ROLE OF NOTCH SIGNALING AFTER TRAUMATIC BRAIN INJURY IN A TRANSGENIC MOUSE MODEL

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ABSTRACT OF THE DISSERTATION

Role of Notch Signaling After Traumatic Brain Injury in A Transgenic Mouse Model

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Traumatic brain injury (TBI) is a leading cause of disability and death in the United States and worldwide. Endogenous neural stem/progenitor cells (NSPCs) in the adult are a potential source for injury recovery. However, much about the response of injury-activated NSPCs is still unknown. Notch signaling is critical for maintaining NSPC status during embryonic development and transiently activated after injury. In the first part of this thesis, the role of Notch signaling after TBI is investigated using a Notch1CR2-GFP transgenic mouse model. During development, GFP mainly marks interneuron progenitor cells. A closed head injury (CHI) in this transgenic mouse was performed to determine the response of injury-activated NSPCs. CHI induces neuroinflammation, cell death, and the expression of typical TBI markers, validating the animal model. In addition, CHI induces cell proliferation in GFP+ cells expressing NSPC markers, e.g., Notch1 and Nestin. A significant higher percentage of GFP+ GABAergic

interneurons was observed in the CHI brain, with no significant change in oligodendrocyte lineage between the CHI and sham animal groups. Since injury is known to activate astrogliosis, these results suggest that injury-induced GFP+ NSPCs preferentially differentiate into GABAergic neurons. Our study establishes that Notch1CR2-GFP transgenic mouse is a useful tool for the study of NSPC behavior in vivo after TBI.

In the second part of this thesis, the role of Gsx1, a neurogenic transcription factor, on promoting Notch1 expression and neurogenesis is investigated. A lentivirus system is used to deliver Gsx1 at the injury site after closed head injury (CHI) in the Notch1CR2-GFP transgenic mice. We identify that CHI increases GFP+ cell, during the acute phase of TBI and increasingly label neurons during the chronic phase of TBI. Lentivirus-mediated Gsx1 overexpression increases Notch1 expressing cells in the cerebral cortex and hippocampus; these virally transduced cells proliferate and mark NSPCs during the subacute phase of TBI and primarily label glutamatergic neurons during the chronic phase of TBI. The role of Gsx1 promoting Notch signaling and neurogenesis after TBI represents a new therapeutic for the treatment of TBI. Unveiling the potential of NSPCs to TBI (e.g., proliferation and differentiation) will identify new therapeutic strategy for the treatment of brain trauma.

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ABBREVIATIONS

Caspase-3	Casp3
Closed Head Injury	CHI
Central Nervous System	CNS
Control	Ctrl
Days	D
Days Post Injury	DPI
Green Fluorescent Protein	GFP
Glasgow Coma Scale	GCS
Immunohistochemistry	IHC
Multiple Comparisons Test	MCT
Neural Stem/Progenitor Cell	NSPC
Notch1CR2-GFP (transgenic mouse)	N1CR2
Red Fluorescent Protein	RFP
Traumatic Brain Injury	TBI
Subgranular Zone	SGZ
Subventricular Zone	SVZ

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All chapters, sections, subsections, and figures are referenced to their original publication source which solely includes work where I am the first author.

It should be noted that Chapters II and III are work in submission during preparation of this thesis:

Chapter II: A novel mouse model for the study of endogenous neural stem and progenitor cells after traumatic brain injury

Chapter III: Gsx1 promotes neurogenesis after traumatic brain injury

The references accompany chapters and subsection titles and also figure legends in the main text, and can be summarized:

Chapter I

1. Traumatic brain injury (TBI)

Blunt or penetrating trauma from falls, sports injuries, vehicle accidents, and violence are the most frequent causes of traumatic brain injury (TBI). A majority of TBI cases are caused by blunt (closed head) trauma, which is estimated to account for 85-89% of injuries (Flierl et al., 2009). Due to the wide range of causes, TBI can affect all ages, genders, and demographics (Taylor et al., 2017). TBI results in temporary or permanent alteration in brain function, including loss of consciousness, memory loss, diminished motor coordination, neurologic deficits, decreased cognitive function, and/or mental impairment (Pavlovic et al., 2019). The severity of TBI is measured using the Glasgow Coma Scale (GCS) score (Teasdale and Jennett, 1974), with injuries ranging from mild to severe (Blennow et al., 2016).

Traumatic brain injury (TBI) is a leading cause of disability and death in young adults worldwide (Needham et al., 2019). There are an estimated 10 million hospitalizations or deaths from TBI-related diseases (Gardner et al., 2017) and an estimated 64-74 million new cases of TBI annually throughout the world (Dewan et al., 2018). TBI is often referred to as a silent epidemic since many cases of TBI go undiagnosed or untreated. The actual number of people affected may be higher, which adds to the scope of the TBI health crisis (Flierl et al., 2009). In the United States, TBI causes approximately 50,000 deaths and affects

approximately 1.4-2.4 million people annually, with 3.5 million people living with chronic TBI disease and 5.3 million people living with TBI-related disabilities (Faul et al., 2007; Masel and DeWitt, 2010; Taylor et al., 2017). It is estimated that TBI-related hospitalization costs are approximately $66,000 per person (Faul et al., 2007) and the annual direct and indirect costs of TBI are estimated to be approximately $50 billion dollars (Flierl et al., 2009).

Mild to severe cases of TBI can be broken down to 2 stages, the primary injury and the secondary injury. The primary injury results in mechanical tissue deformation, causing cell death and blood vessel tearing (Galgano et al., 2017; Xiong et al., 2013). Ongoing damage from the primary injury lead to the secondary injury which includes prolonged swelling, inflammation, cell death, excitotoxicity, ischemia, excitatory amino acid release, mitochondrial damage, increased free radicals, increased cytokines and chemokines, reactive oxygen species, and changes in gene expression (Blennow et al., 2016; Karve et al., 2016; Xiong et al., 2013). Both the primary and secondary injuries result in brain damage and functional deficits from the neuroinflammation and cell death. Neuroinflammation during the secondary phase activates microglia/astrocytes and clears debris after injury, necessary for improving injury recovery (Hailer, 2008; Kamelska-Sadowska et al., 2019; Needham et al., 2019; Neumann et al., 2009). However, prolonged inflammation exacerbates secondary effects and cause adverse effects on neurons and are correlated with long term health conditions including epilepsy, sleep disorders, neurodegenerative diseases,

Alzheimer's disease, and chronic traumatic encephalopathy (CTE) (Hailer, 2008; Masel and DeWitt, 2010; Needham et al., 2019; Neumann et al., 2009). The specific cellular and pathway responses after TBI are still not fully understood. Thus, there is a lack of treatments available for TBI.

There are no available treatments targeting the primary injury of TBI and promoting cellular regeneration, due to the heterogeneity and challenges of cellular regeneration *in vivo*. Current available clinical treatments focus on minimizing effects (e.g., inflammation and additional cell death) from the secondary injury, with a lack of options for regenerating cells lost from the primary injury (Hasan et al., 2017). Medical and surgical interventions for improving TBI recovery include head elevation, hyperventilation, hyperosmolar therapy, and craniotomy (Galgano et al., 2017). Additionally, current preclinical and clinical treatments available targeting the secondary injury of TBI have been minimally effective in improving cognitive, motor, and behavioral outcomes (Karve et al., 2016). Preclinical studies in rodents identified various chemical compounds that improved motor and cognitive responses, but not behavior responses (Wheaton et al., 2011b). Additionally, clinical studies in humans identified only 4 of 30 available treatments had long term improvement effects, often with early and continuous treatment (Wheaton et al., 2011a). Due to this lack of current treatments, current research is looking into using stem cells and/or neural stem/progenitor cells (NSPCs) to improve recovery after TBI. Stem cells are multipotent and have the ability to self-renew and differentiate into one or

more cell types (Xiong et al., 2018; Zhang and Wang, 2008). The goal of stem cell therapy is to use stem cell transplantation or *in vivo* gene overexpression to promote lineage adoption to replace cells lost from the injury.

2. Endogenous neural stem/progenitor cells (NSPCs) in development, adult, and injury response

Neuron migration during development was initially studied in rats, mice, and chicken embryos in the 1960s (Altman and Das, 1966; Johnston, 1966). Brain development during embryonic development uses complex and organized gene expression to define the different brain neural structures (Stiles and Jernigan, 2010). Neural stem/progenitor cells (NSPCs) give birth to all other cells in the brain and have the innate ability to self-renew and differentiate into specific mature cell lineages (Zhang and Wang, 2008).

The chorda dorsalis stimulates the ectoderm to form the neural tube, followed by neural tube compartmentalization of the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain) (Dudok et al., 2017). Neural tube formation occurs during embryonic day (E) E10.5-11 in rats, E9-9.5 in mice (with birth around E21), and E20-21 in humans (with birth around E266-280 days) (DeSesso et al., 1999; Rice and Barone, 2000; Semple et al., 2013). Neuron production in the human brain begins embryonic day 42 (E42) and

in rats around E9.5 (Rice and Barone, 2000; Semple et al., 2013; Stiles and Jernigan, 2010).

During embryogenesis, radial glial NSPCs give rise to B1 cells and E1 cells which are located in the ventricle wall (Merkle et al., 2007; Merkle et al., 2004; Spassky et al., 2005). B1 cells in the ventricular wall are progenitors for neurons and oligodendrocytes during embryonic development (Doetsch et al., 1999; Lin et al., 2015; Menn et al., 2006; Mirzadeh et al., 2008; Ortega et al., 2013) and (primarily quiescent) progenitors for olfactory bulb interneurons during adulthood (Fuentealba et al., 2015; Furutachi et al., 2015; Redmond et al., 2019). The ventricle wall contains B1 cells and E1 cells, and is a region of neurogenesis throughout adulthood (Redmond et al., 2019). A subplate zone formed during development is where glutamatergic and GABAergic neurons provide a source of neurons during development (Kostovic et al., 1989; Semple et al., 2013).

After development, the number of adult NSPCs decrease into adulthood. Although lesser in number, adult NSPCs are required for tissue homeostasis and repair (Miles and Kernie, 2006; Zhang and Wang, 2008). Adult NSPCs in lateral ventricle wall are decreased in number with increased region size as compared to the embryonic brain (Redmond et al., 2019). Endogenous NSPCs in the adult brain are primarily located in the subgranular zone (SGZ) of the dentate gyrus

(DG) in the hippocampus and the subventricular zone (SVZ) of the neocortex (Encinas and Fitzsimons, 2017; Ming and Song, 2011; Tu et al., 2017).

Despite that the majority of adult NSPCs are quiescent, injury has been demonstrated to activate NSPCs (Fuentealba et al., 2015; Wang et al., 2016; Wang et al., 2011; Zhang and Wang, 2008). The balance between quiescent and activated NSPCs are responsible for neural regeneration, injury response, and cell regeneration in healthy and injured brain states (Dixon et al., 2015; Encinas and Fitzsimons, 2017; Ludwig et al., 2018; Weston and Sun, 2018).

Adult neurogenesis has been demonstrated (Kempermann et al., 1997a, b; Kuhn et al., 1996), with cell signaling and environmental cues such as cytokines, bone morphogenetic protein (BMP), Wnt signaling, and cell-to-cell signaling (e.g., Notch) influencing adult neurogenesis and fate determination (Toda and Gage, 2018). Increased NSPC activation and neurogenesis occur after brain injury – and thus NSPCs are often considered as a therapeutic target for increasing neurogenesis post-TBI. Research has identified that injury-activated cells have the potential to differentiate into astrocytes, oligodendrocytes, and neurons (Bohrer and Schachtrup, 2016; Lim and Alvarez-Buylla, 2016; Ludwig et al., 2018; Tian et al., 2018; Vandeputte et al., 2014; Wang et al., 2016; Yan et al., 2018). Although injury-induced neurogenesis has also been identified, the way specific populations of NSPCs (e.g., Nestin+ vs. Sox2+ NSPC subpopulations)

respond (e.g., cell activation and differentiation) to injury is still under investigation. However, the effect of adult neurogenesis and injury-induced neurogenesis is not known as to what extent these newly born neurons have a beneficial or harmful influence on cognitive and behavior (Toda and Gage, 2018). Understanding the populations of NSPCs that are activated upon injury and develop into mature neural lineages is a potential for improving injury recovery. NSPCs are regulated by a variety of genes. Further investigation of the subpopulations of NSPCs and the genes driving activation will provide insight into NSPCs response to injury and treatment options.

Research is primarily focused on stem cells as a source of tissue regeneration in two major aspects, either by 1) transplanting stem cells into the adult brain or by 2) using gene delivery to perform *in vivo* overexpression and activate endogenous stem cells in the adult brain.

Stem cell transplants (e.g., embryonic neural stem cells, induced pluripotent stem cells, mesenchymal stem cells) have been evaluated to improve injury recovery, but these cells have low survival rates and inherent risk after injection into the host due to the stem cell potential to proliferate (i.e., cancer, teratoma) (Reis et al., 2017). These transplants are beneficial because they either integrate into neural networks to promote neurogenesis and improve recovery and/or secrete growth factors to stimulate neurogenesis, angiogenesis, and recovery (Blaya et

al., 2015; Xiong et al., 2009). However, stem cell transplantation treatments face a challenge in gaining regulatory approval (Reis et al., 2017).

Since injury-activated NSPCs respond to injury and integrate into neural networks but are insufficient to recover severe injury, alternative research focuses on utilizing gene delivery to activate endogenous NSPC after TBI (Dixon et al., 2015; Patel and Sun, 2016). Stimulating endogenous NSPCs has improved recovery after TBI (Dixon et al., 2015; Patel and Sun, 2016), but additional knowledge of injury-activated NSPCs and factors driving their lineage development is still needed. Research has identified that overexpression of neural transcription factors can direct neurogenesis and/or reprogram brain cells *in vivo* (Jones and Connor, 2016). It has been demonstrated that overexpression of Sox2, Ascl1, and NeuroD1 reprogram GFAP+ or NG2+ glial to neuroblasts (Jones and Connor, 2016).

Lentivirus gene delivery is commonly used in the adult mammalian brain because lentivirus efficiently deliver genes to dividing and non-dividing cells, including neurons and glial cells (Parr-Brownlie et al., 2015). The recombinant lentivirus construct can integrate into the host genome and maintain the expression in the cells infected and the progeny of dividing neural precursors (Parr-Brownlie et al., 2015). Targeted delivery (lentiviral tropism) is achieved by expression of glycoproteins on the viral surface so the virus can only bind to certain cell types

(Parr-Brownlie et al., 2015). A common pseudotyping method with vesicular stomatitis virus glycoprotein (VSVg) lipoprotein allows the lentivirus to enter most cell membranes including glial and neuron cell types (Finkelshtein et al., 2013)}(Jakobsson et al., 2003). Delivery can be further targeted by using spatial restriction, which injects a small volumes of lentivirus into specific brain regions. Here, the spread of lentivirus is restricted by particle size and diffusion through brain tissue (Cetin et al., 2006; Lerchner et al., 2014; Parr-Brownlie et al., 2015), with the spread of 1 uL of VSVg lentivirus in the brain traveling 1-2mm from the injection site (Desmaris et al., 2001; Linterman et al., 2011). Lentivirus are also good vectors because they have good safety and lower immune response than gamma retroviral vectors and adeno-associated virus (AAV) (Parr-Brownlie et al., 2015).

Many methods of gene delivery exist (e.g., lentivirus, adenovirus, small RNA, small molecule), but we utilize the lentivirus delivery system due to its ability to infect dividing and nondividing cells, safely integrate into the host, and provide continuous expression after host integration (Sakuma et al., 2012). Although used clinically, one drawback of lentivirus vector is the location of integration in the genome, which could interfere with normal gene expression if inserted in the wrong location. Lentiviral delivery provides high gene expression in multiple cell types and is advantageous to use for this proof-of-concept study (Durand and Cimarelli, 2011). This will allow us to study the initial scientific understanding of Gsx1 on the adult brain and after TBI.

The Notch gene was initially discovered to cause wing indentation in Drosophila in the 1910s (Mohr, 1919) and Notch expression was later identified in vertebrates (Coffman et al., 1990; Ellisen et al., 1991). Further studies throughout the 1900s identified the Notch gene to encode a single-pass transmembrane protein (Wharton et al., 1985) that function as a cell surface receptor (Fehon et al., 1990). It was identified that this ligand-receptor interaction affects neuronal differentiation (Sternberg, 1988), required for embryonic development (Artavanis-Tsakonas et al., 1995; Greenwald and Rubin, 1992). The Notch signaling pathway is highly conserved and influences cell differentiation, cell cycle, apoptosis, and stem cell activity in normal and diseased states (Chiba, 2006).

Several key stem cell genes regulate NSPCs during development and in the adult brain, including Notch, Sox, and Nestin (Louvi and Artavanis-Tsakonas, 2006; Tzatzalos et al., 2012; Yoon and Gaiano, 2005). Notch type-1 transmembrane receptors include 4 protein paralogs, Notch1-4 (Chiba, 2006; Steinbuck et al., 2018; Steinbuck and Winandy, 2018). During development, Notch1 is required for brain development and neurogenesis, with homozygous mutations in the Notch1 gene leading to embryo fatality by embryonic day (E) 11 (Chiba, 2006). Additionally, conditional knockout of the Notch1 gene inhibits neurogenesis, demonstrating the importance of Notch1 in embryonic neurogenesis (Chiba, 2006).

The Notch1 signaling pathway is an important regulator of NSPCs (Tu et al., 2017; Wang et al., 2012b). The evolutionarily conserved Notch1 pathways is highly involved in CNS development and in NSPC regulation and injury response in adult brains (Ables et al., 2011; Ables et al., 2010; Tzatzalos et al., 2012; Woo et al., 2009). These Notch signaling pathway also has potential to activates stems cells during recovery, but their role in the adult brain/after injury is still not fully understood (Kamelska-Sadowska et al., 2019; Tu et al., 2017).

Research has shown that Notch activated NSPCs respond to injury (Tian et al., 2018) and Notch1 signaling increases neurogenesis after TBI (Tu et al., 2017). However, the role of Notch signaling in lineage development of injury activated Notch NSPCs, tissue regeneration/repair after injury is not fully studied/understood. Chapter 2 of this thesis investigates the response of Notch1CR2-activated endogenous NSPCs in sham and TBI brains to determine their response and lineage development potential.

