SEX DIFFERENCES IN HIPPOCAMPAL CELL PROLIFERATION AND INFLAMMATION FOLLOWING REPEATED MILD TRAUMATIC BRAIN INJURY IN ADOLESCENT RATS

by

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BSc, McGill University, 2015

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Abstract

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Traumatic brain injury (TBI) is becoming increasingly recognized as a global health issue. Each year over 160,000 Canadians experience some form of TBI, which can be caused by sport-related injuries, motor vehicle accidents, or assault. Adolescents are especially susceptible to repeat head injury and represent an at-risk population for sustaining sports-related concussions. The hippocampus, known for its role in learning and memory, is vulnerable to this injury. Although most TBI studies exclude females, there are important sex differences in outcomes and recovery following brain injury. A greater understanding of how sex differences contribute to the heterogeneity of this disease is critical for clinical care and potential treatments. Currently, few preclinical studies have assessed sex differences in adolescents following repeated mild traumatic brain injury (rmTBI). This study uses an awake closed head injury (ACHI) paradigm in male and female adolescent rats to investigate acute injury-induced changes to the hippocampus after rmTBI. A neurological assessment protocol (NAP) administered immediately after each impact showed that the ACHI acutely alters state of consciousness, and results in deficits after each impact. Following 8 ACHIs spaced 2 hours apart, adolescent rats were injected with the thymidine analogue BrdU and perfused 2 hours later on either post injury day (PID) 1 or 3. BrdU was used to identify cells undergoing DNA synthesis, and Ki-67 – expressed during all active phases of the cell cycle – was used as an endogenous marker for proliferation. Results indicate a robust and diffuse increase in cellular proliferation in male rmTBI animals that was not present to the same extent in female rmTBI animals. Triple labeling experiments revealed a higher proportion of microglia/macrophages in the subgranular zone of rmTBI animals, indicating an immediate inflammatory response in both sexes. This study shows sex differences in the pathophysiology of rmTBI in adolescent rats. Further investigation will reveal the detrimental versus neuroprotective contributions of this effect on learning and memory.

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Table 1: List of Abbreviations

TBI	traumatic brain injury	IEG	immediate early gene
ACHI	awake closed head injury	IPV	intimate partner violence
ACRM	American Congress of Rehabilitation Medicine	LOC	loss of consciousness
ANP	amplifying neural progenitor	MRI	magnetic resonance imaging
BLBP	brain lipid-binding protein	mTBI	mild traumatic brain injury
BrdU	5-bromo-2'-deoxyuridine	MWM	Morris water maze
CA	cornu ammonis	NA	numerical aperature
CC	corpus callosum	NAP	neurologic assessment protocol
CCI	controlled cortical impact	NMDA	N-methyl-D-aspartate receptor
CE	coefficient of error	NPC	neural progenitor cell
CISG	concussion in sport group	PBBI	penetrating ballistic-like brain injury
CNS	central nervous system	PCX	parietal cortex
CT	computed tomography	PFC	prefrontal cortex
CTE	chronic traumatic encephalopathy	PID	post injury day
DAB	3,3-Diaminobenzidine	PND	post natal day
DCX	doublecortin	PTA	post-traumatic amnesia
dDG	dorsal dentate gyrus	p-tau	phosphorylated tau
DG	dentate gyrus	rmTBI	repeat mild traumatic brain injury
DNA	deoxyribonucleic acid	ROS	reactive oxygen species
EC	entorhinal cortex	SCAT	sport concussion assessment tool
FPI	fluid percussion injury	SGZ	subgranular zone
GCS	glasgow coma scale	Sox2	sex determining region Y box 2
GFAP	glial fibrillary acidic protein	vDG	ventral dentate gyrus
Iba1	ionized calcium binding adaptor molecule 1	WHO	World Health Organization
IED	Improvised explosive device		

Territory Acknowledgment

I respectfully acknowledge the Lekwungen-speaking peoples on whose traditional territory the university stands. I extend my gratitude to the Songhees, Esquimalt and WSÁNEĆ peoples for being welcoming and gracious hosts. I acknowledge that I work, live and play as a visitor on these lands and recognize that the Songhees, Esquimalt and WSÁNEĆ people's historical relationships with the land continue to this day.

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This work is dedicated to:

All those affected by TBI,

and

Grandy, who suffered a brain injury during the time I was carrying out this research. I hope you know how much I wish you were here to see it completed – thank you for always supporting my studies.

CHAPTER 1

Introduction

1.1 Rowan Stringer's Story

Rowan Stringer was an avid rugby player who was captain of her high school rugby team. One Friday, Rowan got hit in the head during a game, but returned to the field shortly after. A similar incident happened that following Monday. In a match 2 days later, Rowan lost consciousness after being tackled and landing on her head. Rowan was rushed to the hospital and sadly passed away on Sunday, May 13th, 2013, 4 days after her last rugby game. Her brain had not had time to heal from those first 2 injuries. Rowan suffered multiple concussions: a traumatic brain injury (TBI) caused by an external biomechanical force that results in immediate transient neurologic disruption of the brain tissue. An inquest following her death pointed to a lack of youth sport concussion protocols and in 2018, Rowan's law was passed with new policy aimed to protect youth from repeated concussions. What happened during the last several days of Rowan's life was a lack of understanding about the severity of suffering multiple concussions (Tator et al., 2019). And while Rowan's law and new policy can provide some measure of safety and prevention, we continue to rely on preclinical models to investigate what happens on a cellular level in the brain after multiple concussions. The work presented here will provide insight into the cellular events following multiple concussions in a clinically relevant adolescent model.

1.2 Traumatic Brain Injury

1.2.1 Epidemiology

Traumatic brain injury is becoming an increasingly important health issue worldwide; the global incidence of TBI is estimated to be 10 million deaths and/or hospitalizations annually (Lan-

glois et al., 2006). Severity of TBI varies from mild to moderate to severe; approximately 70-90% of TBIs reported are classified as mild (Cassidy et al., 2004). Severe TBI can result in death and disability, while moderate and mild injuries can lead to long-term cognitive impairments (Cassidy et al., 2004; Langlois et al., 2006; Rutland-Brown et al., 2006). Hospitalizations due to mild traumatic brain injury (mTBI), also referred to as concussion, are estimated at 100-300 cases per 100,000 population (Cassidy et al., 2004). However, because many mild injuries are not treated at hospitals, this is likely an underestimation of the true incidence of these injuries. Common causes of mTBIs include falls, motor vehicle accidents, assault and collisions with moving or stationary objects and assault (Faul et al., 2010). Recently, there has been increased awareness of mTBIs caused by intimate partner violence (IPV). According to a study conducted recently by Valera et al. (2019), 75% of women who experience IPV sustained mTBIs, and 50% sustained repetitive mTBIs. Repeated mTBIs are also common among athletes (especially those engaged in contact sport), and military personnel exposed to improvised explosive devices (IED) and other explosions (Wallace, 2009).

1.2.2 Definitions

Traumatic Brain Injury

TBI is defined as damage to the head caused by an external force that results in immediate disruption of the brain tissue. This disturbance can induce structural injury and sometimes cause axons to become shread or torn (Mckee et al., 2016). The type and magnitude of these external forces vary injury to injury and lead to differences in the severity of TBI. Examples of external forces include penetration (eg. by sharp objects), acceleration and deceleration (eg. from a car accident) or blast waves. These forces lead to focal and/or diffuse traumatic brain injury; penetrating injuries are usually classified as focal injuries while the coup-contre-coup movement of the brain inside the

skull following acceleration/deceleration forces causes a diffuse injury. Acceleration and deceleration forces are common in contact sports where athletes frequently get tackled, as even tackles to the body result in movement of the brain in the skull (Crisco et al., 2012). Injury severity is generally determined by standardized tests including the Glasgow Coma Scale (GCS), which observes ocular, motor and verbal response and scores a patient's ability on a scale up to 15 (mild, GCS 13-15; moderate, GCS 9-12; severe, GCS <9) (Jain & Iverson, 2020). Moderate and severe TBIs may also be diagnosed through gross structural damage, identified by traditional neuroimaging techniques (Fehily & Fitzgerald, 2017). The primary injury caused by these external forces in mild, moderate and severe TBI results in cascades of secondary cellular events that ultimately lead to brain cell death, tissue damage and atrophy.

Mild Traumatic Brain Injury

The World Health Organization (WHO) Collaborative Task Force on mTBI put forward a definition for mTBI derived from both the 1993 American Congress of Rehabilitation Medicine (ACRM; Mild Traumatic Brain Injury Committee) definition and the 2003 Center for Disease Control and Prevention's Mild TBI Working Group (Control, 2003; Kay et al., 1993). The definition described clinical signs of mTBI as loss of consciousness (LOC), post-traumatic amnesia (PTA), confusion and disorientation and neurologic signs (determined by the GCS score). It is worth noting that historically there has been a lack of consensus as to what constitutes mTBI including what range of scores in the GCS assigned to injury severity, as well as the duration of loss of consciousness (Ruff et al., 2009). Although mild implies the absence of overt structural damage (Signoretti et al., 2011), the severity of the secondary injury cascade - the cellular events following the initial injury - may not be "mild". While previous reports showed at least 15% of patients continue to seek health care professionals due to persisting problems following mTBI (Alexander, 1995; Bigler, 2003), more recent studies indicate over 30% of patients were still functionally impaired 3 months after injury,

22% of whom had lasting impairments 1 year after injury (McMahon et al., 2014).

Concussion

The Concussion in Sport Group (CISG) first released a consensus statement on concussion in sport in 2001, aimed at informing athlete care among physicians and healthcare providers. The group has since made four revisions to the consensus statement that detail how to recognize a concussion, when to remove a player from sport, how to evaluate athletes after injury as well as recommendations for rehabilitation, recovery and return to sport protocols. The consensus also clearly states clinical, pathologic, and biomechanical features that define concussion. Of note, in the 2012 consensus statement, the authors state that while concussion and mTBI are often used interchangeably, concussion represents an historical term relating to shaking of the brain, "commotio cerebri". They acknowledge that the term "concussion" is not necessarily related to a pathologic injury and is rather a subset of TBI. In this work, the term concussion will be used when referring to the clinical population involved in sports related injuries. The term mTBI will be used when referring to the pathological consequences of an injury produced in a preclinical model (McCrory et al., 2017).

Repeated mTBI (rmTBI)

As previously mentioned, several populations, including athletes in contact sports, military personnel and victims of intimate partner violence are susceptible to repeated head injuries. While mTBI is generally linked to short-lived symptoms that often resolve within hours to 10 days of injury, the cumulative effects of repeated traumatic brain injuries may increase the severity of symptoms as well as the individual's susceptibility to neuropathological sequelae. rmTBI may increase the likelihood of developing postconcussion syndrome leading to long term cognitive deficits including attention and working memory, and changes in white matter caused by diffuse axonal

injury (Medana & Esiri, 2003; Niogi et al., 2008). Long term outcomes after sustaining rmTBI vary greatly as they are dependent on a multitude of factors. In addition to age at insult, an individual's genetic make up and the injury itself, the number of TBIs, injury severity and time between impacts influence injury outcome (VanItallie, 2019). Furthermore, clinical and preclinical studies have shown that rmTBI increases the likelihood of neurodegenerative disorders including dementia, Parkinson's disease and chronic traumatic encephalopathy (CTE).

Chronic Traumatic Encephalopathy (CTE)

Often, cognitive and behavioural impairments caused by rmTBI do not appear until later in life. In the 1920s, Dr. Harrison Martland observed a condition in boxers, referred to by sports writers of the time as "punch drunk" (later known as "dementia pugilistica") (Millspaugh, 1937):

> *"…the occurrence of the symptoms in almost 50 per cent of fighters who develop this condition in mild or severe form, if they keep at the game long enough, seems to be good evidence that some special brain injury due to their occupation exists."*

Early symptoms of punch drunkenness would appear in the extremities, including "slight staggering", followed by mental confusion or slowing of muscular action. While Martland observed some cases to be mild and not progress further, he noted in severe cases "marked mental deterioration…necessitating commitment to an asylum", (Martland, 1928). In reference to the punch drunk condition, Dr. Martland asserted in his 1928 publication that, "the condition can no longer be ignored by the medical profession or the public".

Nearly a century later, work is still being done to understand the pathological consequences of repeated hits to the head. Recently, studies have shown that repeated head injuries may lead

to pathologies that are associated with CTE, a progressive neurodegenerative disease caused by the hyperphosphorylation of tau (Mckee et al., 2016). The incidence of mTBI leading to CTE is unknown, however – several studies have indicated that the cumulative effects of rmTBI increase the likelihood of developing the pathology, as evidenced first in boxers, and more recently in football players and other contact sport athletes. Some studies have even indicated that a concussive event is not necessary for CTE pathology to arise, as even subconcussive impacts may lead to the disease (Greenwald et al. (2008); for a review on CTE in athletes after rmTBI, see Mckee et al. (2009)). Because a definitive diagnosis can currently only be made post-mortem, research with preclinical models to investigate biomarkers and imaging techniques to aid in diagnosis of CTE is critical (Turner et al., 2013).

1.3 Preclinical Models of TBI

Because mTBIs are not visible using conventional imaging techniques, preclinical models have provided a greater understanding of their pathophysiological and molecular underpinnings. There is a huge heterogeneity in type of injury to the brain which makes the development of a wide variety of preclinical models critical to increasing our understanding of the heterogeneity of pathophysiology that is observed in the clinical population (Xiong et al., 2013). Preclinical models primarily include rodents, however models for animals with gyrencephalic brains including cats, rabbits, sheep, pigs and ferrets have also been developed (Vink, 2018). Experimental TBI can be focal or diffuse injury and result from either an open or closed head injury. Open head injury models involve a craniotomy (removal of part of the bone from the skull), and the injury is applied directly to the intact dura. In closed head injury models, the injury is delivered to the intact (though sometimes exposed) skull.

The most common preclinical models of TBI include: fluid percussion injury (FPI), controlled cortical impact (CCI), penetrating ballistic-like brain injury (PBBI), weight drop models and blast

brain injury. Table 1.1 below illustrates important features of each of these models, though further and more detailed information can be found in the following review papers: Bodnar et al. (2019), Xiong et al. (2013), Turner et al. (2015) (for CTE specifically), Fehily & Fitzgerald (2017) (repeat injuries), and Petraglia et al. (2014). Importantly, even within models there is a variety in how the injury is administered. For example, in the CCI injury model, the velocity of the piston may be altered, the type of impact tip, angle of piston and the impact site (helmet, skull exposed or both). Because each injury model causes different injury biomechanics, it is critical when evaluating studies to look carefully at the mechanism of injury and to take it into consideration when contradictory evidence is observed.

Table 1.1: Experimental models of TBI

Injury Model	Description	Craniotomy	Anaesthesia	Type of Injury	Main Pathology	Reference
Fluid percussion injury	A craniotomy is made around the midline between bregma and lambda and fluid pulse is injected into the epidural space	Yes	Yes	Midline – focal; lateral - mixed	Subdural hematoma, BBB dysfunction, cognitive deficits,	Single: Dixon et al., 1987
					Repeat: Increased microglial activation, persisting cognitive deficits, damage in grey and white matter structures	Repeat: Wright et al., 2019; Shultz et al., 2012; Deross et al., 2002; Aungst et al., 2014
Fenney's Weight Drop (open head)	A guided weight is dropped from a specified height onto exposed dura	Yes	Yes	Focal	Overt tissue damage, astrocyte reactivity, necrosis, cognitive deficits	Single: Morales et al., 2005
					Repeat: mitochondrial dysfunction, oxidative stress	Repeat: Vagnozzi et al., 2005
Marmarou's weight drop (closed head)	A guided weight is dropped from a specified height on a helmet or impact plate	No	Yes	Diffuse	Cognitive and motor deficits, microglial activation, astrocyte activation, axonal swelling/damage, extensive DAI, BBB distruption, edema	Single: Albert-Weissenberger et al., 2012, Marmarou et al., 1994
					Repeat: increased microglial activation, cognitive deficits, axonal damage, BBB damage	Repeat: Weil et al., 2014; Deford et al., 2002; Fujita et al., 2012

Controlled cortical impact – open head	A pneumatic or electromagnetic piston (with precise control of velocity and impact duration and depth) impacts the dura	Yes	Yes	Focal	Overt tissue damage, increase in activated microglia, oxidative stress, BBB dysfunction axonal injury, cognitive and emotional deficits Repeat:	Single: Dixon et al., 1991; Washington et al., 2012,
Controlled cortical impact – closed head; skin intact or skull exposed	A pneumatic or electromagnetic piston (with precise control of velocity and impact duration and depth) impacts the intact skull	No	Yes	Diffuse	No overt tissue damage, astrocyte reactivity, microglial reactivity axonal injury, BBB disruption, transient cognitive deficits, often no motor or emotional impairment Repeat: increased astrocyte reactivity, microglial activation, increased/persisting cognitive deficits, axonal degeneration	Single: Hanlon et al., 2016; Creed et al., 2011 Repeat: Shitaka et al., 2011; Mouzon et al., 2012; Luo et al., 2014
Blast Injury	Simulated blast effects via compression-driven shock tube	No	Yes	Diffuse	DAI, cognitive deficits, chronic neuroinflammation, myelinated axonopathy Repeat: changes in DNA methylation, BBB impairment, microglial activation, prolonged inflammation	Single: Goldstein et al., 2012 Repeat: Skotak et al., 2019
Penetrating ballistic-like	A leading shockwave produces a cavity followed by transmission of high energy projectiles	No	Yes	Focal	Cognitive impairment, inflammation, white and grey matter damage, sensorimotor impairment, extensive hemorrhage, BBB disruption	Williams et al., 2006; Davis et al., 2010; Plantman et al., 2012 Repeat: N/A

Awake closed head injury	A pneumatic piston (with precise control of velocity and impact duration and depth) impacts a helmet on the intact skull in the awake, restrained animal	No	No	Diffuse	Acute sensorimotor deficits, gliosis,	Single: Pham et al., 2019
					Repeat: White matter abnormalities, spatial learning impairments	Repeat: Pinar et al., 2020; Meconi et al., 2018; Petraglia et al., 2014; Christie et al., 2019; Wortman et al., 2018

1.3.1 Experimental mTBI and rmTBI

rmTBI models typically aim to reflect injuries produced in sport, however as it was mentioned previously, repeated head injuries also occur in the clinical population outside of sport due to IPV and military blast-related injuries. These models offer insight into the effects of injury number and frequency compared to single hit models. However, these added variables also present a challenge in the standardization of experimental rmTBI paradigms across laboratories (Turner et al., 2015).

With the exception of the awake closed head injury (ACHI) model and a few early TBI studies, preclinical TBI models administer injuries while animals are anesthetized (in their systematic review, Bodnar et al. (2019) identified six papers classified as "other" models that did not use anesthesia). The use of anesthetics introduces more variables into preclinical injury heterogeneity, including dose (surgical depth), length of exposure and anesthetic agent used. For example, it has been shown in preclinical models that the type of anesthetic agents administered to animals during TBI procedures can have different magnitudes of neuroprotection, including measures of cognitive recovery, neuronal death and inflammation (Luh et al., 2011; Statler et al., 2006). In the clinical population, administration of anesthesia after TBI has been evaluated as a treatment by reducing cerebral metabolic rate and intracranial pressure, thereby preventing secondary injury (Flower & Hellings, 2012). However, in the clinical population, anesthetics are administered exclusively after a TBI, not during (as in preclinical models) which complicates studies looking at, for example, therapeutic time windows. Thus, preclinical models that do not induce injury under anesthesia, such as the one used in this study, are an important addition to clinically relevant experimental models of TBI.

1.3.2 Vulnerable Populations: Age and Sex Differences

According to the Centre for Disease Control, children aged 0-4, adolescents aged 15-19 and adults over the age of 65 are the most likely to sustain a TBI (Faul et al., 2010). Additionally, the incidence of TBI is found to be higher in males than in females, although it has previously been reported that this difference in incidence is based on age, and only seen from puberty until age 45 (Farace & Alves, 2000).

Sports-related injuries account for approximately 50% of all concussions sustained by children and adolescents aged 8-19 (Bakhos et al., 2010; Guskiewicz & Valovich McLeod, 2011). Because a myriad of developmental changes persist into young adulthood - including myelination and synaptic pruning - adolescent populations are intriguing to study (Semple et al., 2013). Puberty occurs within the adolescent period and is marked by neuroendocrinological changes that lead to sexual maturity. An increase in the production of gonadal hormones leads to transient behavioural changes and permanent sexually dimorphic brain development (Spear, 2000). In humans, puberty begins between 11-13 years of age in females and 13-15 years in males (Herting & Sowell, 2017). In rats, onset of puberty occurs between post natal day (PND) 30-39 in females and PND 40-45 in males (Koss et al., 2015).

Functional and psychological outcomes in the clinical population after TBI have shown some symptoms to be worse in males than females (including aggression and some cognitive tasks), though females generally tend to fare worse than males (including anxiety, depression, prolonged symptoms and executive functioning; summarized in Table 1.2, also see Gupte et al. (2019) for an excellent review). Females have been found in some cases to be associated with reduced mortality, but this depends on stage of pubescence. Mortality was not lowered in a prepubescent group, but was in a pubescent group (Ley et al., 2013). In a postpubescent group, Phelan et al. (2007) found

females to be associated with higher survival rates in severe injuries, but less so for moderate and mild injuries. Taken together, this indicates that events surrounding puberty, as well as injury severity, play a role in outcome and sex differences following TBI.

mTBI in the Adolescent Population: Preclinical Models

Preclinical TBI models have largely focused on adult animals, and some studies have shown that as in the clinical population, younger rodents recover to a greater extent than adults and aged animals (Eiben et al., 1984; Gan et al., 2004; Semple et al., 2016). However, it should not be overlooked that injuries in the adolescent brain can have unique long-term consequences. For example, TBI can impair an adolescent's ability to function in a social environment, which can affect educational performance (Beauchamp & Anderson, 2013; Jantz & Coulter, 2007). In preclinical models, impaired play behaviour has been reported in a model of mTBI in male and especially female rats (Mychasiuk et al., 2014). Furthermore, injury-induced changes to hormones during this developmental window can have unique consequences. Greco et al. (2015) found that repeated closed head injuries in adolescent male rats caused a decrease in testosterone, delayed onset of puberty and stunted development of reproductive organs.

Table 1.2: Sex differences in Clincial Outcomes after TBI

Symptom	Evidence for greater or prolonged impairment in males	Evidence for greater or prolonged impairment in females	No sex differences
Anxiety		Farace and Alves 2000[1]; Xiong et al 2016[13]	
Depression		Farace and Alves 2000[1] (but not for mTBI); Schopp et al 2001[11]; Hart et al 2011[12]; Xiong et al 2016[13]	
Cognitive Recovery	Ratcliff et al 2007[2] ; Moore et al 2010[6] (visual memory); Schopp et al 2001[11] (general memory and cognitive flexibility)	Farace and Alves 2000[1]; Niemeier et al 2007[5] (executive functioning); Covassin et al 2007[9] (verbal and visual memory); Sicard et al 2018[14]	Moore et al 2010[6] (executive functioning, processing speed); Berz et al 2013[10] (confusion)
Headache and Fatigue		Colantonio et al 2010[3], Scott et al 2015[4]; Baker et al 2016[8]	
Irritability/ Aggression	McGlade et al 2015[7]	Baker et al 2016[8]	Colantonio et al 2010[3]
Dizziness		Colantonio et al 2010[3]; Baker et al 2016[8]	
Sleep disturbances	Colantonio et al 2010[3], Baker et al 2016[8]		
Substance abuse	Scott et al 2015[4]		

[1] Meta-analysis, males and females aged at least 12 and over;
[2] Males and females 1 year post TBI, 16-45 years;
[3] Males and females aged 14 years and older;
[4] Males and females aged 18-31 with history of childhood TBI;
[5] Males and females aged 18-49 admitted to level I trauma centres;
[6] Males and females, time since injury at least one year aged 19-81;
[7] Male and female veterans aged 18-55;
[8] Student athletes aged 13-19;
[9] Collegiate athletes;
[10] Male and female athletes aged 9-17;
[11] Males and females: outpatients with TBI;
[12] Male and female patients with TBI aged 16 or older;
[13] Males and females at least 15 years of age at time of injury;
[14] Male and female university athletes with sports-related injury;

1.3.3 Translation: From Preclinical Models to Clinical Relevance

Translation from preclinical models to a clinical setting has yet to yield promising therapies following mTBI (Hersh et al., 2018). Despite robust and successful treatments in preclinical models, with drugs such as minocycline, progesterone and erythropoietin, these therapies have all failed in clinical trials (Barha et al., 2011; Kovesdi et al., 2012; Lu et al., 2005). The heterogeneity of both TBI patients and the injuries themselves may be a critical factor in this. Even in the preclinical setting, it is difficult to ensure exact injury replication from one lab to another. Small changes in craniotomy position, differing NSS scoring systems and the multitude of possibilities for injury parameters (eg. Height of weight drop, angle of CCI piston, diameter of rubber tip on piston) increase the variability in injuries and confounds comparison between research groups (Floyd et al., 2002). Another confound in much of the preclinical data is the exclusion of female and non-adult animals (Bodnar et al., 2019). Evidence continues to show that there are differences in reported symptoms and functional outcomes in males and females after TBI (as discussed above in Table 1.2). Failure of clinical trials may be due in part to the lack of sex consideration in animal populations reflected in preclinical models (Maas et al., 2010).

