afflicted individuals, their families, and friends cannot be measured. TBI patients have greater likelihoods of developing drinking problems and depression (Langlois et al., 2006). In a longitudinal study, as much as 48% of TBI patients reported major depressive disorder and 14% reported substance abuse in eight years following injury (Masel & DeWitt, 2010).

Pathology of TBI

TBI is a complex and dynamic series of events that initiates a chronic disease process in a surviving patient. Injuries can be classified as either focal, which includes

hematoma and contusion formation, or diffuse, which includes diffuse axonal injury and diffuse microvascular damage (Povlishock & Katz, 2005). It is often characterized as a biphasic injury, with the primary insult being the mechanical force that causes the tissue distortion, shearing or destruction (Aarabi & Simard, 2009). The secondary injury is composed of the ensuing cellular and biochemical cascade of events that interact and vary depending on the nature and magnitude of the primary insult. These events include changes in ionic balance, ischemia, local and diffuse inflammation, cell death resulting from apoptosis and necrosis, intracranial hypertension, hemorrhage, compromised blood brain barrier, circulating excitatory neurotransmitters, accumulation of free radicals, mitochondrial dysfunction, and diffuse axonal injury (Aarabi & Simard, 2009; Dutton & McCunn, 2003). While inflammation serves a neuroprotective function, in excess it can also conversely exacerbate the neuropathology. In clinical scenarios, current early management of TBI focuses on avoiding hypoxemia and reducing intracranial pressure (ICP) (Aarabi & Simard, 2009).

As the various processes involved in secondary injury are so numerous and intertwined, attempts to develop therapies to improve patients' prognoses by combating a single process have proved to be insufficient in clinical settings (Aarabi & Simard, 2009).

Experimental TBI

While TBI is an extremely complex pathology to reproduce experimentally, fluid percussion injury (FPI) is the most widely used and accepted model (McIntosh et al., 1989). In this experimental model of TBI, using a fluid injury device, a rapid impulse of saline is applied to the intact cortical dura surface through a small craniotomy. This method is shown to produce focal and diffuse injury in the rodent brain. Injury level

varies with increasing applied pressure and leads to increasing magnitudes of injury (Dixon et al., 1987). It is commonly used to model brain injury because it induces a number of physiological and behavioral effects in the injured animal that are observed in clinical studies. Impaired memory is a hallmark clinical feature of TBI and FPI has been shown to result in reproducible memory deficits in animals carrying out Morris water maze (MWM) tests (Smith et al., 1991). It also causes the suppression of postural behavior that suggests a neurological parallel to the loss of consciousness in human patients. Immediate effects of the injury include a brief period of apnea, a compromised blood brain barrier, changes in blood pressure, cerebral blood flow, and vascular resistance. A number of additional effects develop, including diffuse axonal injury, a reduced capacity to autoregulate blood levels of CO₂, altered ion gradients, activated circulating microglia, increases in intracranial pressure and edema, necrosis and apoptosis (Cortez et al., 1989; Hick et al., 1996).

Functional Significance of Hippocampal Neurogenesis

The hippocampus is central to learning and memory (Scoville & Milner, 1957). It is a critical component of the medial temporal lobe, which is known to form spatial and episodic memories (Scoville & Milner, 1957; Snyder et al., 2005). Within the hippocampus, the dentate gyrus houses populations of NS/NPCs that proliferate throughout adult life and produce cells that develop into mature neurons (Lois & Alvarez-Buylla, 1993; Gage, 2000; Kempermann, 2002). Accumulating evidence suggests that adult generated neurons in the dentate gyrus participate in the cognitive functions of the hippocampus. Lesions of the dentate gyrus in rats yield memory deficits similar to those observed in animals with lesions of the hippocampus (Gilbert et al., 2001;

Morris et al., 1982; Mumby et al., 1999).

Moreover, studies that both assess behavioral performance on hippocampal-dependent tasks and quantify levels of neuronal proliferation have demonstrated associations between learning and neurogenesis. There is a positive correlation between neurogenesis and hippocampal-mediated learning capacity in aged rats performing a MWM paradigm (Drapeau et al., 2003). Additionally, training on associative learning and spatial navigation tasks leads to increased numbers of new neurons in the dentate gyrus (Gould et al., 1999a; Dupret et al., 2007). Recent findings suggest that learning induces apoptosis of immature neurons, survival of more mature neurons, and the genesis of new neurons in an important interplay that is required for the formation of new memories and the storage of long-term memory (Dupret et al., 2007).

Other studies have linked neurogenesis and learning by showing that experimental conditions that affect one also affect the other. Both enriched environments and physical exercise have positive effects on learning and levels of adult born neurons. Exercising animals show increased cell proliferation in the SGZ, more new dentate granular neurons, and enhanced performance when compared with non-exercising counterparts on spatial navigation tasks that require the hippocampus (van Praag et al., 1999). The mitogenic factor, basic fibroblast growth factor (bFGF), both enhances injury-induced neurogenesis and improves subsequent performance on the MWM (Sun et al., 2009). Furthermore, conditions that suppress neurogenesis also negatively affect learning, as seen in aged rats and rats that are exposed to prenatal stress (Drapeau et al., 2003; Lemaire et al., 2000).

The functional incorporation of newly generated dentate granular neurons is supported by a number of physiological observations. Firstly, it has been shown that new

neurons in the dentate gyrus are synaptically integrated into existing networks, both receiving input and extending axons to form functional synapses with other neurons (Hastings & Gould, 1999; Emery et al., 2005; Sun et al., 2007). They also take on the morphological and biochemical characteristics of neurons as they mature (Kuhn et al., 1996) and demonstrate electrophysiological properties of neurons through the generation of action potentials (van Praag et al., 1999).

Inhibition studies have more directly implicated adult born neurons in cognition by demonstrating that selectively prohibiting neurogenesis impedes performance on tasks mediated by hippocampus. There are three main methods used to deplete new neuronal populations: anti-mitotic drugs, irradiation, and genetic knockout. In behavioral studies investigating the effects of ablation of neurogenesis, depletion of new neurons must be coincidental with their functional time course, thus these studies pose a number of challenges to researchers.

Arabinofuranosyl cytidine (Ara-C) is a mitogen inhibitor and has been shown to block cell proliferation in rapidly dividing cells. It is the precursor to the active metabolite 1-beta-D-arabinofuranosylcytosine triphosphate (Ara-CTP), which inhibits cell division by blocking adenylate complexes from forming, thus inactivating DNA ligases, and through competitive inhibition of DNA polymerases α and β , thereby halting cell division and modification (Wang et al., 2003; Colon-Cesario et al., 2006). It is shown to block proliferation in the SVZ in models of neonatal hypoxic-ischemic injury, growth factor-promoted neurogenesis in the striatum, as well as the spontaneous behavioral recovery associated with the two (Im et al., 2010). In studies of adult ringdoves, treatment with Ara-C both blocked injury-induced neurogenesis in the ventromedial nucleus

(VMN) of the adult ringdove and abolished recovery of nest coo behavior, which is governed by the VMN (Chen & Cheng, 2007). Another antimitotic agent, methylazomethanol acetate (MAM), is used in a number of studies and has been shown to reduce rats' ability to acquire an eyeblink response in a hippocampal-dependent trace conditioning task and a fear memory task (Shors et al., 2001; Shors et al., 2002). These studies have the drawback that at high dosages, cytostatic agents like Ara-C and MAM reduce the overall health of the animals and such side effects might influence the outcomes of behavioral experiments.

Other groups have utilized irradiation to block adult neurogenesis and to avoid the side effects associated with mitogen inhibitors. Irradiation also has a stronger effect on reducing populations of adult born neurons (Leuner et al., 2006). In adult rodents, irradiation has been shown to impair animals' ability to carry out a hippocampal-dependent place recognition task, but not an object recognition task that does not require the hippocampus (Rola et al., 2004). In one study, rats receiving low doses of irradiation (10 Gw γ to the head for 2 days) performed as well as shams on the MWM during acquisition and the probe trial at 1 week, but had impaired performance on the probe trials at 2 and 4 weeks after training, suggesting a role of these new neurons on long-term spatial memory (Snyder et al., 2005). Inhibition of neurogenesis by irradiation also causes deficits in a non-spatial task mediated by the hippocampus, such as acquisition and extinction in a contextual fear-conditioning paradigm (Ko et al., 2009).

Transgenic techniques are also used to selectively ablate adult generated dentate granular neurons and investigate their role in cognition. Jessberger et al. recently demonstrated that knocking down WNT signaling, which is known to be specific and

critical for the generation of new adult neurons (Lie et al., 2005), inhibited neuronal proliferation and impaired performance on a long-term spatial memory task. While the WNT deficient rats were able to carry out acquisition of the spatial task similar to controls, their performance on the probe trial 2-8 weeks later was significantly weakened. Animals with disrupted WNT signaling and extremely reduced numbers of adult born neurons also showed deficits on the hippocampal-dependent novel object recognition task (Jessberger et al., 2009).

One critical component to this question that has yet to be elucidated is the mechanism by which these neurons contribute to cognitive processes. One possibility is that they are important for future learning once they integrate into existing neurocircuitry or they may contribute to synaptic plasticity in long term potentiation before they are fully mature (Kempermann, 2002). It is also possible that they may function in storage of short-term memories, as a rapidly changing cell population might suggest (Gould et al., 1999). Adding further uncertainty, it is unclear whether the hippocampus retains spatial memory permanently, or if consolidation of these memories ultimately resides in the neocortex (Sutherland et al., 2001; Maviel et al., 2004).

The timing during which new neurons within the dentate gyrus contribute to cognitive processes of the hippocampus is another important aspect to address. One group found that using irradiation to inhibit neurogenesis, new neurons are vital at 4-28 days old for spatial long-term memory (Snyder et al., 2005). This timing is supported by evidence that dentate granular neurons extend their axons into the CA3 region at 4-10 days of age, are functional at 4 weeks, and contribute to other hippocampal dependent studies at 6-21 days of age (Shors et al., 2001). However, other groups have observed that

a variety of time points may be critical for neurogenesis to participate in learning (Sun et al., 2007; Jessberger et al., 2009).

While there is increasing evidence that adult neurogenesis in the hippocampus contributes to its functions in learning and memory, more investigation is required to fully understand the role of injury-induced neurogenesis in cognitive function. This study aims to implicate these newly generated neurons in innate recovery following injury by ablating neurogenesis with intraventricular infusion of Ara-C after LFPI and monitoring performance on spatial memory tasks at different time points post-injury.

TBI-Induced Enhancement of Neurogenesis

NS/NPCs are present in the adult mammalian nervous system and actively divide throughout adult life (Gage, 2000). These cells reside in the SVZ and the SGZ of the dentate gyrus (Altman & Das, 1965; Gage, 2000). They retain the potential to develop into neurons and glia (Lois & Alvarez-Buylla, 1993). As described above, it is thought that these adult-born cells serve a critical role in hippocampal function (Snyder et al., 2005; Shors et al., 2002; Shors et al., 2001; Madsen et al., 2003; Lemaire et al., 2000; Dupret et al., 2007; Gould et al., 1999a; Gould et al., 1999b). Previous studies from this laboratory have demonstrated that 48 hours following moderate LFPI, NS/NPCs in these regions produce a robust proliferative response (**Figure 1.1**, Chirumamilla et al., 2002). Other studies have found that lesions to the dentate gyrus result in cell proliferation in the SGZ (Gould et al., 1997b). It has been additionally shown that in adult ringdoves, electrolytic lesion of the ventromedial nucleus (VMN) of the hypothalamus induces cell proliferation and new neuronal projections in the region. The neurogenic response occurs within the timeframe of the observed behavioral recovery of the nest coo, and conditions

that improve recovery of such courtship behaviors also increased levels of neurogenesis (Chen, et al., 2006; Chen & Cheng, 2007). In studies of rat hippocampal neurogenesis, a proportion of proliferating cells co-localize with mature neuronal markers one month following injury, indicating that these cells develop into dentate granular neurons (Dash et al., 2001). Transgenic studies in mice show that cells produced in response to injury form complex dendritic arbors and are incorporated throughout the dentate gyrus (Blaiss et al., 2011). After selective ablation of these neurons using genetic knockout techniques, animals performed significantly worse than their control counterparts on spatial learning tasks requiring hippocampus (Blaiss et al., 2011). Taken together, these findings strongly suggest that cellular proliferation may be a component of structural plasticity that serves in the brain's endogenous repair process after injury.

Secondary insults from TBI are particularly injurious to the hippocampus, with evidence of cell loss in the CA2 and CA3 regions accompanied by hippocampal-dependent memory dysfunction following moderate to severe TBI (Smith et al., 2001). The proliferative response and enhanced neurogenesis following brain insult may serve as an innate mechanism to restore the functionality of hippocampus. New cells born after injury are shown to integrate as fully functional mature granule neurons in studies using fluorescent tracers and transgenic models to show dendritic arborization (Emery et al., 2005; Blaiss et al., 2010). Importantly, these cells achieve neuronal maturity and anatomic integration at time points when the innate cognitive functional recovery was observed following a moderate LFPI (**Figures 1.2 & 1.3**, Sun et al., 2007).

