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LONG-TERM EFFECTS OF NOTCH1 SIGNALING ON REGENERATIVE NEURAL STEM CELLS IN RESPONSE TO TRAUMATIC BRAIN INJURY

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

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Abbreviations

aCSF – Artificial Cerebrospinal Fluid

ABC – Avidin-Biotin Complex

BrdU – 5-bromo-2-deoxyuridine

CCI – Controlled Cortical Impact

CNS – Central Nervous System

CT – Contralateral

DAB – 3,3’-Diaminobenzadine-tetra-hydrochloride

DAI – Diffuse Axonal Injury

DG – Dentate Gyrus

DCX – Doublecortin

DPI – Days-Post-Injury

GCL – Granular Cell Layer

GZ – Granular Zone

IP - Ipsilateral

LFPI – Lateral Fluid Percussion Injury

MOL – Molecular Layer

NSC – Neural Stem Cell

NICD – Notch Intracellular Domain

NPC – Neural Progenitor Cell

PBS – Phosphate-buffered Saline

SGZ – Sub-Granular Zone

SSC – Sodium Citrate Buffer

SVZ – Subventricular Zone

TBI – Traumatic Brain Injury

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Abstract

LONG-TERM EFFECTS OF NOTCH1 SIGNALING ON REGENERATIVE NEURAL STEM CELLS IN RESPONSE TO TRAUMATIC BRAIN INJURY

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Virginia Commonwealth University, 2019

Advisor: Dong Sun, MD, PhD

Traumatic brain injury (TBI) is a devastating problem which stands as a leading cause of death and disability. The elderly is significantly affected by TBI, typically as the result of falls, and recovery is especially limited. This, in part, is associated with decreased tissue-specific stem cell regeneration and replacement of damaged cells in the aged brain. The diminished ability of the aged brain to recover is especially devastating after TBI, likely leading to permanent loss of sensory, motor, and cognitive functions. Studies have shown that the mature mammalian brain contains Neural Stem Cells (NSCs), found in specific regions of the brain, which can generate functional neurons during normal and pathological conditions. Two of those regions, the Dentate Gyrus (DG) of the hippocampus as well as the Subventricular Zone (SVZ) of the lateral ventricles, have proven to be niches for these multipotent NSCs. A key regulator in the

maintenance of these NSCs is the Notch signaling pathway, shown to control proliferation, differentiation, and apoptosis of NSCs during development and throughout adulthood. In the current study, we assessed the regulatory mechanisms that drive the regenerative functions of NSCs in a neuropathological state following TBI. Using the Lateral Fluid Percussion Injury model, we analyzed the diffuse effects of the injury response on 3-month old male Sprague-Dawley rats. Immediately following TBI, Notch agonist, antagonist or vehicle was infused into the lateral ventricle for 7 days to assess the role of Notch signaling on neural stem cell proliferation/survival and neurogenesis at 30 days post-TBI. Dividing cells during infusion time were labeled with BrdU via single daily intraperitoneal injections for 7 days. Animals were sacrificed at 30 days post-injury and brain tissues were processed then immunolabeling for BrdU and Doublecortin. We found a higher number of BrdU-positive cells in the FPI+Notch1 agonist group when compared to Sham and FPI+Jagged-1 Fc antagonist groups in the contralateral granular zone. A significant increase in proliferation/survival was also seen in FPI+Notch1 versus Sham/FPI+Jagged-1 Fc and for FPI+Vehicle versus Sham animals in both the ipsilateral and contralateral hilus. DCX immunolabeling did not establish a significant difference in FPI+Notch1 compared to Sham animals, nor across any other groups, which is consistent with what we know of activation of the Notch pathway. Our results demonstrate that Notch1 signaling is directly involved in cellular proliferation/survival of NSCs in the DG following TBI at 30 days post-injury, but further work must be done to understand the fate of these cells. Thus, drug treatment targeting Notch1 signaling could serve as a potential therapeutic target following TBI to preserve NSCs and limit long-term cognitive deficits.

Chapter 1 – Introduction

Traumatic Brain Injury – Epidemiology

Traumatic brain injury (TBI) is a devastating problem that stands as a leading cause of death and disability. The World Health Organization recognizes neurotrauma as a critical health problem often leading to permanent health consequences. Due to the variety of ways it can occur, neurotrauma stands as a difficult health issue to address. Within the last decade, there has been a considerable shift in focus on TBI, in part due to advancements in technology capable of identifying this problem. Vehicle accidents, military combat, accidents at home, and helmet-to-helmet contact as seen in the NFL have helped draw warranted attention to assess ways to prevent and respond to neurotrauma.[1,2] In addition, research is actively highlighting differences in the types of TBI, either acute or chronic in nature, to better understand how to deal with specific instances of injury.[2] Specifically, analysis of different biomarkers often seen in the normal inflammatory process has helped to aid in diagnosis and prognosis of TBI.

In addition to the breadth of examples that lead to neurotrauma, there is as wide a spectrum of ages of victims who are at risk or have been reported to suffer from this type of injury. [3–5] Children, adults, and the elderly are all susceptible to lasting physical and intellectual impairments caused by neurotrauma. Pediatric TBI is considered a chronic condition due to long-term cognitive and psychosocial deficits suffers, as survivors of injury get older. [4] Adults, and more specifically athletes, have received highlighted attention to the effects of concussions. One study of former professional athletes demonstrated that the number of sports-related concussions served as a predictor for a number of common mental disorders,

such as anxiety, depression, and sleep disturbances, [6] while others have compared post-mortem, concussed brains to those of Alzheimer's patients. [7] If a patient is fortunate to survive from TBI, they will likely endure large economic costs as a result of the necessary long-term care.[1] The elderly is not only extremely susceptible to TBI, but can suffer worse consequences. A study comparing different aged group mortality rates 6 months post-injury demonstrated that the group of 64-year-olds and older had more than twice the mortality rate of the younger group, aged 20 to 40-years-old. This was in spite of the younger group having a more severe mechanism of injury than the older group.[3] Other studies have confirmed and furthered the correlation of increasing age and increasing mortality rates [8,9] in the elderly and greater functional decline when compared against younger groups. [10] Within the United States, the number of people age 65 or older was roughly 48 million in 2015, making up 15% of the total population, according to the World Health Organization. By the year 2060, that population is expected to more than double to 98.2 million individuals. People are expected to live longer thanks to advancements in modern medicine; however, implications of an aging body, such as pre-existing health conditions, make understanding neurotrauma all the more imperative. [11]

As studies begin to dissect the varying degrees of neurotrauma, more can be understood about how it works and how to treat it. Analysis of different pro-/anti-inflammatory agents across the Central and Peripheral Nervous system [2] demonstrate the range of neuroinflammatory responses that are triggered by TBI. Study of specific genes, such as brain-derived neurotrophic factor (BDNF), [12] highlight the importance of understanding genetic factors and their products that play a role in TBI-related issues.

Our lab has spent the last few years analyzing different aspects of neurotrauma. With a primary focus on TBI, we discovered valuable information about how the brain responds to injury. [13,14] The work we have done, as well as work completed by others, primarily focus on animal models to study these injuries. Short-term consequences of TBI have illuminated mechanisms involved; however, questions about the long-term effects remain.

Biomechanics and Mechanisms of TBI

Traumatic brain injury occurs as the result of an external force acting and altering brain function. [15] Instances of TBI are plentiful; as are signs required for classification: loss of consciousness or memory, disorientation, or loss of balance to name a few. Physical injury fundamentally shifts the normal mechanisms of the brain to varying degrees, depending on the type and severity of injury. In the realm of TBI, damage incurred leads to both primary and secondary injuries. Primary injury is the injury sustained during the traumatic event, often as the result of falls or mechanical insults. Damage resulting from primary injury includes neuronal cell death, subdural and epidural hematomas, and vascular damage.[16] Secondary injury damage can accrue from hours to days after the initial injury and is characterized by metabolic and cellular changes in the brain. [15]

Intracranial damage has been classified in two separate categories: diffuse and focal. As the name implies, diffuse injury to the brain is frequently widespread. Diffuse axonal injury (DAI), is a common example of brain injury that results in damage to white matter in the brain. Focal injuries, in general, involve a pin-pointed focus on certain regions of the brain. These

injury types have been broken down to further clarify and understand the post-traumatic processes following TBI. For example, a proteomic study differentiating the two types of injury in human brain tissue showed increase expression of peptides related to neurodegeneration and decreased expression related to antioxidant defense in diffuse TBI patients versus those suffering focal injuries. [17] This point exemplifies the complexity associated with TBI and why a thorough understanding of the mechanics is so important.

Differences in injury types for TBI have been demonstrated experimentally in rodents to better understand ways TBI can be treated.[18,19] For example, many studies, including those done by our lab, utilize fluid percussion injury (FPI) models to replicate clinical TBI without fracturing the skull.[13,20] Lateral FPI model results in damage directly at the point of fluid impact and diffuse injuries in brain regions far from the impact, whereas midline FPI induces more diffuse injury As its severity increases, this type of FPI can lead to changes in behavior. [21] Other models, such as controlled cortical impact (CCI) [22] and acceleration-impact injury models causing a focal injury are widely used models as well. [23] The overlap between rodent and human injury mechanisms makes it useful for the potential development of therapeutic methods for TBI. It is our hope that deeper understanding of these processes will lead to better long-term care for individuals with TBI.

TBI: Long-Term Consequences

According to the CDC, over 2 million people are thought to be affected each year by TBI.[24] When the brain is physically damaged, the results are not only long-lasting and complex,

but costly as well. Estimates of lifetime costs for those suffering TBI span in the millions of dollars for victims. [1] Besides the immediate damage that results, patients are also subjected to secondary neurological issues such as personality disorders [25], and chronic pain and headaches.[24] Some studies have demonstrated a connection to patients developing psychosis and schizophrenia [26], while others have even linked the offspring of parents who have suffered a TBI to more frequently utilize psychiatric care facilities. [27] Another commonly associated issue following TBI is major depression in patients. [28]

Adult Neurogenesis

The idea of "adult neurogenesis" was previously thought to be somewhat of an oxymoron until the mid-1960's, when a duo of scientists uncovered a proliferative region of the rat brain: the hippocampus; although, Joseph Altman is recognized as the first to discover the neurogenic potential of cells within the hippocampus.[29] He posited that postnatally, there exists an "undifferentiated" pool of cells that gradually declines in number, resulting in a corresponding increase in differentiated granule cells. This idea sparked so much of what we understand about neurogenesis today. Mammalian studies centering around neurogenesis have solidified the idea first posited by half-a-century ago that a pool of multipotent stem cells exists after development, lasting through adult life.[30,31] In humans, studies done in postmortem tissue determined the existence of stem cells within the same regions identified in rodent studies.[32] Of course, humans and rat development and maturation processes are not exactly equal, but have been reviewed and compared showing enough similarity to demonstrate the

value in understanding the mechanisms of the latter to gain insight on the former. [33] Due to restrictions and ethical issues in studying developing human tissue, rat and mouse models are used to investigate the mechanisms that make up this process. Scientists are actively comparing developmental neurogenesis and that seen throughout adult life. [34]

Neural Stem Cells (NSCs) are a recently uncovered tool of significant importance in brain development, maturation, and recovery. Residing within specific regions of the brain, the NSCs are a class of self-renewing cells with the capacity to generate new neurons and other cell types within the nervous system. These regions, often referred to as the neurogenic niches of the brain, are capable of sustaining proliferation and differentiation from the preexisting pool of stem cells. Today, it is well understood that those two regions include the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. [13,29,30,35–37] Stem cells from each specific region have their own capacity to differentiate. For example, *in vivo* progenitor cells that come from the SVZ of the lateral ventricles have a specific path of migration towards the olfactory bulb. These cells travel along what is known as the rostral migratory stream (RMS) and will potentially become mature olfactory neurons.[30] Studies have shown cells of the SVZ will also become olfactory interneurons vital for olfactory discrimination, sensitivity, memory and specificity.[38] In all, these cells relay sensory information to the olfactory cortex and serve a vital role in odor processing.[38,39] Structurally, rodents have a relatively larger volume of the olfactory bulb in comparison to humans, which has made analysis of the SVZ both common and useful.

