2017

The Effects of Alcohol and Traumatic Brain Injury on Neural Stem Cell Responses

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THE EFFECTS OF ALCOHOL AND TRAUMATIC BRAIN INJURY ON NEURAL STEM CELL RESPONSES

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY

SON T. TON

CHICAGO, ILLINOIS

MAY 2017

ACKNOWLEDGEMENTS

First and foremost, I want to thank my adviser Gwendolyn Kartje. Dr. Kartje has been an incredibly supportive mentor. Her advice and insights have been crucial to the success of this dissertation. I appreciate her willingness to give time, ideas and funding to make possible my Ph.D. experience. The members of the Kartje lab have been a source of friendship, ideas and support. I would like to thank Ian Vaagenes, for getting me up to speed with my research project when I first started out in the Kartje lab. Also, Shih-Yen Tsai contributed immensely to my research project with his surgical expertise. Additionally, various summer students in the Kartje lab (Dan Nockels, Kelly Glavin, Hannah Flink, Joanna Wu, Jonathan Hsu) helped in performing experiments and collecting valuable data. My committee has been extremely helpful to me during the dissertation process. Dr. Evan Stubbs has been an excellent program director who is always willing to provide advice. Dr. Jody Martin brings his expertise in viruses. Dr. John Callaci has been very supportive during my training in the Alcohol Research Program. And, Dr. Bill Simmons provided critical evaluation of the manuscript. Finally, I wish to thank my family, especially my wife Ngoc Anh, whose love and support helped to carry me through graduate school.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAL	Blood alcohol level
BrdU	5-bromo-2'-deoxyuridine
BBB	Blood brain barrier
CC	Corpus callosum
CCI	Control cortical impact
CNS	Central nervous system
Contra	Contralateral/Contralesional
CPP	Cerebral perfusion pressure
CsA	Cyclosporine A
CSF	Cerebrospinal fluid
CT	Computed tomography
DA	Dopamine
DAB	Diaminobenzidene
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum

EPO	Erythropoietin
FDA	Food and Drug Administration
FP	Fluid percusion
GABA	Gamma-aminobutyric acid
GCL	Granule cell layer
GF	Growth factors
GFAP	Glial fibrillary acidic protein
GH	Growth hormone
GPCR	G protein-coupled receptor
GSC	Glasgow coma scale
GTPase	Guanosine triphosphatase
HMGA	3-hydroxy-3-methylglutaryl coenzyme A
ICP	Intracranial pressure
IED	Improvised explosive device
IgG	Immunoglobulin G
IL	Interleukin,1b,6,8
Ipsi	Ipsilateral/Ipsilesional
KO	Knockout
LTD	Long term depression
LTP	Long term potentiation
LV	Lateral ventricle
MAP	Mean arterial pressure
MFP	Midline fluid percussion
MRI	Magnetic resonance imaging

MWM	Morris water maze
NAC	N-acetyl cysteine
NeuN	Neuronal nuclei (marker)
NMDA	N-methyl-D-aspartate
NSPC	Neural stem and/or progenitor cell
OB	Olfactory bulb
OCT	Optimum cutting temperature
PNS	Peripheral nervous system
PTSD	Post traumatic stress disorder
ROS	Reactive oxygen species
RMS	Rostral migratory stream
SEM	Standard error of the mean
SGZ	Subgranular zone
ST	Striatum
SVZ	Subventricular zone
TBI	Traumatic brain injury
TNFα	Tumor necrosis factor
WT	Wild type

ABSTRACT

Traumatic brain injury (TBI) is a major source of disability in modern societies. However, there are no good pharmacological strategies for treating long-term TBI complications because we do not fully understand the injury processes that occur afterward. Moreover, a significant percent of patients entering the emergency room with TBI have a blood alcohol level above the legal limit. Alcohol use and intoxication is a widespread issue in our society. Binge drinking is the most common way in which alcohol is consumed, and alarmingly, Americans of all age groups binge drink on a frequent basis. Despite the prevalence of alcohol intoxication seen together with TBI, how it affects the brain injury and recovery process is not well understood. Our laboratory has previously found that a repeated dose of binge alcohol prior to TBI impairs functional sensorimotor recovery. One means by which the brain responds to injury is the mobilization of neural precursor cells produced by neurogenesis. However, the effect of pre-injury binge alcohol exposure on the reactive neurogenesis that occurs after TBI is not known. Adult neurogenesis is an alternative form of plasticity, which involves the production of new neurons and may contribute to functional recovery after TBI. The subventricular zone (SVZ) is one of the brain's main neurogenic niches. The experiments in this dissertation therefore sought to

determine the short and long term effects of pre-TBI binge alcohol on the neural stem cell responses in the SVZ following brain injury. We assessed the SVZ neural stem cell response after binge alcohol and TBI by utilizing the proliferation marker 5-bromo-2'-deoxyuridine (BrdU) along with other markers for neurogenesis such as Doublecortin. We found that binge alcohol did not affect short and long term lesion size (as measured at 24 hours, 7 days and 6 weeks post TBI). As expected, TBI alone significantly increased SVZ proliferation bilaterally 24 hours post injury. Surprisingly, binge alcohol alone also significantly increased proliferation bilaterally in the SVZ at the 24 hour time point. A combined binge alcohol and TBI regimen resulted in decreased SVZ proliferation bilaterally at 24 hours and 7 days post-TBI. Furthermore, when assessed at 6 weeks after TBI, binge alcohol significantly decreased SVZ neuronal differentiation. While we observed that TBI alone increased migration toward the rostral migratory stream (RMS), binge alcohol did not affect either RMS or perilesional migration post-TBI. Taken together, the results from this dissertation suggest that pre-TBI binge alcohol negatively impacts functional recovery by decreasing short-term neural stem cell proliferative responses as well as long-term neuronal differentiation of these proliferative cells in the SVZ.

CHAPTER ONE

OVERVIEW AND HYPOTHESIS

The prevalence of traumatic brain injury (TBI) in our society is striking with 1.7 million occurrences annually (CDC 2010). Many survivors are faced with debilitating outcomes such as deficits in sensory or motor function and increased risk for mood and dementia disorders (Langlois et al 2006). Yet, no pharmacological treatments have been found to be effective at treating long-term TBI effects. Important to this study, up to 43% of TBI patients entering the emergency department have blood alcohol levels above the legal limit of 0.08% (Talving et al 2010). Clinical studies indicate that being intoxicated at the time of head injury leads to delayed recovery (Schutte & Hanks 2010). Binge drinking has become one of the most common ways in which alcohol is abused according to a 2012 CDC report. A recent finding from our group has shown that using a rat model of TBI and binge alcohol, the recovery in skilled sensorimotor task performance is slower in alcohol-exposed animals (Vaagenes et al 2015). The cellular and molecular underpinnings of the impact of this binge pattern of alcohol consumption on TBI recovery has yet to be elucidated, and we believe that a more fundamental understanding of TBI and binge alcohol will help in

designing better therapeutic interventions for patients. One way in which the brain compensates after an injury such as TBI is to increase neurogenesis in various regions such as the subventricular zone (SVZ). Neurogenesis is thought to improve functional recovery by incorporation of new neurons that take on the previous functions of the damaged neurons. The goal of this dissertation was to determine how TBI affects neurogenesis in the SVZ and if alcohol binge prior to TBI leads to a reduction in neurogenesis. The results from this dissertation will show directly how TBI affects the three most important parameters of neurogenesis: proliferation, differentiation and migration.

We therefore hypothesize that: A binge pattern of alcohol consumption prior to traumatic brain injury results in a decrease in the neural stem cell (NSC) response from the SVZ.

This hypothesis will be tested by the following specific aims:

Aim 1: Determine the effect of binge alcohol and TBI on SVZ proliferation: Proliferation will be measured by BrdU staining using unbiased stereology at early time points of 24 hours and 7 days after TBI.

There will be 4 experimental groups: one group will receive binge alcohol (3g/kg/day, 3 days, by oral gavage) and TBI (controlled cortical impact to

sensorimotor cortex); the next group will receive vehicle gavage (isovolumic water) and TBI; another group will receive binge alcohol and sham TBI (craniotomy without cortical damage) and the final group will receive vehicle gavage and sham TBI. Immediately after surgery, all rats will receive bromodeoxyuridine (BrdU) (100mg/kg/day ip for up to 7 days) and survive for 1 day after TBI or 7 days after TBI. At 1 day or 7 days after TBI, rats will be euthanized and transcardially perfused. Brains will be cryosectioned and immunostained for BrdU and visualized by Diaminobenzidene (DAB) labeling. We will be using stereology to assess the number of BrdU positive cells, which is a measure for cellular proliferation.

Aim2: At 6 weeks after TBI, determine the effect of binge alcohol and TBI on neuronal differentiation in the SVZ and the migration of new neurons to the perilesion area and olfactory bulb. Differentiation will be determined by measuring BrdU and Doublecortin (DCX) staining. Migration will be measured by examining BrdU and DCX staining at target migrating sites (perilesional area and rostral migratory stream).

Animals will undergo the same basic experimental conditions as outlined in aim 1. They will receive BrdU (100mg/kg/day, ip) for 7 days following TBI to label

all proliferation and will survive for 6 weeks. At 6 weeks after TBI, rats will be euthanized and brain tissues processed. We will use stereology to assess the number of NSC with a combination of markers including BrdU for proliferation and DCX for differentiation and migration.

CHAPTER TWO

REVIEW OF RELATED LITERATURE

Traumatic Brain Injury

Traumatic Brain Injury (TBI) is one of the major sources of disability in our modern society (Diaz-Arrastia et al 2014). In the United States, about 1.7 million people suffer from TBI every year (Coronado et al 2012) About 3% of these people die due to the severity of the injury or the complication that follows TBI (Corrigan et al 2010). Of the 97% of the people who survive, many face the difficult prospect of living with long-term disability. Additionally, the military faces challenges dealing with TBI, as it had been called the "signature wound of the war in Iraq and Afghanistan"(Lawhorne 2010).

TBI is divided into two distinct phases, the primary and secondary injury processes. Primary injury is the immediate, direct biomechanical insult causing rapid brain tissue deformation and cortical, sub-cortical or axonal tissue damage and death. The results of primary injury are either focal or diffuse lesions. Primary injury often involves vasculature damage leading to bleeding; this type of bleeding can occur anywhere within the head such as epidural, subdural, intracerebral hematoma, contusion and laceration. Epidural hematoma is when a

blood vessel is torn and blood pools between the skull and the meninges (also known as extradural hematoma). When the blood pools between the dura and the arachnoid, it is called subdural hematoma. Subarachnoid hematoma is when blood collects in the space between the arachnoid membrane and the pial mater covering the brain (this space is also known as the subarachnoid space). Usually, subarachnoid hematoma poses poor prognosis for TBI patients especially if co-occurring with loss of consciousness (Servadei et al 2002). Intracerebral hematoma is when blood bleeds into the brain parenchymal space. Hematoma is diagnosed by CT and MRI scans and in certain cases, needs to be evacuated.

Clinical presentation in the emergency room is an important first step toward the treatment of TBI. The presence of symptoms such as loss of or altered consciousness, headaches, nausea, vomiting or dizziness in trauma patients warrants that CT scan should be performed immediately. CT is the procedure of choice to initially assess patients with cranial trauma as it is able to detect skull fractures and hematomas. Patients who present without obvious signs of external trauma still need to be closely evaluated especially if they are intoxicated. Alcohol intoxication can lead to difficulty in diagnosing subdural hemorrhages.

Glasgow Coma Scale (GSC)		
Eye Opening	Best Motor Response	Best Verbal Response
Spontaneous:E4	Obeys commands:M6	Oriented:V5
To voice:E3	Localizes:M5	Confused conversation:V4
To pain:E2	Withdrawal:M4	Inappropriate words:V3
None:E1	Abnormal flexion:M3	Incomprehensible sounds:V2
	Extensor posture:M2	None: V3
	None:M1	
Score=(E+M+V)=3 to 15		

Table 1: The GCS is widely used by clinicians to diagnose the extent of functional deficits after TBI. Adapted from (Teasdale & Jennett 1976).

Once head injury is ascertained in patients, the extent of injury can be further categorized into mild, moderate and severe using the Glasgow Coma Scale (GSC), (see Table 1). The GSC is used by clinicians to communicate the assessment of trauma patients and the GSC score is an important prognosticator of clinical outcome. Mild TBI is classified as GCS 14-15, moderate TBI is 9-13 and severe TBI as 3-8 (Narayan 1997). The GSC relies on the examination of eye, verbal and motor function to evaluate the patients. For instance, a patient with mild TBI would be conscious and responsive to verbal communication; however, this patient might be disoriented and have difficulty sensing painful stimuli. Mild TBI presents a problem in diagnosis, as the patients might not come into the clinic to report it. Additionally, patients with mild TBI might not have any indication of trauma on CT scans and the clinical symptoms might show up over the course of several weeks (Zollman 2011). In contrast, a moderate TBI patient would be

conscious but could be much more disoriented and would have impaired ability to communicate. Finally, severe TBI patients would be unconscious and would not be able to communicate or open their eyes (Zollman 2011).

Depending on the severity of TBI, hypotension and hypoxia might be an issue for the patient; therefore endotracheal intubation might need to be performed. Overall, not much can be done regarding primary TBI injury. Head injuries are stabilized in the emergency room and the hematomas are removed or cerebral spinal fluid evacuated in the case of elevated intracranial pressure (ICP).

The secondary TBI injury process is the main target for therapeutic intervention. Over hours to months and years after the initial primary trauma, additional complications such as intracranial hemorrhage, brain tissue swelling, elevated ICP, hypoxic and ischemic conditions can occur (Morales et al 2005). Various molecular changes occur that characterize the secondary injury process: elevation of pro-inflammatory cytokines, increase in oxidative stress and excitotoxicity. All of these process might culminate in prolonged neuronal inflammation and degeneration.

Elevated Intracranial Pressure and Cerebral Perfusion Pressure

In a normal adult, ICP is around 15mmHg. A sustained increase of over 20mmHg is not well tolerated in the case of injury and is associated with increased mortality (Eisenberg 1988, Narayan 1982). Sustained elevation in ICP after brain injury can lead to cerebral ischemia or even more severely, brain herniation. Ventriculostomy is a procedure in which a silicon catheter is used to measure ICP and to drain the CSF if needed. Additionally, mannitol can be given intravenously to effectively lower ICP.

Cerebral perfusion pressure (CPP) is a measure of how well blood flows in the brain after an injury. CPP is defined as CPP=MAP-ICP; where CPP is MAP (mean arterial pressure) minus ICP (intracranial pressure). CPP below 50mmHg for an extended amount of time is dangerous for the brain because the perfusion cannot match the brain metabolic needs and can lead to cerebral ischemia (Katz et al 2006).

Secondary injury processes start as a consequence of the trauma forces involved in the primary injury process. Calcium homeostasis is first disrupted (Bains & Hall 2012). During normal conditions, calcium is tightly regulated such that its concentration in the cells of the CNS is very low and the extracellular

concentration is much higher. Primary injury leads to cells unable to maintain this gradient and more calcium enters the cell cytosol. This leads to depolarization and release of glutamate which is an excitatory neurotransmitter. An excess of extracellular glutamate leads to a process called excitotoxicity. Excitotoxicity happens when excess extracellular glutamate binds to NMDA and AMPA receptors. These receptors are ligand-gated ion channels which allow even more extracellular calcium to enter the cell leading to more glutamate release. This becomes a self-perpetuating mechanism leading to cell death through the mechanism of apoptosis. The disruption of chemical gradients of different ions in the brain combined with decreased oxygen due to hemorrhage leads to reduced ATP production which can lead to issues like disrupted protein synthesis and disruption of antioxidant systems such as the glutathione peroxidase system. Collectively, the brain experiences oxidative stress because free radicals such as superoxide and peroxinitrite are elevated. These free radicals are extremely harmful to the cell because carbon in lipids, nucleic acids and proteins can react with these reactive oxygen species (ROS) (Hall et al 2010). Oxidative stress, besides affecting neurons and glia, can be detrimental to the blood brain barrier (BBB). The BBB serves as the vascular filtering of the brain; it allows necessary nutrients to enter the CNS and waste products to exit while blocking undesirable

compounds access to the brain (Smith et al 1994). The cellular composition of the BBB is made up of endothelial cells that form tight junctions with each other. Surrounding these endothelial cells are pericytes, smooth muscle cells and astrocytes which form a structure called end feet (Chodobski et al 2011). Following TBI, the BBB breaks down and becomes more leaky. This might be due to an increase in lipid peroxidation, which is a consequence of elevated ROS. Blocking lipid peroxidation was showed to reduce BBB permeability after TBI (Smith et al 1994).

Reduced ATP synthesis after TBI also impairs the active transport of pyruvate into the mitochondria leading to a decrease in the Krebs cycle. As a result, cells enter a Pasteur shift, a process in which normal aerobic respiration is shifted to anaerobic respiration. Glucose is broken down incompletely leading to excess lactic acid accumulation which results in a condition called lactic acidosis (Hall 2012).

Brain edema is a characteristic physiological response of the brain to injury and is thought to have two distinctive components, vasogenic and cytotoxic. Vasogenic edema is a result of increased blood brain barrier (BBB) leakage after TBI. Tight junctions that make up the BBB are broken down after brain injury and

allow extravasations of fluid and proteins into the interstitial space of the brain parenchyma. Cytotoxic edema, also known as cellular edema, happens at the individual cell level, which is due to altered cellular ion homeostasis and cell membrane dysfunction. The time course of vasogenic and cytotoxic edema is different, with vasogenic edema happening early on right after primary TBI injury while cytotoxic edema happens in a much more delayed manner. Brain edema generally is maximal around 24 to 48 hours (Marmarou 1994).

Brain inflammation is an important component of TBI secondary injury and has both acute and chronic aspects. Inflammation is thought to be both beneficial and detrimental and is composed of both released factors (IL-1b, eicosanoids) and cellular factors (neutrophils and macrophages) (Popovich et al 1999, Schoettle 1990, Shapira 1992, Toulmond & Rothwell 1995). IL-1b is an important inflammatory mediator and is found to be elevated in CSF (Marion et al 1997) and contused brain tissue of TBI patients (Clark et al 1999). Additionally, other cytokines have subsequently been found to be playing roles in post-TBI inflammation including IL-6 (Kossmanna 1996), and IL-8 (Kossmann 1997).

Different Types of TBI

There are two main types of TBI, closed, and penetrating brain injury. In closed TBI, the injury can occur due to two primary general means, static and dynamic loading (Graham 2002). Static loading is when a slow moving force is applied to the head; an example of this would be the head being crushed by a heavy object such as a car. In dynamic loading causing TBI, which occurs more frequently, the head is subjected to sudden acceleration and deceleration forces (Stålhammar 1986). Dynamic loading can further be divided into two types, impulsive or impact loading. In the impulsive dynamic loading scenario, the head is set into motion suddenly or brought to a stop suddenly. This is most commonly seen in whiplash type of injury. An example of an impact dynamic loading situation would be a blunt object such as a baseball bat striking the head. Both impulsive and impact loading types of TBI produce tissue deformation due to the mechanical forces involved.

The other major type of TBI is penetrating brain injury. This type of injury is associated with armed combat, such as gunshot and missile wounds. The severity of penetrating TBI is determined by the size of the projectile, shape, mass, velocity and location of impact (Morales et al 2005).

Blast Injury

TBI resulting from blasts from explosive devices (i.e Improvised explosive devices or IED's) is increasingly recognized as being mechanistically and physiologically distinct compared to traditional penetrating and closed head TBI (Ling et al 2009). It is thought that up to 60% of deployed military personnel are potentially exposed to blast TBI. Furthermore, there are some important links between blast TBI and post traumatic stress disorder (PTSD). Primary blast results in forces emanating from the explosion in the form of pressure wave transients causing injuries to the brain much like closed TBI would. Interestingly, common diagnoses among blast TBI patients are diffuse brain edema, hyperemia, and vasospasm.

Concussive Sport Head Injury

It had been recognized that repetitive exposure to mild TBI that is experienced by certain athletes such as boxers and football players could result in a condition called chronic traumatic encephalopathy (CTE). Signs and symptoms of this condition are somewhat similar to Parkinson's disease in which the patients have gait disorder, slurred speech, neuropsychiatric and behavioral symptoms (Gardner et al 2013).

Animal TBI Models

Modeling of human TBI in laboratory animals requires that the clinical symptoms seen in human patients be replicated in the model. For instance, primary injury to the cortex must be the same as seen in humans and secondary injury processes should closely mimic that of the human condition. Additionally, a good model needs to have parameters of the injury adjusted so that it can produce graded severity of TBI injury (mild, moderate and severe). Furthermore, a good model needs to be reproducible between different laboratories. To this end, models that had been shown to produce focal brain injury are midline fluid percussion (MFP), controlled cortical impact (CCI) and weight drop (Figure 1). However, in addition to the focal nature of the injuries that these models produce, there is an additional axonal injury when the injury severity reaches a threshold.

The weight drop model relies on gravitational force of a free falling object to produce a focal TBI (Feeney et al 1981). In this model, rat or mice are anesthetized and restrained to stabilize the head during impact as to ensure reproducibility (Figure 1A). The skull of the animal is exposed and craniotomy to expose the brain can be performed; however, another variation on this model is

without craniotomy. The severity of TBI can be adjusted by changing the mass of the weight or its height in which it is dropped from. A strong advantage of the weight drop model is its convenience; it is very easy and quick to set up for the investigator. The weight drop model is able to reproduce clinical symptoms of concussive TBI such as cortical cell loss, axonal injury and in more severe TBI parameters, contusions and hemorrhages. Additionally, behavioral dysfunctions are reproduced in animals with the weight drop models such as impairments in motor function as well as cognitive deficits and these behavioral deficits do correlate to injury severity (Chen et al 1996, Tang et al 1997). However, there are also some strong drawbacks of the weight drop model: the lesion produced can be inaccurate (especially when no craniotomy is performed) and there are chances the animals might get a secondary “rebound” injury. Furthermore, this model had not been evaluated in rodents for long-term effects or recovery making it less applicable to human TBI.

Three Widely Used Animal Models of TBI

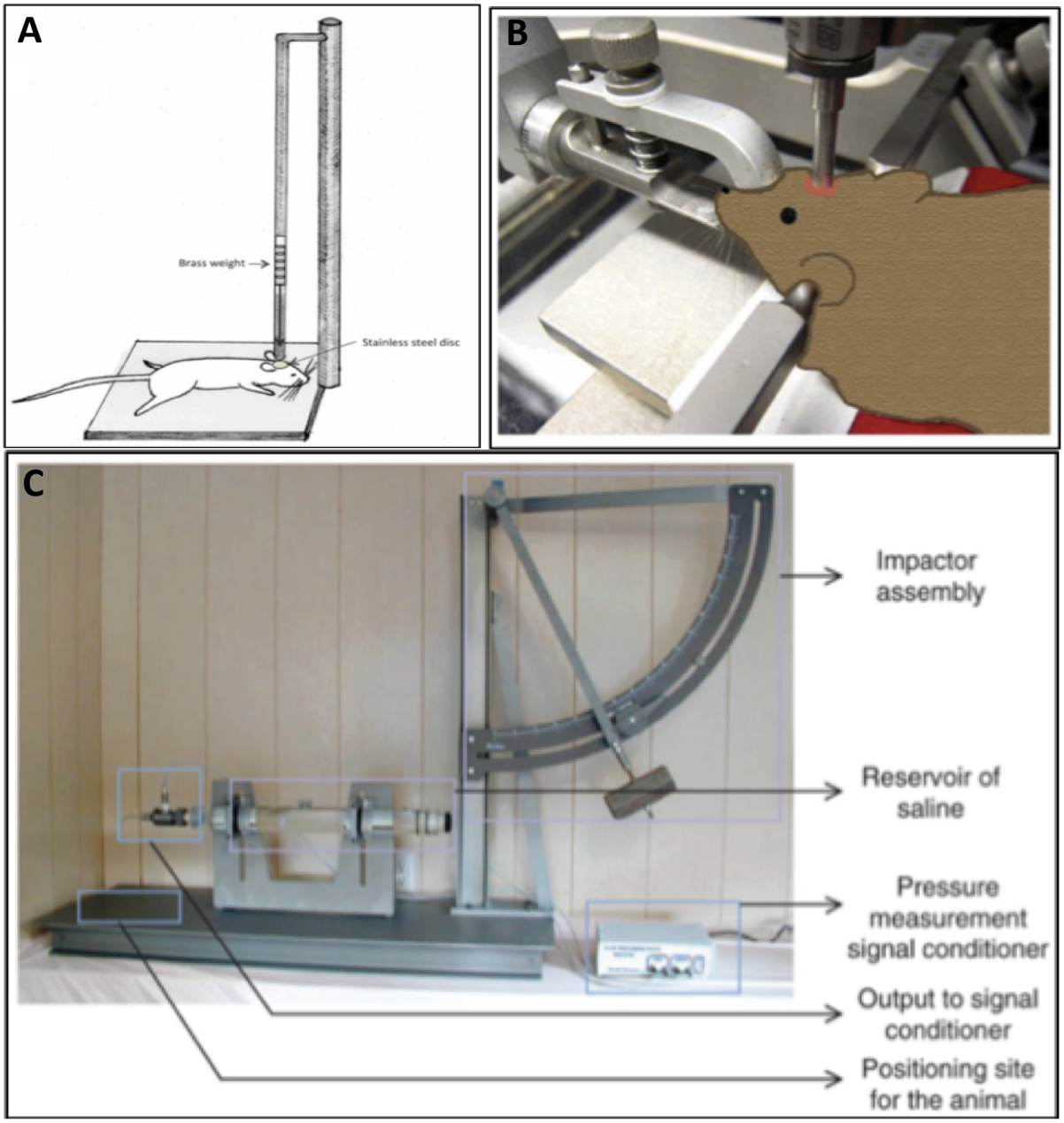


Figure 1: Three well characterized models of TBI. A. The weight drop model; adapted from (Michael 2016). B. The controlled cortical impact model; adapted from (Michael 2016). C. The lateral fluid percussion model; adapted from (Kabadi et al 2010).

The midline fluid percussion (MFP) TBI model was first demonstrated in cat and rabbit (Härtl et al 1997, Hayes et al 1987); however, it was later adapted for use in rat (Dixon et al 1987, McIntosh et al 1987). In the MFP model, the skull is exposed and a craniotomy performed (at the midline) to expose the brain. A pendulum is the source of kinetic energy that causes a fluid bolus to strike the dural surface of the brain (Figure 1C). The fluid force extends into the epidural space causing diffuse loading of the brain (Sullivan et al 1976). The severity of MFP TBI can be varied by the height at which the pendulum is released. The MFP model leads to focal injury in the gray matter of areas directly in and around the site of contact and can lead to intraparenchymal and subarachnoid hemorrhage (Graham et al 2000). Additionally, depressed cerebral blood flow is observed for around 24 hours post TBI (Ozawa et al 1991). Animal models of MFP TBI have deficits in cognition and motor functions (Dixon et al 1987). A major disadvantage of MFP is the high mortality rate because the percussive force acting on the brain often leads to herniation and brainstem damage.