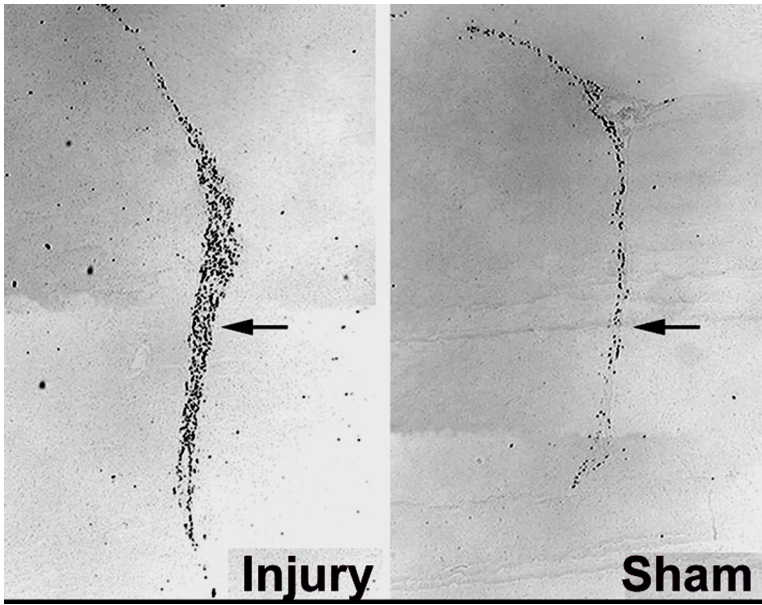
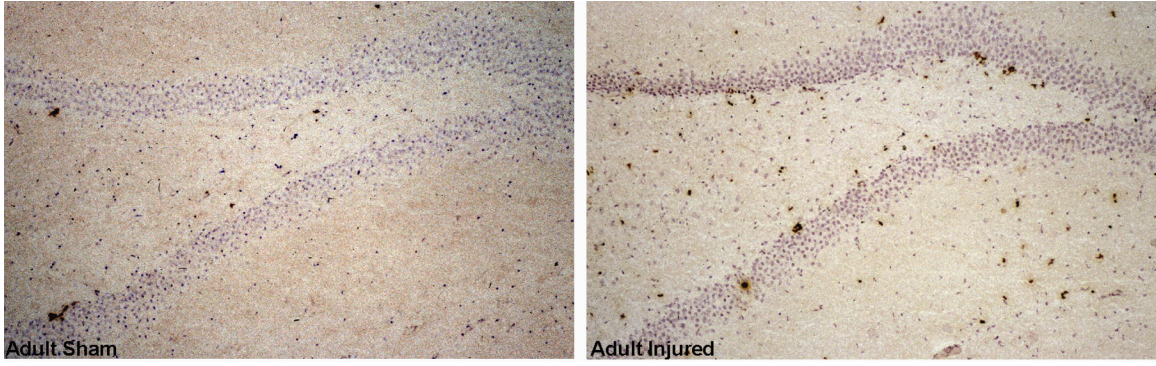


Figure 1.1. The effect of lateral fluid percussion injury on cell proliferation in the dentate gyrus 48 hours post-injury. Increased BrdU staining was observed in the injured animals compared to sham at 2 days post-injury in the dentate gyrus (top panel) and the SVZ (bottom panel).

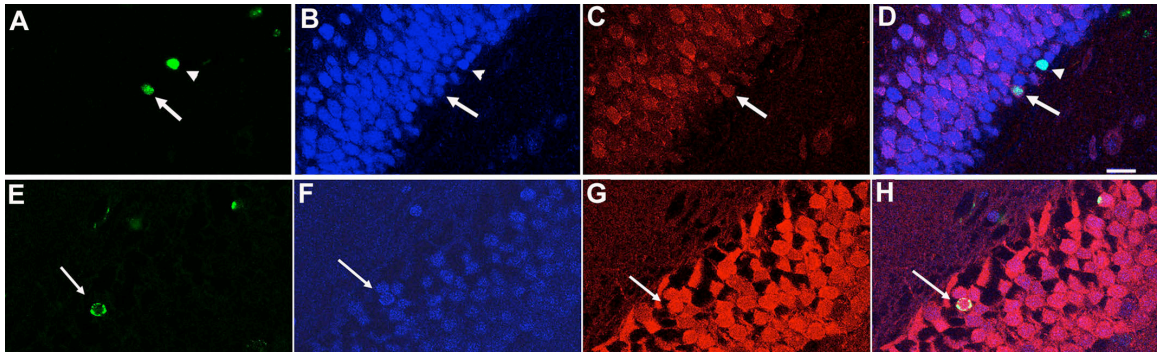


Figure 1.2. Newly generated cells display markers for mature granule cells. (A) Confocal micrograph showing newly generated BrdU+ cells within the granule cell layer (green) at ten weeks post injury. In (B) the same cells are labeled with the mature neuronal marker, NeuN (blue). (C) Granule neurons retrogradely incorporated with FG (red). (D) Merged image of A, B and C. The arrow points to one of the newly generated cells labeled with BrdU, NeuN and FG while the arrow head points to a cell labeled with BrdU and NeuN only. Scale bar: 30µm. (E-H) Confocal photomicrograph showing newly generated cells express the granule cell marker, calbindin. The arrow indicates a BrdU-labeled cell (green, E), which expressed calbindin (blue, F) and was retrogradely incorporated with FG (red, G). (H) Merged image of E, F, and G. Scale bar: 30µm.

MWM Performance

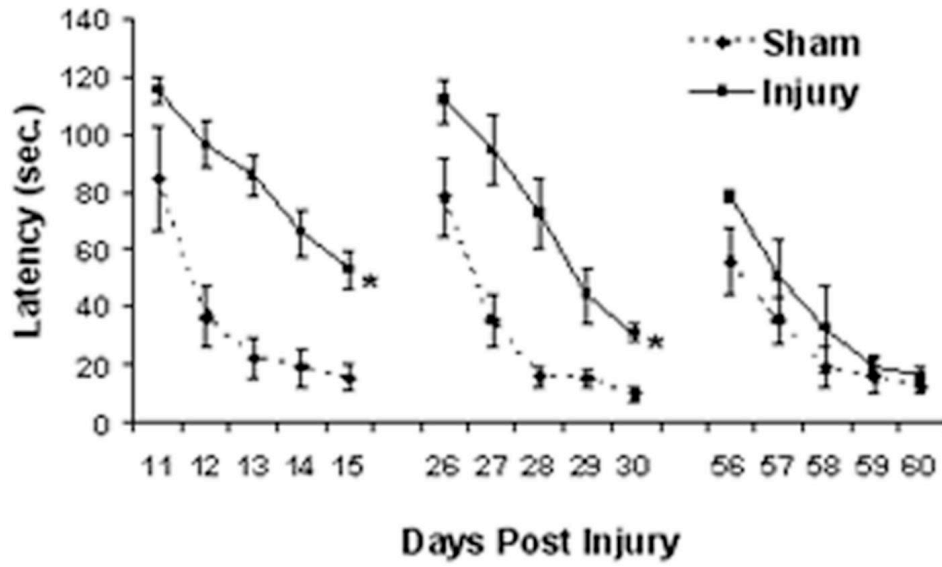


Figure 1.3. Cognitive deficits following TBI recover over time. Graph comparing Morris water maze performance of injured rats to sham animals during trials at days 11-15, 26-30 or days 56-60 following injury. Injured animals displayed significant cognitive deficits, as characterized by a longer latency, at 11-15 days post injury when compared to sham animals (* $p < 0.05$). These deficits persisted at 26-30 days (* $p < 0.05$). At days 56-60, injured animals showed cognitive recovery with a shorter latency, which was not significantly different to sham animals

Additionally, in studies comparing young and old animals, young subjects demonstrate increased levels of injury-induced cellular proliferation in the SGZ, a greater proportion of which develop into mature neurons, as well as a greater capacity for cognitive recovery (Sun et al., 2005). Young animals also have higher basal levels of adult neurogenesis, which may underlie the difference observed in TBI induced cellular proliferation (Kuhn et al., 1996). These findings strongly suggest that the age-related distinction in cognitive recovery may be dependent on differences in the neurogenic response to injury.

Growth Factors and Neurogenesis

Neurogenesis occurs throughout life in the mammalian brain and there is increasing evidence that new neurons produced within the dentate gyrus contribute to hippocampal function. It has also been shown that SGZ cell proliferation significantly increases following TBI and a number of studies strongly suggest that this response serves in endogenous cognitive recovery (Chirumamilla et al., 2002; Dash et al., 2001; Sun et al., 2007; Sun et al., 2009). The precise mechanism governing hippocampal neuronal proliferation is still unresolved, but a number of studies point to growth factors as key mediators of this process. One growth factor in particular, basic fibroblast growth factor (bFGF), appears to contribute as a mitogenic factor both *in vitro* and *in vivo* (Palmer et al., 1999; Kumon et al., 1993; Kuhn et al., 1997; Yoshimura et al., 2001). *In vivo* it is shown to promote cell proliferation among neural progenitor cells in the SVZ, many of which developed into fully functional neurons of the olfactory bulb (Kuhn et al., 1997). In animal models of ischemia, bFGF increases transiently in neurons and astrocytes, indicating that it is endogenously released in response to insult (Kumon et al.,

1993; Yoshimura et al., 2001). Further, in bFGF genetic knockout models, the cell proliferative response to injury is diminished, but retrieved by exogenous administration of bFGF (Yoshimura et al., 2001).

Previous studies in this laboratory have shown further that TBI-induced neurogenesis is enhanced by intraventricular infusion of bFGF (**Figure 1.4**, Sun et al., 2009). This enhancement is seen in combination with improved performance on cognitive tasks known to require the hippocampus (**Figure 1.5**, Sun et al., 2009). Taken together, these results suggest a critical role for adult-born neurons in hippocampal activity and the therapeutic potential for improving cognitive recovery following injury through manipulating injury-induced proliferative response in the dentate gyrus.

While there is increasing evidence that adult neurogenesis in the hippocampus contributes to its functions in learning and memory, studies have not demonstrated a direct role of injury-induced neurogenesis in cognitive function. To further understand the role of neurogenesis in the restoration of hippocampal function following TBI, we utilized mitogen inhibitor Ara-C to inhibit injury-induced cell proliferation, administered both independently and with bFGF. We postulate that inhibition of injury-induced cell proliferation by Ara-C treatment will cause diminished innate cognitive recovery following TBI. Furthermore, inhibition of bFGF enhanced post-TBI neurogenesis by Ara-C will abolish bFGF improved cognitive recovery following injury.

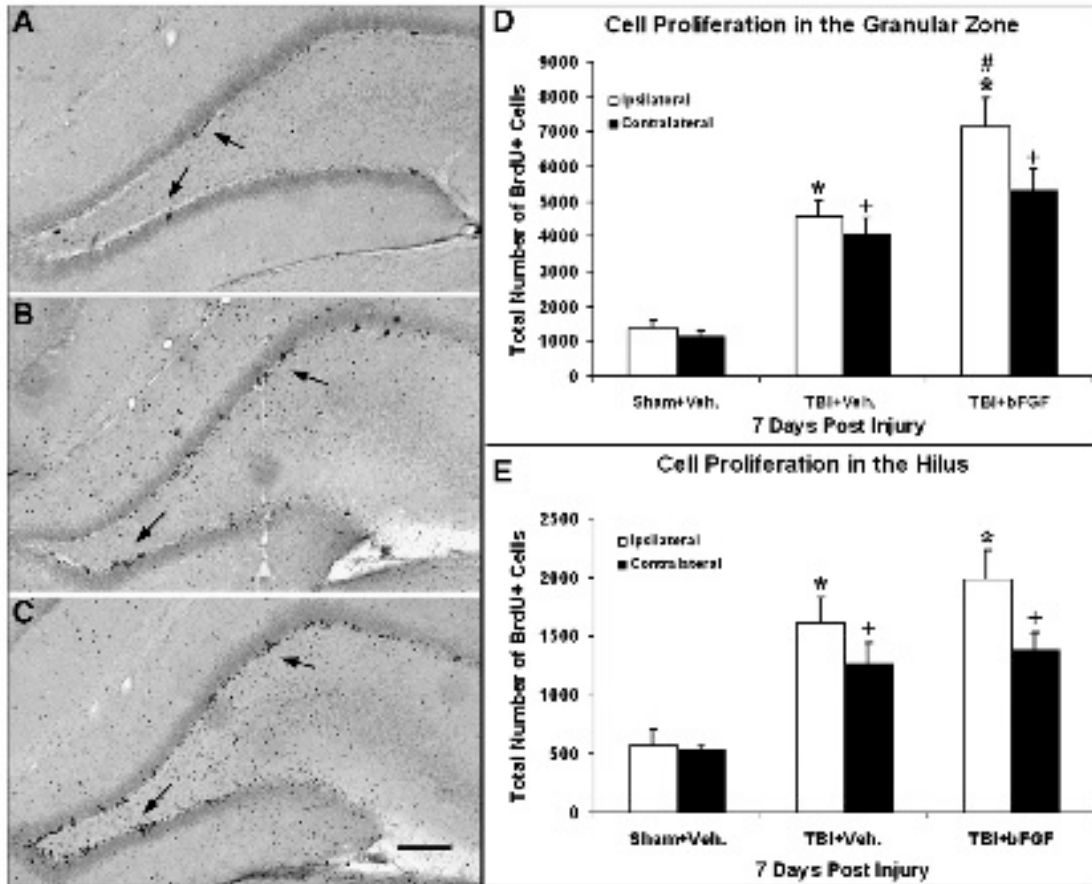


Figure 1.4. Enhancement of cell proliferation in the dentate gyrus. Coronal sections of ipsilateral dentate gyrus 7 days post-injury: (A) sham-injured animal with vehicle infusion; (B) injured animal with vehicle infusion; and (C) injured animal with bFGF infusion. More BrdU+ cells (black dots indicated by arrows) are seen in the injured animal compared to the sham and this number is increased with infusion of bFGF. (D) Quantification of BrdU+ cells in the granular zone (GCL and SGZ) for each group for ipsilateral and contralateral sides. There were significantly more BrdU+ cells in the ipsilateral and contralateral granular zone of the injured animals treated with vehicle and injured animals treated with bFGF compared to sham animals ($*p<0.05$). Injured animals treated with bFGF had significantly enhanced levels of BrdU+ cells compared to vehicle treated injured animals ($\#p<0.05$). (E) Quantification of BrdU+ cells in the hilus for each group for ipsilateral and contralateral sides. BrdU+ cells were significantly more abundant in the ipsi- and contralateral hilus hilus of both vehicle and bFGF treated injured animals compared to shams ($*/+p<0.05$). (Sun et al., 2009).