In both rodents and humans, the stem cells residing within DG of the hippocampus play a different role than those in the SVZ. Studies have demonstrated the NSCs of the DG to have a

significant role in learning, memory, and cognitive function. [36,40] Cells residing within the DG can become excitatory granule cells whose axons form the mossy fiber tract.[40] This tract connects the DG to the CA3 region of the hippocampus. The dentate gyrus **(Figure 1.1)** has been broken down further into subregions, including the molecular layer (MOL), the granular cell layer (GCL), and the hilus or polymorphic layer. The granular layer, often referred to as the granular zone, consists of a subregion called the subgranular zone (SGZ) which is now known to be the site of origin of stem cells. Cells within the SGZ can travel through the granular cell layer toward the molecular layer as they differentiate. [41] While the final location differs for stem cells within the different neurogenic niches, both processes have been determined to be controlled in part by molecules in the extracellular matrix and ligands found on other cells.[42]

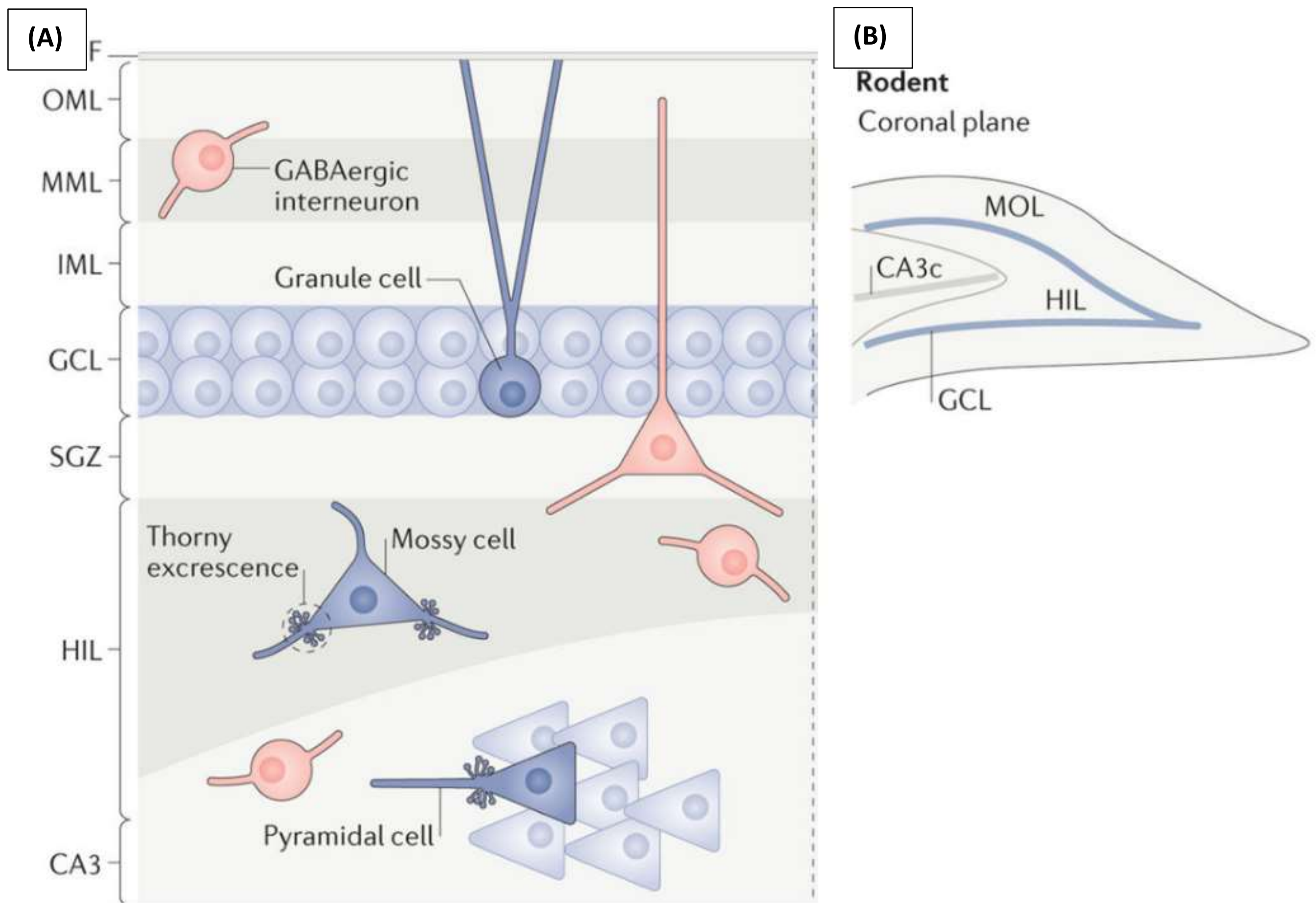


Figure 1.1 The Dentate Gyrus of the Hippocampus. Image extracted from Scharfman 2016. **(A)** Depicts a breakdown of the layers of the dentate gyrus DG of the hippocampus. The molecular layer, the granular layer, and the hilus are all pictured. **(B)** Demonstrates a coronal section of the same DG structures.

Stem cells progress through different phases **(Figure 1.2)** as they mature. In general, NSCs will differentiate to neural progenitor cells (NPCs) before becoming mature neurons. A thorough background of adult hippocampal neurogenesis has been established, exposing distinct phases and corresponding cell types.[35] These phases are triggered by specific signals and as the cells progress through the stages and can be classified by differences in morphology. The first stage of neurogenesis consists of Neural Stem Cells (NSCs), which differentiate into Type 1 Neural Progenitor Cells (NPCs). The latter cell type then differentiates into a Type 2 NPC before differentiating further into a Neuroblast. [34]

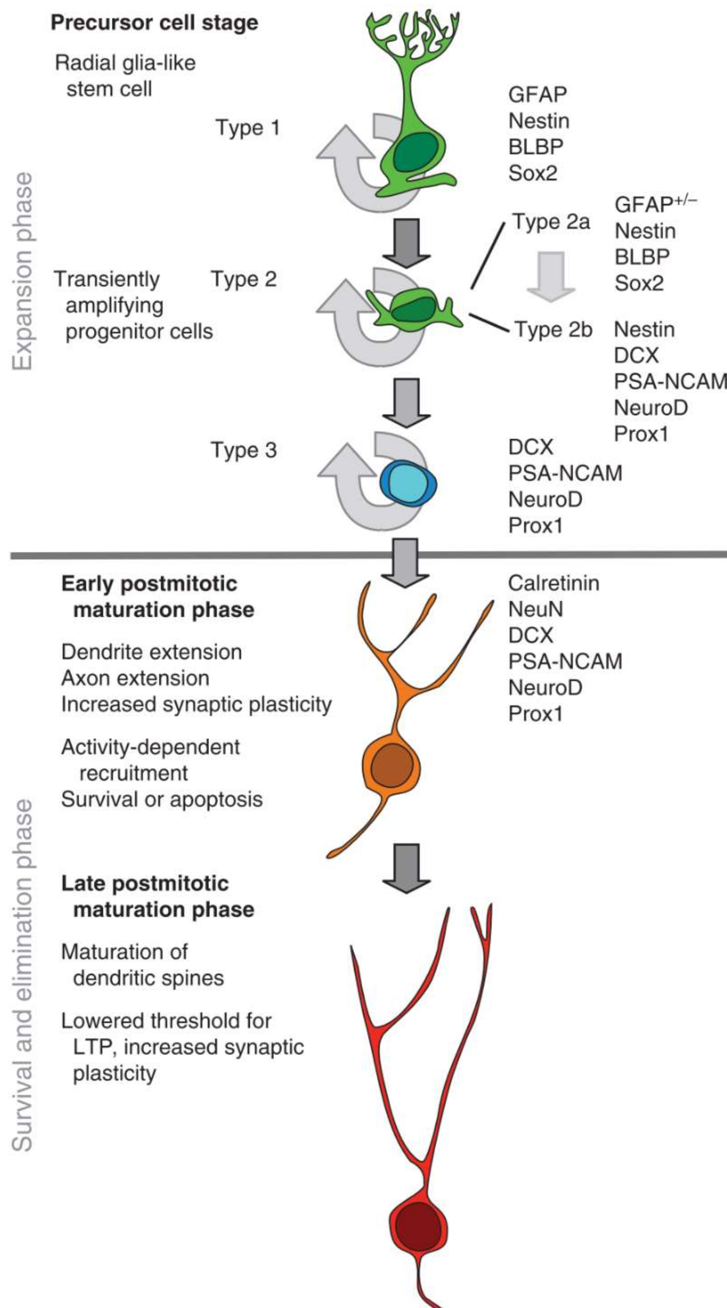


Figure 1.2 Differentiation of NSC's in the DG. Image extracted from Kempermann et al., 2015. This visual represents the progression of neurogenesis through various phases, from NPC to a mature neuron. Additionally, stage-specific markers are noted on the right.

While studies have demonstrated differences within the makeup of young/mature NSCs, the overarching view of adult neurogenesis has been one of a continuation of development.[34] This has made the understanding and investigation of the developmental process key to providing insight on how the adult brain functions. Neural stem cells are also known to be an active component of regenerative processes, as well as homeostatic conditions. Character traits of stem cells have allowed for manipulation of growth to better understand the proliferation process. One study identified a certain type of stem cell within the SVZ to contain epidermal growth factor receptor (EGFR). When presented with EGF, this receptor signals for proliferation of the NSC. [43] Knowledge of the intrinsic properties of stem cells has allowed for comparisons in other drug treatment studies, such as these experiments. Although there is an overlap in the developmental process and adult neurogenesis, there exists a difference in the responsiveness of tissue that is aged dependent. Dr. Sun et. al, completed work that demonstrated specifically how aged tissue is worse at recovering from TBI than its younger counterpart. Specifically, we demonstrated that aged tissue has a higher apoptotic response and reduced levels of proliferation within the DG of the hippocampus. [14,44] There exists an age-related decline of a tissue's capacity, which is due to NSC's gradual decline in potency. Further, there is an age-related impairment of hippocampal cognition found in both humans and primates.[33] Interestingly, the result of reduced neurogenesis in aging tissue does not occur from loss of NSCs, but rather a transition of the stem cells from a potent-to-quiescent state.[45] This was demonstrated via a correlation between a loss of active horizontal NSCs in aged mice, rather than a decrease in quantity of the observed NSCs. Establishing an understanding of how aging

tissue behaves can further aid in the process of treating both the elderly population, as well as those who suffer neurotrauma.

Notch Signaling Pathway

Current research has begun to unveil the mechanisms in which NSCs function. The Notch Pathway has been implicated as a key regulator in neurogenesis. [46–48] Our lab and others have begun mapping the downstream effects of Notch activation and demonstrating its role on for NSCs. Notch is thought to be critical for NSC maintenance and differentiation as well as learning and memory.[49] Serving as a regulator for the timing of motor neuron genesis as well as maintaining the progenitors in a proliferative state under homeostatic conditions[50], the role and importance of Notch is likely to carry over into recovery following neurotrauma.

The pathway is a simple, well preserved process which is associated with other well studied regulatory mechanisms in tissues that undergo neurogenesis, hematopoiesis, and more.[51,52] Through interactions with exposed ligands, such as Jagged 1/2 and Delta-like ligands 1/3/4, from neighboring cells, the Notch receptor itself is cleaved via gamma-secretase, resulting in the release of the Notch intracellular domain (NICD) **Figure 1.3**. This portion will then translocate into the nucleus and interacts with a class of proteins in humans known as CSL (CBF1, Suppressor of Hairless, and Lag-1). These proteins act as transcriptional activators, producing a series of products dependent on the type of Notch receptor activated. Although there are several different Notch receptors which have been discovered, it appears that Notch 2/3 have similar, repetitive functions.[53] Commonly seen products are Hes1/Hes5, or hairy and enhancer of split family.[52] *Hes* genes have been demonstrated to be important for

transcriptional repression in one study of a loss-of-function hematopoietic stem cell line.[52] In the nervous system, experiments have shown that Hes is vital for maintaining the pool of neural stem cells by inhibiting neuronal transcriptional effectors (Neurogenin/Mash1).[48] A knockout study done in mice demonstrated that upregulation of Hes1 resulted in differentiation of NPCs, while its downregulation caused the opposite effect. [54] In a simplified sense, Notch signaling products act on the existing pools of NSCs to prevent differentiation and thereby maintaining them in their proliferative state. Current studies have helped understand the role of Notch on NSCs during development and adult maturation. What remains unclear, however, is the extent that Notch plays in recovery following TBI.