While preclinical models aim to relate to certain aspects of TBI, there is no one model that is able to accurately portray the heterogeneity of injury observed in the clinical population. Therefore, an awareness of the strengths and weaknesses of each preclinical model is critical in the translational capacity of experimental TBI. Still, these preclinical models are essential for understanding the cellular and molecular aspects of human TBI and understanding how they relate to behavioural outcomes.

1.4 mTBI Pathophysiology

A head impact results in both primary and secondary injuries. The mechanical disruption to the head, be it a blow, an impact or a blast wave, causes immediate disruption of the brain tissue and can cause axons to become sheared, torn or stretched. Following this primary injury, cascades of cellular and molecular events take place that can ultimately lead to cell damage, atrophy and death (see Figure 1.1). While the primary injury is immediate, this secondary injury cascade may develop over hours to days and even months. The events comprising secondary damage are a focus of many animal studies as this pathophysiological response may indicate pathways that can be targets for therapeutic interventions (Kumar & Loane, 2012; Pearn et al., 2017).

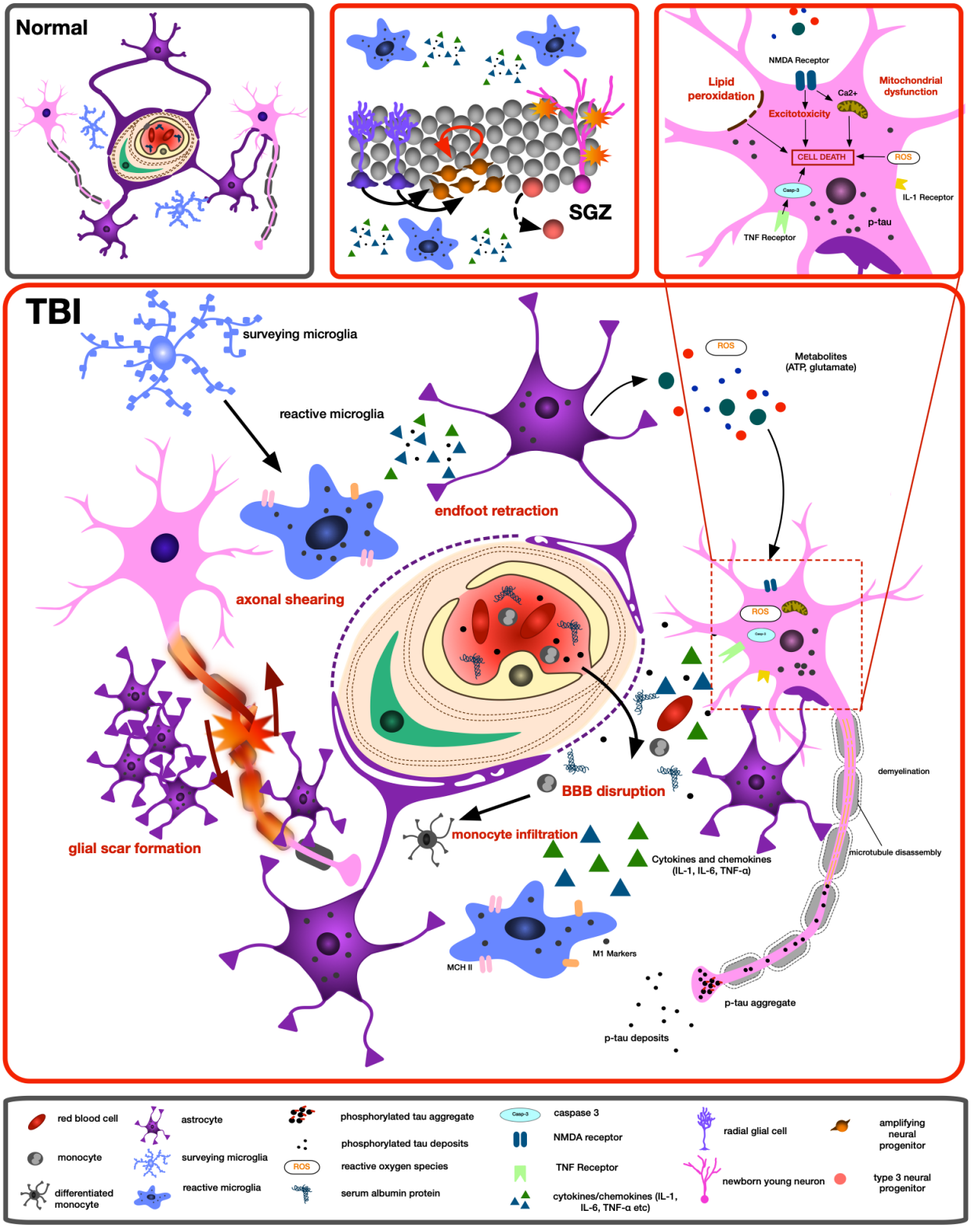

Normal
SGZ
NMDA Receptor
Lipid peroxidation
Mitochondrial dysfunction
Ca2+
Excitotoxicity
CELL DEATH
ROS
Casp-3
IL-1 Receptor
TNF Receptor
p-tau
TBI
surveying microglia
reactive microglia
Metabolites (ATP, glutamate)
endfoot retraction
axonal shearing
BBB disruption
monocyte infiltration
glial scar formation
Cytokines and chemokines (IL-1, IL-6, TNF-α)
demyelination
microtubule disassembly
M1 Markers
MCH II
p-tau aggregate
p-tau deposits
red blood cell
monocyte
differentiated monocyte
astrocyte
surveying microglia
reactive microglia
phosphorylated tau aggregate
phosphorylated tau deposits
reactive oxygen species
serum albumin protein
caspase 3
NMDA receptor
TNF Receptor
cytokines/chemokines (IL-1, IL-6, TNF-α etc)
radial glial cell
newborn young neuron
amplifying neural progenitor
type 3 neural progenitor

Figure 1.1: mTBI Pathophysiology. Under normal conditions (top left), astrocyte endfeet ensheathe blood vessels and microglia actively survey the environment. The blood brain barrier (BBB) is intact and blood vessel contents remain contained. Pericytes adjacent to endothelial cells surround the blood vessels and regulate the BBB. The axons of neurons are intact and microtubules in axons and dendrites are stabilized by tau. After TBI (centre), the primary injury can cause axons of neurons to become shread or torn leading to axonal dysfunction. Tau dissociates from microtubules and becomes abnormally phosphorylated (p-tau). Hyperphosphorylated tau aggregates begin to form. (Top right) Membrane disruption caused by the injury leads to a disruption in the ionic equilibrium and a cascade of changes in glucose metabolism, free radical production and mitochondrial dysfunction. Free radicals react with polyunsaturated fatty acids in the cell membrane and leads to increased membrane permeability (lipid peroxidation). Microglia become activated, change morphology and increase secretion of cytokines and chemokines. They attract peripheral monocytes to the injured region and increase production of ROS and RNS. Activated cells become phagocytic, and begin to scavenge and clear debris. Astrocytes also change morphology and become activated. Reactive perivascular astrocytes compromise BBB integrity; BBB breakdown causes further infiltration of peripheral immune cells. (Top middle) Cell proliferation and neurogenesis in the subgranular zone (SGZ) is altered. Neural stems cells are activated out of quiescent and begin to proliferate. Proliferation of amplifying progenitor cells is also increased. There is aberrant migration of immature neurons to the hilus and newborn granule cells display abnormal connectivity. Figure adapted from Tagge et al 2018.

Mechanical damage to cells and blood vessels after an mTBI causes disruption to cell membranes which results in a myriad of neurochemical changes. There is an immediate redistribution of ionic balance and an efflux of K^+ ions through voltage-gated ion channels which causes unregulated neuronal depolarization (Giza & Hovda, 2014). Depolarization leads to the release of neurotransmitters from presynaptic terminals including excitatory amino acids like glutamate which further potentiates this response by binding to N-methyl-D-aspartate (NMDA) receptors. NMDA receptors are ionotropic glutamate receptors whose excessive activation can lead to increased calcium influx and cellular excitotoxicity (Ladak et al., 2019). Animal studies have observed a transient and immediate increase in extracellular glutamate concentrations that recover within hours after mTBI (Giza & Hovda, 2014).

Na^+/K^+ pumps normally maintain membrane potential between -40 and -70mV so when the efflux of ions occurs, they act to restore normal ionic gradients. Because Na^+/K^+ pumps are ATP-dependent, this requires high levels of glucose metabolism causing a depletion in energy stores. Following this increase in glucose consumption there is a general metabolic depression, which has been observed in both clinical and pre clinical models of TBI (Prins et al., 2013). Interestingly, studies have shown that younger rats are able to reverse the glucose metabolic depression more quickly than older animals (Prins & Hovda, 2009).

In addition to efflux of ions that result in depolarization, membrane disruption also causes accumulation of intracellular calcium. This happens within hours but has shown to return to control levels between 4-7 days after injury (Deshpande et al., 2008; Fineman et al., 1993). Calcium also accumulates in mitochondria, causing mitochondrial calcium overloading which leads to oxidative stress (Peng & Jou, 2010). Increased intracellular reactive oxygen species (ROS) lead to further mitochondrial dysfunction and lipid peroxidation (Uryu et al., 2002). Altogether, membrane disruption potentiates secondary damage including blood brain barrier (BBB) dysfunction

and activation of apoptotic and necrotic pathways (Fehily & Fitzgerald, 2017; Hsiang et al., 1997; Marmarou et al., 1994).

These secondary metabolic cascades also lead to an increase in proinflammatory molecules; neuroinflammation is another key component of the pathophysiologic response to mTBI and will be covered in the next section. Briefly, pro inflammatory molecules like cytokines and chemokines are released after TBI, which trigger a response from microglia – key mediators of the immune response that become activated after injury (Kumar & Loane, 2012). Evidence suggests prolonged inflammation after TBI plays a role in the development of CTE pathology and neurodegenerative disease like Alzheimer's Disease and Parkinson's Disease (Smith et al., 2013; Xiong et al., 2018).

The secondary injury phase of mTBI leads to a multitude of cellular and molecular events including excitotoxicity, energy crisis, mitochondrial dysfunction, oxidative stress and neuroinflammation. While each metabolic disruption has been studied in isolation, it is important to recognize that they are not isolated events in response to an injury but rather collectively constitute the secondary injury that leads to neurological deficits after mTBI. Notably, mTBI pathophysiology has primarily been characterized in single hit animal models. However, recent studies show that time between injuries and number of injuries play a role in rmTBI pathophysiology (Fehily & Fitzgerald, 2017). Continuing investigation on how mTBI and rmTBI pathophysiology relate to symptoms and functional outcomes will help identify therapeutic opportunities.

1.5 Inflammatory Response

Neuroinflammation is part of the immediate response and secondary cascade; it may last for years after a primary injury is sustained (Wofford et al., 2017). This inflammatory response after injury is complex as it is triggered to promote neurological recovery, but excessive inflammation has been shown to lead to cognitive impairments and increased risk for developing neurodegen-

erative pathologies (Kumar & Loane, 2012). Because of this dual response, interventions that target neuroinflammation with anti-inflammatory agents may fail because the protective effects of inflammation are inhibited. Therefore, the timecourse of neuroinflammation and a thorough understanding of when a heightened response is beneficial to the injured brain and when inflammation is maladaptive is key to developing successful therapeutics (Xiong et al., 2018).

1.5.1 The Major Players in the Inflammatory Response

Neuroinflammation after TBI is primarily mediated by glial cells: astrocytes and microglia. As the main immune cells of the brain, microglia make up 10-12% of cells in the central nervous system (CNS), and actively survey their environment with ramified processes that respond to chemotactic signals (Nimmerjahn et al., 2005). Microglia are highly mobile (Nimmerjahn et al., 2005); they act as the first response to injury, migrating to the injury site to scavenge cellular and molecular debris from damaged cells (Donat et al., 2017). Initially, microglia exert their response in a neuroprotective capacity. Microglia of an M1-like phenotype produce pro-inflammatory cytokines, chemokines and iNOS (and unregulated can worsen the injury) while an M2-like phenotype release neurotrophic factors that promote repair and play a phagocytic role (See Figure 1.2). However, recent reports argue against an M1/M2 classification of microglia/macrophage phenotype and suggest a spectrum of macrophage/microglial polarization (Ransohoff, 2016). When microglia are in a prolonged state of activation, they become pathologically proinflammatory which has been shown to lead to worse functional outcomes in clinical and preclinical studies (Aungst et al., 2014; Johnson et al., 2013; Ramlackhansingh et al., 2011). Additionally, following an increase in cytokine production, microglia will target and recruit surrounding glia, neurons and peripheral immune cells like macrophages. These peripheral immune cells infiltrate the brain, as TBI can damage the BBB, contributing further to secondary damage. Macrophages can be difficult to distinguish from microglia as they both share similar markers. However, a common and widely used marker for microglia/macrophages

in TBI studies is ionized calcium binding adaptor molecule 1 (Iba1). Iba1 is involved in membrane ruffling, which plays a role in the morphological changes associated with macrophage/microglial functions including phagocytosis (Kanazawa et al., 2002).

Astrocytes are another important cell type in the inflammatory response and outnumber microglia, monocytes and lymphocytes in the brain. These glial cells support neuronal function and play a role in BBB maintenance (Acosta et al., 2013) Like microglia, astrocytes become activated after injury and increase production of cytokine and chemokines in addition to changing their morphology (more hypertrophic morphology with cell body swelling and extending processes). Astrocytes also play a key role in glial scar formation; glial scars act as a barrier, and encapsulate damaged tissue, preventing toxic molecules from affecting surrounding healthy tissue (Karve et al., 2016).

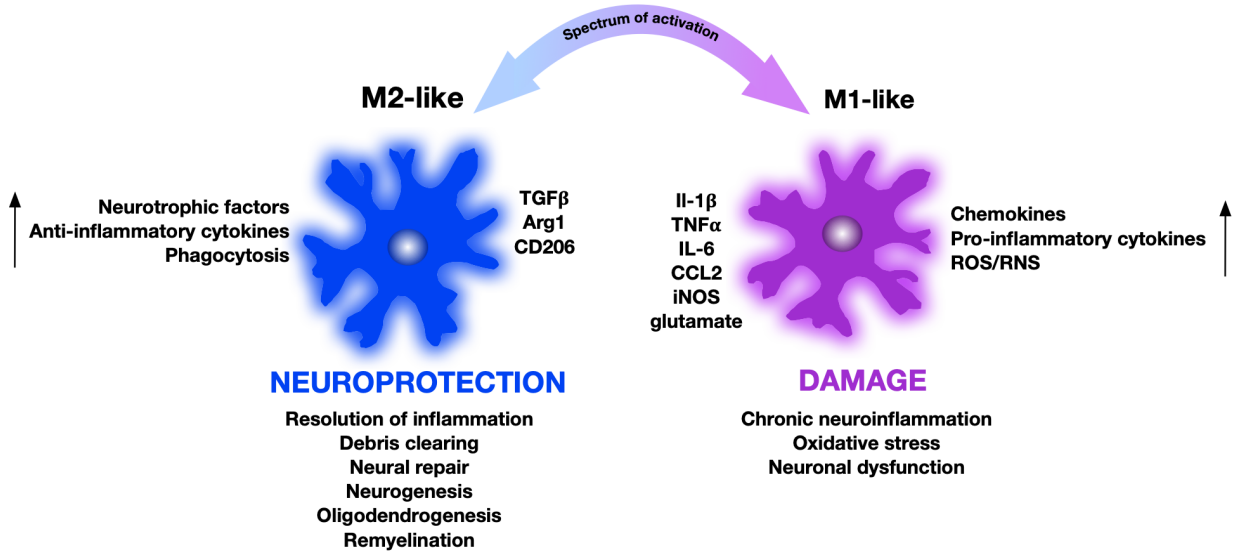


Figure 1.2: Pro and anti-inflammatory polarization of M1 and M2-like microglia/macrophages. Cytokines and chemokines produced by microglia can be pro-inflammatory or anti-inflammatory (pro-inflammatory IL-1β, IL-6, IL-17, TNFα or anti-inflammatory IL-4, IL-10, IL-13). These elevated markers have been measured in the clinical and preclinical populations (review on preclinical populations see Chiu et al 2016; clinical see Ziebell and Morganti-Kossmann 2010). Figure adapted from Loane and Kumar 2016.

1.5.2 Inflammation after rmTBI

Because the inflammatory response can be prolonged after injury, multiple head injuries can exacerbate the response before the brain has had time to heal. After a single TBI and once microglia are activated, they can become primed and have a lower threshold for activation (Witcher et al., 2015). In a rat model of repeated mild FPI (3 injuries 2 days apart), Aungst et al. (2014) found increased numbers of activated microglia in the ipsilateral and contralateral hippocampus 28 days after injury compared to shams and single mTBI animals. Furthermore, increased numbers of microglia have been associated with impaired spatial learning after rmTBI (Shitaka et al., 2011). Exacerbated neuroinflammation after rmTBI may provide an explanation for why multiple injuries can lead to increased risk for neurodegenerative diseases (Kumar & Loane, 2012).

1.5.3 Sex Differences in Inflammation

Studies have shown that microglia differ in number in female vs male rodents; males tend to have higher number of microglia in several regions of the brain including the hippocampus (Mouton et al., 2002). In control animals, save for some differences during development, there are no differences in cytokine expression between males and females (Crain et al., 2013). For a review on microglia sex differences during development, refer to Mosser et al. (2017) and McCarthy et al. (2015). Evidence for the influence of sex hormones on the microglia response after TBI is conflicting, and few studies have performed thorough investigations (see Caplan et al. (2017) for review; specifically, their Table II). Some report that sex hormones are important mediators of the microglial response to TBI (Barreto et al., 2014) while others do not find a protective or harmful role in sex hormones after TBI (Bruce-Keller et al., 2007).

There is evidence in the clinical and preclinical population that sex differences exist in the inflammatory response to TBI. In a mouse model of CCI injury, Villapol et al. (2017) found an in-

crease in Iba1$^+$ cells in the cortex, dentate gyrus (DG) and thalamus in male TBI animals compared to females at post-injury day (PID) 1 and PID 3. The morphology of Iba1$^+$ cells in male mice also showed more of a hypertrophy/bushy morphology at PID 1 and PID 3, with more amoeboid morphologies at PID 7 compared to female TBI mice who maintained more of the surveying ramified morphology. Furthermore, there were sex differences in the pro-inflammatory cytokine expression after TBI where females had higher levels of Il-1β and TNFα at 4 hours, but males had higher levels at PID 1 and PID 3. There were sex differences in anti-inflammatory cytokine expression as well, where males had increased TGFβ compared to shams at PID 1, but females did not. The astroglial response was also different in males and females, showing a rapid response in males but not in females in the cortex, DG and thalamus. Similarly, 55 days after injury, Yamakawa et al. (2017) found that there were increased numbers of Iba1$^+$ cells in the ventromedial hypothalamus of males but not females (compared to sex-matched shams) after 3 lateral closed-head impacts starting at PND 30 administered three days apart. Studies in stroke models have found similar sex differences; a lower inflammatory profile in microglia of female mice was present after stroke compared to males (Bodhankar et al., 2015). Taken together, these studies show that the inflammatory response to TBI by microglia and astrocytes is more robust in males than in females. It also provides further evidence that it is important to consider sex as a biological variable when studying TBI.

In summary, the inflammatory response is a major component of the brain's reaction to TBI. The acute response is necessary for recovery after injury, however prolonged inflammation and unregulated pro-inflammatory cytokines and chemokines can result in long term cognitive impairment, and even lead to neurodegenerative disease. Therapies targeting the inflammatory response must take into consideration the dual role of the key players, astrocytes and microglia, in order to control neuroinflammation while preserving the critical neuroprotective components of the response. Most studies of TBI have focused only on males. However, increasing evidence in the

clinical and preclinical literature that sex differences exists in the inflammatory response indicates a need to continue to include both sexes in studies to help understand which differences lead to improved outcomes in either sex.

1.6 Hippocampus

1.6.1 Anatomy and Circuitry

The hippocampus is a bilateral structure in the limbic system that plays an important role in learning and memory, and mood regulation. Its name was coined by Arantius in 1587, who saw the resemblance in shape of the hippocampus to a sea horse (hippocampus is derived from the Greek word for sea horse). The structure and subregions are maintained from human to rodent, though the human hippocampus is 100 times larger in volume than rats and some regions like the entorhinal cortex have more subdivisions. Additionally, in rodents the hippocampus stays dorsal and is more vertically oriented, while in humans the hippocampus gets displaced (due to more complex cerebral cortical development) into a ventral and more horizontal position in the temporal lobe (Figure 1.3).

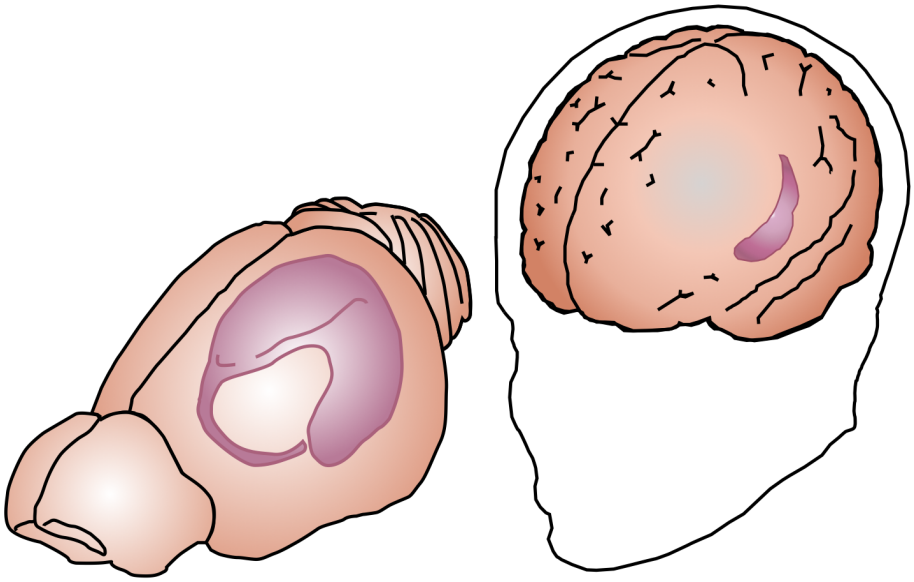


Figure 1.3: Position of hippocampus in the human and rat brain. The hippocampus is located in the medial temporal lobe. The rat hippocampus runs rostrocaudally and dorsoventrally in a vertical orientation. In humans the hippocampus runs anterior to posterior and sits in a more horizontal position.

A brief note on terminology: *hippocampus* refers to the cornu ammonis (CA) subregions of the *hippocampal formation* which is comprised of the DG, the subicular complex (subiculum, presubiculum and parasubiculum) and the entorhinal cortex (EC). The hippocampus is divided into three subregions: CA1, CA2 and CA3 and with the entorhinal cortex and DG make up the trisynaptic circuit (Figure 1.4). This circuit is unique in that it is largely a unidirectional passage of information: when information is projected from one region to another, it is not projected back as in other cortical areas of the brain.