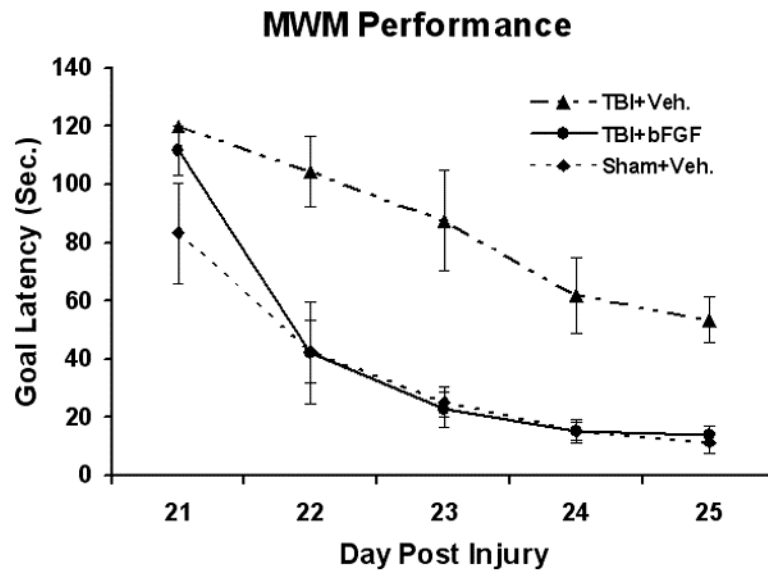


Figure 1.5. Improved performance of bFGF-infused animals on MWM 22-25 days post-TBI. Injured animals that received infusion of the growth factor bFGF showed shorter latencies to find the goal platform in the MWM when compared with their vehicle infused counterparts, and performed much more like sham-injured animals ($*p<0.01$, $n=10$ for each group). Improved MWM performance is an indicator of enhanced cognitive recovery following injury (Sun et al., 2009).

Chapter 2 – Materials and Methods

Subjects

A total of 92 three-month old male Sprague-Dawley rats (Harlan Inc., Indiana) weighing approximately 300g at the beginning of this study were used. The animals were housed in the animal facility, two per cage with food and water *ad libitum*. The animal facility where the rats were kept was maintained on a 12-hour light/dark cycle and at a temperature of 20°C. All animal protocols followed guidelines established in the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services) and were approved by Institution of Animal Care and Use Committee (IACUC) of Virginia Commonwealth University.

Surgical Preparation and Procedure

Animals were subjected to a moderate lateral fluid percussion injury (LFPI) following a standard protocol (Sun et al., 2005). All surgical tools were sterilized in an autoclave and aseptic procedures were used throughout surgery. Each subject was anesthetized in a Plexiglas chamber with 5% isoflurane. The top of the animal's head was shaved, the animal was intubated, and maintained on 2.5% isoflurane in a gas mixture (30% O₂, 70% N₂) throughout surgery. The head was then secured in a stereotaxic apparatus. The surgical site was sterilized with Betadine, and Paralube ointment was applied to each eye. A midline incision was made and the skull was exposed, using hemostats to retract connective tissue from the surgical site. If the animal received an intraventricular cannula connected to a mini-osmotic pump seven days prior, the cannula and pump were carefully removed before the surgical procedure for FPI. A

trephine and a Dremel drill fitted with a small dental drill bit were used to place 4.9 mm craniotomy on the left parietal bone, half way between the lambda and bregma sutures (**Figure 2.1**). The bone flap was removed and remaining bone shards were removed from the edges of the craniotomy. A Luer lock hub was placed over the site of the craniotomy and cemented with cyanoacrylic. Dental acrylic was then applied around the base of the hub, in order to secure the seal, and allowed to dry. The hub was then filled with 0.9% saline to test the seal between the hub and the skull and as a medium for the fluid percussive device.

Lateral fluid percussion injury (LFPI)

Once the Luer lock hub was fitted and secured with acrylic, the isofluorane rate was reduced to 1% for about 4 minutes. Concurrently, the fluid percussion injury device (**Figure 2.2**) was calibrated by repeated use, once every 30 seconds, to ensure it consistently produced pressures within the target range. The isofluorane was then completely shut off for one minute and then the animal was disconnected from anesthesia and the Luer lock hub was securely connected to the injury device. A moderate injury of 1.9 ± 0.04 atm was then administered. Sham animals received the same surgical procedure without the fluid pulse.

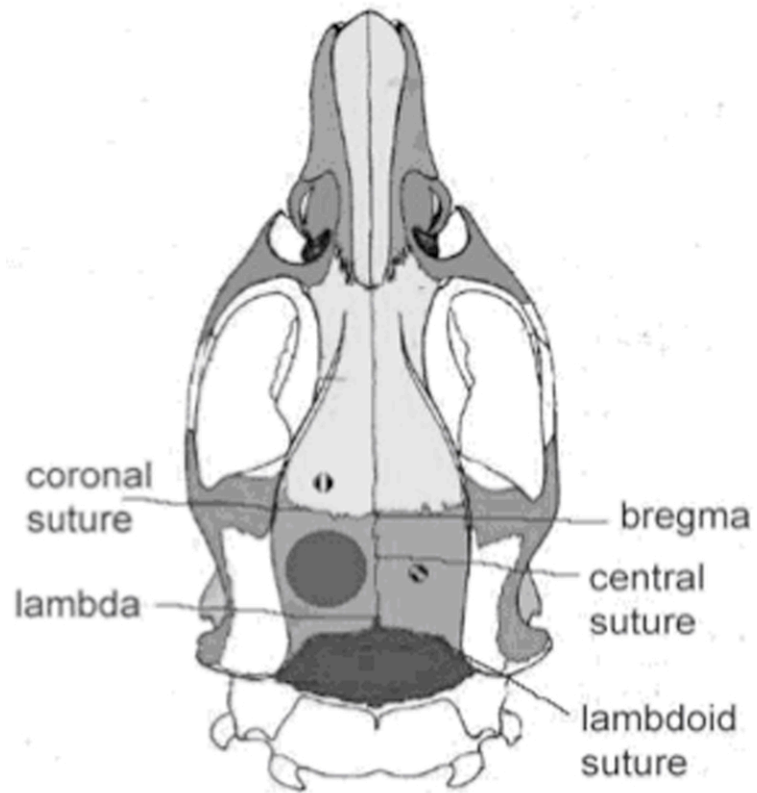


Figure 2.1. Craniotomy and injury site. A 4.9mm craniotomy was placed on the left parietal bone, halfway between the lambda and bregma sutures, which served as the opening for the water blast for the fluid percussion injury.

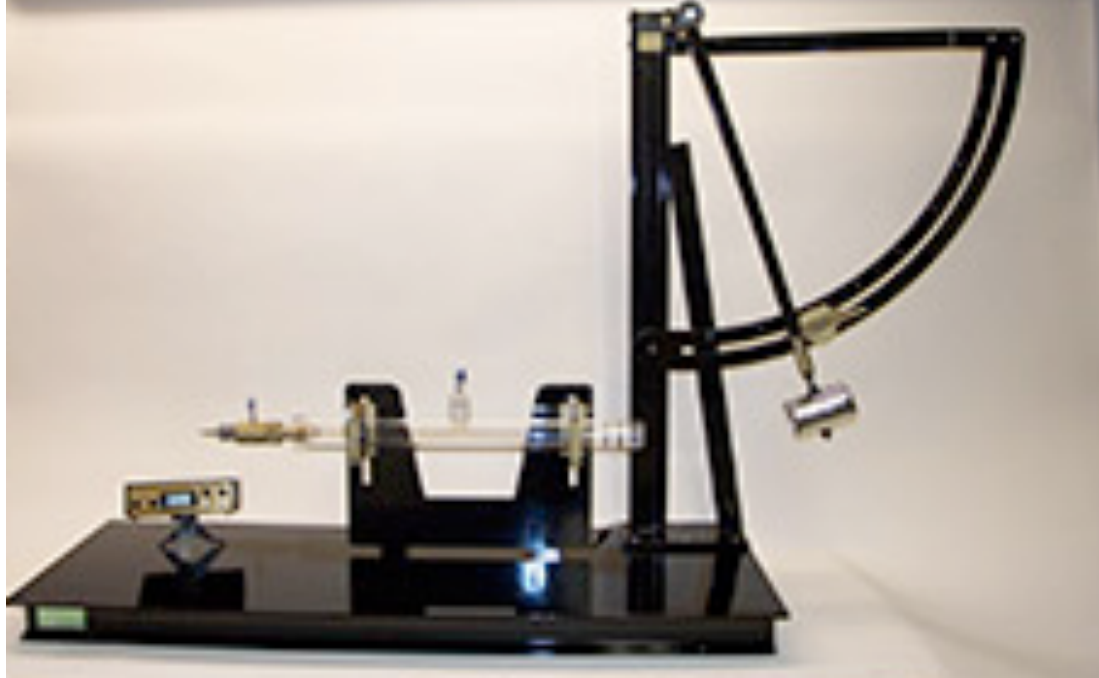


Figure 2.2. Photograph depicting the fluid percussion injury device.

Following injury, the Luer lock and acrylic base were removed and the animal's breathing tube was reattached to ventilation without isoflurane. The time it took for the animal to regain paw, tail, and righting reflex were recorded. The animal was observed until it regained a normal breathing pattern.

Intraventricular infusion

Implantation of the Alzet brain infusion cannula (Brain Infusion Kit II, DURECT, Cupertino, CA) connected to a Alzet mini-osmotic pump (Model 1007D) for intraventricular infusion took place either 15 minutes after injury (or sham surgery), or seven days prior to injury (or sham surgery), depending on the experimental group. The animal was again deeply anesthetized via the ventilation tube with 5% isoflurane then the isoflurane concentration was dropped to 2.5%. The cannula connected to the mini-osmotic pump was then stereotactically implanted into the posterior lateral ventricle ipsilateral to the injury site (coordinates: AP -1.0 mm, ML 1.4 mm, and 3.5 mm beneath the pial surface) and was held in place with cyanoacrylic adhesive. The connective tissue between the skin and the back of the neck was retracted and the mini-osmotic pump was placed subcutaneously. 20 injured rats and 13 sham surgery rats received Ara-C infusions after LFPI. 20 injured rats and 15 sham surgery rats were infused with a vehicle. 11 injured rats received Ara-C/bFGF infusions. 5 rats received Ara-C vehicles seven days prior to LFPI. 8 injured animals received bFGF infusions post-TBI. (Table 2.1).

The surgery site was then closed using a sterilized stainless steel suture needle and a 5-0 polyamide surgical suture in a simple continuous pattern. Triple antibiotic and local anesthetic were then applied to the surgical site and the isoflurane was terminated.

TABLE 1. ANIMAL GROUPINGS

<i>Days post injury</i>	<i>Injury & Infusion</i>						
	<i>TBI +Ara-C</i>	<i>Sham +Ara-C</i>	<i>TBI+ Veh</i>	<i>Sham +Veh</i>	<i>TBI +Ara-C/ bFGF</i>	<i>Ara-C +TBI</i>	<i>TBI +bFGF</i>
7	5	4	4	3	4	3	0
28	9	6	3	2	7	0	8
62 or 74	6	3	13	10	0	2	0

Table 2.1. Groupings of animals used in this study. Animals are organized by date of sacrifice relative to the date of injury as well as injury/sham and infusion treatment type. 20 injured rats and 13 sham surgery rats received Ara-C infusions after LFPI. 20 injured rats and 15 sham surgery rats were infused with a vehicle. 11 injured rats received Ara-C/bFGF infusions. 5 rats received Ara-C vehicles seven days prior to LFPI. 8 injured animals received bFGF infusions post-TBI.

The animal was observed until it regained a normal breathing pattern and the intubation was removed. It remained on a heating pad until it righted and then it was returned to its home cage, lined with a surgical drape. Each rat was observed for three hours and then returned to the animal housing facility.

The cannulae and osmotic mini-pumps were left in place for seven days. During the seven-day infusion period, animals were monitored for body weight and other indicators of health, including lethargy, prophyrin staining, and wound healing. Each of these measures was noted on a scale of 0-4. All cannulae and pumps were removed on post-injury day 7. For animals sacrificed on day 7, the removal took place after the animal was transcardially perfused. Animals that survived for behavioral tasks were placed under 2.5% isoflurane anesthesia and the cannulae and attached mini-pumps were carefully removed. The surgery site was again closed followed by antibiotic and local anesthetic. The animal was monitored until it righted, and then it was returned to its home cage, lined with sterile surgical draping, and returned to the animal facility.

BrdU injections

To label dividing cells, all subjects received single daily intraperitoneal (i.p.) injections of 5-bromo-2'-deoxyuridine (BrdU, Sigma) from days 2-7 post-injury. BrdU is a thymidine analog that is incorporated into the DNA of a dividing cell. Injections were given at a 50 mg/kg dosage based on body weight.

Tissue Processing

Animals were sacrificed on day 7, 28, 62 or 74 post-injury, depending on the experimental group. Each animal was deeply anesthetized with isoflurane until

breathing ceased. The animals were then transcardially perfused, first with 400 mL PBS followed by 400 mL fixative, composed of 4% paraformaldehyde in PBS. The brains were dissected out and stored in fixative at 4°C. After at least 48 hours, the brains were transferred to a solution containing PBS and .01% sodium azide. Brains were then rinsed in PBS and then sliced into 60 µm coronal sections with a vibratome. Sections were placed in individual wells in a 24-well tray containing PBS .01% sodium azide and stored at 4°C until staining.