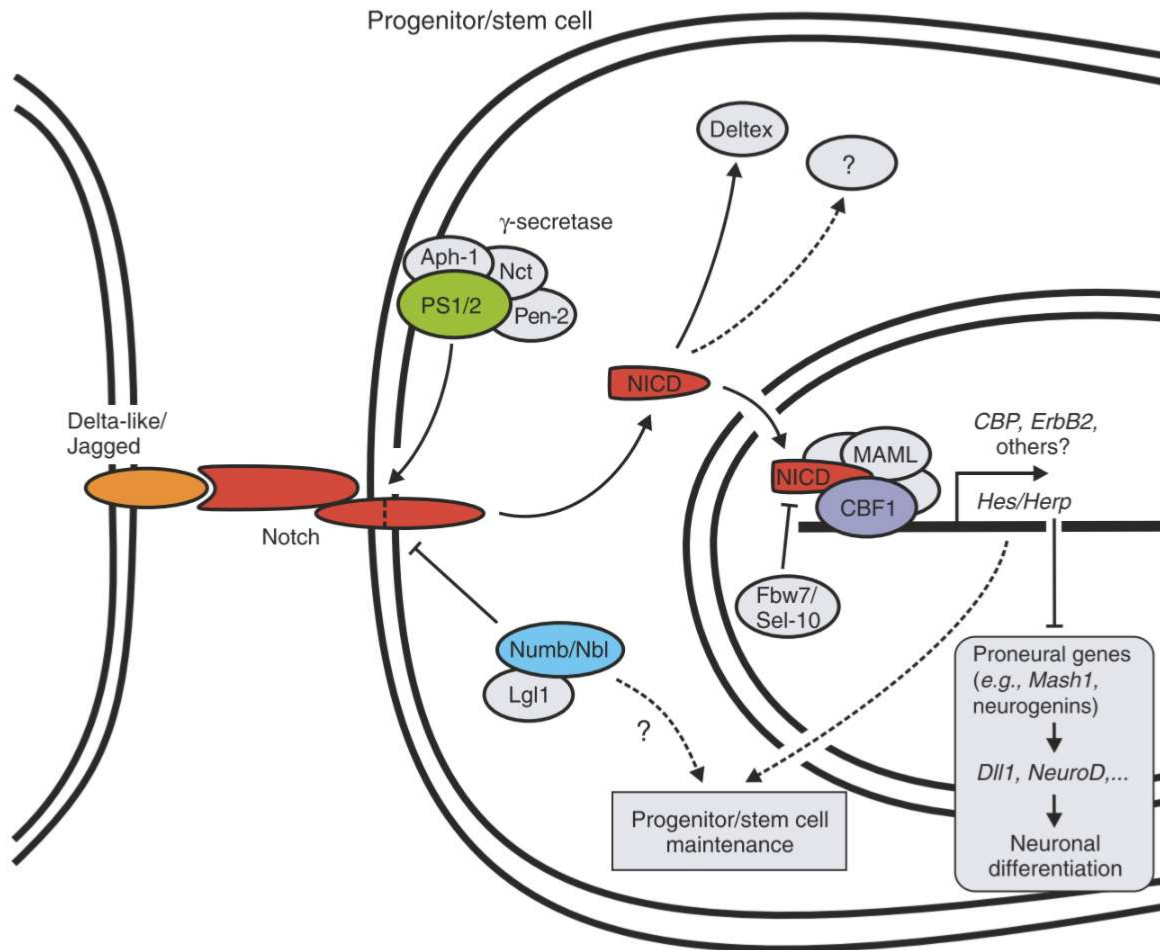


Figure 1.3 The Notch Signaling Pathway. Image extracted from Yoon et al., 2005.[55] Notch receptor activation requires an extracellular protein from another cell to trigger the signaling cascade. ɣ-secretase cleaves a portion of the Notch receptor, the NICD, which translocates into the nucleus, resulting in transcription of genes like *Hes*.

TBI-induced neurogenesis (TBI and the Notch pathway)

Animal models are currently the most popular options to replicate TBI because of standardized outcome measurements, low costs, and other factors, although some studies have utilized larger animals.[18] In Dong Sun's lab, work has been completed to establish the neurogenic niches in the adult brain and to demonstrate that Notch signaling is a key regulatory mechanism which mediates regenerative NSC response post-TBI.[56] Increased levels of Notch-specific proteins were also observed within the SVZ of a 3-month TBI rat. It was noticed that in comparing the levels of those same proteins in aged versus young rats, the levels were markedly lower in the former.[14] Generally, the older the tissue becomes, the worse it becomes at recovery. Within the nervous system, when compared to younger tissue, aged tissue is associated with decreased tissue-specific stem cell regeneration and replacement of damaged cells[56]. Thus, this aging-related character of tissue compounded with TBI frequently results in permanent loss of sensory, motor, and cognitive functions as the result of brain injury.

Presently, there is little-to-no knowledge available about the role of Notch following TBI; however, we speculate that Notch could likely play a role in regulating TBI-induced NSC regulation based on its role in development.

Hypothesis

Our focus in this study was to analyze the longer-term effect of Notch signaling on neurogenesis following traumatic brain injury. By manipulating the Notch signaling pathway through administering Notch agonist (Notch1 antibody) or Notch antagonist (Jagged1 Fusion

protein), we set out to assess how NSCs from the neurogenic niches would respond following a moderate TBI. With our previous work as a foundation, we expanded upon a 7-day study that involved administration of the same drug treatment groups to determine the difference at 30 DPI. We hypothesized that increased activity of the Notch signaling pathway through the Notch1 antibody would result in increased proliferation/survival and decreased differentiation of NSCs within the DG. Contrarily, we expected inhibition of the same pathway to result in decreased proliferation and increased differentiation of stem cells in the hippocampus.

Chapter 2 – Materials and Methods

Experimental animals

The focus of this experiment is to determine the effects of Notch Signaling on long-term endogenous neural stem cell proliferation, survival and differentiation in the dentate gyrus of the hippocampus following traumatic brain injury. Adult rats were exposed to a moderate traumatic brain injury, followed by an intraventricular administration of a Notch agonist (Notch1), Notch antagonist (Jagged-1 Fc), or Vehicle infusion for 7-days via osmotic minipump. A total of n=21, male, 3-month old Sprague-Dawley rats (ENVIGO Corporation) were utilized. Weighing roughly 300 grams, the rats were provided food and water *ad libitum* while housed in pairs in a Plexiglas cage on a 12-hour light cycle. Procedures and handling followed the ethical care guidelines and were approved by the Institution of Animal Care and Use Committee (IACUC) of Virginia Commonwealth University, the Guide for Care and Use of Laboratory Animals and by the U.S. Department of Health and Human Services.

Experiment Setup

Animals were randomly separated into four groups. Three of those four groups were exposed to traumatic brain injury (TBI) and were further separated based on drug treatment. All animals received the same care and housing. Group one, the Sham group, was composed of five animals, did not receive TBI, but was exposed to the same surgical procedures as the other groups. Sham group did not receive any drug treatment. Group two, the Vehicle group, had five animals, underwent surgery, and received artificial CSF (aCSF) infusion for 7 days-post-injury.

Group three, the Notch antagonist group, had five animals, underwent surgery, and was infused with Jagged-1 Fc for 7 DPI. Group four, the Notch agonist group, had six animals, underwent surgery, and received the Notch agonist infusion for 7 DPI. Each group of animals was given single, daily intraperitoneal injections of 50 mg/kg BrdU for 7 DPI. All animals were sacrificed at 30 days-post-injury (DPI).

Table 2.1 Animal Grouping

	N=	Injury	Drug Treatment	Dosage
Group 1 - Sham	5	No	No	N/A
Group 2 – FPI + Vehicle	5	LFPI	Yes, aCSF (Vehicle)	100ul
Group 3 – FPI+ Jagged-1 (Notch antagonist)	5	LFPI	Yes, human recombinant Jagged-1 fusion protein	50µg/mL
Group 4 – FPI + Notch1 (Notch agonist)	6	LFPI	Yes, Notch1 antibody	2µg/mL

Table 2.1 Animal Grouping. Animals were split into 4 groups. Group 1 was the only group not exposed to a Lateral Fluid Percussion Injury (LFPI) or drug treatment. Group 2, 3, and 4 all were subjected to Lateral Fluid Percussion Injury and received Vehicle (aCSF), Notch1 antagonist (Jagged-1 Fc) and Notch1 agonist (Notch1 antibody) infusions for 7 days, respectively. All groups were given intraperitoneal injections of BrdU at 50mg/kg of body weight for 7 days post-injury.

Surgical procedures

Animal groups were dividedly at random to undergo LFPI or Sham injury. Sterilization of all surgical instruments was complete prior to surgery. During surgery, aseptic procedures were followed through completion to minimize risk. All adult rats were individually contained within a Plexiglas holding chamber and anesthetized by exposure to 5% isoflurane until unconscious and breathing rate significantly slowed. The animal's head was then shaved to expose an area for incision. Next, it was placed within a stereotaxic apparatus which stabilized the position of the animal for surgery. Continuous treatment of isoflurane (2.5%) in a 70% Nitrogen and 30% Oxygen gas mixture was given through a nose piece to keep the animal anesthetized. To sterilize the surgical site along the animal's head and to prevent the animal's eyes from drying, Betadine and Paralube ointments were applied, respectively. From here, the animal was incised along the midline of the skull using a scapula and the skin was retracted on both sides using a hemostat. Excess connective tissue was removed using sterilized cotton swabs. This exposed the area of the skull for a 5 mm craniotomy to take place. With the lambdoid and sagittal sutures as landmarks, the craniotomy was carried out approximately 1.5 mm anterior to lambdoid suture and 2 mm lateral to the midline suture on the left parietal bone using a trephine and a Dremel drill. An outline created by the trephine allowed for a clean circular area of skull to be removed. If necessary, small shards of bone were carefully removed to create a clear exposure to the brain. This area was utilized for placement of the hub cap, which connected to the Fluid Percussion injury instrument. In addition, a burr hole was created approximately 1.5 mm laterally and 1 mm posteriorly from bregma and was the point for implantation of the intraventricular infusion cannula. After implantation of the infusion cannula

into the injury ipsilateral side lateral ventricle, the cannula was secured with dental acrylics. The cannula connected osmotic min-pump was then embedded subcutaneous at the back of the neck. Once surgery was complete, the animal was sutured with a 4-0 monofilament in a running locking pattern. Application of lidocaine jelly and a triple antibiotic ointment were generously applied to the closed wound and isoflurane treatment ceased. From here, the animal was monitored to ensure normal breathing began and continued. Animals were returned to their recovery cage and placed over a warming pad. They were observed for 15 to 20 minutes post-operatively to ensure normal functions returned. The pump was removed at 7 DPI.

Lateral Fluid Percussion Injury

Moderate Lateral Fluid Percussion Injury (LFPI) was utilized for this project. Once the region of the parietal bone was carefully removed, a hub connector was cemented to the skull using cyanoacrylic, then dental acrylic was mixed and applied around the base of the hub and allowed to harden. To ensure proper alignment, it was noted that no acrylic leaked within the space of the hub and the area was rinsed with 0.9% saline solution with the expectation that the solution would not diffuse through the area of exposed brain tissue. Once the safety checks were considered, isoflurane was gradually decreased to 1.5% while prepping and testing the Fluid Percussion instrument. To establish a precise administration of a moderate injury within the range of 2.05 ± 0.05 ATM, the height of the pendulum was adjusted such that upon release and impact, the associated calibration devices displayed an output of the desired pressure. Those devices were a pressure transducer amplifier and a Tektronix oscilloscope. **Figure 2.1 (A)**

and **(B)** display the FPI instrument and calibration devices, respectively. After appropriate pressure readings were measured, the animal was removed off isoflurane until tail reflex was back, then connected via the hub to the Fluid Percussion injury instrument. The pendulum was released, inducing injury, and the exerted pressure was recorded. Upon injury, a timer was started to record the subsequent responses. Once the animal was removed from the device, it was immediately placed in a supine position on the table and assessed for breathing. If a respiratory response was not present, manual stimulation was given. Once breathing returned, animals were monitored for paw, tail, and righting reflex in that order. An external physical stimulus was applied to the animal's hindlimb and tail until flexion occurred in both. Righting time was determined as the time it took the animal to bring itself upright from its side. These reflexes have been established to determine completeness of injury. [57] After, the animal was brought back to the surgical area and placed back under anesthesia with 5% isoflurane to remove the hub and dental acrylic. In addition, a burr hole was created for insertion of the infusion cannula for intraventricular drug infusion. Observing the intersection between the coronal and midline sutures allowed for identification of bregma. From the established 0 point, the stereotaxic arm was moved 1.5 mm laterally and 1 mm posteriorly to locate the ipsilateral ventricle. The depth of the implanted infusion cannula was 3mm from the dura surface. Dental acrylic was applied around the base of the cannula to secure it. The connected osmotic mini pump was embedded subcutaneously. Finally, the surgical site was closed.