Genomic Screened Homeo Box (Gsh or Gsx) is a Notch1 transcription factor. There are 2 forms, Gsx1 and Gsx2, which have similar structural homeodomains but different expression patterns (Li et al., 1996). Gsx1 and Gsx2 were initially identified to be important in brain development (Li et al., 1996). It has been identified that both Gsx1 and Gsx2 regulates proliferation and differentiation of

neuronal progenitors (Szucsik et al., 1997; Toresson and Campbell, 2001), and that loss of Gsx1 and Gsx2 interferes with neurogenesis (Bergeron et al., 2015). Additional studies have identified that Gsx plays a role in neuron differentiation of GABAergic and glutamatergic neurons (Mizuguchi et al., 2006; Satou et al., 2013; Seto et al., 2014).

While Gsx2 has been identified to control activation of NSPCs and neurogenesis after adult brain injury (Lopez-Juarez et al., 2013), the role of Gsx1 has not been identified after adult brain injury. Due to the role Gsx1 plays in regulating Notch1 signaling, NSPCs development, and neuron development, we hypothesize Gsx1 also regulates adult NSPCs and neurogenesis. Chapter 3 of this thesis investigates the response of Gsx1 overexpression in endogenous NSPCs in sham and TBI brains as a potential therapeutic. Here, we use targeted lentivirus delivery to deliver Gsx1 to the hippocampus and cerebral cortex of the brain to study the effect of Gsx1 on NSPC/injury response.

3. Animal models for studying TBI and NSPCs

The use of animal models of TBI is advantageous because of the challenges of studying human TBI. Due to the lack of clinical treatments available, new animal models have been developed for studying TBI response (e.g., cellular response, molecular response, stem cell response). TBI animal models include controlled cortical impact (CCI), rotational acceleration (RA), fluid percussion injury (FPI),

weight drop injury (WDI), and blast injury (Galgano et al., 2017; Masel and DeWitt, 2010). Although these models are designed to mimic clinical TBI, no model can fully replicate the human TBI (Galgano et al., 2017). Our research uses a variation of the WDI, the closed head injury (CHI) weight drop model, to replicate closed head trauma as it is the most clinically relevant model of TBI. Since an experimental model of closed head injury (CHI) in rodents was first established to mimic TBI (Chen et al., 1996), the CHI injury model has been the most commonly used model in TBI research that is advantageous because it is not an invasive model of TBI (Bodnar et al., 2019). This injury model replicates injuries caused by sports and vehicle accidents that lead to neurological defects, inflammation, neurodegeneration, and microglial activation (Galgano et al., 2017).

A lot has been discovered with neuron development, migration, gene regulation, and injury response. However, NSPC response to injury/disease is a complex field with more to learn. Genetic tools such as knockout mouse, in utero electroporation, and Crispr-Cas9 have furthered our understanding of neurons and gene regulation (Dudok et al., 2017).

Clinical studies have failed because of the heterogeneity of the NSPC population affects their response to injury. The lack of animal models to identify/understand how the heterogeneous NSPC population responds to injury is a major gap in the

field. Recent analysis has identified that different subpopulations of NSPCs and injury-activated NSPCs have differential responses (Lugert et al., 2010). Commonly used animal models for the study of NSPCs include Nestin-GFP (Kawaguchi et al., 2001; Mignone et al., 2004; Yamaguchi et al., 2000) and Sox2-GFP (Arnold et al., 2011) transgenic mouse models which target Nestin-activated NSPCs and Sox2-activated NSPCs respectively. Although these models allow for the characterization of Nestin+ and Sox2+ NSPCs, these are only 2 distinct subpopulations of NSPCs in the adult brain responsible for NSPC regulation and injury response. In addition to Nestin and Sox2, Notch is also a key regulator of NSPCs in development and injury response (Ables et al., 2011; Ables et al., 2010; Tzatzalos et al., 2012; Woo et al., 2009). Since there are no other Notch transgenic mouse models to identify and characterize Notch1-activated NSPCs, our lab has developed a Notch1CR2-GFP transgenic mouse model where Notch1CR2-activated cells are tagged with GFP (Tzatzalos et al., 2012). This Notch1CR2-GFP mouse model labels NSPCs and neuroblasts with GFP (Tzatzalos et al., 2012), allowing for identification of these cells *in vivo* to better characterize the role/response of Notch1CR2-activated NSPCs after TBI. Here, we utilize this Notch1CR2-GFP transgenic mouse model to identify Notch1CR2-activated (GFP+) NSPCs, to characterize the role of Notch1 activation and lineage development after TBI, an under characterized subpopulation of NSPCs. Here, we combine the Notch1CR2-GFP transgenic mouse model with a CHI model of TBI to study Notch1CR2-activated NSPC

respond after injury. Chapter 2 of this thesis investigates the potential and response of Notch-activated endogenous NSPCs after TBI.

My research targets two gaps in the field of TBI, including a) utilization of a novel animal model for identification of Notch1CR2-activated NSPCs and studying TBI *in vivo*, an understudied subset of NSPCs in TBI (Chapter II), and b) evaluation of a novel Gsx1 gene therapy to promote neurogenesis *in vivo*, identifying therapeutic targets for the development of future TBI therapeutics (Chapter III). The Notch1 NSPC population is a population that highly important for NSPC maintenance and injury response, but the lineage development of these activated cells is not well characterized. Only one study has identified that Notch1-activated NSPCs migrate and differentiate into neurons at the injury site, but this was demonstrated in Zebrafish (Kishimoto et al., 2012). Out study is the first to demonstrate Notch1-activated NSPCs increase GABAergic and glutamatergic neuron populations after brain injury. Gsx1 is also an important Notch1 transcription factor that is necessary for the development of NSPCs and neurons, but the role is not known in the adult brain after TBI. Gsx2 has been demonstrated to be important for NSPC regulation in the adult brain, but this study is the first to demonstrate the role of Gsx1 on NSPC activation and lineage development in the adult brain after TBI.

One peer reviewed paper describing the first study has been submitted for publication. One manuscript describing the second study has been submitted for publication. The following review literature regarding the background of these studies, discussing gaps and significance to the field.

Chapter II

A novel mouse model for the study of endogenous neural stem and progenitor cells after traumatic brain injury

1. Prologue

The Notch1 pathway regulates NSPCs during development, in the adult brain, and after injury response. However, the specific response of Notch1-activated NSPCs after injury is not well characterized. Here, we examine the response of Notch1-activated NSPCs to identify their potential for injury recovery.

The remainder of this chapter is reproduced verbatim from a manuscript that has been submitted for publication with minor modifications.

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2. Abstract

Traumatic brain injury (TBI) is a leading cause of death and disability in the US. Neural stem/progenitor cells (NSPCs) persist in the adult brain and represent a potential cell source for tissue regeneration and wound healing after injury. The Notch signaling pathway is critical for embryonic development and adult brain injury response**.** However, the specific role of Notch signaling in the injured brain is not well characterized. Our previous study has established a Notch1CR2-GFP reporter mouse line in which the Notch1CR2 enhancer directs GFP expression in NSPCs and their progeny. In this study, we performed closed head injury (CHI) in the Notch1CR2-GFP mice to study the response of injury-activated NSPCs. We show that CHI induces neuroinflammation, cell death, and the expression of typical TBI markers, validating the animal model. In addition, CHI induces cell proliferation in GFP+ cells expressing NSPC markers, e.g., Notch1 and Nestin. A significant higher percentage of GFP+ astrocytes and GABAergic neurons was observed in the injured brain, with no significant change in oligodendrocyte lineage between the CHI and sham animal groups. Since injury is known to activate astrogliosis, our results suggest that injury-induced GFP+ NSPCs preferentially differentiate into GABAergic neurons. Our study establishes that Notch1CR2-GFP transgenic mouse is a useful tool for the study of NSPC behavior *in vivo* after TBI. Unveiling the potential of NSPCs response to TBI (e.g., proliferation and differentiation) will identify new therapeutic strategy for the treatment of brain trauma.

3. Introduction

Human traumatic brain injury (TBI) is the leading cause of death and disability in children and young adults in the United States. TBI results in temporary or permanent neurological damage including loss of memory, cognitive function, and motor function. Currently, there is no effective treatment for TBI since little can be done to reverse the tissue damage caused by trauma (Hasan et al., 2017). It is estimated that 5.3 million individuals in the United States are living with disabilities from TBI. TBI is responsible for approximately 282,000 emergency room visits and 56,000 deaths annually (Gardner et al., 2017). TBI is defined as a blow to the head that disrupts brain function and results in temporary or permanent neurological damage including loss of memory, cognitive function, and motor function (Chen et al., 2017). TBI causes cell death from the direct mechanical injury to the head (primary injury) followed by additional cell death from inflammation and swelling (secondary injury). Although the secondary injury is often reduced with anti-inflammatory medicines, cell death from the primary injury is not recoverable.

Several animal models have been established for studying TBI (Ma et al., 2019; Phipps, 2016), including the closed head injury by weight drop (CHI) (Flierl et al., 2009), lateral fluid percussion (LFP) (Van and Lyeth, 2016), a controlled cortical impact (CCI) or impact acceleration models (Campolo et al., 2018). Although no model can fully represent the spectrum of human TBI, the CHI model mimics the majority of clinical cases.

Neural stem/progenitor cells (NSPCs) are multipotent, making them a useful cell source to repair damaged and lost cells after injury (Dixon et al., 2015; Encinas and Fitzsimons, 2017; Ludwig et al., 2018; Weston and Sun, 2018). In the adult brain, endogenous NSPCs are present in the subgranular zone (SGZ) of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Encinas and Fitzsimons, 2017; Ming and Song, 2011; Tu et al., 2017). Injury induces neurogenesis in the SVZ of the cerebral cortex (Bohrer and Schachtrup, 2016; Ludwig et al., 2018; Yan et al., 2018) by stimulating NSPC proliferation and differentiation (Wang et al., 2016). NSPCs in the adult SGZ and SVZ have shown to proliferate and differentiate into various mature neural lineages, including astrocytes, oligodendrocytes, and various neuronal cell lineages (Lim and Alvarez-Buylla, 2016). However, the composition of specific cell types derived from injury-induced NSPCs has not been clearly determined (Patel and Sun, 2016; Reis et al., 2017; Zhao et al., 2008).

Several reporter mouse lines (e.g., Nestin-GFP, Pax6-GFP, Sox1-GFP, and Sox2-GFP) have been established to characterize NSPCs in the development of the CNS (Mignone, Kukekov et al. 2004, Barraud, Thompson et al. 2005, Arnold, Sarkar et al. 2011, Gao, Zhang et al. 2018). Adding to this pool of NSPC report mouse lines, we have previously established a Notch1CR2-GFP mouse line and characterized GFP+ cells as NSPCs in the developing brain (Tzatzalos, Smith et al. 2012) and spinal cord (Li, Tzatzalos et al. 2016). Notch

signaling is highly involved in the development of the central nervous system (CNS) (Louvi and Artavanis-Tsakonas, 2006; Tzatzalos et al., 2012; Yoon and Gaiano, 2005). The evolutionarily conserved Notch signaling pathway is involved in stem cell regulation and differentiation not only during development, but also in the adult brain (Ables et al., 2011; Ables et al., 2010; Woo et al., 2009). Notch1 also regulates adult NSPC quiescence and proliferation, and overexpression of Notch1 has been implicated to promote neural proliferation and self-renewal (Ables et al., 2011; Chapouton et al., 2010; Imayoshi and Kageyama, 2011; Zhou et al., 2010). Notch1 deficiency decreases NSPC number and neurogenesis (Ables et al., 2010). Studies have shown that the astrogliogenic response of the SVZ to injury is accompanied by activation of the Notch signaling pathway (Benner et al., 2013; Carlen et al., 2009; LeComte et al., 2015). Notch signaling is also known to regulate adult endogenous NSPC proliferation and neurogenesis after brain injury (Chojnacki et al., 2003; Puhakka et al., 2017). Notch1 ligands, Jagged1 and Delta1, are co-expressed in the SVZ and SGZ of the injured brain with proliferative NSPCs (Tatsumi et al., 2010; Wang et al., 2009b; Wang et al., 2012a). In addition, γ-secretase inhibitor of Notch signaling disrupts the maintenance and proliferation of NSCPs (Chojnacki et al., 2003). Different subpopulations of NSPCs exist based on their markers (e.g., Sox2, Nestin, Pax6, and Notch1), neurogenic region, cell state (e.g., quiescent vs. activated), and propensity to differentiate into mature lineages (Artegiani et al., 2017; Dulken et al., 2017). Although NSPCs are heterogeneous, many current studies have either characterized NSPCs as a homogeneous population (e.g.,

Nestin+). The Notch signaling is a key regulator of NSPCs (Giachino and Taylor, 2014). In our previous established Notch1CR2-GFP reporter line, the Notch1 enhancer CR2 directs GFP expression mainly in interneuron progenitors (Tzatzalos et al., 2012). Thus, this animal model allows the easy tracking of these GFP+ NSPCs in the developing and adult brains.

In this study, we establish a CHI model in Notch1CR2-GFP reporter mouse to examine the behavior of a unique subpopulation of Notch1+ NSPCs in response to TBI. The use of GFP reporter mouse provides the ease of identification and characterization of injury-activated NSPCs during the acute phase of TBI. During the chronic phase of TBI, NSPCs of Notch1CR2-GFP subpopulation preferentially differentiate into GABAergic interneurons in mice, suggesting an important role of Notch1 signaling in neural differentiation after injury. Thus, our study establishes that Notch1CR2-GFP transgenic mouse is a useful tool for the study of NSPC behavior *in vivo* after TBI.

4. Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee at Rutgers University. All animal work was conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Transgenic Animal

Notch1CR2-GFP (N1CR2) transgenic mouse line was generated and maintained in our lab (Tzatzalos et al., 2012). Animals of 8-12 weeks of age and with equal number of males and females were used for the experiments.

CHI Procedure

Mice 8-12 weeks of age were subjected to CHI using the protocol previously described (Flierl et al., 2009). Briefly, mice were anesthetized with 5% Isoflurane for 3-5 minutes (min) and kept under 3% Isoflurane for experimental procedure. The parietal bone was exposed by a midline scalp incision after shaving and cleaning the skin with 3 sets of betadine scrub, followed by 70% ethanol. A free-falling rod (327 g weight) with a blunt tip of 3.0 mm diameter dropped onto mouse skull 3.0 mm anterior to the lambda suture and 2.0 mm lateral to the middle line and the falling height was 3.0 cm over the skull to induce

injury without cracking the skull. After surgery, mice were allowed to recover on a heating pad until fully awake.

Behavior

Mice were evaluated beginning at 1 hour (hr) post-CHI using mNSS behavior tests by two blinded, trained observers (Flierl et al., 2009).

Tissue Preparation

At designated time points after injury, animals were euthanized, and brains were removed for tissue processing. Brains were washed in 1X PBS, and fixed with 4% (w/v) PFA for 1 day. Fixed brains were then washed four times with 1X PBS before being added to 30% (w/v) sucrose for 1-2 days. Next, the brain tissue was embedded in cryopreservation solution (Tissue Tek OCT compound) and frozen at -80°C until cryosectioning.

Immunohistochemistry (IHC)

Frozen brain tissue was cryosectioned at 12 μm using a cryostat (Thermo Shandon Cryostat Cryotome) and air dried. Tissues sections were frozen at -80°C until staining. Before staining, tissue sections were removed and stored at ambient room temperature for 30 min until beginning procedure. Sections were antigen retrieved with methanol for 10 min at room temperature, blocked and

permeabilized for 1 hour at room temperature in blocking buffer (10% donkey serum, 0.1% Triton-100, 0.1% Tween 20), then incubated with primary antibody overnight at 4°C. The next day, samples were washed with 1X PBS, then incubated with corresponding fluorophore-conjugated secondary antibodies for 1 hour at room temperature. Slides were washed with PBS, DAPI was added, then slides were dried before adding Cytoseal20 mounting media prior to fluorescent imaging. The primary antibodies used include: GFP (Abcam ab5450), Ki67 (Abcam ab15580), Nestin (Abcam ab6142), Sox2 (Millipore MAB4343), Caspase3 (Cell Signaling 661S), CD68 (Millipore mab1435), Notch1 (Abcam ab8925), DCX (Cell Signaling 4604S), NG2 (Millipore MAB5384), S100b (Abcam ab52642), Olig2 (Millipore AB9610), NeuN (Millipore MAB377), vGlut2 (Abcam ab79157), and GABA (Abcam ab8891).

Cell counting

Cell counting was performed manually on 8-12 weeks-old mouse brain sections. For each marker, 3-5 sections from at least 3 animals of each gender were counted at each time point. Since some markers were nuclear and others cytoplasmic, DAPI nuclei staining was necessary to confirm co-expression of markers. Data was presented as dot plots, where each dot represents an animal with the average result of ≥ 3 quantified cryosections from that animal. Statistics was performed on each group and error bars are represented as Mean±SEM. For statistical significance, students t-test was performed with * = $P<0.05$

significance, ** = P<0.01 significance, *** = P<0.0005 significance, and ****=P<0.0001.

Quantitative real-time PCR (qPCR)

For qPCR, total RNA was extracted from mouse SVZ, hippocampus, and cerebral cortex at specified time points with Tri Reagent. RNA was isolated, and cDNA was synthesized using qSCRIPT cDNA SuperMix. qPCR was performed using the Roche 480 LightCycler platform with SYGR Green FastMix and primers for genes of interest and GAPDH as a reference gene. Analysis was performed using the Livak method (Livak and Schmittgen, 2001).

5. Results

1) CHI induces substantial inflammation and cell death in the cerebral cortex and hippocampus

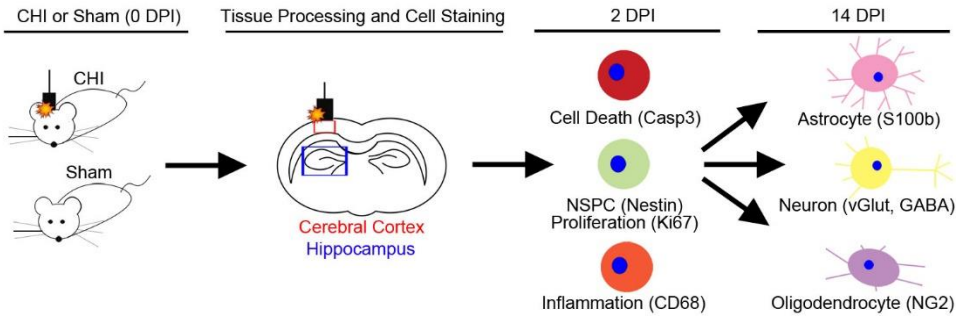

Figure 1. Experimental Design

Closed head injury (CHI) was induced by weight-drop impact in Notch1CR2-GFP mice after scalp skin opening. Sham mice did not receive weigh-drop impact injury but received an incision on the scalp to expose the skull. At 2 days post injury (DPI) and 14 DPI, brain tissue was harvested and cryosectioned in coronal orientation. At 2 dpi, markers for cell death, inflammation, cell proliferation, and TBI were examined by IHC and qPCR analysis. At 14 dpi, neural lineage markers were analyzed to determine the cell fate for injury-induced GFP+ cells. Imaging analysis was performed in both the cerebral cortex (red box) at the injury site and hippocampus (blue box) beneath the injured cerebral cortex.