The controlled cortical impact (CCI) TBI model relies on a rigid impactor that is either pneumatic or electromagnetically powered (Figure 1B). The CCI was first demonstrated in ferret (Lighthall 1988) and subsequently adapted for use in rat (Dixon et al 1991) and mouse (Smith et al 1995). The cortical locations of the CCI

model can be varied; for instance, midline CCI has been performed (Lighthall 1988) and lateral cortices (Chen et al 2003). Importantly, CCI TBI produces less mortality due to minimal brain stem damage. The severity of TBI can be greatly varied by changing the parameter of the impactor such as time, velocity and depth. There is also no chance of secondary damage due to “rebound” injury. The CCI TBI model can recreate a variety of clinical signs observed in human TBI patients such as contusion, intraparenchymal, epidural and subdural hematomas (Dixon et al 1991, Lighthall 1988), gray and white matter damage, ventricular enlargement (Dixon et al 1999), and hippocampal and thalamic degeneration (Hall et al 2005). These widespread effects due to CCI TBI correlate to behavioral deficits, as impairments in sensorimotor (Fox et al 1998) and cognitive functions (Scheff et al 1997) are observed for extended periods of time after TBI for up to one year. An additional and important benefit of the CCI TBI model is its reproducibility among different laboratories. Due to these reasons, the CCI model was used in the experiments in this dissertation.

More recently, the military conflicts in the Middle East have demonstrated the need for developing an animal model for blast injury to study the effect produced by improvised explosive devices (IED). The injury characteristic of blasts is rapid pressure waves moving through the brain tissue (Risling & Davidsson

2012). This could also be accompanied by further non-blast injuries from blunt trauma either from shrapnel or environmental objects (Sundaramurthy et al 2012). Rodents have been used to model this blast injury, which utilize a unique shock wave generator. In this model, anesthetized mice are placed in a metallic tube and subjected to pressure waves, which closely resemble the force produced by an IED. Diffuse cortical damage, edema and intracranial hematoma are commonly seen in this model (Svetlov et al 2010).

Current and Potential Treatments for TBI

The Neurotrauma Pharmacology Workgroup in 2012 (Diaz-Arrastia et al 2014), reviewed the current possible treatments for TBI to identify strengths and limitations in these drugs. Their findings are summarized below:

Acetylcholinesterase inhibitors (AchEI) increase the concentration of acetylcholine at the synapse by inhibiting its breakdown. Studies using various AchEI such as physostigmine (Cardenas et al 1994, McLean et al 1987), donepezil (Ballesteros et al 2008), and rivastigmine (Blount et al 2002), have shown some potential benefits for chronic TBI patients with cognitive deficits. Additionally, pre-clinical trials have shown beneficial effects of AchEI including decreased neuronal cell death, decreasing hippocampal neural degeneration, reduced BBB breakdown, decreased vasogenic brain edema and preservation of neurological functions (Ballesteros et al 2008, Chen et al 1998). However, the majority of clinical studies on the efficacy of AchEI for TBI were not randomized controlled trials, and has issues with methodology and small sample sizes; these important limitations prevented a good assessment of these AchEI drugs. Additionally, there is no evidence in pre-clinical studies in animals showing the efficacy of AchEIs

given after an extended period of time after TBI (i.e. delayed administration paradigm).

Amantadine is a FDA approved antiviral used for the treatment of influenza A. Additionally, it had some efficacy in the treatment of Parkinson's disease but the exact mechanism is not fully elucidated. Studies have suggested that amantadine increases dopamine (DA) concentration either by decreasing DA reuptake or increasing DA synthesis (Bales et al 2009). Additionally, there is some evidence suggesting amantadine increases post-synaptic DA receptor expression (Gianutsos et al 1985). An interesting piece of evidence that might explain amantadine's beneficial effect for TBI patients is its role as a noncompetitive antagonist of NMDA receptors (Stoof et al 1992), which is the main mediator of excitotoxicity. One study using CCI as a TBI model and amantadine treatment commenced one day after TBI showed a modest beneficial effect in the Morris water maze (MWM) (Dixon et al 1999). However, the pre-clinical animal studies for amantadine as a treatment for TBI is still lacking; more research needs to be done on the time-course and duration of amantadine treatment. In addition, the different types and severity of TBI paradigms need to be tested in amantadine research.

Cyclosporine A (CsA) maintains mitochondrial membrane potential and prevents mitochondrial induced damage after TBI. In various animal models of TBI, cyclosporine had shown positive effects in axonal injury and learning and memory (Alessandri et al 2002, Heuvel et al 2004). CsA also has immuno-modulating effects, which is another aspect in which it could be potentially beneficial for TBI patients. There are several clinical studies that showed CsA improves histological outcomes such as decreased axonal damage and decreased contusion size. However, there are fewer studies that showed improved functional outcomes. One study using CCI as a TBI model did not show CsA significantly improved cognitive outcome in rat (Baki et al 2010). However, more research is needed in regard to the CsA administration route because CsA is thought to have poor BBB permeability. Additionally, more information about the ideal time-course of CsA treatment, and different outcome measures are needed to completely evaluate CsA efficacy as a treatment for TBI.

Erythropoietin (EPO) is a cytokine that plays an important role in erythropoiesis and has been found to have positive effects after TBI such as decreasing excitotoxicity and nitric oxide toxicity, decreasing cell death, increasing neurogenesis and angiogenesis (Ozisik et al 2007, Xiong et al 2011, Zhang et al 2010). Additionally, EPO has antioxidant and anti-inflammatory effects (Ozisik et

al 2007). Erythropoietin acts through the EPO receptors, which are present in many cell type of the CNS. However, EPO might act through other mechanisms as EPO receptors are not needed to mediate some of the effects of erythropoietin. EPO has been extensively studied in the rodent system and using different models of TBI, and its dosing and route of administration have been well characterized. There are different on-going clinical trials using EPO as treatment for severe TBI. One study recruiting patients with subarachnoid hemorrhage showed significant positive effects with regards to vasospasm, ischemic deficit and improved discharge outcome when EPO was administered acutely (Tseng et al 2009). Another non-randomized retrospective study again with severe TBI patients showed that EPO administered sub-acutely (within 14 days of TBI) resulted in lower hospital mortality (Heeschen et al 2003). However, more pre-clinical studies are needed to explore EPO administration for moderate or mild cases of TBI or more delayed administration.

Glyburide is a sulfonylurea that binds to Sur1 and thus blocks the K_{ATP} channels. It is approved for use as an insulin secretagogue and is used to treat patients with adult onset diabetes. Sur1 is an adenosine triphosphate binding cassette transporter; Sur1 associates with other pore forming subunits to form ion channels (Diaz-Arrastia et al 2014). After TBI, non-selective cation channels

($NC_{Ca\text{-}ATP}$) in which Sur1 is a component of, are increased in expression levels in neurons and endothelial cells of the CNS. Pre-clinical studies have found beneficial effects of glyburide in animal TBI models, including reduced inflammation, hemorrhage and vasogenic edema. One study showed glyburide treatment led to reduction in secondary hemorrhage (Simard et al 2009); and another study showed improved performance in the Morris water maze (Patel et al 2010). These studies administered glyburide shortly after TBI (within 10 hours) so more studies that use a delayed treatment paradigm are needed to fully evaluate glyburide. Additionally, glyburide has been used in several retrospective clinical studies for the treatment of ischemic stroke, where glyburide treated patients suffered less hospital mortality and decreased edema. A phase II trial of glyburide for moderate to severe TBI has started. In this study, glyburide treatment commences at 8 hours after TBI and continues for 72 hours.

Growth hormone (GH) is secreted by the anterior pituitary gland and has important anabolic effects by acting through the GH receptors. About 20% of TBI patients have GH deficiencies, which might be due to direct or indirect injury to the pituitary or hypothalamus (Benvenga et al 2000, Wilkinson et al 2012). Using a rat model of TBI, one study showed that increasing GH leads to increased motor and cognitive recovery (Saatman et al 1997). Clinical studies looking at TBI

patients with GH insufficiency/deficiency have found that administration of recombinant human GH at the chronic stage by daily subcutaneous injections for up to 12 months showed improved cognitive outcomes (Devesa et al 2013, Reimunde et al 2011). However, other clinical studies suggest that GH given in the acute stage is detrimental (Demling 1999, Takala et al 1999). Overall, GH administration still holds strong promise as an effective chronic TBI treatment. However, more preclinical studies are needed to study the exact molecular mechanism in which GH acts.

Lithium is an effective pharmacological treatment for bipolar disorder. It had been found to be neuro-protective by reducing excitotoxicity, decreasing ischemia related injury and decreasing cell death by apoptosis, attenuates pro-inflammatory cascades and preserves the function of the BBB (Dash et al 2011, Shapira et al 2007, Yu et al 2012a). For instance, in an animal model of TBI, lithium was given shortly after injury and continued for 3 weeks showed reduced TBI lesion size and improved behavioral recovery (Yu et al 2012b). Despite favorable preclinical studies, no well-controlled clinical trials have test lithium for the treatment of TBI. There are some small case studies in which lithium was given to bipolar patients after TBI of varying severity; however, the primary outcome measurements were aggression and agitation. Overall, lithium is a well-tolerated

drug that needs further clinical testing as a treatment for TBI. Therapeutic windows and effective outcome measurements need to be used to effectively evaluate the usefulness of lithium.

Methylphenidate is used to effectively increase synaptic concentration of DA and norepinephrine; it block DA transporters and inhibits DA and norepinephrine reuptake (Moeller et al 2012). Atomoxetine also increases synaptic concentration of DA and norepinephrine through similar mechanisms as methylphenidate (Swanson et al 2006). Both of these drugs are FDA approved for the treatment of attention deficit hyperactivity disorder. In animal TBI models, methylphenidate treatment leads to increased striatal dopaminergic neurotransmission and subsequently increased spatial learning and motor recovery (Wagner et al 2009). Similarly, atomoxetine leads to better Morris water maze performance after TBI (Reid & Hamm 2008). In the clinic, methylphenidate is well tolerated in patients with TBI (Alban et al 2004). The treatment windows for several studies started at several weeks to years after injury (sub-acute to chronic phase of TBI), and the doses ranged from a single one-time dose to up to 30 days. Overall, these studies were not conclusive about the effects of methyphenidate due to small sample size, limited outcome measures and not well-controlled injury and administration parameters. Furthermore, there is

currently no completed clinical study looking at the effect of atomoxetine on post TBI recovery. Overall, methylphenidate and atomoxetine have promising effects on TBI recovery in experimental animal TBI models, yet further clinical work that utilizes large sample sizes are needed to evaluate its true therapeutic potential in human TBI patients.

Minocycline is a member of the tetracycline family of drugs. Minocycline exhibits antimicrobial properties and due to its lipophilic property, it readily crosses the BBB (Kim & Suh 2009). Minocycline also has anti-inflammatory, anti-apoptotic and antioxidant activity at larger doses (at six time the normal dose used to elicit antimicrobial action) making it an attractive candidate for use in TBI patients who experience polytrauma. Minocycline was used in several animal models of TBI, stroke and different neurodegenerative diseases with beneficial effects shown. It is thought that minocycline's positive effects after TBI are due to its ability to block caspase dependent and independent apoptosis events, inhibition of microglia activation and direct action as an antioxidant by scavenging reactive oxygen species and decreasing the activity of metalloproteinases (Kim & Suh 2009). In several animal TBI models of CCI and weight drop, minocycline was given acutely at 45 minutes and up to 4 hours post injury. Lesion volume was smaller in minocycline treated animals (Bye et al 2007). In the clinics, high dose

minocycline is well tolerated in patients with spinal cord injuries, and markers for inflammation were decreased in these patients. However, no completed clinical studies have looked at Minocycline as a treatment for acute or chronic TBI.

NAC (N-acetyl cysteine) is a potent antioxidant that had been approved for the treatment of acetaminophen overdose. NAC is processed in the liver to become cysteine, which readily crosses the blood brain barrier. In the CNS, cysteine increases the level of glutathione, which is a potent endogenous antioxidant. Cysteine can also increase the concentration of extracellular glutamate (Olive et al 2012). In pre-clinical animal TBI studies, NAC was shown to decrease markers of oxidative damages (Hicdonmez et al 2006), and decrease inflammatory mediators IL-1b and TNF-a via an NF-kB dependent mechanism (Chen et al 2008a). Functionally, NAC was shown to decrease TBI lesion volume (Yi & Hazell 2005) but other measures of functional recovery were less conclusive (Thomale et al 2006). Interestingly, NAC appears to have synergistic effects when combined with minocycline treatment when used in experimental TBI (Baki et al 2010). A recent randomized clinical trial using NAC (dosed for 7 days) to treat soldiers exposed to blast TBI injury showed significant improvement in recovery. Therefore, NAC should be further explored in more clinical studies as treatment

for other types of TBI and in a wider population. Additionally, detailed therapeutic windows of NAC need to be established.

Progesterone is an endogenous steroid produced in the CNS, reproductive organs and the adrenal glands. Furthermore, it is found in high concentration in the brain of both males and females. Progesterone has several potential mechanisms in which its effect might be beneficial to TBI patients. It has been shown to enhance myelination (Koenig et al 1995), increase neurogenesis (Liu et al 2009) and modulate aquaporin expression, which potentially decreases the aquaporin induced edema post TBI. Additionally, progesterone serves as a precursor to other neurosteroids with neuroprotective effects. For instance, allopregnanolone is elevated after oral progesterone administration, which exhibits potent neuro-protective effects (Sayeed et al 2006). In animal models of TBI including CCI, progesterone treatment showed decreased edema(Cutler et al 2007) reduced cell death by apoptosis (Cekic et al 2012) and attenuation of pro-inflammatory mediators such as IL-6, IL-1b, and TNFa (Pan et al 2007). It also was shown to exhibit antioxidant properties with progesterone treatment decreasing lipid peroxidation (Roof et al 1997). Interestingly, progesterone affects the Toll-like receptor signaling pathway (Chen et al 2008b), which is the primary mechanism of alcohol induced CNS inflammation. These animal studies also

demonstrated enhanced behavioral recovery post TBI as a result of progesterone treatment (Djebaili et al 2005, Wali et al 2011). In clinical studies using progesterone or its allopregnanolone metabolite, it is very well tolerated and significantly reduced mortality when it was given in the acute phase of TBI (Wright et al 2007). Overall, progesterone holds exciting potential as one of the most effective treatment for TBI. However, more basic research into the mechanism of its therapeutic effects is needed. Furthermore, additional therapeutic windows of progesterone should be tested.

Simvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A (HMGA) reductase inhibitor used widely for the reduction of serum cholesterol. However, there is experimental evidence for simvastatin and other statins being beneficial to treat both acute and chronic TBI. Beneficial effects include reduced brain edema, improved BBB function, increased cerebral blood flow, attenuated CNS inflammation, axonal injury and cell death (Diaz-Arrastia et al 2014). Simvastatin and other statins have been extensively investigated in pre-clinical animal models of TBI primarily using CCI. Most studies have used oral as the method of administration and treatment started as late as 24 hours post injury and for 7 to 14 days (Abrahamson et al 2009, Li et al 2009). There is currently an on-going clinical phase II study using atorvastatin to treat adults with mild TBI

(NCT01013870). Given the large body of evidence for the beneficial effect of statins in animal studies, more clinical studies are needed to address other aspects of statins in TBI treatment such as using more delayed administration and treating different severity of TBI.

Rehabilitation

Given the limitation in current pharmacological approaches to the treatment of TBI, especially chronic TBI dysfunction such as sensorimotor deficits, cognitive and behavioral impairments, rehabilitation remains the most effective method to treat chronic TBI. Rehabilitation is a holistic approach that involves the patients, their family and the health care team. According to (Barnes 1999), rehabilitation consists of three approaches: to reduce disability, acquire new skills and strategies to reduce the impact of disability, and to alter the environment as to reduce the impact of the disability as much as possible. For instance, the approach to reduce disability after TBI could be medications to control spasticity or bladder control problems. An example of acquiring new skills to cope with the TBI-acquired disability could be physical therapies designed to improve gait or to increase stamina so the patient could use the wheelchair for an extended period of time. The last approach, to modify the environment to reduce the impact of a

patient's disability could be modification to the patient home to include a powered lift or grab rails in the toilet. Goal setting is an important part of rehabilitation. For instance, setting goals that are realistic and can be broken down into smaller component goals that can be measured. Valid and reliable outcome measures also need to be used in evaluating the progress of the rehabilitation process.

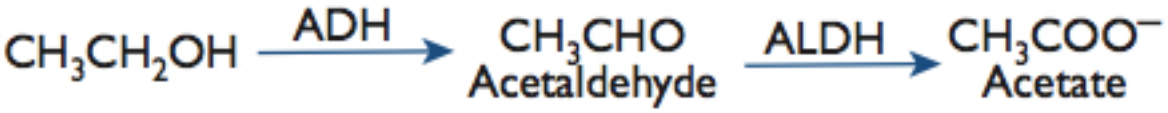
Alcohol and The Brain

Alcohol Use Disorder (AUD) is a major problem in our society with about 7.2% of adults in the United States diagnosed with this disorder. This population is approximately 17 million people. AUD is twice as prevalent in men as compared to women (NIAAA 2012). Additionally, approximately 855,000 adolescents from 12-17 years of age are also diagnosed with AUD. The Diagnostic and Statistical Manual of Mental Disorders (DSM) guide recently released a new classification for AUD in the new DSM-5. AUD is categorized by DSM-5 based on the symptoms reported by the patients. Out of the 11 possible symptoms, AUD is defined based on severity. Mild is having a combination of 2 to 3 symptoms, moderate as having the presence of 4 to 5 symptoms and severe as having 6 or more symptoms (Table 2).

1	Had times when you ended up drinking more, or longer, than you intended?	The presence of at least 2 of these symptoms indicates an **Alcohol Use Disorder (AUD).** The severity of the AUD is defined as: **Mild:** The presence of 2 to 3 symptoms **Moderate:** The presence of 4 to 5 symptoms **Severe:** The presence of 6 or more symptoms
2	More than once wanted to cut down or stop drinking, or tried to, but couldn't?	
3	Spend a lot of time drinking? Or being sick or getting over other aftereffects?	
4	Wanted a drink so badly you couldn't think of anything else? ****This is new to DSM-5****	
5	Found that drinking-or being sick from drinking-often interfered with taking care of your home or family? Or caused job troubles? Or school problems?	
6	Continued to drink even though it was causing trouble with your family or friends?	
7	Given up or cut back on activities that were important or interesting to you, or gave you pleasure, in order to drink?	
8	More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex)?	
9	Continued to drink even though it was making you feel depressed or anxious or adding to another health problem? Or after having had a memory blackout?	
10	Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?	
11	Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?	

Table 2: Guideline for the diagnosis of Alcohol Use Disorder (AUD). Adapted from NIH Publication No.13-7999.

The effects of alcohol are dependent on the amount that is ingested, its absorption, metabolism and its level in different organs. Alcohol's main site of metabolism is in the liver where two different enzymes are mainly responsible for its breakdown. First, alcohol is oxidized into acetylaldehyde by the enzyme alcohol dehydrogense (ADH); acetyladehyde is further oxidized into acetate by the enzyme aldehyde dehydrogenase (ALDH) (Edenberg 2007).



There are multiple variations of both enzymes ADH and ALDH; these different variations of enzymes have different efficiency for converting alcohol into its by-products. Because of this, a person is more or less at risk of developing alcoholism and dependence depending on which variants of ADH and ALDH they express (Hurley 2002). For instance, there are a few factors that determine a person's ability to handle alcohol and affect his or her intoxication level and these include ADH and ALDH alleles, their liver size, and the enzyme expression levels. All these factors might be one of the reasons why alcohol researchers looking into its impact on post TBI recovery in patients are so different in their conclusions.

Binge Drinking

One of the most common ways in which alcohol is misused is by binge drinking. According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), binge drinking is defined as having 5 or more drinks for men and 4 or more drinks for women within a 2 hour time window to reach a blood alcohol level (BAL) of 0.08-gram percent or more. Binge drinking is very prevalent, as it is estimated that 1 in 6 adults in the USA binge drink (38 million people) and they do

so very often (approximately 4 times a month). On average, each person has 8 drinks each time they binge (CDC Vitalsigns, 2012). While most binge drinkers are not technically categorized in the DSM-5 as having AUD, it is still a major problem because intoxication due to binge drinking leads to increased chances of health problems. For instance, one study indicated that alcohol consumption leads to a greater chance of dying from injuries and sustaining nonfatal injuries (Miller & Spicer 2012). Furthermore, recent statistics indicated that 88,000 deaths annually are alcohol related and about 31% of driving deaths are due to alcohol-impaired driving (NIAAA Alcohol Facts and Statistics 2015).

A high level of alcohol in the human body is very toxic especially to the liver, which performs most of the alcohol metabolism in the body. According to a recent report, alcohol is the leading cause of liver cirrhosis (Musso et al 2014). Alcohol exposure during the developmental stage of human life leads to fetal alcohol spectrum disorders (FASD) and is estimated to be as prevalent as 20 to 25 cases per 1,000 births (May et al 2009). In terms of the CNS, high levels of alcohol have been found to have various negative effects. For instance, (Collins et al 1996), found that binge administration paradigm of alcohol 3 times/day continuous for 4 days which achieved BAL higher than 300mg/dL in rats leads to neuronal degeneration in several key cerebral and olfactory brain regions.

Interestingly, the degeneration in the olfactory region of the brain might explain why olfactory memory deficits are observed in human alcoholic patients (Shear et al 1992). Additionally, alcohol has been shown *in vitro* to increase reactive oxygen species (ROS) in brain endothelial cell lines, which might indicate that alcohol decreased BBB functions (Haorah et al 2005). In fact, the metabolism of alcohol via ADH has been shown to generate free radicals and increase ROS in neuronal cell lines (Haorah et al 2008) and lead to lipid peroxidation (Altura et al 2002) a definitive molecular marker for the presence of elevated ROS. High levels of alcohol administration in rats also lead to neuro-inflammation characterized by elevated inflammatory mediators such as arachidonic acid and TNFa (Crews et al 2004). However, it is not known whether this neuro-inflammation is a response to the preceding neuronal degeneration or the cause of the degeneration. Interestingly, after administration of ethanol into organotypic slice cultures, one study observed that there were apparent edema effects, which could be blocked by the diuretic furosemide (Collins et al 1998). Therefore, aquaporin is suspected to be playing a role in ethanol induced edema in the brain. Alcohol induced neuro-inflammatory responses such as elevated TNFa and arachidonic acid might be due to glial TLR-4 activation by alcohol (Alfonso-Loeches et al 2010). The important process of neurogenesis, in which the brain generates new cells, is also

potently affected by the presence of alcohol. For Instance, one study found that using *in vitro* cultures, ethanol decreased neural stem cell proliferation (Crews et al 2003). Another study found that in rats, in response to a binge paradigm of 5g/kg alcohol, hippocampal neurogenesis was inhibited (Nixon & Crews 2002).

Alcohol and TBI in The Human Population

Because intoxicated people are more likely to get into dangerous situations that might result in injuries, it is not surprising that data from trauma centers indicated that up to 43% of patients entering the emergency department for severe TBI have blood alcohol levels above 0.08% (Talving et al 2010). Several researchers studied a group of patients having mild to severe TBI with and without having been alcohol intoxicated at the time of injury. They used functional and cognitive outcome measurements that were known to be sensitive to the effect of TBI. They found that having alcohol intoxication significantly decreased the Glasgow coma scale score, a functional measure for the severity of TBI (Schutte & Hanks 2010). Short-term (measured at the time of admission to inpatient rehabilitation) functional measures showed a significant negative effect of alcohol intoxication. However, functional and cognitive measures looking at post rehabilitation or at 1 year post-TBI showed no alcohol effects. One clinical

study pointed out that alcohol intoxicated patients might preferentially have a history of other substance abuse, thus the negative functional effects might not only be due to alcohol intoxication at the time of injury (Corrigan 1995). On the contrary, some other reports on the effect of alcohol on TBI arrived at different conclusions. One example is a recent clinical study showing evidence for the positive impact of BAL at the time of trauma center admission on recovery (Mohseni 2016). However, there were some important issues with the patient population included in this study. For instance, the patients with positive BAL where significantly younger and had less comorbidity.

Animal Models of Alcohol and TBI

Due to the prevalence of alcohol intoxication in TBI patients seen in the clinics, a number of laboratories had tried to develop a clinically relevant model to study the biological effect of alcohol on TBI. For instance, one group found some interesting effects of a chronic alcohol paradigm on recovery after TBI (Zhang et al 1999). Rats were fed with a liquid diet containing ethanol (6.6%). Importantly, at the time of TBI, the animals had been withdrawn from the alcohol diet for 2 days. The average daily ethanol consumption was 11.2-13g/kg/day, which was very high compared to the acute model that our laboratory used (3g/kg/day). Additionally,

the BAL that was attained at the end of alcohol feeding (6 weeks) was 154mg/dL, which indicated there was significant tolerance to alcohol in these animals. There were no significant overall differences in the beam walk scores after TBI between alcohol/TBI versus vehicle/TBI groups with the exception that on day #2 after surgery, the vehicle/TBI group did significantly worse. In a spatial learning test, there was a significant main effect of injury in that TBI lowered the performance score; however, having alcohol with TBI did not significantly affect performance in comparison to the control diet.

A different group investigated the effect of longer chronic ethanol administration (CEAn). They increased the length of time to three months, and in addition, made the animals intoxicated at the time of injury (Masse et al 2000). Yet, they observed no significant effect of ethanol on the post-TBI behavioral task of the Morris water maze (MWM). However, ethanol fed groups appeared to do better in certain parameters of the MWM. There were no other differences in TBI parameters such as survival rate or cortical lesion volume. Interestingly, there was significant BAL tolerance when examining the BAL level at different time points during the CEAn paradigm.

Another group investigated the effect of binge ethanol consumption on the outcome of TBI using the lateral fluid percussion model (LFP) (Prasad et al 2001). Alcohol was given intragastrically at 3g/kg, Thursday and Friday for four consecutive weeks follow by moderate injury (animals are intoxicated at time of injury). Then as before, they used MWM to analyze post injury recovery. There were no noticeable histological differences between alcohol/TBI and vehicle/TBI groups. Interestingly, in certain measures of the MWM such as mean search latency, alcohol TBI group did worse than vehicle TBI. One of the drawbacks of the studies performed by the Prasad group is the use of the MWM; this behavioral task is mainly to assess deficits in spatial memory and learning; to detect finer deficit in motor skills, other test should have been used. What this group had shown is that acute binge alcohol consumption was more detrimental to post injury recovery than an “alcoholic”-type of consumption.

Acute single dose alcohol had some interesting effects when given shortly before a fluid percussion model of TBI (Yamakami et al 1995). In this study, the dose of alcohol was divided into low (1.5g/kg) and high (3g/kg) and given to rats 2 hours before TBI. For either moderate or high severity TBI, there were 3 conditions, saline, low alcohol and high alcohol. Interestingly, significantly prolonged hypotension was observed in both alcohol level conditions.

Neurological motor function was assessed and there was no significant difference observed between either the alcohol group or vehicle group in moderate TBI. However, following more severe TBI, the high alcohol level group experienced higher mortality and worse motor recovery at 24 hours post injury. Furthermore, post-traumatic cerebral alkalosis was observed in both ethanol treated groups.

Our laboratory used an acute binge alcohol administration paradigm to investigate its effect on behavioral recovery after TBI (Vaagenes et al 2015). Alcohol was given at 2g/kg/day (i.p.) for three consecutive days. At the time of TBI injury, rats were intoxicated and had a blood alcohol level of more than 200mg/dL. Rats were given a TBI in the forelimb area of the sensorimotor cortex using the CCI model. We used the skilled forelimb reaching task to assess the recovery of injured rats for up to 7 weeks after TBI and found that the alcohol administered group had a significantly slower recovery curve with a significantly lower recovery plateau compared to the vehicle administered group.