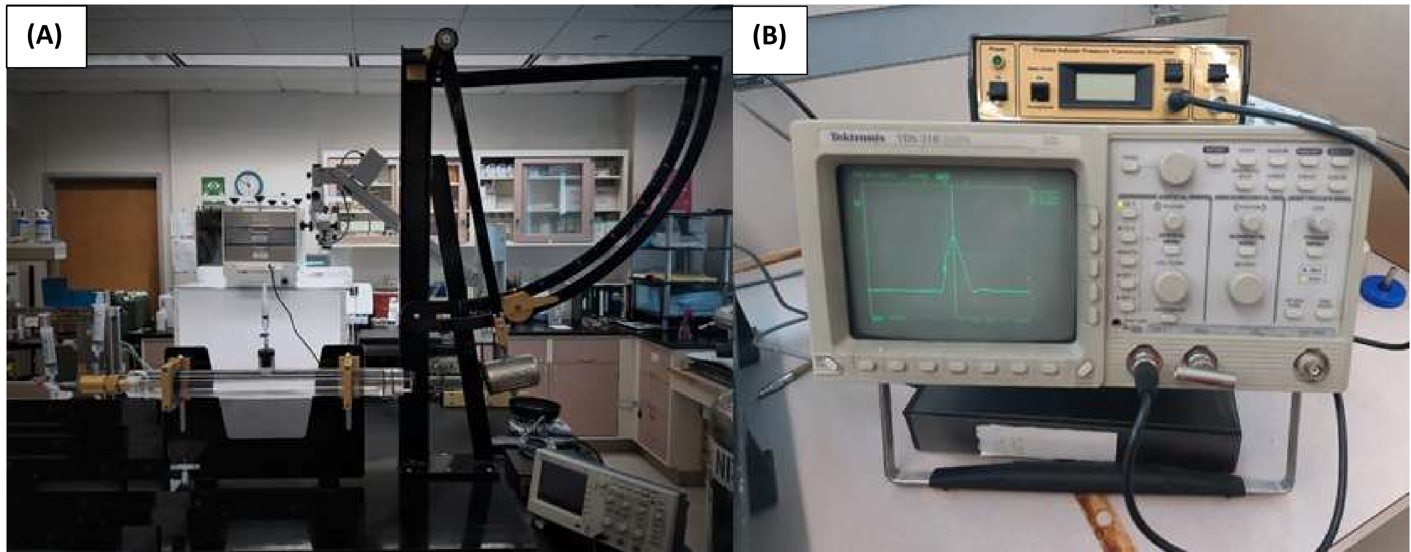


Figure 2.1 The LFPI instrument and oscilloscope. (A) Shows the Lateral Fluid Percussion Instrument used to deliver injury through a fluid-pulse. **(B)** Demonstrates a recorded pulse from the oscilloscope, which displays a frequency (Hz) based on the current of the fluid flowing through the tube. Conversion of Hz to pressure (atm) was calculated after.

Drug Dosage Determination

The three LFPI groups were separated to receive Vehicle, Notch1 antagonist, or Notch1 agonist infusions. The 7-day infusion mini-pump contains 100ul of infusion solution. The vehicle group was infused with 100ul of aCSF. As previously described by Sun et al. 2010, the composition of aCSF consisted of 148mM NaCl, 3mM KCl, 1.4mM $CaCl_2$, 0.8mM $MgCl_2$, 1.5mMNa_2HPO_4, and 0.2mMNaH_2PO_4 at a pH 7.4. The Jagged-1 Fc and Notch-1 groups were selected to receive a pre-determined concentration of the selected drug, at 50µg/mL and 2µg/mL, respectively. These doses were selected based on the dose response study examining DG neural stem cell proliferative response following Notch1 agonist and antagonist infusion determined previously in our lab.

BrdU Injections

To label dividing cells, animals were intraperitoneally injected with bromodeoxyuridine (BrdU) at 50 mg/kg body weight, single, daily for 7 days during the infusion period. BrdU is a thymine analog, which incorporates into the DNA of all proliferating cells during S-phase. Thus, BrdU serves as a crucial indicator of quantity and timing of newly formed DNA/replicating cells within the specific timepoint of injury, as seen in previous studies. [20]

Sacrificing and Tissue Preparation

At 30 DPI, all animals were sacrificed by transcardic perfusion. Animals were placed in a Plexiglass container and deeply anesthetized with an overdose of isoflurane inhalation. A gauze pad soaked in isoflurane was used to cover the animal's facial area to maintain that the animal stayed unconscious. An incision was made along the midline of the thoracic cavity, exposing the heart. The heart was cross-clamped using rubber tipped hemostats, then a blunt-tip, 16-gauge needle was inserted through the left ventricle into the aorta. Next, the right atrial appendage was cut to allow the exit of blood. A transcardial perfusion was completed, using 150 mL of Phosphate-buffered Saline (PBS) and 150 mL of 4% Paraformaldehyde in PBS fixative. PBS flowed through the needle, circulating throughout the animal's vascular system. The flow of PBS was continued until the fluid exiting the right atrium was clear and the liver, as well as other organs, had lost their dark color. From here, Paraformaldehyde flowed through the animal's circulatory system to serve as a fixative for the tissues. After fixing the tissue, the animal was decapitated, and the brain was carefully extracted out from beneath the skull. Brains were kept in 4% paraformaldehyde at 4°C until they were sliced. Using a Vibratome **(Figure 2.2)**, the brains were sliced coronally into 60 µm slices throughout the ventricles and hippocampus. Sequential sections were collected in 4 sets into 24-well plates that were filled with PBS and 0.01% sodium azide, then stored in the 4ºC for preservation until further processing. Brain sections were used accordingly for immunostaining.

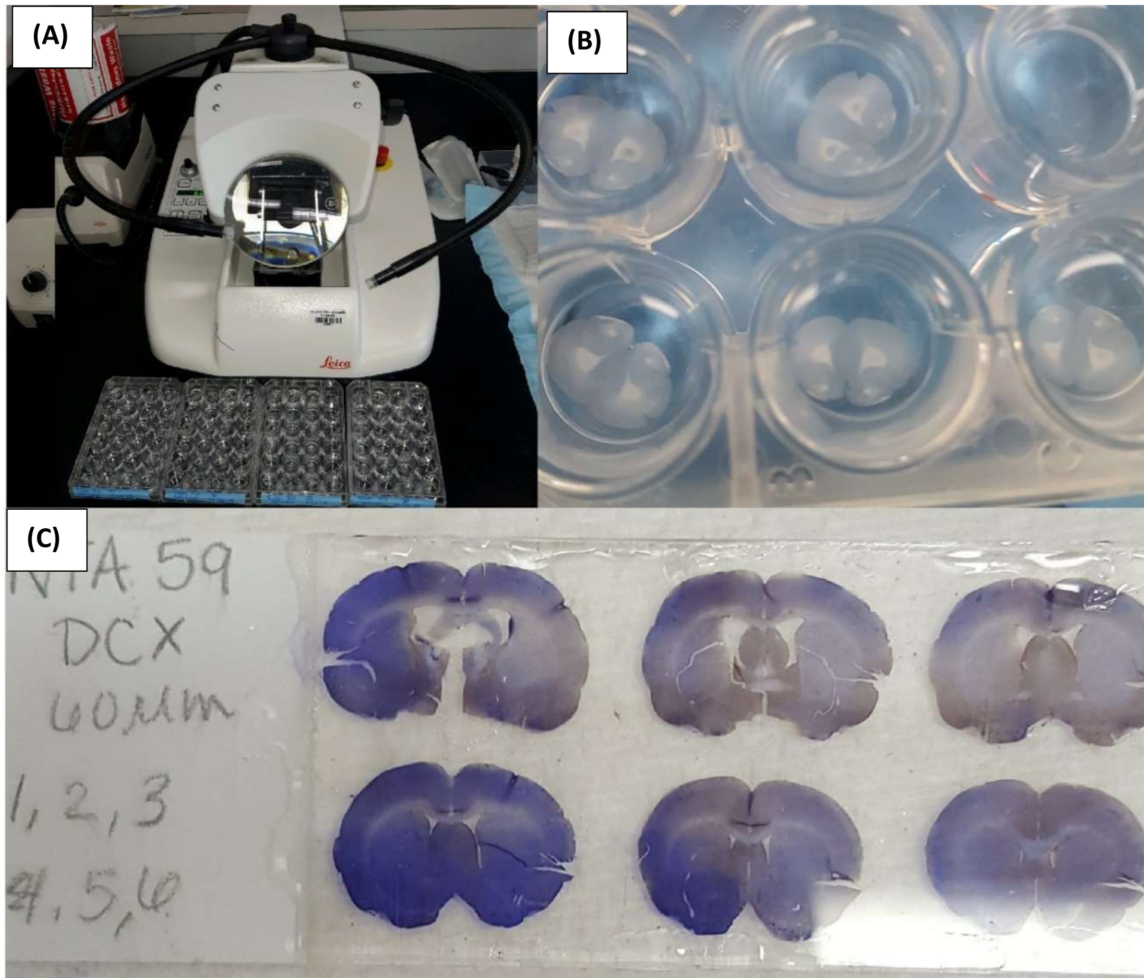


Figure 2.2 Tissue slicing and processing. (A) This image captures the Vibratome, used for slicing tissue samples. **(B)** Rat brains were sliced coronally into 60 µm slices throughout the ventricles and hippocampus and collected in 24-well plates filled with PBS and 0.01% sodium azide. **(C)** Tissue samples were immunostained and mounted on *Superfrost* slides, counterstained with Crystal Violet, then covered using *Permount* solution and coverslips.

Immunohistochemistry

Two separate immunostaining techniques were utilized for this experiment. For each brain, 12 sequential sections spanning 480 µm throughout the lateral ventricle and hippocampus were selected and were processed for immunostaining of a specific marker. This ensured that equal and complete coverage of the hippocampus and ventricles were accounted for each animal. Our goal was to identify the presence of BrdU and doublecortin (DCX), an immature neuronal marker. The protocol for staining was similar for both markers except the DNA denaturing steps for BrdU. Briefly, sections were rinsed in 1X PBS two times for five minutes each. For BrdU staining, a denaturing solution composed of 50% formamide, 37.5% Nanopure H_2O, and 12.5% 20X Sodium Saline Citrate Buffer (SSC) was used. The sections were denatured for one hour in an oven set at 65°C. Next, sections were removed from the oven and rinsed with 2X SSC for five minutes, two times. After, sections were further denatured in 2N Hydrochloric acid (HCl) for 30 minutes in a 37°C oven. Sections were then rinsed again in 1X PBS for five minutes, twice. From the previous step onward, the protocols for BrdU and DCX were identical. Following the wash, the trays were quenched in 3% hydrogen peroxide for 1 hour. A mixture of 1X PBS with 0.3% Triton X-100 was placed in the trays for 10 minutes. Sections were incubated with a blocking buffer, consisting of 5% horse serum in 1X PBS + 0.3% Triton, overnight at 4°C. The following day, the blocking buffer was removed, and the specific primary antibody solution was prepared: for BrdU, primary monoclonal mouse antibody solution (*Life Technologies Corp*) was set at a dilution of 1:2000 in blocking buffer. For DCX, primary goat anti-DCX (*Santa Cruz Biotech)* was made at 1:1000 dilution in blocking buffer. Sections were incubated with the primary antibody solution for 48 hours at 4°C on shaker. Sections were then

brought back to room temperature and rinsed with 1X PBS + 0.3% Triton for 10 minutes, three times. After, they were placed in blocking buffer for 3 hours at room temperature before being placed in secondary antibody solutions overnight at 4°C. The secondary antibody solution for BrdU was prepared as follows: A Biotin-conjugated anti-mouse antibody, measured at 1:200 dilution in blocking buffer, from horse (*Vector Laboratories, Inc)* was prepared. The DCX secondary antibody (*Vector Laboratories, Inc)* solution was anti-goat, biotinylated IgG, prepared at 1:200 dilution in blocking buffer. Sections were incubated with the secondary antibody for overnight at 4°C on shaker. On the last day, the sections were brought back to room temperature and washed in 1X PBS for 10 minutes, three times. Because our secondary antibodies were Biotin-conjugated antibodies, the sections were further incubated with an Avidin-Biotin Complex (ABC) solution for two hours at room temperature. This solution works by associating with Biotin-conjugated secondary antibody, increasing staining intensity of the tissue. The ABC solution was prepared at 1:200 dilution in 1x PBS 30 minutes prior to use. Following 2 hours of exposure, the sections were washed with 1X PBS three times for 10 minutes. Concurrently, DAB solution, consisting of dry diaminobenzidine tetra-hydrochloride (DAB) (*Sigma)* was prepared. With the help of a peroxidase, DAB can be oxidized in the presence of hydrogen peroxide, forming a brown precipitate that is easily visualized under the microscope. For every 10mg of DAB, 40 mL of Phosphate Buffer and 17.5 µL of 30% hydrogen peroxide was added. Tissue sections sat in DAB solution for 5-15 minutes and allowed to react under the microscope. **(Figure 2.3)** After the precipitate was visualized, the sections were washed a final time in 1X PBS for 5 minutes, three times, and carefully mounted on *Superfrost* slides to be stored until further processing.