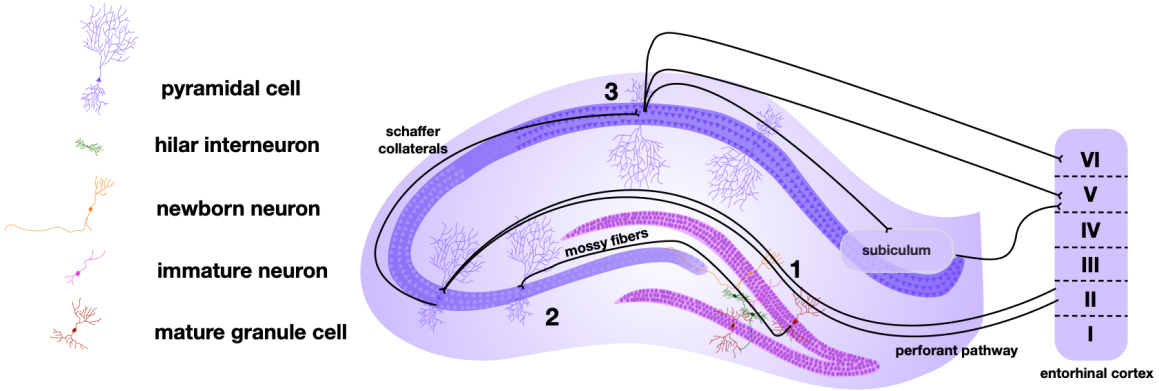


Figure 1.4: The trisynaptic circuit Axons projecting from layer II of the entorhinal cortex (EC) synapse on granule cells in the DG (synapse 1). Mossy fibers (axons projected from granule cells) project to the CA3 where they synapse on pyramidal cells (synapse 2). CA3 pyramidal cells then project to CA1 via schaffer collaterals (synapse 3) which subsequently project to the subiculum and EC.

The hippocampus can be divided along several axes. Numerous studies suggest that along the septotemporal axis, the hippocampus can be divided in dorsal and ventral regions, and subsequently different functions (Figure 1.5). Generally, the dorsal hippocampus plays a role in spatial learning and memory, while the ventral hippocampus is involved in anxiety and emotional behaviour (Bannerman et al., 2004; Kheirbek & Hen, 2011). Arguments have also been made for an intermediate region between the two with overlapping characteristics; dorsal, ventral and intermediate regions can be defined based on lesion studies, gene expression and neural connectivity (see Fanselow & Dong (2010) for review).

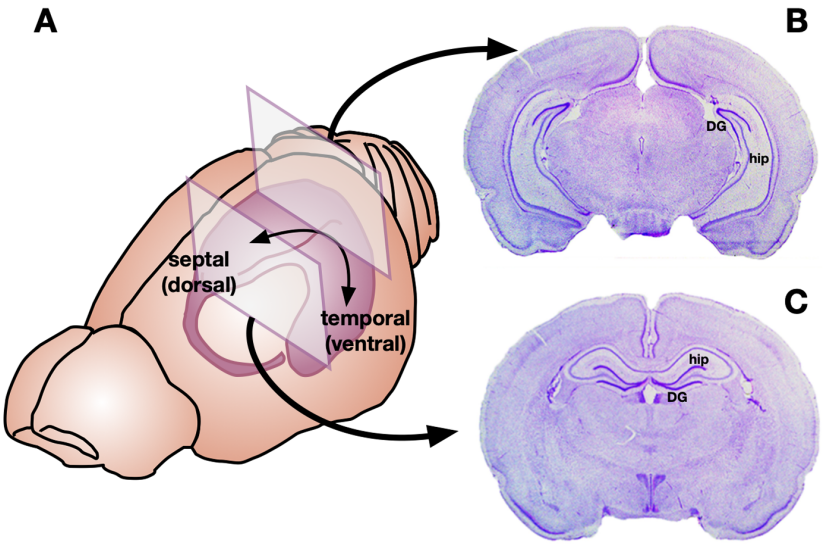


Figure 1.5: Septotemporal axis of the hippocampus. Function of the rat hippocampus differs along the septotemporal axis. A. Most commonly referred to as the dorsal (septal pole) and the ventral (temporal) hippocampus, the dorsal hippocampus plays a role in spatial learning and memory while the ventral hippocampus is involved in anxiety and emotional behaviour. B,C. Coronal sections of a nissl stain show the change in structure of the hippocampus along the septotemporal axis. Abbreviations: DG, dentate gyrus; hip, hippocampus.

1.6.2 The Dentate Gyrus (DG)

The DG is a subregion in the hippocampal formation that is comprised of three layers: the molecular layer, the granule cell layer (GCL) and the polymorphic layer (or the hilar region/hilus). Between the GCL and the hilus is the subgranular zone (SGZ), which is a unique microenvironment, also called the neurogenic niche, that contains neural stem cells, intermediate progenitors and immature neurons (Figure 1.6). Immature neurons migrate out of the neurogenic niche and differentiate and mature into granule cells (Aimone et al., 2014; Kempermann et al., 2015). This

process is known as adult neurogenesis and will be covered later in further detail.

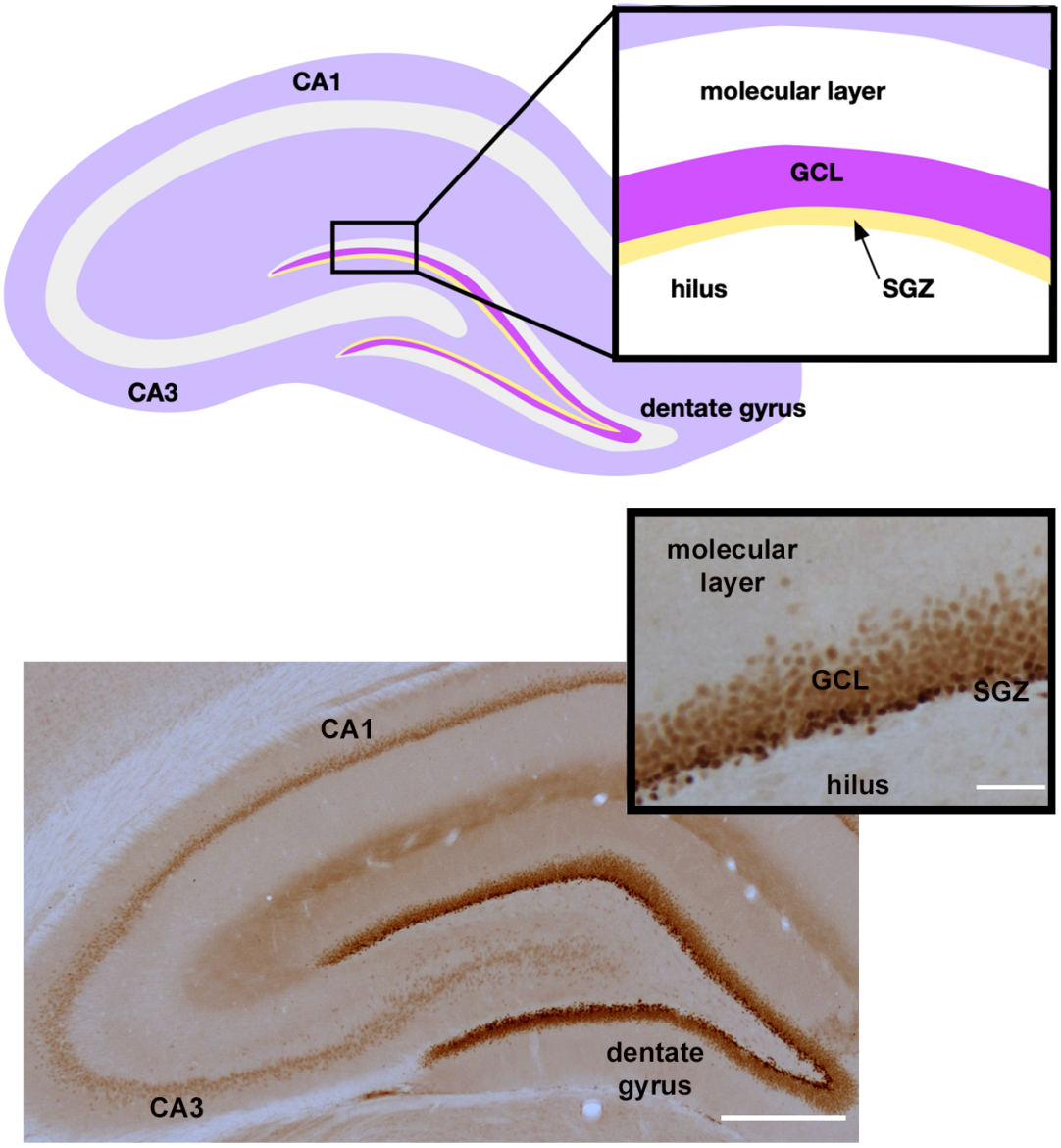


Figure 1.6: Subdivisions of the DG seen schematically and stained with NeuroD, a marker of immature neurons. The SGZ is located between the GCL and the hilus. As immature neurons have not all migrated into the GCL, the majority of NeuroD immunopositive cells are located in the SGZ. Scale bar is 100 μm, inlay scale bar is 50 μm. Abbreviations: SGZ, subgranular zone; CA, cornu ammonis; GCL, granule cell layer.

1.6.3 Hippocampal Vulnerability to TBI

The hippocampus plays an important role in learning, memory consolidation and mood regulation (see Andersen et al. (2006) for extensive review). Hippocampal lesion studies show impairment in episodic memories (patient H.M. is the most famous example; (Scoville & Milner, 1957)), fear and anxiety-related behaviour (Kjelstrup et al., 2002; Richmond et al., 1999) and spatial learning ((Moser et al., 1993); also see Bannerman et al. (2004) for review). Electrophysiological and behavioural studies looking at hippocampal synaptic plasticity have further proven the role of the hippocampus in learning and memory (Bannerman et al., 2004; Pinar et al., 2017). Importantly, neurogenesis, the development and integration of new neurons in the adult brain, has been shown to play a role in spatial learning and memory. Studies that use interventions like exercise to enhance neurogenesis have shown improved learning ability (VanPraag, 2005). Learning and memory deficits due to decreased neurogenesis have been shown in disease models including traumatic brain injury (Sun et al., 2015), fetal alcohol exposure (Gil-Mohapel et al., 2010) and depression (Song & Wang, 2011).

Because learning and memory deficits are commonly seen after TBI, it can be inferred that there is a role for the hippocampus in TBI pathology. Selective neuronal loss in the hippocampus and changes in hippocampal excitability as well as impairment in hippocampal synaptic plasticity have all been observed in various models of experimental TBI (Girgis et al., 2016). Loss of DG neurons, CA1, CA3 pyramidal neurons and selective death of newborn neurons in the hippocampus have all been reported (Gao et al., 2008; McCullers et al., 2002; Soares et al., 1995). Prins & David (1998) saw loss of hippocampal dentate hilar cell projections associated with memory dysfunction in adult, but not young, rats. Additionally, reduced dendritic complexity on newborn neurons in a mouse model of CCI (S. Ibrahim et al., 2016a) and decreased spine density and number of spine

branches on DG granule cells (Gao et al., 2011) may contribute to changes in neuronal excitability in the hippocampus and subsequent function. In a study using the ACHI model administering 8 repeated injures over 4 days to juvenile rats saw impairment one week after injury in spatial memory with the novel location recognition task (Pinar et al., 2020). These results indicate that across various injury models and ages, the hippocampus is particularly vulnerable to TBI.

1.7 Neurogenesis

Adult neurogenesis is the process by which new neurons are generated and incorporated into pre-existing networks in the adult brain. The phenomenon occurs primarily in two specific regions – the subventricular zone in the olfactory bulb and the SGZ of the DG in the hippocampus. Although there has been recent controversy on the extent to which human adult hippocampal neurogenesis occurs ((Lima & Gomes-Leal, 2019) for overview), it is widely appreciated that several species, including humans, undergo development of new neurons into adulthood (Toda & Gage, 2018).

Adult neurogenesis was first observed by Joseph Altman and Gopal Das in 1962 in rats (Altman, 1962). The animals were injected with thymidine-H3, a radiolabeled DNA precursor, which is incorporated into DNA during cell division thus labelling the nuclei of dividing cells (Das, 2008). Following lesion in the lateral geniculate body, they found radioactive labeling of glial cells around the lesion, but also neurons and neuroblasts in the thalamus and cortex. In a subsequent study, Altman looked to see if this phenomenon was present in non-lesioned rats (2 weeks after thymidine H3 injection). He observed glial proliferation in all parts of the brain and labelled granule cells in the DG of the hippocampus (Altman, 1963). In his 1965 study, Altman observed radiolabelled cells in rats aged 1-8 months primarily in the "basal region of the granular layers of the DG" (the SGZ). Since Altman's autoradiography studies in rodents, immunohistological techniques for studying neurogenesis have harnessed the ability to trace diving cells using nucleotide analogues

such as 5-bromo-2'-deoxyuridine (BrdU).

Neurogenesis in the hippocampus is a multi-step process (Figure 1.7). It spans from the proliferation of neural stem cells to their maturation into fully functional dentate granule cells. In the rat hippocampus, this process takes approximately 5 weeks. Radial glial cells (Type 1) residing in the SGZ, an approximately 40-50 μm band of cells between the GCL and the hilus, can divide asymmetrically to produce an amplifying progenitor cell (Type 2) and another radial glial cell. The progenitor cells will divide in a limited capacity and can differentiate into neurons or glia (Encinas et al., 2011). These type 2 progenitor cells give rise to type 3 neuroblasts which will eventually exit the mitotic stage and give rise to immature neurons which will no longer divide. Not all newborn cells will survive; in fact, most will be eliminated before becoming mature neurons (Dayer et al., 2003). Over the next few weeks, surviving cells will develop dendritic arborizations and axonal projections (towards CA3) and migrate from the SGZ into the GCL. As they continue to mature, they will integrate excitatory and inhibitory inputs and become fully functioning granule cells. Distinction of cell types involved in neurogenesis can be done using confocal microscopy to look for colocalization of specific markers, shown in Figure 1.7.

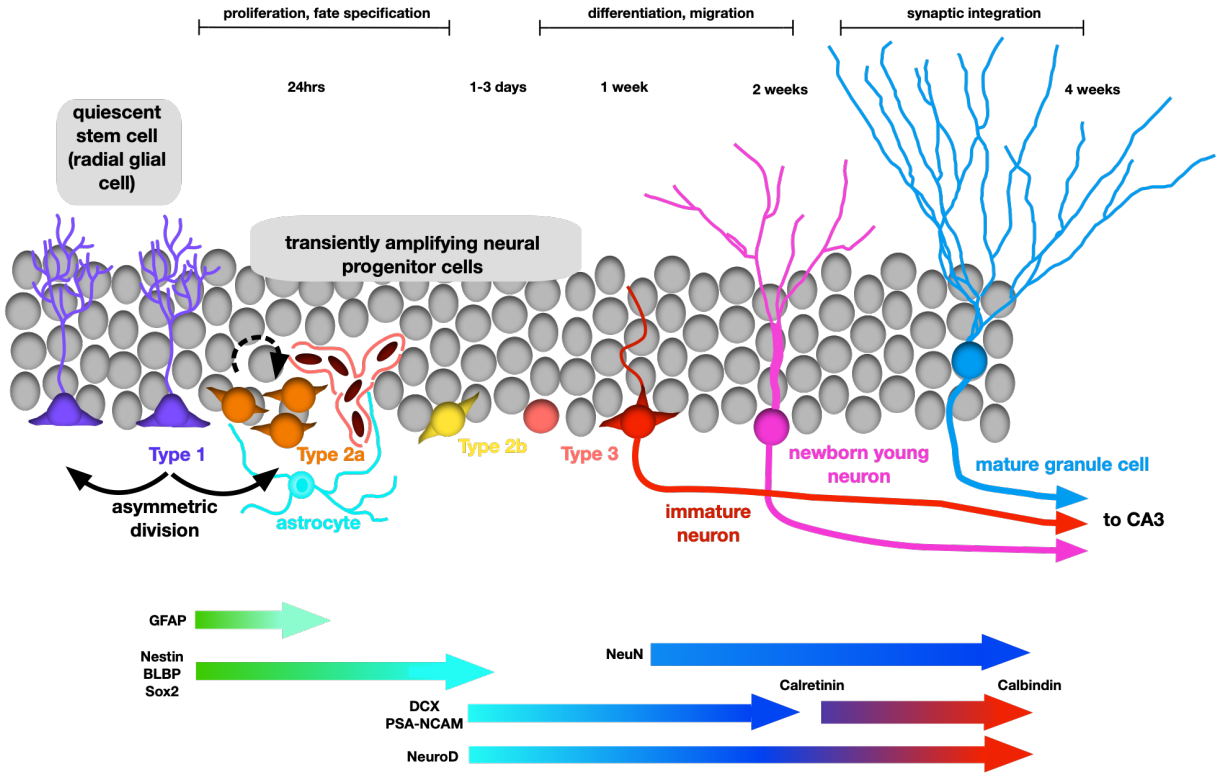


Figure 1.7: Cell proliferation and neurogenesis in the SGZ. Type I quiescent stem cells divide asymmetrically to produce more stem cells and amplifying progenitors (Type 2a). Amplifying neural progenitors proliferate and are increasingly determined to neuronal cell fate (Type 2b, Type 3). Progenitors continue to differentiate and enter a postmitotic period. Immature neurons begin to develop axonal projections and dendritic arborizations. Newborn granules cells continue to mature and become incorporated into pre-existing networks. Commonly used endogenous markers for cell types at each stage of neurogenesis are shown below. Adapted from Aimone et al 2014. Abbreviations: BLBP, brain lipid-binding protein; CA, cornu ammonis; DCX, doublecortin; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; PSA-NCAM, polysialylated-neural cell adhesion molecule; Sox2, sex determining region Y-box 2

1.7.1 Function of Adult Neurogenesis

In addition to exciting debates regarding the extent to which adult neurogenesis occurs in the human adult DG, the function of neurogenesis in humans is also widely debated (Aimone et al., 2010; Kempermann et al., 2018). In rodent studies, it is understood that interventions that increase neurogenesis like exercise or environmental enrichment improve spatial memory (VanPraag,

2005). Conversely, events like stress and aging that decrease neurogenesis have shown impairment in memory functions (Bettio et al., 2017; Lucassen et al., 2015). Furthermore, as a structure in the limbic system, the hippocampus plays a role in anxiety, stress response and social behaviour, and so neurogenesis may play a role in these functions as well (Cope & Gould, 2019). Still, the exact function and role played by newborn cells remains unclear. Immunohistochemistry studies using immediate early genes (IEGs, highly expressed in and a marker of neuronal activity) and birth-date markers like BrdU have shown that newborn neurons do not express IEGs until a certain point in their maturation (2 weeks in rats). Once mature, adult born neurons display greater IEG expression when animals are exposed to novel environments or subjected to spatial memory tasks (Deng et al., 2010; Kee et al., 2007). Behavioural studies are the main evidence that adult hippocampal neurogenesis is important in spatial learning and memory and dentate-specific working memory tasks involving pattern separation ((Aimone et al., 2014; Deng et al., 2010) for reviews). Pattern separation is the integration of new, similar, inputs into pre-existing networks in such a way that the output successfully encodes differences. This phenomenon has been studied in clinical and preclinical settings. In a human fMRI study, an incidental encoding task, in this case identifying whether a picture is novel or repeated, has been shown to elicit and increase in brain activity in the CA3/DG when slightly difference versions of a picture were shown (Bakker et al., 2008). A spatial separation radial arm maze task requires subjects to distinguish novel arm to collect a reward when given a choice of two arms; one familiar and one novel. C. D. Clelland et al. (2009) found that when neurogenesis was ablated with focal x-ray irradiation, mice had a more difficult time choosing the correct arm when the separation between correct and incorrect arms was small. Because similar studies involving manipulations to neurogenesis cannot be informed in humans, preclinical models have been crucial in understanding the role of neurogenesis in pattern separation and spatial learning and memory (see Gonçalves et al. (2016) for review). And while the exact

function of adult-born neurons is still under investigation, changes to any part of the neurogenic process may result in altered cognitive functioning.

1.7.2 Sex Differences in Cell Proliferation and Neurogenesis

Sex differences occur in the hippocampus in terms of structure and function and are not constricted to, or explained solely by, sex steroid hormone fluctuations. For example, dendritic morphology and dendritic spines (see Sheppard et al. (2019), their Table 1), neurogenesis and synaptic plasticity have all been shown to contribute to hippocampal sex differences and differences in spatial learning and memory (Triviño-Paredes et al., 2016). Sex differences related to the hippocampus have been well reviewed in Koss & Frick (2017), Choleris et al. (2018) and Sheppard et al. (2019).

Behaviour

There is evidence for sex differences in hippocampal-related spatial learning and memory and tasks that involve adult-born neurons. For example, Chow et al. (2013) showed an increase in cell survival in males but not females following spatial training; male animals outperformed females during the spatial probe trials in the Morris Water Maze (MWM). Yagi et al. (2016) also found increased neurogenesis in males that employed spatial strategies in a pattern separation task, and better performance than females when separating similar patterns. Taken together, these studies confirm reports that males generally outperform females on spatial learning and memory tasks and is found to be related to differences in neurogenesis (Epp et al., 2013).

Cell Numbers

Interestingly, sex differences in rodents may vary in an age-dependent manner. Siddiqui & Romeo (2019) found an increase in the number of Ki-67^{+} cells (proliferating cells) in the DG of male rats compared to females at PND 30, but not at PND 45 or PND 70. However, Perfilieva et

al. (2001) did find a male-dependent increase in proliferating cells 1 day after BrdU injections in adult rats. This may highlight the importance of combining birth-dating and endogenous proliferation techniques. Although behavioural and/or hormonal modulation of neurogenesis results in sex differences, there have been mixed reports as to whether baseline sex differences in neurogenesis exist. In the study above (Yagi et al., 2016), sex differences were apparent only with specific behavioural tasks. Siddiqui & Romeo (2019) saw an increase in immature neurons (DCX^+ cells) in adult females compared to males; cell survival studies have also shown an increase in females compared to males (Perfilieva et al., 2001).

Sex Hormones

Sex hormones in both males and females have been shown to influence cell proliferation and neurogenesis (see Triviño-Paredes et al. (2016) for review). In males, Spritzer et al. (2007) saw a decrease in cell survival after castration, and an increase following injection with testosterone. Differences in cell proliferation, however, were not observed. In female rodents, the 4-5 day estrous cycle is marked by peak level of estradiol and progesterone during proestrus. During proestrus, several studies have observed higher levels of cell proliferation and increased survival of newborn neurons (Mahmoud et al., 2016). Furthermore, ovariectomy reduces cell proliferation acutely, though the brain can recover to normal levels within a few weeks (Tanapat et al., 1999, 2005). This brings to light an important consideration which is sex steroids estradiol and testosterone, in addition to being secreted from the gonads, can also be synthesized in the brain: estradiol can be synthesized from cholesterol or converted from androgens by aromatase (Hojo et al., 2004).

1.8 Cell Proliferation

1.8.1 Cell Types

Neural stem/progenitor cells reside in the SGZ of the DG. They form the population of cells that have the capacity to divide and mature into adult-born granule cells; without an adequate pool of neural stem/progenitor cells, neurogenesis can be impaired (Encinas et al., 2011; Gonçalves et al., 2016). Division patterns (symmetric vs asymmetric) as well as cell protein markers and morphology allow classification of these neural stem/progenitor cells as quiescent or amplifying. Quiescent neural stem cells are also known as radial glial cells, or Type 1 cells. As the name implies, they are normally quiescent; only a small fraction of them are usually labelled with a marker of cell division like BrdU (Cameron & Mckay, 2001; Encinas et al., 2006; Gao et al., 2009). They express nestin, glial fibrillary acidic protein (GFAP), vimentin and brain lipid binding protein (BLBP). They have an apical process that extends into the GCL, ending in the molecular layer. Radial glial cells divide asymmetrically; division produces another radial glial-like cell and small round/oval cells. These small round cells no longer express GFAP or vimentin and divide at a much higher frequency. These are the amplifying neural progenitor (ANPs), which can be further classified as Type 2a, 2b and Type 3 cells depending on protein markers they express (see Figure 1.7). Because neural stem/progenitor cells have the capacity for self renewal, they have been investigated for their potential to repair the damaged hippocampus after TBI.

1.8.2 Regulation of Neurogenesis

The SGZ is one of two neurogenic niches in the brain; a microenvironment that supports differentiation of neural progenitor cells (NPCs) into neurons. While NPCs exist in other areas of the brain, it is the unique environment in the SGZ that promotes differentiation into granule cells; NPCs that are transplanted into the DG from non-neurogenic regions gain the ability to differentiate

into neurons (Shihabuddin et al., 2000). Growth factors, neurotrophic factors, neurotransmitters and cytokines make up this unique environment (for review, see Gonçalves et al. (2016)). Astrocytes and microglia are a key component of the neurogenic niche. While anti-inflammatory cytokines released by these cells can enhance neurogenesis (Barkho et al., 2006; Battista et al., 2006), their secretion of proinflammatory cytokines can inhibit differentiation of NSCs into neurons (Ekdahl et al., 2003; Monje et al., 2003). Microglia also phagocytose NPCs that do not survive past their first week (Sierra et al., 2010). Considering the involvement of inflammation in secondary damage following TBI, regulation of cell proliferation and neurogenesis by astrocytes and microglia in the neurogenic niche may be altered after injury.