We performed a closed head injury (CHI) on Notch1CR2-GFP transgenic mice using a weight-drop device (Chen et al., 1996; Flierl et al., 2009) with a drop weight of 327g at a height of 3.0 cm (Chen et al., 1996; Evanson et al., 2018; Khalin et al., 2016; Leinhase et al., 2006; Sun et al., 2018) to determine the effects of TBI on inflammation, cell death, and NSPC behavior after injury (Figure 1).

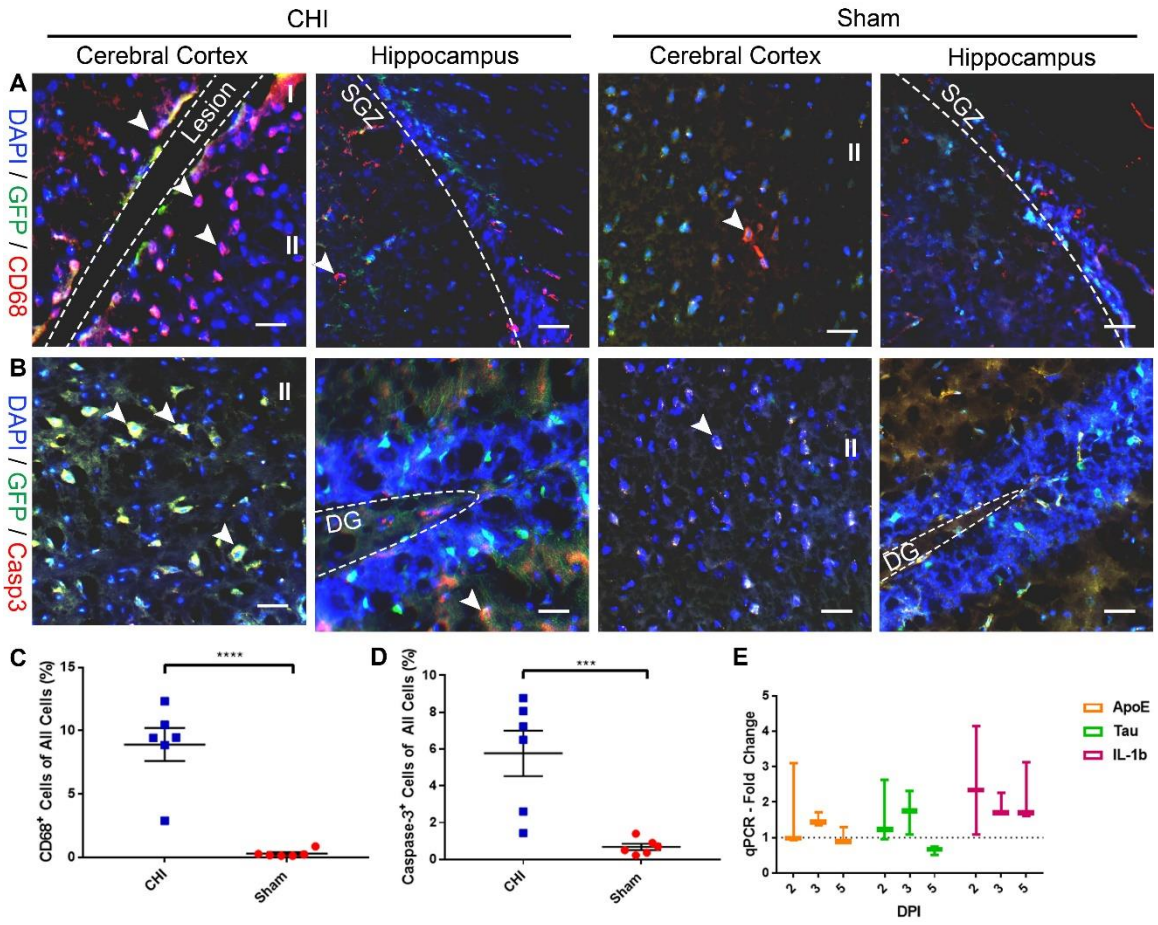

Figure 2. CHI induces substantial inflammation and cell death in the cerebral cortex and hippocampus

Injury-induced inflammation and cell death in the cerebral cortex and hippocampus were determined by immunostaining and qPCR. Cells expressing macrophage marker CD68 (**A**) and cell death marker Caspase-3 (Casp3) (**B**) were detected in coronal sections at 2 days post injury (DPI). Quantification shows a significant increase in the number of CD68+ macrophages ($p<0.0001$) (**C**) and Caspase-3+ cells ($p<0.0005$) (**D**) in the cerebral cortex of injured animals as compared to the sham animals. Scale bar=30 µm, Mean ± SEM, n=6. (**E**) qPCR analysis shows fold-change increase in markers of TBI (ApoE, Tau) and inflammation (IL-1b) at 2, 3, and 5 DPI compared to sham animals.

It has been established that TBI induces substantial neuroinflammation and cell death (Hsieh et al., 2013; Schwab et al., 2002; Stoica and Faden, 2010; Turtzo et al., 2014). Neuroinflammation involves the activation of glia (e.g.,

microglia and astrocytes) to release inflammatory mediators within the brain, and the subsequent recruitment of peripheral immune cells (Chiu et al., 2016; Schwab et al., 2002; Stoica and Faden, 2010). An increase in the number of macrophages and cell death has been observed in the cerebral cortex near the injury site after TBI (Ray et al., 2002). We thus examined neuroinflammation and cell death to confirm the extent of injury and validate the cellular response in the cerebral cortex and hippocampus by immunohistochemistry (IHC) analysis. Marker gene expression for neuroinflammation (CD68, Figure 2A) and cell death (Caspase-3 or Casp3, Figure 2B) was observed in injured and sham brain tissues at 2 days post injury (DPI). The increased number of CD68+ cells were found mostly in the ipsilateral hemisphere of the injured brain compared to the contralateral hemisphere of the brain. In the injured brain tissues, a majority of CD68+ cells were found in the ipsilateral cerebral cortex of the CHI brain at or near the site of impact compared to the sham brain (Figure 2A). A majority of Casp3+ cell death was found in the ipsilateral hemisphere of the cerebral cortex and hippocampus of the CHI brain compared to the sham brain (Figure 2B). Both GFP+ cells and non-GFP+ cells express cell death marker Casp3 near the injury site in both the cerebral cortex and hippocampus (Figure 2A) indicating that the injury is substantial. The increase in the number of cells with inflammation marker CD68 (8.91±1.30% in CHI (n=6) vs. 0.30±0.12% in sham (n=6); $p<0.0001$; Figure 2C) and cell death marker Casp3 (5.77±1.24% in CHI (n=6) vs. 0.68±0.17% in sham (n=6); $p<0.0005$; Figure 2D) was significant in injury brain tissues at 2 DPI. Antibody staining confirms that there was an increase of

markers for cell death and inflammation after CHI compared to the sham animals. The minimal expression of these markers in the sham animals confirms that the surgical procedure did not induce injury in the brain. To further confirm the CHI model, we performed qPCR analysis on TBI and inflammation markers, e.g., ApoE, Tau, and IL-1b, in the injured brain. When compared to sham animals, we found that there was an increase for TBI markers ApoE (1.67 ± 0.71 fold increase at 2 DPI) and Tau (1.72 ± 0.36 fold increase at 3 DPI) after injury (Figure 2E). The expression level of Tau was similar to a previous study in brain tissue (Pluta et al., 2018). Additionally, we also observed an increase of inflammation marker IL-1b (2.52 ± 0.89 fold increase at 2 DPI) after injury (Figure 2E). The expression level of these inflammation markers are consistent to the previous published data (Newell et al., 2018); (Kumar et al., 2016; Ma et al., 2017). Differences in NSS scores were also observed between sham and CHI animals, with CHI animals scoring higher than sham mice (Figure 2F). Thus, our results confirm that the CHI model induces substantial cell death and neuroinflammation.

2) CHI increases the number of GFP+ cells in the cerebral cortex and hippocampus

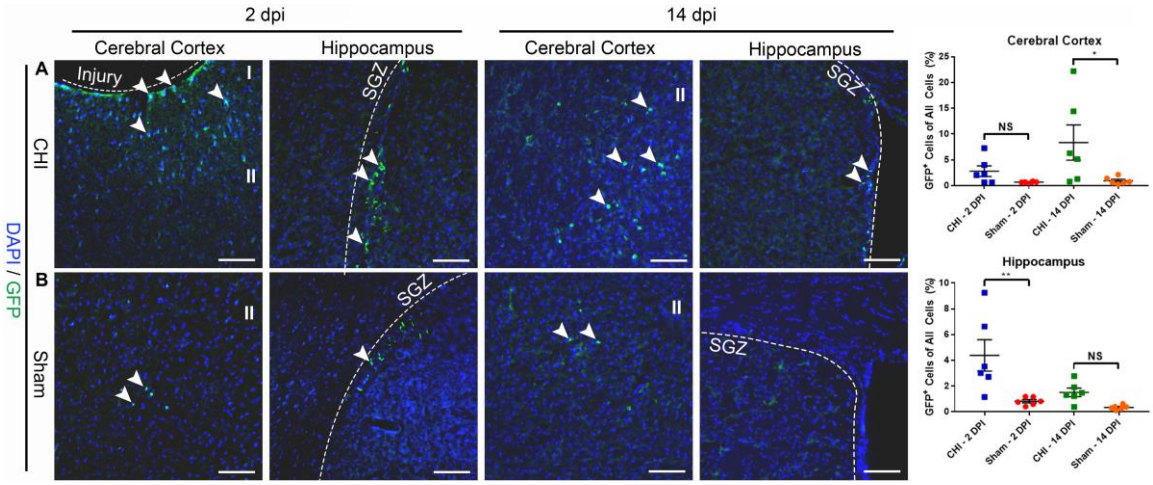

Figure 3. CHI increases the number of Notch1CR2-GFP+ cells in the cerebral cortex and hippocampus

Injury-induced GFP+ cells in the cerebral cortex and hippocampus were determined by immunostaining in a mouse model of closed head injury (CHI). GFP+ cells in the injured cerebral cortex and hippocampus (SGZ) were detected in coronal sections of the brain at 2 DPI and 14 DPI. An increased number of GFP+ cells was observed in the injured hippocampus at 2 DPI ($p<0.01$) and in injured cerebral cortex at 14 DPI ($p<0.05$) (**A**) as compared to sham mice (**B**). Scale bar=50 µm, Mean ±SEM, n=6.

With confirmation of the CHI model by markers of cell death, inflammation, and TBI, we next performed cellular and molecular characterization of GFP+ cell behavior after injury. Our previous study has shown that GFP+ cells label interneuron progenitor cells in Notch1CR2-GFP animals (Tzatzalos et al., 2012). It has been also shown that injury induces NSPCs in the SGZ of the hippocampus and the SVZ in the cerebral cortex (Bohrer and Schachtrup, 2016; Ludwig et al., 2018; Yan et al., 2018). To identify brain regions with GFP+ cells in the Notch1CR2-GFP transgenic mice, we performed IHC in the ipsilateral cerebral cortex and hippocampus. We compared the CHI (n=6, Figure 3A) and sham (n=6, Figure 3B) mice after CHI. A majority of GFP+ cells were located in

the ipsilateral cerebral cortex and hippocampus, with an increased GFP+ cell expression after injury. At 2 DPI, there was an increased number of GFP+ cells at cerebral cortex (injury site) and the SGZ, with a significant increase of GFP+ cells located in the SGZ ($p<0.01$), a known region of NSPCs in the adult brain. By 14 DPI, there was still increased GFP+ cells, but with a significant increase of GFP+ cells located in the ipsilateral cerebral cortex ($p<0.05$), indicating injury-activated NSPCs either at the site of injury or migrating to the site of injury are increased after CHI. IHC confirms that injury consistently induces GFP+ cell activation in the ipsilateral cerebral cortex and the hippocampus of the CHI animal compared to the sham animal.

3) GFP+ cells express Notch1 in the injured brain

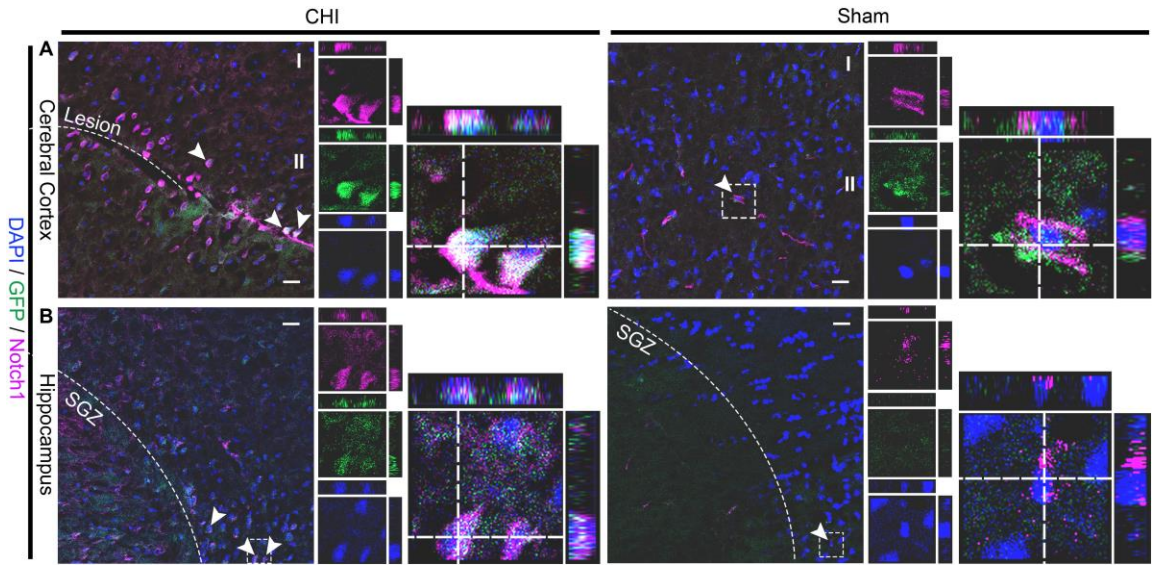

Figure 4. CHI increases the number of Notch1+ cells in the cerebral cortex and hippocampus

Injury-induced Notch1+ cells in the cerebral cortex (**A**) and hippocampus (**B**) were determined by immunostaining in a mouse model of closed head injury (CHI). Notch1+ cells in the injured cerebral cortex and hippocampus (SGZ) were detected in coronal sections of the brain at 2 DPI. An increased number of Notch1+ cells was observed in the injured hippocampus and cerebral cortex at 2 DPI as compared to sham mice. Scale bar=50 µm.

To determine whether CHI upregulates Notch1 signaling in injured brain tissue, we performed IHC on tissue sections from the cerebral cortex (Figure 4A) and hippocampus (Figure 4A) using antibody against Notch1 at 2 DPI. We found there was in increased number of Notch1+ cells after CHI in injured mice (n=6) compared to sham mice (n=6). A majority of GFP+ cells co-label with Notch1, confirming the increase of Notch1 signaling in GFP+ cells after CHI. To determine the identity of CHI-induced GFP+ cells, we next analyzed the early

response to injury (i.e., proliferation and cell death) and the lineage development (i.e., astrocytes, neurons, oligodendrocytes).

4) CHI induces cell proliferation in the hippocampus

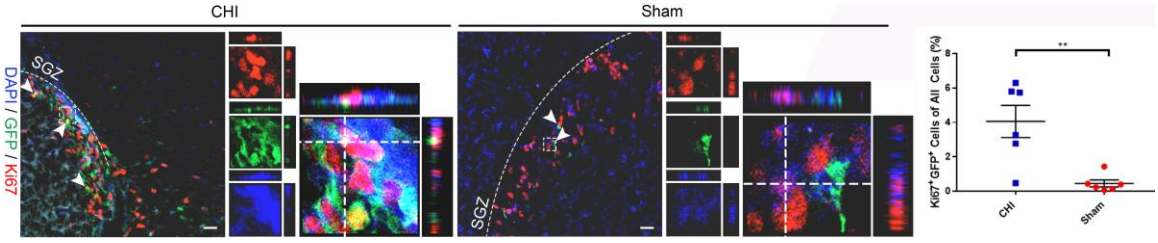

Figure 5. CHI increases cell proliferation in the hippocampus

Injury-induced GFP+ cells in the hippocampus were determined by immunostaining in a mouse model of closed head injury (CHI). Confocal images show GFP+ cells expressing cell proliferation marker Ki67 (red) in coronal sections of the hippocampus at 2 DPI. Arrowheads indicate co-labeled cells. Dash line boxed region shows an example of Ki67+GFP+ co-labeled cells in separate image channels and in a higher magnification with orthogonal views of Z-stack. Quantification shows a significant increase in the number of Ki67+ GFP+ cells (4.06±0.93% in CHI vs. 0.45±0.21% in sham; $p<0.01$) out of all the cells in the hippocampus of injured mice as compared to sham mice. Scale bar=20 µm, Mean ±SEM, n=6.

To analyze the cellular response and GFP+ cell identity after injury, we performed IHC using cell proliferation marker Ki67 at 2 DPI in the SGZ (Figure 5). The expression of Ki67 in the SGZ of the injured mouse brain was detected, a known region of NSPC proliferation (Lim and Alvarez-Buylla, 2016; Sibbe et al., 2012). Increased number of Ki67+ cells was observed in the ipsilateral hippocampus of injured mice compared to the contralateral hippocampus of injured mice and the hippocampus of sham mice. Ki67+/GFP+ co-expression

was detected in all tissue sections, with a majority of the signal observed in the SGZ. Ki67+/GFP+ co-labeled cells are significantly increased in the ipsilateral hippocampus at 2 DPI (4.06±0.93% in CHI (n=6) vs. 0.45±0.21% in sham (n=6); p<0.01; Figure 5) based on quantification of co-labeling merged fluorescence images. The most profound increase in Ki67 expression is in the SGZ, a known region where active NSPCs reside. Ki67 staining in GFP+ cells indicate injury induces cell proliferation.