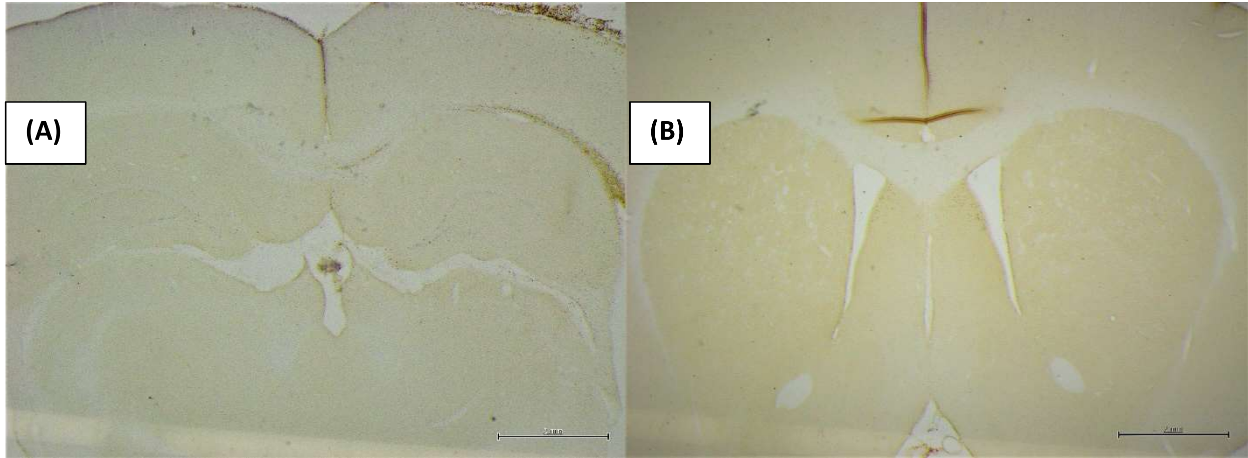


Figure 2.3 Positive DAB staining for BrdU. (A) and (B) Positive DAB staining for BrdU was observed under a microscope within the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles, respectively. The reaction was visualized by a darkening of these regions of interest. **(A)** shows an example slice of the hippocampus while **(B)** shows staining in the SVZ. Scale bar = 2mm.

Crystal Violet Counter Staining

The Crystal Violet stain (*Biochemical Sciences,* Inc) was used to help to identify the morphology of brain sections. Crystal violet binds to Nissl substance, which is found in the soma and dendrites of neurons and glial cells. Nissl is composed from the cell's rough endoplasmic reticulum thus can be closely associated with the soma. Axons, on the other band, have little Nissl present so therefore appear lighter in color after exposure. Crystal violet assays have been demonstrated to help quantify samples based on staining intensity.[58] During cell death, a typically adherent cell will dissociate from culture and is lost from that sample, thus decreasing the amount of crystal violet staining. The overall staining of the tissue created a purple backdrop to quantify cells within the regions of interest. After hydrating in distilled water for 3 minutes, a tray of slides was placed in a 1:100 dilution of Crystal Violet in distilled H_2O for 35 seconds, then immediately placed back in pure diH_2O. Next, the tray was placed in two separate containers of 75% ethanol for 2 minutes, then placed in 95% ethanol for 3 minutes. Following, the tray was moved into two 100% alcohol containers for 3 minutes each. Ethanol steps were intended to slowly dehydrate the tissue sections. Then, they were placed in one container of Xylene (*Citrisolv, Deacon Lab*) for 3 minutes, and another container of Xylene for 5 minutes. Xylene serves the role of a defatting agent. All containers housed a volume of solution of 250mL. Finally, coverslips were placed over the slides and sealed using *Permount* solution.

Stereology

After crystal violet staining was complete, all slides containing regions of the hippocampus were analyzed. Five sections, spanning 480 µm, throughout the hippocampus were selected for each brain. Sections were observed under an inverted light microscope, *Olympus DP71,* which was paired with the *Visiopharm Integrator System (*Olympus) program. These tools allowed for cell quantification and tissue thickness calculation of the Dentate Gyrus (DG). The DG was broken down into the following areas: The Molecular Layer (MOL), the Granular Cell Layer (GCL) and the Hilus. Between the GCL and Hilus exists a layer called the Subgranular Zone (SGZ), which is a roughly two-cell thick layer, staining prominently with crystal violet. Cell bodies are easily identifiable within the region of the SGZ, and extensions of the cells typically extend through the GCL to the MOL, as seen experimentally by our lab and others [44,59] To start, sections were focused under a 4x objective lens and the outlines were made around the DG and Hilus excluding the region of the hippocampus identified as CA3. Every positive BrdU cell was counted across the entire height of the optical dissector, using the optical fractionator method. The dissector height (h) was set to 15 µm and sections were analyzed across that range. Cells outside the height were not included in the total. The total number of cells were counted on both the ipsilateral and contralateral side of the tissue of both the Granular Zone and Hilus. The values reported for BrdU cells represents the sum of all cells for each animal, and the total number of stained cells was calculated to give the estimated number of cells throughout the DG of the hippocampus. Using the Optical Fractionator (3D) equation $n = \Sigma Q \cdot (t/h)(1/asf)(1/ssf)$, where the sum Q equals the total number of particles, (t/h) is defined as the average section thickness divided by counting frame height, *asf* represents the

area sampling fraction and was equal to 1, and *ssf* was valued at 1/8 and represented the section sampling fraction. The latter value was determined by calculating the total number of sections collected for each brain and assessing that every eighth tissue sample was used in this methodology. To determine thickness of the tissue, 5 separate measurements of the focal plane of each section were taken. These numbers were averaged to determine the average tissue thickness value for each animal.

Statistical Analysis

To determine significance, a one-way ANOVA test was run with the SPSS software. A multi-group comparison was complete across the four groups introduced. Included in the analysis was a look at the difference in cellular proliferation of the GZ and Hilus, on both the ipsilateral and contralateral sides of injury. A Bonferroni test was used with a significant p value of less than 0.05.

Chapter 3- Results

In this study, we focused on the longer-term effects of the Notch signaling in regulating survival of proliferating neural stem cells and generation of new neurons in the DG of the hippocampus following TBI. In this experiment, we quantified the number of cells representing stem cell proliferation/survival (BrdU-positive cells) and neural differentiation (DCX-positive cells) at 30 days post-injury across Sham, FPI+Vehicle, FPI+Jagged-1 Fc (Notch1 antagonist), and FPI+Notch1 antibody (Notch1 agonist) groups. . All four groups were analyzed to determine the difference in proliferation/survival and differentiation within the same region of the brain: the dentate gyrus of the hippocampus. For cell counting, the DG **(Figure 3.2)** was further divided into the granular zone (GZ) and the hilus region. An example of a BrdU+ cell, located within the subgranular zone of this section, was identified by its circular nucleus and dark color. **Figure 3.3** compares all four animal groups and demonstrates the noticeable difference in proliferation in the hippocampus. To analyze our results, we ran a one-way ANOVA multi-group comparison. We specifically ran a Bonferroni Post Hoc, comparing mean differences with a 95% confidence interval to identify significant differences between groups. Our threshold for significance was set to $p<0.05$.

Determining the longer-term role of Notch1 signaling in survival of proliferated cells following TBI.

To study the effects of activation of Notch signaling on NSCs within the neurogenic region of the hippocampus, we used a Notch1 antibody (anti-Notch Extracellular clone 8G10)

which acted as an agonist on the Notch1 receptor. Conversely, to assess the effect of Notch signaling inhibition on post-TBI neurogenesis, we used human recombinant Jagged-1 Fc, a receptor blocking fusion protein. This fusion protein works to inhibit Notch signaling by binding to a ligand of the Notch1 receptor, Jagged1.[56] Previous studies have shown that activating Notch results in proliferation of stem cells not only in the CNS, but stem cells of non-neural origin as well.[60,61] More recent work has focused on Notch manipulation by blocking the Notch pathway using siRNA against Notch or a ɣ-secretase inhibitor, leading to significantly reduced downstream transcriptional effectors. [62] These data, along with our preliminary work, solidified our use of these drug treatments. In the current study, animals were infused with 2μg/mL of Notch1 or 50 μg/mL of Jagged-1 Fc intraventricularly through an osmotic pump immediately following injury for 7 days. As previously demonstrated by our lab, we analyzed completeness of injury across the vehicle and drug infusion groups by comparing the righting response time of animals post-injury. [20] Righting time **(Figure 3.1)**, among other neurobehavioral measures, were measured following lateral FPI to establish a baseline level of injury amongst animal groups.[63]

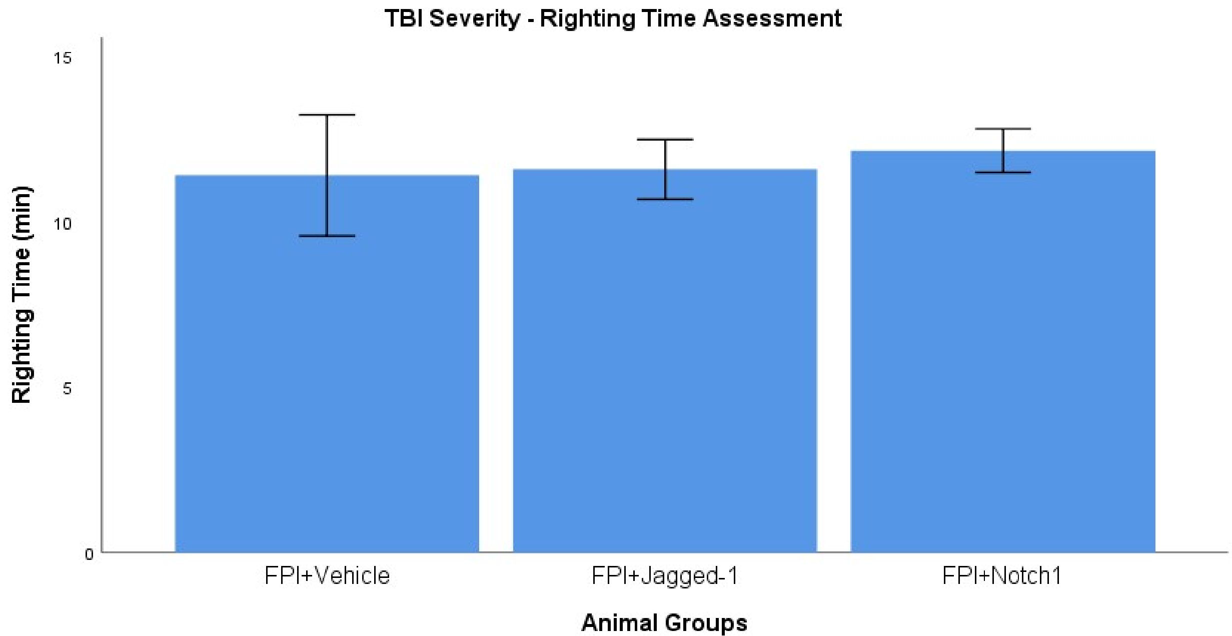


Figure 3.1 Animal Righting Time Following Injury. The righting time of each animal was recorded following LFPI. No significant differences were seen across groups. Error bars = 2x SE.

Analysis of Cellular Proliferation/Survival at 30 DPI

Intraperitoneal BrdU injections (50mg/kg) were given once daily following injury for 7 days to timestamp proliferating cells for all groups. Due to its similar structure to the DNA base thymine, BrdU is a trusted immunohistochemical tool for labeling proliferating cells. The thymine analog incorporates into DNA of dividing cells during S-phase of the cell cycle. [64] As BrdU was only available for 2 hours after injection to incorporate into DNA of proliferating cells during the 7 days injection period, and since animals were sacrificed at 30 days, the number of BrdU-labeled cells in this study represents proliferating cells during the injection period that survived at 30 days post-injury. As shown in **Fig. 3.3**, BrdU staining was mostly observed in the

granular cells layer of the DG. Intensity of staining represents different stages of division. Darker staining represented a more stable state in which BrdU was not diluted due to division, whereas lighter staining represented that the cell went through multiple divisions, thus BrdU was more diluted. BrdU+ cells were also observed not only in the subgranular zone where the DG neurogenesis occurs, but also into the middle and outer layers of the GZ indicating that BrdU-labeled dividing cells migration from the SGZ to the GZ.