1.8.3 Quantifying Cell Proliferation - BrdU

One of the most widely used techniques in studying cell proliferation and neurogenesis is the use of 5-bromo-2'-deoxyuridine (BrdU). BrdU is a thymidine analogue and is incorporated into DNA when cells are replicating (during the S phase of the cell cycle). BrdU provided the first evidence of neurogenesis in humans (Eriksson et al., 1998) and has since become a widely used technique in preclinical cell proliferation and neurogenesis studies (Kee et al., 2002). The bioavailability of BrdU is 2 hours and the average cell cycle in rats is 24 hours, approximately 9 hours of which is the S phase (Cameron & Mckay, 2001). If BrdU has been taken up by a cell during the S phase, its progeny will also have BrdU incorporated in its DNA. Therefore, the population of cells that is $BrdU^+$ is highly dependent on the number of injections, the time between injections, and the dose; many paradigms exist for the use of BrdU and each paradigm will answer different questions. For example, a single dose of BrdU injected at least 2 hours prior to perfusion will answer questions about cell proliferation. With that same injection paradigm, an animal perfused 1 month later will answer questions about cell survival: how many of the cells that incorporated BrdU were able to survive and become mature granule cells? Because BrdU is only bioavailable for

2 hours, many studies will give multiple injections of BrdU and assess proliferation or cell survival on a larger (and therefore less temporally resolved) population of cells. While this makes it a very versatile technique, it is important to note that when comparing studies answering questions about proliferation and/or neurogenesis, one must take into consideration the injection paradigm the study has used. Additional endogenous markers are often used in conjunction with BrdU, including Ki-67 to identify proliferating cells in not only the S-phase of the cell cycle, but all other active stages of division (Figure 1.8).

Cell proliferation is an important part of the neurogenic process. It is regulated by many factors including cytokines released by microglia and astrocytes and as such is vulnerable to changes after mTBI. It is possible to investigate specific temporal windows of cellular proliferation using different BrdU injection paradigms and further identify progenitor and glial cell type with colocalization of other cell-type specific markers.

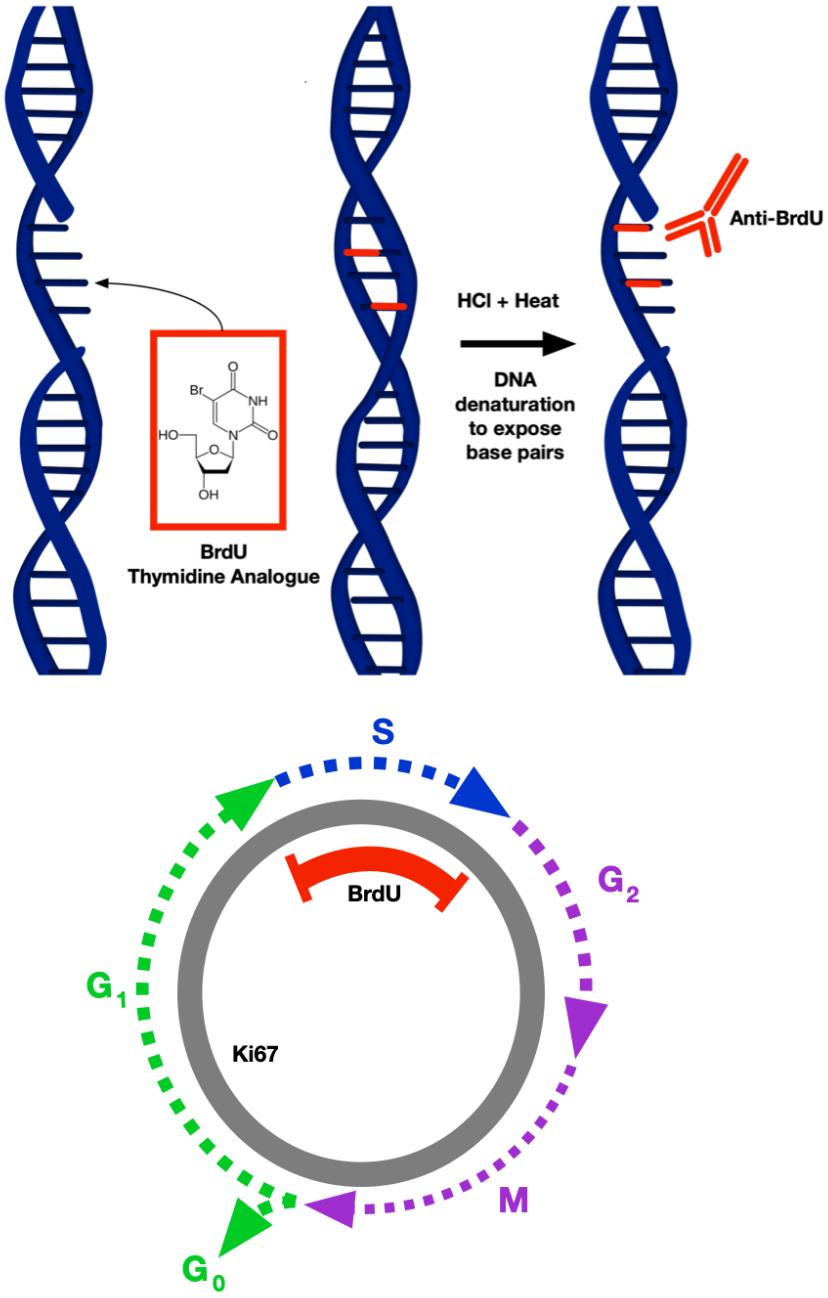


Figure 1.8: Incorporation of BrdU into DNA; BrdU and Ki-67 in the cell cycle. BrdU is a thymidine analogue and gets incorporated into the DNA of dividing cells. For immunohistochemistry, double stranded DNA must be denatured for antibodies to access incorporated BrdU. BrdU and endogenously expressed Ki-67 are both commonly used markers of cell proliferation; BrdU labels cells undergoing DNA synthesis, while Ki-67 labels actively dividing cells in all phases of the cell cycle except the resting phase, G0.

1.9 Cell Proliferation and Neurogenesis after TBI

Following TBI, all stages of neurogenesis can be affected. It has been shown to result in an increase in proliferation, though the survival of these adult born neurons after injury has been mixed. Studies have shown increases, decreases and no change in neurogenesis (Yu et al., 2016). An important consideration in addressing the outcome of neurogenesis after mTBI is the heterogeneity in the injury produced.

Most studies have found an increase in cell proliferation after TBI or mTBI. One study investigated whether QNPs of ANPs were responsible to the increase in proliferation and found that while ANPs were not affected by TBI, QNPs were being induced to enter the cell cycle. Other studies have shown that while there is an increase in proliferation after TBI in the DG, proliferation in TBI animals is not restricted to the SGZ (ie. the neurogenic niche where one would expect to find dividing neural stem/progenitor cells). Instead, proliferation occurs diffusely throughout the SGZ, GCL and molecular layer (Barha et al., 2011; Gao et al., 2009; Gao & Chen, 2013). While some studies have found that increased cell proliferation is associated with impairments in cognitive tasks, Sun et al. (2015) found that inhibiting cell proliferation impaired cognitive recovery in the MWM task. In animal models that show a decrease in neurogenesis after experimental TBI, there is also impairment in learning and memory tasks (Yu et al., 2016).

Neural stem cells have the potential to differentiate into the three basic cell types that exist in the CNS: neurons, oligodendrocytes and astrocytes. Glial cells may also be induced to divide in response to injury. Indeed, numerous studies have investigated whether injury-induced cell proliferation is due to proliferating neural stem/progenitor cells or astrocytes, microglia, oligodendrocytes or endothelial cells. Using co-labeling immunofluorescence, most of these studies have found that proliferating cells outside of the SGZ are astrocytes or microglia (See Appendix Table 5.3).

Several studies in preclinical models have investigated the effects of therapeutic inhibition of injury-induced proliferation. For example, Zhang et al. (2014) found that enhancing proliferation and differentiation of NPCs in the DG by downregulation of Hes1, a downstream target of Notch signaling and a regulator of neurogenesis, resulted in an increase in differentiation of NPCs and improvement on the MWM. Kleindienst et al. (2005) looked at the effects of injection of S100B, which increased the proliferative response in the DG of TBI animals at 5 days after injury. They found an increase in the number of $BrdU^+$ cells that differentiated into neurons rather than glia and improved cognitive function (assessed in MWM on PID 30-34) in TBI animals that had been injected with S100B. This provides evidence that increased numbers of functioning newly generated neurons after injury can improve learning and memory after TBI; injury-induced neurogenesis may result in increased numbers of functionally integrated new neurons. For further review on therapies targeting endogenous cell proliferation see Patel & Sun (2016).

With the exception of Slemmer (2002), who studied repeated mild injury in cell culture, there is no existing evidence for acute hippocampal proliferation after rmTBI in animal models, and there is a paucity of data examining sex differences in hippocampal cell proliferation in experimental models of all injury severity.

1.10 Summary and objectives

The cellular cascades after mTBI contribute to secondary damage such as neuroinflammation. The hippocampus is a vulnerable region to injury as evidenced by learning and memory impairment in the clinical population as well as various preclinical models. While single hit experimental TBI models conclusively demonstrate an increase in proliferation, there is still a paucity of data on injury-induced cell proliferation after rmTBI in the juvenile rat (Yu et al., 2016). The juvenile rat brain undergoes important neurodevelopmental and organizational processes and while there is

some evidence for an increased capacity to recover after injury, the young brain may be especially sensitive to secondary damage after insult (Semple et al., 2016). Importantly, most of these single-TBI models administer open-head injuries while subjects are under anesthesia which may exert confounding neuroprotective effects. To date, there are no studies on injury-induced cell proliferation that investigates sex differences at a juvenile age. Thus, this work seeks to determine whether there are sex differences in the acute proliferative response in the neurogenic niche following rmTBI. To answer this question, a model of awake closed head injury was used to induce rmTBI in male and female adolescent rats. The objectives and hypotheses are outlined below:

Objective 1: To investigate acute neurological impairment and structural damage in male and female adolescent rats after rmTBI.

Hypothesis: It is hypothesized that rmTBI will result in acute neurological impairment in both sexes, but without overt structural damage to the hippocampus.

Objective 2: To investigate whether rmTBI results in cellular proliferation in the hippocampus and the extent to which it may differ between sexes. To determine whether the magnitude of the response differs between immediate and acute timepoints.

Hypothesis: It is hypothesized that rmTBI will cause an increase in cellular proliferation in the DG and it may be greater in males than in females, and more robust in both sexes at an acute rather than immediate timepoint.

Objective 3: To characterize the cell type involved in the proliferative response.

Hypothesis: It is hypothesized that the proliferative response after rmTBI will involve a combination of amplifying progenitors and glial cells and that this response may be more robust in males than females.

CHAPTER **2**

Materials and Methods

2.1 Animals

56 Long Evans rats were used in this study (28 females, 28 males). Dams with litters of 10-12 pups aged post natal day (PND) 13-15 were purchased from Charles River Laboratories (St. Constant, PQ) and given one week to acclimatize prior to being weaned at PND 21. Subjects were housed in standard cages and given *ad libitum* access to food and water. Room temperature was maintained at 22.5°C ± 2.5°C with a 12 hour light/dark cycle. Animals were randomly assigned to either sham or repeat TBI groups and kept with 2-3 sex-matched and condition-matched littermates. All awake closed head injuries were administered on PND 28 and body weights were recorded before baseline testing. All animal procedures performed in this study were approved by the University of Victoria Animal Care Committee and are in compliance Canadian Council on Animal Care (CCAC) standards.

2.2 Awake Closed Head Injury

2.2.1 Injury Model

Animals were subjected to repeated mild awake closed head injuries (ACHI) according to the protocol described in detail by Christie et al. (2019). Briefly, animals were impacted using a modified Leica Impact One controlled cortical impactor (Impact One, Leica Biosystems Inc., ON, Canada) at a velocity of 6.0 m/s (injury depth 10 mm, dwell time 10 ms) on their left parietal cortex (Figure 2.1). To ensure accuracy of the injury site and to diffuse the force of the impact, a 3D printed plastic helmet with a 7 mm target location was placed on the animal's head. As each

impact was administered without the use of anaesthesia, all animals were restrained using a rodent restraint bag with an opening at the front to allow for air flow (Model DC-200, Braintree Scientific, Braintree, MA). Each animal was given a restraint score to indicate how well they were habituated to the restraint bag. Sham animals were subjected to the same procedure but were moved out of the path of impact so they would hear the piston without sustaining an injury.

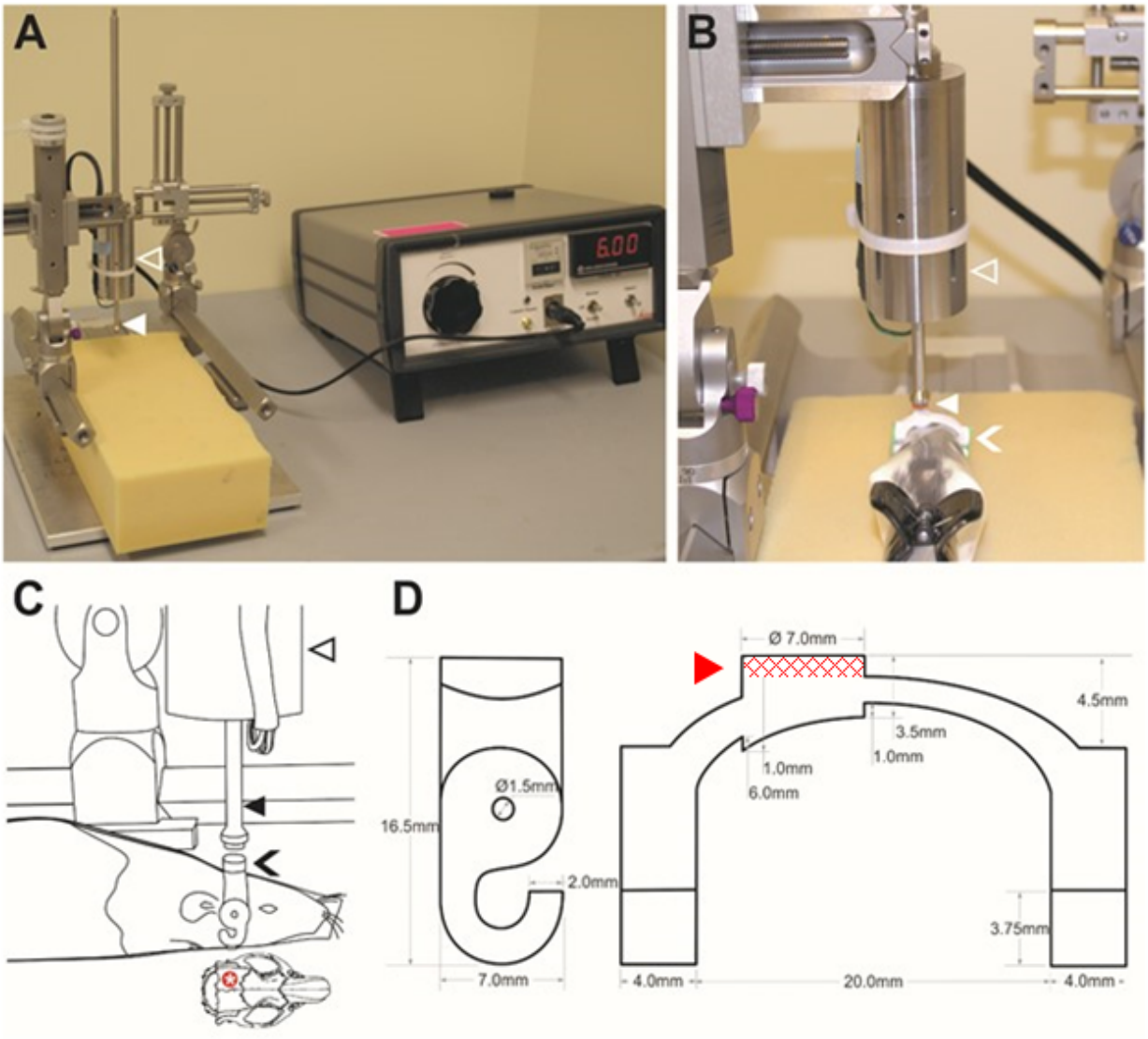


Figure 2.1: Awake closed head injury (ACHI) model to induce rmTBI in juvenile rats A. A modified Leica Impact One controlled cortical impactor with an electromagnetic piston (open arrowhead) and rubber tip (7 mm diameter, solid arrowhead) is used to deliver an impact at velocity of 6 m/s and dwell time of 10 ms. **B.** Animals are restrained and placed on a soft foam platform with plastic 3D printed helmet (arrow) placed over the restraint bag and positioned so the 7 mm target site (red arrowhead) is aligned over the left parietal cortex **(C, D).** Used with permission from Christie et al., 2019.

2.2.2 Injury Paradigm

Animals received 8 ACHIs in 1 day, with 2 hours between each impact. Before the first ACHI or sham procedure, animals were weighed, and a baseline NAP score was recorded. Animals were also weighed prior to perfusion on PID 1 or PID 3. The first 5 impacts were administered during the light cycle while the last 3 impacts were administered during the dark cycle (see Figure 2.2). All injuries were administered between the hours of 7:30 am and 11:30 pm.

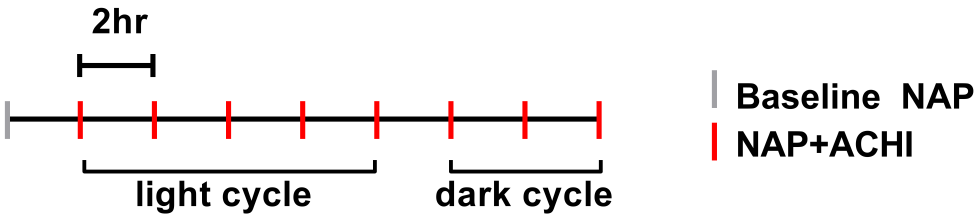
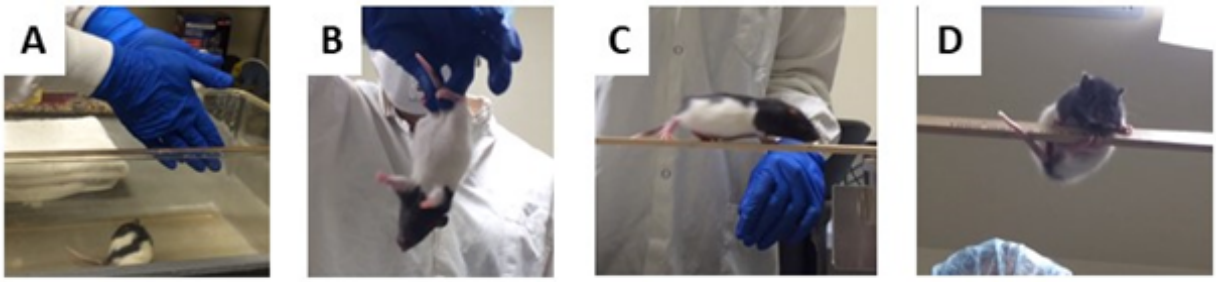


Figure 2.2: Timeline of awake closed head injuries and neurological assessment protocol (NAP) testing.

2.2.3 Neurologic Assessment Protocol

Diagnosis of TBI and assessment of injury severity is primarily tested in the clinical population by the Glasgow Coma Scale (GCS), where higher scores are indicative of milder injuries. In preclinical models, neurological severity scores (NSS) are used to evaluate immediate neurological deficits after impact (Xiong et al., 2013). There is currently no standardized NSS test used in animals models, so our lab has developed a neurological assessment protocol (NAP) to evaluate level of consciousness, basic reflexes and sensorimotor function in our preclinical model (Christie et al., 2019). After every ACHI, animals were immediately assessed with the NAP. The presence or absence of apnea (cessation of breathing) was recorded, followed by toe pinch reflex and righting reflexes to assess level of consciousness. If apnea was observed, latency to breath recovery was recorded. Toe pinch was assessed by the animal's latency to retract their contralateral hind limb

after it was extended and pinched. Righting reflexes were tested by placing subjects on their back and recording latency to right themselves. Next, the animals underwent four simple reflex and sensorimotor tasks: startle reflex, limb extension, beam walk and rotating beam (Figure 2.3). All four tests were scored on a scale from 0-3 with 0 being the lowest score (greatest impairment) and 3 being the highest score (no impairment). The scoring for each test is detailed in Figure 2.3. All tests are described in detail in (Christie et al., 2019). Briefly, animals were first placed in a clean cage facing away from the observer who then clapped both hands above the animal's head. The response to the observer's clap was assessed. Animals were then picked up by the base of their tail and lowered slowly towards a metal beam to test limb extension. Their ability to extend both limbs towards the metal beam was recorded. Animals were lowered onto the metal beam (100 cm x 2 cm x 0.75 cm) and their balance and ability to cross the beam was assessed. Lastly, animals were placed to the centre of the metal beam which was then raised by the observer to approximately 50 cm above the cushioned surface. Their ability to stay on the beam as it was rotated at a speed of 1 rotation per second for a total of 4 rotations was observed. Animals were monitored in their home cages after each ACHI to identify any abnormal behaviour such as isolation from cage mates or laboured breathing. Animals were also observed for indications of abnormal behaviour prior to every ACHI. No animals were removed from this study due to indications of abnormal behaviour.



Task	0	1	2	3
Startle Response	No reaction to clapping sound	Only ears react to clapping sound	Slow reaction or slight freezing reaction to clapping sound	Ears react quickly and whole body jumps and freezes
Limb Extension	Complete absence of limb extension; limp body	Intermittent retraction/extension of limbs	Only one limb properly extends	Full extension of both paws, animals grasps beam successfully
Beam Walk	Complete absence of movement with all limbs hanging off beam	Non-locomotive movement ("swimming" or "rowing" motion of limbs without movement across the beam)	Animal walks on beam, but more than 2 limb slips are observed	Animal walks across beam to home cage with 2 or fewer foot slips
Rotating Beam	Animals falls during the 1st rotation	Animal falls during 2nd or 3rd rotation	Animal falls during 4th rotation	Animal successfully completes 4 rotations

Figure 2.3: Photographs of each task in the NAP A. Startle response B. Limb extension C. Beam walk D. Rotating Beam. D. Detailed scoring rubric used for NAP to assess basic reflexes and sensorimotor function after mTBI. Startle response, limb extension, beam walk and rotating beam were all scored on a scale of 0 to 3, with 0 being the lowest score and 3 being the highest. Scores for all tests were combined to give a total NAP score out of 12. Adapted with permission from Christie et al 2019.

2.3 Tissue Preparation

2.3.1 BrdU Injections

Animals were given single intraperitoneal injections of bromodeoxyuridine (BrdU; 200 mg/kg), a thymidine analogue, 2 hours prior to perfusion on PID 1 or PID 3. Stock solution of BrdU was prepared in 0.1 M phosphate buffered saline (PBS) at 20 mg/ml. On PID 1, injections were administered approximately 12 hours after the final injury (Figure 2.4).

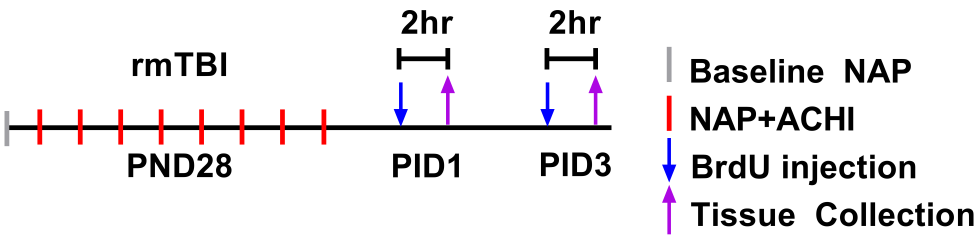


Figure 2.4: Timeline of BrdU injections and tissue collection following ACHI and NAP.