5) CHI increases the number of GFP+ NSPCs in the hippocampus

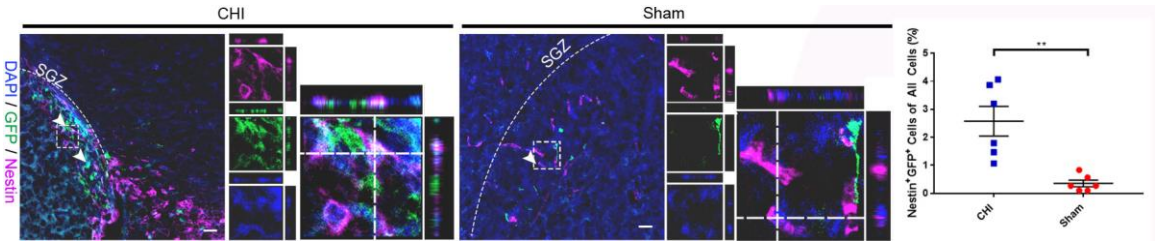

Figure 6. CHI induces Nestin+ cells in the hippocampus

Injury-induced GFP+ cells in the hippocampus were determined by immunostaining in a mouse model of closed head injury (CHI). Confocal images show GFP+ cells co-labeled with NSPC marker Nestin (purple) in the hippocampus in coronal sections at 2 DPI. Arrowheads indicate co-labeled cells. Dash line boxed region shows an example of Nestin+ GFP+ co-labeled cells in separate image channels and in a higher magnification with orthogonal views of Z-stack. Quantification shows an increase in the number of Nestin+/GFP+ cells (2.58±0.53% in CHI vs. 0.36±0.12% in sham; p<0.01) out of all the cells in the ipsilateral cerebral cortex of injured mice compared to sham mice. Scale bar=20 µm, Mean ±SEM, n=6.

To determine whether the injury-induced GFP+ cells are NSPCs, we examined the co-expression of GFP with a NSPC marker Nestin by IHC at 2 DPI in the SGZ of the hippocampus. We examined the expression levels of these injury-induced GFP+ cells and their co-labeling with Nestin in both injured and sham mice (Figure 6). Nestin+/GFP+ co-labeled cells are significantly increased in the ipsilateral hippocampus at 2 DPI (2.58±0.53% after CHI (n=6) vs. 0.36±0.12% in sham (n=6); $p<0.01$; Figure 6). This result indicates that GFP+ cells are NSPCs. The increased number of Nestin+/GFP+ cells indicates that CHI activates NSPCs and induces neurogenesis.

6) Injury-induced GFP+ NSPCs differentiate into astrocytes in the cerebral cortex

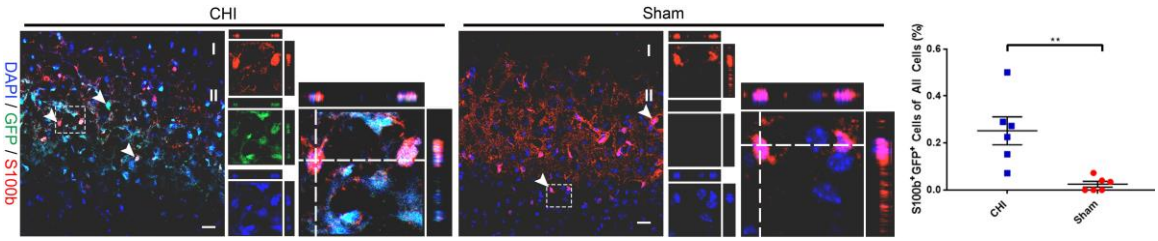

Figure 7. CHI increases GFP+ astrocytes in the cerebral cortex

The lineage adoption of injury-induced Notch1CR2-GFP+ cells in the cerebral cortex was determined by immunostaining in a mouse model of closed head injury (CHI). Confocal images show GFP+ cells expressing astrocyte lineage marker S100b in coronal sections of the cerebral cortex at 14 DPI. Arrowheads indicate co-labeled cells. Dash line boxed region shows an example of S100b+ GFP+ cells in separate image channels and in a higher magnification with orthogonal views of Z-stack Quantification shows a significant increase in the number of S100b+GFP+ (co-labeled) astrocytes (0.25±0.06% in CHI vs. 0.02±0.01% in sham; $p<0.01$) out of all the cells in the cerebral cortex of injured mice compared to sham mice. Scale bar=20 μm, Mean ±SEM, n=6.

Studies have shown that TBI-/stroke-activated NSPCs mainly give rise to astrocytes (Carlen et al., 2009; Givogri et al., 2006; LeComte et al., 2015; Makara et al., 2003; Shimada et al., 2011). It is also found that Notch signaling plays a role in astrocyte proliferation after stroke/ischemic injury (LeComte and Spees, 2016; Zhang et al., 2015). However, the extent of astrogliogenesis in injured brain is not well examined. We next determined the percentage of astrocytes express GFP by IHC at 14 DPI using astrocyte marker S100b. S100b is an astrocyte-specific CNS protein upregulated after TBI (Kim et al., 2018). IHC analysis shows that an increased number of GFP+ cells co-labeled with astrocyte marker S100b was observed in the injury site of the cerebral cortex compared to sham mice (0.25±0.06% in CHI (n=6) vs. 0.02±0.01% in sham (n=6); $p<0.01$) at 14 DPI (Figure 7). The most profound increase in S100b expression was in the cerebral cortex surrounding the injury site. The increased number of S100b+/GFP+ co-labeled astrocytes indicate injury induced enhancer Notch1CR2 activation in NSPCs, and Notch1CR2 plays an important role astrogliosis in injured brain tissue.

7) Injured-induced GFP+ NSPCs preferentially differentiate into GABAergic neurons in the cerebral cortex

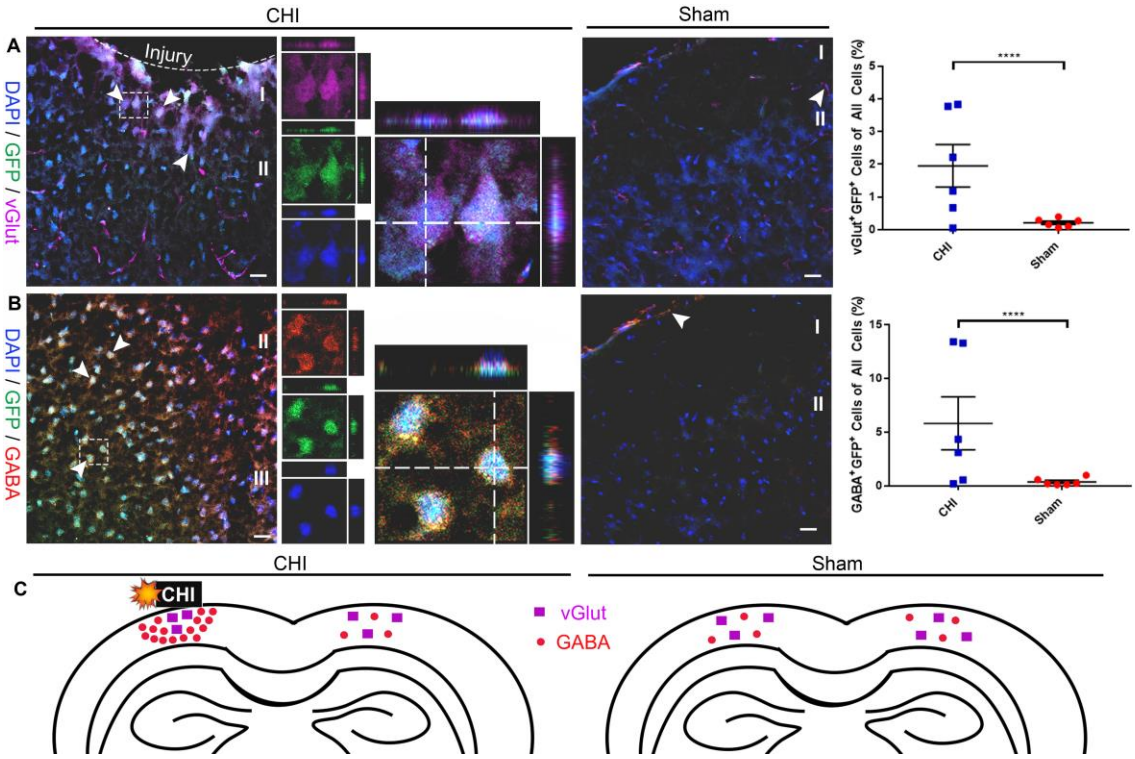

Figure 8. CHI-induced GFP+ cells contain increased number of glutamatergic and GABAergic neurons in the cerebral cortex

Lineage adoption of injury-induced Notch1CR2-GFP+ cells in the cerebral cortex was determined by immunostaining in a mouse model of closed head injury (CHI). Confocal images show GFP+ cells expressing excitatory neuronal marker vGlut (**A**) and inhibitory neuronal marker GABA (**B**) in coronal sections of the cerebral cortex at 14 DPI. Arrowheads indicate co-labeled cells. Dash line boxed region shows an example of vGlut+/GFP+ (**A**) and GABA+ GFP+ cells (**B**) in separate image channels and in a higher magnification with orthogonal views of Z-stack. Quantification shows a significant increase in the number of vGlut+/GFP+ neurons (1.95±0.65% in CHI vs. 0.21±0.05% in sham; $p<0.0001$) and GABA+GFP+ neurons (5.82±2.47% in CHI vs. 0.37±0.14% in sham; $p<0.0001$) out of all the cells in the ipsilateral cerebral cortex of injured brains compared sham brains. The approximate distribution of vGlut+ excitatory neurons and GABA+ inhibitory neurons (**C**) in the injured and sham brain is shown. Scale bar=20 µm, Mean ±SEM, n=6.

The extent of injury-induced neurogenesis has not been well characterized. Thus, the potential of injury-activated GFP+ NSPCs to differentiate into neuronal lineages was examined by IHC using various markers for neurons (GABA and vGlut2). GABA+/GFP+ and vGlut2+/GFP+ cells at 14 DPI were analyzed by confocal imaging and quantification. We found that co-labeled cells were mainly localized near the injury site in the cerebral cortex (Figure 8). The percentage of vGlut+/GFP+ neurons (1.95±0.65% in CHI (n=6) vs. 0.21±0.05% in sham (n=6); p<0.0001; Figure 8A) and GABA+/GFP+ neurons (5.82±2.47% in CHI (n=6) vs. 0.37±0.14% in sham (n=6); p<0.0001; Figure 8B) was significantly higher in CHI animas as compared to sham group (Figure 8A).

8) CHI does not affect GFP+ oligodendrocyte differentiation

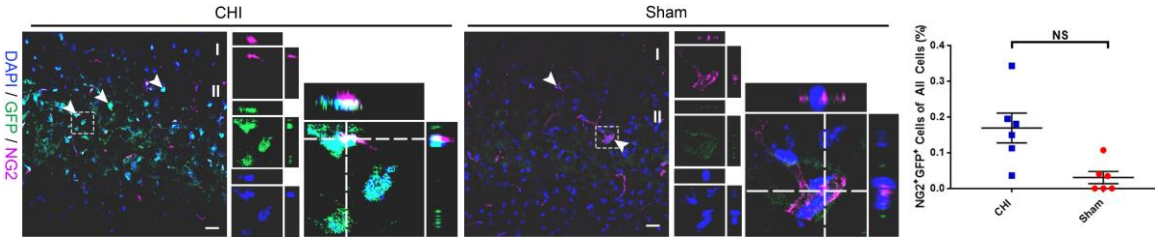

Figure 9. CHI does not alter the number of GFP+ oligodendrocyte progenitors in the cerebral cortex

The lineage adoption of injury-induced Notch1CR2-GFP+ cells in the cerebral cortex was determined by immunostaining in a mouse model of closed head injury (CHI). Confocal images show GFP+ cells expressing oligodendrocyte progenitor marker NG2 in coronal sections of the cerebral cortex at 14 DPI. Arrowheads indicate co-labeled cells. Dash line boxed region shows an example of NG2+GFP+ cells in separate image channels and in a higher magnification with orthogonal views of Z-stack. Quantification shows no significant (NS) increase in the number of NG2+GFP+ (0.17±0.04% in CHI vs. 0.03±0.02% in sham; NS) oligodendrocyte progenitors out of all the cells in the cerebral cortex of injured animals compared to sham animals. Scale bar=20 µm, Mean±SEM, n=6.

To evaluate the potential for oligodendrocyte lineage development of injury-induced GFP+ cells, we determined the fate of GFP+ cells in the ipsilateral cerebral cortex. Co-expression of NG2+/GFP+ cells was determined by IHC at 14 DPI using an oligodendrocyte progenitor cell marker NG2. GFP+ cells were found to be co-labeled with NG2 in the ipsilateral cerebral cortex after CHI (Figure 9). Although co-labeling of GFP+ oligodendrocyte progenitors was observed after injury, the number of NG2+/GFP+ co-labeled cells (0.17±0.04% in CHI (n=6) vs. 0.03±0.02% in sham (n=6); Figure 9) was not statistically significant compared to the sham at 14 DPI. These results indicate that Notch1-activated NSPCs (GFP+) have the potential to differentiate into oligodendrocytes in the injured and uninjured cerebral cortex, but injury-response does not alter oligodendrocyte lineage adoption.

9) Differences in cell type distribution of the GFP+ cells

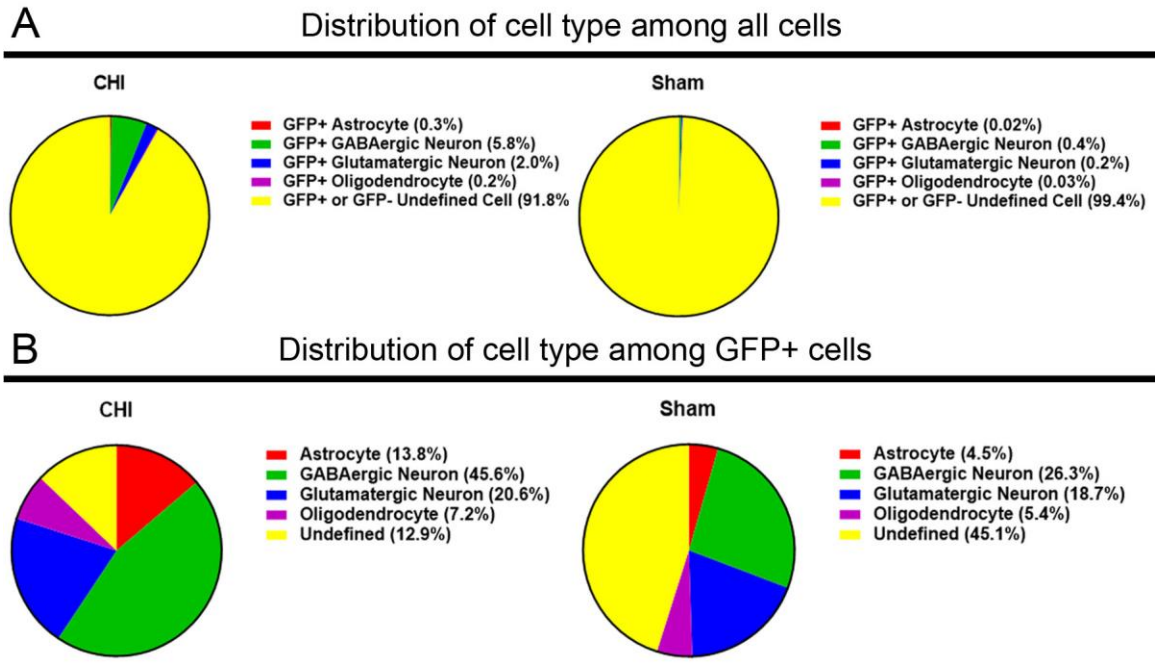

Figure 10. Distribution of the GFP+ cell population in CHI and sham brains

Pie charts (**A**) show distribution of various sham and injury-induced GFP+ neural cell populations out of all cells in CHI and sham transgenic mouse brains at 14 DPI. There is an increase of GFP+ cell types after CHI compared to sham animals. Pie charts (**B**) show distribution of various sham and injury-induced GFP+ neural cell populations out of all GFP+ cells in CHI and sham transgenic mouse brains at 14 DPI. In injured brain regions, there was an increased proportion of GFP+ astrocytes, GABAergic neurons, and glutamatergic neurons after CHI compared to sham animals.

Although the number of GFP+ cells is increased after CHI, the GFP+ cells represent a small cell population compared to the total cells in the brain (Figure 10A). Focusing only on the GFP+ cell population, we wonder whether the cellular distribution of GFP+ cells is different between the CHI and sham animal groups. Since the cellular identities (e.g., astrocytes, GABAergic neurons, glutamatergic neurons, and oligodendrocytes) of all GFP+ cells were determined by IHC, we

then calculated the percentage of GFP+ cells in each different cell type (Figure 10B). Compared with the sham group, there was a significant difference in the cellular distribution of the GFP+ cells in CHI group. Notablly, there was a significantly higher percentage of GFP+ astrocytes (13.8% in CHI vs. 4.5% in sham) and GFP+ GABAergic neurons (45.6% in CHI vs. 26.3% in sham) at the injury site in mice with CHI.

Although there was no major increase in the total number of GFP+ glutamatergic neurons between CHI (20.6%) and sham (18.7%), the location of these cells was different with a majority of the glutamatergic neurons being centralized at the injury site after injury (Figure 8C). The difference in the distribution of GFP+ glutamatergic neurons suggests a possibility that the microenvironment at the injury site may favor the glutamatergic neurons vs. GABAergic neurons.

There was no significant changes in the number of GFP+ oligodendrocytes between the CHI brain (7.16%) and the sham (5.40%) brain. The number of NG2+/GFP+ co-labeled oligodendrocyte progenitors were 7.16% in the brain with CHI compared to 5.40% in sham animals, indicating that Notch1CR2 activity may not have an effect on NSPC differentiation to the oligodendrocyte lineage.

The undefined GFP+ cells may be representative of quiescent populations of NSPCs or other specific neural lineages not evaluated in this study.