There are several mechanisms that could support why alcohol consumption prior to TBI and intoxication during TBI could result in worse functional outcomes. For instance, there is evidence that alcohol intoxication could lead to aquaporin channel dysregulation, which might potentiate edema processes after TBI

(Sripathirathan et al 2009). Furthermore, alcohol administration can elicit an inflammatory response, and when combined with TBI, further potentiate that inflammatory cascade in the secondary injury process (McClain et al 2011). Another possible mechanism in which alcohol might negatively synergize with TBI to further worsen TBI outcomes is elevated reactive oxygen species (ROS). Alcohol metabolism is characterized by the production of ROS. TBI inflammatory cascades produce elevated ROS, so it is possible that the ROS produced by alcohol metabolism in combination with the ROS produced by TBI is responsible for the negative effects of alcohol on post-TBI functional recovery (Le Belle et al 2011). Another possible CNS pathway which both alcohol intoxication and TBI share is neurogenesis. High levels of alcohol alone negatively impact neurogenesis (Hansson et al 2010). We know that after TBI, there is a natural boost in subventricular and hippocampal-derived neurogenesis and these newly produced cells might contribute to the recovery process. It is possible that alcohol, by decreasing neurogenesis, leads to worse or delayed functional recovery as shown previously in our lab.

Neurogenesis

For a long time it was thought that we are born with all the neurons that we would have in our entire lifetime, and this dogma was partly solidified by Cajal and other neuroscientists of the nineteenth century. However, in 1912, it was noted by Ezra Allen that there is a zone of proliferation in the area adjacent to the cerebral lateral ventricle (Allen 1912). However, it was not until the 1960's when Altman's lab released a seminal paper documenting the existence of postnatal neurogenesis in the mammalian brain using tritiated thymidine autoradiography (Altman 1962). Since then, many studies have confirmed these two original observations that there are continual new cells being produced (neurogenesis) in the adult mammalian brain in mainly two sites, the subventricular zone (SVZ), and the hippocampal sub-granular zone (SGZ) (Ihrie & Alvarez-Buylla 2011, Spalding et al 2013).

SVZ Neurogenesis

Particularly important in the context of disease and injuries to the CNS is that these newborn cells from the SVZ have been detected in the injury sites (Young et al 2011). The main purpose of SVZ neurogenesis in the rodent brain is to produce cells to replace those in the olfactory bulb (OB). However, the

connection between proliferation in the SVZ and the presence of new cells in the OB was not made until relatively recently (1994) by the laboratory of Arturo Alvarez-Buylla. Before that, it was believed that the SVZ is a vestigial structure and new cells generated from it were meant to die off as these cells are not well retained in the SVZ or structures nearby such as the striatum (Morshead & van der Kooy 1992). One important study showed that cells labeled by the proliferation marker BrdU do not remain in the SVZ for an extended amount of time, but instead of dying, they migrate to the OB and mature into neurons (Lois & Alvarez-Buylla 1994). Additional work since then has clarified the SVZ to OB migration process (figure 2).

It has been estimated that tens of thousands of interneurons are replaced daily by this neurogenesis mechanism (Chang et al 2016). The SVZ is the thin layer of cells covering the lateral ventricles; newborn cells in the SVZ migrate toward the OB via a well-characterized path called the rostral migratory stream (RMS). The SVZ is considered a niche, a unique environment that is home to quiescent, neural stem cells (referred to as type B cells) with astrocytic features. Type B neural stem cells become activated and divide in a process called asymmetric division to generate two daughter cells. One daughter is another type B stem cell and another is a transit-amplifying progenitor called type C cell. Type C cells can

divide many rounds by symmetric division to give rise to a much larger population of another cell type called neuroblasts (type A cells). Type A cells are committed to a neuronal lineage but are not terminally differentiated. Neuroblasts migrate through the RMS toward the OB in a process called tangential migration. The distinct migration path of neuroblasts is well characterized, and is termed “chain migration” because chains of neuroblasts in close contact move forward by contacting each other in a chain like fashion (Lois et al 1996). Once neuroblasts reach the OB core, they migrate radially into the OB parenchyma. Here, they finish their maturation and terminally differentiate into GABAergic and periglomerular interneurons. A very small percentage also become glutamatergic juxtaglomerular cells (Lazarini & Lledo 2011).Besides producing new neurons, SVZ neurogenesis can generate new oligodendrocytes and astrocytes. New oligodendrocytes migrate dorsally and are found in the corpus callosum (Menn et al 2006). The newly generated astrocytes have been found to migrate toward injured areas of the brain (Benner et al 2013). SVZ neurogenesis has also been detected in the adult human brain and other primates; however, its purpose is less well known (Chang et al 2016).

SVZ Neurogenesis and Its Contribution to OB Neuro-circuitry

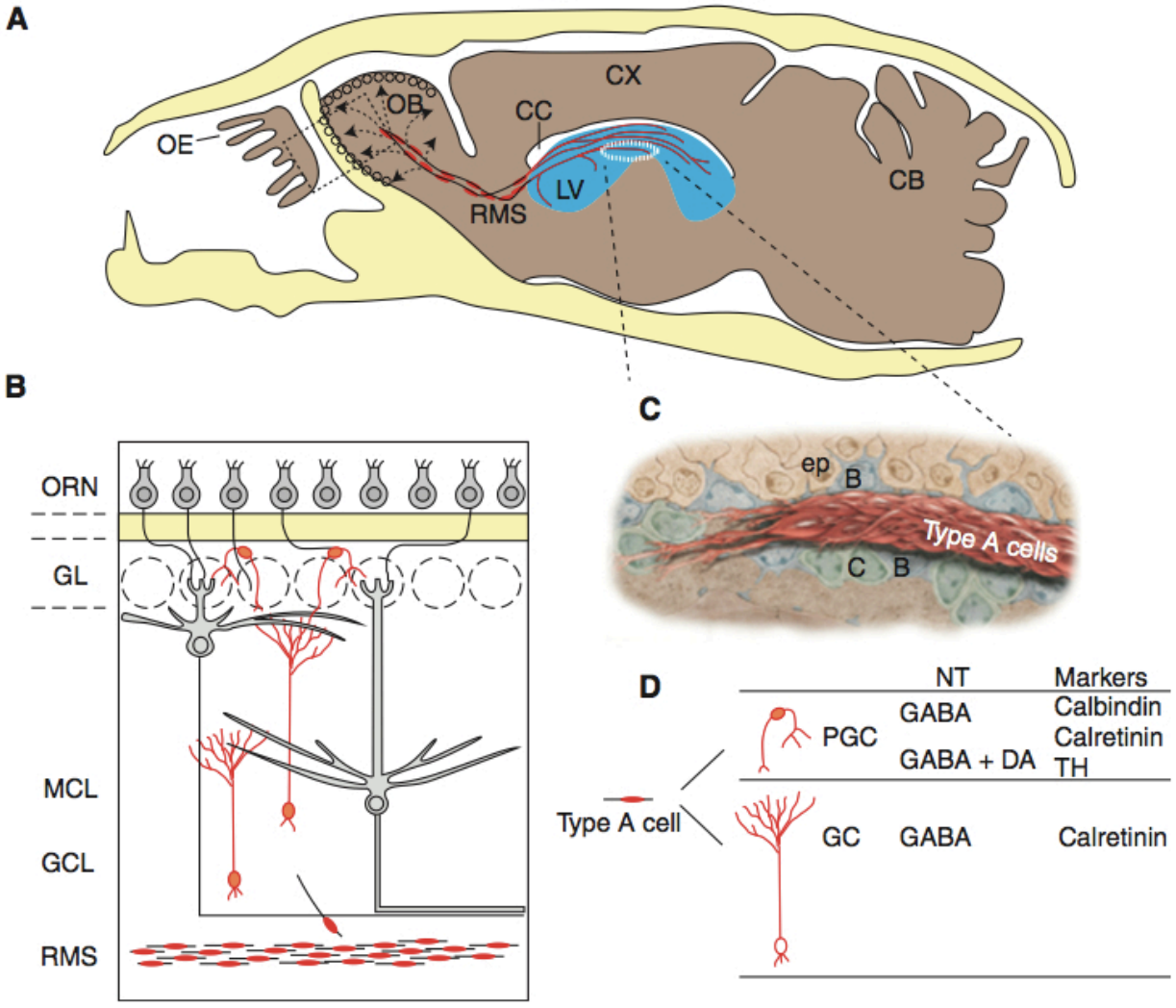


Figure 2: subventricular zone (SVZ) neurogenesis and olfactory bulb (OB) migration, differentiation and maturation. **A** Sagittal view of the rat brain showing the connection between the SVZ, rostral migratory stream (RMS) and OB. **B** Enlargement of the boxed area in part A, showing the different neuronal layers of the OB. The neuroblasts leave the RMS in single cell fashion and differentiate into granule cells (GC) and periglomerular cells (PGC). **C** Depiction of the chain migration of type A cells also known as neuroblasts. **D** Type A cells differentiate into either GC or PCG; each can be distinguished by a unique combination of cellular markers and their neurotransmitter phenotype. Adapted from (Lim & Alvarez-Buylla 2016).

Migration is an important component of neurogenesis during development and the adult. Migration allows cells to move from the place where they were born to their eventual destinations. Along their migratory path, they encounter different interactions such as cell-to-cell, trophic factors and extra-cellular matrix; these interactions play a large role in the development of new neural cells. There are three distinct migratory modes that can be seen during adult neurogenesis, i.e., tangential, radial, and chain migration.

Chain migration is the unique migratory pattern seen in the RMS. Type A cells produced in the SVZ traverse the millimeters-long RMS pathway that connects the SVZ and the OB. These migratory cells use each other as tracks to move forward (Lois & Alvarez-Buylla 1994). This pattern of migration is referred to as homotypic because the type A cells slide along each other. The leading cell will extend a growth cone that serves as a track for the few following cells (Kempermann 2011). Interestingly, in rodent, the RMS is ensheathed in a tube-like structure made up of glial cells; however, functionally, little is known about this glial structure (Gritti et al 2002). One study disrupted this glial structure by manipulating the Beta1 Integrin level and found that chain migration still occurred; however, type A cells moved away from the stream prematurely (Belvindrah et al 2007). In humans, chain migration and the existence of the RMS

had not been extensively confirmed due to the difficulty in observing this anatomical feature. Human neuroblasts have been seen migrating along the obliterated olfactory ventricle in a manner different than that of rodents and without the characteristic cluster of migrating cells (Curtis et al 2007).

Radial migration is seen both in developing CNS as well as in the adult brain. During development, radial migration is seen when new neurons from the ventricular plate migrate out to their terminal location in the different cortical layers. Radial migration during the developmental phase relies on radial glia as the guiding tracks (Poluch & Juliano 2007). In adult neurogenesis, once type A cells reach the core of the olfactory bulb, these immature neurons switch their mode of migration and start moving radially toward the granular and periglomerular layers. In the OB, blood vessels serve as radial scaffold for the migration of type A cells (Bovetti et al 2007). On the other hand, tangential migration is the process in which interneurons move in parallel direction to the plane of the ventricular plate; this migratory process is independent of radial glia. In adult rodents, it has been shown that GABAergic neurons are generated in the SVZ and migrate tangentially toward key structures of the brain such as the cortex, striatum and nucleus accumbens (Inta et al 2008).

SGZ Neurogenesis

The hippocampus is another brain structure that was found to produce new brain cells. Specifically, the subgranular zone (SGZ) of the dentate gyrus, which is at the junction of the hilus and the granule cell layer, is the niche for neural stem cells. Neural stem cells in the SGZ have a different cellular organization compared to their SVZ equivalents. SGZ stem cells are not located next to a ventricle and are surrounded by other neurons (Kempermann 2011). Interestingly, SGZ stem cells have long processes that extend to the granular and molecular layers of the hippocampus. These quiescent stem cells in the SGZ are called type 1 cells, which in turn divide asymmetrically to produce progenitors called type 2a and 2b cells. Type 2a and 2b progenitors divide symmetrically to produce neuroblasts which eventually mature and terminally differentiate into glutamatergic dentate granule cells (figure 3). Newborn hippocampal neurons are hyperexcitable during the critical maturation period of 3-6 weeks after their birth. These new cells receive excitatory glutamatergic inputs from the entorhinal cortex, however, without having inhibitory GABAergic inputs, they have a low threshold for the induction of long-term potentiation (LTP).

Hippocampal Neurogenesis

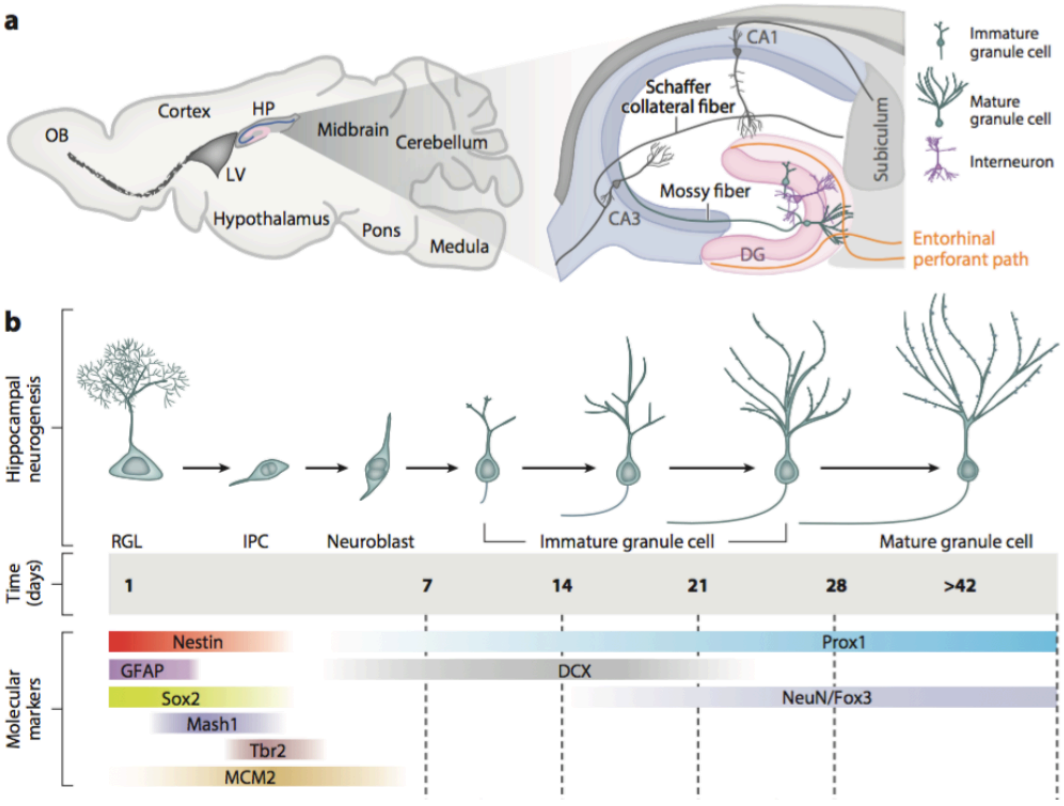


Figure 3: Neurogenesis, differentiation and maturation in the dentate gyrus of the hippocampus. A. Sagittal view of the rodent brain (left) showing the relative location of the dentate gyrus of the hippocampus (HP) to that of the SVZ and lateral ventricle (LV) and the olfactory bulb (OB). Right: enlargement of hippocampus showing cellular connections between the sub-structures of the hippocampus. Perforant path compose of entorhinal cortex input signal by forming synapse with dentate granule cells, which are continually generated by neurogenesis. Granule cells form synapses with pyramidal cells of CA3, and these cells in turn form synapses with CA1 neurons. B. Summary of neurogenesis in the hippocampus. Radial glia-like (RGL) neural stem cells divide to become intermediate progenitor cells (IPCs), which in turn proliferate into neuroblasts. These new neuroblasts undergo several immature stages to finally become mature granule cells. Adapted from (Christian et al 2014).

Molecular Regulators of Neurogenesis

Neural stem cells and progenitors in the SVZ and dentate gyrus are exposed to complex microenvironments composed of local cells and diffusible molecules. Many of the common neurotransmitters in the brains have regulatory functions in neurogenesis. For instance, NMDA receptor dependent activity is inversely correlated with hippocampal proliferation (Zhao et al 2008). Glutamate, the predominant excitatory neurotransmitter in the CNS is the main ligand for NMDA receptors. Furthermore, an *in vitro* study using hippocampal stem/progenitor cells showed glutamate increasing neuronal differentiation (Deisseroth et al 2004). Similarly, the neurotransmitter GABA binds to type 2 progenitor cells in the hippocampus leading to calcium influx and depolarization and subsequent increased expression of NeuroD, a neuronal differentiation factor (Tozuka et al 2005).

The growth factor family of molecules also potently affects neurogenesis. *In vivo* studies demonstrated that both epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) increase SVZ stem cell proliferation (Kuhn et al 1997). Additionally, FGF2 administration leads to increased incorporation of new neurons to the olfactory bulb. FGF2 signaling might also play a crucial role in

hippocampal neurogenesis as one study showed that by deleting its receptor fgfr1, SGZ neurogenesis was decreased (Zhao et al 2007). Another key regulator of neurogenesis is brain-derived neurotrophic factor (BDNF). Transgenic animals lacking BDNF receptor p75 have smaller olfactory bulbs and impaired SVZ neurogenesis. Interestingly, BDNF appears to mediate antidepressant-induced increase in hippocampal neurogenesis. One study showed that using transgenic mice with deficits in TrkB signaling (the main receptor for BDNF), antidepressant treatment failed to increase SGZ cell proliferation (Duman & Monteggia 2006).

ROS have traditionally been thought to be detrimental to cellular activities. ROS accumulation is a result of oxidative stress and is a contributor to many diseases and neurodegeneration in the brain. The effect of ROS on stem cells is intriguing; for instance, hematopoietic stem cells (HSCs) maintain a low endogenous level of ROS, which is critical for the maintenance of quiescence. This is important to prevent the exhaustion of the available pool of stem/progenitor cells (Jang & Sharkis 2007). Intracellular ROS had emerged to be a critical second messenger in a tightly controlled signal transduction system to control cellular proliferation (Kwon et al 2004). Endogenous ROS levels are controlled by mitochondrial activity and several NADPH oxidase (NOX) enzymes (Lambeth et al 2008). In contrast to the HSCs, neural stem cells (NSCs) are dependent on the ROS

as the mediator of the proliferative response (Figure 4). One important study found higher ROS levels in the neural stem/progenitor cells in the SVZ (Le Belle et al 2011). Additionally, when ROS was artificially increased by the addition of non-toxic concentration of H_2O_2 *in vitro*, SVZ stem cell proliferation significantly increased. This same study also showed that NOX2 mutant mice had impaired SVZ proliferation and smaller olfactory bulbs (Le Belle et al 2011).

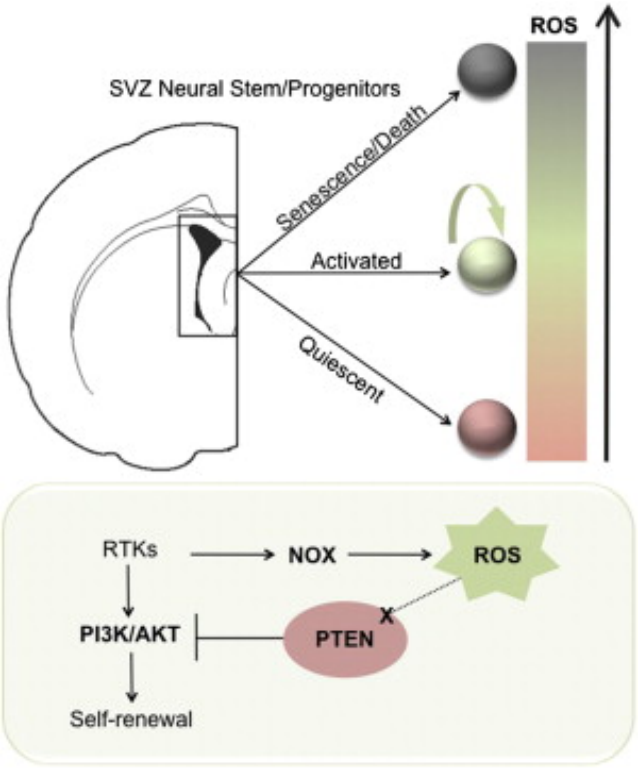


Figure 4: Reactive Oxygen Species (ROS) regulation and its role in SVZ neurogenesis. Adapted from (Le Belle et al 2011).

Systemic Regulators of Neurogenesis

It is well known that aging is associated with a progressive decrease in cognitive functions such as memory formation. Furthermore, strong evidence exists in animal models for the decline in total neurogenesis in aged animals (Zhao et al 2008). The hippocampus is the brain structure most often associated with memory formation. In aged animals, both hippocampal volume and the level of neurogenesis significantly decrease (Driscoll et al 2006). Other reports utilizing transgenic mice demonstrated that in the SGZ of aged animals, there were significant reductions in both the number of stem/progenitor cells and the proliferative rate of those cells (Walter et al 2011). However, cognitive impairment in the aged animals did not necessarily correlate with decreased neurogenesis. One study used the MWM to demonstrate that in aged rats, animals with impaired learning ability have higher proliferative rate in the SGZ compared to a similarly aged group with intact learning ability (Bizon et al 2004). Additionally, aging also affects the SVZ-OB area and its function in both humans and experimental animal models. For instance, in humans, olfactory discrimination abilities decrease with age (Kaneda et al 2000). One important study using a mice model of aging found that aged animals exhibit decreased SVZ proliferation and less OB new interneurons compared to younger mice (Enwere et

al 2004). Additionally, the aged mice performed the same as young mice when discriminating two discrete odors; but performed significantly worse at discriminating similar odors. This finding indicates that SVZ-OB neurogenesis is important in fine olfactory discrimination tasks. This same group also found that the OB of aged mice expressed less epidermal growth factor (EGF) and its corresponding receptor (EGFR).

Stress is another potent negative regulator of neurogenesis. Increased stress leads to decreased cell proliferation in the dentate gyrus of the hippocampus. This phenomenon had been reported in various species including mouse, rat, tree shrew and marmoset (Mirescu & Gould 2006). Various stressors were used in these animal models to demonstrate this effect including subordination, resident-intruder, foot-shock, restraint, isolation, cold immobilization, cold swim and predator odor (Gould et al 1997, Gould et al 1998, Malberg & Duman 2003, Pham et al 2003). Furthermore, both acute and chronic stressors cause decreased hippocampal neurogenesis (Mirescu & Gould 2006). The cause for decreased hippocampal neurogenesis following stress is linked to stress hormones like glucocorticoids. Glucocorticoids directly regulate hippocampal neurogenesis. For instance, the cells of the hippocampus express adrenal steroid receptors. Furthermore, modulating circulating levels of these

stress hormones changes hippocampus structure and functions (McEwen 1999). Studies in which either the adrenal gland was removed or pharmacological methods were used to inhibit the HPA axis (CRF-1 and V1b receptor antagonists) showed an increase in hippocampal neurogenesis (Cameron & McKay 1999); (Alonso et al 2004). In contrast, administering exogenous corticosterone to postnatal and adult rodents leads to an opposite decrease in hippocampal neurogenesis (Cameron & Gould 1994, Gould et al 1991). Taken together, these findings indicate that glucocorticoids are the major mediator of stress-induced effects on hippocampal neurogenesis.

In contrast to age and stress as two of the most characterized negative regulators of neurogenesis, various environmental factors contribute positively to neurogenesis. For instance, voluntary running had been shown to promote SGZ cell proliferation (Kee et al 2007). Exposure to an enriched environment was shown to increase new hippocampal neuron survival (Olson et al 2006). Importantly, both voluntary exercise and environmental enrichment lead to an increase in performance of animals in the MWM (Zhao et al 2008). One study used a transgenic strain of mice lacking presenilin-1, which is a protein critical to the memory function of the hippocampus and showed that environmental enrichment did not elicit a response in neurogenesis in these animals (Feng et al

2001). Taken together, these studies demonstrate the hippocampal functional implications of increased neurogenesis caused by positive environmental regulators.

Neural activity has also been shown to positively regulate neurogenesis. The relationship between learning and SGZ neurogenesis is complex with specificity toward experimental tasks, phases of learning and SGZ stem/progenitor cell types (Zhao et al 2008). The enhanced survival of new hippocampal neurons is only seen in learning tasks that are specifically dependent on the hippocampus. For instance, trace eyeblink conditioning and the Morris water maze lead to higher survival of newborn SGZ neurons while the active shock avoidance and delayed eyeblink conditioning did not (Leuner et al 2006). Hippocampal learning does not simply lead to a general increase in SGZ proliferation and survival. One interesting study showed that training in the MWM increased apoptosis of the early proliferating cells and that blocking this apoptosis event led to worse subsequent performance (Dupret et al 2007). Additionally, learning tasks such as the MWM can be broken down into specific stages, such as the early phase where rapid improvement of performance is seen and late asymptotic phase. Each of these phases affects hippocampal neurogenesis differently. No SGZ cell proliferation is observed during the early

phase while during the late asymptotic phase, increased hippocampal cell proliferation is observed (Döbrössy et al 2003). These findings illustrate the complexity and specificity of learning-induced increases in hippocampal neurogenesis and suggest the functional importance of integration of only a subset of newborn neurons.

Functions of SVZ and SGZ Neurogenesis

The field of neurogenesis is still in its infancy; however, the roles of new neurons that are produced by neurogenesis are starting to be clarified by studies. While neurogenesis is not required for survival in laboratory animals, functional deficits are evident using sensitive behavioral tasks. For instance, new neurons that are produced in the SVZ and migrate to the OB integrate into the existing interneuron circuits and significantly contribute to the network activity. The olfactory system processes odorant signals from the environment. Specifically, odorant molecules enter the nasal cavity and bind to olfactory receptors, which are expressed by olfactory sensory neurons (OSNs). These sensory neurons have axons that pass through the cribiform plate and innervate the OB. Mitral and tufted cells are the main projection neurons that receive the signals from OSNs and send them to higher olfaction processing centers (Munger et al 2009).

Periglomerular and granule cells are GABAergic inhibitory neurons that are being continually generated by neurogenesis in the SVZ. These cells make contact with OSN axon terminals and the dendrites of mitral and tufted cells and modulate the incoming olfactory signals. In effect, these new inhibitory interneurons play important roles in fine tuning the olfaction signals (Lazarini & Lledo 2011). These new neurons are not necessary for the detection or discrimination of odorants, but seem to have some roles in the cognitive processing of odorant context. An example of this process is learning to discriminate two or more similar odors (Moreno et al 2009). Intriguingly, when OB neurogenesis is prevented, which leads to the depletion of OB interneurons, no sensory deficits were observed with tasks such as smell discrimination (Imayoshi et al 2008). Yet, similar irradiations of the OB resulted in significant impairments in olfactory fear conditioning (Lazarini et al 2009).