2.3.2 Perfusions and Brain Extraction

Animals were administered an overdose of isoflurane (>5%, inhalant) and, once unresponsive to toe pinch, were transcardially perfused using a gravity system 2 hours after receiving BrdU injections on PID 1 or PID 3. Following perfusion with 0.1 M PBS, animals were fixed with 4% PFA made from a 32% stock (Electron Microscopy Science 100504-858). Brains were carefully extracted and placed in 4% PFA solution overnight prior to being cryoprotected in 30% sucrose solution (prepared in 0.1 M PBS). Once fully saturated with sucrose, brains were kept in 0.1 M PBS until they were sliced.

2.3.3 Tissue Collection and Storage

Brains were sliced using a Leica vibratome (Leica Biosystems 31 Inc, ON, Canada). 50 µm sections were collected beginning at bregma -2.28 mm until bregma -6.48 mm (Paxinos & Watson, 2006). For all immunohistochemistry experiments, dorsal hippocampus was designated as bregma -2.28 mm to -4.68 mm and the ventral hippocampus as -4.8 mm to -6.48 mm. Sections were placed sequentially in a 96 well plate containing Walter's antifreeze solution and kept in the freezer until use.

2.4 Immunohistochemistry and Histology

2.4.1 Cresyl Violet Staining

Representative dorsal and ventral slices were chosen for cresyl staining to examine structural damage after ACHI. Slices were mounted onto Superfrost Plus slides and dried at room temperature prior to staining. Sections were rinsed in ethanol of decreasing concentrations (100% 5 min, 95% 1 min, 70% 1 min) then stained with 0.5% cresyl violet (pH 6). Sections were then rinsed in distilled water and de-stained in 1% acetic acid solution before dehydration in ethanol. Sections were cleared in xylene then coverslipped with permount and left to dry overnight prior to imaging.

2.4.2 3,3' - Diaminobenzidine tetrahydrochloride (DAB) Immunohistochemistry

Cell proliferation after rmTBI was assessed at PID 1 and PID 3 using BrdU and an endogenous marker of proliferation, Ki-67. For both Ki-67 and BrdU staining, a 1 in 6 series of coronal slices containing dorsal and ventral dentate gyrus was used. For BrdU staining, slices were rinsed 3 times in 0.1 M Tris Buffered Saline (TBS), quenched in 0.6% H_2O_2 for 30 minutes then rinsed again in TBS. DNA was denatured by incubation in 10 mM sodium citrate and 50% formamide at 65°C for 1 hour, rinsed briefly in sodium citrate only, then incubated for 30 minutes in 2 N Hydrochloric acid (HCl) at 37°C. Following denaturation, sections were neutralized in 0.1 M borate buffer, rinsed in 0.1 M TBS and preincubated in blocking buffer (see Table 2.1) for 1 hour at room temperature. They were then incubated overnight in primary antibody at 4°C (details in Table 2.1). Following washes in blocking buffer and then TBS, sections were incubated in biotinylated secondary antibody for 2 hours. With washes between each step, sections were incubated in an avidin-biotin peroxidase complex (Vectastain ABC Elite) which was detected using DAB with nickel (Vector laboratories Burlingame, CA). Sections were then mounted on Superfrost Plus slides, dehydrated in increasing concentrations of ethanol, and coverslipped with paramount. For Ki-67 staining, all steps were the

same with a few exceptions. Antigen retrieval was done in 10 mM sodium citrate at 85°C for 30 minutes and the quenching was performed prior to secondary incubation on day 2 in 3% H_2O_2 for 15 minutes.

Table 2.1: Source, concentration and protocol details for primary and secondary antibodies used to assess cellular proliferation after rmTBI.

Primary Antibody	Company	CAT#	Concentration Used (%Serum)	Time in Primary	Secondary (Source)
Mouse anti-BrdU	Millipore Sigma	MAB3424	1:4000 (3%)	16h	Goat anti Mouse (Millipore Sigma B6649)
Rabbit anti-Ki67	Abcam	Ab15580	1:1000 (5%)	48h	Goat anti Rabbit (Millipore Sigma B8895)

2.4.3 Immunofluorescence Triple Staining

To determine whether the $BrdU^+$ cells were glial cells, sections from n = 3 animals/group were triple stained for BrdU; ionized calcium binding adaptor molecule (Iba1), expressed by macrophages and microglia; and glial fibrillary acidic protein (GFAP), a type III intermediate filament protein expressed by astrocytes. A 1 in 12 series of tissue sections including dorsal and ventral hippocampus were stained. DNA denaturation followed the same protocol as for DAB staining, however washes were done in 1X PBS. Following denaturation, sections were blocked in 3% bovine serum albumin (BSA) with 0.25% Triton X-100 in 1X PBS for 1 hour at room temperature. They were then incubated overnight in primary antibody at 4°C (details in Table 2.2). Following washes in PBS and blocking solution, sections were incubated first in the biotinylated goat anti-sheep antibody for 2 hours, then in fluorophore secondaries for 2 hours at room temperature. Sections were rinsed

and mounted in 60% 2,2'- thiodiethanol (TDE).

Table 2.2: Source, concentration and protocol details for primary and secondary antibodies used in triple labeling experiment to assess cell type after rmTBI.

Primary Antibody	Company	CAT#	Concentration Used (%Serum)	Time in Primary	Secondary (Source)
Sheep anti-BrdU	Abcam	Ab1893	1:1000	16h	Goat anti Sheep Biotin (Invitrogen A24565) Streptavidin Alexa Fluor 488 conjugate (ThermoFisher S11223)
Rabbit anti-Iba1	Wako	019-19741	1:1000	16h	Donkey anti Rabbit, Alexa Fluor 568 (ThermoFisher A10042)
Mouse anti-GFAP	Millipore Sigma	G3893	1:1000	16h	Donkey anti Mouse Alexa Fluor 647 (Millipore Sigma, AP192SA6)

2.5 Cell Counting and Quantification

2.5.1 Profile Counting in the SGZ

$BrdU^+$ and $Ki\text{-}67^+$ cells were quantified using exhaustive profile counting using an Olympus brightfield CX21 microscope (Olympus Corporation, Center Valley, PA, USA) and a 100X oil immersion lens. Only cells in the SGZ (within 2-3 cell diameters of the granule cell layer; approximately 50 µm) were counted and the counter was blind to animal group, PID and sex. Because $Ki\text{-}67^+$ and $BrdU^+$ cells tend to be distributed heterogeneously and often in clusters, extra care was taken to distinguish clear cell bodies when cells were overlapping. The average of all slices

was calculated and multiplied by the estimated number of 50 µm dorsal or ventral DG sections (48 slices to represent total cells per dorsal DG, 33.6 slices to represent total cells per ventral DG). Cell counts are presented as mean $BrdU^+$ or $Ki\text{-}67^+$ cells per entire dorsal or ventral DG ± SEM. Representative images were taken using an Olympus brightfield BX51TF microscope (MBF Bioscience, Williston, VT, USA) and StereoInvestigator software version 11.03 (MBF Bioscience, Williston, VT, USA) using 10X, 40X and 100X objectives.

2.5.2 Qualitative/threshold assessment of BrdU the hippocampus

To quantify $BrdU^+$ cells in the whole hippocampus, a custom macro in FIJI-Image J (Version 1.52p, National Institutes of Health, USA) was used to threshold images and count particles. The macro was applied to images that were taken on an Olympus brightfield BX51TF microscope with a 10X objective. Images of whole tissue sections were compiled using StereoInvestigator software version 11.03 (MBF Bioscience, Williston, VT, USA) for n = 5 animals/group. The hippocampus was segmented carefully in dorsal and ventral sections as shown in (Figure 2.5). Briefly, the contrast of images were enhanced, the background was subtracted and local maxima (pixel values of 255) were found. Particles were made solid by running the fill holes command before running the analyze particles command of a specific size (0-60 $pixels^2$). Data is presented as mean $BrdU^+$ cells per mm^2 ± SEM.

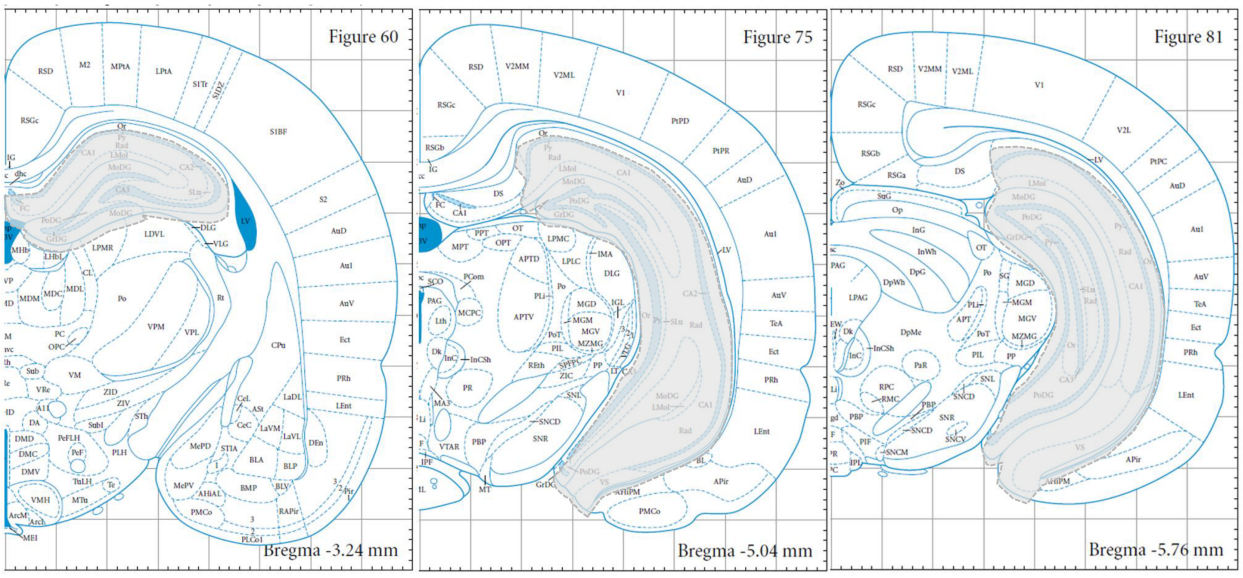


Figure 2.5: Representative slices throughout the hippocampus to depict thresholding segmentation for particle analysis. Overlays were applied to selective images from *The Rat Brain in Stereotaxic Coordinates* (Paxinos and Watson 2006).

2.5.3 Cell type characterization

To determine whether the $BrdU^+$ cells were glial cells, sections from n = 3 animals/group were triple stained and analyzed for colocalization of BrdU with either Iba1 or GFAP. Two slices in the 1:12 series were selected and the entire DG was imaged using an Olympus BX61WI confocal laser scanning microscope and Olympus FluoView FV10-ASW 1.7c software (Olympus Corporation, Center Valley, PA, USA) with a 20X objective at 1.5X zoom (NA = 0.75, 1024x1024, 0.41 µm/Pixel), 1.0 µm z steps and Kalman filtering (mean = 2). Because of the low number of $BrdU^+$ cells in sham animals, all $BrdU^+$ cells were identified in each stack, then characterized as $BrdU^+/Iba1^-/GFAP^-$, $BrdU^+/Iba1^+/GFAP^-$ or $BrdU^+/Iba1^-/GFAP^+$.Data is presented as % Cell Type of Total $BrdU^+$ cells ± SEM.

2.6 Statistical Analysis

All statistical analysis was conducted using R (R Core Team, Vienne, Austria, 2019) or GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Graphs were generated exclusively

using Graphpad Prism 8. Statistical tests and means are detailed in tables throughout the text; each test is identified within the text using superscript letters. Appropriate assumptions for each test were checked, using appropriate tests (Levene's test for homogeneity of variance; Shapiro Wilk or QQ plots for normality); decisions to use parametric or non parametric tests were decided based on these tests and type of data. *Loss of consciousness*: differences in toe pinch and righting reflex between sexes in rmTBI animals was tested using either a two-tailed Mann Whitney U Test or two-tailed Unpaired t test. *NAP scores*: data from rmTBI 1-8 was assessed using a two-way repeated measures ANOVA with the Geisser-Greenhouse Correction for sphericity with group and rmTBI as factors. Group differences between specific tasks were analysed using one-tailed Mann Whitney U Tests. *Subdural Hematoma*: Differences in rmTBI animals with versus without subdural hematoma were analysed using one-tailed Mann Whitney U Tests. *Immunohistochemistry and immunofluorescence*: three-way mixed effects ANOVAs were performed with group and sex as between groups factors and side as a within groups factor. Two-way mixed effects ANOVAs were performed for fold-change from sham with sex (between groups) and side (within groups) as factors. For all statistical analyses $\alpha = 0.05$.

CHAPTER **3**

Results

3.1 mTBI results in loss of consciousness

At PND 28, animals were subjected to sham or repeated mild traumatic brain injury procedures using an awake closed head injury model. After each ACHI, subjects were tested for loss of consciousness by their toe pinch reflex and ability to right (righting reflex). No sham animals experienced loss of toe pinch or righting reflex (data not shown). Male and female rmTBI animals experienced an average latency to recover toe pinch reflex of 5.26 s and 7.21 s respectively. An unpaired t-test revealed no significant difference ($p = 0.526$) in latency to recover toe pinch reflex between male and female rmTBI animals (Figure 3.1A). The average latency to recover righting reflex was 1.32 s for males and 0.80 s for females. 64% of male rmTBI animals and 79% of female rmTBI animals experienced acute loss of toe pinch reflex at least one time (Table 3.1). These animals lost toe pinch reflex an average of 3.8 and 4.2 out of 8 times for males and females respectively. An unpaired t-test revealed no significant difference ($p = 0.680$) in average frequency of loss of toe pinch reflex between male and female rmTBI animals (who experienced loss of toe pinch reflex at least once) (Figure 3.1B). An unpaired t-test revealed no significant difference ($p = 0.439$) in latency to recover righting reflex between male and female rmTBI animals (Figure 3.1D). 93% of (male and female) rmTBI animals experienced acute loss of righting reflex; an average frequency of 3.13 and 4.00 times out of 8 for males and female respectively (Table 3.1). An unpaired t-test revealed no significant difference ($p = 0.267$) in average frequency of loss of righting reflex between male and female rmTBI animals (who experienced loss of righting reflex at least once) (Figure 3.1E).

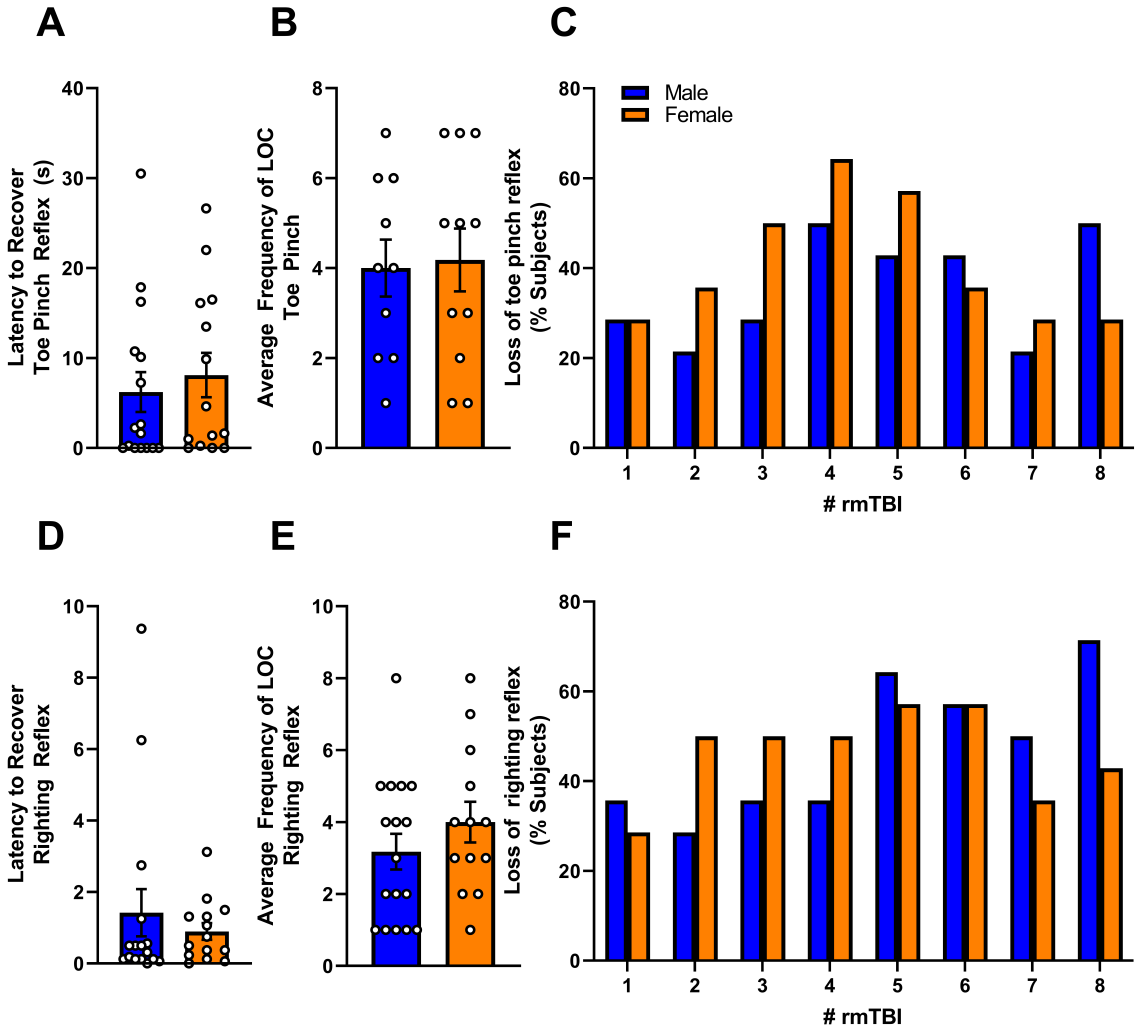


Figure 3.1: mTBI induced by awake closed head injury causes loss of consciousness in males and females. Shams are not shown, but did not experience loss of consciousness after sham ACHI. There were no sex differences in the latency to recover either toe pinch reflex **(A)** or righting reflex **(D)**. **B,E.** For rmTBI animals that did experience LOC events at least once, there were no sex differences in the average frequency of these events across all 8 ACHI procedures. **C,F.** Analysis of whether these events occur evenly across all number of injuries, showed that for loss of toe pinch reflex, there may be a peak of LOC frequency at 4 injuries . Data is presented as mean ± SEM.

Table 3.1: Loss of consciousness following rmTBI. Following sham ACHI procedures, animals did not experience loss of consciousness. After rmTBI ACHI procedures, both male and female rats experienced loss of consciousness events including loss of toe pinch reflex and ability to right.

	Toe Pinch				Righting Reflex			
	Freq = 0 (%)	Freq = 1 (%)	Freq > 1 (%)	Average Freq (Max 8/8 ACHI)	Freq = 0 (%)	Freq = 1 (%)	Freq > 1 (%)	Average Freq (Max 8/8 ACHI)
Sham Male	14 (100)	0 (0)	0 (0)	0	14 (100)	0 (0)	0 (0)	0
rmTBI Male	5 (35.71)	1 (7.14)	8 (57.14)	4.00	1 (7.14)	3 (21.43)	10 (71.43)	3.18
Sham Female	14 (100)	0 (0)	0 (0)	0	14 (100)	0 (0)	0 (0)	0
rmTBI Female	3 (21.43)	2 (14.29)	9 (64.29)	4.18	1 (7.14)	1 (7.14%)	12 (85.71)	4.00

Table 3.2: Statistical Analsysis for Loss of Consciousness

Superscript in Text	Figure	Test	Factors	Result	Mean Male ± SEM	Mean Female ± SEM
a	3.1A	Mann Whitney Test, two tailed	sex	U = 96, p = 0.512, n1 = 16, n2 = 14	6.22 ± 2.22	8.11 ± 2.47
b	3.1B	Unpaired t test, two tailed	sex	t = 0.1916, df = 19, p = 0.850 n1=10, n2 = 11	4.00 ± 0.63	4.18 ± 0.70
c	3.1D	Mann Whitney Test, two tailed	sex	U = 95.5, p = 0.503, n1 = 16, n2 = 14	1.42 ± 0.66	0.90 ± 0.23
d	3.1E	Unpaired t test, two tailed	sex	t = 1.096, df = 28, p = 0.282 n1=17, n2 = 13	3.18 ± 0.49	4.00 ± 0.57

3.2 Repeat ACHI causes acute neurological deficits

Immediately following each ACHI and recovery from loss of consciousness (if applicable), sham and rmTBI animals were subjected to 4 sensorimotor and reflex tasks: startle response, limb extension, beam walk and rotating beam. Each task was scored from 0-3, with 3 being a perfect score. A total NAP score was given out of 12. Baseline scores were recorded for all subjects prior to sham or rmTBI procedures. There were no sex differences or differences between groups at baseline (Figure 3.2A and Figure 3.2B). After each ACHI, both male and female rmTBI animals were significantly impaired compared to their sham groups[e,f] ($p < 0.001$, Figure 3.2A and Figure 3.2B). Averaged total scores across all 8 ACHIs showed male and female rmTBI animals were significantly impaired compared to shams[g] (Figure 3.2B). Male and female rmTBI animals showed impairment on all 4 tests[h-o] ($p < 0.001$), with the exception of female performance on rotating beam ($p = 0.184$, Figure 3.2 D-F (males), G-J (females)).

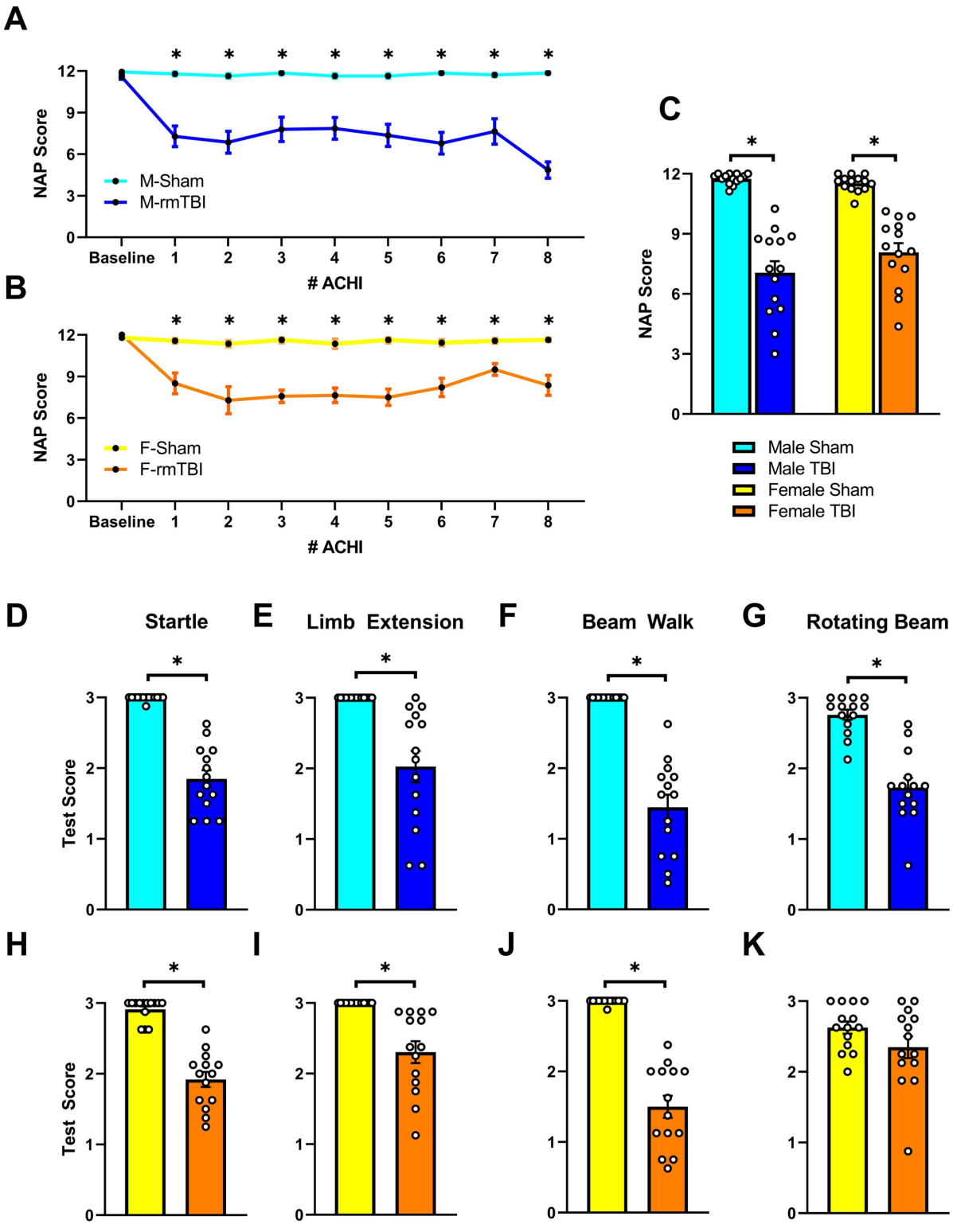


Figure 3.2: mTBI induced by awake closed head injury causes acute neurological impairment. Male **(A)** and female **(B)** rmTBI animals had significantly lower NAP scores across all 8 ACHIs (Two-way repeated measures ANOVA with Geisser-Greenhouse correction for sphericity). **C.** The average NAP scores for male and females were significantly lower than sex-matched sham scores ($p < 0.001$). **D-K.** Males and females showed significant neurological impairment based on the startle response, limb extension and beam walk, however only males showed impairment on the rotating beam. Data is presented as mean ± SEM.