Immunohistochemistry

All brains were divided into four sets of 30 sections, with each set used for different stains. For animals sacrificed at 7 days post-injury, one set was stained for BrdU. In TBI animals receiving either Ara-C or vehicle infusion and sacrificed at 7 days post-injury, brains were also double-labeled for BrdU and one of a number of biomarkers, including Doublecortin (DCX), Sox2, Iba1, PSA-NCAM and GFAP. For animals sacrificed at any other time point, 12 sections were selected from one set and stained for Ki67.

BrdU immunostaining

Sections were washed with PBS twice for five minutes each, then denatured in 50% formamide (50% formamide, 37.5% H₂O, 12.5% 20X saline sodium citrate (SSC) buffer) for one hour at 65°C. They were then rinsed in 2X SSC two times for five minutes. The sections were then placed on 2N HCl for 30 minutes at 37°C. Then they were rinsed twice with 1XPBS for five minutes, and then quenched with 3% hydrogen peroxide for one hour on the shaker. Following a PBS rinse (1X PBS + 0.3% Triton100)

for 10 min, the sections were blocked overnight in a blocking buffer (5% Normal Horse Serum in 1X PBS + 0.3% triton) at 4°C. The blocking buffer was replaced with the primary antibody and the sections incubated for 48 hours at 4°C on the shaker. The primary antibody was prepared in a 1:200 concentration in the same blocking buffer as the one previously described. The sections were then brought back to room temperature and rinsed in 1X PBS + 0.3% triton for 10 minutes three times. They were blocked for three hours at room temperature in the blocking buffer described above, on the shaker. The blocking buffer was removed and replaced with the secondary antibody, which was prepared in a solution containing Biotin-conjugated anti-mouse IgG in a 1:200 dilution in the blocking buffer. The sections were incubated overnight at 4°C on the shaker. They were brought back to room temperature, then washed in 1X PBS + 0.3% triton for 10 minutes three times. Then they were incubated in Avidin-biotin complex (ABC) for two hours at room temperature. ABC was prepared in a 1:200 concentration in 1X PBS 30 minutes before use. The sections were then washed in PBS for ten minutes three times. Liquid 3-3' diaminobenzadine (DAB) (Vector Laboratories) was prepared by mixing buffer stock solution in nanopure water (2 drops/5mL water), followed by DAB stock solution (2 drops/5mL water) and hydrogen peroxide solution (2 drops/5mL water). The solution was mixed well and added to the sections briefly. The reaction was observed under the microscope and when the sections were adequately stained, they were quenched with 1X PBS. The sections were rinsed again with 1X PBS and stored in fresh 1X PBS at 4°C until they were mounted to glass slides.

Nissl Counterstain

Sections mounted on glass slides were rehydrated in water for two minutes, then

stained in 0.1% cresyl violet for one minute. The slides were rinsed in running water for about one minute, then placed in 75% ethanol for dehydration for about two minutes. They were transferred to 95% ethanol for further dehydration for another minute, and to 100% ethanol for another minute. They were briefly placed in acid alcohol for differentiation. The sections were transferred to Citrosolv for 5 minutes, then placed in fresh Citrosolv for another 5 minutes or until they were cover slipped using Permount.

Ki67 Immunostaining

To assess whether TBI, Ara-C treatment or Ara-C/bFGF combination treatment affect cell proliferation in the dentate gyrus at later time points, immunohistochemistry was performed for the proliferation marker Ki67 on animals that completed the water maze on days 21-24. Animals were sacrificed at 62 days post-injury and every fourth section was processed for Ki67 expression. The immunohistochemistry procedure was similar to that used in BrdU staining, with the exception that the denaturation step with 50% formamide and 2N HCL was omitted. The primary antibody used was rabbit anti-Ki67 (1:500 Abcam) and the secondary antibody was biotinylated goat anti-rabbit IgG (1:200 Jackson lab). These were followed by application of the ABC kit and DAB substrate. Three animals from each experimental group were used and 5 sections were quantified. Every Ki67+ cell within the GZ and hilus was counted and the totals were averaged across the animals.

Stereological quantification

Stereological quantification was carried out in dentate gyrus (DG) of the hippocampus for each animal. The Visiopharm program (Olympus) was utilized to

quantify BrdU and Ki67 positive cells within the granular cell layer (GCL, including the granular zone and the subgranular zone) and hilus of the DG. For BrdU counts, ten 60µm thick sections containing hippocampus were selected for each animal. For Ki67 counts, five sections containing hippocampus were selected. For each section, the area of interest was outlined at 4x magnification and cells were counted at 60x under oil immersion. The dissector height (h) was 15µm and only cells within h were counted, cells above and below were excluded. The average thickness (t) of the sections was calculated for each animal by measuring the focal plane in five random locations of each section and then averaging across the ten sections. The total number of cells (N) was estimated to be $N = \frac{\Sigma Q^{-}}{t/h} \cdot (1/asf)(1/ssf)$, where the total number of cells counted was ΣQ^{-} . The average sampling fraction (asf) was equal to one, as the entire region was counted. The section-sampling fraction (ssf) was 0.25 for the BrdU counts and .0125 for the Ki67 counts, as ten sections were used in the former and five in the latter, representing one quarter and one eighth of the total hippocampus, respectively.

Behavioral assessment: Morris water maze

The Morris water maze (MWM) was utilized to assess spatial cognition following TBI (**Figure 2.3**, Morris et al., 1982; Smith et al., 1991). All animals surviving beyond 7 days post-injury were tested. For the purpose of investigating innate cognitive recovery after TBI, animals were tested at two key time points: 21-24 days post-injury and 56-60 days post-injury, with an additional probe trial two weeks at 25 days post-injury for the former group. For all animals undergoing the MWM, the procedure was identical. The maze consisted of a large, circular tank, 180cm in diameter and 45cm in height. The tank was filled with water to a 30cm depth. The water was constantly maintained at 25-28°C

and white latex paint was added to the water for opacity. The goal platform was located 45cm from the edge in the SE quadrant of the tank. The room that the tank occupied contained constant external visual cues.

Latency

Subjects completed four trials per day for four or five consecutive days. On the first day of testing before the first trial, each animal received a period of habituation in which they were placed the goal platform for 30 seconds. For each trial, the subject was placed in a randomized quadrant, N, S, E or W, facing the wall. Once the animal reached the goal, they remained on the platform for 30 seconds, then placed in an incubator until the next trial. A total of 120 seconds was allotted for the animal to reach the goal platform and when the maximum time was reached, the animal was manually moved to the platform and remained there 30 seconds. The latency and path length were recorded with a video tracking system (Videomex, Columbus Instruments, Columbus, OH). Using latency and path length, swim speed was calculated for each animal to rule out the possibility of motor deficits affecting performance.

Probe trial

To test the memory function, probe trials were performed at 24 hours after the last latency training. The platform was removed from the tank. The animal was placed in the quadrant where the platform was placed before facing the wall and given 60 seconds to swim in the pool. Using the video tracking system, the time spent in the goal platform quadrant and the average proximity to the goal location were recorded.

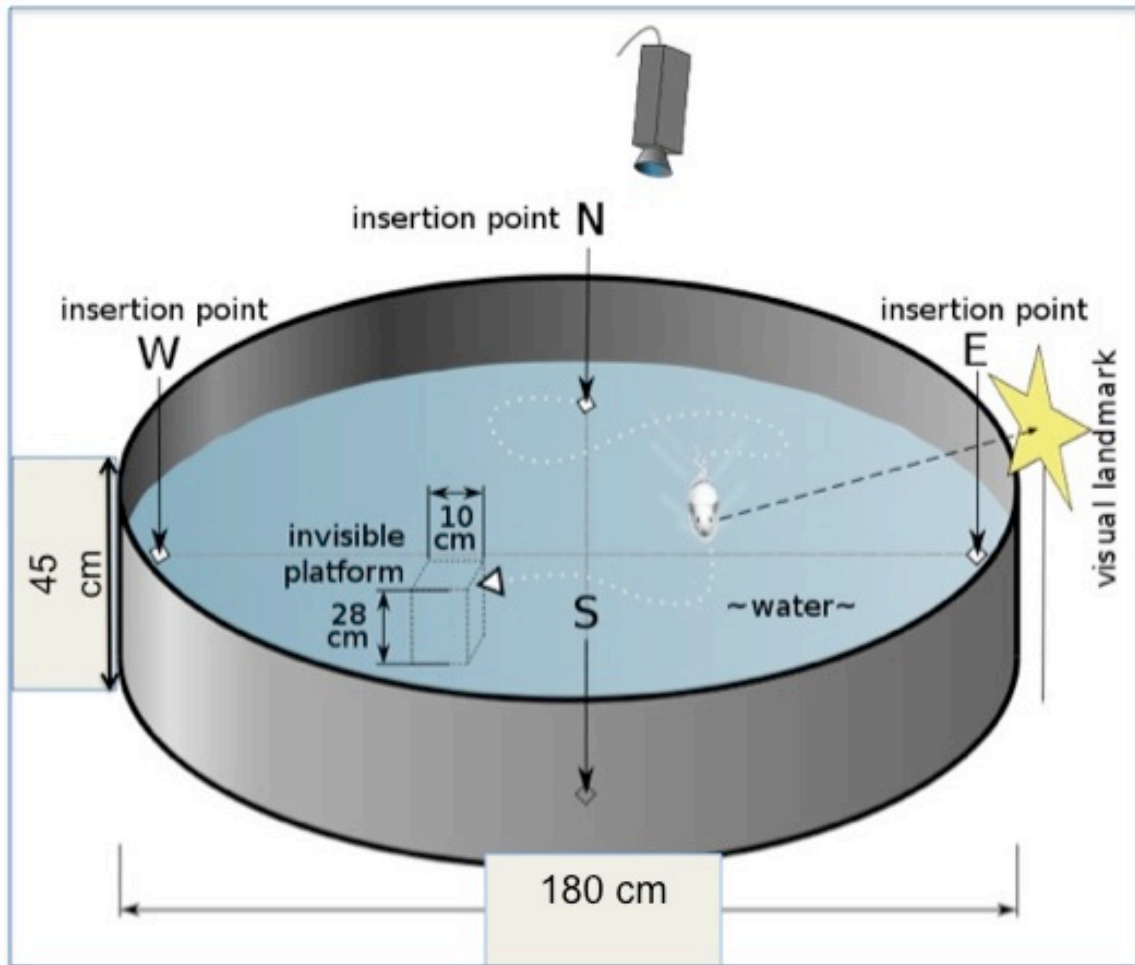


Figure 2.3. Illustrated depiction of the Morris water maze (MWM). In the MWM task, the subject is placed in a large circular tank filled with water. A hidden platform is placed 2cm below the water line. A video tracking system is used to record the behavior of animals in the maze.

Statistical analysis

Statistical analyses were performed using SPSS software. A one-way ANOVA was used to determine statistically significant differences in cell quantification, probe trial data, and swim speed data. A post-hoc LSD test was utilized to determine differences within groups and a *p* value of less than 0.05 was considered statistically significant. A repeated ANOVA was used to analyze latency data from the MWM. All data are presented as the group mean \pm SEM.

Chapter 3 - Results

Previous research in this laboratory has demonstrated that cell proliferation in neurogenic regions of the rodent brain increases significantly after TBI. This response is further enhanced by intraventricular infusion of bFGF, which also improves cognitive function following injury (Sun et al., 2007; Sun et al., 2005; Sun et al., 2009). The purpose of this study was to validate the direct association of injury-induced neurogenesis with cognitive functional recovery observed following TBI and bFGF treatment. To this end, we utilized the anti-mitotic agent Ara-C to block injury-induced neurogenesis, and examined cell proliferation and cognitive function in those animals.

Experiment 1: The effect of intraventricular infusion of Ara-C and Ara-C/bFGF on injury-induced cell proliferation

Intraventricular infusion of Ara-C blocks TBI and bFGF-enhanced cell proliferation in the dentate gyrus

This laboratory has previously shown that moderate LFPI leads to increased levels of neurogenesis in the dentate gyrus. Additionally, bFGF administration significantly increases injury-induced cell proliferation. To deplete this TBI-induced neurogenesis or bFGF-enhanced neurogenesis, we subjected animals to moderate LFPI, followed by a 7-day intraventricular infusion of Ara-C with or without bFGF to the ipsilateral lateral ventricle via a cannula and osmotic mini-pump. To test the efficacy of Ara-C on cell inhibition, BrdU was administered intraperitoneally daily during the period of Ara-C infusion.

Following a 7-day Ara-C treatment, we found that in the dentate gyrus granular

zone (GZ), which includes the granule cell layer (GCL) and the subgranular zone (SGZ), infusion of Ara-C significantly reduced the number of BrdU positive cells in the injured animals with or without bFGF treatment when compared with injured animals receiving vehicle treatment (**Figure 3.1**). Specifically, stereological quantification analysis demonstrated that LFPI increased levels of BrdU+ cells in vehicle-treated animals both ipsilateral and contralateral to the injury site compared to sham animals ($p<0.01$, **Figure 3.2**), whereas injured animals receiving Ara-C or Ara-C/bFGF infusions did not show significant increases in cell proliferation compared with sham animals (TBI+Ara-C vs sham, $p=0.729$; TBI+Ara-C/bFGF vs sham, $p=0.974$, **Figure 3.2**). Among injured animals, TBI vehicle-infused animals had significantly more BrdU+ cells compared to injured animals with Ara-C or Ara-C/bFGF infusion, revealing a reductive effect of Ara-C treatment on TBI-induced proliferation ($p<0.05$, **Figure 3.2**). In shams, reduced cell proliferation was observed in animals with Ara-C infusion compared to vehicle-treated animals, but the reduction was not statistically significant ($p=0.34$, **Figure 3.1&3.2a**). With respect to findings in previous studies that demonstrate TBI and TBI/bFGF increase levels of newly generated cells in the dentate gyrus at 7 days post-injury, the current results suggest that Ara-C treatment abolishes the effect of TBI and/or bFGF on cell proliferation in the GZ, reducing the number of BrdU+ cells at this time point to sham levels.