In the hippocampus, newly produced granule neurons participate in a similar process that is called pattern separation. This is when similar inputs are differentiated and processed as distinct conditions (Clelland et al 2009). Pattern separation manifests as the ability to discriminate subtle differences in spatial patterns. This can be traditionally assessed by the spatial memory task, the MWM, in which animals have to use environmental visual cues to find the hidden

platform in a pool of water (Garthe & Kempermann 2013). Studies that ablate hippocampal neurogenesis consistently resulted in deficits in the MWM performance. In contrast, studies that use methods to stimulate hippocampal neurogenesis lead to improvement in the MWM task (Lacar et al 2014, Sahay et al 2011a). These studies demonstrate that hippocampal neurogenesis in rodents is important for acquisition of the platform location, probe trial performance (when the platform is removed to access the length of time animals are in the goal quadrant), and reversal (when the platform is move to a random new location and the animals have to re-learn) (Garthe & Kempermann 2013).

While the functional relevance of adult neurogenesis in rodents has been well established, their roles in adult humans are less well understood. However, adult neurogenesis is implicated in a number of diseases such as epilepsy and major depression, (Braun & Jessberger 2014). A decrease in adult hippocampal neurogenesis does not contribute to the development of depression. However, hippocampal neurogenesis is necessary for the efficacy of antidepressants (Sahay & Hen 2007). On the other hand, aberrant hippocampal neurogenesis has been implicated in epileptogenesis (Parent & Murphy 2008). New hippocampal neurons after either seizure or traumatic brain injury can form abnormal networks that can play a role in future propagation of seizure activity. Furthermore, striatal

interneurons are being generated throughout the human adult lifetime by adult neurogenesis. This same population of interneurons has been found to be depleted in Huntington's disease (HD) patients raising the possibility that the lack of adult neurogenesis might contribute to the HD disease state (Jessberger & Gage 2014).

SVZ Neurogenesis in TBI

Mechanical forces involved in primary TBI can affect the SVZ directly and therefore affect neurogenesis. For instance, cranial compressive force can tear the lateral ventricle lining and increase intracranial pressure. Hemorrhages (epidural, intracranial, extradural) can cause a persistent increase in intracranial pressure and increase in lateral ventricular pressure. Hemorrhages can lead to obstructive hyrocephalus and lead to lateral ventricular hydrostatic pressure. Hydrocephalus has been shown to cause mechanical damage to the lateral ventricular wall (Bruni et al 1985, Sarnat 1995). Additionally, brain edema, which is part of the mid-term secondary injury process, leads to elevated intracranial pressure. In contrast, cortical lesions with extensive brain matter loss lead to enlarged ventricle volume (Reider-Groswasser et al 2002). These are the possible mechanical stresses that can affect the SVZ stem cell environment.

The mechanism for stem cell responses to mechanical forces has been described: for instance β-catenin/Wnt signaling : β-catenin can translocate to the nucleus in response to increased mechanical force. In the nucleus, β-catenin inhibits cell cycle exit and this leads to an increase in proliferation (Chenn & Walsh 2002).

Ependymal cells are ciliated cells lining the lateral ventricle wall and are the first part of the SVZ structure. These ciliated cells affect SVZ fluid dynamics and form a barrier that separates the fluid of the lateral ventricle and the SVZ cells by tight junctions with each other (Mitro & Palkovits 1981). It is thought that TBI affects the strength of these ependymal tight junctions in addition to other extracellular interactions, which can lead to enlarged ventricles. Ventricular enlargement can be modeled in the rat by kaolin administration, which leads to communicating hydrocephalus. This model demonstrated that there is a transient increase in the number of SVZ Nestin^{+} cells, which are mature neurons, in communicating hydrocephalus.

There is some evidence to suggest that more neuroblasts survive after TBI compared to normal animals. For instance, in a mouse transgenic model of TBI using CCI, SVZ proliferative cells were labeled by yellow fluorescence protein (YFP). After a period of 6 weeks post-TBI, the number of YFP^{+}/DCX^{+} double

positive cells was increased in the dorsolateral SVZ but few were found in the TBI lesion area (Mierzwa et al 2014). Another separate study found very few mature neurons in the perilesional area 28 days after injury (Goodus et al 2014). Furthermore, the SVZ is a distinct niche compared to areas that the neuroblast migrate to such as the corpus callosum or the cortex; and these different niches affect differentiation of newly proliferated cells differently. For instance, one interesting study found that newly generated SVZ cells that migrated to the corpus callosum after TBI became oligodendrocytes. In contrast, those that migrated to the perilesional area became astrocytes (Goings et al 2004). Another study found that most BrdU positive cells in the perilesional area were either positive for Iba1 or GFAP but not NeuN (Radomski et al 2013). Overall, there is strong evidence for TBI increasing the survival and migration of SVZ derived cells; yet evidence for the differentiation of these new SVZ-derived cells into functionally relevant new cortical neurons is limited.

Detection of Neurogenesis by Measuring Cell Proliferation

Cell proliferation is a critical parameter of neurogenesis: Cell proliferation gives rise to new cells in the SVZ because it contains cells with proliferative capacity (stem cells). Cell proliferation is most commonly assessed using 5-Bromo-

2”-deoxyuridine (BrdU) labeling. BrdU is injected into an animal or introduced into the media of an *in vitro* system, and cells that are actively dividing will take up this DNA nucleotide analog and incorporate it into the genome. Monoclonal antibodies against BrdU are used to detect it in cells. BrdU labeling detects cells that are in the S-phase of the cell cycle; however, there are some thoughts that reporting a pure number of BrdU+ cell is less useful than measuring what is called the “labeling index” which is basically the percentage of cells that are BrdU+ (Chang et al 2016). In addition to using BrdU to detect the population of cells that have undergone proliferation, intrinsic markers of the cell cycle can also be used. For instance, proliferative cell nuclear antigen (PCNA) and Ki67 are the two most widely used markers.

There are some interesting differences in how the SVZ of rat and mouse response to a cortical injury with the caveat that these results are from multiple labs using different models of TBI. Generally, proliferation increases after TBI, but only transiently before going back to baseline. There are several studies in the mouse that indicate that proliferation decreases after TBI (Goings et al 2002, Jankovski et al 1998). In the rat, multiple studies looking at proliferation at different time points after TBI seem to indicate that there are four distinct phases in the SVZ proliferative response (Chang et al 2016). During the first five days,

proliferation appears to be unchanged; between 5 days and seven days, proliferation peaks with about 100% increase over baseline; from the peak at 7 days, proliferation remains high but slowly declines and comes back to baseline at 4 weeks after TBI (Bye et al 2011). However, by 8 weeks after TBI, baseline proliferation is decreased by about 50% (Acosta et al 2013).

SVZ Proliferation in Humans After TBI

There is considerable evidence for SVZ proliferation in humans. For instance, children two years old and younger have a high level of constitutive SVZ proliferation. However, in adults, evidence has not been as strong. One study found that after 18 months of age, proliferation along the RMS and SVZ are not detectable (Sanai et al 2011). On the contrary, another study found that there are migrating doublecortin positive cells (DCX^{+}) in the RMS and olfactory bulb in adults (Wang et al 2011). Furthermore, using a novel technique of carbon (^{14}C) dating, proliferative SVZ and striatal neurogenesis was detected in adult brains (Bergmann et al 2012, Ernst et al 2014). ^{14}C is an isotope of carbon that is elevated following terrestrial nuclear bomb testing. The use of this carbon dating technique is very useful in birth dating neural cells; however, it requires highly specialized equipment. Other alternative techniques that had recently been used

to investigate human neurogenesis are non-invasive magnetic resonance imaging (MRI) and positron emission tomography (PET) (Jessberger & Gage 2014). However, these imaging techniques at this point still lack specificity and resolution to accurately detect neurogenesis in humans.

Following TBI, one post-mortem study looked at brain tissue of a group of children; 6 had TBI and 7 were non-TBI controls. They detected no differences in the number of DCX^+ cells (Taylor et al 2013). On the contrary, a separate group found that adult patients following TBI expressed a higher number of cortical DCX^+, PSA-$NCAM^+$ and $SOX2^+$ cells, all of which are markers indicative of neurogenesis (Zheng et al 2013). Overall, more complete human studies are needed to definitively evaluate if TBI has an effect on SVZ proliferation; for instance, better use of markers for proliferation, better control of TBI type, age, and timing are needed for conclusive results.

It had been thought that for the therapeutic potential of stem cells to be fully realized, the newly produced cells must migrate from the site that they are produced (in this case the SVZ) to the lesion area. In adult rodents, newly produced immature neurons, i.e., neuroblasts, migrate from the SVZ to the OB along the RMS in strings in chain migration, which is in part assisted by a network

of astrocytes (Jankovski & Sotelo 1996, Lois et al 1996). In the event of a CNS injury, the stream of migrating neuroblasts are induced to migrate out in both chains and as individual cells. Diffusible protein signaling is thought to be playing a role in attracting neuroblast migration toward the lesion area (chemoattractants). Astrocytes in the lesion area secrete SDF-1, which has been shown to increase mobility of neuroblasts (Kokovay et al 2010). In the mouse, OB transection experiments in which the RMS is cut has shown that neuroblasts accumulate posterior to the cut and that these cells migrate toward the cortical lesion and striatum (Jankovski et al 1998). It was found that in response to cortical TBI, neuroblasts migrate out of the RMS in chain formation and enter into the corpus callosum; however, when they go into the cortex, they migrate as individual cells which suggest there are multiple mechanisms of migration of neuroblasts after injury (Kempermann 2011). The evidence for neuroblast migration toward the injury site in humans is less convincing. One post-mortem study looking at brain tissue from children less than 10 years old detected the presence of DCX^+ cells that had the morphology of migratory cells; these DCX^+ cells were negative for astrocyte, microglia or macrophage markers (Taylor et al 2013).

The limited endogenous stem cell response means that finding a pharmacological means of increasing this intrinsic stem cell response might be

useful in treating TBI. To this end, there are many potential therapies that have shown good promise in experimental models. A study using activated protein C delivered intraperitoneally to mice increased post-TBI neuroblast proliferation and migration to the perilesional area (Petraglia et al 2010). Furthermore, administration of colony-stimulating factor (GCSF) after TBI was shown to lead to increased DCX^+ cells in the SVZ (Yang et al 2010). One group showed that either GCSF and/or human umbilical cord blood cell (hUBC) significantly increased SVZ and hippocampal DCX^+ cells 8 weeks after TBI (Acosta et al 2014). Infusion of growth factors was shown to increase some aspect of SVZ neurogenesis and could theoretically be a potential treatment for TBI. For instance, administration of BDNF and its molecular mimetic resulted in SVZ derived cells migrating toward adjacent nuclei (Benraiss 2001). EGF and FGF2 administration led to higher SVZ proliferation (Kuhn et al 1997). TGF-a administration resulted in higher striatal accumulation of SVZ derived cells (Craig et al 1996). Extra-cellular matrix (ECM) molecules and cell surface protein can also play a role in neurogenesis. Matrix metalloproteinase (MMP) regulates ECM molecules by degrading them and also plays a role in regulating neurogenesis. The cell surface proteins EphrinB3 and EphB3 can control SVZ neurogenesis via a p53 dependent mechanism (Theus et al 2010). While these experimental means to increase neurogenesis have yielded

interesting cellular responses, further work needs to be done to evaluate their functional relevance and to identify any negative outcomes. Overall, in order to identify worthwhile therapies for use in the clinics, significant improvements in the standardization of animal models for TBI and the behavioral tasks used to evaluate these animals are needed.

CHAPTER THREE

METHODS

Animal Subjects

Male Sprague Dawley rats (Harlan, IN), 2 month old at the beginning of the experiments, were used in our study (total animal number:76) (Table 3 and 4). Rats were housed as a pair in each cage in a fully accredited animal care facility with a 12-hour light/dark cycle. Food and water was available ad libitum. Experiments were approved by the Hines VA Institutional Animal Care and Use Committee. All animals were number coded and investigators were blinded to the animal treatment groups.

Alcohol Administration

Rats were administered three consecutive doses of alcohol by gastric gavage at a dose of (3g/kg, i.g. of 40% alcohol) once per day for three days (Figures 5 and 6). A curved stainless steel gavage needle with a smooth rounded ball head was used along with a 5cc syringe to administer the alcohol (Oregon State University,Laboratory Animal Resources Center, 2011). Additionally, the gavaging needle was lubricated with food-safe lubricant; all animals tolerated the

gavage procedure with no adverse effects. Control animals received an equal volume of water, given using an identical dosing schedule. This alcohol dose leads to blood alcohol levels typical of a TBI patient entering the emergency room (150mg/dl) (Bombardier & Thurber 1998) and was well tolerated by these rats which lead to no adverse weight loss during the three days administration window. Furthermore, this dosing regimen has been adapted from one previously used to mimic binge drinking (Przybycien-Szymanska et al 2010). One hour after the last gavage administration, animals were either subjected to a moderate brain injury using the controlled cortical impact model (CCI) of TBI or given a sham surgery (Figures 5 and 6).

Blood Alcohol Level Quantification

Approximately 250ul to 500ul of blood was drawn for the tail vein of each rat immediately following the TBI surgery. Blood was then allowed to clot on ice for five minutes and centrifuged, to separate the serum. Alcohol concentration was measured using alcohol reagent set (Pointe Scientific, Canton MI, USA). The alcohol reagent set relies on an enzymatic method to detect ethyl alcohol:

$$\text{Ethanol} + NAD^{+} \xrightarrow{\text{ADH}} \text{Acetaldehyde} + NADH + H^{+}$$

Table 3. Experimental Groups for Aim 1				
Group	Alcohol	TBI	24 hrs	1 week
1	-	-	6	6
2	-	+	6	7
3	+	-	6	6
4	+	+	6	7
Subtotal			24	26
Total			50	

Table 4. Experimental Groups for Aim 2			
Group	Alcohol	TBI	6 weeks
1	-	-	6
2	-	+	7
3	+	-	6
4	+	+	7
Total			26

Table 3 and 4: Animal numbers and groups for Aim 1 and Aim 2 respectively. Animals were randomly assigned to each of the 4 experimental groups, either with alcohol or vehicle and TBI or sham.

Alcohol dehydrogenase (ADH) catalyzes the reaction of ethanol to acetaldehyde with the concomitant reduction of NAD+ to NADH; this leads to a change in absorbance at 340 nm which is directly proportional to the ethanol concentration in the sample. The serum samples were de-proteinized using 6.25% trichloroacetic acid; 0.9% sodium chloride was used as the non-serum blank. Samples were loaded onto 96 well plates at a sample/reagent ratio of 1:201, incubated at 30°C for 5 minutes, and the absorbance was read at 30°C. A standard curve was generated for each assay run from the known ethanol samples. Sample measurements were done in duplicate and the values averaged

for each animal. Known high and low alcohol controls were always run along with unknown samples and the resultant alcohol concentration values for the controls were always within acceptable range ($^{+}$/- 5%).

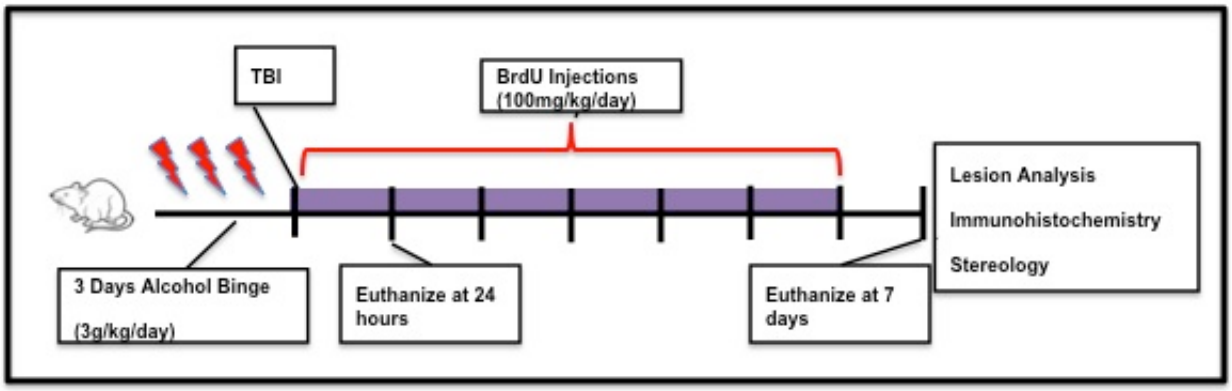


Figure 5: Experimental design for Aim 1. Rats experienced 3 consecutive daily doses of alcohol binge. One hour after the last alcohol dose, animals undergo TBI surgery. Animals sacrificed at 24 hours after TBI received only 1 dose of BrdU injection by i.p at the time of TBI. Animals sacrificed at 7 days received 7 consecutive daily doses of BrdU.

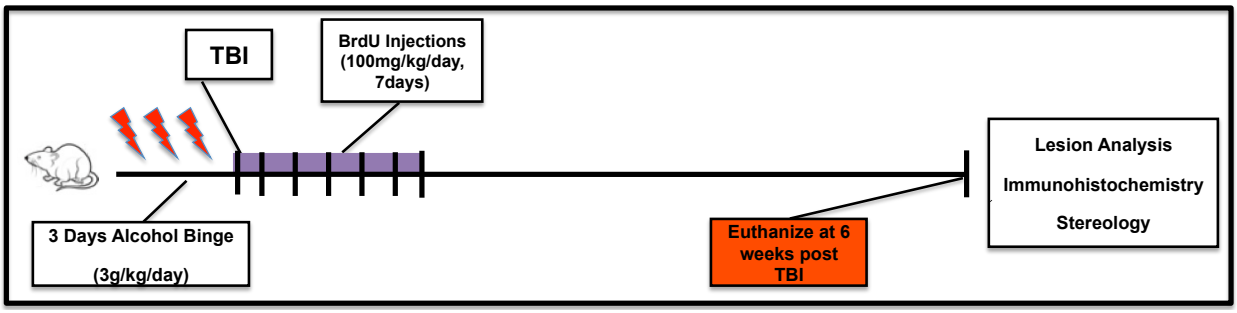


Figure 6: Experimental design for Aim 2. Rats experienced binge alcohol, TBI and BrdU injections identically to those in Aim 1. Animals survived for 6 weeks following TBI.

TBI Model

All rats were anesthetized using 75mg/ml of isoflurane delivered as an inhalant with 5% oxygen. The TBI was delivered using a double acting magnetic piston mounted on a stereotaxic crossbar, angled for cortical impact (diameter: 3 millimeters, velocity: 2.5 meters/second, depth: 2 millimeters, dwell time: 250 milliseconds). Animals were secured in a stereotaxic frame and a midline incision was made and the skin was pulled back. The analgesic buprenorphine was administered locally near the skin incision (0.1cc). Using a trephine, a disc of skull bone was removed above the forelimb area of the sensorimotor cortex (1 millimeter anterior, 1.5 millimeter lateral from bregma) on the right hemisphere. Saline solution (0.9%) was used during the trephine process to minimize heat. Before the impactor was placed in position, the dura layer covering the brain was carefully removed. This impact resulted in a reproducible lesion, typified by both focal and diffuse brain damage. Sham animals were treated identically (anesthetized, skin incision, etc), apart from the piston. The CCI model has the advantage that it allows the experimenter to control precisely the physical/kinetic variables of the injury (velocity, dwell-time, diameter, etc). In our model, this injury leads to prolonged deficit on the skilled forelimb-reaching task. Most importantly, the CCI model is clinically relevant, leading to the histological

sequelae often seen in humans after TBI: epidural and subdural hematoma as well as intraparenchymal petechial hemorrhages. Following the surgery, rats were closely monitored for complications; rats were able to ambulate and eat normally in less than three hours post injury.

Bromodeoxyuridine (BrdU) Administration

Bromodeoxyuridine (BrdU) is an analog of the nucleoside thymidine that can be incorporated into the DNA during DNA synthesis. During cell division, extensive DNA synthesis occurs in the genome prior to physical cell division thus BrdU incorporation is a stable marker for proliferation (Taupin 2007). Commonly, BrdU is administered through intraperitoneal (i.p.) injection, and it is estimated that BrdU is available for labeling proliferating cells in the brain for approximately two hours after administration (Taupin 2007). BrdU can be detected histologically using monoclonal antibodies and combined with labeling various cell phenotype markers; this essentially allows for the birthdating and cell-type determination of newborn cells.

There is a dose-dependent effect of BrdU administration on the number of labeled cells in the proliferative brain regions saturating at around 200mg/kg. This dose has been found to maximally label proliferating cells in the dentate gyrus

after stimulating proliferation with exercise (Cameron & Mckay 2001, Eadie et al 2005). For long-term assessment of newborn cell phenotypes, it is necessary to administer multiple doses of BrdU spaced further apart to mitigate the effects of BrdU dilution over successive cell divisions.

In this dissertation, two separate BrdU injection paradigms were used. In both cases, BrdU was prepared at 20mg/mL in sterile saline. The solution was warmed in a water bath and repeatedly vortex-mixed until dissolved and then sterile-filtered through a 0.22 um syringe filter. To measure short-term cellular proliferation, a single injection of BrdU at 100mg/kg body weight (i.p.) was injected immediately after TBI followed by sacrifice 24 hours after. For mid-term determination of cell proliferation and long-term determination of newborn cell phenotypes, BrdU was injected at 100mg/kg (i.p.) starting immediately following TBI and continuing once a day for the next six days. Rats were anesthetized with isoflurane during BrdU injection to minimize discomfort.

BrdU labeling is advantageous compared to staining for endogenous proliferation markers such as Ki67 or PCNA (Proliferating Cell Nuclear Antigen) because of its short half-life; this property of BrdU essentially allows us to birth-

date the new cells. BrdU was injected after TBI, labeling only the proliferative response after TBI.

Perfusion, Tissue Processing, and Histology

At various time points, rats were overdosed with Euthasol (phenytoin/pentobarbital; 390 mg/kg i.p.) and transcardially perfused with cold heparinized saline followed by 4% paraformaldehyde (PFA). Brains were extracted and post-fixed overnight at 4°C in 4%PFA, then cryoprotected in 30% sucrose in phosphate buffer pH 7.4 until sinking. Brains are then frozen in Optimal Cutting Temperature Compound (OCT) for cryosectioning. Sections (40 µm) were cut on a cryostat and stored in ethylene glycol-based antifreeze cryoprotectant at -20°C until use. BrdU requires an antigen retrieval procedure to be identified immunohistochemically. Our method was adapted from (Tang et al 2007), where tissue sections were subjected to high heat (99-100° C) sodium citrate solution for 10-15 min. This method was preferable to hydrochloric acid-based procedures since this severely weakens subsequent DNA staining, such as with DAPI or Hoechst. However, incubation of free-floating sections in boiling sodium citrate leads to excessive wrinkling of the tissue that impairs downstream processing steps. Therefore, we adapted a staining method where the tissue sections were

extensively washed in phosphate buffer pH 7.4, mounted on plus-charged slides, and then allowed to dry overnight at room temperature. The following day, the slides were incubated in 99-100° 10 mM sodium citrate pH 6, which had been placed in a container in a boiling water basin, for 10 minutes. The slides were then removed and immediately immersed in room temperature phosphate buffer pH 7.4. From there, the tissue sections were carefully removed from the slides using a razor blade and rinsed in phosphate buffer. We found that using this strategy, tissue wrinkling was nearly eliminated, while still allowing us to perform free-floating tissue staining (which allows for better antibody penetration) on thick tissue sections (which are required for the optical fractionator stereology probe).

This procedure led to bright BrdU staining that was compatible with general DNA labeling using DAPI, as described by Tang and colleagues (Tang et al 2007). However, we experienced very high white matter nonspecific fluorescence, as if the white matter had become extremely “sticky” to antibodies. This was traced to the use of high concentrations of the detergent Triton X100 in the incubation buffers. Switching to Tween-20 greatly reduced the background fluorescence and was therefore used in all steps after high heat antigen retrieval.

Immunostaining was performed by diluting primary antibodies in phosphate- buffered saline (PBS) pH 7.4 plus 0.2% Tween-20 (when high heat antigen retrieval was used) or 0.3% Triton-X100 (when no antigen retrieval was used). Tissue sections were then incubated in primary antibody solution overnight at 4° C with gentle agitation. The following day, the tissue sections were extensively washed in PBS/0.2% Tween 20 (for high heat antigen retrieval) or phosphate buffer pH 7.4 (when antigen retrieval was not performed). The sections were then incubated in fluorophore- or biotin-conjugated secondary antibodies in the same dilution buffers listed above for 2 hours at room temperature with shaking, and then washed. For fluorescent imaging, nuclei were counterstained with DAPI, then mounted on gelatin-subbed slides and coverslipped with Fluoromount G anti-fade mounting media. For signal detection with the chromogenic substrate diaminobenzidine (DAB), tissue sections were incubated in avidin-biotin- peroxidase complex (Vector Laboratories, 1 drop component A plus 1 drop component B per 5 mL dilution buffer, as above) for 1 hour at room temperature. Lastly, sections were reacted in nickel-enhanced DAB in the presence of hydrogen peroxide to visualize the target antigens.

Marker/Antigen	Abbreviation	Class or function	Associated cell type	Antibody source; host; concentration
Glial fibrillary acidic protein	GFAP	Intermediate filament	Astrocytes; neural stem cells in SVZ and DG	DAKO Z0334; Rabbit; 1:1000 (IF)
Doublecortin	DCX	Microtubule-associated protein	Immature neurons in SVZ and DG	CellSignaling; Rabbit; 1:500 (IF)
Ionized binding adapter molecule 1	Iba1 (aka AIF1)	EF-hand containing calcium binding protein	Microglia, macrophages	Wako 019-19741; Rabbit, 1:5000 (IHC)
5-bromo-2'-deoxyuridine	BrdU	Thymidine analog	Incorporate into cell during DNA synthesis, a marker of proliferation	Thermo MA3-071;IgG2a; 1:500(IF); 1:4000 (IHC)
Neuronal nuclei	NeuN (aka Fox3)	RNA splicing factor	Mature neurons	Millipore MAB377 (Ms IgG1); ABN78 (rabbit); 1:1000 (IF)
4',6-diamidino-2-phenylindole	DAPI	DNA minor groove binder	DNA	IF

Table 5: Summary of cell-type markers and antibodies used in this dissertation. IF: immunofluorescence; IHC: Immunohistochemistry (with avidin-peroxidase complex and DAB detection).

Unbiased stereology

Unbiased stereology is a method of estimating properties (number, length, volume, etc.) of three-dimensional structures from tissue sections without making assumptions about the size, shape, or orientation of the objects of interest (Peterson 1999). Various stereology "probes" can be applied to tissue sections to estimate these quantities (Table 6). One of these probes, the optical fractionator, is designed to quantify the number of features (for example, cells) within a region of interest. The software-based optical fractionator probe superimposes a grid with counting boxes at systematically-spaced intervals, and features within the box are counted with specific counting rules after excluding "guard zones" at the tissue surfaces (Figure 7). An estimate of the total number of features within the brain structure of interest is calculated by:

$$N = \sum Q^{-} \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}$$

Where 'N' is an estimate of the total number of features, ΣQ- is the the sum of the counted features, *ssf* is the section sampling fraction (for example, 1/6 if 1 of every 6 sections is examined), *asf* is the area sampling fraction (the proportion

of the initially defined area that is sampled), and *tsf* is the thickness sampling fraction (the fraction of the tissue section thickness that is sampled).

Use of the optical fractionator is warranted in studying cellular proliferation in the subventricular zone, as the number of proliferating cells is high and generally evenly distributed, especially in the dorsolateral SVZ. However, the optical fractionator is less useful for studies of neural precursor proliferation in the dentate gyrus, where proliferating cells are often found in discrete clusters with large spaces in between (Noori & Fornal 2011). For this reason, and because of the lower level of cellular proliferation in the DG versus the SVZ, absolute counting has been advised (Noori & Fornal 2011).