6. Discussion

In the Notch1CR2-GFP mouse line, the reporter GFP marks NSPCs for interneurons (Li et al., 2016; Tzatzalos et al., 2012). Using this reporter line, we characterize GFP+ cells in the young adult mouse after TBI. Injury induces NSPC proliferation in response with an expansion of Notch1CR2-GFP+ NSPCs at the acute phase of injury. During the subchronic phase of TBI at 14 DPI, GFP+ cells constitute a higher percentage of GABAergic neurons and astrocytes around the impact site (mainly in the SVZ and SGZ) in the injured animals. Since injury is known to induce astrocytes, it is thus suggested that Notch1CR2-GFP+ NSPCs preferentially differentiate into GABAeregic neurons.

The increased percentage of GFP+ GABAergic neurons confirms that the Notch signaling is preferentially active in GABAergic progenitors as we have reported in our previous studies (Li et al., 2016; Tzatzalos et al., 2012). These findings establish that Notch1CR2-GFP transgenic animal is a valuable model for the study of NSPC behavior after brain injury. Since our published data show that manipulating transcription factors (e.g., Nkx6.1) regulates Notch1 signaling (Li et al., 2016), our mouse line would make it simple to monitor this important

signaling pathway by tracking the GFP activity during normal and pathological conditions.

The Notch signaling pathway is essential for continuous production of NSPCs/neuroblasts and neural differentiation during development (Ables et al., 2011; Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005). It has been shown that increased Notch1 signaling activity increases NSPC proliferation, whereas inhibiting Notch1 signaling results in a reduction of proliferating cells in the SVZ (Benner et al., 2013; Oya et al., 2009; Tatsumi et al., 2010; Wang et al., 2009b). Transient activity of Notch signaling regulates proliferation and differentiation of NSPCs was observed in the injured brain (Tatsumi et al., 2010). However, its specific role in neuroregeneration after injury is still need to be investigated.

Injury-induced Notch signaling is known to contribute mainly to astrogliosis in mammals (Benner et al., 2013; Chojnacki et al., 2003; Givogri et al., 2006; LeComte et al., 2015; Tanigaki et al., 2001; Zhang et al., 2015). Consistent with the role of Notch signaling in astrogliosis, we found that CHI increases S100b+/GFP+ astrocytes. The increased number of astrocytes could be due to the following two processes: 1) injury-induced reactive astrogliosis; and/or 2) NSPC differentiation after injury.

In adult brain, NSPCs in the SVZ and SGZ generate neuroblasts that migrate and integrate into olfactory bulb circuitry or dentate gyrus (Braun and Jessberger, 2014; Ming and Song, 2011). However, the generation of new neurons at the injury site has not been well characterized. One study in zebrafish shows that the injury-induced NSPCs differentiated into Tbr1+ neurons in the regions surrounding the injury site (Kishimoto et al., 2012). However, the injury-induced neurons in mammalian brain were not well characterized. Our study reveals that mature neurons (GABAergic and glutamatergic neurons) can be induced by CHI with a significantly higher percentage of GFP+ GABAergic neurons at the injury site in Notch1CR2-GFP animals. These findings suggest that 1) Notch signaling promotes the generation of neurons after CHI; and 2) Notch signaling pathway provides a potential target for the development of TBI therapeutics/regenerative medicine.

The Notch1CR2-GFP mouse model is complementary to other animal models (e.g., Nestin-GFP) which identify the subset of Nestin+ NSPCs (Gao et al., 2009; Wang et al., 2016). Our previous studies established that GFP marks NSPCs for interneurons in the developing mouse brain (Tzatzalos et al., 2012) and spinal cord (Li et al., 2016). The higher percentage of GFP+ GABAergic neurons in the injured animals has not been reported in Nestin-GFP animals. It is not clear to what extent the cellular composition of the Notch1CR2-GFP+ cells are different from that of the Nestin-GFP+ cells. Thus, Notch1CR2-GFP mouse line provides a useful model for the study of interneurons and other neural types

(e.g., glutamatergic neurons and astrocytes) in the adult brain in normal and pathological conditions.

In summary, our cellular and molecular analysis of NSPCs in Notch1CR2-GFP animals identify a subpopulation of NSPCs that respond to injury and the extent of the Notch1-activated cellular response (e.g., activation, lineage adoption). Future studies should further investigate this response by examining the molecular mechanisms driving the injury response in specific subtypes of NSPCs that benefit functional and behavioral recovery after TBI.

7. Acknowledgements

This work was supported by the grants from the New Jersey Commission on Spinal Cord Research [08–3074-SCR-E0; 10–3091-SCR-E-0; 15IRG006]; Busch Biomedical Award [659218]; and the NIH Biotechnology Training Program [NIH T32 GM00839].

8. Supplemental Materials

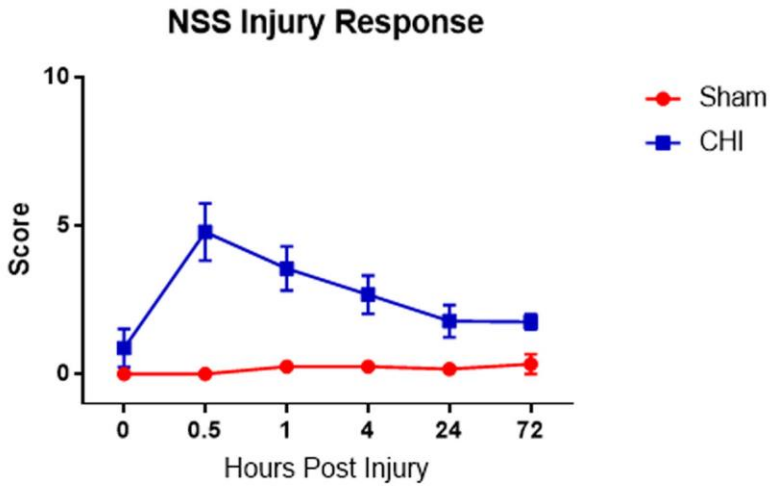

Figure 11. NSS behavior response of mice after injury

NSS assessment of mice was performed before surgery/injury (t = 0) and after sham or injury (t = 30 min, 1 hour, 4 hours, 24 hours, and 72 hours). On a scale where 0 is normal behavior and 1 is not normal (impaired) behavior, CHI mice demonstrated impaired responses after trauma. Mean±SEM, n≥3.

Chapter III

Gsx1 promotes neurogenesis after traumatic brain injury

1. Prologue

Gsx1 is a Notch1 transcription factor that is required for NSPC and neuron development. Although Gsx2, a close homologue of Gsx1, has been demonstrated to affect neural lineage development in the adult brain, the role of Gsx1 in the adult brain is not identified. Here, we identify that Gsx1 increases proliferation and NSPCs after brain injury, and that Gsx1 overexpression increases glutamatergic neurons after TBI in the adult brain. Gsx1 may represent a new therapeutic target for inducing neurogenesis after TBI.

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2. Abstract

Homeobox gene Gsx1 regulates neural stem/progenitor cells (NSPCs) in the developing central nervous system. However, the role of Gsx1 in injury response is not well defined. We show that lentivirus-mediated Gsx1 overexpression (Gsx1 treatment) enhances Notch signaling and promotes the generation of neurons after closed head injury (CHI) using a Notch1CR2-GFP transgenic mouse model. Injury activated Notch1-expressing cells and neurons were identified in the cerebral cortex and hippocampus after injury. Specifically, in the acute stage, Gsx1 treatment enhances Notch1 expression near the injury site and increases the number of NSPCs in the hippocampus. In the chronic stage of CHI, Gsx1 treatment promotes the generation of GABAergic and glutamatergic neurons. These results suggest that Gsx1 may represent a therapeutic gene for tissue damage repair and neural regeneration after traumatic brain injury.

3. Introduction

Traumatic brain injury (TBI) is one of the major causes of death and lifelong disability worldwide, with estimated 76 million new cases occur globally each year (Dewan et al., 2018). On clinical ground, TBI is categorized in mild, moderate and severe (Galgano et al., 2017), and leads to irreversible cell death, adverse effects in memory, emotional problems and impaired motor coordination (Blennow et al., 2016). To date, the main approaches to TBI are focused on minimizing neuronal cell death and the lesion size, however the neuroprotective treatments showed poor efficacy in the clinical trials (Wheaton et al., 2011a). An alternative and promising approach is the neuroregenerative strategy, which aims to increase the neurological recovery of cells in terms of neurogenesis, axonal remodeling, and angiogenesis, by leveraging the protective capacity of neural stem/progenitor cells (NSPCs) in the brain.

NSPCs reside in the subventricular zone (SVZ) of the lateral ventricles and in the subgranular zone (SGZ) of the hippocampus (Encinas and Fitzsimons, 2017; Ming and Song, 2011; Tu et al., 2017) and participate both in embryonic development and adult neurogenesis. After tissue damage, NSPCs undergo a transient expansion and migrate to the injury site where they have the potential to differentiate into neurons, astrocytes, and oligodendrocytes (Dixon et al., 2015; Encinas and Fitzsimons, 2017; Ludwig et al., 2018; Weston and Sun, 2018). This unique ability is of paramount importance to target neurogenesis following TBI.

The regulation of NSPCs in the embryonic and adult central nervous system (CNS) is orchestrated by multiple genes, in particular Notch, Sox, and Nestin (Flierl et al., 2009; Louvi and Artavanis-Tsakonas, 2006; Tzatzalos et al., 2012; Yoon and Gaiano, 2005). Notch1 is a member of Notch family and is critical in the development of CNS (Steinbuck et al., 2018; Steinbuck and Winandy, 2018). It mediates glial fate, maintains multipotency, inhibits neuronal progenitor differentiation, regulates oligodendrocyte differentiation and myelin formation. The most prominent impacts of Notch1 pathway in NSPCs are the regulation of neuronal differentiation (Lin et al., 2018) and the ability to maintain NSPC quiescence and activation in the adult brain and after injury response (Tian et al., 2018).

In search of neurogenic factors that affect Notch signaling, NSPCs, and neurogenesis, we identified a transcription factor, Gsx1, that activates the Notch1 expression during spinal cord neurogenesis via its interaction with an enhancer in the second intronic region of Notch1 gene (Tzatzalos et al., 2012). Gsx1, together with its homolog Gsx2, regulate telencephalic progenitor maturation (Pei et al., 2011) and control region-specific activation of NSPCs and injury-induced neurogenesis in the SVZ of the cerebral cortex (Lopez-Juarez et al., 2013). Gsx1 expression was dramatically downregulated in the adult brain (Gong et al., 2003). Given the critical role of Gsx1 in regulating neurogenesis (Bergeron et al., 2015; Chapman et al., 2018; Szucsik et al., 1997; Toresson and Campbell, 2001) and

specification of GABAergic and glutamatergic neurons in the CNS (Bergeron et al., 2015; Mizuguchi et al., 2006; Satou et al., 2013; Seto et al., 2014), we postulate that the upregulation of Gsx1 in the adult injured brain activates endogenous NSPCs and promotes the generation of new neurons for injury repair and regeneration.

We test the effect of Gsx1 on NSPC activation and neurogenesis after closed head injury (CHI) with lentiviral delivery of Gsx1 with an RFP reporter in Notch1CR2-GFP transgenic mice, where GFP tags the Notch1 activated neural progenitors (Tzatzalos et al., 2012). Using this approach, we found that Gsx1 activates the Notch1 signaling pathway and increases NSPC proliferation during the acute stage of CHI, and increases the generation of glutamatergic neurons in the chronic stage of CHI. These results provide important insights on the role of Gsx1 and Notch signaling in neurogenesis after TBI.

4. Materials and Methods

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biosafety Committee at Rutgers University. All animal experiments were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

Overview of experimental procedure

In injured (CHI) and sham mice, stereotaxic injection was performed to deliver lentivirus encoding Gsx1 and a reporter RFP (lenti-Gsx1-RFP) or only a reporter RFP (lenti-ctrl-RFP) to target NSPCs in the subventricular zone (SVZ) of the cerebral cortex and the subgranular zone (SGZ) of the hippocampus followed by CHI (CHI mice) (Figure 12). Injected brains were examined at 7 days post-injury (DPI) and 21 DPI in the following four animal groups: 1) CHI animals with lenti-Gsx1-RFP treatment (CHI+Gsx1), 2) CHI animals with lenti-Ctrl-RFP treatment (CHI+Ctrl), 3) Sham animals with lenti-Gsx1-RFP treatment (Sham+Gsx1); and 4) Sham animals with lenti-Ctrl-RFP treatment (Sham+Ctrl). The comparison between the two sham and two CHI groups were used to establish the role of Gsx1 in the uninjured and injured brains. At the given 7 DPI and 21 DPI, brain tissue was harvested and cryosectioned in a coronal orientation. Lentivirus transduced (RFP+) cells were characterized by IHC to determine the effects of Gsx1 treatment on neuroinflammation, cell proliferation,

and NSPC activation at 7 dpi. Lineage development of Gsx1-transfected cells (RFP+) cells were analyzed by IHC at 21 dpi.

Transgenic animal

The Notch1CR2-GFP transgenic mouse was generated and maintained in the Cai Lab as previously described (Tzatzalos et al., 2012). Transgenic mice 8-12 weeks old of both male and female genders were used for the animal experiments.

Lentivirus prep

pLenti-GIII-CMV-RFP-2A-Puro lentiviral vector with Gsx1 and an RFP reporter was purchased from ABM (LV465366). pLenti-CMV-RFP-2A-Puro-Blank lentiviral vector with a blank gene and an RFP reporter was also purchased from ABM as a control (LVP691). Human Embryonic Kidney (HEK293T) cells were cultured in high glucose Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (FBS), 1% Glutamax, and 1% nonessential amino acids (NEAA) until approximately 60% confluent before transfection with either the Gsx1 or blank (no gene) lentiviral vector, packaging plasmids (pMDLg/pRRE), and envelope plasmid (pMD2.G/VSVG). The supernatant containing the virus was removed and saved two and four days after transfection. Viral particles from the combined supernatant from both collections were concentrated by centrifugation in polyethylene glycol 6000 (PEG6000) and frozen in aliquots prior

to use. The Gsx1 virus had a titer of 1x10^8 iU/1mL and the Control (Blank) virus had a titer of 1x10^7 iU/1mL.

Procedures for closed head injury (CHI) and lentiviral injection

Transgenic mice were subject to CHI using a Nature published protocol (Flierl et al., 2009). Mice were anesthetized and the scalp was shaved, cleaned, and opened by midline scalp incision to expose the parietal bone. If receiving CHI, injured mice were subject to weight drop by a (327 g) free-falling rod from 3.0 cm from above the skull with a blunt tip 3.0 mm diameter, positioned 3.0 mm anterior to the lambda suture and 2.0 mm lateral to the midline. Weight drop created injury without cracking the skull. Sham and injured mice were then placed in earbars to receive stereotactic lentiviral injection of Gsx1-RFP or Control-RFP at a volume of 0.75µL at both the hippocampus (from bregma: -1.9 AP, -1.5 ML, -2.5 DV) and cerebral cortex (from bregma: -1.9 AP, -1.5 ML, -1.5 DV) (Jeon et al., 2018). Before injections and after injections, the needle was kept in place for 3 min to minimize bleeding and ensure delivery of the lentivirus to the targeted area. Mice were observed during recovery on a heating pad after surgery until awake.

Behavioral tests

Mice were evaluated prior to procedure and post-CHI using mNSS-R behavior test by two blinded and trained observers. There were n=6 animals tested for each condition at each time point (Yarnell et al., 2016).

Tissue preparation

At 7 DPI and 21 DPI, mice were euthanized, perfused with 4% (w/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS), and brains were removed for tissue processing. Brains were further held in 4% PFA for two days, washed with four times 30 min of 1X PBS, and then preserved in 30% (w/v) sucrose for two days. Brains were embedded in cryopreservation solution (Tissue Tek OCT) and stored at -80°C until use for cryosectioning.

Immunohistochemistry (IHC)

OCT embedded brains were cryosectioned using a cryostat (Thermo Shandon Cryostat Cryotome) set to 12 µm. Sectioned brain slices were stored at -80°C until immunohistochemistry (IHC) staining. During IHC staining, frozen tissue sections were allowed to equilibrate to ambient room temperature for 30 min. During IHC staining procedure, antigen retrieval was performed using methanol at ambient room temperature for 10 min, followed by blocking and permeabilization was performed using blocking buffer (10% donkey serum, 0.1%

Triton-100, 0.1% Tween-20) at ambient room temperature for 60 min, and primary antibody (1:50-1:1000 dilution) was added to the tissue at 4°C overnight. The next day, samples were washed using 1X PBS at ambient room temperature for 3 x 10 min prior to fluorophore-conjugated secondary antibodies (1:200 dilution to make 7.5 µg/mL) being added to the tissue sections at ambient room temperature for 60 min. Lastly, slides were washed using 1X PBS at ambient room temperature for 3 x 10 min prior to addition of DAPI for 5 min and a final washing using 1X PBS at ambient room temperature for 3 x 10 min prior to drying the slides and mounting the slides with Cytoseal20.

The primary antibodies used include: GFP (Abcam ab5450), Ki67 (Abcam ab15580), Nestin (Abcam ab6142), Sox2 (Millipore MAB4343), Caspase3 (Cell Signaling 661S), CD68 (Millipore mab1435), Notch1 (Abcam ab8925), DCX (Cell Signaling 4604S), NG2 (Millipore MAB5384), S100b (Abcam ab52642), Olig2 (Millipore AB9610), NeuN (Millipore MAB377), vGlut2 (Abcam ab79157), GABA (Abcam ab8891), and Gsx1 (Sigma SAB2104632-100µL).

Cell quantification

Manual cell counting was performed on brain sections distributed along the anterior-posterior axis with quantification of three representative sections of the injection area in both the cerebral cortex and hippocampus region per animal. For the n=6 at each time point per condition, three animals of each gender were

assessed. Cell counting was performed in a region (300 µm by 300 µm) for each of the three representative images, with consistency between the anterior to posterior region of the brain including the lentivirus injection area being analyzed. The data is presented using dot plots, where each dot represents the average of the three counted sections from a given region of a given animal. Statistics are represented as Mean±SEM. For statistical significance, ANOVA followed by Multiple Comparisons Test (MCT) was performed with * = $P<0.05$ significance, ** = $P<0.01$ significance, *** = $P<0.0005$ significance, and ****=$P<0.0001$.