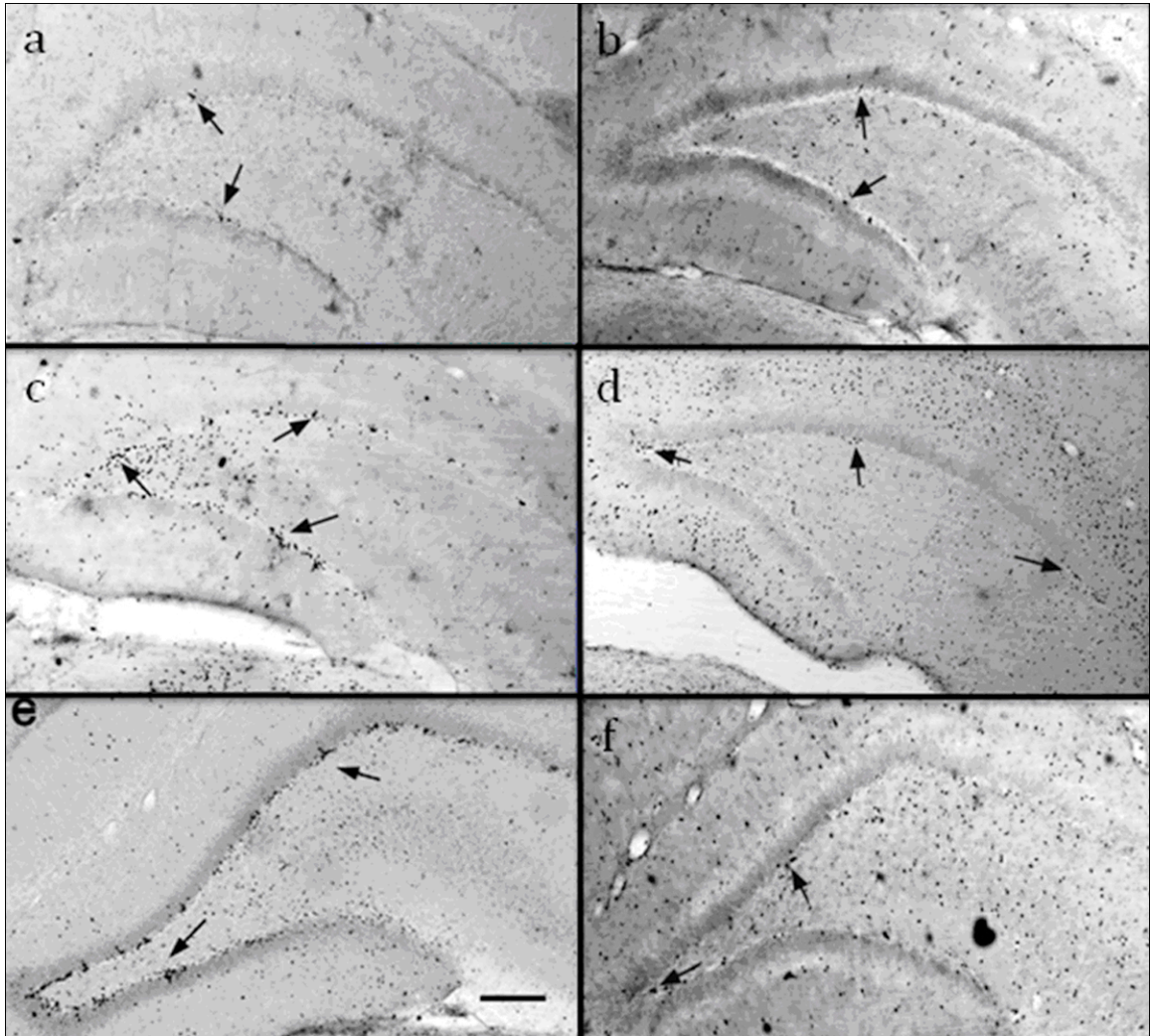


Figure 3.1. Cell proliferation at 7 days post-injury. Micrographs taken from BrdU stained coronal sections in the ipsilateral dentate gyrus from (A) sham with vehicle treatment, (B) sham with Ara-C treatment, (C) injured with vehicle treatment, (D) injured with Ara-C treatment, (E) injured with bFGF treatment and (F) injured with Ara-C/bFGF treatment. (A-B). Sham animals show a small number of BrdU⁺ cells, most located in the SGZ of the dentate gyrus; C) injured animal receiving vehicle shows a greatly increased number of BrdU⁺ cells in the GCL and the hilus, and especially in the SGZ; D) reduced number of BrdU⁺ cells is seen in injured animals that received Ara-C infusions in the SGZ and GCL when compared with (C); E) enhanced cell proliferation in bFGF treated animal but not in animals received both Ara-C and bFGF infusion (F). Brown dots indicated BrdU⁺ cells. Bar scale = 500 μ m.

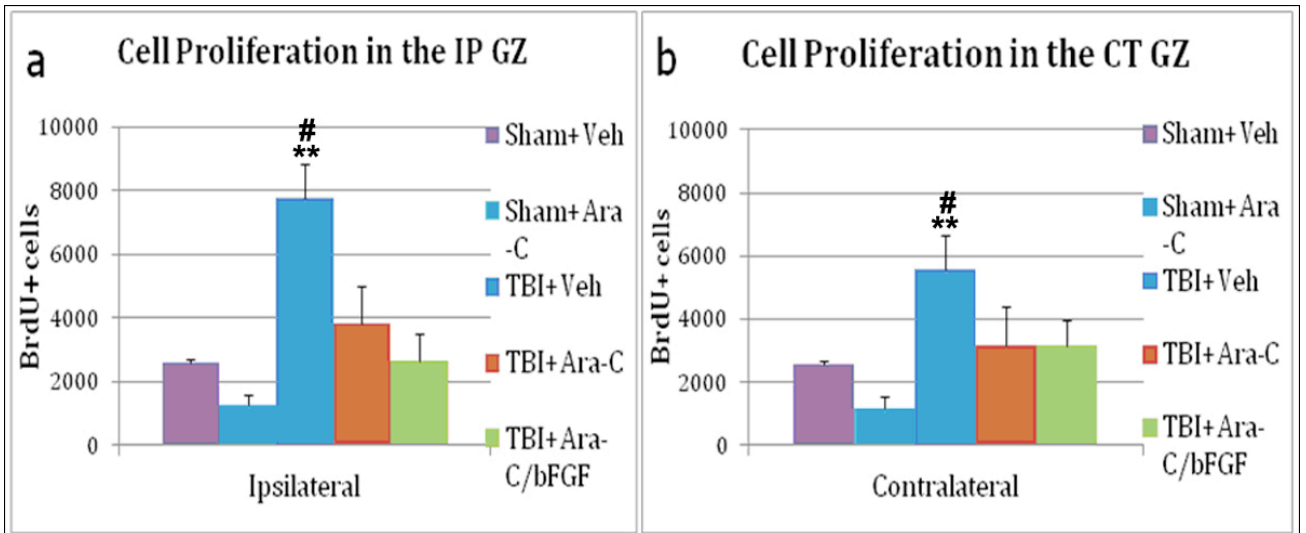


Figure 3.2. Quantitative analysis of cell proliferation in the GZ of the dentate gyrus 7 days post-injury. In both the (A) ipsilateral and (B) contralateral GZ (SGZ and GCL combined), TBI vehicle-treated animals had significantly higher numbers of BrdU-labeled cells in comparison to sham animals (** $p < 0.01$), and injured animals receiving Ara-C or Ara-C/bFGF infusion ($\#p < 0.05$). The number of BrdU+ cells between Sham+vehicle, Sham+Ara-C, TBI+Ara-C, TBI+Arc-C/bFGF were not significantly different.

In the hilus region, increased cell proliferation was observed in all injured groups and sham animals receiving Ara-C (**Figure 3.1**). Stereological quantification showed an increased number of BrdU-labeled cells for all three TBI groups and the sham+Ara-C group compared to the sham animals receiving vehicle in both the ipsilateral and contralateral sides, though this increase was only significant in vehicle-treated animals in ipsilateral hilus and in Ara-C/bFGF treated animals in contralateral hilus (**Figure 3.3**, IP: TBI+vehicle vs. sham, $p<0.05$; TBI+Ara-C vs. sham, $p=0.053$; TBI+Ara-C/bFGF vs. sham, $p=0.08$. CT: TBI+vehicle vs. sham, $p=0.44$; TBI+Ara-C vs. sham, $p=0.06$; TBI+Ara-C/bFGF vs. sham, $p<0.05$).

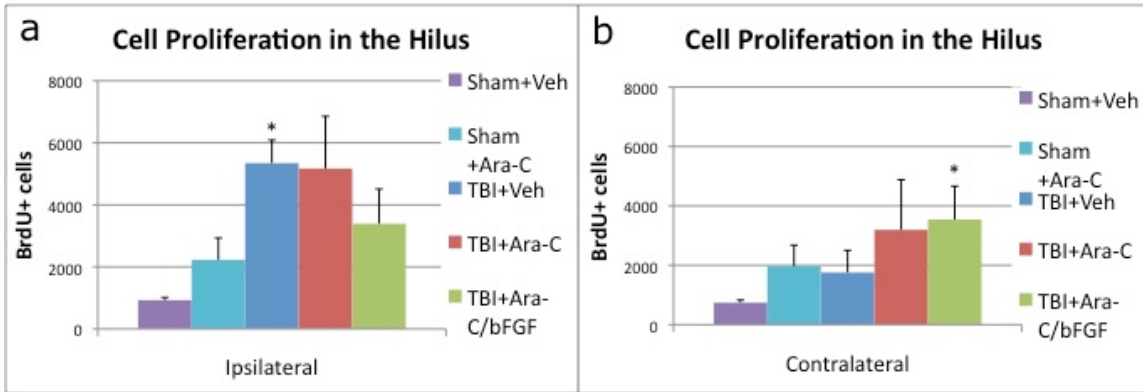


Figure 3.3. Cell proliferation in the hilus 7 days post-injury. In the ipsilateral hilus (A), sham animals showed the least amount of BrdU+ cells, with increased numbers of cells in Ara-C-infused shams and injured animals receiving vehicle, Ara-C and Ara-C/bFGF infusions. The only significant difference was in vehicle-treated LFPI animals compared to shams $(p<0.05)$ There was also increased cell proliferation indicated by BrdU+ cells in the (B) CT hilus of injured animals receiving Ara-C/bFGF, as well as animals receiving Ara-C. These trends were significant only in TBI animals with Ara-C/bFGF infusions $(p<0.05)$.

Pre-TBI Ara-C treatment does not significantly affect TBI-induced cell proliferation

To test whether focal delivery of the mitogen inhibitor Ara-C has a long-lasting effect on cell proliferation, we infused Ara-C prior to TBI. After a 7-day infusion period, Ara-C was withdrawn and animals were subjected to a moderate LFPI, then received BrdU injections from days 2-7 and then were sacrificed at 7 days post-injury. Quantification analysis revealed that Ara-C treatment prior to injury did reduce BrdU-positive cells in the ipsilateral and contralateral GZ and the ipsilateral hilus, but this effect was not significant for any region (IP GZ, $p=0.14$; CT GZ, $p=0.91$; IP hilus, $p=0.50$; CT hilus, $p=0.39$; **Figure 3.4**). These data suggest that Ara-C exerts its inhibition effect only at the time when it is administered.

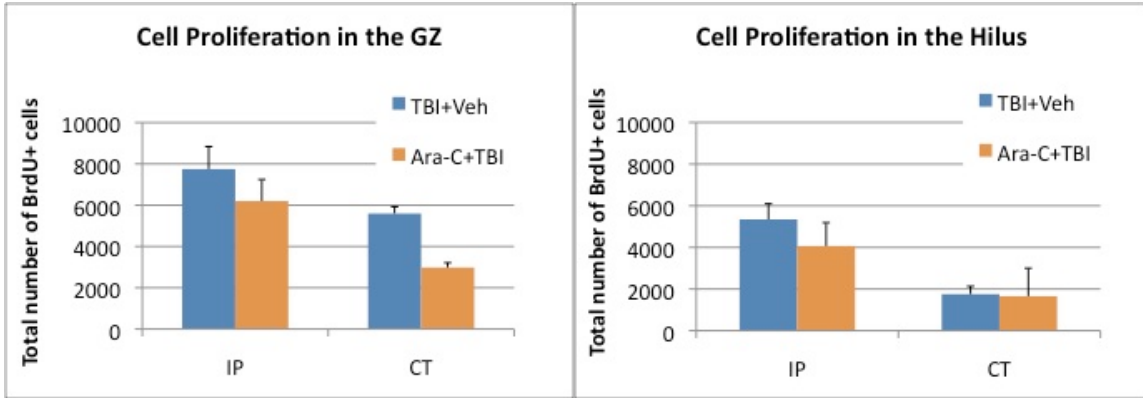


Figure 3.4. Quantitative analysis of the effect of Ara-C pre-treatment on injury-induced cell proliferation. To investigate whether or not intraventricular infusion of Ara-C would have a prolonged effect on injury-induced neurogenesis, we implanted osmotic mini-pumps and cannulae that deliver the antimitotic agent for 7 days prior to LFPI. While there appears to be a trend toward diminished proliferation in the contralateral GZ, there was no statistical difference between injured animals receiving a vehicle following injury and injured animals receiving Ara-C prior to injury in either the ipsilateral or contralateral GZ or hilus at 7 days post-injury (IP GZ, $p=0.14$; CT GZ, $p=0.091$; IP hilus, $p=0.50$; CT hilus, $p=0.39$.)