Type of Measurement	Probe Used
Cell Size	Nucleator
Cell Shape	Isotrophic Fakir
Cell Number	Optical Fractionator

Table 6: Different types of probes commonly used in unbiased stereology.

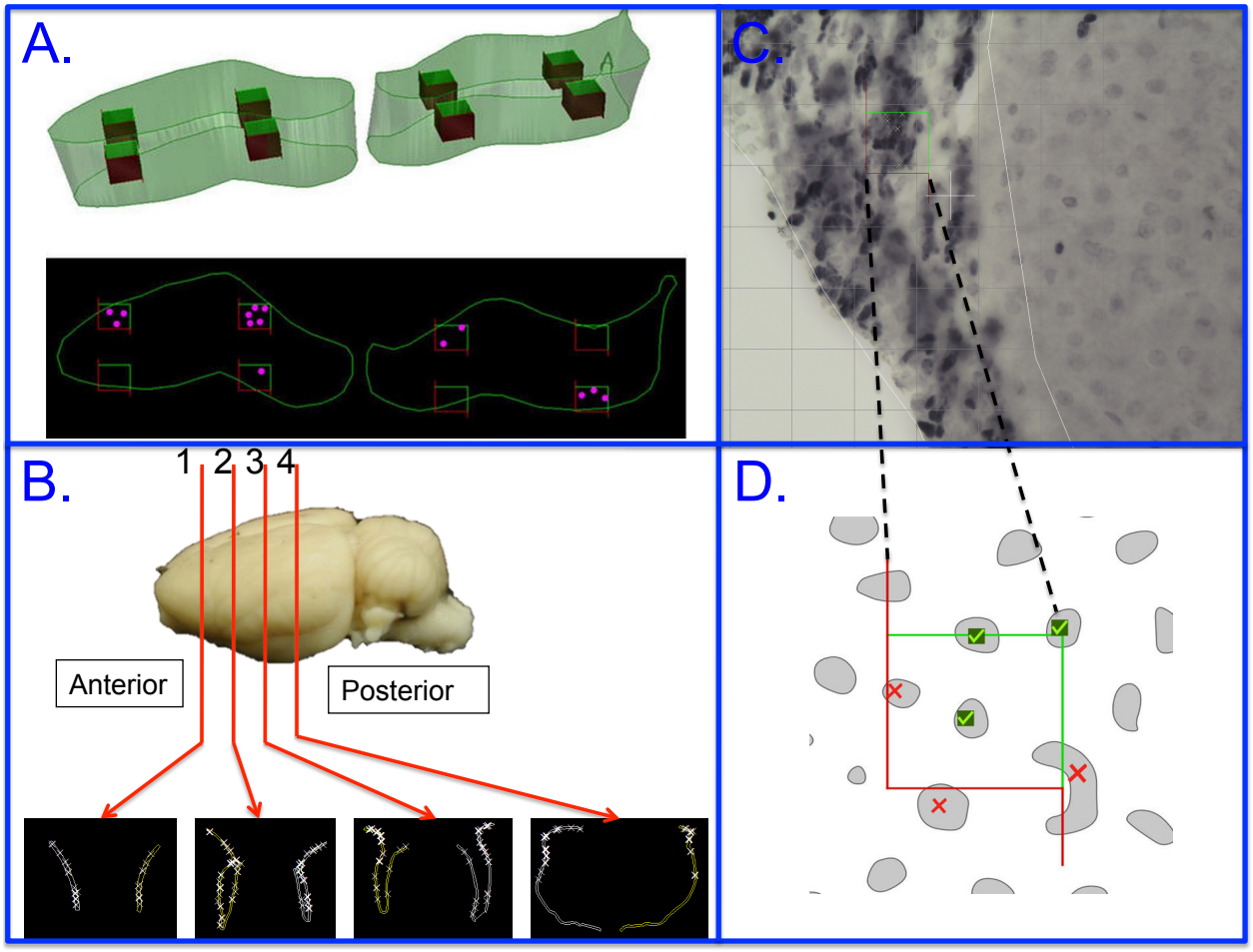


Figure 7: Stereology method for counting cells in the SVZ. A. Schematic of a theoretical structure demonstrating random unbiased sampling. The sampled volumes are the four solid cubes within the structure. The lower figure demonstrate the 2-D cross section of the above theoretical structure with example cell number represented by the purple colored dots. The total number of cells in the theoretical structure is a function of mean cell density and volume of the structure (described by the stereology equation). B. Our approach to quantifying SVZ cells by stereology. Sections were collected from anterior to posterior SVZ and the outline tracings of the representative SVZ are shown bellow. C. High magnification view of the SVZ with DAB staining for BrdU. The colored box is called the optical dissector window. D. Schematic representation of the optical dissector window illustrating the stereology counting rules. All cell counting was performed on MBF Bioscience Leica DM400B with Stereo Investigator version 9.0.

Lesion analysis

Brains were coronally cryosectioned at 40 µm and alternating sections were mounted on gelatin-coated slides and stained for Nissl substance (excluding olfactory bulbs and cerebellum). The TBI lesion volume was quantified from 1.4mm to 2.5mm relative to bregma using the method outlined in (Kawamata et al 1997), (the total area of the uninjured contralateral hemisphere-total area of the injured (ipsilesional) hemisphere, divided by the intact hemisphere). The lesion size was thus expressed as a percentage of the unlesioned contralateral hemisphere. Nissl stained slides were then scanned at high resolution using a flatbed scanner and imported into Adobe Photoshop CS5, where the number of pixels in the intact and lesioned hemispheres were measured.

Statistics

All data analysis was performed using either Minitab version 17 (Minitab, Inc. State College, Pennsylvania, USA) or Graphpad Prism version 5.0 (GraphPad Software, San Diego California, USA). Daily animal weights were fitted using a nonlinear, third order polynomial (cubic) regression. Lesion volume comparisons were done using Student's t-test with $\alpha=0.05$ as the cutoff for statistical significance. BrdU stereological counting results was analyzed by using general

linear model two-way ANOVA to detect interactions between alcohol treatment and TBI injury; to compare if means of treatment groups were significantly different, WITH-IN group analysis was performed using regression analysis with $\alpha=0.05$ as the cutoff for statistical significance. For 7 days post TBI groups, cell count data was transformed on the natural log scale to stabilize variance and achieve normality. For 6 weeks post TBI groups, the cell count data was transformed using the optimal (Box-Cox) transformation to stabilize variance and achieve normality.

CHAPTER FOUR

RESULTS

AIM 1: THE SHORT-TERM EFFECTS OF BINGE ALCOHOL AND TRAUMATIC BRAIN INJURY ON STEM CELL RESPONSES

Gavage results in intoxicating BAL and decreased animal weight

Blood Alcohol Level (BAL)

Rat blood alcohol levels were determined by taking 500ul of blood via tail veinipuncture one hour after the last dose of 3g/kg; this was the same time that the animals received an experimental TBI by the CCI method. The mean BAL level of this cohort of animals was 156.1mg/dL with a standard error of 8.3mg/dL (figure 8A).

Rat Weights

Rats in the TBI groups were weighed during a 14 day period during which they underwent acclimatization, binge alcohol administration, and experimental TBI (Figure 8B). During the acclimatization period (day 1 to day 4), there were no significant differences between treatment groups ($p>0.05$). During the three days of binge alcohol administration (day 5 to day 7), there were also no significant

differences between treatment groups ($p>0.05$). During the recovery period after TBI (day 8 to day 14), the weights of the alcohol binge group were significantly lower compared to the vehicle administration group at two time points, day 11(means 306 grams versus 318 grams respectively) and day 13 (310 grams versus 319 grams respectively), ($p<0.05$).

Lesion Analysis

The lesion was confined to the right unilateral cortical region of the sensorimotor cortex, with the center of the lesion approximately at 1.5mm anterior, 2.5mm lateral from bregma. Lesion size was quantified for both vehicle and binge alcohol groups at 24 hours after TBI and 7 days after TBI. At 24 hours post TBI, lesion size was not significantly different between vehicle TBI and alcohol TBI groups (mean lesion size was 7.2% and 5.5% respectively) (Figure 9A). At 7 days post TBI, lesion size was also not significantly different between vehicle TBI and alcohol TBI groups (mean lesion size was 6.4% and 9.4% respectively) (Figure 9B).

Acute Repeated Binge Alcohol Causes Intoxicating BAL and Transient Weight Loss After TBI

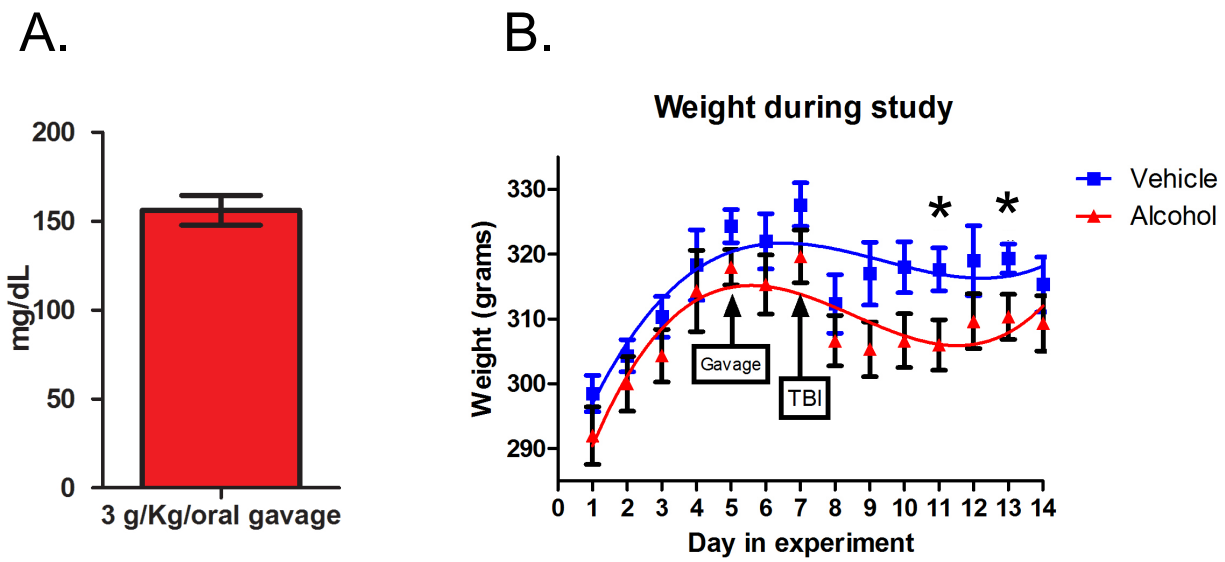


Figure 8 A. Alcohol administration by gastric gavage produces a mean BAL of 156.1mg/dL,+/- 8.3 SEM (n=13) B. Weight of animals before, during and after alcohol administration and experimental TBI by controlled cortical impact (CCI). The average weight of the alcohol group was significantly less compared to the vehicle group on day 11 (306 vs. 318 respectively) and day 13 (310 vs. 319 respectively) (student t test, n=6, error bars=SEM).

Acute Repeated Binge Alcohol Had No Effect on Lesion Size

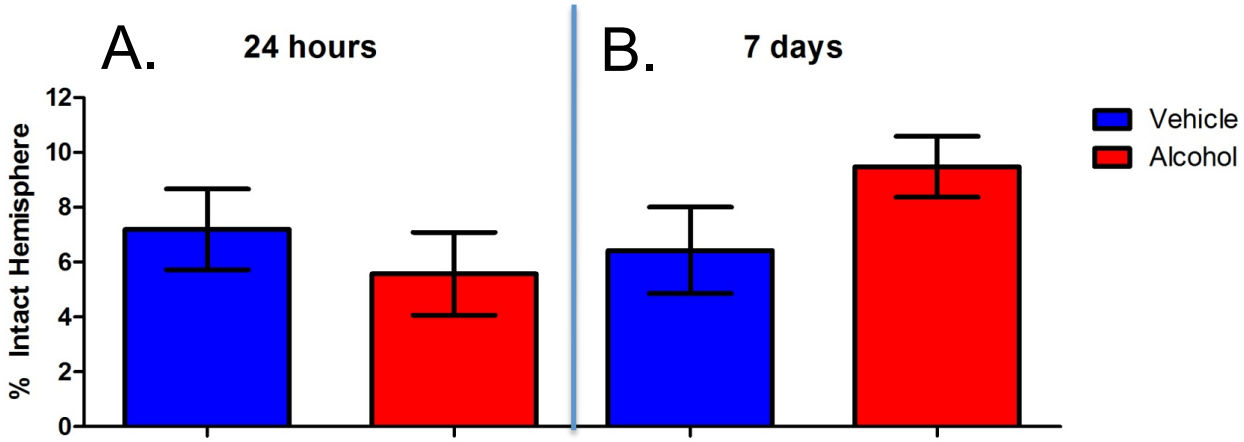


Figure 9: Alcohol had no effect on lesion size at early time points. Lesion size is expressed as the percentage of intact contralesional brain hemisphere. A. At 24 hours after TBI, the mean lesion size was 7.2+/- 1.4%, n=6 and 5.5+/-1.5, n=7 for Vehicle and Alcohol groups respectively. B. At 7 days after TBI, the mean lesion size was 6.4+/-1.5,n=5 and 9.4+/-1.1,n=10 for Vehicle and Alcohol groups respectively. Statistical analysis was performed by Student's t-test with $p \leq 0.05$ as significance criteria, error bars=SEM.

Binge Alcohol Affects Proliferation within the SVZ at 24 hours Post TBI

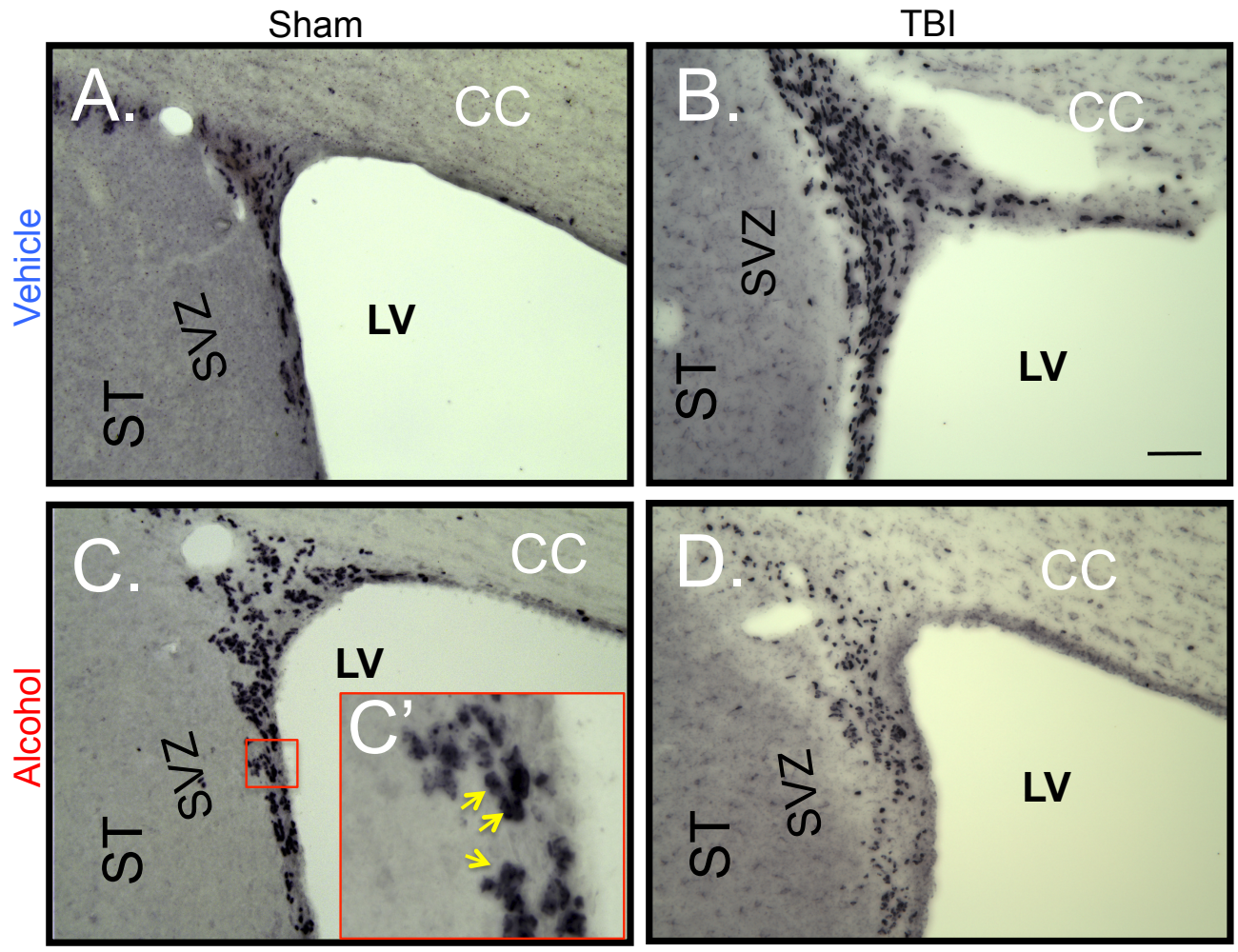


Figure 10: At 24 hours after injury, binge alcohol significantly increased SVZ proliferation in sham groups (A,C) and decreased proliferation in TBI groups (B,D). The ipsilesional SVZ is shown. LV=lateral ventricle, CC=corpus callosum, ST= striatum, SVZ=subventricular zone. C' is magnified view of the red boxed area in C. Yellow arrows point to $BrdU^{+}$ nuclei of SVZ cells. Scale bar=50µm.

Binge Alcohol Affects Proliferation within the SVZ at 24 hours Post TBI

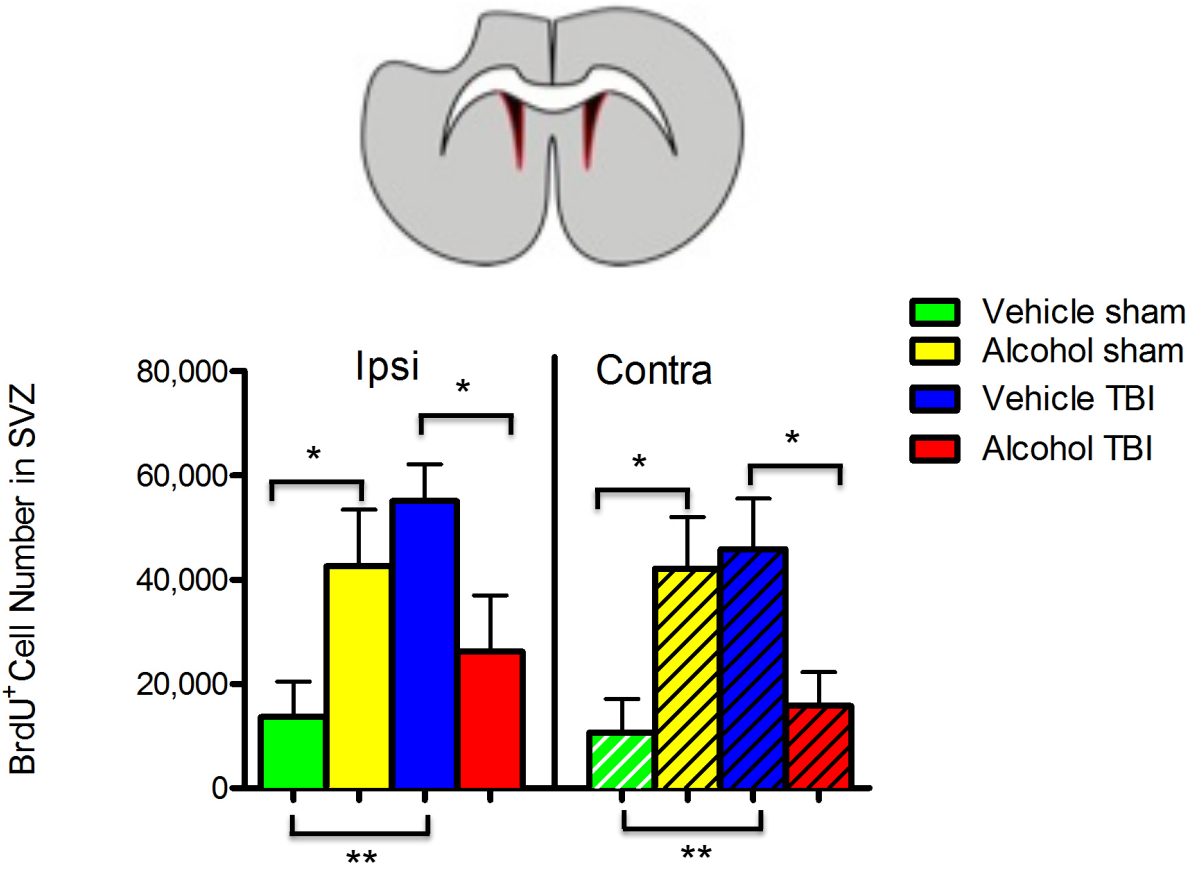


Figure 11: A. At 24 hours after injury, binge alcohol significantly increased SVZ proliferation in sham groups and decreased proliferation in TBI groups bilaterally**.** Statistical analysis was performed by two-way ANOVA, post-hoc within-group analysis performed, * denotes p≤0.05, ** denotes p≤0.01, error bars=SEM.

SVZ Proliferation Cell Counting by Stereology

24 hours: The ipsilesional (injured side) and contralesional (uninjured side) were examined separately due to the unique proximity of these two SVZ environments to the lesion (Figure 10). First, we looked at the number of $BrdU^+$ cells in the SVZ of the ipsilesional side to detect if there was main effect of Injury (TBI, Sham), Treatment (alcohol, vehicle) or if there was interaction between them (Figure 11). We found that there was statistically significant effect of Injury x Treatment interaction (table 7) ($p= 0.005$). Next we performed within group analysis to determine if the means of certain groups were statistically different. First, the mean of Vehicle TBI was significantly higher than that of Vehicle Sham (table 8, $p=0.03$); TBI induced approximately a 4 fold increase in cell proliferation. Next, when comparing Alcohol TBI against Vehicle TBI group, there was a significant effect of alcohol reducing proliferation by approximately 2 fold (table 8, $p=0.039$). Interestingly, when comparing Vehicle Sham against Alcohol sham groups, there was a significant effect of alcohol increasing proliferation by 3 fold (table 8, $p=0.038$).

Then, we looked at the number of $BrdU^+$ cells in the SVZ of the contralesional side to detect whether there was a main effect of Injury (TBI,

Sham), Treatment (alcohol, vehicle) or if there was an interaction between them (Figure 11). Similar to the ipsilesional SVZ, there was a statistically significant effect of Injury x Treatment interaction (table 7) ($p = 0.001$). Next we performed within group analysis to determine if the means of certain groups were statistically different. Again, the mean of Vehicle TBI was significantly higher than that of Vehicle Sham (table 8, $p=0.006$); TBI induced approximately a 4 fold increase in the number of proliferating cells. Next, when comparing Alcohol TBI against Vehicle TBI group, there was a significant effect of alcohol reducing proliferation by almost 3 fold (table 8, $p=0.016$). Again, when comparing Vehicle Sham against Alcohol sham groups, there was a significant effect of alcohol increasing proliferation by almost 4 fold (table 8, $p=0.012$).

Effect	Treatment	Injury	Treatment*Injury
Ipsilesional BrdU+ SVZ cell number	$F(1,23) = 0.00$, $p = 0.996$	$F(1,23) = 1.90$, $p = 0.183$	$F(1,23) = 10.14$, $p = 0.005$
Contralesional BrdU+ SVZ cell number	$F(1,23) = 0.01$, $p = 0.931$	$F(1,23) = 0.28$, $p = 0.601$	$F(1,23) = 13.77$, $p = 0.001$

Table 7. $BrdU^+$ Cell Number in The SVZ at 24 hours two-way ANOVA Results

Effect	Within TBI	Within Sham	Within Vehicle	Within Alcohol
Ipsilesional $BrdU^+$ cell #	F(1,23) = 4.85, p =0.039	F(1,23) = 4.88, p =0.038	F(1,23) = 10.94, p =0.003	F(1,23) = 1.71, p =0.205
Contralesional $BrdU^+$ cell #	F(1,23) = 6.79, p =0.016	F(1,23) = 7.47, p =0.012	F(1,23) = 9.44, p =0.006	F(1,23) = 5.30, p =0.032

Table 8. $BrdU^+$ Cell Number in The SVZ at 24 hours two-way ANOVA within Analysis

Binge Alcohol Affects Proliferation within the SVZ at 7 Days Post TBI

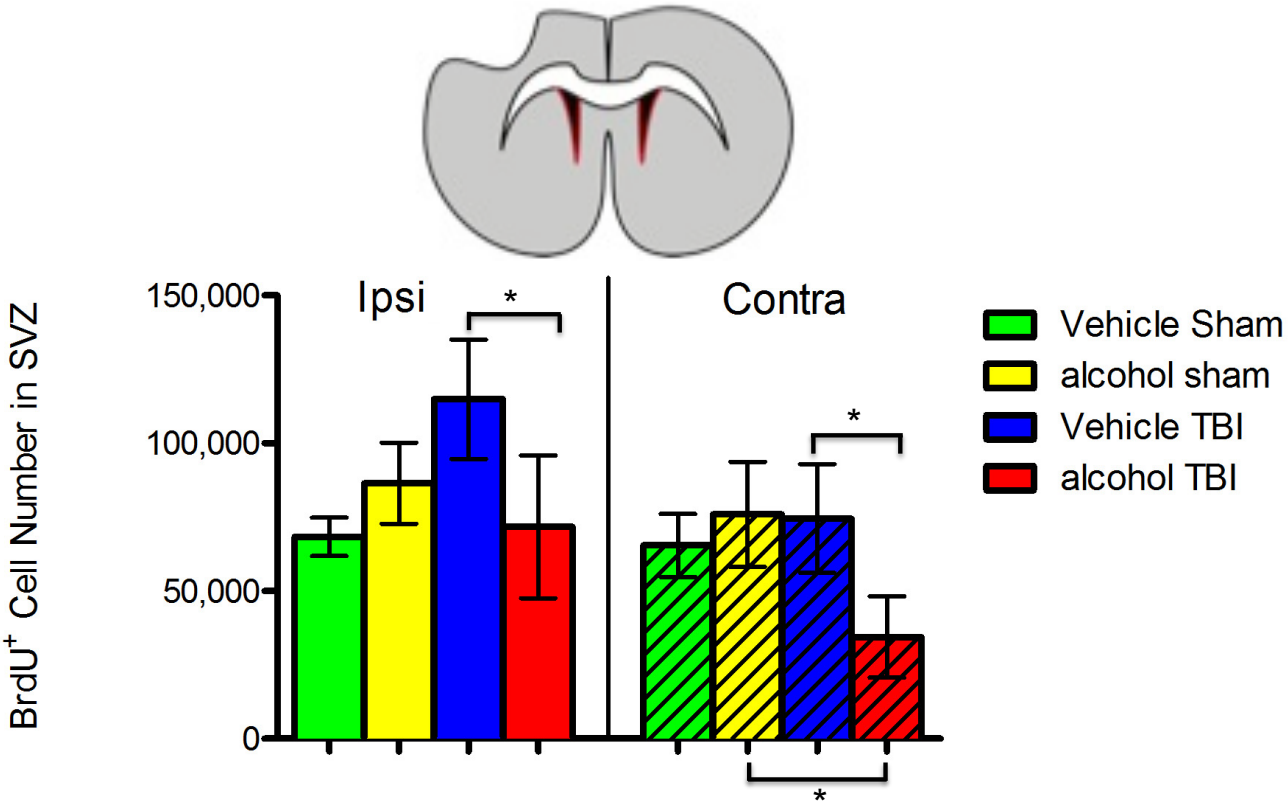


Figure 12: At 7 days after injury, binge alcohol significantly decreased proliferation in the TBI group bilaterally. Statistical analysis was performed by two-way ANOVA, post-hoc within-group analysis performed,* denotes $p \leq 0.05$, error bars=SEM.