In counting the total number of BrdU+ cells using unbiased stereology method, we found that the number of BrdU+ cells was significantly higher in both the granular zone and hilus of the dentate gyrus in animals with Notch1 agonist infusion when compared to Sham animals. **(Figure 3.4)** Specifically, the number of BrdU+ cells in the Notch1 agonist group was significantly higher on the contralateral side of GZ (p=0.015) and was trending towards significance on the ipsilateral side of injury (p=0.057) when compared to Sham levels. On the contralateral GZ, the number of BrdU+ cells were also significantly higher in the Notch1 infused group than the Jagged-1 Fc group (p=0.046). In the hilus, the FPI+Notch1 agonist group had a significant increase in the number of BrdU+ cells on the ipsilateral side (p=0.001) and contralateral side of injury (p=0.000024) versus Sham. In addition, BrdU+ cell in the Notch1 group was increased significantly against the Jagged-1 Fc antagonist group on the ipsilateral (p=0.049) and contralateral (p=0.000216) side of injury within the Hilus. Lastly, comparison of the Vehicle group versus Sham demonstrated that injury alone resulted in a significant increase on the ipsilateral Hilus (p=0.019) and was trending towards significance contralaterally (p=0.055). Collectively, the BrdU data suggested that activation of Notch1 signaling following TBI enhances survival of proliferated neural stem cells at the chronic stage when compared to

Sham animals following TBI, whereas Notch1 signaling inhibition decreases survival of proliferated cells when compared to the Notch agonist group.

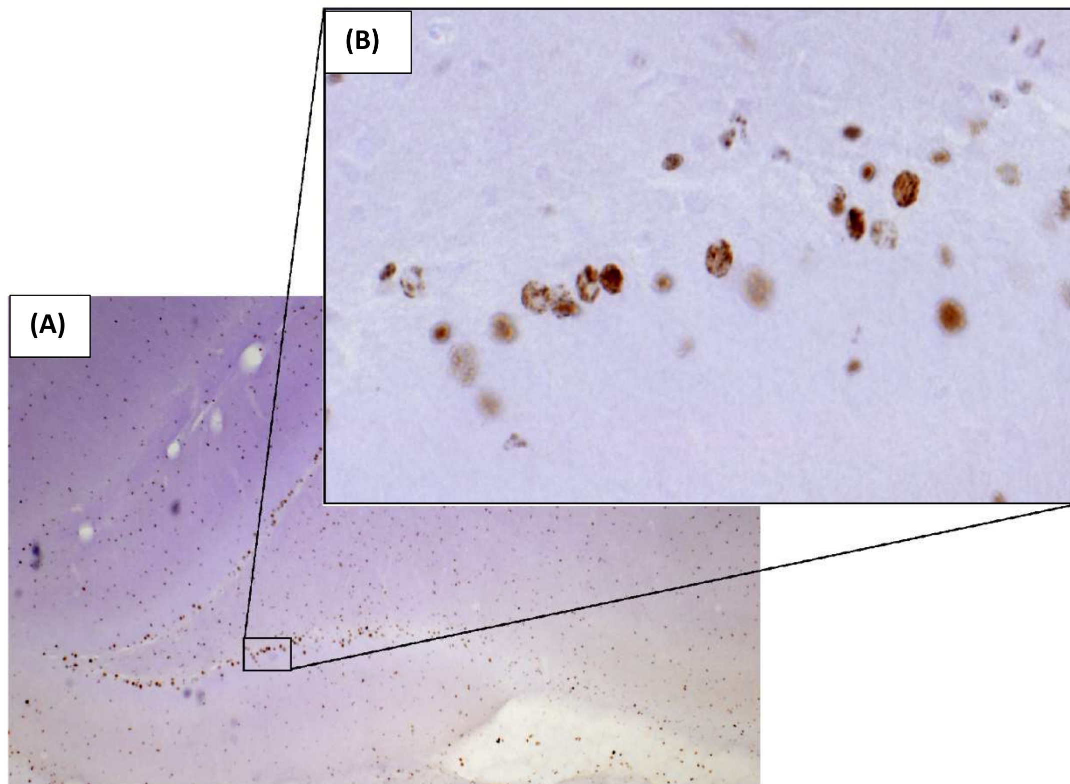


Figure 3.2 Visualization of BrdU+ staining along the GZ and hilus. In this image, we demonstrate what qualified as a positively stained BrdU+ cell. Morphologically, BrdU+ cells were identified as the round nucleus of the cells and the darkened color in contrast to background staining. BrdU+ cells were typically found along the Subgranular Zone (SGZ) and GZ of the DG while fewer were found within the polymorphic layer (hilus). **(A)** Shows a section at 4x magnitude, while **(B)** is shown at 40x.

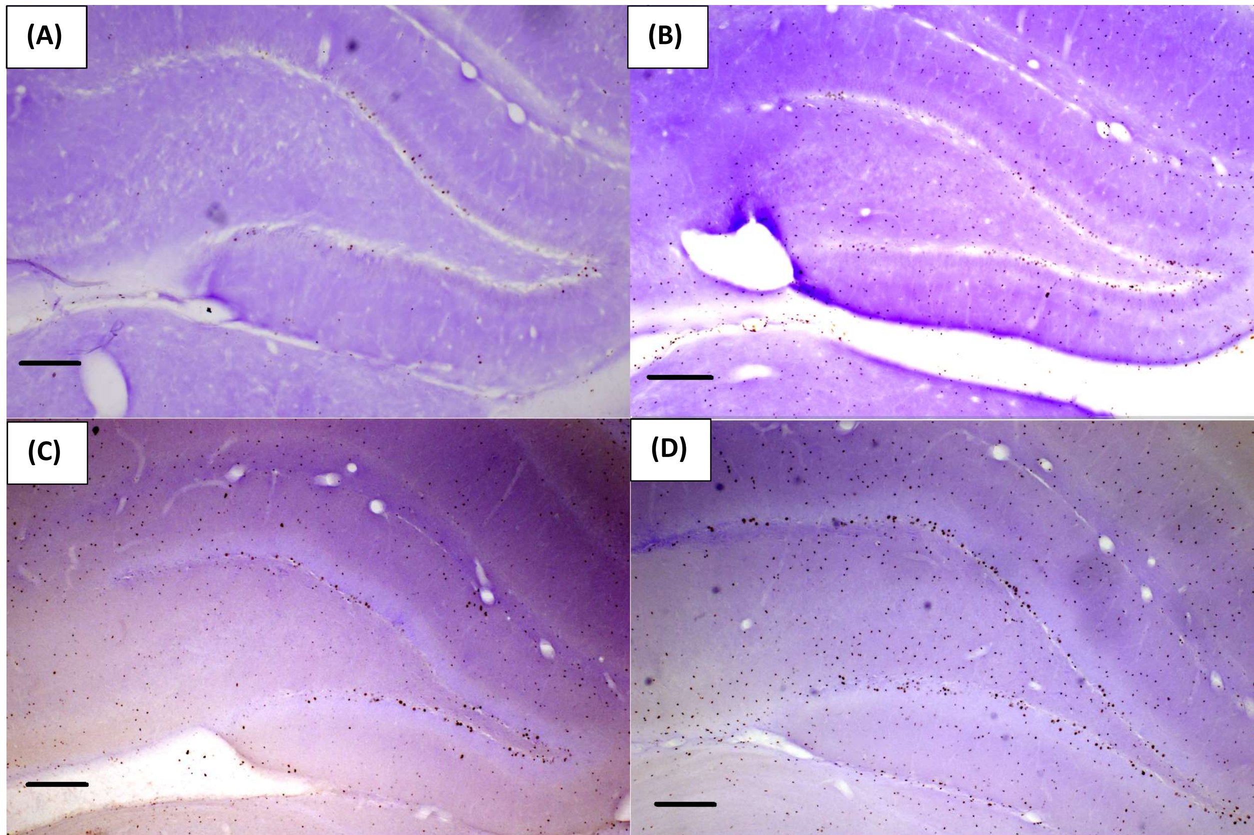
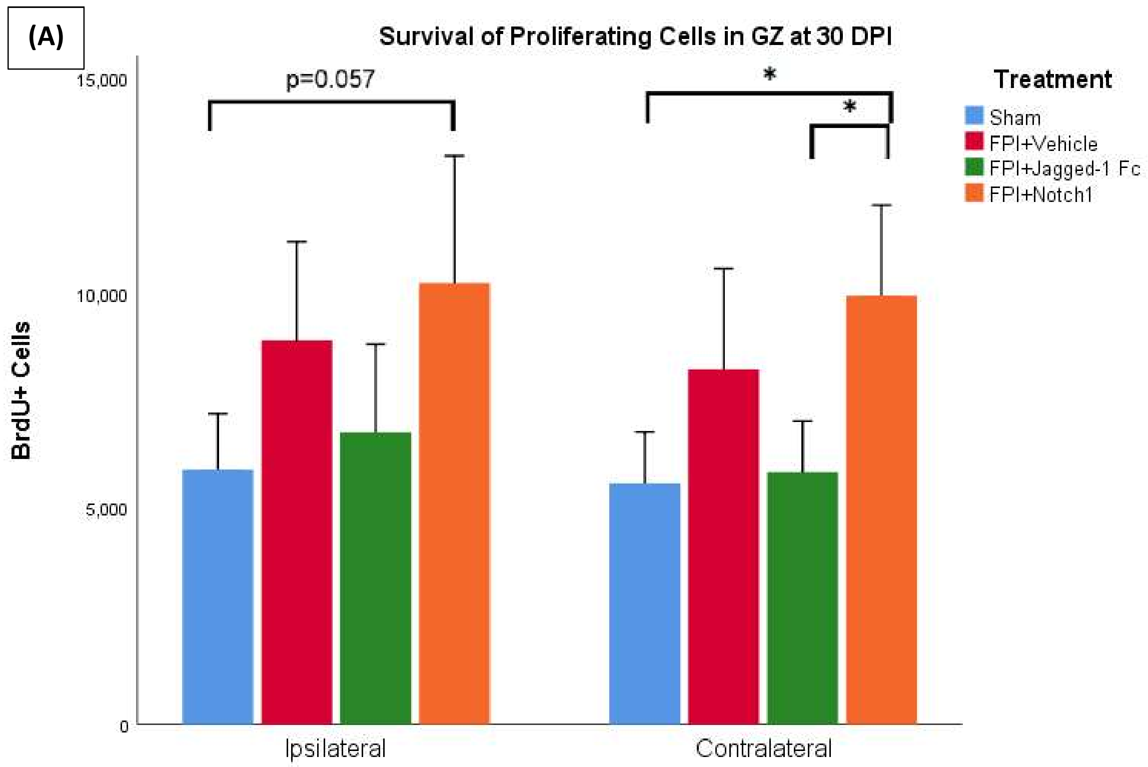


Figure 3.3 Visualization of BrdU+ staining across treatment groups. Representative images of 60µm thick coronal hippocampal tissue sections processed for immunostaining against BrdU and counterstained with Crystal Violet Nissl stain. Sections were observed under a 4x lens to map the GZ and Hilus of the DG. **(A)** Sham **(B)** FPI+Vehicle **(C)** FPI+Jagged-1 Fc and **(D)** FPI+Notch1. Scale bar = 100 µm.