Ara-C treatment does not have a significant prolonged effect on inhibition of cell proliferation

To determine if Ara-C treatment continued to affect cell proliferation, we examined cell proliferation in animals sacrificed at 62 days post-injury using Ki67. Ki67 is a cell cycle related nuclear protein that is expressed in all phases of the active cell cycle. Previous studies from this laboratory show that injury-induced cell proliferation following a moderate LFPI peaks at 48 hours post-injury and returns to near sham levels by 2 weeks post-injury using BrdU labeling (Sun et al., 2005). Conversely, the long-term effect of Ara-C on TBI-induced cell proliferation is not well studied. In the current study, Ki67 labeling revealed that the number of Ki67+ cells in the ipsilateral and contralateral GZ was slightly lower in both sham and injured animals that received Ara-C infusion. Statistical significance was only observed between sham+vehicle animals and TBI+Ara-C animals in the ipsilateral GZ ($p < 0.05$, **Figure 3.5**). The number of Ki67+ cells in injured animals receiving Ara-C treatment was reduced compared to those receiving vehicle treatment, though this difference was not statistically significant.

In the hilus region, a trend of higher levels of Ki67 expression was found in the ipsilateral hilus in sham+vehicle animals and in the contralateral hilus of TBI+Ara-C animals compared to other groups, but the differences were not statistically significant (**Figure 3.6**).

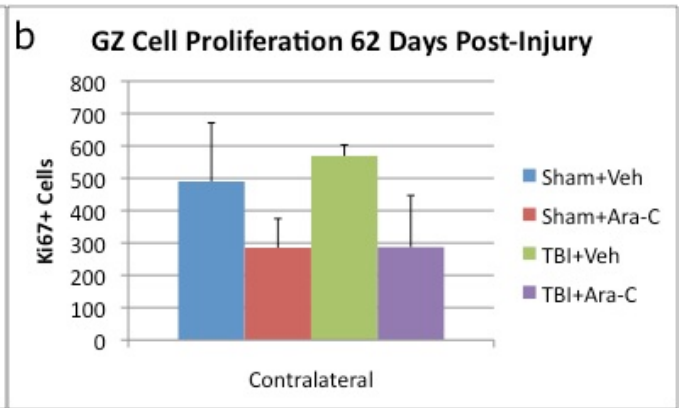
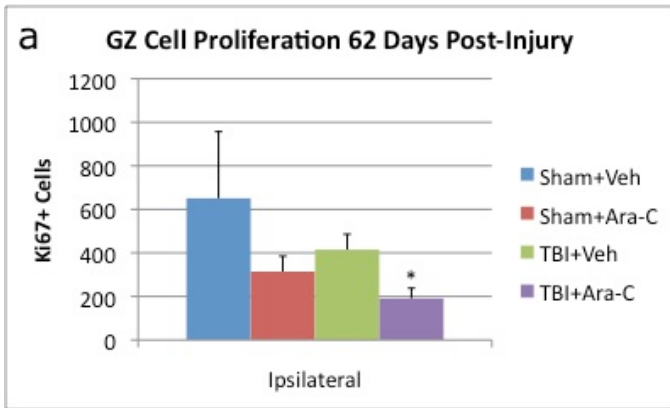


Figure 3.5. Quantitative analysis of Ki67 labeling in the GZ at 62 days post-injury.

Tissue was processed for the cell cycle marker, Ki67, at 62 days post-injury. Animals treated with Ara-C demonstrated reduced expression of Ki67 compared to vehicle treated animals for both injured and sham groups. Statistical analysis only showed a significant difference in the number of Ki67+ cells in the ipsilateral GZ between sham vehicle animals and TBI animals with Ara-C treatment (* $p < 0.05$).

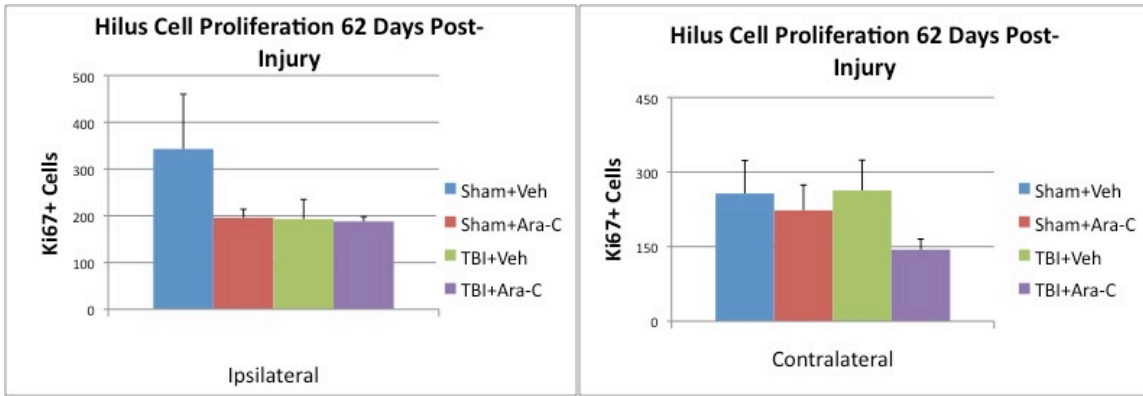


Figure 3.6. Quantitative analysis of Ki67 labeling in the hilus at 62 days post-injury.

Tissue was processed for the cell cycle marker, Ki67, at 62 days post-injury. Statistical analysis did not show a significant difference in Ki67 expression in either ipsilateral or contralateral hilus.

Experiment 2: The effect of intraventricular infusion of Ara-C and Ara-C/bFGF on innate cognitive recovery following TBI

Moderate LFPI leads to impaired cognitive function. Previous studies in this laboratory have demonstrated that moderate LFPI initiates increased cell proliferation in the dentate gyrus and that the recovery of cognitive capacity coincides with the time course of the integration of newly generated neurons into existing neuronal networks within the hippocampus (Sun et al., 2007). It has been further shown that intraventricular infusion of bFGF post-TBI enhances hippocampal neurogenesis and significantly improves cognitive recovery on the MWM (Sun et al., 2009). To determine whether injury/bFGF enhanced hippocampal neurogenesis plays a pivotal role in improving cognitive recovery following injury, we evaluated the learning and memory function of injured animals after depleting injury/bFGF-enhanced cell proliferation using the MWM. Animals were infused with either Ara-C alone or a combination of Ara-C/bFGF for seven days post-injury to deplete cell injury-induced proliferation, followed by MWM tests at 21-25 or 56-60 days post-injury in two separate groups of animals.

Righting Response

To assess whether subjects included in behavioral tasks received similar levels of injury severity, we compared the spontaneous righting response post-injury in injured animals with vehicle, Ara-C, or Ara-C/bFGF infusion, as well as animals infused with Ara-C prior to injury. The length of suppression of the righting reflex is a reflection of neuromotor damage and is an accepted measure of the extent of injury (Hamm et al., 1991; Hamm et al., 2001). The mean (\pm SEM) duration of suppression of the righting response was 14.93 ± 1.56 for vehicle-infused animals, 13.36 ± 1.02 for Ara-C-treated

animals, 12.29 ± 1.21 for Ara-C/bFGF-treated animals, and 13.25 ± 0.75 for animals infused with Ara-C prior to TBI. A t-test was performed on the righting times and found that there was no significant difference in the righting times between any of the groups (Ara-C, $p=0.45$; Ara-C/bFGF, $p=0.21$, pre-treated Ara-C, $p=0.52$). This suggests that there is no significant difference in injury severity level between groups.

7-day infusion post-injury of Ara-C does not affect cognitive function at 21-25 days post-injury

To test whether inhibition of injury-induced cell proliferation has a further detrimental effect on cognitive function, a group of animals was tested on MWM tasks at 21-25 days post-injury. The mean latency (s) to reach the platform at 21-24 days was used as a measure of cognitive function. The data were analyzed using a split-plot analysis of variance (repeated measures ANOVA, Group x Day). The results of the ANOVA revealed significant differences in Group effect ($p < 0.001$) and Day x Group interaction ($p < 0.001$) between sham and injured groups. Post-hoc analysis revealed that TBI animals in both vehicle and Ara-C-infused groups had significantly longer latencies to reach the goal platform compared to sham-injured animals treated with vehicle ($p < 0.05$, **Figure 3.7**). There was no significant difference in the performance of Ara-C-treated sham animals compared to vehicle-treated sham animals ($p = 0.538$), or injured animals receiving vehicle or Ara-C infusion (**Figure 3.7**).

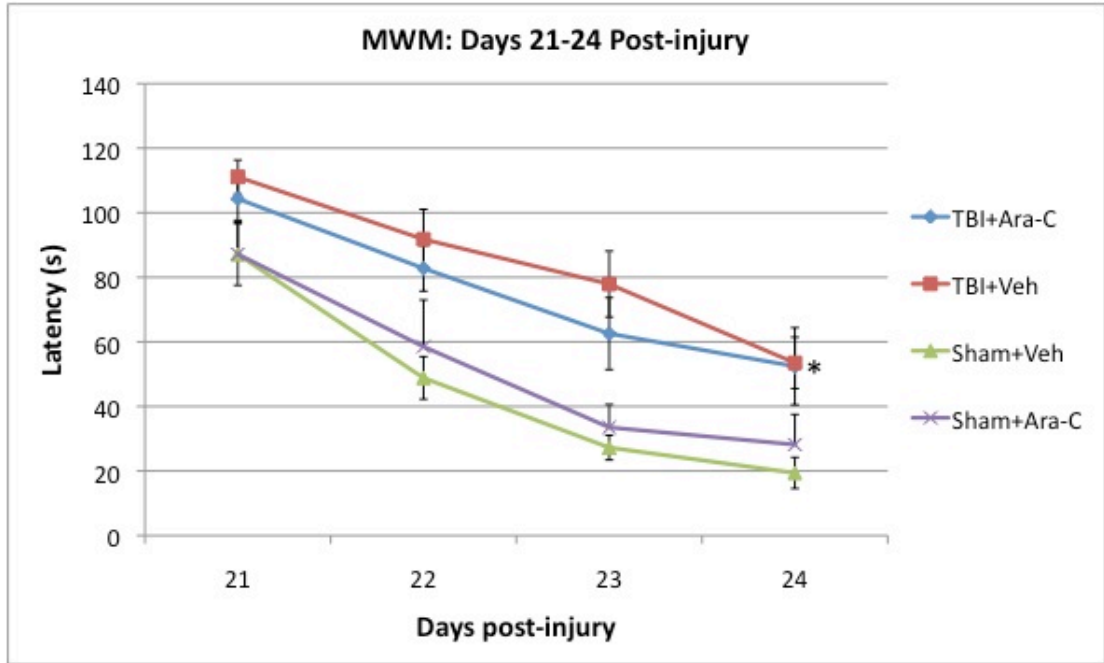


Figure 3.7. Ara-C infusion does not exacerbate MWM performance at 21-24 days post-injury. Animals were tested on the MWM on days 21-24 post-injury for goal latency. All groups showed significant improvement in goal latency by the fourth day of training ($p<0.01$). Group comparison revealed that injured animals, either with vehicle or Ara-C infusion, had significantly longer goal latency compared to sham animals with vehicle or Ara-C infusions at 21-24 days post-injury ($*p<0.01$). No significant difference was found between sham vehicle and sham Ara-C animals.

A probe trial was conducted at 24 hr after the last day of latency testing. Probe trial latency data analysis showed that injured animals in both vehicle and Ara-C groups spent less time searching for the removed platform compared to sham animals, suggesting memory deficits in the injured animals. This difference was only significant in Ara-C-treated animals (**Figure 3.8**, * $p < 0.05$). Proximity data analysis showed that compared to sham groups, both injured groups were on average further from the goal quadrant throughout the probe trial (**Figure 3.8**, * $p < 0.05$, ** $p < 0.01$).

The results from the probe trial tests implicate short-term memory deficits in LFPI animals with or without Ara-C infusion.

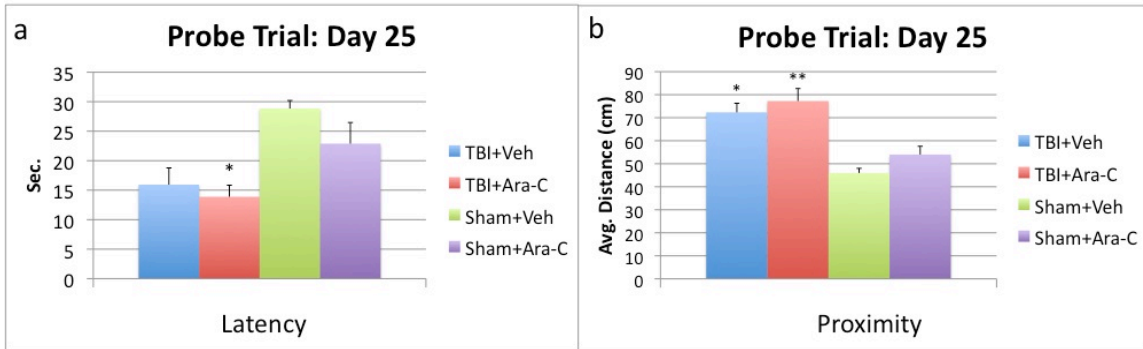


Figure 3.8. Morris water maze probe trial performance on day 25 post-injury. (Left) Time spent in the goal quadrant, and (Right) the average proximity to the goal location. Injured animals from both infusion groups demonstrated reduced latency in the goal quadrant during the probe trial compared to sham vehicle animals ($*p<0.05$). They were also on average at a significantly greater distance from the goal platform in the probe trial (Ara-C, $**p<0.01$; and vehicle, $*p<0.05$). Among injured groups, they did not perform significantly different from each other for either of these measures (Latency, $p=0.698$; and proximity $p=0.582$).