5. Results

1) Gsx1 treatment increases Notch1 signaling

Given the important function of Gsx1 embryonic brain development, we postulate that Gsx1 promotes neuroregeneration after TBI. To test this hypothesis, lentivirus encoding Gsx1 with a RFP reporter (lenti-Gsx1-RFP) or control lentivirus (lenti-Ctrl-RFP) was delivered by stereotactic microinjection to the cerebral cortex and hippocampus of young adult (8-12 week old) Notch1CR2-GFP mice (Figure 12). A closed head injury (CHI) was introduced by a 327 g weight-drop at 3.0 cm height to the skull. The following groups were analyzed: Sham with 1) lenti-Gsx1-RFP or 2) lenti-Ctrl-RFP injection, and CHI with 3) lenti-Gsx1-RFP or 4) lenti-Ctrl-RFP injection. Lentivirially transduced cells (RFP+) were analyzed and compared between the Gsx1 and control groups to determine the effect of Gsx1 treatment in the brain. The viral infection rate of the lenti-Gsx1-RFP and lenti-Ctrl-RFP at 7 days post injury (DPI) was 41.4±0.9% (n=3) and 37.7±6.2% (n=3) at the injection sites, respectively (Figure 13). Lentivirus-mediated Gsx1 expression (Gsx1 treatment) was confirmed by immunohistochemistry (IHC) using anti-Gsx1 antibody. Animals receiving lenti-Gsx1-RFP injection showed high co-expression of Gsx1 and RFP (48.6±5.7%, n=3), whereas animals receiving lenti-Ctrl-RFP injection showed low levels of co-expression of Gsx1 and RFP (9.7±2.5%, n=3), indicating the Gsx1 lentivirus induced Gsx1 expression (Figure 13).

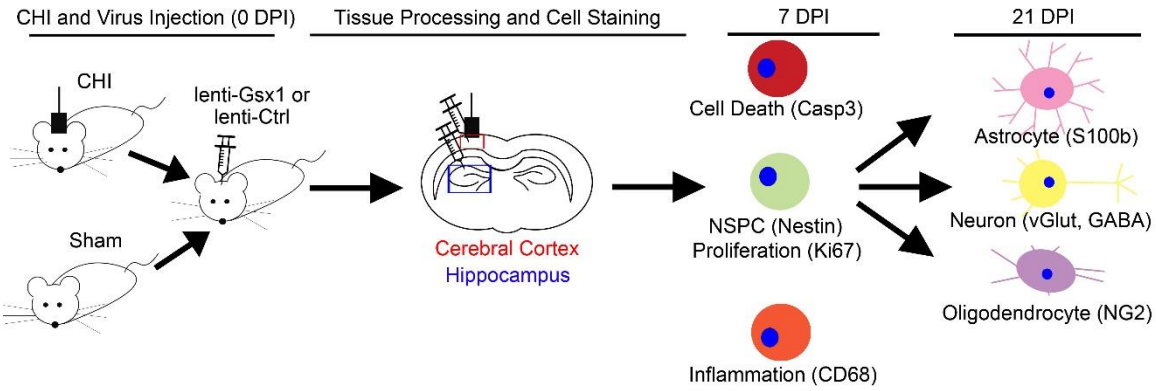


Figure 12. Experimental design. Lentivirus encoding lenti-Gsx1-RFP or lenti-ctrl-RFP was delivered by stereotactic injection into the brains (sham and CHI mice) followed by CHI (CHI mice), At 7 days post injury (DPI) and 21 DPI, brain tissue was harvested and cryosectioned in a coronal orientation. Neuroinflammation, cell proliferation, and NSPC activation was analyzed by IHC at 7 DPI. Lineage development of Gsx1-transfected cells (RFP+) cells was analyzed by IHC at 21 DPI.

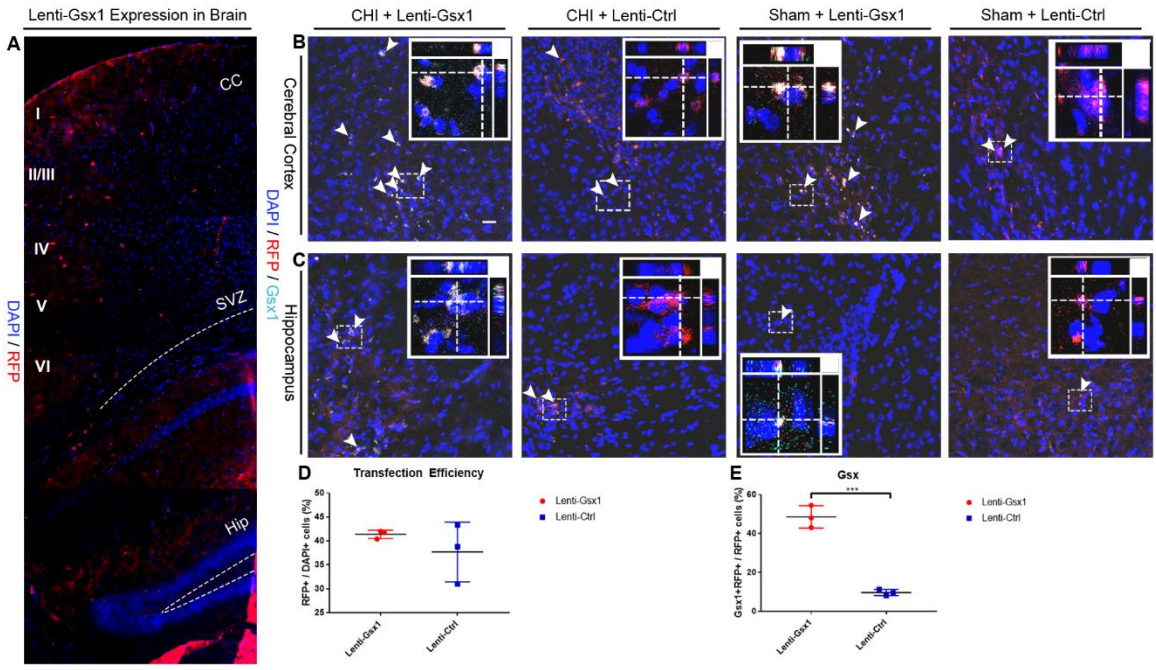


Figure 13. Gsx1 lentivirus induces RFP and Gsx1 expression. A) IHC of brain after lentivirus treatment demonstrating RFP expression at day 7. Both viral vectors achieved over 35% transfection. B,C) Lenti-Gsx1-RFP or lenti-ctrl-RFP expression is demonstrated in the uninjured and injured cerebral cortex and hippocampus. Gsx1 and RFP co-expression is demonstrated after lenti-Gsx1-RFP treatment compared to the lenti-ctrl-RFP treatment. D) Transfection efficiency of the lenti-Gsx1-RFP and lenti-ctrl-RFP is demonstrated. E). Gsx1 co-expression with RFP after treatment of lenti-Gsx1-RFP or lenti-ctrl-RFP is shown, demonstrating that Gsx1 treatment induces Gsx1 expression in over 45% of cells whereas the blank virus induces Gsx1 expression in 10% of cells.

Increased levels of Notch1 expression have been observed after brain injury (Tian et al., 2018). To determine the effect of Gsx1 treatment on Notch1 expression, IHC was performed using anti-Notch1 antibody on brain tissues at 7 DPI and 21 DPI. Gsx1 treatment increased the number of Notch1 positive cells in the cerebral cortex of both the CHI and sham mice, but only in the hippocampus of the CHI mice at 7 DPI (Figure 14, Table 1). At 21 DPI, the number of Notch1 positive cells was not significantly different between Gsx1 treatment and the

control group (Table 1). These results confirm that Gsx1 treatment increases Notch signaling in the acute stage of CHI at 7 DPI but not in a chronic stage at 21 DPI.

To further analyze changes in the Notch1 signaling pathway, the expression of Jagged1 and Hes1 was analyzed by IHC after Gsx1 treatment at 7 and 21 DPI. Jagged1 (Jag1), a ligand upstream the Notch signaling pathway, is important for NSPC maintenance, proliferation, and neurogenesis in the embryonic and adult hippocampus (Lavado and Oliver, 2014). We noticed that Gsx1 treatment increased the number of Jagged1 positive cells (except in the hippocampus of sham mice) at 7 DPI, but this increase was no longer observed at 21 DPI (Figure 21, Table 1). These results indicate that Gsx1 increases Jag1 signaling in the acute stage of CHI at 7 DPI, while the effect of Gsx1 was diminished by 21 DPI.

Hes1, a protein downstream the Notch signaling pathway, is important for regulating NSPC proliferation and differentiation in the embryonic and adult hippocampus (Zhang et al., 2014). Gsx1 treatment increased the number of Hes1 positive cells in the injured brain at 7 DPI, while at 21 DPI, Gsx1 treatment increased the Hes1 positive cells only in the cerebral cortex (Figure 22, Table 1). This indicates that Gsx1 treatment affects Notch1 downstream signaling in response to injury.

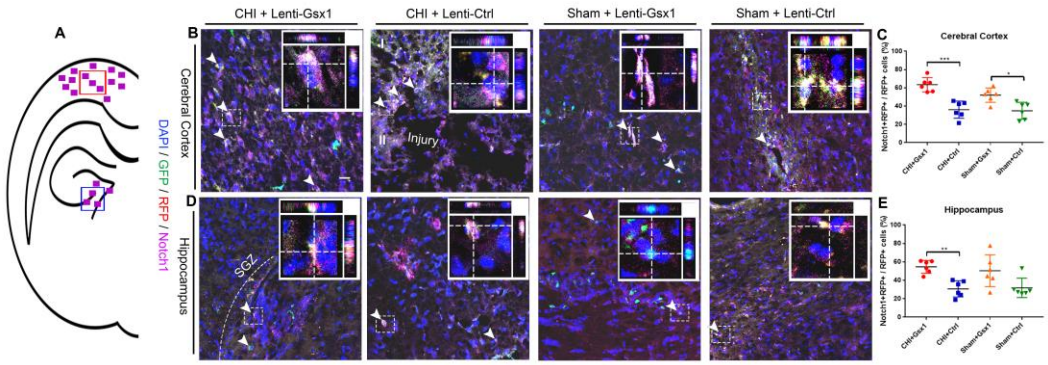


Figure 14. Gsx1 increases the number of Notch1+ cells in the uninjured and injured brain. Gsx1 treatment significantly increases the number of Notch1 positive cells in the sham and injured cerebral cortex. Although Gsx1 treatment increases the number of Notch1 positive cells in the sham and injured hippocampus, this increase was only significant after injury. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 7. Notch1 positive cells in the cerebral cortex indicated by the arrow are Notch1 positive cells (pink). B) Quantification of number of Notch1 positive cells in the cerebral cortex. C) Notch1+ cells in the hippocampus indicated by the arrow are Notch1 positive (pink). D) Quantification of number of Notch1 positive cells in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *p < 0.05, ANOVA followed by multiple comparisons test.

2) Gsx1 treatment increases macrophages, cell proliferation, and NSPC activation in the acute phase of CHI at 7 DPI

Macrophage populations within the CNS include microglia and macrophages; these brain immune cells play a pivotal role in the NSPC pool maintenance and are activated after injury (Colonna and Butovsky, 2017; Li and Barres, 2018; Sierra et al., 2010). To investigate the effect of Gsx1 on macrophage activation, we examined the number of CD68 positive cells. Gsx1 treatment significantly increased the number of macrophages in the injured cerebral cortex and

hippocampus, while no significant changes were observed in the sham mice (Figure 15, Table 1). These results suggest that Gsx1 increases macrophage activation in injured tissue but does not substantially affect macrophages in healthy tissue.

Increased cell death is a major repercussion of TBI that negatively affects memory and motor function (Clark et al., 2000; Han et al., 2001; Zhang et al., 2005). We examine the effect of Gsx1 on cell death by IHC with cell death marker, activated Caspase3 (Casp3), a protease activated during cell death and neuronal cell apoptosis (D'Amelio et al., 2010; Porter, 1999). Gsx1 treatment did not significantly alter the number of Casp3 positive cells in the cerebral cortex and hippocampus of the sham and CHI mice (Figure 23, Table 1). These results indicate that Gsx1 treatment does not affect cell survival in both the uninjured and injured adult brains.

Gsx factors are known to regulate embryonic and postnatal NSPC proliferation (Chapman et al., 2018; Lopez-Juarez et al., 2013; Mendez-Gomez and Vicario-Abejon, 2012). We used Ki67 as the cell proliferation marker to examine the number of proliferating cells after Gsx1 treatment. In the sham brains, Ki67 positive cells were primarily observed in the hippocampus and in the region surrounding the injection needle tract in the cerebral cortex. Gsx1 treatment did not significantly alter the number of Ki67 positive cells (Figure 16,

Table 1). In CHI mice, Ki67 positive cells were primarily observed in the ipsilateral cerebral cortex and hippocampus (Figure 16, Table 1). Different from Gsx1 treatment in the sham brain, Gsx1 treatment in the injured brain significantly increased the number of Ki67 positive cells in the hippocampus, suggesting that Gsx1 treatment further increases cell proliferation in the hippocampus after CHI.

A majority of adult NSPCs are quiescent (Fuentealba et al., 2015) and become activated after brain injury (Wang et al., 2016; Wang et al., 2011; Zhang and Wang, 2008). To determine the effect of Gsx1 treatment on NSPC activation, we used the NSPC marker, Nestin. In the sham brains, Nestin positive cells were primarily observed in the hippocampus and in the region surrounding the injection needle tract in the cerebral cortex. Gsx1 treatment had no significant effect on the number of Nestin positive NSPCs. After CHI, Nestin positive cells were similarly observed in the hippocampus and in the region surrounding the needle tract in the cerebral cortex. Gsx1 treatment in the injured brain significantly increased the number of Nestin positive NSPCs in the hippocampus (Figure 17, Table 1), suggesting that Gsx1 treatment further increases NSPC activation after CHI.

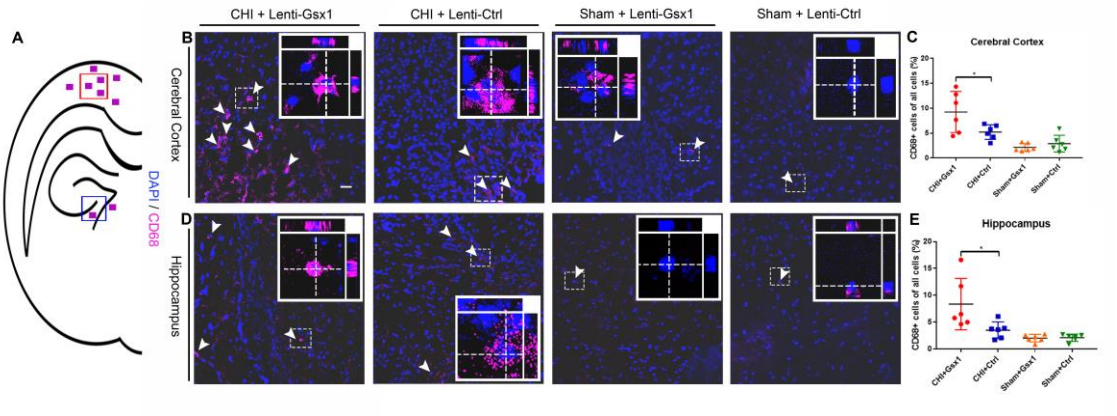


Figure 15. Gsx1 increases the number of macrophages in the subacute phase of CHI. Gsx1 treatment does not affect the number of CD68 positive macrophages in the sham cerebral cortex, but Gsx1 treatment significantly increase the number of CD68 positive macrophages in the injured cerebral cortex. Gsx1 treatment significantly increases the number of CD68 positive macrophages in the hippocampus after CHI. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 7. Macrophages in the cerebral cortex indicated by the arrow are CD68 positive cells (pink). B) Quantification of number of CD68 positive macrophages in the cerebral cortex. C) Macrophages in the hippocampus indicated by the arrow are CD68 positive cells (pink). D) Quantification of number of CD68 positive macrophages in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *p < 0.05, ANOVA followed by multiple comparisons test.

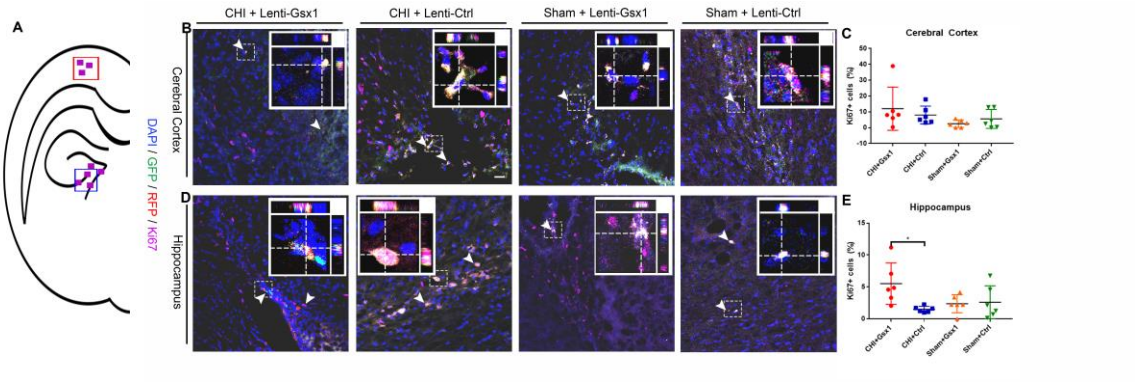


Figure 16. Gsx1 increases the number of proliferating cells in the subacute phase of CHI. Gsx1 treatment does not affect the number of Ki67 positive cells in the sham or injured cerebral cortex. Gsx1 treatment significantly increases the number of Ki67 positive cells in the hippocampus after CHI. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 7. Cell proliferation in the cerebral cortex indicated by the arrow are Ki67 positive cells (pink). B) Quantification of number of Ki67 positive cells in the cerebral cortex. C) Cell proliferation in the hippocampus indicated by the arrow are Ki67 positive cells (pink). D) Quantification of number of Ki67 positive cells in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *$p < 0.05$, ANOVA followed by multiple comparisons test.