(A)
Survival of Proliferating Cells in GZ at 30 DPI
p=0.057
*
*
Treatment
Sham
FPI+Vehicle
FPI+Jagged-1 Fc
FPI+Notch1
BrdU+ Cells
15,000
10,000
5,000
0
Ipsilateral
Contralateral

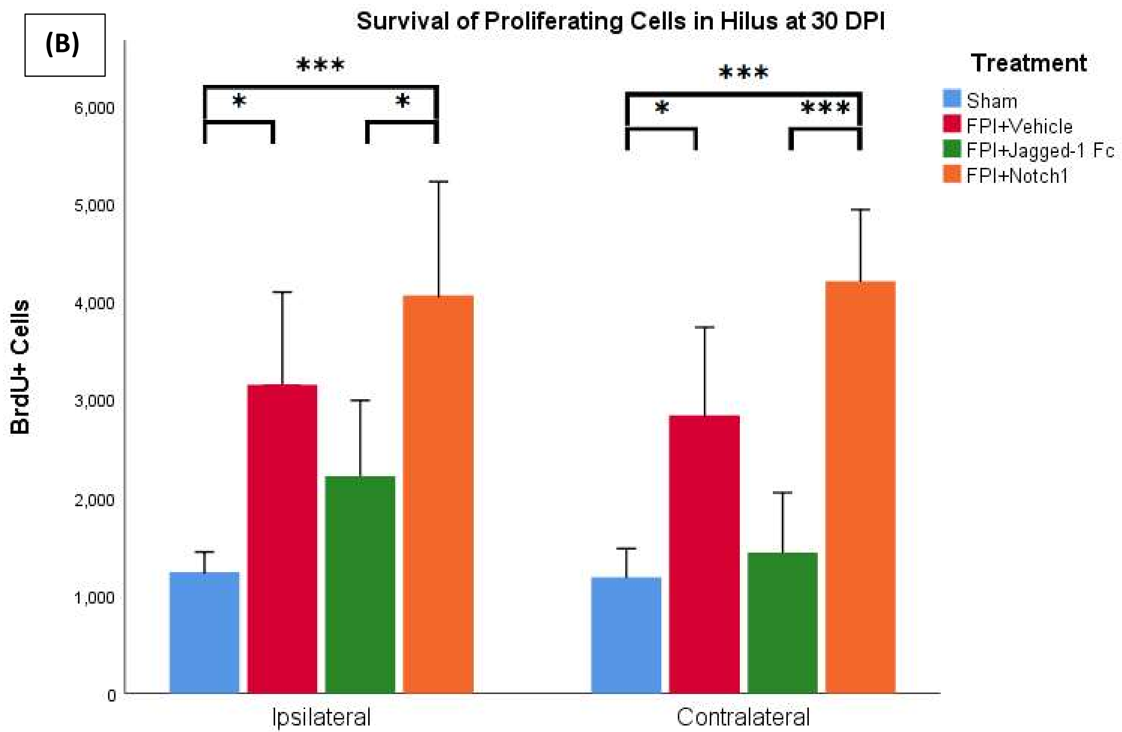

(B)
Survival of Proliferating Cells in Hilus at 30 DPI
***
*
*
***
*
***
Treatment
Sham
FPI+Vehicle
FPI+Jagged-1 Fc
FPI+Notch1
BrdU+ Cells
6,000
5,000
4,000
3,000
2,000
1,000
0
Ipsilateral
Contralateral

Figure 3.4 Quantification of BrdU positive cells at 30 Days Post-Injury. Proliferation/survival of DG neural stem cells was determined by quantifying the number of BrdU+ cells for Sham, FPI+Vehicle, FPI+Jagged-1 Fc, and FPI+Notch1 groups at 30 days after TBI. Counts were measured on both the ipsilateral and contralateral side of injury in the hilus and granular zone (GZ) of the hippocampus using stereological principles. **(A)** In the GZ, the levels of BrdU+ cells in the FPI+Notch1 agonist group was significantly higher in the contralateral side (p=0.015) and was trending toward significance in the ipsilateral side (p=0.057) when compared to Sham. The number of BrdU+ cells in the FPI+Notch1 group was also significantly higher compared to the FPI+Jagged-1 treated group. **(B)** In the ipsilateral hilus, the number of BrdU+ cells in the FPI+Notch1 agonist group was significantly higher compared to Sham (p=0.0001) and FPI+Jagged-1 Fc (p=0.049) groups. FPI+Vehicle group had a significantly higher number of BrdU+ cells when compared to Sham in the ipsilateral (p=0.019) and contralateral (p=0.011) hilus. In the contralateral hilus, the number of BrdU+ cells in the FPI+Notch1 agonist group was also significant higher compared to Sham (p=0.000024) and FPI+Jagged-1 Fc (p=0.000216) groups. Note that: (*p<0.05, ***p<0.0001) and Error bars set to 2x SE.

Analysis of Neural Differentiation 30 DPI

In addition to examining Notch signaling manipulation on cell proliferation and survival within the dentate gyrus of the hippocampus, we also assessed changes in Notch1 signaling in the generation of new neurons at the chronic stage following TBI using DCX as the marker. Doublecortin X (DCX) was discovered as a microtubule associated protein involved in migration of neural progenitor cells (NPC's). [65] DCX is a marker commonly used to identify the presence of immature granule cells and migrating neuroblasts [66–68]. In **Figure 3.5 (A-D),** example sections for each group are shown to demonstrate the staining patterns seen in the DG. Typically, dark-staining regions of cell bodies could be identified around the SGZ of the DG. Less frequently would cells be found in the hilus region. Additionally, cells were categorized by morphology of their dendrites and identified as vertical, horizontal, or mixed dendrites. This marked their stage of maturation stage, with vertical dendrites indicating a more mature stage, as shown in **Figure 3.5 (E-G)**.

For quantification, the total number of DCX+ cells regardless of their dendritic morphology were lumped together. In both the ipsilateral and contralateral GZ, the number of DCX+ cells were slightly higher in the sham and TBI-Vehicle group compared to TBI-Notch1 agonist and TBI-Notch1 antagonist groups. However, the difference did not reach statistical significance. There is no difference in the number of DCX+ cells between the Notch1 agonist and antagonist group as well. In the Hilus, the mean value of DCX+ cells for the FPI+Vehicle group was higher than the other three groups on both sides of injury but did not reach statistical significance in the multi-group comparisons of the one-way ANOVA test. Overall, no group difference was found in the GZ and hilus regions across all groups in both ipsilateral and

contralateral side of injury **(Figure 3.6)**. This data suggests that Notch transient signaling manipulation does not have prolonged effect on hippocampal neurogenesis.

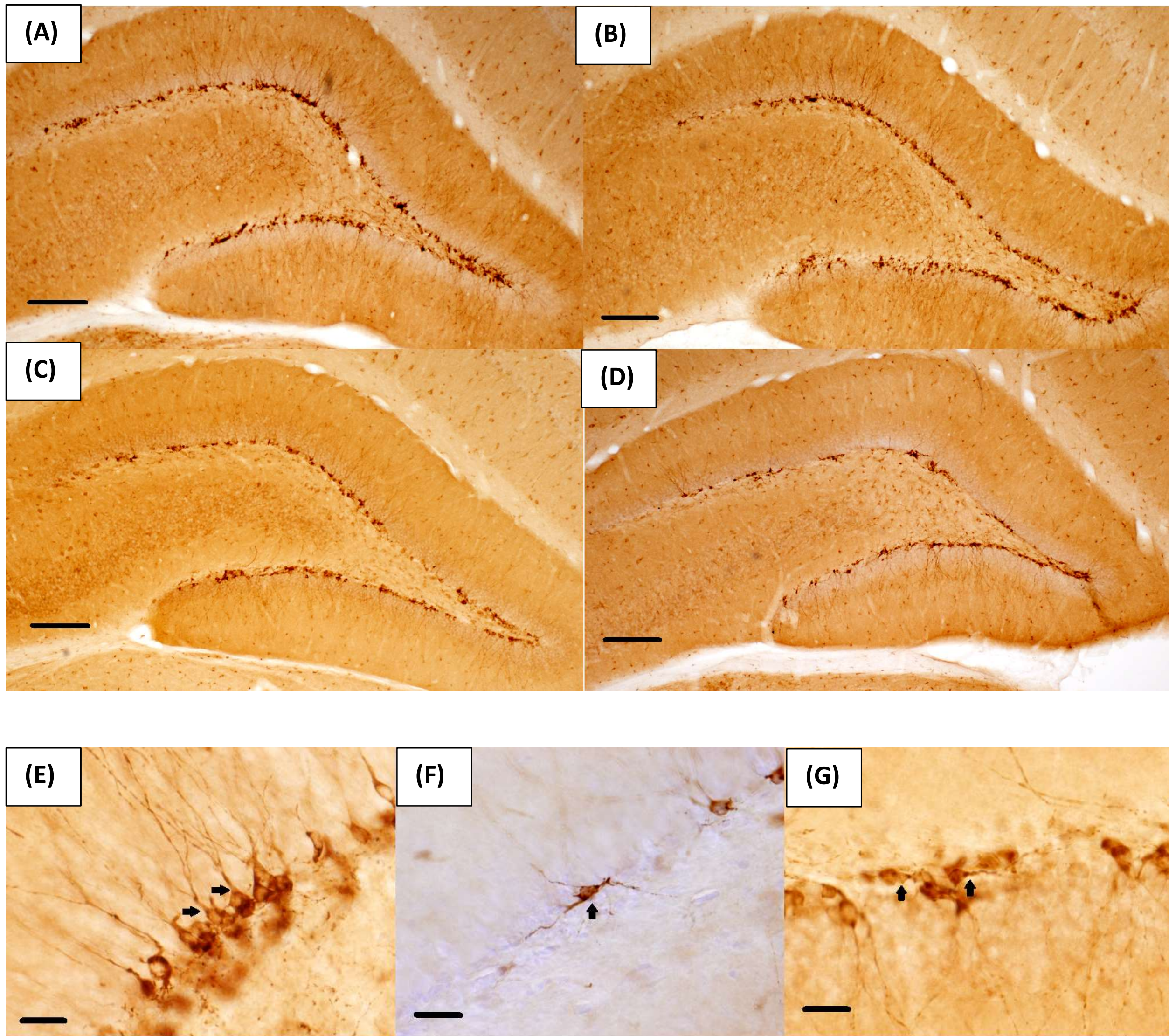
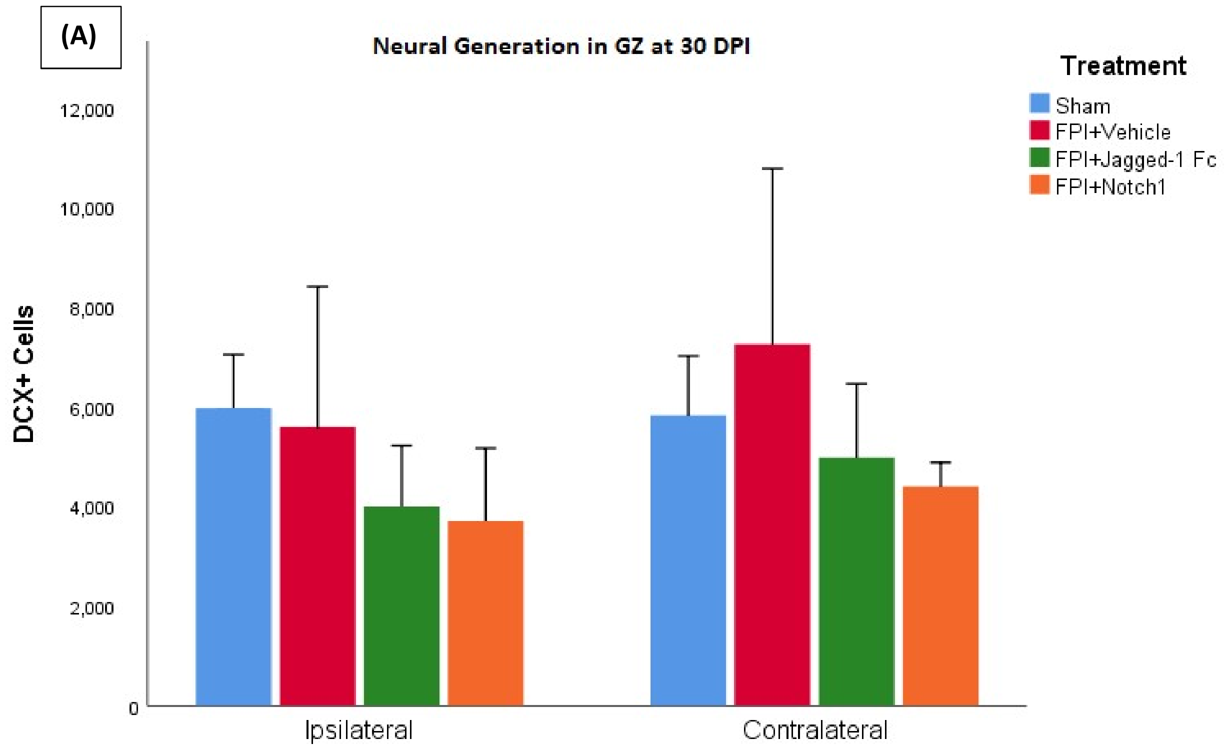


Figure 3.5 Visualization of DCX+ cells in the GZ and Hilus regions. DCX immunostaining was carried out in **(A)** Sham, **(B)**FPI+Vehicle, **(C)** FPI+Jagged-1 Fc, and **(D)** FPI+Notch1 groups. Scale bar = 100 µm. Different dendritic morphology was shown: **(E)** vertical, **(F)** mixed, or **(G)** horizontal. The arrows identify corresponding examples of positively stained DCX cells. Scale bar = 20 µm.