Inhibition of injury-induced cell proliferation with Ara-C infusion impedes innate cognitive recovery following injury

Studies have found that following a moderate LFPI, cognitive deficits in MWM tasks last for several weeks. Spontaneous cognitive recovery has been observed around 2 months post-injury, a time point corresponding to the integration of injury-induced newly generated dentate granular neurons in existing neural networks (Sun et al., 2007). To confirm that injury-induced neurogenesis plays a direct role in innate post-injury cognitive recovery, MWM tests were performed at 56-60 days post-injury. ANOVA revealed significant differences in Group effect ($p < 0.001$) but not in Day x Group interaction ($p = 0.74$). Post-hoc analysis revealed that the injured animals with Ara-C infusion had significantly longer latencies to reach the goal platform, whereas the vehicle treated injury group performed similar to sham animals (TBI+Ara-C, $p < 0.05$; TBI+vehicle, $p = 0.26$, **Figure 3.9**). Taken together, this finding suggests that Ara-C inhibition of injury-induced cell proliferation significantly disrupted the innate recovery of hippocampal function following TBI. This effect is seen most clearly at time points that correlate with observations of newly generated dentate granular neurons integrating into existing neuronal circuitry (Sun et al., 2007).

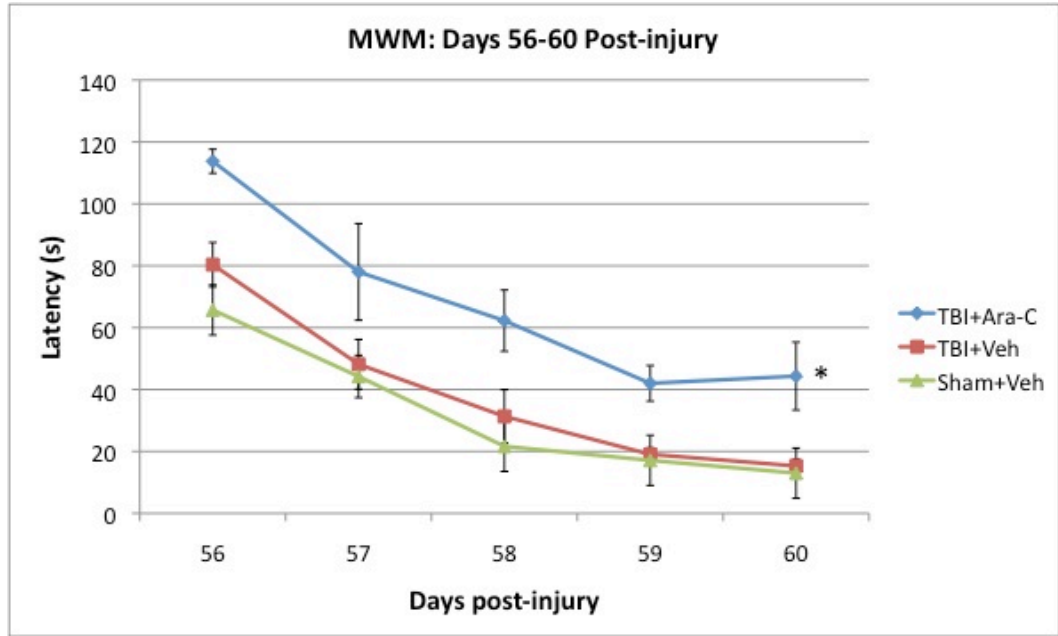


Figure 3.9. Ara-C infusion abolishes innate cognitive recovery following TBI at 56-60 days post-injury. Animals were tested on the MWM on days 56-60 post-injury for goal latency tests. All groups showed significant improvement in goal latency by the fourth day of training ($p<0.01$). Group comparison revealed that injured animals with vehicle infusion perform similar to sham vehicle animals. However, injured animals with Ara-C infusion had significantly longer goal latencies compared to injured- or sham-vehicle animals ($*p<0.01$).

Inhibition of injury- and bFGF-enhanced cell proliferation with Ara-C infusion abolishes bFGF-improved cognitive recovery following injury

We have previously found that infusion of bFGF infusion post-TBI can significantly enhance injury-induced cell proliferation in the dentate gyrus and improve cognitive functional recovery in injured animals (Sun et al., 2009). To test whether bFGF-improved cognitive recovery is due to the neurogenic effect of bFGF, we infused Ara-C with bFGF into the lateral ventricle in injured animals and compared their cognitive function to animals that received bFGF infusion using the MWM. When tested at 21-25 days post-injury, injured animals which received vehicle or Ara-C/bFGF had significantly longer goal latencies compared to injured animals treated with bFGF or sham animals (**Figure 3.9**, $p < 0.01$). This result indicates that Ara-C infusion blocks the improved cognitive recovery seen in bFGF-treated animals.

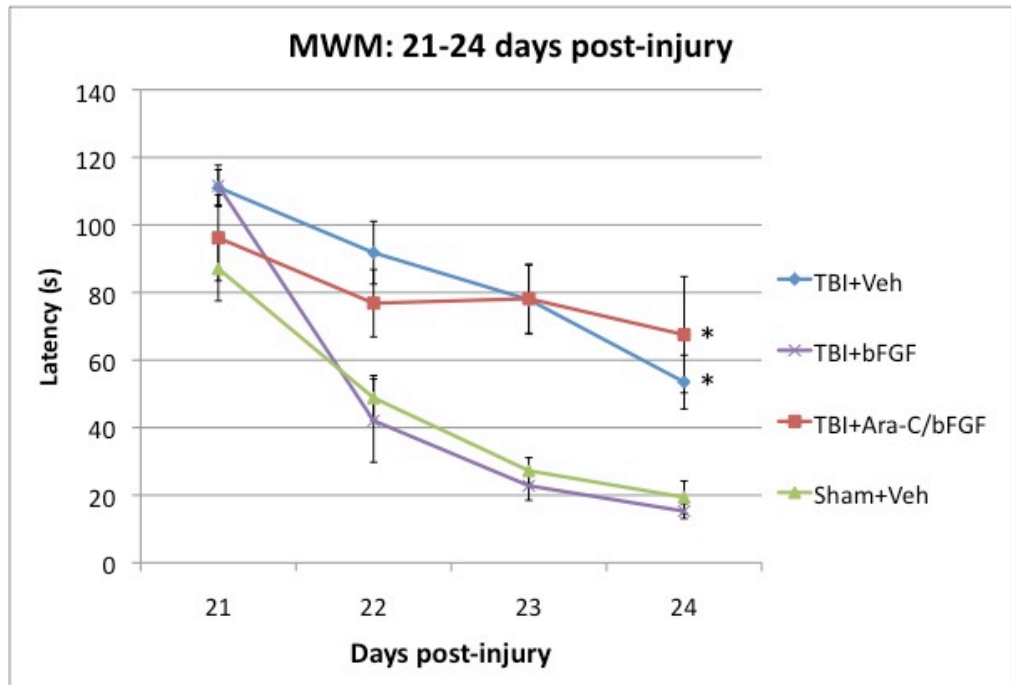


Figure 3.10. Ara-C infusion abolishes bFGF-enhanced cognitive recovery following TBI. Animals were tested on the MWM on days 21-24 post-injury. Injured animals that received Ara-C/bFGF treatment significantly increased the goal latency compared to vehicle-treated sham and bFGF-treated injured animals ($p<0.01$). These results suggest that infusion of Ara-C in combination with bFGF abolishes the beneficial effects of intraventricular infusion of bFGF on cognitive recovery post-injury.

Chapter 4 – Discussion

TBI continues to be a profound clinical problem facing health care providers and researchers. There are minimal current treatments available, as the central nervous system is known to retain limited regenerative capacity once it has matured. Learning and memory deficits are a hallmark characteristic of TBI and prove to be a central challenge to individuals with this type of injury (Smith et al., 1991; Masel & DeWitt, 2010). Therefore it is an important research goal to potentially restore cognitive function in individuals with TBI. Research has demonstrated that some of the endogenous cellular responses to TBI may have therapeutic potential. Studies show cell proliferation and generation of new neurons in the dentate gyrus of the hippocampus following TBI may contribute to cognitive recovery (Sun et al., 2007). While an accumulating number of studies implicate hippocampal neurogenesis in cognitive function, it is important to provide direct evidence that TBI-induced neurogenesis is responsible for innate cognitive repair. Additionally it is critical to further investigate agents that have been shown to enhance cognitive recovery. Pursuing these research goals will lead to improved therapies addressing the cognitive deficits that afflict TBI patients. Recently, there have been a number of encouraging findings from investigation into growth factors that promote neurogenesis and cognitive recovery.

A number of studies demonstrate a central role of neurogenesis in the dentate gyrus in hippocampal-dependant learning and memory. It has been shown that conditions that promote neurogenesis also improve cognitive abilities, from enriched environments to exercise and growth factors (van Praag et al., 1999a & b; Sun et al., 2009). Learning in

spatial navigation tasks, an example of a hippocampal-mediated function, has also been shown to lead to increased neurogenesis within the SGZ. And conversely, conditions that suppress neurogenesis also seem to impair performance on hippocampal-dependent tasks (Drapeau et al., 2003; Lemaire et al., 2000). A number of ablation studies have further confirmed the role of hippocampal neurogenesis in spatial learning and memory.

Irradiation to the hippocampus blocks adult neurogenesis and impairs performance on spatial learning tasks (Rola et al., 2004; Snyder et al., 2005). Ara-C, an anti-mitotic agent, has been used to block neurogenesis and is shown to impair learning as well (Wang et al., 2003; Colon-Cesario et al., 2006; Chen & Cheng, 2007). With a growing body of research supporting the direct role of adult neurogenesis in hippocampal function, we carried out experiments investigating the role of TBI-induced neurogenesis in cognitive recovery following TBI using the mitotic inhibitor Ara-C.

Results summary

In this study, rats were subjected to a moderate LFPI followed by infusion of Ara-C with or without bFGF into the lateral ventricle for a 7-day period. Stereological analysis of BrdU-labeled dividing cells at seven days post-injury demonstrated that LFPI significantly increased levels of cell proliferation in the dentate gyrus of the hippocampus in both the ipsilateral and contralateral GZ and the ipsilateral hilus regions. Infusion of Ara-C significantly reduced the injury-induced cell proliferation within the dentate gyrus of injured animals to the sham level. Ara-C infusion also abolished bFGF-enhanced cell proliferation in the dentate gyrus. Infusion of Ara-C prior to LFPI did not significantly inhibit injury-induced proliferation, although animals with Ara-C pre-treatment did show decreased BrdU staining compared to vehicle-treated animals. To determine whether

inhibition of injury-induced cell proliferation in the dentate gyrus is associated with spontaneous cognitive recovery following injury, we evaluated the cognitive function in animals at 21-24 or 56-60 days post-injury using the MWM. We found that inhibition of injury-induced cell proliferation does not significantly affect the performance of animals in MWM tests at 21-24 days post-injury, a period in which injury-induced cognitive deficits were present. However, inhibition of injury-induced cell proliferation in the dentate gyrus abolished the innate cognitive recovery at 56-60 days post-injury, the time when the recovery was observed in injured vehicle-treated animals. Furthermore, inhibition of TBI and bFGF-enhanced cell proliferation completely eliminated bFGF-improved cognitive recovery in injured animals 21-24 days post-injury. Collectively, the results from the current study suggest that injury and bFGF-enhanced cell proliferation in the neurogenic region of the hippocampus is directly associated with innate cognitive recovery following TBI.

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

In this study, we found that TBI significantly increased cellular proliferation in the dentate gyrus of the hippocampus at seven days post-injury in vehicle-treated groups using BrdU labeling. This effect was shown to be significant in both the ipsilateral and contralateral GZ and the ipsilateral hilus. These results are consistent with previous studies in this laboratory and others (Chirumamilla et al., 2002; Sun et al., 2009; Sun et al., 2010; Dash et al., 2001; Emery et al., 2005; Zheng et al., 2011). We anticipated these findings, as they have been replicated in a number of previous studies using the LFPI model of TBI.

Infusion of Ara-C following TBI significantly reduces TBI-induced cell proliferation in

the DG of the hippocampus

The mitotic inhibitor Ara-C may serve as a powerful tool in understanding the cell proliferative response following TBI by suppressing this injury-induced proliferation. A structural analog of deoxycytidine, it functions as a pyrimidine antimetabolite that prevents proliferation by inhibiting the S-phase of the cell cycle (Baker et al., 1991). Once within a cell, it is converted to Ara-CTP and then inhibits DNA synthesis by inhibiting DNA polymerases and terminating DNA chain elongation (Hamada et al., 2002). It has been widely shown to inhibit neurogenesis in vivo (Breton-Provencher et al., 2009; Li et al., 2010; Yau et al., 2011, Zhang et al., 2012). Intraventricular infusion was the chosen method of delivery because Ara-C is taken up by CSF and is known to affect cell populations in contact with CSF and the dentate gyrus is in close proximity to the ventricles. We found that intraventricular administration of Ara-C significantly reduced the cell proliferation observed in response to TBI when compared with vehicle-treated animals. Other studies have previously shown that Ara-C abolishes injury-induced proliferation, and this anti-mitotic effect within the brain has been demonstrated repeatedly in a number of studies, from models of ischemia (Li et al., 2010; Im et al., 2010), to models of electrolytic lesions in ringdoves (Chen & Cheng, 2007) and in CCI models in the rat (Zhang et al., 2012). Proliferation in these studies has not only been blocked within the dentate gyrus, but also in the hypothalamus in ring doves, growth factor-promoted neurogenesis in the neostriatum, and the SVZ. Ara-C is also shown to undo the positive effects of exercise on hippocampal neurogenesis and the restoration of spatial learning capacities in animal models of stress (Yau et al., 2011).

In this study, Ara-C did not significantly reduce hippocampal proliferation in sham animals. Ara-C affects cell populations that are rapidly dividing more than populations that are dividing more latently. Accordingly, the rapid and robust proliferative response would be more severely impacted by the infusion of the drug when compared to basal levels of proliferation. One drawback to using an anti-mitotic inhibitor over irradiation is that while Ara-C significantly reduces cell proliferation in the dentate gyrus, irradiation comes closer to depleting it, with levels of reduction up to 90% in irradiated mice (Rola et al., 2004). Therefore, the less powerful form of ablation used here may have not had the strength to significantly diminish levels of proliferating cells in sham animals.