7 Days: The ipsilesional (injured side) and contralesional (uninjured side) were analyzed separately (Figure 12). For the Ipsilesional SVZ, there was no statistically significant main effect of either Injury (TBI, Sham) or Treatment (Alcohol, Vehicle). Also, there was no significant Injury x Treatment interaction (table 9) (p= 0.052). Within group analysis showed that when comparing Alcohol TBI against the Vehicle TBI group, a significant effect in alcohol reducing proliferation by approximately 1.6 fold was seen (table 10, p=0.024).

Then, we looked at the number of $BrdU^+$ cells in the SVZ of the contralesional side. Similar to the ipsilesional SVZ, we found that there was no statistically significant main effect of either Injury (TBI, Sham) or Treatment (Alcohol, Vehicle). Also, there was no significant Injury x Treatment interaction (table 9). Within group analysis showed that Alcohol TBI and Vehicle TBI groups were significantly different; alcohol reduced proliferation by approximately 2 fold (table 10, p=0.028).

Effect	Treatment	Injury	Treatment*Injury
Ipsilesional BrdU+ SVZ cell number	F(1,25) = 1.36, p =0.256	F(1,25) = 0.03, p =0.855	F(1,25) = 4.21, p =0.052
Contralesional BrdU+ SVZ cell number	F(1,25) = 2.58, p =0.122	F(1,25) = 3.37, p =0.080	F(1,25) = 3.07, p =0.094

Table 9. BrdU+ Cell Number in The SVZ at 7 days two-way ANOVA Results

Effect	Within TBI	Within Sham	Within Vehicle	Within Alcohol
Ipsilesional BrdU+ SVZ cell number	F(1,25) = 5.85, p =0.024	F(1,25) = 0.38, p =0.543	F(1,25) = 2.67, p =0.116	F(1,25) = 1.89, p =0.182
Contralesional BrdU+ SVZ cell number	F(1,25) = 5.54, p =0.028	F(1,25) = 0.01, p =0.926	F(1,25) = 0.00, p =0.978	F(1,25) = 6.46, p =0.018

Table 10. BrdU+ Cell Number in The SVZ at 7 days two-way ANOVA within Analysis

In summary, the results from Aim #1 are:

1) Binge alcohol had no effect on lesion size.
2) Binge alcohol alone significantly **increased** proliferation bilaterally in the SVZ at 24 hours only.
3) TBI alone significantly **increased** proliferation bilaterally in the SVZ at 24 hours after injury.
4) However, when binge alcohol was combined with TBI, binge alcohol significantly **decreased** proliferation bilaterally in the SVZ of TBI injured animals at 24 hours and 7 days after injury.

AIM 2: THE LONG-TERM EFFECTS OF BINGE ALCOHOL AND TRAUMATIC BRAIN INJURY ON STEM CELL RESPONSES

Lesion Analysis

As in Aim 1, the lesion was confined to the right unilateral cortical region of the sensorimotor cortex, with the center of the lesion approximately at 1.5mm anterior, 2.5mm lateral from bregma (Figure 13A,B). Lesion size was quantified for both vehicle and binge alcohol groups at 6 weeks after TBI. Lesion size was not significantly different between vehicle TBI and alcohol TBI groups (mean lesion size was 9.7% and 8.3% respectively).

SVZ Proliferation Cell Counting by Stereology

6 weeks Total $BrdU^+$ Cell in SVZ: We examined the ipsilesional (injured side) and contralesional (uninjured side) separately due to the unique proximity of these two SVZ environments to the lesion (Figure 14). First, we looked at the number of $BrdU^+$ cells in the SVZ of the ipsilesional side to detect if there was a main effect of Injury (TBI, Sham), Treatment (alcohol, vehicle) or if there was interaction between them (Figure 15). We found that there was a statistically significant main effect of Injury (TBI, Sham) ($p=0.000$), but no statistically significant effects of Treatment or Injury x Treatment interaction (table 11). Next we performed within

group analysis to determine if the means of certain groups were statistically different. First, we saw that the mean of Vehicle TBI was significantly higher than that of Vehicle Sham (table 12, p=0.000); TBI induced more than a 4 fold increase in the number of proliferated cell in the SVZ. The mean SVZ $BrdU^+$ cell number of Alcohol TBI group was significantly higher than Alcohol Sham group (more than a 4 fold increase) (table 12, p=0.000).

In contrast to the ipsilesional SVZ, no statistically significant effects of Injury, Treatment or Injury x Treatment interaction in the contralesional SVZ was found (table 11).

Effect	Treatment	Injury	Treatment*Injury
Ipsilesional $BrdU^+$ SVZ cell number	F(1,25) = 1.40, p =0.249	F(1,25) = 137.54, p =0.000	F(1,25) = 0.02, p =0.904
Contralesional $BrdU^+$ SVZ cell number	F(1,25) = 0.38, p =0.542	F(1,25) = 0.34, p =0.566	F(1,25) = 0.03, p =0.854

Table 11. $BrdU^+$ Cell Number in The SVZ at 6 weeks two-way ANOVA Results

Effect	Within TBI	Within Sham	Within Vehicle	Within Alcohol
Ipsilesional $BrdU^+$ SVZ cell number	F(1,25) = 0.61, p =0.443	F(1,25) = 0.79, p =0.383	F(1,25) = 67.34, p =0.000	F(1,25) = 70.22, p =0.000
Contralesional $BrdU^+$ SVZ cell number	N/A	N/A	N/A	N/A

Table 12. $BrdU^+$ Cell Number in The SVZ at 6 weeks two-way ANOVA within Analysis

Repeated Binge Alcohol Had No Effect on Long-term Lesion Size

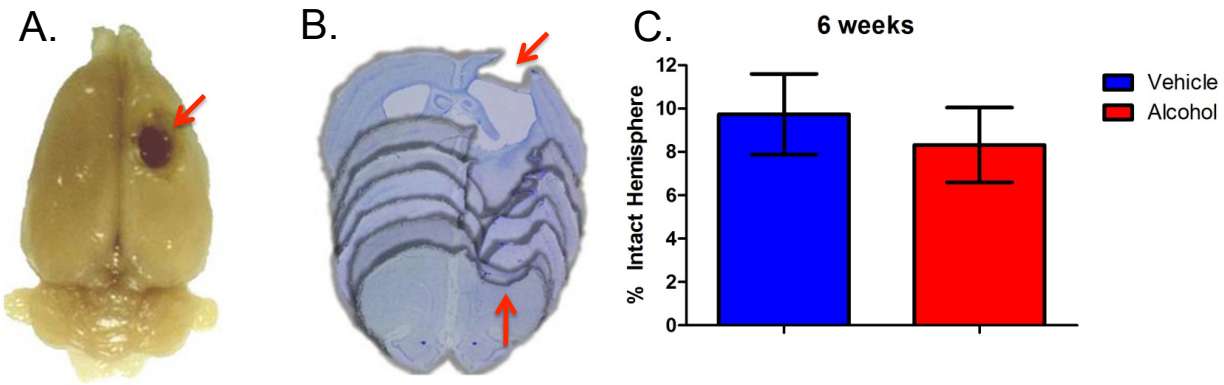


Figure 13: Alcohol had no effect on lesion size at the 6 weeks time point. A. Gross rat brain with a TBI in the right sensorimotor cortex at 6 weeks post TBI. B. Coronal brain sections of a rat with TBI stained for Nissl. Red arrows point to the location of the TBI lesion. C. At 6 weeks after TBI, the mean lesion size was 9.7+/-1.8%, n=9 and 8.3+/-1.7, n=9 for Vehicle and Alcohol groups respectively. Statistical analysis was performed by Student's t-test with $p \geq 0.05$ as significance criteria.

At 6 Weeks Post-TBI, TBI Increased Proliferation within the Ipsilesional SVZ; Binge Alcohol Had No Effect on SVZ Proliferation

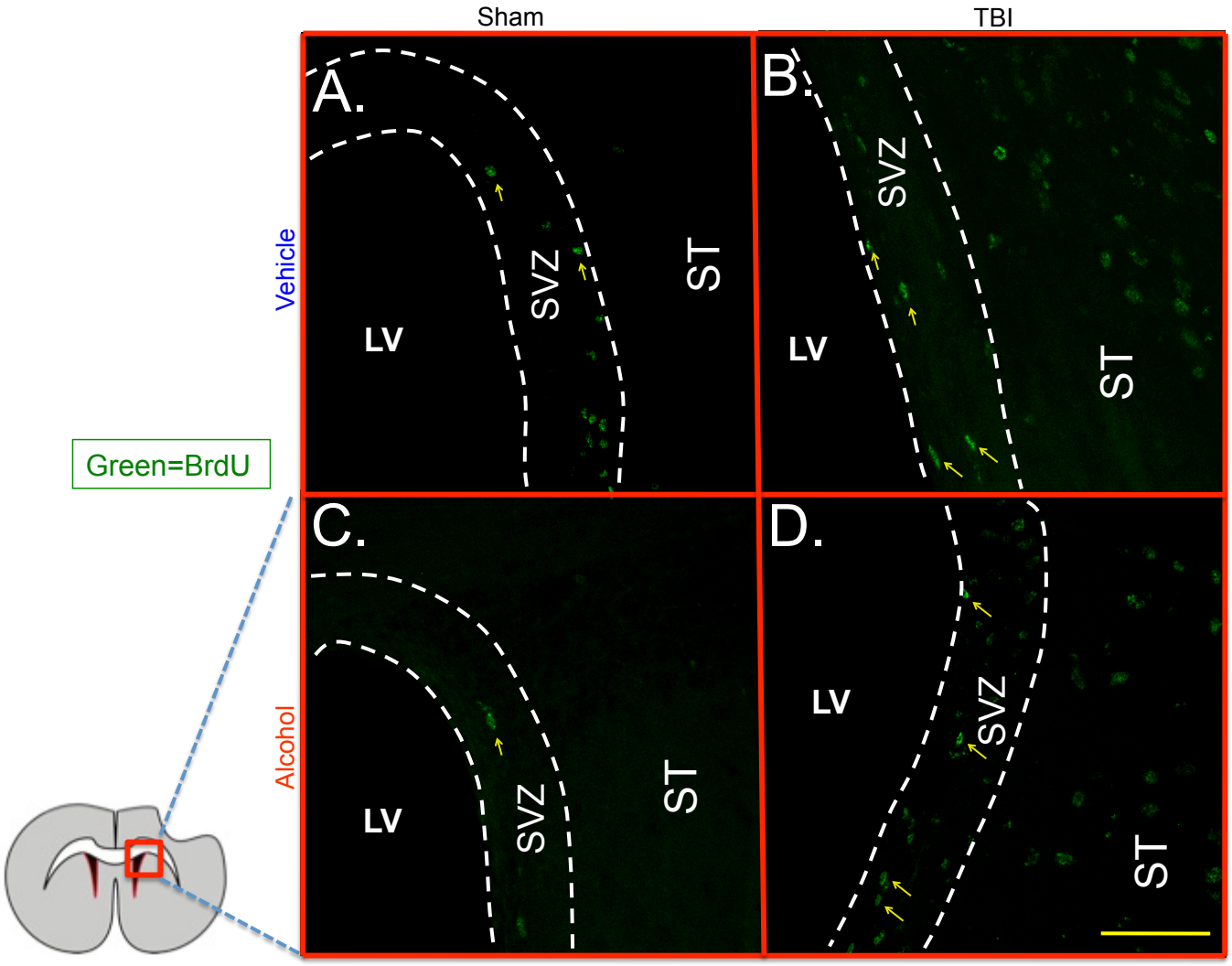


Figure 14: BrdU immunofluorescence showing at 6 weeks after injury, TBI significantly increased proliferation in the ipsilateral SVZ. A-D. Enlargements of the dorsolateral SVZs on the ipsilesional side of the brain. Binge alcohol had no effect on SVZ proliferation after TBI. LV= lateral ventricle, ST= striatum, SVZ= subventricular zone. Yellow arrows point to $BrdU^{+}$ nuclei of SVZ cells. Scale bar = 50µm.

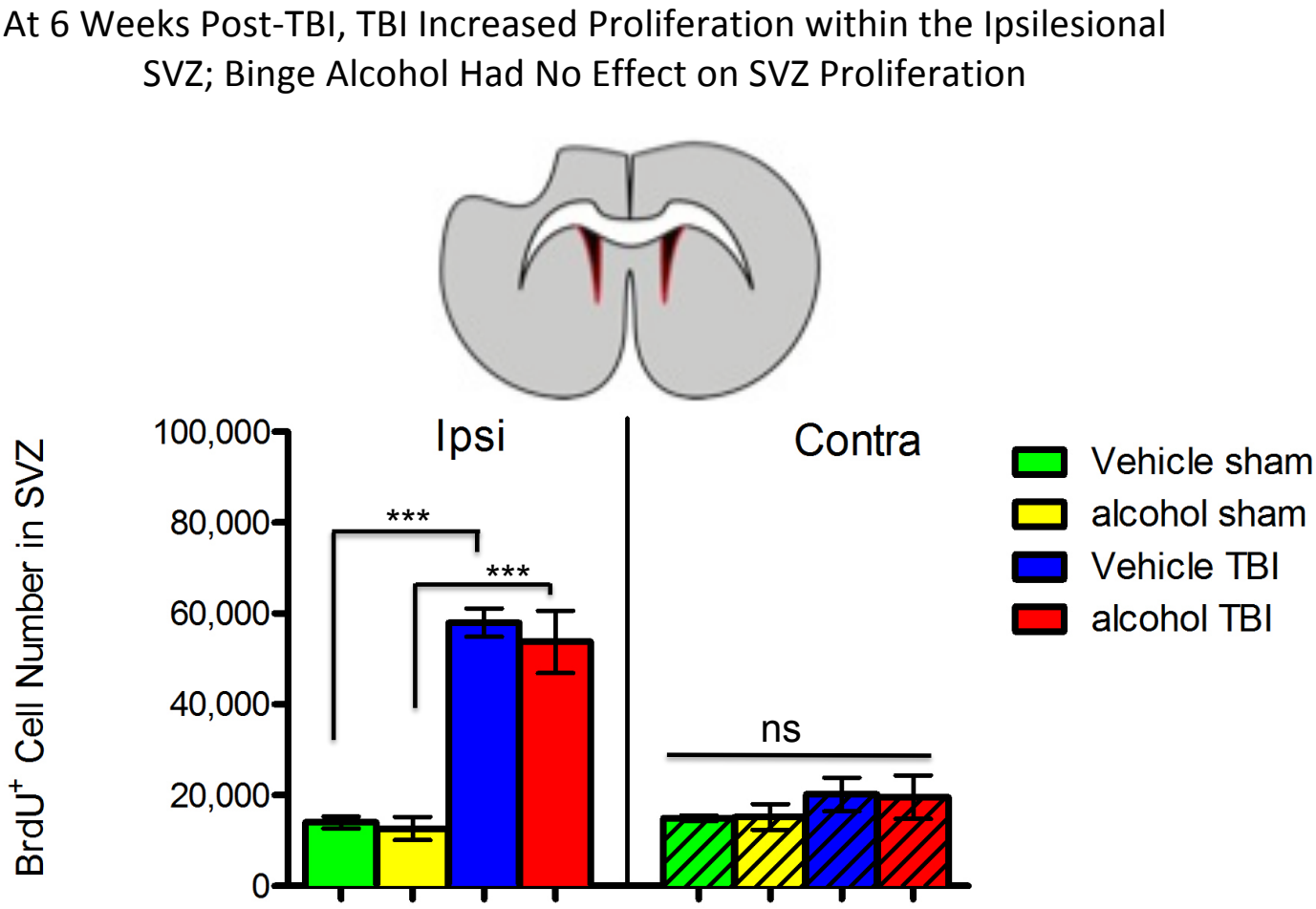


Figure 15: Statistical analysis was performed by two-way ANOVA, post-hoc within-group analysis performed, *** denotes p≤0.001, error bars=SEM.

6 weeks ($BrdU^+$/ DCX^+) Double-labeled Cell in SVZ (Figure 16, 17): For the ipsilesional SVZ, there was a statistically significant main effect of Injury (TBI, Sham) but not Treatment (Alcohol, Vehicle); additionally, there was a significant Injury x Treatment interaction (table 13, p= 0.029). Within group analysis showed that when comparing Vehicle Sham against Vehicle TBI, there was a significant effect in TBI increasing the number of double-labeled ($BrdU^+$ /DCX^+) cells by more than 11 fold (table 14, p=0.000). When comparing Alcohol Sham against Alcohol TBI, a significant effect in TBI increasing the number of double-labeled cells by about 7 fold was detected (table 14, p=0.000). When comparing Vehicle TBI against Alcohol TBI, there was a significant effect of alcohol decreasing the number of double-labeled cells by about 38% (table 14, p=0.006).

Then, the number of double-labeled ($BrdU^+$/ DCX^+) cells in the SVZ of the contralesional side was examined (Figure 17). There was a statistically significant main effect of Injury (table 13, p=0.000) but not Treatment. Furthermore, there was a significant Injury x Treatment interaction (table 13, p=0.021). Within group analysis showed that Vehicle TBI and Vehicle Sham groups were significantly different; TBI increased the number of double-labeled ($BrdU^+$/ DCX^+) cells by more than 7 fold (table 14, p=0.000). Alcohol Sham and Vehicle Sham groups were also

significantly different, with alcohol increasing the number of double-labeled ($BrdU^+$/ DCX^+) cells by more than 2 fold (table 14, p=0.019).

Effect	Treatment	Injury	Treatment*Injury
Ipsilesional double labeled cell number	F(1,25) = 3.18, p =0.088	F(1,25) = 117.12, p =0.000	F(1,25) = 5.43, p =0.029
Contralesional double labeled cell number	F(1,25) = 1.50, p =0.234	F(1,25) = 18.50, p =0.000	F(1,25) = 6.16, p =0.021

Table 13. ($BrdU^+$/ DCX^+) Double Labeled Cell Number in The SVZ at 6 weeks two-way ANOVA Results

Effect	Within TBI	Within Sham	Within Vehicle	Within Alcohol
Ipsilesional double labeled cell number	F(1,25) = 9.17, p =0.006	F(1,25) = 0.14, p =0.713	F(1,25) = 86.49, p =0.000	F(1,25) = 36.06, p =0.000
Contralesional double labeled cell number	F(1,25) = 0.86, p =0.365	F(1,25) = 6.38, p =0.019	F(1,25) = 23.00, p =0.000	F(1,25) = 1.65, p =0.212

Table 14. ($BrdU^+$ & DCX^+) Double Labeled Cell Number in The SVZ at 6 weeks two-way ANOVA within Analysis

Repeated Binge Alcohol Decreased Neuronal Differentiation within the SVZ at 6 Weeks Post-TBI

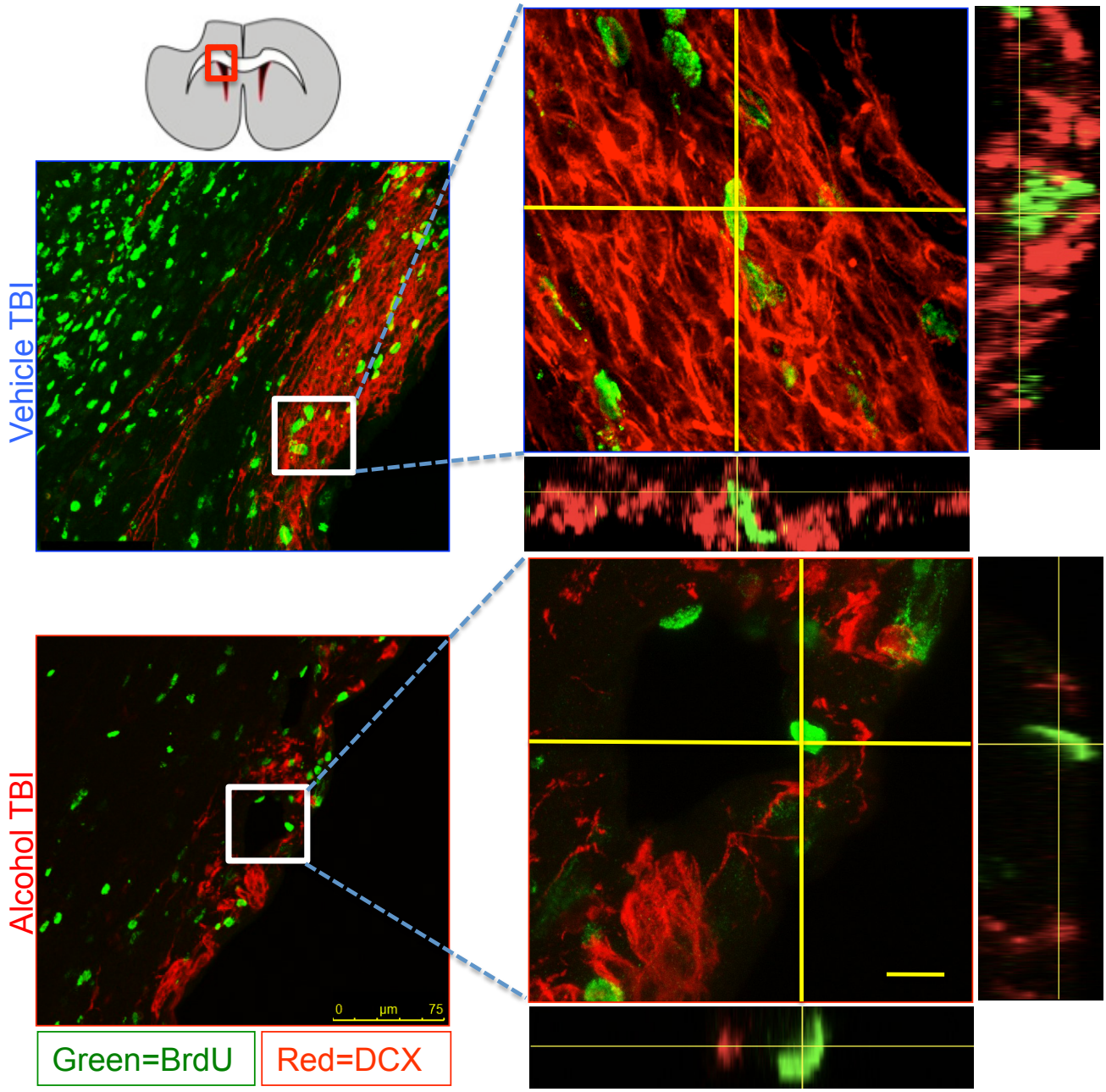


Figure 16: At 6 weeks after injury, binge alcohol significantly decreased neuronal differentiation in the ipsilesional SVZ. Several sections were chosen for immunofluorescence to demonstrate labeling. First column imaged at 400x magnification, scale bar = 75 µm. Second column imaged at 1000x, scale bar=10µm.

Repeated Binge Alcohol Decreased Neuronal Differentiation within the SVZ at 6 Weeks Post-TBI

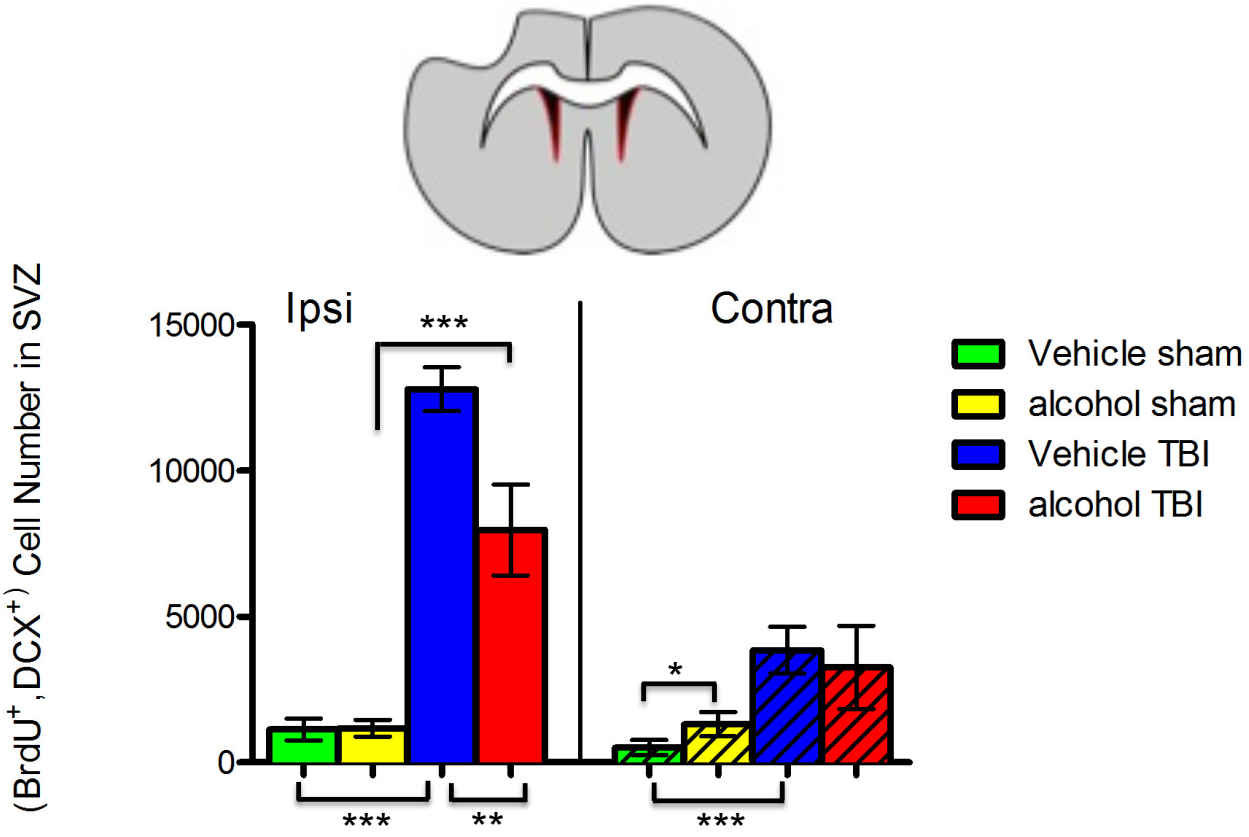


Figure 17: At 6 weeks after injury, binge alcohol significantly decreased neuronal differentiation. Statistical analysis was performed by two-way ANOVA, post-hoc within-group analysis performed,* denotes $p \leq 0.05$, ** $p \leq 0.01$,and *** $p \leq 0.001$, error bars=SEM.