(A)
Neural Generation in GZ at 30 DPI
Treatment
Sham
FPI+Vehicle
FPI+Jagged-1 Fc
FPI+Notch1
DCX+ Cells
12,000
10,000
8,000
6,000
4,000
2,000
0
Ipsilateral
Contralateral

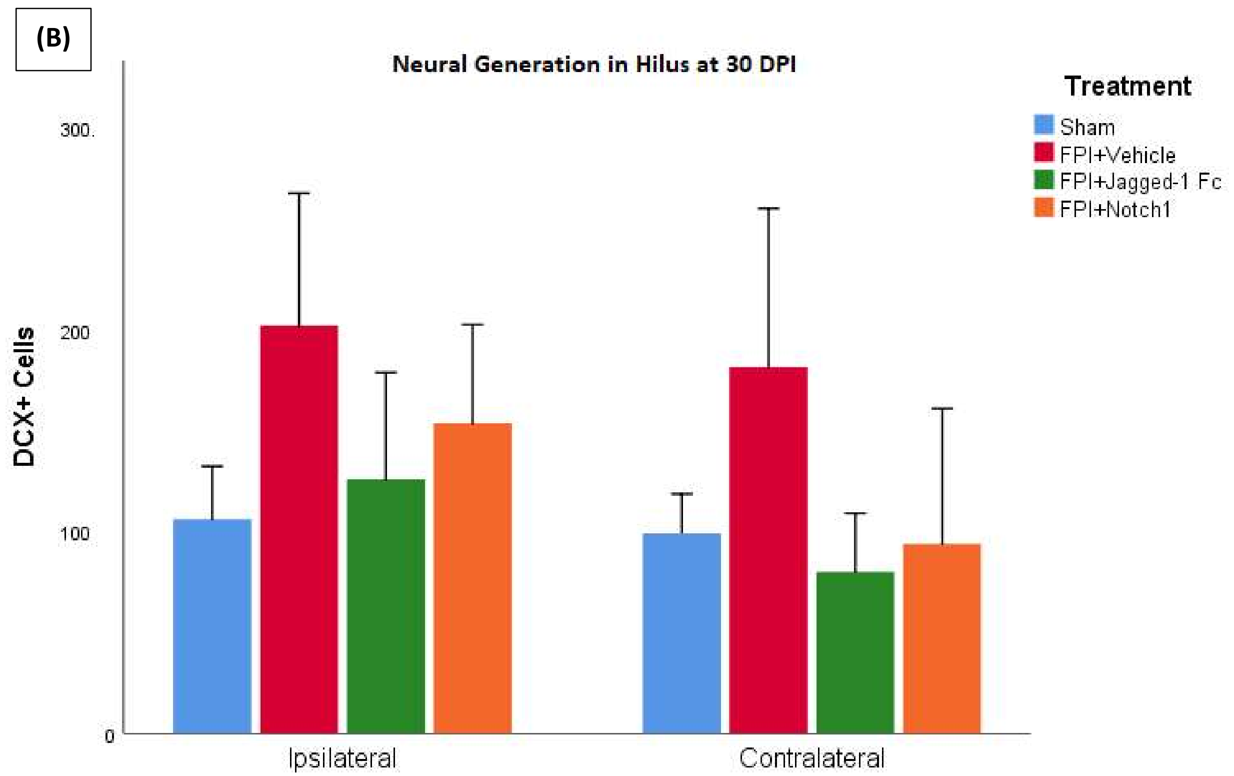

(B)
Neural Generation in Hilus at 30 DPI
Treatment
Sham
FPI+Vehicle
FPI+Jagged-1 Fc
FPI+Notch1
DCX+ Cells
300.
200
100
0
Ipsilateral
Contralateral

Figure 3.6 Quantification of DCX positive cells at 30 Days Post-Injury. Stereological counts were completed to determine the number of DCX+ cells across all four treatment groups. Counts were completed across the ipsilateral and contralateral **(A)** GZ and **(B)** Hilus for all animals. No significant differences were found in the multi-group comparisons. Error bars set to 2x SE.

Chapter 4 – Discussion

Traumatic brain injury stands as a global health issue affecting millions of people each year. In the United States alone, TBI is estimated to have affected over a million people from 1995 through 2001 according to the CDC. [69] Of that group, 50,000 individuals died from the event, while 1.1 million were treated and released. As technology continues to improve, a greater number of people will survive TBI-related incidents, therefore creating greater risk of post-TBI complications over time. It is our hope that increasing the understanding of how TBI affects the brain will allow for immediate, efficient treatment to limit, or perhaps even prevent, the outstanding secondary injuries commonly associated with this injury model.

In this dissertation, we have discussed a very brief overview of the study of neurogenesis, the Notch signaling pathway, TBI, and how all three of those are intertwined. Neurogenesis is a crucial process during homeostatic and pathological conditions. Notch signaling, one of the key mechanisms in regulating neurogenesis, plays a vital role in normal development of the brain [70] and maintenance in later stages of adulthood.[48] Specifically, the Notch1 signaling pathway plays a role in proliferation, differentiation, and apoptosis of NSCs in direct association with specific cellular signals. Our lab has explored the role of Notch signaling in regulating the regenerative neural stem cell response following TBI. [20,44,71] Thus far, we have analyzed the short-term effects of Notch manipulation; this study questioned the long-term effects of Notch manipulation in the neurogenic niches of the brain during the chronic stage following TBI to gain insight on how post-injury regenerative stem cells are affected by Notch signaling.

Summary of Results

In this study, we analyzed the significance of the Notch signaling pathway manipulation in adult neurogenesis following TBI. Notch pathway manipulation was achieved by intraventricular infusion of Notch1 inhibitor Jagged-1 Fc or Notch1 activator Notch1 antibody for 7 days immediately following a lateral fluid percussive injury. Animals were sacrificed at 30 days post-injury. The number of BrdU-positive cells that were labeled via intraperitoneal BrdU injection during the infusion period was utilized as the indicator of Notch manipulation on neural stem cell proliferation and survival, whereas immature neuronal marker DCX was used as the indicator of generation of new neurons. We found that at 30 days post-injury, the FPI+Notch1 agonist group had a significant increase in survival of proliferated cells, as measured with BrdU+ cell quantification, when compared to Sham groups in both the granular zone (GZ) and hilus region of the dentate gyrus (DG) in the hippocampus. The FPI+Notch1 group also had significantly higher number of BrdU+ cells compared to FPI+Jagged-1 Fc group on both the ipsilateral and contralateral Hilus, as well as the contralateral GZ. In addition, there was a significant increase in proliferation in FPI+Vehicle versus Sham groups throughout the Hilus.

In the assessment of DCX+ cells, we found that no significant differences were established when comparing Sham, FPI+Vehicle, FPI+Jagged-1 Fc, and FPI+Notch1 groups. Within the ipsilateral GZ, Sham and FPI+Vehicle maintained roughly the same mean values of DCX+ cells, although both FPI+Jagged-1 Fc and FPI+Notch1 had a tendency for a slightly lower mean value in this region. On the contralateral side of injury, elevated levels of DCX+ cells were seen in the FPI+Vehicle group when compared against other groups in the GZ of the hippocampus. FPI+Notch1 maintained a lower value of doublecortin-positive cells throughout

the GZ. In the hilus, DCX+ cells were highest in the FPI+Vehicle group on both the ipsilateral and contralateral side of injury. There was a tendency for a slight increase in DCX-positive cells for both the FPI+Notch1 and FPI+Jagged-1 Fc groups when compared to sham in the ipsilateral hilus as well, but no corresponding trend on the contralateral side of injury.

Cellular Proliferation at 30 DPI

In a previous experiment carried out in our lab, cellular proliferation following TBI was analyzed at 7 DPI in the GZ and hilus regions of the hippocampus. Comparatively, in the current study we set out to determine the longer-term effects of Notch signaling following TBI at 30 DPI. This experiment helped further the understanding what happens in the DG following traumatic brain injury and what effect manipulation of the Notch signaling pathway has on cellular proliferation at 30 DPI.

Pharmacological manipulation to upregulate the Notch1 signaling pathway was directly responsible for increased cell proliferation in the hippocampus. In the current study, a significant increase in the number of BrdU+ cells was observed in the GZ and the hilus region of the contralateral side of injury, and close to significant increase in the GZ of the ipsilateral side of GZ (p=0.057) in animals that received Notch1 activator treatment compared to Sham and injured animals with Notch1 inhibitor treatment. Additionally, the FPI+Vehicle group had a significant increase of positive BrdU cells when compared to Sham animals. These data suggest that injury alone was enough to cause a significant increase in cellular proliferation/survival in the DG of animals, which is in agreement with our previous observation.[72] Our data also suggest

that activation of the Notch1 pathway on top of injury does not provide further enhancement of cell proliferation/survival as no difference was observed between injured vehicle group and Notch1 activator group. Our previous study found that TBI can enhance Notch pathway activation by upregulation of the Notch1 pathway protein expression levels during the acute post-injury stage. At this timepoint, Notch ligand and receptor binding may have already reached maximal efficiency, thus supplementation with exogenous Notch1 activator does not have any additional effect. However, in this study, inhibition of the Notch pathway using Jagged-1 Fc prevented the proliferative response, suggesting that Notch signaling is uniquely involved in the endogenous proliferative response.

Neural Generation at 30 DPI

Identification of doublecortin-positive cells has served as an important tool for observing adult neurogenesis in mammals. [73] DCX is a known microtubule associated protein that stabilizes the cellular skeleton of immature and migrating neurons. [65] In analyzing neural differentiation of our tissue, we ran the sections through a series of immunological steps using an antibody against DCX to mark newly generated neurons.

In this experiment, we observed slightly higher number of DCX-positive cells in FPI+Vehicle when compared to Sham animals. Interestingly, we saw a relative decrease in DCX-positive cells in both the FPI+Notch1 and FPI+Jagged-1 groups. In part, these finding remain consistent with what we anticipated to be true; for our FPI+Notch1 agonist group, higher levels

of proliferation were expected to result in a relative inhibition of levels of differentiation. We observed this trend throughout the granular zone and in portions of the hilus.

This trend corresponds with what we know about Notch activation and what previous studies have demonstrated as well. Upregulation of the Notch pathway should result in increased negative transcription regulators like *Hes* genes, which are known to antagonize pro-neural genes, thus preventing differentiation.[46] Other studies demonstrated that inhibition of the Notch pathway in stem cells, both in vivo and in vitro, resulted in accelerated neural differentiation.[74] DCX is a widely used marker for detecting of newly generated neurons during the 2-week period following generation. As the Notch1 activator and inhibitor were administrated only for the first 7 days post-injury, whereas the animals were sacrificed at 30 days post-injury, DCX+ cells that we observed were new neurons generated around 15-30 days post-injury, well after the termination of Notch1 manipulation. Our data suggested that short-term modulation of Notch1 signaling with pharmacological agents does not have prolonged effect on neurogenesis. Our data showed the trend of a lower number of DCX+ cells in the GZ and hilus region in animals that received Notch manipulation compared to Sham and injury-Vehicle groups. That the statistical difference is not significant is possibly due to the big variable. Increasing animal number for each group may reduce the variable and provide more accurate information.

Our data allows us to further understand how TBI affects the neurogenic regions of the brain. Upregulation of the Notch1 signaling pathway resulted in an overall increase in survival of proliferated cells in response to injury when compared to Sham animals; however, what is not yet understood is the outcome of these proliferated cells. Further studies using double-

labeling techniques, examining the fate of differentiation of the BrdU-labeled cells with different cell markers, such as mature neuronal marker, astrocytes, and other glial cell markers as well as neural stem cell markers will answer this question.

Conclusion and Future Direction

Much has been done to understand, prevent, and treat TBI over the last few decades, but much work remains. In this study, we have analyzed a portion of the long-term role that the Notch signaling pathway plays in regulating endogenous neurogenesis in the adult brain following TBI. We found that upregulation of Notch signaling, through intraventricular infusion of Notch1 antibody, causes a significant increase in cell proliferation/survival at 30 DPI in the DG of the hippocampus. While significant cellular proliferation was observed in the DG, upregulation of the Notch pathway at the acute stage post-injury does not have prolonged effect in generation of new neurons during the chronic stage following injury. As the current study lacks examination of the fate mapping of Notch1 activation-induced proliferated cells, also taking into consideration the relative low number of animals included in this study, the results of the current study are not conclusive. In order to solidify our understanding of what occurred, increasing animal numbers across groups could allow for greater clarity. Additionally, further experimentation using double-labeling to determine quantity of mature neurons (using NeuN) or astrocyte proliferation (with GFAP) could help expose wherein lies the difference in cell type.

Vita

Cruz Sevilla Jr. was born in Chicago, Illinois on April 25th, 1992. Cruz spent the first 13 years of his life living in Chicago until moving with his family to Orland Park, IL in 2005. There, he graduated from Carl Sandburg High School in 2010, before moving to Champaign, IL. He received his Bachelor of Science degree in Molecular and Cellular Biology at the University of Illinois in Urbana/Champaign in 2014. After graduation, Cruz moved to Falls Church, Virginia, and worked for three years in the fitness industry. In 2017, he found himself in pursuit of post-baccalaureate degree at Virginia Commonwealth University in Richmond, Virginia. Additionally, he received a Master of Science degree from the department of Anatomy and Neurobiology in 2019 from VCU. In August 2019, he will matriculate at Drexel University's School of Medicine as a member of the Class of 2023.

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