Additionally, Ara-C has been shown to specifically target proliferating populations of cells. Previous studies of the effect of Ara-C on adult neurogenesis have shown that while Ara-C functions as a mitotic inhibitor, it does not affect existing synapses or the neurites of mature neurons within neurogenic regions (Breton-Provencher et al., 2009). It also does not appear to impact membrane properties of these cells, suggesting that Ara-C suppresses neurogenesis but does not influence the function of these networks by altering existing functional neurocircuitry.

Intraventricular infusion of Ara-C does not have a significant continued impact on TBI-induced cellular proliferation following cannula removal

As this study is focused on blocking injury-induced neurogenesis, it is critical to validate that Ara-C is effective at the time points surrounding the injury-induced proliferative response, which peaks at 2 days post-injury and lasts for about 7 days (Sun et al., 2005). In order to verify that the Ara-C infusion does not continue to block

neurogenesis once the osmotic mini-pump and cannula have been removed, we included a group of animals that received Ara-C treatment for 7 days prior to injury. If the prior treatment had similar effects on proliferation as Ara-C treatment beginning immediately after injury, we would conclude that the drug was continuing to block proliferation. We found that animals pre-treated with Ara-C showed reduced BrdU-staining in the dentate gyrus compared to vehicle-treated animals, however this did not prove to be significant within any of the individual regions. While there is some effect of Ara-C pretreatment on injury-induced proliferation, Ara-C exerts the strongest antimitotic influences on proliferating cells at time points when it is administered via the cannula.

One reason why Ara-C was chosen as the antimitotic agent for this study was for its rapid half-life. The plasma concentration of Ara-C declines with a biphasic disappearance curve, with the initial half-life being 10-15 minutes and a second, slower phase of about 2-3 hours. Within the CNS, its half life is about 2-4 hours (Hamada et al., 2002). It is rapidly deaminated into the inactive metabolite, uracil arabinoside (Ara-U), by cytidine deaminase (Baker et al., 1991). Within 24 hours, about 80% of administered Ara-C is excreted in the urine as Ara-U. Cytidine deaminase is ubiquitous in the intestine, liver, and kidney, but absent within the CNS (Baker et al., 1991; Hamada et al., 2002). It is possible that for this reason, Ara-C has a longer presence within the CNS and was affecting proliferation at time points shortly after cannula removal when cells within neurogenic regions initially began their proliferative response to the LFPI. Irradiation, on the other hand, is shown to produce 70% reductions in cell proliferation at 1 and 3 months post-irradiation, a significant continued effect.

Increased BrdU staining surrounding the dentate gyrus in Ara-C-treated animals

One observation that was not quantified within this study and requires further investigation was the increased levels of BrdU-labeled cells surrounding the dentate gyrus in Ara-C-treated animals. This increase indicates that Ara-C is affecting the physiology of the rats beyond the scope of suppressing neurogenesis within the dentate gyrus.

There are a number of potential explanations for this increased staining. Firstly, as a thymidine analog, BrdU-labeling indicates both proliferating cell populations and cells undergoing DNA repair. It is possible that the Ara-C treatment may be damaging cells, thus cells outside the dentate gyrus are being labeled with BrdU as they undergo DNA repair. Another possibility is that there is an increased number of microglia and monocytes being recruited following Ara-C treatment. To further probe this question, it is essential to carry out double labeling to learn more about the identity of these cells. Ara-C was chosen to suppress cell proliferation in this study because other methods such as irradiation have been shown to not only deplete neurogenic populations, but also elicit strong inflammatory responses in the brain (Rola et al., 2004). However, Ara-C has been also shown to produce negative systemic effects at high dosages, stemming partially from a reactive proliferation of glial cells and astrocytes within the cerebellum (Baker et al., 1991). In this study, a lower dosage was used because it showed significantly diminished proliferation in the dentate gyrus, without ataxia and other signs of adverse effects of Ara-C systemically. Animals were monitored for weight loss and fur loss, and swim speeds were calculated in the MWM to ensure that Ara-C animals did not show changes in measures of health compared to vehicle-treated animals. While these observations are

not reported here, the animals receiving Ara-C were comparable to vehicle animals in these evaluations.

LFPI followed by Ara-C treatment reduces cell proliferation at 62 days post-injury in the ipsilateral GZ of the hippocampus

The cell-division marker Ki67 was used to test whether proliferation would be affected by TBI or Ara-C treatment at more distant time points post-TBI. Our results revealed that the Ara-C treatment and TBI together reduced cell proliferation in the ipsilateral GZ of the hippocampus. This effect was not seen in any other region or in any other experimental group. One possible explanation for this data is the notably high variance due to the small number of animals used within the study. The standard error in the sham animals receiving vehicle treatment was especially high. This high degree of variance may have been caused by human error in stereological quantification or immunohistochemistry procedures. To reduce the variance, more animals should be incorporated into each group.

Ara-C was employed in this study to suppress cell proliferation because irradiation had previously been shown to lead to a persistent decrease in the production of new neurons (Rola et al., 2004) and this study was aimed at understanding the contribution of injury-induced proliferation alone to cognitive recovery. A continued blockade of proliferation would not clearly demonstrate the role played by this specific population of cells. While the results from this study do not provide a clear answer as to whether Ara-C continues to affect cell proliferation at later time points, the addition of more animals to the study may provide more evidence as to its long-term effects.

Inhibition of injury/bFGF-induced cell proliferation with Ara-C impairs cognitive

recovery in rats following TBI

This study monitored cognitive recovery following TBI using the MWM at two time points: 21-25 and 56-60 days post-injury. We found that compared with sham animals, animals receiving TBI and either Ara-C or vehicle treatment showed longer goal latency performance 21-24 days post-injury. The injured groups also showed impaired short-term memory performance at the 24-hour probe trial test in measures of both average proximity to the goal platform and time spent in the goal quadrant. We anticipated these results, as a number of studies suggest that TBI impairs an animal's ability to perform spatial learning tasks at this time point (Hamm, 2001; Sun et al., 2009). Additionally, we found that Ara-C abolished the positive effect of bFGF on functional recovery post-TBI. We expected these results as well, because previous studies in this laboratory show that bFGF promotes both functional recovery at these time points and cell proliferation among dentate granular neurons (Sun et al., 2009). Ara-C has been shown in other laboratories to ablate populations of new neurons within the dentate gyrus (Yau et al., 2011; Zhang et al., 2012) as well as abolish innate cognitive recovery following injury (Im et al., 2010; Li et al., 2010). We hypothesized that these two agents would inversely affect mitotic activity within the same cell populations and thus counteract each other on both a cellular and behavioral level. In other studies, agents that produce both increased TBI-induced hippocampal proliferation and positive effects on cognitive recovery are inhibited in both these respects following Ara-C treatment (Zhang et al., 2012). The precise physiological basis for these effects is still uncertain and our results pose additional questions about the mechanism behind TBI-induced cell proliferation.

The exact role of adult-born neurons in learning and memory is still unknown. One hypothesis is that newly generated neurons are recruited into existing circuits and thus provide more opportunity for future learning (Kempermann, 2002). It is also possible that they contribute to these processes before they are fully mature, as newly born cells demonstrate enhanced characteristics of synaptic plasticity that may make them adept for processing new associations. Young granule cells show a lower induction threshold for LTP, which is also insensitive to GABAergic inhibition (Snyder et al., 2001; Leuner et al., 2005). As newly born neurons, they may be responsible for detection or processing of novel stimuli, a function often ascribed to the hippocampus (Kempermann, 2002; Lemaire et al., 1999). Another possible role for new neurons is the temporary storage of information. As a rapidly changing population of cells, they may be especially suited for maintaining memories for a short period of time (Gould et al., 1999). Ultimately, the details of the physiological processes that underlie the contribution of newly born cells to cognitive recovery remains unknown.

Importantly, we observed that Ara-C-treated animals with TBI performed significantly worse than vehicle-treated injured animals on the MWM at 56-60 days post-injury. At this time point, the vehicle-treated injured animals carried out the water maze with latencies similar to uninjured animals. These findings are supported by other studies, which demonstrate Ara-C treatment has negative effects on behavioral recovery following brain insult (Chen & Cheng, 2007; Im et al., 2010; Li et al., 2010). Ara-C has been further demonstrated to significantly impair performance on the MWM task following TBI and administration of a therapy that has been shown to improve spatial memory performance (Zhang et al., 2011).

These findings suggest that Ara-C does indeed block spontaneous functional recovery from TBI as a result of its anti-mitotic effects as an inhibitor of DNA ligases and polymerases. This interpretation is supported by studies that show that the cell populations affected by Ara-C would have reached functional maturity by this time point. Investigations of the neural progenitor cells within the dentate gyrus targeted by Ara-C show that these cells begin to show mature axons and dendrites around 3-4 weeks after their birth (Hastings & Gould, 1999; Esposito et al., 2005). These cells are only able to generate action potentials in response to excitatory stimulation at 24-28 days old (Mongiat & Schneider, 2011). Retrograde tracing with Fluorogold at 56 days post-injury reveals anatomical integration of 30% of surviving neurons and double-labeling with BrdU and synaptophysin shows that some of these cells receive synaptic input at this time point (Sun et al., 2007). Taken together, the behavioral differences recorded between Ara-C- and vehicle-treated TBI animals at different time points and their correlation to evidence of anatomical integration, support the role of these cells in the functional recovery of spatial memory post-TBI.

Conclusions and future directions

This study demonstrated multiple mitigating effects of Ara-C on cell proliferation and cognitive recovery following TBI. It also showed an aversive effect of Ara-C on bFGF-promoted cognitive recovery. Therefore, these findings have a number of important potential therapeutic implications. Further investigation is needed to yield a more complete understanding of the direct role that neurogenesis plays in cognitive recovery and how bFGF improves the restoration of hippocampal function by leading to amplified levels of neurogenesis.

Adult neurogenesis is shown to be critical to hippocampal function. Accumulating evidence implicates post-TBI cell proliferation in restoring hippocampal-mediated cognitive capacities (Sun et al., 2009; Li et al., 2010; Blaiss et al., 2011). In a number of different injury models, inhibition of cell proliferation by transgenic methods, irradiation, and anti-mitotic administration all reduce behavioral recovery post-insult. Ara-C is widely shown to block hippocampal cell proliferation and disrupt performance on tasks requiring hippocampus (Wang et al., 2003; Snyder et al., 2005; Jessberger et al., 2009). Our results add to this body of evidence, implicating the new neurons born post-injury, with or without bFGF enhancement, in cognitive function.

Our findings that Ara-C blocks this effect of bFGF are in accord with what is currently known about this growth factor. This laboratory has previously shown that bFGF augments injury-induced neurogenesis in the dentate gyrus and improves performance on spatial learning tasks after TBI (Sun et al., 2009). Basic FGF is a well-known mitogen for both neuronal and non-neuronal cells, stimulating cell division *in vitro* and *in vivo*. It provides extracellular signals that help determine proliferation and maturation of stem and progenitor cells of the CNS (Calof, 1995). *In vitro* it regulates the differentiation and mitotic activity of neuroblasts and glial cells and promotes the survival and proliferation of neural stem cells (Gritti et al., 1996). *In vivo* promotes cell proliferation among neural progenitor cells in the SVZ (Kuhn et al., 1997). Basic FGF increases transiently in neurons and astrocytes in animal models of ischemia, which yield cell proliferation in the CNS (Kumon et al., 1993; Yoshimura et al., 2001). In this study, we showed further that the bFGF enhanced cognitive performance on the MWM was

abolished using an anti-mitotic agent, supporting its role in neurogenesis rather than neuroprotection in restoring cognitive function following TBI.

While the findings presented within this study strongly support the direct role of TBI/bFGF-promoted neurogenesis in the spontaneous functional recovery of spatial learning, it is incomplete for a number of reasons. Firstly, the cell types that are affected by Ara-C are yet to be described. This study only shows that cell proliferation in this region is blocked by Ara-C and that this treatment also reduces the animals' ability to perform spatial learning tasks at two time points post-injury. The specific types of cell populations that are targeted by intraventricular Ara-C infusion are not shown here. However, a great deal of evidence from other studies indicates that neural progenitor cells are indeed those that are ablated by Ara-C treatment (Im et al., 2010; Yau et al., 2011). In one study, Ara-C treatment after CCI significantly reduced the number of cells double-labeled with BrdU and the mature neuronal marker NeuN 35 days after injury (Zhang et al., 2012). They also observed significantly reduced numbers of GFAP-labeled astrocytes and CD68-labeled microglia and macrophages.

We also have yet to look at cell survival of BrdU-positive cells in animals sacrificed at 62 and 74 days post-injury. This information could be useful in determining the effect of Ara-C and Ara-C/bFGF on long-term cell survival and differentiation and would give further information regarding the behavioral results observed in the study. Thirdly, we have observed many more BrdU+ cells in regions outside the SGZ in the Ara-C. It seems that Ara-C induces glial response, and the type of glial cells needs to be identified.

Injury-induced neurogenesis provides a promising avenue for researchers seeking to develop new therapies for TBI patients. This study contributes to the understanding of the direct role of cell proliferation in cognitive recovery post-TBI and the data presented here will help to inform future research in this field.

Vita

Teresa Daniels was born in Alexandria, Virginia, on October 20, 1985. She graduated from Oakton High School in Oakton, Virginia. She is an alumnus of the University of Virginia, where she graduated magna cum laude receiving a Bachelor of Arts in May of 2008. She received a post-baccalaureate research fellowship at the National Institutes of Health in August 2008 and carried out research in the Laboratory of Neuropsychology until August 2010. She attended VCU in Richmond, Virginia to continue her graduate education in the Department of Anatomy and Neurobiology. In August 2012, she will be matriculating to the MD program at the VCU School of Medicine as a member of the Class of 2016.

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