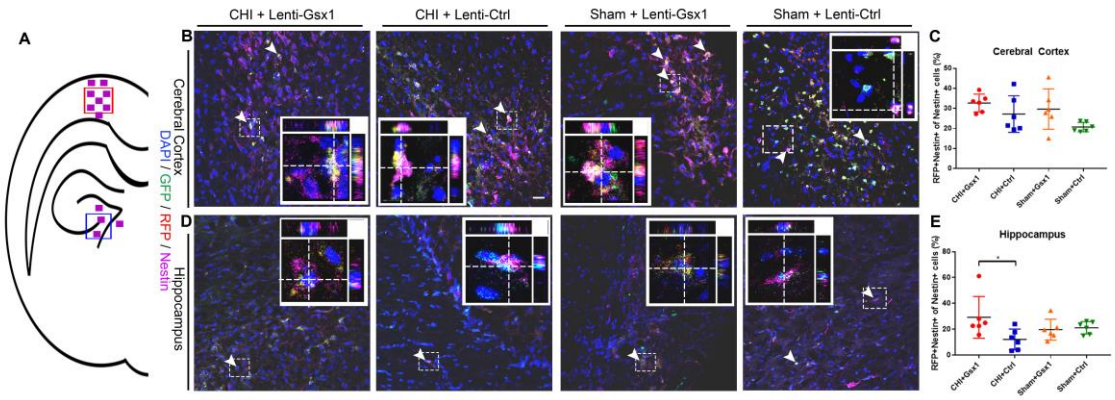


Figure 17. Gsx1 increases the number of NSPCs in the subacute phase of CHI. Gsx1 treatment does not affect the number of Nestin positive NSPCs in the sham or injured cerebral cortex. Gsx1 treatment significantly increases the number of Nestin positive NSPCs in the hippocampus after CHI. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 7. NSPCs in the cerebral cortex indicated by the arrow are Nestin positive cells (pink). B) Quantification of number of Nestin+ NSPCs in the cerebral cortex. C) NSPCs in the hippocampus indicated by the arrow are Nestin positive cells (pink). D) Quantification of number of Nestin positive NSPCs in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *p < 0.05, ANOVA followed by multiple comparisons test.

3) Gsx1 treatment promotes neurogenesis but not gliogenesis in the chronic phase of CHI at 21 DPI

Gsx factors affect differentiation of glutamatergic and GABAergic neurons during embryonic development (Mizuguchi et al., 2006; Satou et al., 2013; Seto et al., 2014). To determine the effect of Gsx1 treatment on neurogenesis in the adult mouse brain, we performed IHC using glutamatergic neuron marker, vGlut2, and GABAergic neuron marker, GABA on brain sections at 21 DPI. Gsx1 treatment significantly increased the number of vGlut2 positive cells in the CHI

hippocampus and GABA positive neurons in the sham hippocampus (Figure 18, Table 1). Overall, the RFP+ cells represent primarily GABAergic and glutamatergic neurons in the cerebral cortex and hippocampus (Figure 20). Gsx1 treatment primarily increases glutamatergic neurons in the injured hippocampus and GABAergic neurons in the uninjured hippocampus.

While increased Gsx2 expression suppresses gliogenesis in the embryonic (Chapman et al., 2018) and adult brain (Mendez-Gomez and Vicario-Abejon, 2012), the role of Gsx1 expression on gliogenesis is unclear. To evaluate the number of adult glial cells, we used astrocyte marker, S100b, and oligodendrocyte marker, Olig2. Gsx1 treatment did not have an effect on the number of astrocytes (Figure 24, Table 1) or oligodendrocytes (Figure 25, Table 1) in the cerebral cortex and hippocampus in both the sham and CHI brains. Between these two glial lineages, the RFP+ cells represent primarily oligodendrocytes over astrocytes (Figure 20). Together, these results indicate that Gsx1 treatment does not exert an effect on gliogenesis in the adult brains of either sham or CHI conditions.

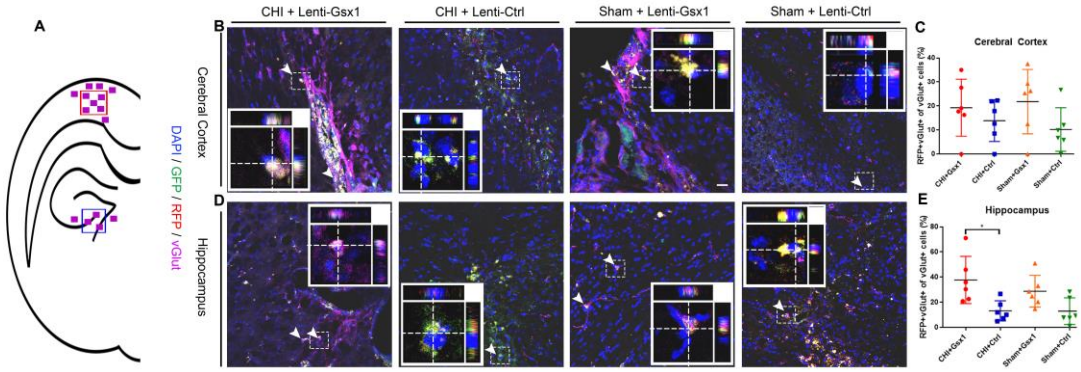


Figure 18. Gsx1 increases the number of glutamatergic neurons in the chronic phase of CHI. Although Gsx1 treatment increases the number of vGlut positive neurons in the sham and injured cerebral cortex, this increase was not significant. Although Gsx1 treatment increases the number of vGlut positive neurons in the sham and injured hippocampus, this increase was only significant after injury. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 21. Glutamatergic neurons in the cerebral cortex indicated by the arrow are vGlut positive cells (pink). B) Quantification of number of vGlut positive neurons in the cerebral cortex. C) Glutamatergic neurons in the hippocampus indicated by the arrow are vGlut positive cells (pink). D) Quantification of number of vGlut positive neurons in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *p < 0.05, ANOVA followed by multiple comparisons test.

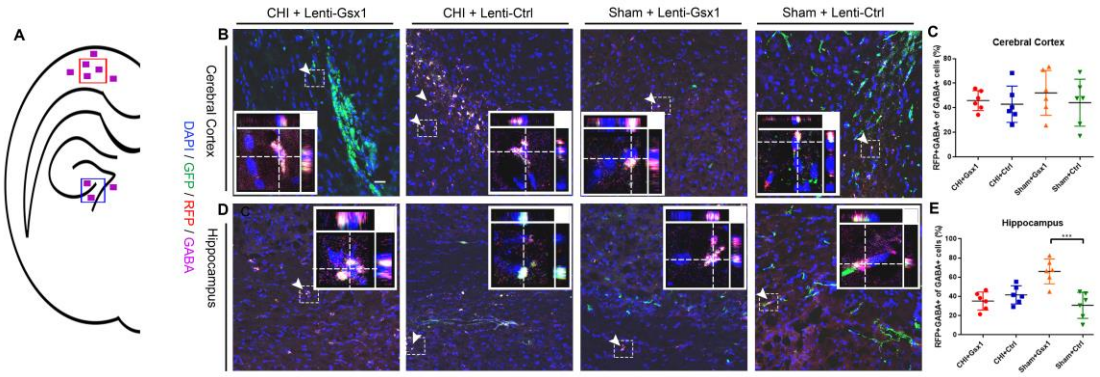


Figure 19. Gsx1 increases the number of GABAergic neurons in the uninjured brain. Gsx1 treatment does not affect the number of GABA positive neurons in the sham or injured cerebral cortex. Gsx1 treatment significantly increases the number of GABA positive neurons in the uninjured hippocampus. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 21. GABAergic neurons in the cerebral cortex indicated by the arrow are GABA positive cells (pink). B) Quantification of number of GABA positive neurons in the cerebral cortex. C) GABAergic neurons in the hippocampus indicated by the arrow are GABA positive cells (pink). D) Quantification of number of GABA positive neurons in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *p < 0.05, ANOVA followed by multiple comparisons test.

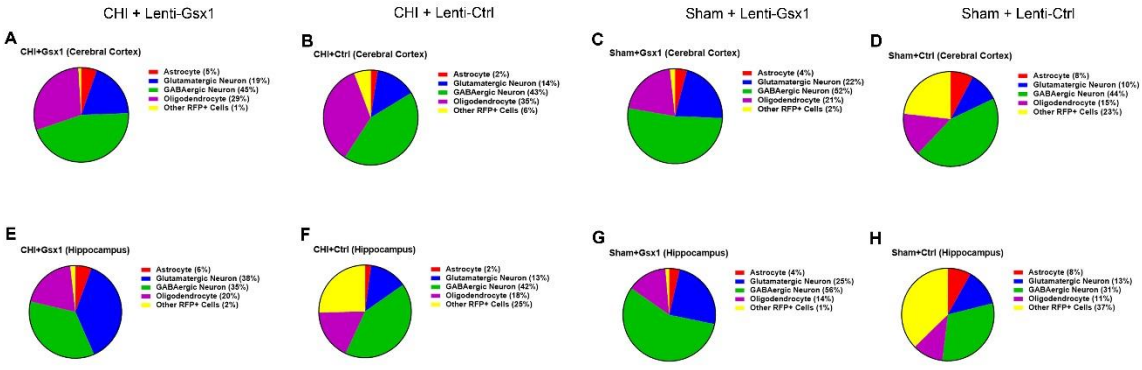


Figure 20. Distribution of RFP+ cells after injury and or Gsx1 treatment. The distribution of RFP+ cells by lineage after Gsx1 or Ctrl treatment in sham and injured mice is depicted in the A-D) cerebral cortex and E-H) hippocampus. RFP+ cells primarily co-label with GABAergic and glutamatergic neurons, and also preferentially label oligodendrocytes over astrocytes.

4) Gsx1 treatment does not alter behavioral response after CHI

TBI is known to negatively affect motor, behavior, sensory, and cognitive behavior. As Gsx1 promotes neurogenesis, we next evaluate if the treatment improves sensory and behavioral functions during the first three weeks of treatment. Modified neurological severity score (mNSS) common behavior tests were used to evaluate the effect of Gsx1 treatment on motor, behavior, and sensory outcomes. We found that the injured animals, regardless of treatment, performed worse than sham animals, due to the injury causing negative repercussions. However, Gsx1 treatment did not have a significant impact on behavior outcome (Graph 1), suggesting that Gsx1-induced neurons may not be sufficient to improve behavior functional recovery.

6. Discussion

The initial injury and subsequent inflammatory response can lead to loss of neural function. Regenerating neural connections in damaged brain tissue is a key goal following TBI. We demonstrate that Gsx1 treatment transiently up-regulates Notch signaling, activates NSPCs, and promotes the generation of glutamatergic and GABAergic neurons in a CHI mouse model. Our data demonstrate the therapeutic potential of Gsx1 gene therapy for neural regeneration after TBI.

We previously established the role of Gsx1 in Notch1 activation in the developing mouse brain and spinal cord (Tzatzalos et al., 2012; Li et al., 2016). In our current study, Gsx1 treatment increased the number of Notch1 positive cells (Figure 14), suggesting that Gsx1 functions in a similar way by inducing Notch1 signaling necessary for NSPC activation in the adult brain. Gsx1 treated brains exhibited increased RFP co-labeling with Notch1 positive cells, indicating that Gsx1 treatment induces Notch1 expression in virally infected cells (Figure 14). Gsx1 also affects the expression of Jagged 1 and Hes1 (Figure 21, 22). Since Jag1 is upstream of Notch1, the increase of Jag1 indicates a cell feedback mechanism or non-cell autonomous activation of upstream Notch pathway components, whereas the upregulation of Hes1 (downstream target) indicates a direct cell autonomous activation of Notch signaling by Gsx1. Studies have shown that Jag1 expression in epidermal growth factor receptor (EGFR) activation may play a role in NSPC activation (Choi et al., 2009), and Hes1 activation is known to activate other critical cell signaling pathways, including Hedgehog and Wnt (Liu et al., 2015). In addition, an increased neuronal migration has been identified after injury (Kishimoto et al., 2012), suggesting that the increased expression of Jag1 and Hes1 in the cerebral cortex may be a molecular mechanism for NSPC activation and increased cell migration after TBI. Gsx2, a close homolog of Gsx1, has been identified to regulate NSPC activation, proliferation and injury-induced neurogenesis (Pei et al., 2011; Mendez-Gomez and Vicario-Abejon, 2012; Lopez-Juarez et al., 2013). Here, we demonstrate that Gsx1 increases proliferation of RFP+ cells (Figure 16) and RFP+ NSPCs (Figure

17) during the acute stage of TBI at 7 DPI, indicating that a cell autonomous role of Gsx1 in promoting cell proliferation and NSPC activation. Gsx1-induced activation of NSPCs after injury suggests an important role of Gsx1 in injury response.

TBI triggers the activation and migration of macrophages (Sierra et al., 2010; Colonna and Butovsky, 2017; Li and Barres, 2018). We show that Gsx1 treatment increases the number of macrophages after TBI (Figure 15), indicating that the treatment may support the recruitment of macrophages during the acute response of TBI. Studies noted macrophage presence impacted the proliferation of neural precursor cells (Goings et al., 2006) and identified that depletion of Jag2 enhances chemoattraction of THP-1 human monocytes (Choi et al., 2009). Thus, it is possible that the role of Jag1 after TBI may also contribute to the increase of macrophages identified in this study. In addition, we noticed that Gsx1 treatment does not have an effect on cell survival (Figure 23). Consistent to these findings, a role of Gsx1 in cell apoptotic pathways has not been reported.

A major challenge for the injury recovery is the loss of neurons and lack of regeneration of neurons after injury. Identification of a factor that promotes neurons after injury is important for TBI recovery. Gsx1 has been previously identified to promote neurogenesis (Pei et al., 2011; Alvarez-Bolado, 2019) of interneurons in the CNS (Mastick et al., 1997; Mizuguchi et al., 2006), and excitatory and inhibitory neurons in the spinal cord (Mizuguchi et al., 2006). Gsx1

treatment primarily increased the number of RFP+ glutamatergic neurons (Figure 18). These findings align to other studies, which identified Gsx1 expression in glutamatergic neurons (Szucsik et al., 1997; Toresson and Campbell, 2001; Bergeron et al., 2015; Chapman et al., 2018). Our study demonstrated that Gsx1 treatment induces Notch signaling and preferential differentiation of glutamatergic neurons in the uninjured and injured brain (in a cell autonomous fashion), indicating that Gsx1 is a potential therapeutic gene for neural regeneration after TBI.

It has been demonstrated that Gsx transcription factors control neuronal versus glial specification in ventricular zone progenitors during embryonic development of the mouse brain (Chapman et al., 2018). Gsx1 misexpression in the embryonic neural progenitors resulted in a significant reduction of cortical oligodendrocyte progenitors, suggesting that Gsx factors suppress gliogenesis (Chapman et al., 2018). In contrast, our Gsx1 treatment in the adult mouse brain did not have a significant effect on the glial lineages (Figures 24-25, Table 1), indicating that Gsx1 functions differently in the adult brain. It may be beneficial for TBI recovery that Gsx1 treatment does not promote astrocytes. Reactive astrocytes form glial scar, which leads to long term adverse effects (Burda et al., 2016; Karve et al., 2016; Shinozaki et al., 2017).

TBI negatively affects behavioral responses, but Gsx1 treatment did not improve behavioral outcomes indicating that Gsx1-induced neurons alone may

not be sufficient to recover behavior damage after injury. Consistent with this notion, it has been identified that glutamatergic neurons play a role in the sensorimotor pathway and regulate the startle sensitivity response (Tabor 2018). Alternatively, more specialized tests may be necessary to further evaluate changes in memory and cognitive ability after injury (e.g., Morris water maze). In addition, the evaluation of the functionality and connectivity of the Gsx1-induced neurons (e.g., electrophysiological testing). Finally, three weeks may not be sufficient for neurons to be functional and to provide behavior recovery.

7. Acknowledgements

This work was supported by the grants from the New Jersey Commission on Spinal Cord Research [08–3074-SCR-E0; 10–3091-SCR-E-0; 15IRG006]; Busch Biomedical Award [659218]; and J.A. and M.N.P are fellows of the NIH Biotechnology Training Program [NIH T32 GM00839].

8. Supplemental Materials

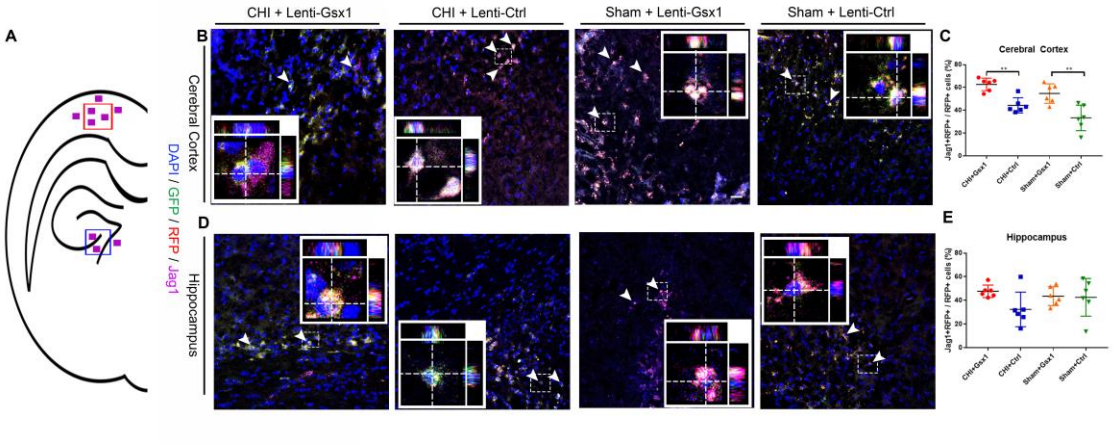


Figure 21. Gsx1 increases the number of Jagged1+ (Jag1+) cells in the uninjured and injured cerebral cortex. Gsx1 treatment significantly increases the number of Jag1 positive cells in the sham and injured cerebral cortex. Gsx1 treatment did not significantly increase the number of Jag1 positive cells in the sham and injured hippocampus. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 7. Jag1+ cells (pink) in the cerebral cortex indicated by the arrow. B) Quantification of number of Jag1 positive cells in the cerebral cortex. C) Jag1 positive cells (pink) in the hippocampus indicated by the arrow. D) Quantification of number of Jag1 positive cells in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *$p < 0.05$, ANOVA followed by multiple comparisons test.