6 weeks ($BrdU^+$/ DCX^-) Single-labeled Cell in SVZ (Figure 19): For the ipsilesional SVZ, we found that there was a statistically significant main effect of Injury (TBI, Sham), (table 15, p=0.000), but not Treatment (Alcohol, Vehicle); we did not detect a significant Injury x Treatment interaction. Within group analysis showed that when comparing Vehicle Sham against Vehicle TBI, there was a significant effect of TBI increasing the number of single-labeled ($BrdU^+$/ DCX^-) cells by more than 3 fold (table 16, p=0.000). When comparing Alcohol Sham against Alcohol TBI, there was a significant effect of TBI increasing the number of single-labeled cells by about 4 fold (table 16, p=0.000).

On the contralesional side, we did not detect any statistically significant main effect of Injury or Treatment (Figure 19). Furthermore, there was no significant Injury x Treatment interaction.

Effect	Treatment	Injury	Treatment*Injury
Ipsilesional single labeled cell number	F(1,25) = 0.71, p =0.408	F(1,25) = 83.19, p =0.000	F(1,25) = 0.11, p =0.740
Contralesional single labeled cell number	F(1,25) = 0.16, p =0.692	F(1,25) = 0.06, p =0.811	F(1,25) = 0.09, p =0.765

Table 15. ($BrdU^+$ & DCX^-) Single Labeled Cell Number in The SVZ at 6 weeks two-way ANOVA Results

Effect	Within TBI	Within Sham	Within Vehicle	Within Alcohol
Ipsilesional single labeled cell number	F(1,25) = 0.14, p =0.712	F(1,25) = 0.65, p =0.430	F(1,25) = 38.59, p =0.000	F(1,25) =44.72, p =0.000
Contralesional single labeled cell number	N/A	N/A	N/A	N/A

Table 16. ($BrdU^+$ & DCX^-) Single Labeled Cell Number in The SVZ at 6 weeks two-way ANOVA within Analysis

Repeated Binge Alcohol Does Not Affect Non-Neuronal Differentiation within the SVZ at 6 Weeks Post-TBI

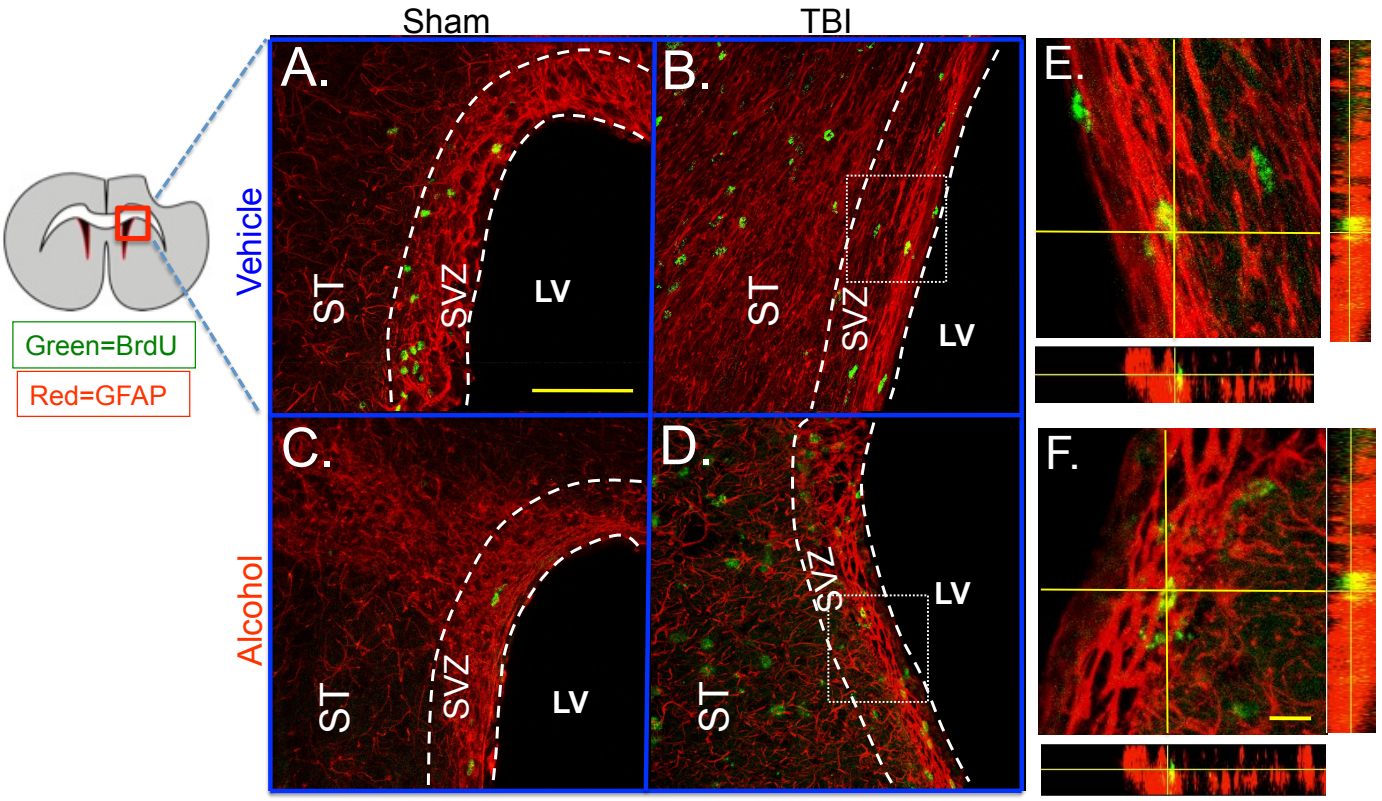


Figure 18. Immunofluorescence of BrdU and the astrocyte marker GFAP. A-D. Enlargements of the dorsolateral SVZs of the ipsilesional side of the brain. There appears to be little co-localization of BrdU signal and GFAP signal in the sham groups. In TBI groups, more BrdU co-localize with GFAP signal; however, there was no noticeable difference between vehicle and alcohol groups. LV= lateral ventricle, ST= striatum, SVZ= subventricular zone. Scale bar=150µm.

Repeated Binge Alcohol Did Not Affect Non-Neuronal Differentiation within the SVZ at 6 Weeks Post-TBI

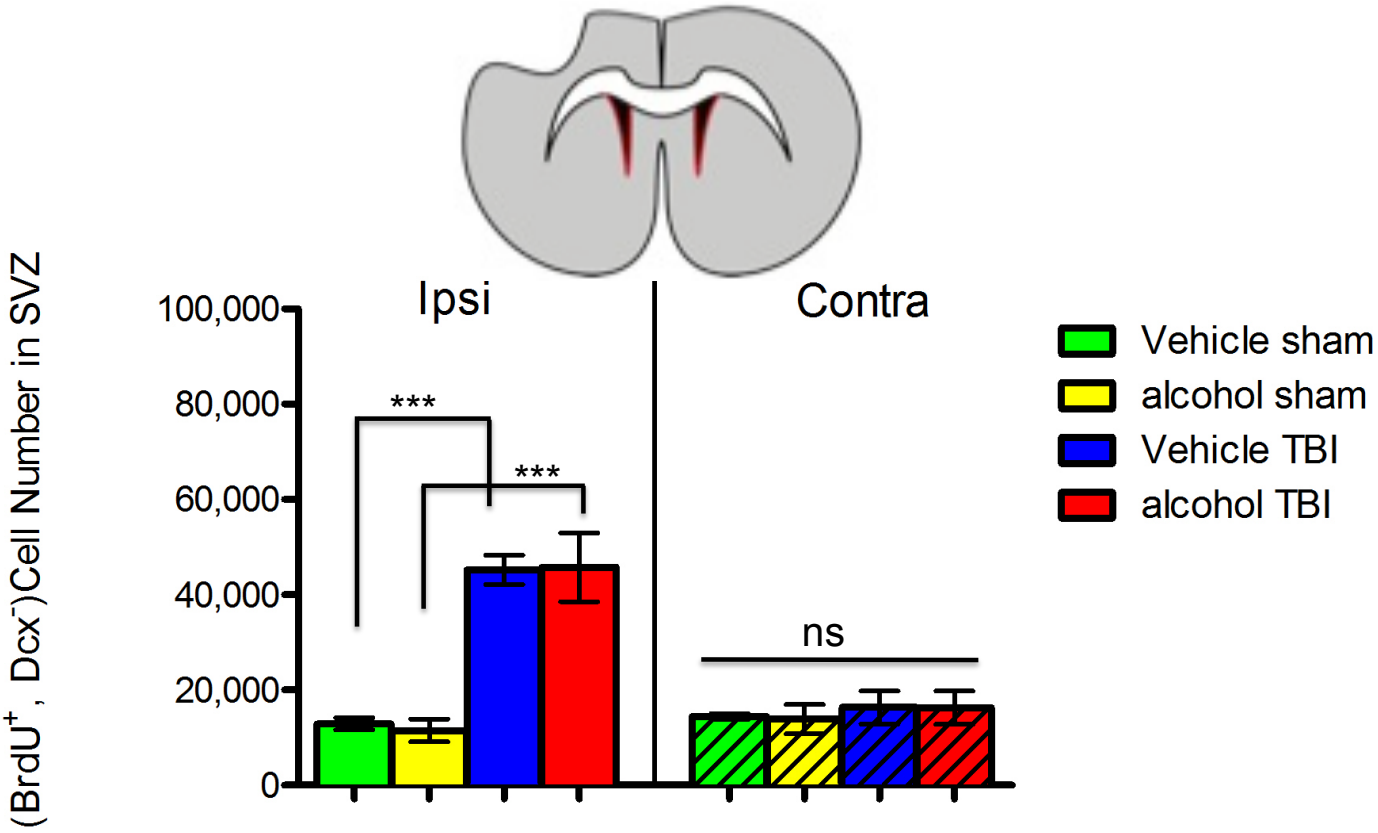


Figure 19: Repeated binge alcohol did not affect glial proliferation as measured at 6 weeks after injury. Statistical analysis was performed by two-way ANOVA, post-hoc within-group analysis was performed,*** denotes p≤0.001, error bars=SEM.

Repeated Binge Alcohol Does Not Appear to Affect the Number of Neurons with Mature Phenotypes Within the SVZ and Perilesional Area at 6 Weeks Post-TBI

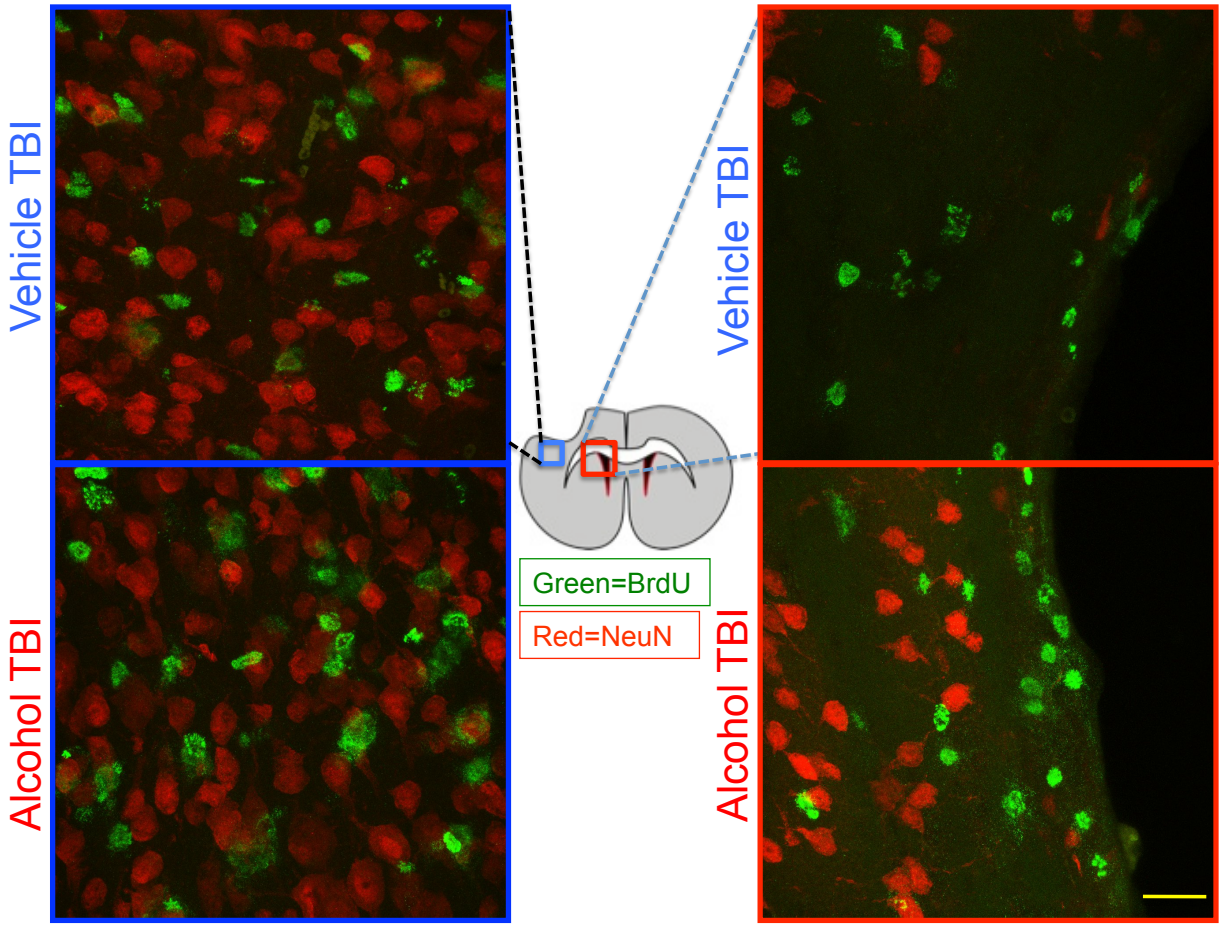


Figure 20: Immunofluorescence of BrdU and the mature neuronal marker NeuN. Images in the left column are from the perilesional cortical area (blue box). Images in the right column are from the dorsolateral SVZ area (red box). There appeared to be little co-localization of BrdU and NeuN signal in either the SVZ or the cortical area. No noticeable differences between vehicle and alcohol groups were observed. Scale bar=10µm.

6 weeks ($BrdU^+$/ DCX^+) Double-labeled Cells in the Perilesional Area (figure 21): There was no statistically significant difference between Vehicle TBI and Alcohol TBI groups.

6 weeks ($BrdU^+$/ DCX^+) Double-labeled Cell in RMS (figures 22 and 23): We found that there was a statistically significant main effect of Injury (TBI, Sham), (table 17, p=0.000), but not Treatment (Alcohol, Vehicle); we did not detect a significant Injury x Treatment interaction. Within group analysis showed that when comparing Vehicle Sham against Vehicle TBI, there was a significant effect of TBI increasing the number of double-labeled ($BrdU^+$/ DCX^+) cells by about 2 fold (table 18, p=0.001). When comparing Alcohol Sham against Alcohol TBI, we also saw a significant effect of TBI increasing the number of double-labeled cells (table 18, p=0.037).

Effect	Treatment	Injury	Treatment*Injury
RMS double labeled cell number	F(1,26) = 1.12, p =0.302	F(1,26) = 19.48, p =0.000	F(1,26) = 1.87, p =0.185

Table 17. Neuronal Migration in the RMS at 6 weeks two-way ANOVA Results

Effect	Within TBI	Within Sham	Within Vehicle	Within Alcohol
RMS double labeled cell number	F(1,26) = 3.62, p =0.070	F(1,26) = 0.04, p =0.843	F(1,26) = 15.85, p =0.001	F(1,26) = 4.91, p =0.037

Table 18. Neuronal Migration in The RMS at 6 weeks two-way ANOVA within Analysis

Repeated Binge Alcohol Did not Affect Peri-lesional Migration of Immature Neurons

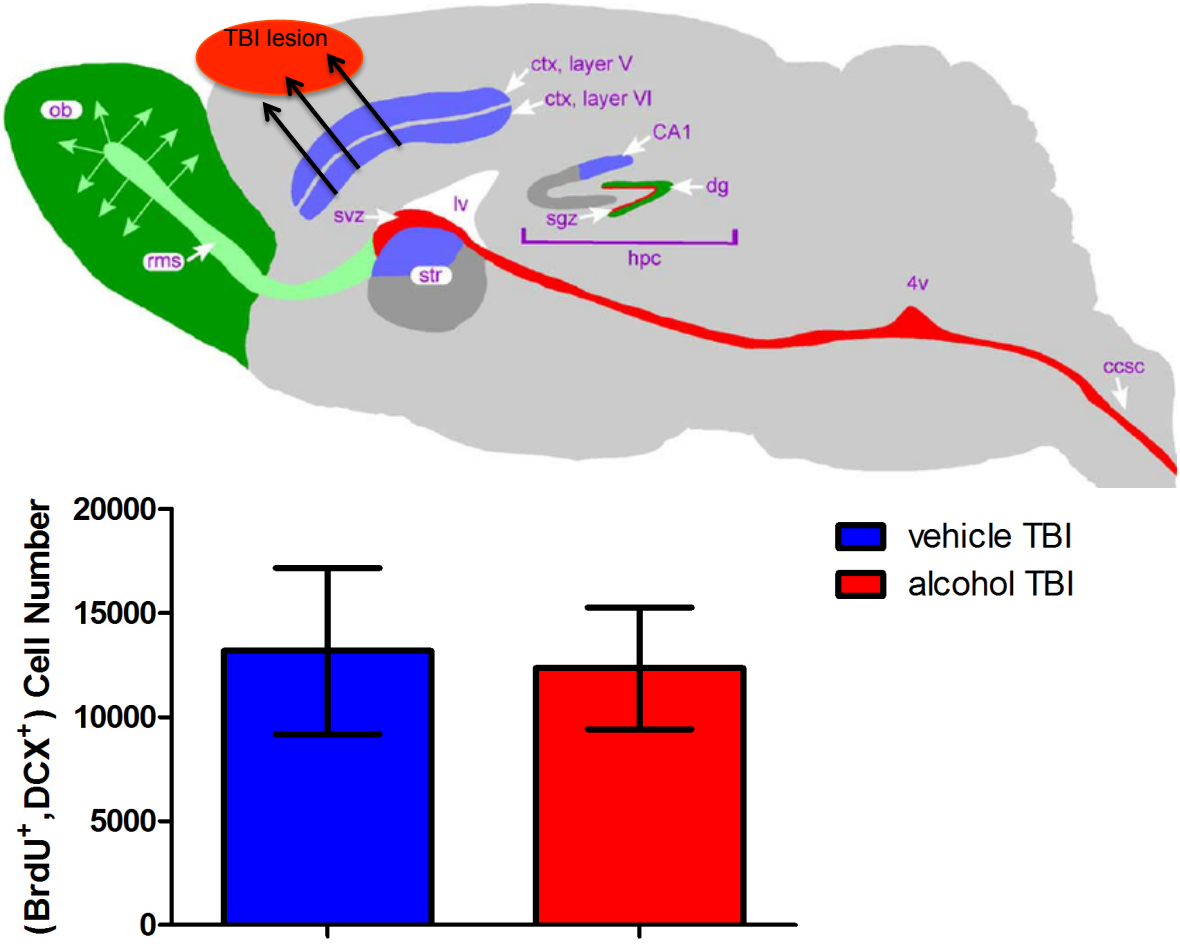


Figure 21: At 6 weeks after injury, binge alcohol did not affect the migration of neuroblasts toward the peri-lesional area. Statistical analysis was performed by Student's t-test with significant criteria of $p \leq 0.05$, error bars=SEM. OB: Olfactory bulb, RMS: Rostral migratory stream, Str: striatum, SVZ: subventricular zone, LV: lateral ventricle, HPC: hippocampus, DG: dentate gyrus, SGZ: subgranular zone, 4V: 4th ventricle, CCSC: central canal of the spinal cord, CTX: cortex. Diagram adapted from (Emsley et al 2005).

TBI increased RMS Migration

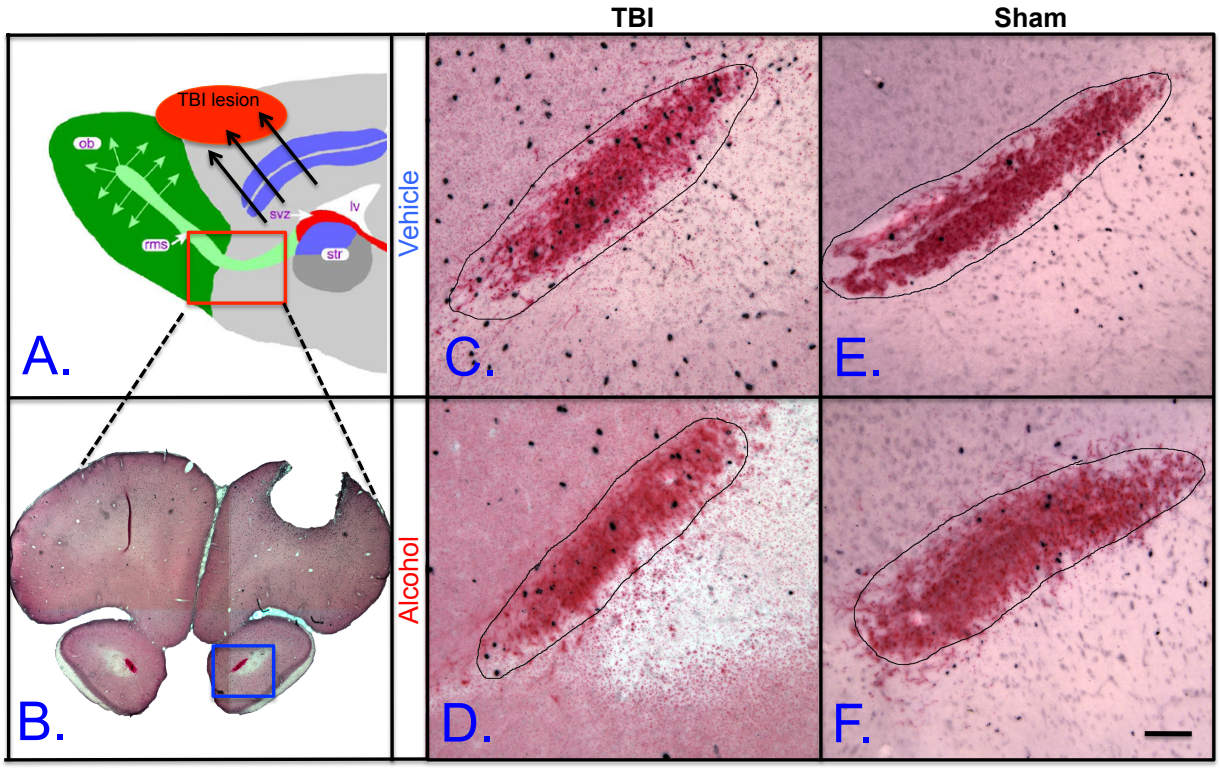


Figure 22: At 6 weeks after injury, TBI significantly increased migration of SVZ derived neuroblasts toward the olfactory bulb. A. Diagram of the rostral migratory stream (RMS) the pathway that neuroblasts take toward the olfactory bulbs, shown in green. B. Coronal section of rat brain immunolabeled for Doublecortin (DCX) and BrdU. Area in the blue box is the olfactory bulb nucleus (OBN), which is a part of the RMS. C-F. Enlargements of the ipsilateral OBN, with $BrdU^{+}$ cells as black and DCX^{+} cells as dark red. Diagram in A was adapted from (Emsley et al 2005). Scale bar=50µm.

TBI increased RMS Migration and Binge Alcohol Did Not Affect This Process

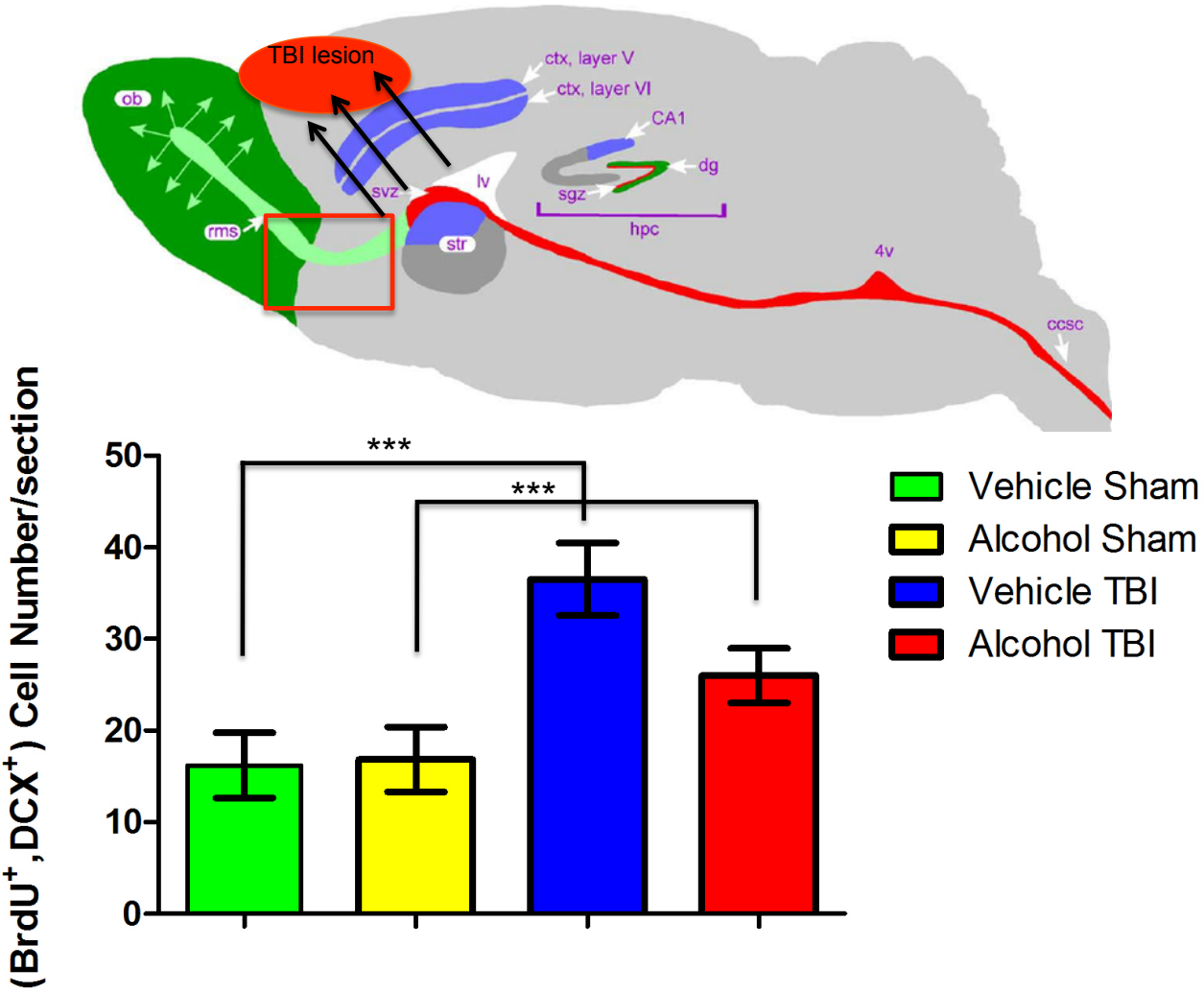


Figure 23: At 6 weeks after injury, binge alcohol did not affect migration of SVZ derived neuroblasts toward the olfactory bulb. Statistical analysis was performed by two-way ANOVA, post-hoc within-group analysis performed, *** denotes $p \leq 0.001$, error bars=SEM. OB: Olfactory bulb, RMS: Rostral migratory stream, Str: striatum, SVZ: subventricular zone, LV: lateral ventricle, HPC: hippocampus, DG: dentate gyrus, SGZ: subgranular zone, 4V: 4th ventricle, CCSC: central canal of the spinal cord, CTX: cortex. Diagram adapted from (Emsley et al 2005).