Table 3.3: Statistical Analsysis for NAP Scores

Superscript in Text	Figure	Test	Factors	Result	Mean Male ± SEM	Mean Female ± SEM
e	3.2A	Two-way RM ANOVA with Geisser-Greenhouse Correction	Main effect Group	F (1, 26) = 66.17, p < 0.001		
			Main effect ACHI	ACHI: F (5.220, 135.7) = 9.172, p < 0.001		
			group*ACHI	F (8, 208) = 8.571, p < 0.001		
f	3.2B	Two-way RM ANOVA with Geisser-Greenhouse Correction	Main effect Group	F(1,26) = 51.99. p < 0.001		
			Main effect ACHI	F(5.290, 137.5) = 8.453, p < 0.001		
			group*ACHI	F (8, 208) = 6.592, p < 0.001		
g	3.2C	Mann Whitney Test, one tailed	group	Males: (U = 0, p < 0.0001, n1=14, n2=14)	11.75 ± 0.07	7.05 ± 0.58
				Females: (U = 10, p < 0.0001, n1=14, n2=14)	11.53 ± 0.11	8.07 ± 0.46
h	3.2D	Mann Whitney Test, one tailed	group	U = 0, p = < 0.0001, n1 = 14, n2 = 14	2.99 ± 0.009	1.85 ± 0.12
i	3.2E	Mann Whitney Test, one tailed	group	U = 7, p = < 0.0001, n1 = 14, n2 = 14	3.00 ± 0.00	2.03 ± 0.22
j	3.2F	Mann Whitney Test, one tailed	group	U = 0, p = < 0.0001, n1 = 14, n2 = 14	3.00 ± 0.00	1.45 ± 0.18
k	3.2G	Mann Whitney Test, one tailed	group	U = 7, p = < 0.0001, n1 = 14, n2 = 14	2.76 ± 0.07	1.73 ± 0.13
l	3.2H	Mann Whitney Test, one tailed	group	U = 1.5, p = < 0.0001, n1 = 14, n2 = 14	2.91 ± 0.04	1.92 ± 0.11
m	3.2I	Mann Whitney Test, one tailed	group	U = 0, p = < 0.0001, n1 = 14, n2 = 14	3.00 ± 0.00	2.30 ± 0.15
n	3.2J	Mann Whitney Test, one tailed	group	U = 0, p = < 0.0001, n1 = 14, n2 = 14	2.99 ± 0.009	1.50 ± 0.16
o	3.2K	Mann Whitney Test, one tailed	group	U = 69, p = 0.1838, n1 = 14, n2 = 14	2.63 ± 0.09	2.35 ± 0.15

3.3 No major signs of tissue damage or loss following rmTBI

One of the main features of mTBI is normal imaging and the absence of intracranial lesions, indicating a functional rather than structural disturbance (McCrory et al 2017, Dewitt et al 2013 for review). To assess whether rmTBI induced by the ACHI model results in tissue damage, representative tissue slices for the dorsal (approx. Bregma -3.36 mm) and ventral (approx. Bregma -5.28 mm) hippocampus were stained with a nissl stain. There were no major signs of tissue damage or tissue loss in cresyl violet stained sections following rmTBI under the impact site or other regions (Figure 3.3). Because the profile counting conducted in this study was focused on the SGZ of the DG, area measurements for the DG (granule cell layer and SGZ) were taken for a series of 1 in 6 sections to determine whether there was a change based on sex or injury. There were no significant differences in DG area in males or females at PID 1 or PID 3 (Appendix Figure 5.5).

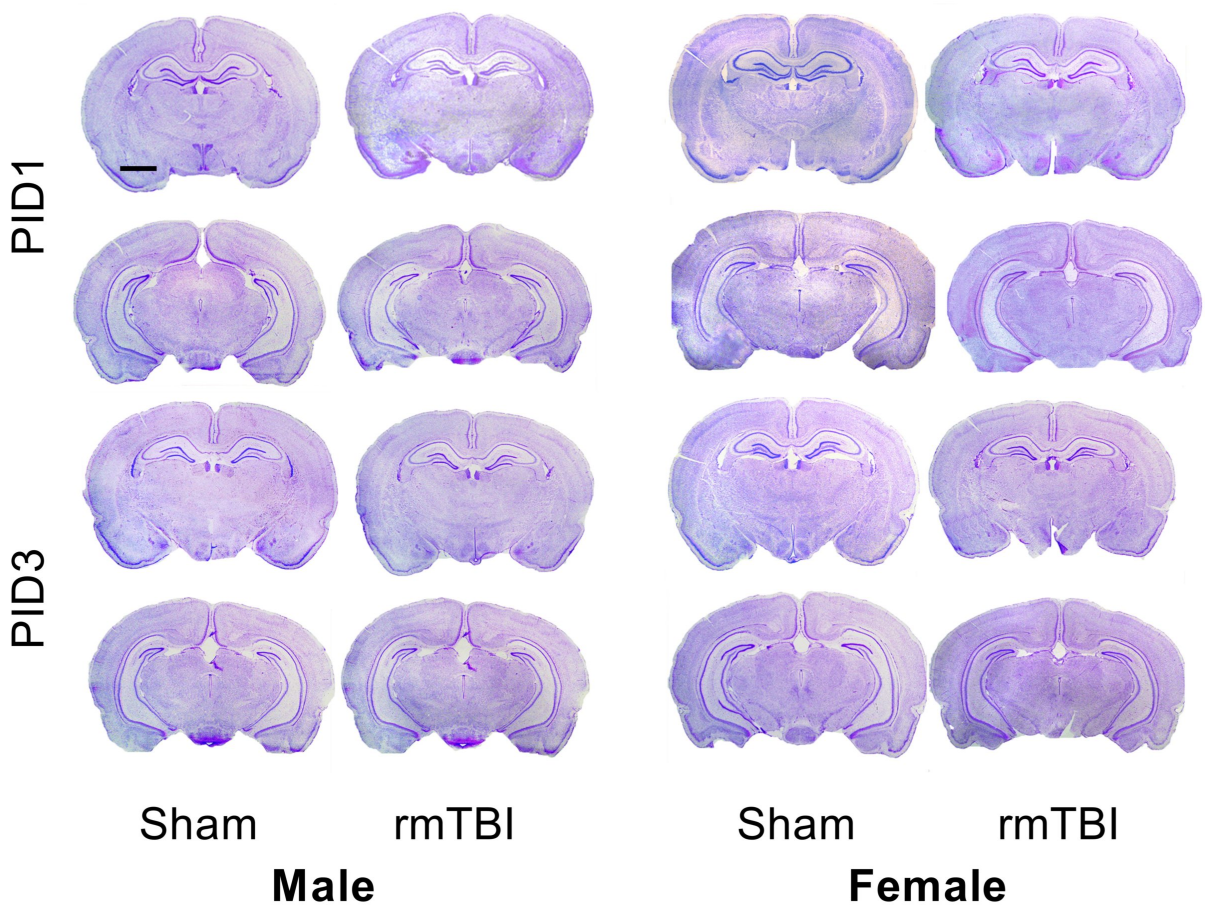


Figure 3.3: No major signs of tissue damage or loss following rmTBI. Cresyl violet stain on representative coronal sections displaying the dorsal and ventral hippocampus show no signs of cortical contusion of major tissue damage in male or female rmTBI animals. Scale bar is 2 mm.

3.4 rmTBI may result in subdural hematoma

Some animal models of mTBI have shown evidence of subdural hematoma (due to ruptured bridging veins) as a result of primary injury (DeWitt et al., 2013; Nyanzu et al., 2017; Orlando et al., 2019; Viano et al., 2009). Upon brain extraction following transcardial perfusion, subdural hematoma (at approximately the impact site) were observed in 4 male rmTBI animals (29%) and 2 female rmTBI animals (14%). In one animal, bleeding in the folliculus was also observed (Figure 3.4F). Subdural hematoma were defined as visible bleeding on the surface of the brain tissue that could be seen macroscopically. All subdural hematoma were imaged using a stereoscope. The average total NAP scores for rmTBI animals with and without subdural hematoma were compared (Figure 3.4C and Figure 3.4D). Non-parametric Mann Whitney U tests were performed and there was no significant difference in total NAP score for males or females based on absence or presence of bleeds. However, the unequal sample sizes (and violation of homogeneity of variance) should be noted and taken into consideration when drawing conclusions about the effect of subdural hematoma after rmTBI.

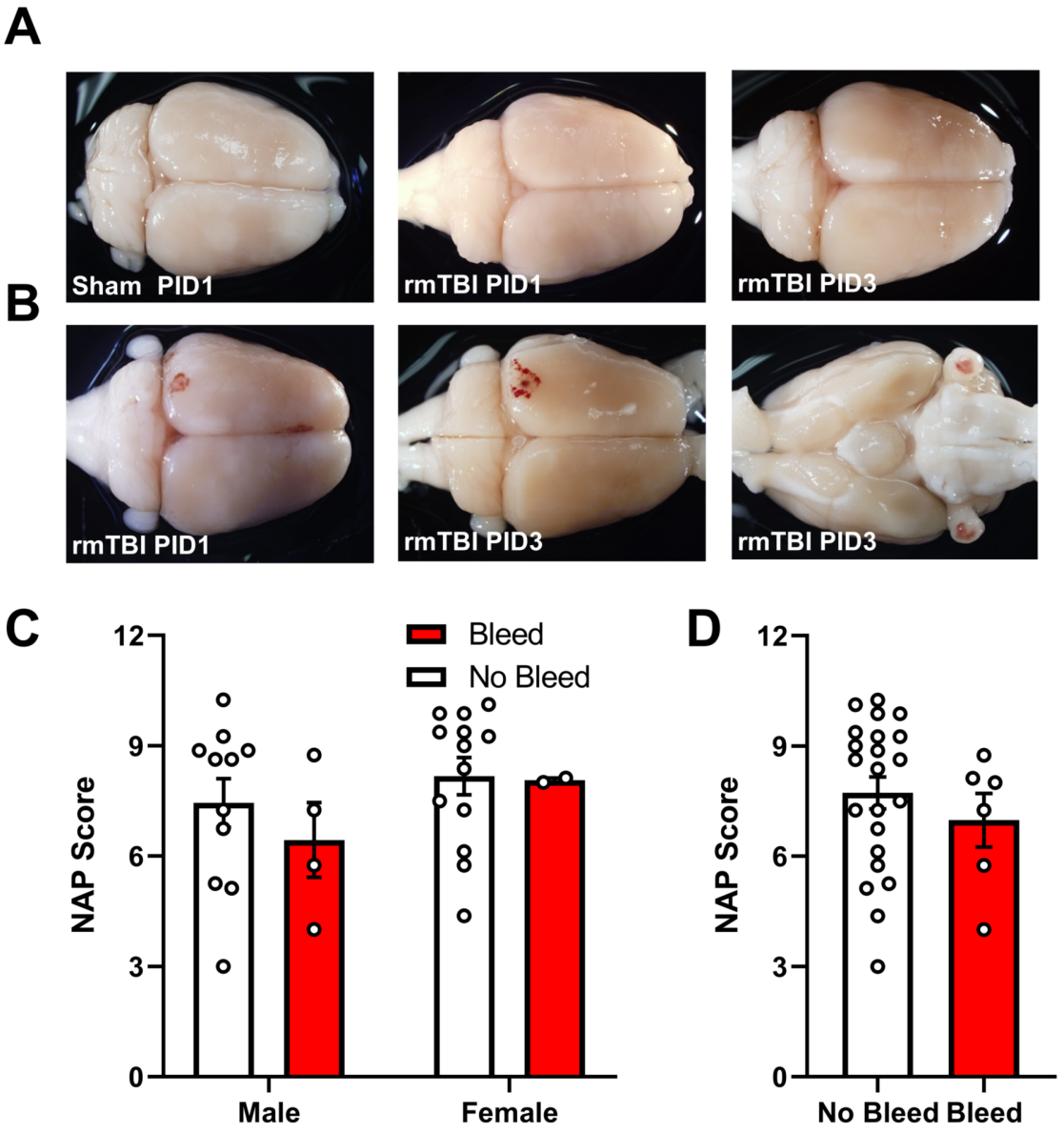


Figure 3.4: rmTBI may result in subdural hematoma. A. All sham and most rmTBI animals did not show signs of subdural hematoma. **B.** However, in 4 male and 2 female rmTBI animals, there were subdural hematomas approximately under the impact site and, in one case, on the cerebellar folliculus. **C,D.** Mann Whitney U tests showed no difference in NAP scores between rmTBI animals with and without subdural hematoma.

Table 3.4: Statistical Analysis for Subdural Hematoma

Superscript in Text	Figure	Test	Factors	Result	Mean Male ± SEM	Mean Female ± SEM
p	3.4A	Mann Whitney U Test, one tailed	bleed, no bleed	Males: (U = 15.5, p = 0.283, n1=10, n2=4)	7.30 ± 0.72	6.44 ± 1.02
				Females: (U = 10, p = 0.385, n1=12, n2=2)	8.07 ± 0.54	8.06 ± 0.63
q	3.4B	Mann Whitney U Test, one tailed	bleed, no bleed	U = 46.5, p = 0144, n1 = 22, n2 = 6	7.72 ± 0.44	6.98 ± 0.73

3.5 Detection of BrdU and Ki-67 cells

On PID 1, animals were injected with one dose of BrdU (200 mg/kg) at least 2 hours prior to transcardial perfusion. BrdU is a thymidine analogue and is therefore incorporated into the DNA of cells undergoing DNA synthesis (S phase of the cell cycle). DAB immunohistochemistry was used to identify $BrdU^+$ cells in a 1 in 6 series of tissue sections. Because the BrdU injection paradigm used in this study captures a very specific window of cell division, an antibody against a commonly used endogenous cell proliferation marker expressed during all active phases of the cell cycle, Ki-67, was used in another subset of 1 in 6 tissue sections. $BrdU^+$ and $Ki\text{-}67^+$ cells were identified (Olesen et al., 2017; Perfilieva et al., 2001) as medium-sized circular/oval or elongated nuclei (Figure 3.5). Many cells appeared in clusters, as is characteristic of cells dividing from a single stem cell (Perfilieva et al., 2001). Some cells, especially in rmTBI animals, had morphology consistent with glial cells: smaller, with more irregular-shaped nuclei. Others had triangular soma that could indicate radial glial cells (Figure 3.6). All cells lying within the SGZ were counted, regardless of morphology. Thus, numbers of $BrdU^+$ and $Ki\text{-}67^+$ cells include a variety of cell types. Cell type was considered in a later experiment using triple immunofluorescent labeling to provide a more robust and reliable characterization of $BrdU^+$ cells.

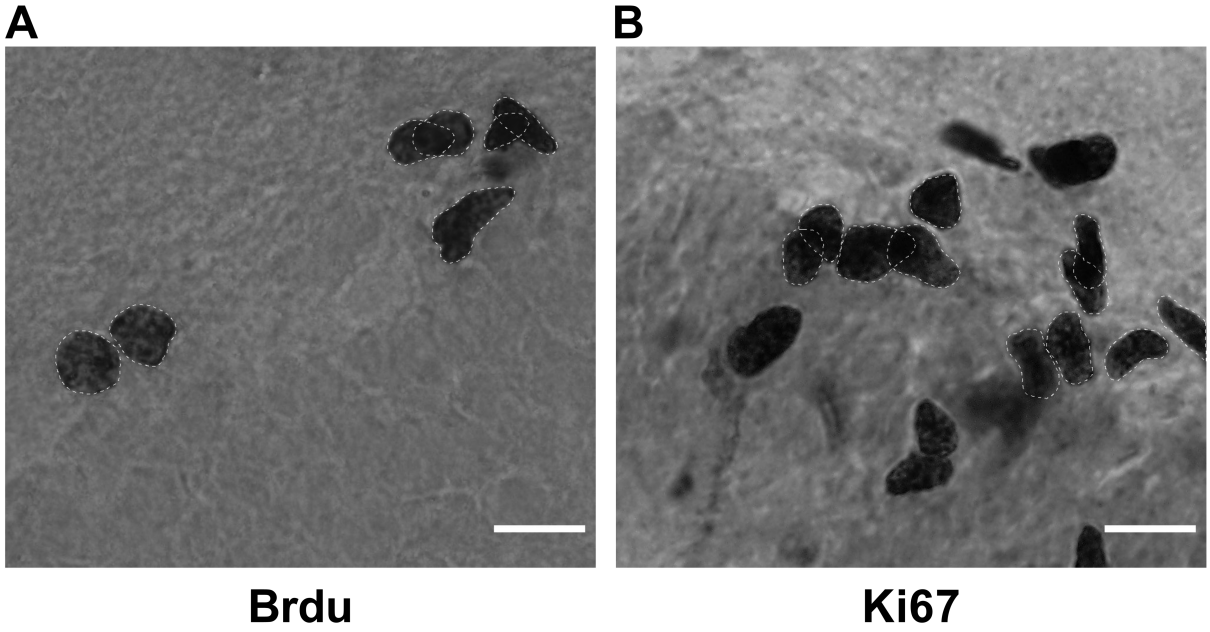


Figure 3.5: BrdU$^+$ and Ki-67$^+$ cells were found in all animals. Representative images of BrdU **(A)** and Ki-67 **(B)** in the SGZ taken with a 100X objective: white dotted outlines delineate cells that would be counted as positive in the SGZ. Overlapping cells can be distinguished from one another by scrolling through the z axis at high magnification. Scale is 10 μm.

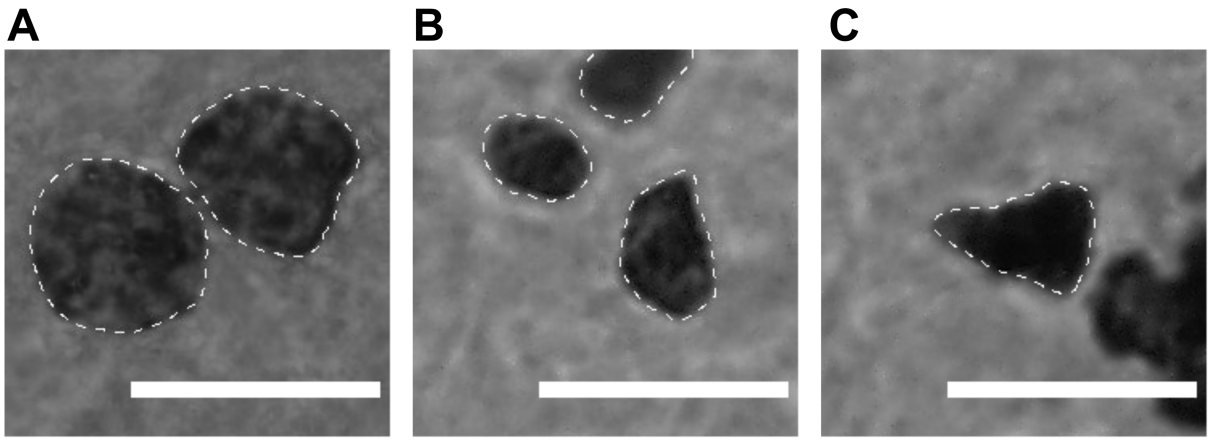


Figure 3.6: A variety of cell morphologies were observed, especially in rmTBI animals. A. BrdU$^+$ and Ki-67$^+$ cells were mostly circular round/oval or elongated in morphology. Other cell soma were smaller and more irregular in shape **(B)** or triangular **(C)**. Scale is 10 μm.

3.6 Robust increase in BrdU$^+$ cells at PID 1 in the SGZ of male rmTBI animals but not females

To investigate the effects of rmTBI on cellular proliferation in the neurogenic niche, BrdU$^+$ cells were counted in the SGZ of the dorsal dentate gyrus (dDG) and ventral dentate gyrus (vDG) at PID 1 in the contralateral and ipsilateral hemispheres. In a three-way mixed effects ANOVA looking at the effects of group, sex and side on BrdU$^+$ cells, there was a main effect of group[r-i,t-i] and a main effect of sex[r-ii,t-ii], but not side[r-iii,t-iii] in the dDG and vDG.

In the dDG, there was a significant interaction between group and side[r-v] and in the vDG there was a significant interaction between group and side[t-v] and a significant three way interaction between group, side and sex[t-vii] (Figure 3.7A and Figure 3.8A). Tukey post hoc tests revealed that BrdU$^+$ cells in the dDG were significantly higher in male rmTBI animals compared to male sham animals in the contralateral and ipsilateral hemispheres ($p = 0.037$ and $p < 0.0001$ respectively). This effect was not seen in female animals. Post hoc tests also revealed that BrdU$^+$ cells were significantly higher in males than females in both sham and rmTBI groups in the dDG ($p = 0.03$ and $p = 0.043$ respectively; sham data also presented in Appendix Figure 5.4). In the vDG, post hoc tests revealed a significantly higher number of BrdU$^+$ cells in the ipsilateral SGZ in male rmTBI animals compared to male shams ($p = 0.008$), but not in the contralateral hemisphere and in neither hemisphere for females (Figure 3.8A). There was also a significantly higher number of BrdU$^+$ cells in the ipsilateral SGZ of male rmTBI animals compared to their contralateral SGZ ($p = 0.016$). The fold change in BrdU$^+$ cell number from sex and PID-matched shams was evaluated to show the extent of an increase in BrdU$^+$ cells from sham animals. A two-way mixed effects ANOVA with sex and side as factors revealed a main effect of side[s-ii] in the dDG and vDG (Figure 3.7B) and (Figure 3.8B).