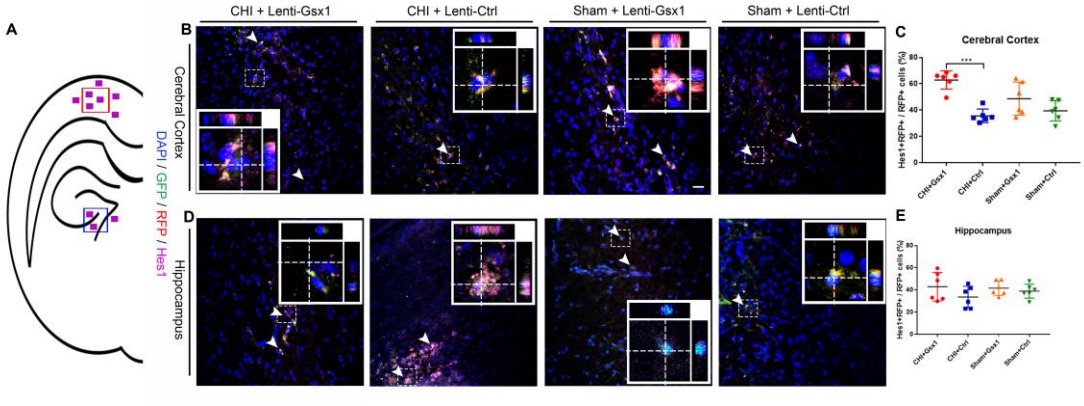


Figure 22. Gsx1 increases the number of Hes1+ cells in the uninjured and injured cerebral cortex. Although Gsx1 treatment increases the number of Hes1 positive cells in the sham and injured cerebral cortex, this increase was only significant after injury. Gsx1 treatment did not affect the number of Hes1 positive cells in the sham and injured hippocampus. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 7. Hes1 positive cells (pink) in the cerebral cortex indicated by the arrow. B) Quantification of number of Hes1 positive cells in the cerebral cortex. C) Hes1 positive cells (pink) in the hippocampus indicated by the arrow. D) Quantification of number of Hes1 positive cells in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *$p < 0.05$, ANOVA followed by multiple comparisons test.

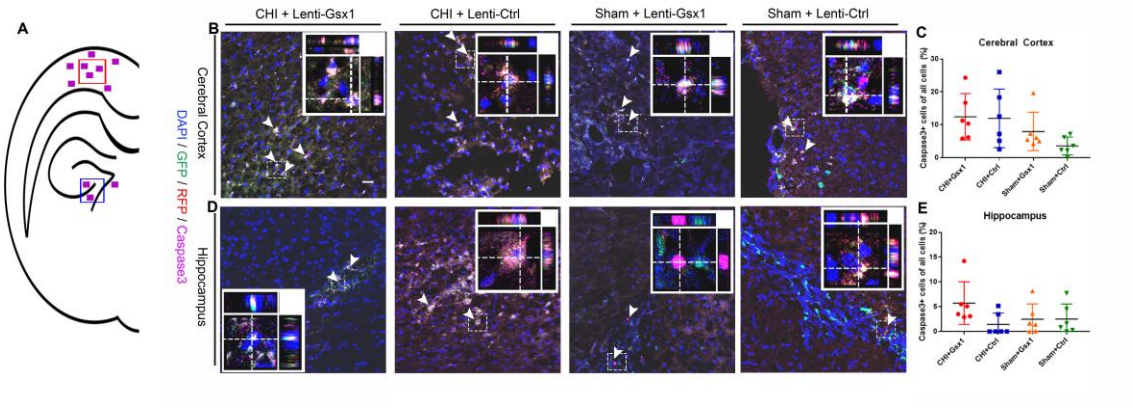


Figure 23. Gsx1 does not affect cell death in the sham or injured brain. Gsx1 treatment does not affect the number of Caspase3 positive cells in the sham or injured cerebral cortex. Gsx1 treatment does not affect the number of Caspase3 positive cells in the sham or injured hippocampus. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 7. Cell death in the cerebral cortex indicated by the arrow are Caspase3 positive cells (pink). B) Quantification of number of Caspase3 positive cells in the cerebral cortex. C) Cell death in the hippocampus indicated by the arrow are Caspase3 positive cells (pink). D) Quantification of number of Caspase3 positive cells in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *$p < 0.05$, ANOVA followed by multiple comparisons test.

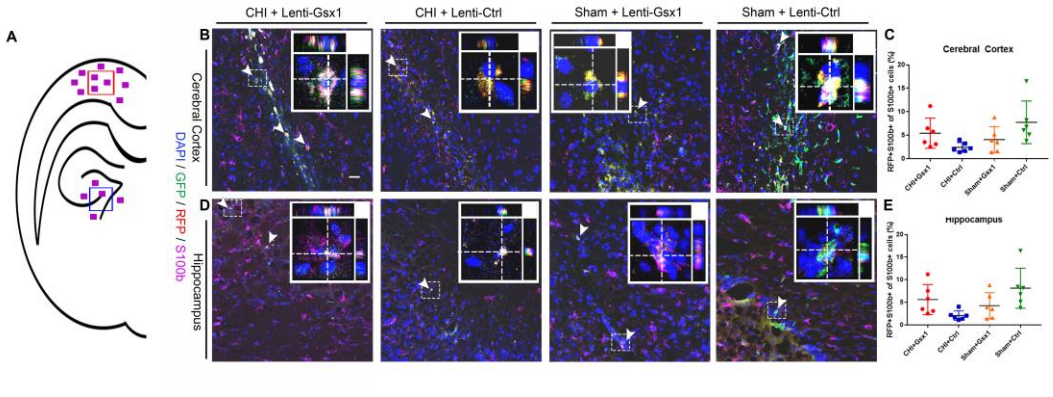


Figure 24. Gsx1 does not affect the number of astrocytes in the uninjured and injured brain. Gsx1 treatment does not affect the number of astrocytes in the sham or injured cerebral cortex. Gsx1 treatment does not affect the number of astrocytes in the sham or injured hippocampus. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 21. Astrocytes in the cerebral cortex indicated by the arrow are S100b positive cells (pink). B) Quantification of number of S100b positive astrocytes in the cerebral cortex. C) Astrocytes in the hippocampus indicated by the arrow are S100b positive cells (pink). D) Quantification of number of S100b positive astrocytes in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *$p < 0.05$, ANOVA followed by multiple comparisons test.

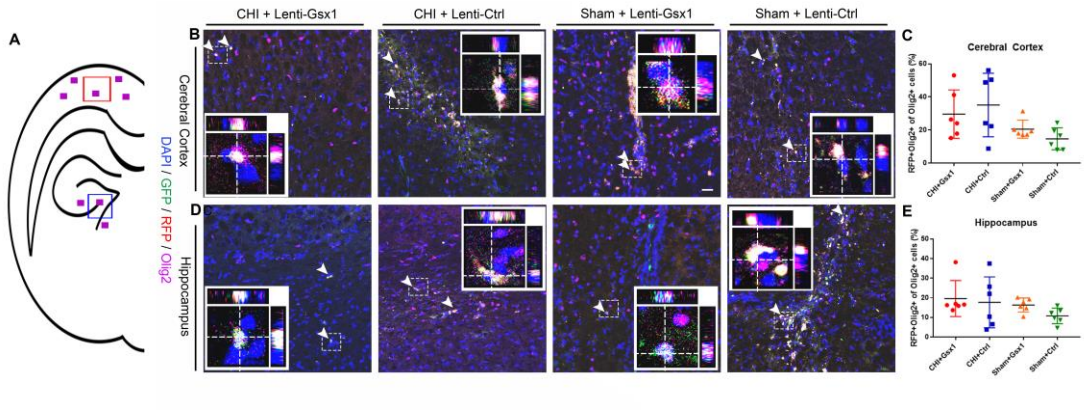
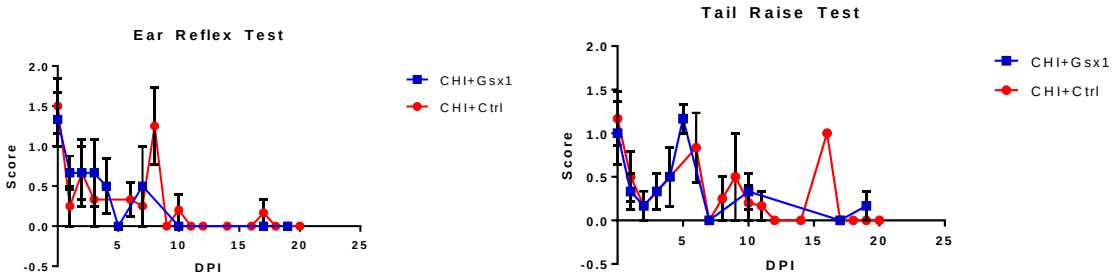


Figure 25. Gsx1 does not affect the number of oligodendrocytes in the uninjured and injured brain. Gsx1 treatment does not affect the number of oligodendrocytes in the sham or injured cerebral cortex. Gsx1 treatment does not affect the number of oligodendrocytes in the sham or injured hippocampus. A) IHC of sham or injured mouse brains were treated with lenti-Gsx1-RFP or with lenti-ctrl-RFP at day 0 and harvested at day 21. Oligodendrocytes in the cerebral cortex indicated by the arrow are Olig2 positive cells (pink). B) Quantification of number of Olig2 positive oligodendrocytes in the cerebral cortex. C) Oligodendrocytes in the hippocampus indicated by the arrow are Olig2 positive cells (pink). D) Quantification of number of Olig2 positive oligodendrocytes in the hippocampus. Error bars represent Mean Percent ± SEM, n = 6 animals per condition, *$p < 0.05$, ANOVA followed by multiple comparisons test.



Graph 1. Gsx1 does not improve behavior response after treatment. Gsx1 treatment does not increase the behavior response after injury in mice. A) mNSS cumulative scores of each condition after treatment and/or injury. Sham mice with both treatment conditions (Gsx1 and Ctrl) exhibit good behavior response since there is no injury. CHI mice with both treatment conditions (Gsx1 and Ctrl) exhibit impaired behavior response due to the injury. Gsx1 treatment did not improve behavior response in sham or injured mice. Error bars represent Mean Percent ± SEM, n = 6 animals per condition.

Table 1. Summary of Gsx1 effect in Sham and CHI mouse brains							
7 DPI							
Cellular/molecular target (marker)	***Brain Region***	***Sham***			***CHI***		
		Gsx1	**Ctrl**	**p-value**	**Gsx1**	**Ctrl**	**p-value**
Macrophage/ Microglial (CD68)	CC	2.1±0.3	2.9±0.7	p > 0.05	9.2±1.7%	5.2±0.6%	**p < 0.05**
	Hip	2.0±0.3	2.1±0.3	p > 0.05	8.4±2.0%	3.5±0.6%	**p < 0.05**
Cell Death (activated-Casp3)	CC	8.0±2.4	3.6±1.1	p > 0.05	12.4±2.9	12.0±3.6	p > 0.05
	Hip	2.5±1.3	2.5±1.2	p > 0.05	5.7±1.8	1.4±0.9	p > 0.05
Proliferation (Ki67)	CC	2.5±1.0	5.6±2.5	p > 0.05	12.0±5.6	8.0±2.3	p > 0.05
	Hip	2.4±0.6	2.6±1.1	p > 0.05	5.5±1.3%	1.5±0.2%	**p < 0.05**
NSPC (Nestin)	CC	29.6±4.1	20.7±1.0	p > 0.05	32.7±1.8	27.1±3.7	p > 0.05
	Hip	19.7±3.3	21.2±2.0	p > 0.05	29.2±6.6%	12.1±3.3%	**p < 0.05**
Notch (Notch1)	CC	52.0±3.2%	34.9±3.8%	**p < 0.05**	63.4±3.2%	36.1±3.8%	**p < 0.005**
	Hip	50.4±7.0	31.8±4.3	p > 0.05	54.6±2.9%	30.7±3.6%	**p < 0.01**
Notch upstream (Jag1)	CC	54.7±3.5%	33.3±4.6%	**p < 0.01**	62.7±2.3%	44.3±2.7%	**p < 0.01**
	Hip	43.6±3.3	42.6±6.5	p > 0.05	47.7±2.1	32.8±6.0	**p < 0.05**
Notch downstream (Hes1)	CC	48.7±5.1	39.5±3.2	p > 0.05	62.9±2.8%	35.6±2.1%	**p < 0.01**
	Hip	41.6±2.7	38.9±2.6	p > 0.05	42.9±5.3	33.6±4.0	**p < 0.05**
21 DPI							
Excitatory Neuron (vGlut2)	CC	21.6±5.5	10.2±3.7	p > 0.05	19.3±4.8	13.9±3.5	p > 0.05
	Hip	28.8±5.2	13.0±4.3	p > 0.05	37.8±7.7%	13.2±3.3%	**p < 0.05**
Inhibitory Neuron (GABA)	CC	52.1±7.5	44.2±7.8	p > 0.05	45.9±3.3-	42.9±6.0	p > 0.05
	Hip	66.1±5.3	30.8±5.5	**p < 0.005**	35.2±3.8%	41.6±3.9%	p > 0.05
Astrocyte (S100b)	CC	5.5±1.3%	2.4±0.4%	p > 0.05	4.1±1.1%	7.8±1.9%	p > 0.05
	Hip	4.3±1.2	8.1±1.8	p > 0.05	5.6±1.4	2.1±0.4%	p > 0.05
Oligodendrocyte (Olig2)	CC	20.5±2.2%	14.7±2.7%	p > 0.05	29.6±6.0%	35.1±7.8%	p > 0.05
	Hip	16.3±1.5	10.8±1.6	p > 0.05	19.6±3.7%	17.7±5.3%	p > 0.05
Notch (Notch1)	CC	46.6±7.3	38.9±5.7	p > 0.05	50.0±6.7	35.5±3.6	p > 0.05
	Hip	43.2±4.4	40.6±4.9	p > 0.05	50.8±4.5	36.3±5.0	p > 0.05
Notch upstream ligand (Jag1)	CC	32.1±3.4	43.4±5.0	p > 0.05	42.6±4.2	42.6±4.9	p > 0.05
	Hip	33.0±2.9	34.8±3.8	p > 0.05	39.0±5.9	36.7±3.9	p > 0.05
Notch downstream (Hes1)	**CC**	44.7±2.5%	33.1±3.0%	**p < 0.05**	56.6±2.3%	32.6±2.8%	**p < 0.005**
	Hip	42.4±4.1	29.7±5.4	p > 0.05	44.0±2.4	31.5±4.1	p > 0.05

Table 1. Counts of RFP+ co-labeled cells after injury and or Gsx1 treatment. The counts of RFP+ co-labeled cells after Gsx1 treatment and/or injury is shown.

Chapter IV

Conclusions and Future Direction

TBI is a major health crisis with a lack of available treatments. Research in the field of NSPC response to TBI will provide a greater understanding required for the development of future therapeutics.

Due to the complexity of NSPCs, there is a lack of understanding regarding the different responses of various subpopulations of NSPCs after injury. We utilized a Notch1CR2-GFP transgenic mouse model to characterize Notch1CR2 NSPC activation and differentiation after TBI. Although other transgenic animals exist (e.g., Sox2-GFP, Nestin-GFP), these models characterize different subsets of the heterogeneous NSPC population. Since Notch1 is a key regulator of NSPCs in development and injury response, we characterize their role in lineage development after TBI. Here we examined Notch1CR2-activated NSPCs to characterize this subpopulation of NSPCs after TBI. Other studies have identified that injury activates Notch1 cells (Miles and Kernie, 2006; Zhang and Wang, 2008); with one study reveals that Notch1-activated cells differentiate into neurons after TBI in zebrafish (Kishimoto et al., 2012). Our study (Chapter II) identified that Notch1CR2-activated cells increasingly differentiate into neurons, specifically GABAergic and glutamatergic neurons, after TBI in a mouse model. Understanding how this Notch1CR2 NSPC population responds to disease and

increases neuron populations make them a candidate for future studies of brain disease.

There is also a lack of treatments for improving cellular recovery after TBI. Studies have identified that the transcription factor, Gsx1, is important in regulating NSPC activation and differentiation during development (Lopez-Juarez et al., 2013). Although Gsx2 has been identified to regulate injury-activated NSPCs and neurogenesis in the adult brain (Lopez-Juarez et al., 2013; Pei et al., 2011)(Alvarez-Bolado 2019), the role of Gsx1 in the adult brain and after TBI is not characterized. This innovative study is the first to identify the role of a novel transcription factor, Gsx1, on proliferation, macrophage recruitment, and NSPC activation and differentiation after TBI (Chapter III). This research demonstrated that Gsx1 treatment increases NSPCs and (GABAergic and glutamatergic) neurons in the adult brain and after TBI. Overexpression of Gsx1 is a useful therapeutic for increasing NSPC activation and neurogenesis after brain disease. Additionally, this Notch1CR2 animal model is a useful tool in the study of other aspects of CNS development and disease states (e.g., ischemia, stroke, neurodegeneration).

This study identifies that TBI increases Notch1CR2 (GFP+) expression and Gsx1 treatment increases Notch1+ cells after injury, leading to NSPC activation and neurogenesis. Our study evaluated the motor and sensory response of mice to

Gsx1 treatment and did not identify a significant increase in behavior response. Additional cognitive testing should be evaluated, including tests such as Morris Water Maze that evaluate memory and learning in mice. Future studies should additionally further characterize the response of these activated cells using lineage tracing to demonstrate neuronal migration and electrophysiology testing to determine functionality of injury-activated neurons. Since it is unclear if NSPC proliferation results in mature neurons and how functional/integrative into neural networks these neurons are (Gao and Chen, 2013). Further evaluation of other cell/pathway changes can also highlight therapeutic targets for future TBI therapies.

Additional considerations for future directions include evaluation of dosage, clinically relevant delivery methods, and timing of treatment. Studies have identified that dose and time of treatment greatly influence success of stem cell therapies after injury (Kamelska-Sadowska et al., 2019).

Overall, stem cells provide good therapeutic potential – whether it is injected or manipulated *in vivo*. Due to the complexity of stem cells, further evaluation into the gene and protein expression changes after treatment are also necessary to be investigated (e.g., single-cell sequencing). A greater understanding though research regarding NSPC development, adult regulation, and injury response will

allow researchers to further understand their potential and develop new therapeutics.

Current standards of care (e.g., medical and surgical) manage TBI patients rather than promote recovery. With TBI being a widespread disease and more survivors requiring better treatments, research into TBI will continue to focus on neurorestoration and neurorehabilition to improve outcome for these patients and their quality of life (Galgano et al., 2017; Teasdale and Jennett, 1974).

Chapter V

Appendix:

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