In Summary, the results from Aim #2 are:

1) Binge alcohol had no effect on lesion size as measured at 6 weeks after TBI.
2) Binge alcohol decreased neuronal differentiation of SVZ derived cells at 6 weeks after TBI.
3) TBI alone increased RMS migration to the olfactory bulb.
4) Binge alcohol did not affect migration of immature neurons to the perilesional area or to the olfactory bulb.

CHAPTER FIVE

DISCUSSION

TBI is a significant health crisis as many survivors of TBI face disabilities and a difficult path to recovery. Alcohol consumption and intoxication is a lifestyle factor that strongly contributes to suffering a TBI. In fact, a large fraction of TBI patients are positive for blood alcohol levels higher than the 0.08 legal limit at the time of injury (Parry-Jones et al 2006). Binge drinking is the most common way in which alcohol is consumed and it has been shown to be extremely widespread in the adolescence and adult populations in our society (CDC Vitalsigns, 2012). Despite the strong association of alcohol consumption to TBI injuries, the biological effect of alcohol consumption and intoxication on the TBI recovery process is not well understood. An earlier behavioral study using a rat model for binge alcohol consumption and TBI indicated that a three day binge alcohol administration prior to cortical injury by CCI significantly decreased fine motor skill recovery when measured 8 weeks after TBI (Vaagenes et al 2015). However, the mechanism behind this alcohol-induced impairment is not known. Therefore, in this dissertation, we used the same binge alcohol and TBI model previously

developed in our lab to explore the combined effect of binge alcohol and TBI on the neural stem cell response.

Our results showed that alcohol given for three consecutive days (a binge pattern of alcohol consumption) before a moderate TBI to the right forelimb motor cortex leads to a significant reduction in short term SVZ proliferation as measured at 24 hours and 7 days post injury. Binge alcohol did not affect the retention of proliferated cell in the SVZ as measured at 6 weeks post injury. However, binge alcohol significantly reduced the number of proliferated cells that differentiated into neurons in the SVZ. Binge alcohol did not have significant effects on lesion size, or migration of new neurons to the perilesional area and the olfactory bulb. Additionally, we confirmed earlier literature that our model of TBI using the controlled cortical impact method produced a significant increase in the number of proliferating cells bilaterally in the SVZ when measured at 24 hours and 7 days post injury and ipsilateral at the 6 week time point. TBI also induced a significant increase in migration of new neurons toward the perilesional area and the olfactory bulb via the RMS pathway. An interesting effect of binge alcohol alone was its effect on increasing SVZ proliferation when measured 24 hours after the last binge episode. Overall, we found that binge alcohol potently affected neurogenesis after TBI, and this might be the reason behind the reduced

functional recovery as observed in the same animal model as we reported previously (Vaagenes et al 2015).

Alcohol had been found by various groups to have detrimental effects on the lesion size of animals (Kelly et al 1997). Additionally, there is evidence to suggest that alcohol affects aquaporin expression, which could lead to elevated edema in animals intoxicated with alcohol, thus leading to an increase in lesion size (Sripathirathan et al 2009). However, we found that lesion size was not affected in our rat model of binge alcohol and TBI at the acute time point of 24 hours, sub-acute time point of 7 days or the chronic time point of 6 weeks. This suggests that alcohol intoxication at the level experienced in our model does not affect the edema process after TBI. Furthermore, our results suggest that binge alcohol consumption did not have detrimental effects on perilesional cell death (apoptotic or necrotic) or lesion site degeneration. However, to further investigate this process, we would need to perform staining using specific markers for apoptosis, necrosis or degeneration.

Additionally, we also confirmed observations from past studies regarding the **general** increase in SVZ proliferation post-TBI. However, we observed some differences regarding the time-course of this proliferative response. One recent

study noted that there were significant variations in the SVZ proliferative response following a TBI (Chang et al 2016). For instance, species differences (rats versus mice) and different TBI methods (CCI versus MFP versus weight drop…etc) can account for the variation in the SVZ proliferative response as observed in these studies (Tzeng & Wu 1999), (Szele & Chesselet 1996),(Goings et al 2002). However, the consensus is that in rats, during the first five days after injury, SVZ proliferation is unchanged. However, we saw that TBI alone induced up to a 4 fold increase in SVZ proliferation at the 24 hour time point (Figure 10 and 11). The difference in this result might be due to the difference in the TBI model used, for instance, (Tzeng & Wu 1999) used the stab wound model to inflict TBI while (Goodus et al 2014) used a pediatric rat TBI model. While another group used a thermal coagulation model to produce a cortical lesion that was distal to the SVZ to show that within the first 5 days after injury, there was no change in proliferation (Gotts & Chesselet 2005). The model used in this dissertation, i.e., CCI, is thought by others to be a more appropriate model for closed-head TBI with contusion (Dixon et al 1991), produces a focal TBI lesion that is proximal to the SVZ, and thus might explain the marked and almost immediate elevation in SVZ proliferation.

Furthermore, according to two groups which used aspiration lesion and diffuse TBI models, SVZ proliferation reached a maximum of around 2 fold above baseline at 5-7 days post-TBI (Szele & Chesselet 1996), (Bye et al 2011). In our model, at 7 days post injury there was no significant difference in SVZ proliferation in TBI versus the sham group (Figure 12). This result indicates that the proliferative maximum had been reached between days 1 and before day 7, and that the rate of new cell incorporation into the SVZ equals the rate of migration of cells away from the SVZ. Again, as in the 24 hours post-TBI result, we feel that the difference in our TBI model is able to explain why we observed SVZ proliferation reaching a maximum before 7 days.

At 7 days after TBI, the total number of BrdU labeled cells in the SVZ of animals in all the groups was dramatically higher compared to the 24 hour time point because of the accumulation of these cells during the 7 days of BrdU injection/labeling. The distribution of proliferated cells was dramatically different when we observed the 7 day survival animals compared to the 24 hour animals. At 24 hours, we only observed $BrdU^+$ cells in the SVZ and perilesional area; at 7 days, we observed $BrdU^+$ cells in a number of other brain structures such as the striatum and RMS. This indicated that newborn cells from the SVZ were migrating toward other brain structures. It is possible that alcohol could increase SVZ cell

death and that is why we observed less $BrdU^+$ cells in the SVZ of TBI animals exposed to alcohol compared to vehicle. This is unlikely, however, due to the relatively low dose of alcohol that we used and thus less likely to induce neurodegeneration/cell death. Furthermore, alcohol/sham animals in our study had significantly more proliferation compared to vehicle/sham animals and thus we do not believe that alcohol caused an increase in cell death.

A novel result from this study was that binge alcohol alone elicited a proliferative response from the SVZ. Alcohol treated sham animals exhibited up to a 4 fold increase in $BrdU^+$ cells compared to vehicle treated sham animals. This increase is in the same magnitude as an increase after TBI alone (Figure 10 and 11). This is in particular contrast with previous studies using different alcohol intoxication models in rodents (either acute or chronic) indicating that alcohol caused significant depression in SVZ and SGZ proliferation (Anderson et al 2012) and (Nixon & Crews 2002). One report found that after discontinuing chronic alcohol administration, there was an increase in SGZ cell number at day 2 and 7 after cessation (Anderson et al 2012). Similar studies have found similar proliferation bursts in the SVZ (Hansson et al 2010); however, these studies used chronic doses of alcohol for extended periods of time (up to 7 weeks). We believe that we are the first to observe this SVZ burst of proliferation at 24 hours after the

last dose of alcohol following three days of moderate binge. It is possible that the three days moderate binge paradigm that we used was enough to cause neurodegeneration in the CNS, which in turn acts as an injury to stimulate an increase in neurogenesis. Additionally, reactive oxygen species (ROS) are elevated in the enzymatic degradation of alcohol. There is strong evidence that moderate ROS increase can stimulate neurogenesis (Le Belle et al 2011). Therefore, it will be important to further characterize this unique SVZ response by employing different markers to detect neurodegeneration, ROS and cell death in different brain regions in future studies.

Important to the goal of this dissertation, we found that at the 24 hour time point, in animals that received binge alcohol and TBI, there was a 3 fold decrease in proliferating cells in the SVZ. To our knowledge, we are the first group to show that alcohol in the context of TBI resulted in decreased overall SVZ proliferation. This is what we predicted in our hypothesis based on our previous observation that alcohol/TBI rats performed worse in the skilled forelimb-reaching task compared to vehicle/TBI rats. It remains to be determined whether SVZ proliferation contributed significantly to the performance on the skilled forelimb-reaching task. Although not examined explicitly in this dissertation, this may occur through the production of new interneurons in the olfactory bulb

(granular cells and perigranular cells, Figure 2). New OB granular and perigranular cells are inhibitory interneurons that have modulatory functions in the olfaction neural circuitry that is important in a process called pattern separation (Sahay et al 2011b).Furthermore, one important study showed that the skilled forelimb reaching task relies heavily on olfaction (Whishaw & Tomie 1989).This group used rats that were trained to reach food through a small opening; when these animals had their vision blocked by the mean of eye patches, their reaching performance was not affected. However, when the olfactory bulb was removed, they experienced profound impairments in the reaching task. These findings point to the possibility that reduced SVZ-OB neurogenesis due to alcohol could lead to impaired olfaction pattern separation and in turn cause deficits in the skilled forelimb reaching task.

Binge alcohol and TBI alone increased proliferation, but the combination of the two factors led to decreased proliferation in the SVZ. There are several mechanisms that might explain this observation:

Inflammatory response: TBI has an important inflammation component in the primary injury phase. Alcohol is known to increase CNS inflammation via its effect on microglia (Marshall et al 2013). It is possible that the

combined magnitude of inflammation in TBI/binge alcohol animals is higher than in TBI or binge alcohol animals alone. A certain amount of inflammation can stimulate neurogenesis, however, if inflammation reaches a high threshold, it may cause the SVZ microenvironment to be non-supportive for neurogenesis. For instance, there is strong evidence for pro-inflammatory cytokines such as IL-1b suppressing hippocampal neurogenesis (Goshen et al 2008).

Reactive oxygen species (ROS): Alcohol metabolism produces ROS and primary events following TBI also lead to high ROS levels. Therefore, in TBI and alcohol administered animals, ROS levels could become additive from the two conditions. This combined elevated ROS from TBI and alcohol could be inhibitory to proliferation. As previously noted, low concentration of ROS can stimulate neurogenesis, likely through a mechanism involving elevated NF-kB (Ruiz-Ramos et al 2009). However, extremely high levels of ROS could inhibit NSC proliferation (Limoli et al 2006). Furthermore, extreme levels of ROS could lead to an increased rate of cell death of recently proliferated cells due to an increased rate of DNA damage (Figure 4).

BDNF availability: Growth factors are crucial to the neurogenesis process. For instance, BDNF enhances survival and in certain cases serves as a

chemokine to attract new neurons toward lesion areas (Rostami et al 2014). TBI increases BDNF expression through several cellular mechanisms: astrocytes, microglia and vascular cells all secrete BDNF. Alcohol however, decreases BDNF expression and thus lowers the potential neurogenesis that is possible if there is only TBI alone.

Technical Issues:

BrdU injection allows us to label and birth-date the proliferating cells; however, there are some drawbacks of this technique that deserve some thought when interpreting the data. For instance, while BrdU readily crosses the BBB and becomes available to CNS cells for incorporation into their DNA, the bioavailability of BrdU is short. It is rapidly degraded and only available to cells for about 2 hours after injection. This likely means that we were only seeing a fraction of proliferation that actually happened during the observed time interval. Although we did use daily injections of BrdU for the 7 day group of animals, we would still not be able to label all proliferating events. An alternative approach would be to inject BrdU more frequently, multiple times per day; however, the drawback of such an approach would be increased animal stress and exposure to anesthesia. Additionally, there are some concerns that after TBI, there is an increase in BBB

leakiness, which might lead to higher BrdU permeability into the brain parenchyma. However, we did used a relatively high BrdU dose of 100mg/kg which means the available concentration in the brain reached the saturation point, so any further increase in BrdU permeability after TBI should not affect the number of $BrdU^+$ cells. Due to the short bioavailability of BrdU, in future studies, it would be interesting to include a group of animals that would be sacrificed at the 2-3 hour post-TBI and BrdU injection. This approach would enable us to rule out the possibility that cell death or migration of recently proliferated cells might impact the number of $BrdU^+$ cell in the SVZ.

An important consideration when looking at our results showing alcohol decreased the SVZ proliferative response after TBI is how this translates to human patients. While production of new brain cells by neurogenesis was observed in the human brain, no conclusive evidence exists for their roles (Chang et al 2016). Additionally, neurogenesis in the human brain happens at a much lower level compared to the rodent brain (Curtis et al 2007), thus limiting the translatability of rodent neurogenesis findings. Also, newly proliferated cells must become functionally relevant in the adult brain post TBI for them to have any role in the recovery process. These newly generated cells from the SVZ would need to undergo the differentiation process to become either neurons or glia and they

must also migrate to different brain regions. Therefore, we sought to explore these aspects of neurogenesis in our second aim.

One of the most interesting aspects of neurogenesis is whether the newborn cells become functionally relevant in the CNS. In the rodent system, the roles of newborn SVZ and DG cells are perhaps the best studied and understood. Yet their roles after CNS injury are less well known and this area of research is still very active as the roles of NSC are hotly debated (Garthe & Kempermann 2013). The goal for this part of the dissertation was to determine the NSC responses after TBI in the chronic TBI phase. These long-term responses include the survival of proliferated cells, their differentiation into neuronal and glial lineages and the migration of neuronal differentiated cells to various areas of the CNS. To this end, we performed BrdU injections for the first 7 days following TBI to label all proliferating cells within this period of time. The animals were allowed to survive for 6 weeks. According to previous behavioral studies in our lab, this is sufficient time for animals to reach a functional plateau, which in human corresponds to the chronic phase of TBI.

We quantified the total number of $BrdU^+$ cells in the SVZ of the prolonged survival time point; $BrdU^+$ cells are those that had been labeled during the first 7

days after injury. We found at the acute time point of 24 hours and sub-acute 7 days after TBI, the contralesional SVZ robustly responded to the TBI by increasing proliferation by up to 5 fold; however, at 6 weeks after TBI, the number of $BrdU^+$ cells retained in the contralesional SVZ decreased to baseline levels, essentially indistinguishable from sham groups (irrespective of alcohol or vehicle treatment). It is possible that these $BrdU^+$ cells seen at the earlier time points had migrated out from the SVZ to other parts of the CNS such as the striatum, corpus callosum, the cortex or toward the olfactory bulb.

Interestingly, we found significant retention of $BrdU^+$ cells in the ipsilesional SVZ in TBI animals and this level of retention was virtually the same across alcohol and vehicle treated groups. There were around 60,000 cells in the ipsilesional SVZ of TBI animals, which is around 4 fold higher than baseline levels (sham groups). This is not surprising considering the strong evidence in the literature suggesting that newly proliferated cells could survive better in the SVZ after TBI. For instance, one group found that there was a decrease of TUNEL positive cells in the SVZ of animals after CCI (Theus et al 2010). TBI might have altered the ipsilesional SVZ microenvironment to support more proliferating cells. By contrast, another group reached different conclusions in their model of TBI and neurogenesis (Acosta et al 2013). This group examined the long-term effect of TBI on SVZ neurogenesis. They

found that at 8 weeks post TBI, there was significant reduction in $Ki67^+$ cells in the SVZ of TBI animals. This is in stark contrast to our finding of elevated $BrdU^+$ in TBI animals at 6 weeks. It is possible that the use of different cell proliferation markers could partially explain the discrepancy. Ki67 is expressed during all phases of mitosis and is necessary for cell cycle processes (Mandyam et al 2007). Ki67 has been shown to correlate with BrdU staining; however, Ki67 has a half-life of 17 hours, which make it difficult to ascertain when the cell proliferation event happens.

Binge alcohol did not affect the long-term survival of proliferated cells in the SVZ in our model. This result is somewhat unexpected and is in contrast with previous studies on alcohol binge. For instance, in a prominent study, alcohol was given to rats 4 days in a row; they detected significant reduction in the survival of new hippocampal cells 4 weeks after the binge episodes (Nixon & Crews 2002). It is possible there are regional differences in response to binge alcohol, i.e., hippocampus being more sensitive to binge alcohol than the SVZ. Additionally, the binge alcohol given to the animals in that study was a very high dose of 5g/kg for 4 days (versus our model of 3g/kg for 3 days) which could contribute to the differences observed.

Neuronal differentiation was measured as the number of cells that expressed both BrdU and DCX (doublecortin). DCX is expressed by developing neurons and is thus a reliable marker for neuronal differentiation (Francis et al 1999). We found that TBI alone significantly induced an increase in neuronal differentiation bilaterally in the SVZ. This observation is in agreement with the literature on neuronal differentiation after TBI. For instance, one group used transgenic mice that had YFP tagged to new neurons to show that new neurons were populated in the dorsal SVZ and expressed DCX; they used the same CCI model and detected these cells at 6 week post TBI similar to our approach (Mierzwa et al 2014). It is possible that the microenvironment of the SVZ is changed after TBI and thus more permissive to the neuronal differentiation of new proliferating cells; for instance, one study found BDNF expression to be increased in the hippocampus after TBI (Hicks et al 1997).

Intriguingly, we found that binge alcohol decreased the number of early SVZ proliferating cells differentiated into the neuronal lineage 6 weeks after TBI. Stereological quantification of the SVZ area showed that alcohol significantly reduced the number of $BrdU^{+}/DCX^{+}$ double-labeled cell by 38% on the ipsilesional side (Figure 17). Immunofluorescence imaging confirmed our counting data showing reduced co-localization of BrdU and DCX signaling in TBI animals that

were alcohol treated versus vehicle (Figure 16). We suspect that binge alcohol might have affected neuronal differentiation by changing expression levels of trophic factors in the brain, for instance, BDNF, insulin, GDNF and EGF (Janak et al 2006). One group found that high dose alcohol administration lead to depressive like behavior and reduction in hippocampal BDNF (Hauser et al 2011). One clinical study looking at serum levels of several trophic factors found that alcohol dependent patients had reduced GDNF and BDNF (Heberlein et al 2010).

We did not expect our binge alcohol administration paradigm to have such long-term effect on neuronal differentiation post TBI. It is possible that alcohol is acting on neuronal differentiation via epigenetic mechanisms. One study found that there was reduced methylation of the CpG (Cytosin-phosphatidyl-Guanin) of the promoter of NGF (nerve growth factor) in the serum of alcohol dependent patients (Heberlein et al 2013). Furthermore, BDNF, a trophic factor important in the neuronal differentiation process is found to be affected by alcohol by epigenetic regulation in mice (Stragier et al 2015).

Another finding from this dissertation was that binge alcohol affects neuronal differentiation. New SVZ derived cells can differentiate into the three main cells of the CNS, i.e., neurons, astrocytes and oligodendrocytes. We

quantified the number of cells in the SVZ at 6 weeks that were positive for BrdU and negative for DCX. $BrdU^+/DCX^-$ cells are early-proliferated cells that differentiated into the non-neuronal lineage, either astrocytes or oligodendrocytes. Alcohol did not change the number of $BrdU^+/DCX^-$ cells in the SVZ of TBI animals (Figure 19). It appears that the vast majority of $BrdU^+/DCX^-$ cells were astrocytes according to qualitative microscopic observation of GFAP and BrdU staining (Figure 18). However, it is also possible that $BrdU^+/DCX^-$ cells might be microglia or infiltrating blood-borne macrophages. These types of immune cells have been found to proliferate after TBI (Lalancette-Hébert et al 2007). While alcohol did not have an effect on total glial differentiation, it could have effects on specific types of glia; for instance, there was qualitatively more $BrdU^+/GFAP^+$ cells in our microscopic observation of the Alcohol TBI compared to Vehicle TBI groups (Figure 18).

Migration is an important aspect of SVZ neurogenesis. For instance, in the SVZ-OB system, new putative neurons travel through a network of astrocytes and vascular cells to reach their destination in the OB. The molecular and cellular interactions that these migratory cells experienced during their excursion play an important role in their eventual differentiation into functional neurons. Alcohol exposure had been found to profoundly affect neuronal migration. For instance,

in a model of prenatal alcohol exposure, alcohol was found to decrease migration of serotonin neurons (Zhou et al 2001). Furthermore, after TBI, neuronal migration toward the perilesional area was increased; for instance, more DCX^+ cells were detected in the TBI lesion area in a model of TBI (Chang et al 2016)

First, we determined whether binge alcohol differentially affected TBI induced perilesional migration of new neurons. TBI itself induced robust migration of new cells toward the perilesional area (defined as 100um from the edge of the lesion). The total number of $BrdU^+/DCX^+$ cells in the perilesional area was around 12,000 cells which is the same magnitude as the number of $BrdU^+/DCX^+$ in the SVZ. However, the total volume of the perilesional area is much larger than that of the SVZ so the effective density of new neurons in the perilesional area is much less. Furthermore, qualitative microscopic observation of sham animals showed there were little to no $BrdU^+/DCX^+$ cells in the neocortical area dorsal to the SVZ. However, there was no evidence for binge alcohol having any effect on perilesional area migration of new neurons in TBI animals (Figure 21). In addition to the presence of new migratory neurons in the perilesional area, there was a large population of proliferating cells in this area. However, these $BrdU^+$ cells did not express markers for mature neurons ($BrdU^+/NeuN^+$, Figure 20). Our observation is in line with the literature on post-TBI

migration to the cortical area in that there are rarely any new cells expressing markers of mature cortical neurons (Chang et al 2016).

Migration from the SVZ to the cortex has important implications in the recovery post-TBI. The presence of new neurons in the perilesional area raises the possibility that these new cells can become functionally integrated and can take over the role of previously dead neurons. This event however has not been well documented. There is also the possibility that newborn neurons contribute to functional recovery in structures other than the perilesional area. There is strong evidence that olfactory function contributes greatly to the skilled forelimb reaching task performance (Whishaw & Tomie 1989). We quantified the migration of new neurons in the SVZ-OB pathway by counting the number of cells expressing both BrdU and DCX (Figure 22). This pathway is the well-characterized rostral migratory stream. We found TBI significantly elevated SVZ-OB migration (Figure 23). However, alcohol did not have a significant effect on SVZ-OB migration.

Future Directions

Although we did not examine neurogenesis in the hippocampus, it is possible that binge alcohol might also affect hippocampal neurogenesis. In future

studies, we could first use a hippocampal sensitive functional task to assess binge alcohol impact on the hippocampal dependent task post TBI. However, we know that in the hippocampus, reduced NSC proliferation or ablation was shown to lead to inhibition of LTP in the perforant pathway (Kempermann 2011). Other evidence suggests that reduced NSC responses can lead to worse performance on hippocampal dependent tasks such as the MWM, contextual fear conditioning, and trace conditioning. Additionally, we could extend our work by using the BrdU injection paradigm to study different temporal responses of neurogenesis to TBI and alcohol in the hippocampus.

BDNF is responsible for the survival of new neurons. A large number of newly generated cells die due to insufficient trophic support. Alcohol potently affects BDNF expression and could affect how many newly generated cells in the SVZ survive. Our future study could explore how alcohol affects BDNF expression in TBI animals and in turn how that affects the survival of those new neurons. We would need to utilize markers of neurogenesis (such as BrdU) along with markers of cell death (such as Caspase3). Furthermore, our lab is in the process of refining the method to alter the BDNF level in the SVZ. This would allow us to modulate the endogenous BDNF and to assess its contribution to neurogenesis after TBI.

While BrdU has been a useful tool in the study of neurogenesis, there are important limitations to this technique. One new technology that has been shown to be useful in the study of neurogenesis is conditional lentivirus expressing Cre-Flex, a bioluminescence marker in addition to green fluorescence protein (GFP). This technique utilizes lentivirus and can be used to label neural stem cells in the SVZ as has been demonstrated recently in a stroke model (Vandeputte et al 2014). The lentivirus vector is introduced into the SVZ by stereotactic injection, and becomes incorporated into the stem cells of the SVZ. The markers carried by the lentivirus become stably expressed in the stem cells which allow us to track the migration pattern as well as determine the eventual phenotype of these cells.

Inflammation is a large component of TBI pathology, and alcohol has a pronounced inflammatory modulating effect. One question is how binge alcohol affects inflammatory cells such as infiltrating macrophage, and microglia activation. In this regard, large amounts of adenosine triphosphate (ATP) is produced in the cells of TBI lesion sites after CNS injury and released to the extracellular space and serves as an inflammatory signaling molecule. ATP has been found to be a potent chemo-attractant to microglia, the resident inflammatory cell of the CNS (Ohsawa & Kohsaka 2011). Additionally, in the hematopoietic system, ATP has a chemo-attraction role for stem cells (Rossi et al

2012). It is likely that ATP also is a homing signal for migrating neuroblasts (i.e. DCX^+ cells). Extracellular ATP is recognized by cells via the purinergic cell surface receptors such as P2X4 and P2X7. Interestingly, P2X7 receptors are also affected by alcohol. For instance, alcohol is an antagonist for purinergic receptors P2X4 and P2X7 (Ostrovskaya et al 2011).

Finally, we could expand our binge alcohol model to more closely mimic human alcohol consumption. Typically, TBI patients do not get a TBI during their first binge episode. It is likely that they would have a more prolonged history of alcohol consumption. Since laboratory animals such as rats can experience tolerant effects to alcohol when given repeatedly over time, this may affect the outcome of repeated binge alcohol studies. Alcohol is an antagonist for NMDA receptors and repeated alcohol administration can lead to an increase in expression of NMDA receptors. We know that NMDA receptors are the main perpetrator for excitotoxicity via high levels of extracellular glutamate after TBI. Therefore, it is possible that a more prolonged binge alcohol administration paradigm could lead to different SVZ response outcomes as compared to what we observed in our current model. As there is evidence in human TBI patients of a strong likelihood of increase in intake of alcohol post-TBI (Bombardier et al 2003),

examining how alcohol given after TBI affects neurogenesis is an interesting avenue of research.

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VITA

Son Trung Ton was born on December 14th, 1988 in Binh Thuan, Vietnam to Steaven Ton and Hoang Van. Son's family now resides in Chicago, Illinois.

As an undergraduate student at Loyola University in Chicago, Son majored in biochemistry and spent 4 years involved in basic neuroscience research in the field of gustatory neuronal guidance. Due to this experience, Son realized he wanted to pursue a research career in neuroscience.

After graduation, Son entered the graduate program in Neuroscience at Loyola University Chicago. In the spring of 2012, he joined the lab of Dr. Gwendolyn Kartje at Loyola University to study traumatic brain injury. In 2013, Son was awarded a Predoctoral Fellowship in the Alcohol Research Program, at which point he studied the effects of alcohol on the neural stem cell response after traumatic brain injury. Son has presented his work at the Annual Society for Neuroscience and at the Annual Research Society on Alcoholism.