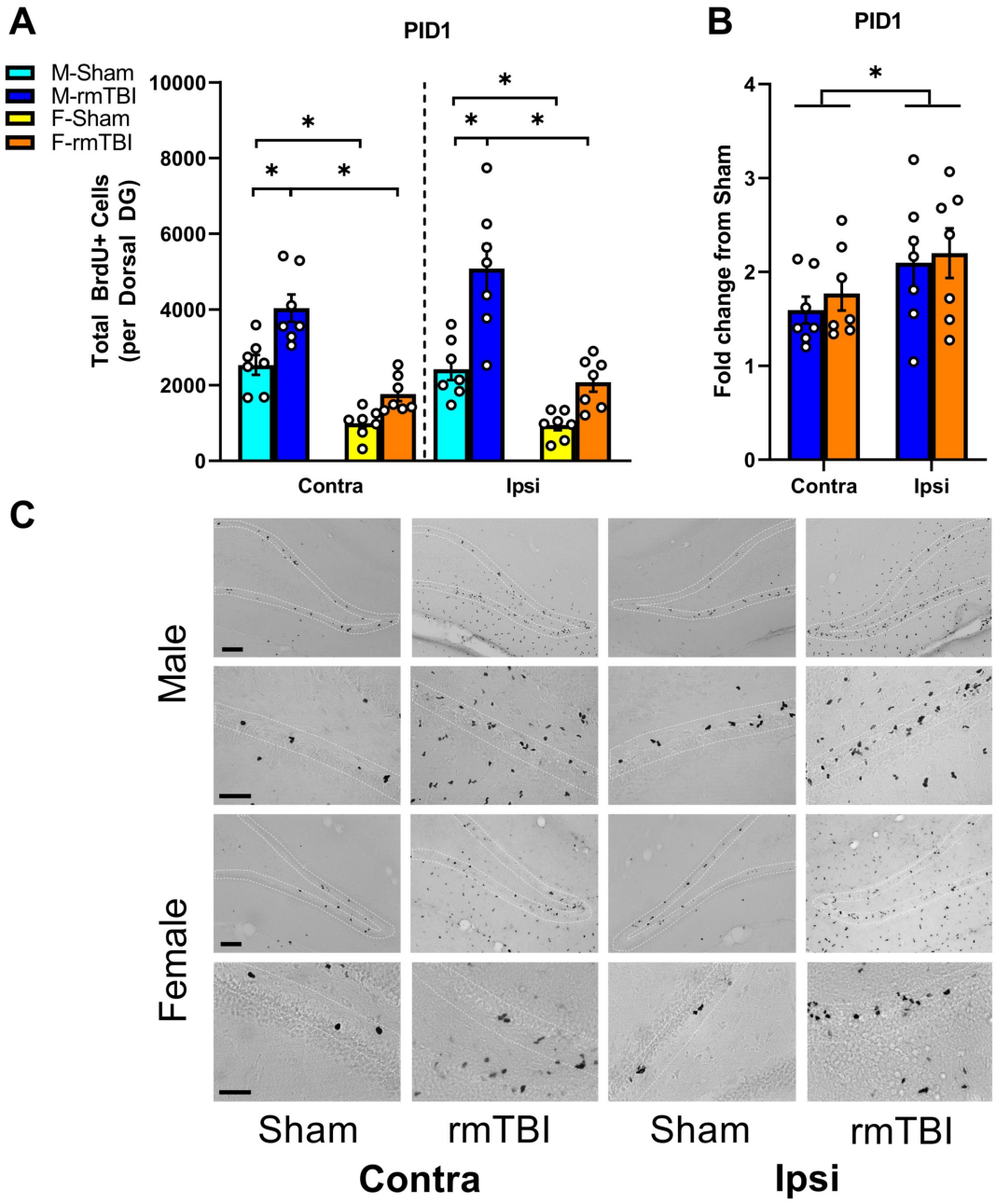


Figure 3.7: Increase in BrdU$^+$ cells at PID 1 in male rmTBI animals, but not females in the contralateral and ipsilateral dorsal SGZ. A. A three-way mixed effects ANOVA revealed a main effect of group ($F(1,24) = 28.08$, $p < 0.0001$) and a main effect of sex ($F(1,24) = 52.45$, $p < 0.0001$) and a significant interaction of group and side ($F(1,24) = 6.374$, $p = 0.019$). Post hoc analysis revealed an increase in BrdU$^+$ cells in contralateral and ipsilateral SGZ of male rmTBI animals compared to shams, but not in females. Male rmTBI animals showed a significantly higher number of BrdU$^+$ cells compared to female rmTBI animals in the contralateral and ipsilateral SGZ. There was also a significant difference in BrdU$^+$ cells in male and female sham animals in contralateral and ipsilateral hemispheres. **B.** Fold change from sham revealed no sex differences, but an overall significantly greater fold change from sham in the ipsilateral hemisphere (Two-way mixed effects ANOVA ($F(1,12) = 9.667$, $p = 0.009$). **C.** Representative images taken at 10X and 40X: scale bars are 50 μm and 25 μm respectively. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

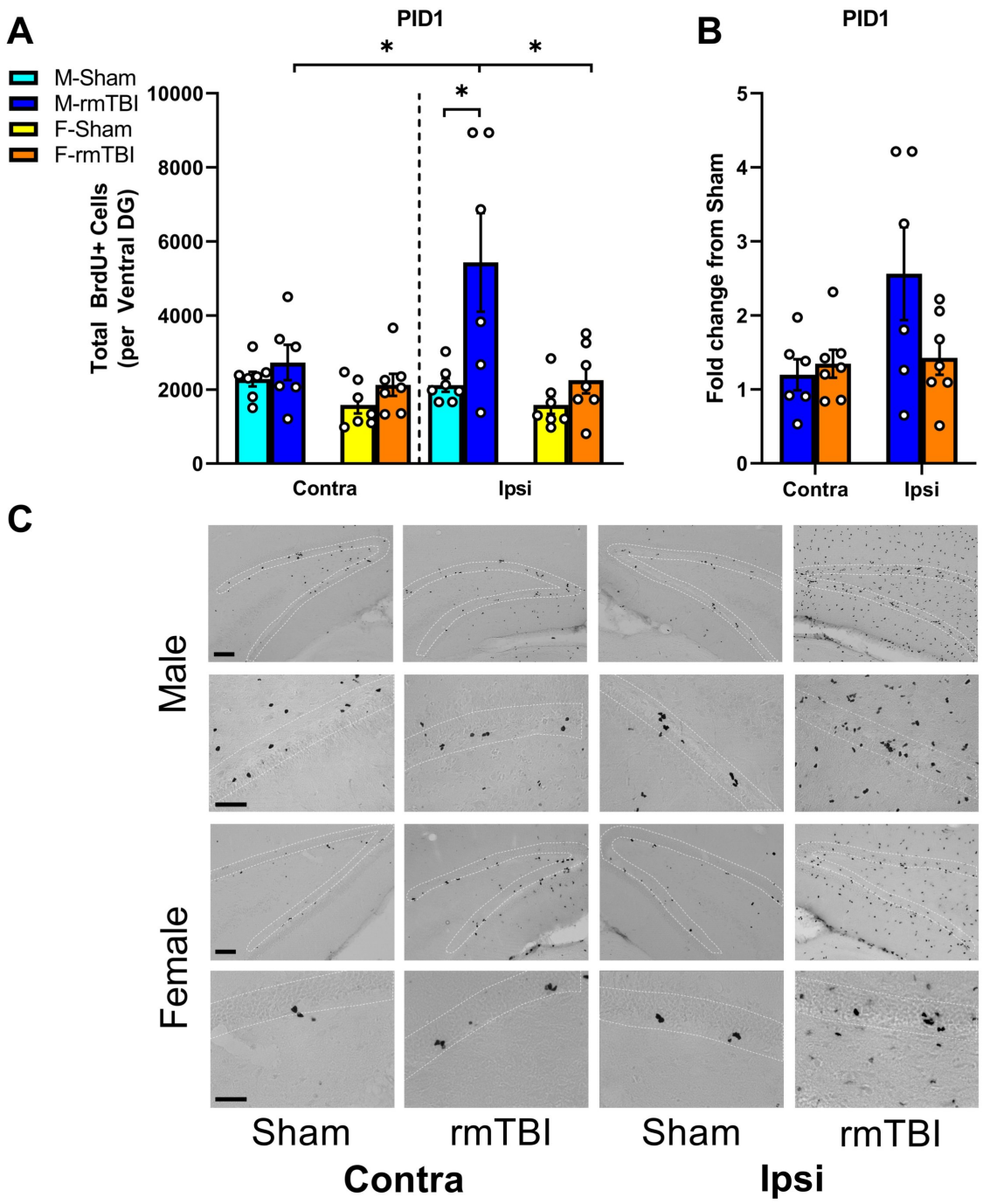


Figure 3.8: Increase in BrdU$^+$ cells at PID 1 in male rmTBI animals but not females in the ipsilateral ventral SGZ. A. A three-way mixed effects ANOVA revealed a main effect of group ($F(1,24) = 11.28$, $p = 0.003$) and a main effect of sex ($F(1,23) = 11.45$, $p = 0.003$). Post hoc analysis revealed an greater number of BrdU$^+$ cells in male rmTBI animals in the ipsilateral SGZ compared to their contralateral SGZ and compared ipsilateral SGZ of male shams and female rmTBI animals. **B.** A two-way mixed effects ANOVA revealed a main effect of side ($F(1,11) = 5.064$, $p = 0.046$) in fold change from sham. **C.** Representative images taken at 10X and 40X: scale bars are 50 μm and 25 μm respectively. Data is represented as mean ± SEM and individual points represent 1 animal.

Table 3.5: Statistical Analysis of Dorsal and Ventral BrdU Profile Counts on PID1

Superscript in Text	Figure	Test	Factors	Result
r	3.7A	Three factor mixed ANOVA	i-Main effect Group	F (1, 24) = 28.08, p < 0.0001
			ii-Main effect Sex	F (1, 24) = 52.45, p < 0.0001
			iii-Main effect Side	F (1, 24) = 3.940, p = 0.059
			iv-Group*Sex	F (1, 24) = 3.900, p = 0.060
			v-Group*Side	F (1, 24) = 6.374, p = 0.019
			vi-Sex*Side	F (1, 24) = 1.235, p = 0.277
			vii-Group*Sex*Side	F (1, 24) = 1.719, p = 0.202
s	3.7B	Two factor mixed ANOVA	i-Main effect Side	F (1, 12) = 9.667, p = 0.009
			ii-Main effect Sex	F (1, 12) = 0.2606, p = 0.619
			iii-Side*Sex	F (1, 12) = 0.06393, p = 0.805
t	3.8A	Three factor mixed ANOVA	i-Main effect Group	F (1, 23) = 11.28, p = 0.003
			ii-Main effect Sex	F (1, 23) = 11.45, p = 0.003
			iii-Main effect Side	F (1, 23) = 4.021, p = 0.057
			iv-Group*Sex	F (1, 23) = 2.948, p = 0.099
			v-Group*Side	F (1, 23) = 5.036, p = 0.035
			vi-Sex*Side	F (1, 23) = 3.290, p = 0.083
			vii-Group*Sex*Side	F (1, 23) = 4.228, p = 0.051
u	3.8B	Two factor mixed ANOVA	i-Main effect Side	F (1, 11) = 5.064, p = 0.046
			ii-Main effect Sex	F (1, 11) = 1.841, p = 0.202
			iii-Side*Sex	F (1, 11) = 4.019, p = 0.070

Table 3.6: Mean Values for BrdU Profile Counts on PID 1

Sex	SGZ	Dorsal Mean ± SEM	Ventral Mean ± SEM
Male	Sham Contra	2533 ± 259.5	2281 ± 198.1
	TBI Contra	4036 ± 361.8	2731 ± 478.6
	Sham Ipsi	2421 ± 291.4	2121 ± 181
	TBI Ipsi	5079 ± 646.4	5437 ± 1332
Female	Sham Contra	996 ± 142.5	1582 ± 226.1
	TBI Contra	1764 ± 182.4	2128 ± 297.6
	Sham Ipsi	945 ± 137.1	1583 ± 240.6
	TBI Ipsi	2079 ± 249.7	2255 ± 359.1
Fold Change	Male Contra	1.593 ± 0.143	1.197 ± 0.21
	Female Contra	1.771 ± 0.183	1.345 ± 0.188
	Male Ipsi	2.098 ± 0.267	2.563 ± 0.628
	Female Ipsi	2.2 ± 0.264	1.424 ± 0.227

3.7 Robust increase in BrdU$^+$ cells in the SGZ is transient

To determine whether this increase in BrdU$^+$ cells in the SGZ of male rmTBI is maintained throughout the acute phase after injury, BrdU$^+$ cells were counted in animals injected with BrdU at PID 3 and transcardially perfused a minimum of 2 hours later. A three-way mixed effects ANOVA looking at the effects of group, sex and side on BrdU$^+$ cell counts in the dDG revealed a significant interaction between group and side[v-v] (Figure 3.9A).

In the vDG, a three-way mixed effects ANOVA revealed a main effect of group[x-i] (Figure 3.10A). Because post hoc analysis did not reveal significant differences in comparisons of interest, data was pooled and an unpaired t-test revealed sham animals had a significantly higher number of BrdU$^+$ cells than rmTBI animals ($p = 0.029$)(Appendix Figure 5.3A). BrdU$^+$ cell numbers return to sham levels in the dDG, and sham levels surpass those of rmTBI animals in the vDG. A two-way mixed ANOVA revealed no main effects or significant interactions for fold change in BrdU$^+$ cells in rmTBI animals from shams in the dDG[w]. In the vDG, a main effect of sex was found[y-ii], but no comparisons of interest were found to be significant in post hoc analysis.

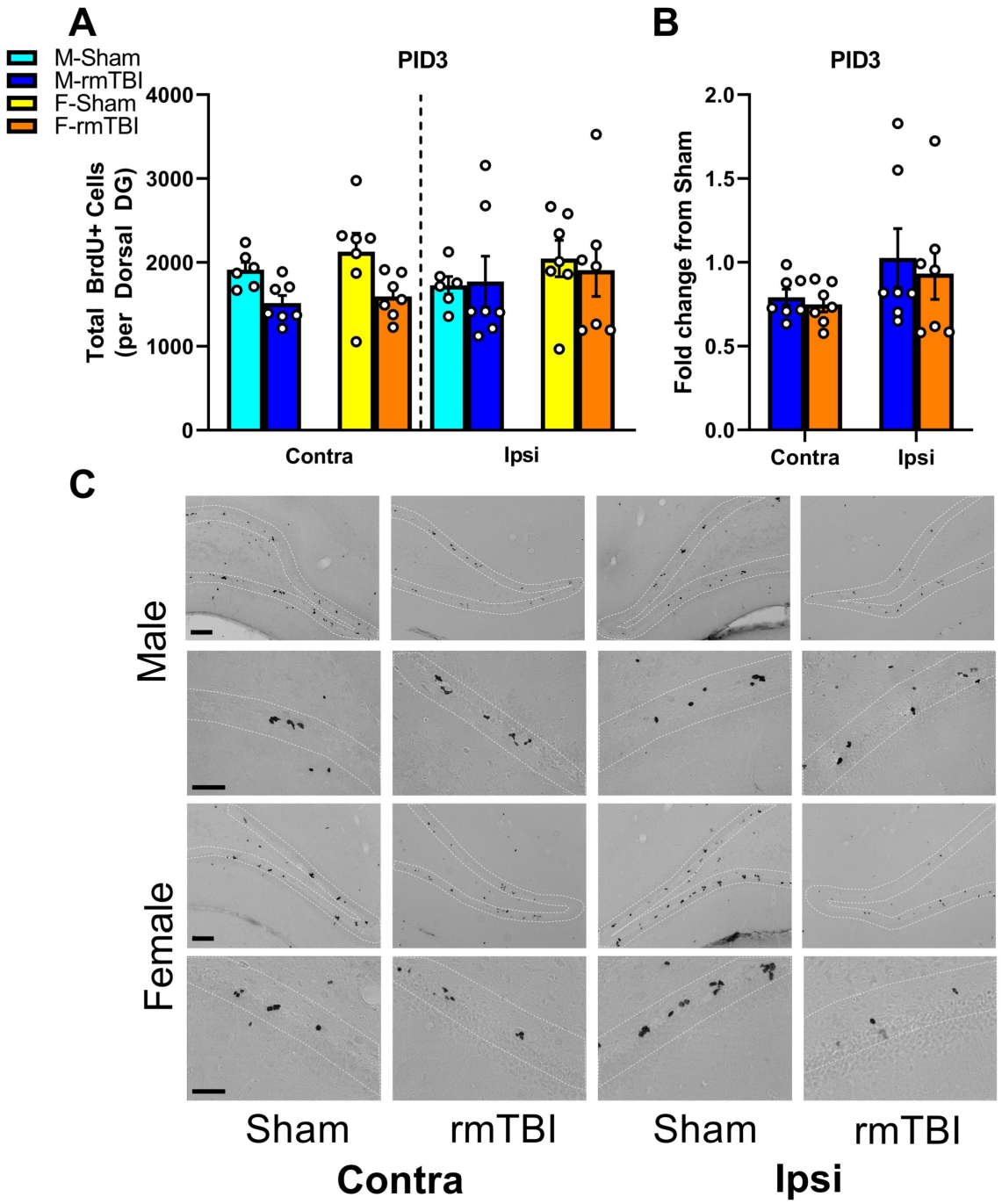


Figure 3.9: BrdU$^+$ cells in the dorsal SGZ of male and female rmTBI animals are comparable to shams at PID 3. A. A three-way mixed effects ANOVA revealed a main interaction between of group and side ($F(1,23) = 4.591$, $p = 0.043$) in the number of BrdU$^+$ cells in the ipsilateral and contralateral dorsal SGZ. **B.** A two-way mixed effects ANOVA revealed no main effects of side or sex in fold change from sham. **C.** Representative images taken at 10X and 40X: scale bars are 50 μm and 25 μm respectively. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

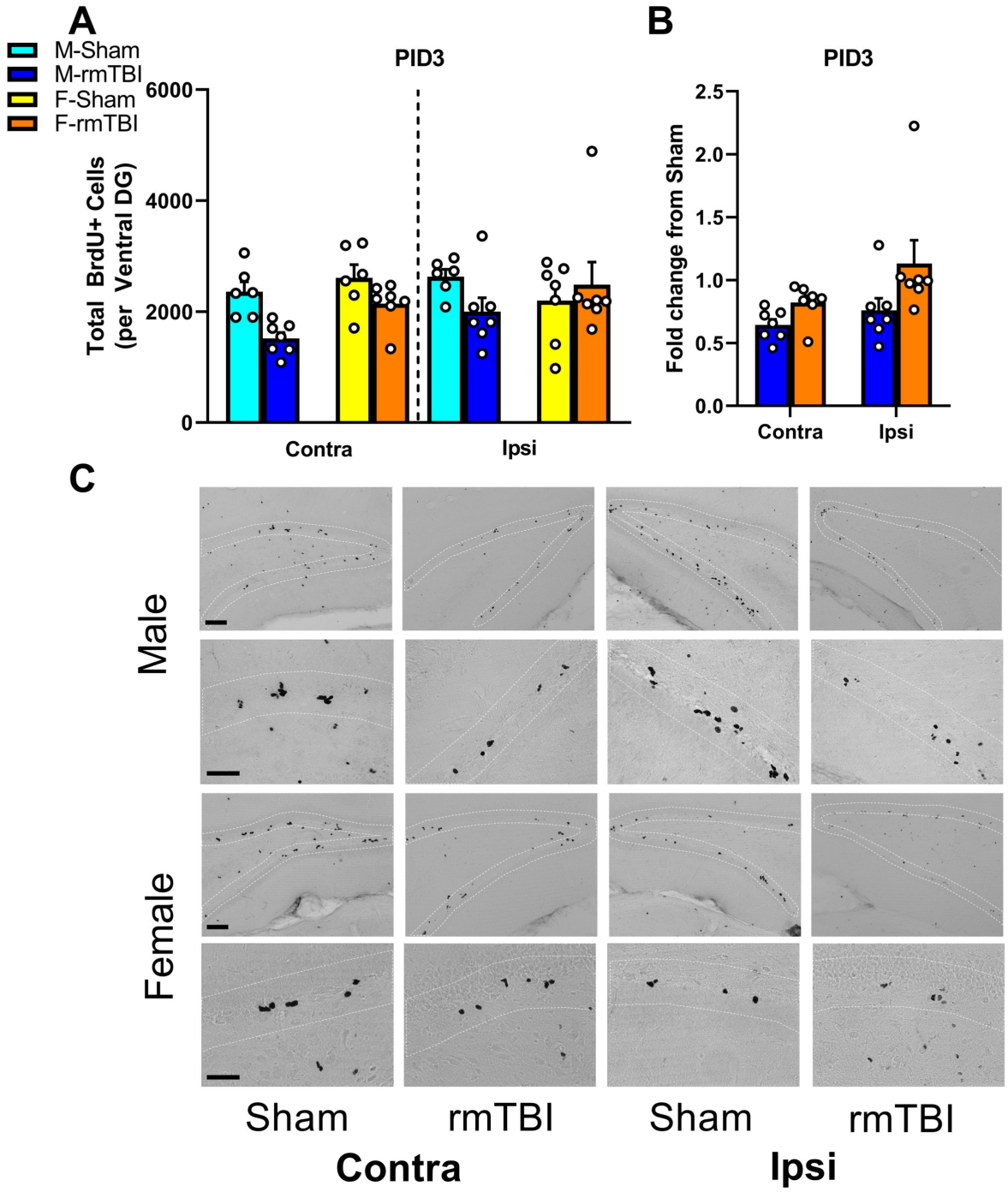


Figure 3.10: BrdU$^+$ cells in the ventral SGZ of male and female rmTBI animals are comparable to shams at PID 3. A. A three-way mixed effects ANOVA revealed a main effect of group ($F(1,23) = 4.749$, $p = 0.040$) in the number of BrdU$^+$ cells in the ipsilateral and contralateral ventral SGZ. **B.** A two-way mixed effects ANOVA revealed a main effect sex in fold change from sham ($F(1,12) = 5.946$, $p = 0.031$). **C.** Representative images taken at 10X and 40X: scale bars are 50 μm and 25 μm respectively. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

Table 3.7: Statistical Analysis for Dorsal and Ventral BrdU Profile Counts on PID 3

Superscript in Text	Figure	Test	Factors	Result
v	3.9A	Three factor mixed ANOVA	i-Main effect Group	F (1, 23) = 1.960, p = 0.175
			ii-Main effect Sex	F (1, 23) = 1.048, p = 0.317
			iii-Main effect Side	F (1, 23) = 0.587, p = 0.451
			iv-Group*Sex	F (1, 23) = 0.186, p = 0.670
			v-Group*Side	F (1, 23) = 4.591, p = 0.043
			vi-Sex*Side	F (1, 23) = 0.175, p = 0.690
			vii-Group*Sex*Side	F (1, 23) = 0.015, p = 0.903
w	3.9B	Two factor mixed ANOVA	i-Main effect Side	F (1, 12) = 4.950, p = 0.046
			ii-Main effect Sex	F (1, 12) = 0.221, p = 0.646
			iii-Side*Sex	F (1, 12) = 0.074, p = 0.791
x	3.10A	Three factor mixed ANOVA	i-Main effect Group	F (1, 23) = 4.749, p = 0.040
			ii-Main effect Sex	F (1, 23) = 1.542, p = 0.227
			iii-Main effect Side	F (1, 22) = 1.280, p = 0.270
			iv-Group*Sex	F (1, 23) = 2.776, p = 0.109
			v-Group*Side	F (1, 22) = 2.775, p = 0.110
			vi-Sex*Side	F (1, 22) = 2.058, p = 0.165
			vii-Group*Sex*Side	F (1, 22) = 0.915, p = 0.349
y	3.10B	Two factor mixed ANOVA	i-Main effect Side	F (1, 12) = 3.908, p = 0.0715
			ii-Main effect Sex	F (1, 12) = 5.946, p = 0.031
			iii-Side*Sex	F (1, 12) = 0.801, p = 0.389

Table 3.8: Mean Values BrdU Profile Counts PID 3

Sex	SGZ	Dorsal Mean ± SEM	Ventral Mean ± SEM
Male	Sham Contra	1915 ± 87.51	2358 ± 180.9
	TBI Contra	1515 ± 92.06	1517 ± 107.7
	Sham Ipsi	1727 ± 105	2631 ± 129.9
	TBI Ipsi	1772 ± 303.2	1999 ± 251.7
Female	Sham Contra	2128 ± 219.3	2609 ± 235.3
	TBI Contra	1595 ± 96.86	2144 ± 143.9
	Sham Ipsi	2046 ± 217.2	2198 ± 279.4
	TBI Ipsi	1909 ± 315.1	2486 ± 407
Fold Change	Male Contra	0.791 ± 0.048	0.643 ± 0.046
	Female Contra	0.749 ± 0.046	0.822 ± 0.055
	Male TBI	1.026 ± 0.176	0.76 ± 0.096
	Female TBI	0.933 ± 0.154	1.131 ± 0.185

3.8 Increase in Ki-67$^+$ cells at PID 1 in the dorsal ipsilateral SGZ in male rmTBI animals compared to shams.

As mentioned previously, the BrdU injection paradigm used in this study captures a very specific (approx. 2 hour) window of cell division. To investigate levels of cell proliferation without a specific temporal paradigm, an antibody against Ki-67 was used in another subset of 1 in 6 tissue sections from animals that had been injected with BrdU at PID 1 or PID 3. A three-way mixed effects ANOVA with group, sex and side as factors revealed significant main effects of group[z-i] and sex[z-ii] and a significant interaction of side and sex[z-vi] on Ki-67$^+$ cell number in the dDG (Figure 3.11A). Follow up post-hoc analysis revealed a significant increase in Ki-67$^+$ cells in the ipsilateral SGZ of male rmTBI animals ($p = 0.032$). No differences in Ki-67$^+$ cell number were observed in female rmTBI animals compared to shams. However, there were sex differences in sham levels of Ki-67$^+$ cells in contralateral and ipsilateral hemispheres ($p = 0.016$ and $p = 0.043$ respectively). Similarily, there were sex differences observed in Ki-67$^+$ cell number for rmTBI animals in both hemispheres (contralateral and ipsilateral $p < 0.001$).

In the vDG, main effects of group[ab-i] and sex[ab-ii] were observed as well as a significant interaction between side and sex[ab-vi]. Post hoc analysis revealed a significantly higher number of Ki-67$^+$ cells in the contralateral SGZ of male rmTBI animals compared to sham ($p = 0.045$), but not in females or in either sex in the ipsilateral hemisphere (Figure 3.12A). There were significant differences between male and female rmTBI animals in both SGZs (contralateral and ipsilateral $p < 0.001$ and $p = 0.013$ respectively). A two-way mixed effects ANOVA with sex and side as factors for fold change from shams in Ki-67$^+$ cells in rmTBI animals revealed no main effects or significant interactions in the dDG[aa] or vDG[ac] at PID 1 (Figure 3.11B, Figure 3.12B).

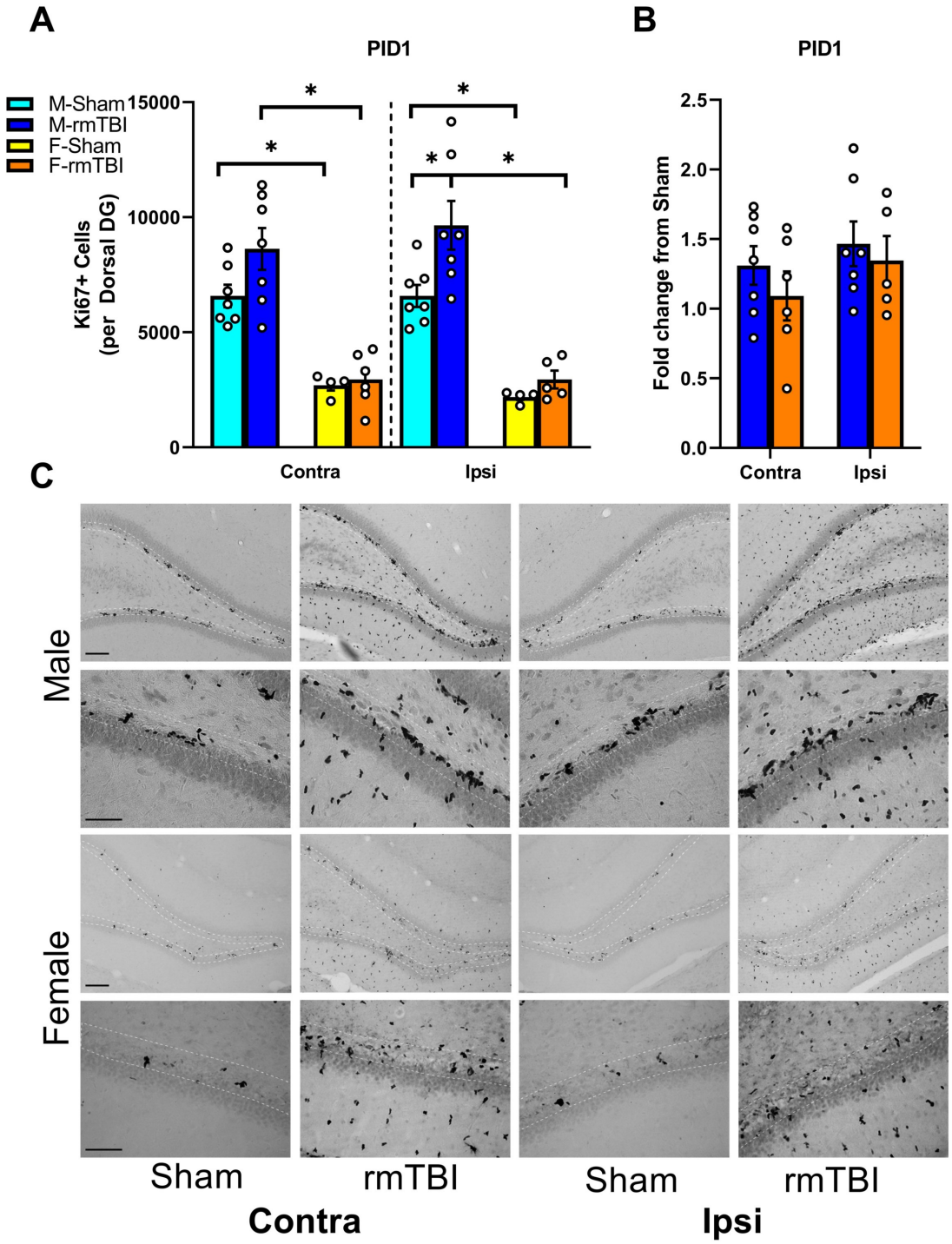


Figure 3.11: Increase in Ki-67$^+$ cells in the ipsilateral dorsal SGZ of rmTBI animals at PID 1 in males but not females. A. A three-way mixed effects ANOVA revealed a main effect of group ($F(1, 20) = 4.419$, $p = 0.048$) and a main effect of sex ($F(1,20) = 57.61$, $p < 0.0001$) and a significant interaction of side and sex ($F(1,19) = 6.351$, $p = 0.021$). Post hoc analysis revealed an increase in BrdU$^+$ cells in the ipsilateral SGZ of male rmTBI animals compared to shams, but not in the contralateral SGZ. Male rmTBI animals exhibited significantly higher numbers of Ki-67$^+$ cells in both hemispheres compared to female rmTBI animals. There were no significant differences between female rmTBI animals and female shams in either hemisphere. Similar to BrdU staining, there were significantly higher numbers of Ki-67$^+$ cells in male compared to female sham animals in contralateral and ipsilateral hemispheres. **B.** Fold change from sham revealed no main effects of sex or side. **C.** Representative images taken at 10X and 40X: scale bars are 50 μm and 25 μm respectively. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

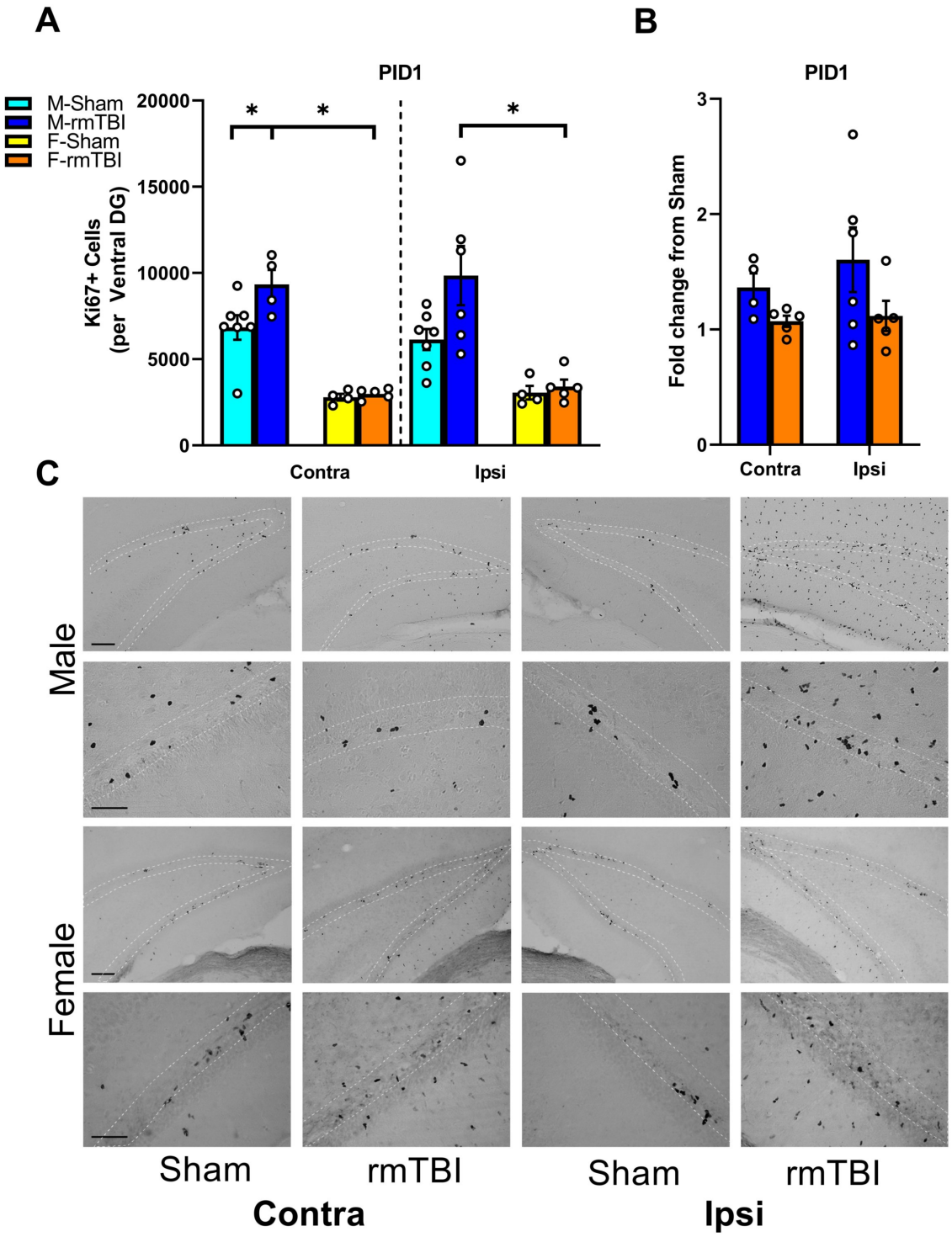


Figure 3.12: Increase in Ki-67$^+$ cells in the ipsilateral ventral SGZ of rmTBI animals at PID 1 in males but not females. A. A three-way mixed effects ANOVA revealed a main effect of group ($F(1,18) = 5.001$, $p = 0.038$), and a main effect of sex ($F(1,18) = 34.42$, $p < 0.0001$) as well as a significant interaction between side and sex ($F(1,14) = 5.457$, $p = 0.035$). There was a significant increase in Ki-67 cells in the contralateral SGZ of male rmTBI animals. Post hoc analysis revealed an increase in BrdU$^+$ cells in the ipsilateral and contralateral SGZ of male rmTBI animals compared to female rmTBI animals. **B.** Fold change from sham revealed no main effects of sex or side. **C.** Representative images taken at 10X and 40X: scale bars are 50 μm and 25 μm respectively. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

Table 3.9: Statistical Analysis for Ki-67 Profile Counts PID 1

Superscript in Text	Figure	Test	Factors	Result
z	3.11A	Three factor mixed anova	i-Main effect Group	F (1, 20) = 4.419, p = 0.048
			ii-Main effect Sex	F (1, 20) = 57.61, p <0.0001
			iii-Main effect Side	F (1, 19) = 0.072, p = 0.791
			iv-Group*Sex	F (1, 20) = 2.559, p = 0.125
			v-Group*Side	F (1, 19) = 2.778, p = 0.112
			vi-Sex*Side	F (1, 19) = 6.351, p = 0.021
			vii-Group*Sex*Side	F (1, 19) = 1.281, p = 0.272
aa	3.11B	Two factor mixed anova	i-Main effect Side	F (1,10) = 7.253, p = 0.023
			ii-Main effect Sex	F (1,11) = 1.001, p = 0.338
			iii-Side*Sex	F (1,10) = 0.041, p = 0.843
ab	3.12A	Three factor mixed anova	i-Main effect Group	F (1, 18) = 5.001, p = 0.038
			ii-Main effect Sex	F (1, 18) = 34.42, p <0.0001
			iii-Main effect Side	F (1, 14) = 1.494, p = 0.242
			iv-Group*Sex	F (1, 18) = 3.756, p = 0.069
			v-Group*Side	F (1, 14) = 0.032, p = 0.860
			vi-Sex*Side	F (1, 14) = 5.457, p = 0.035
			vii-Group*Sex*Side	F (1, 14) = 0.190, p = 0.67
ac	3.12B	Two factor mixed anova	i-Main effect Side	F (1,7) = 0.0002, p = 0.988
			ii-Main effect Sex	F (1,9) = 3.584, p = 0.091
			iii-Side*Sex	F (1,7) = 0.178, p = 0.685

Table 3.10: Mean Values Ki-67 Profile Counts PID 1

Sex	SGZ	Dorsal Mean ± SEM	Ventral Mean ± SEM
Male	Sham Contra	6581 ± 486.9	6840 ± 486.9
	TBI Contra	8623 ± 911.3	9334 ± 911.3
	Sham Ipsi	6579 ± 478.7	6136 ± 478.7
	TBI Ipsi	9647 ± 1057	9847 ± 1057
Female	Sham Contra	2700 ± 234.4	2788 ± 234.4
	TBI Contra	2949 ± 474.5	2988 ± 474.5
	Sham Ipsi	2189 ± 127.2	3055 ± 127.2
	TBI Ipsi	2948 ± 384	3413 ± 384
Fold Change	Male Contra	1.31 ± 0.138	1.365 ± 0.122
	Female Contra	1.092 ± 0.176	1.072 ± 0.048
	Male Ipsi	1.466 ± 0.161	1.605 ± 0.28
	Female Ipsi	1.347 ± 0.175	1.117 ± 0.13

3.9 Proliferation of Ki-67$^+$ cells persists: Increase in SGZ of male rmTBI animals but not females in both hemispheres of dDG and vDG at PID 3

A three-way mixed effects ANOVA with group, sex and side as factors revealed a main effect of group[ad-i], a main effect of sex[ad-ii] and significant interactions between group and sex[ad-iv] and group and side[ad-v] on Ki-67$^+$ cells in the dDG at PID 3 (Figure 3.13A). Tukey post hoc analysis revealed a significant increase in Ki-67$^+$ cells in the contralateral and ipsilateral SGZs of male rmTBI rats (contralateral $p = 0.003$; ipsilateral $p < 0.0001$). There was a significant increase in Ki-67$^+$ cells in male rmTBI animals as compared to female rmTBI animals in contralateral and ipsilateral SGZs (contralateral and ipsilateral $p < 0.0001$).

In the vDG, main effects of group[af-i] and sex[af-ii] were observed as well as significant interactions between group and sex[af-iv], group and side [af-iv] and sex and side[af-vi] (Figure 3.14A). Post hoc analysis revealed similar differences as in the dDG: an increase in Ki-67$^+$ cells in the contralateral and ipsilateral SGZs of male rmTBI animals (contralateral $p = 0.0003$; ipsilateral $p < 0.0001$) and significantly higher numbers of Ki-67$^+$ cells in male rmTBI animals as compared to female rmTBI animals (contralateral $p = 0.0007$; ipsilateral $p < 0.0001$). Additionally, there was a significantly higher number of Ki-67$^+$ cells in the ipsilateral SGZ in male rmTBI animals compared to the contralateral SGZ ($p = 0.007$).

A two-way mixed effects ANOVA with sex and side as factors for fold change in Ki-67$^+$ cells in rmTBI animals compared to shams revealed main effects of sex and side as well as significant interactions between sex and side in the dDG[ae] and vDG[ag] (Figure 3.13B) and Figure 3.14B). Post hoc analysis in the dDG revealed a significantly higher fold change in Ki-67$^+$ cell number from sham in male rmTBI animals compared to females in both SGZs (contralateral $p = 0.004$; ipsilateral $p < 0.0001$) and a significantly greater fold change from sham in the ipsilateral SGZ compared to the

contralateral SGZ in males ($p = 0.002$). Post hoc analysis for the vDG revealed similar increases: an increase in both SGZs in male rmTBI animals compared to females (contralateral $p = 0.0006$; ipsi $p < 0.0001$) and a greater fold change from sham in the ipsilateral SGZ in male rmTBI animals compared to the contralateral SGZ ($p < 0.0001$).

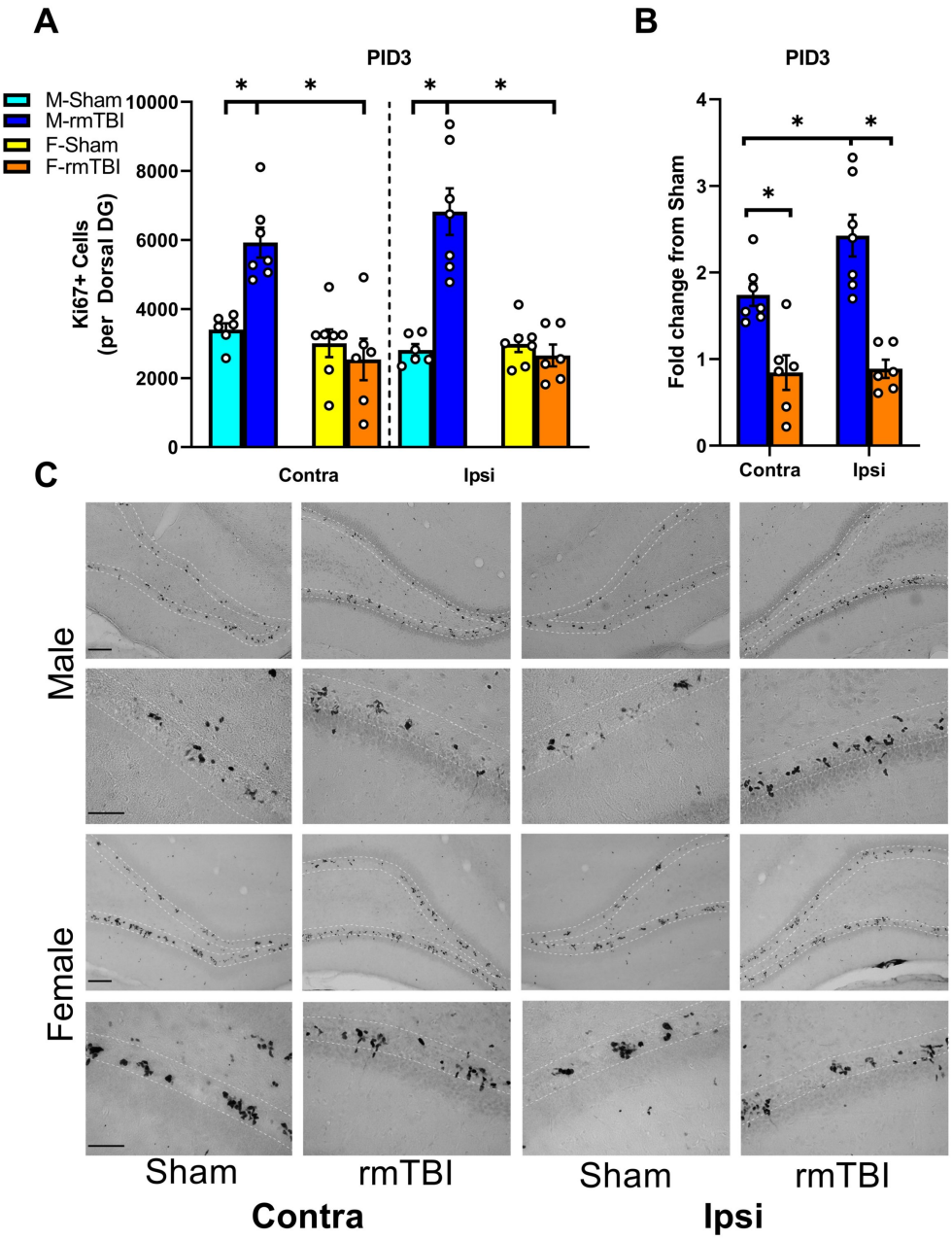


Figure 3.13: Increase in Ki-67$^+$ cells in the contralateral and ipsilateral dorsal SGZ of rmTBI animals at PID 3 in males but not females. A. A three-way mixed effects ANOVA revealed a main effect of group ($F(1,22) = 13.69$, $p = 0.001$) and a main effect of sex ($F(1,22) = 25.19$, $p < 0.0001$). There were significant interactions between group and sex ($F(1,22) = 22.50$, $p < 0.0001$) and group and side ($F(1,22) = 4.935$, $p = 0.037$). Post hoc analysis revealed an increase in BrdU$^+$ cells in the ipsilateral and contralateral SGZ of male rmTBI animals compared to shams. Male rmTBI animals exhibited significantly higher numbers of Ki-67$^+$ cells in both hemispheres compared to female rmTBI animals. There were no significant differences between female rmTBI animals and female shams in either hemisphere. **B.** A two-way mixed effects ANOVA revealed a main effect of side ($F(1,11) = 9.732$, $p = 0.010$), a main effect of sex ($F(1,11) = 28.65$, $p = 0.0002$) and a significant interaction between sex and side ($F(1,11) = 7.528$, $p = 0.019$). Post hoc analysis revealed greater fold change from sham in male rmTBI animals compared to females and a greater fold change in the ipsilateral SGZ compared to the contralateral SGZ. **C.** Representative images taken at 10X and 40X: scale bars are 50 μm and 25 μm respectively. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

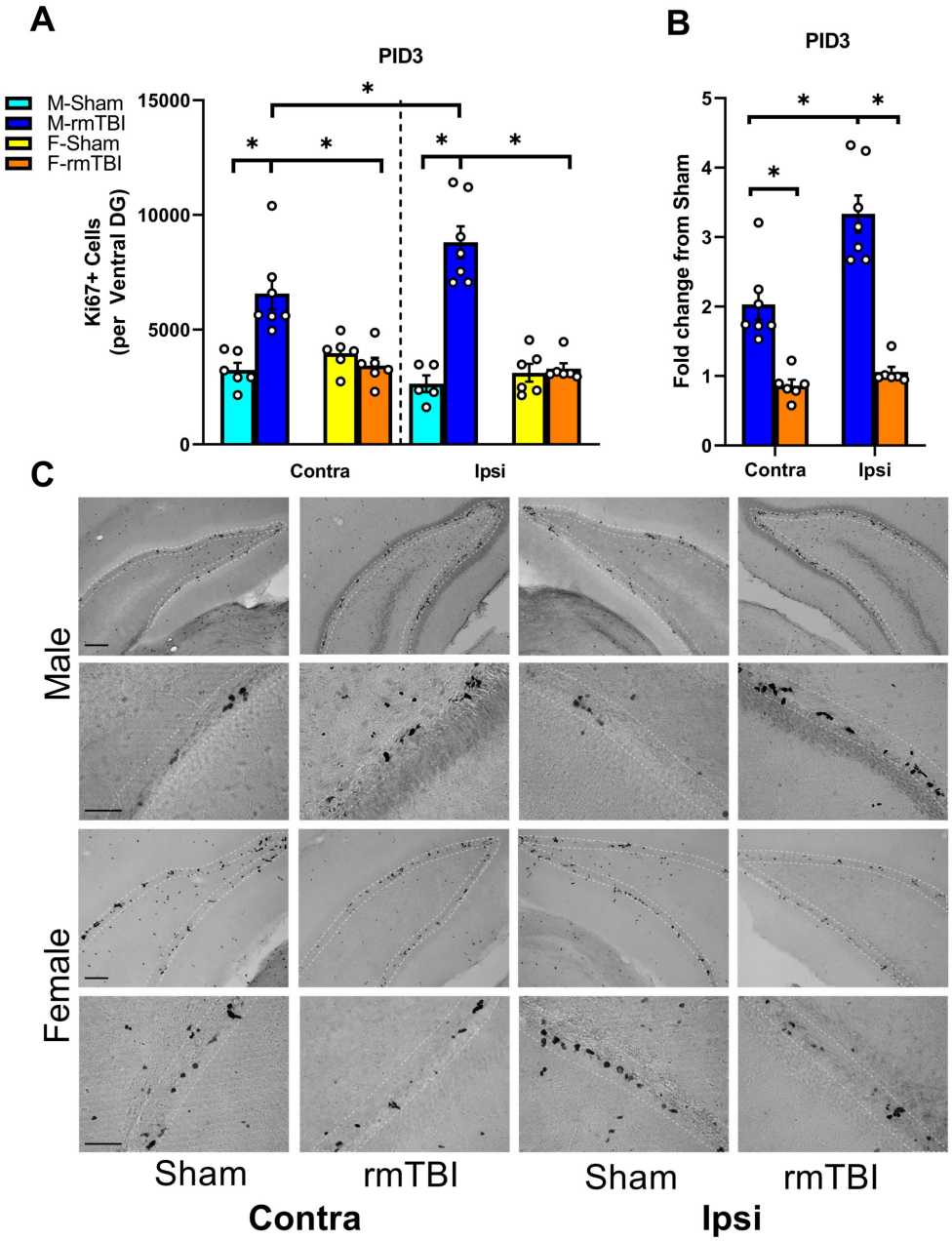


Figure 3.14: Increase in Ki-67$^+$ cells in the contralateral and ipsilateral ventral SGZ of rmTBI animals at PID 3 in males but not females. A. A three-way mixed effects ANOVA revealed a main effect of group ($F(1,21) = 33.31$, $p < 0.0001$) and a main effect of sex ($F(1,21) = 21.83$, $p = 0.0001$). There were significant interactions between group and sex ($F(1,21) = 38.80$, $p < 0.0001$), group and side ($F(1,20) = 10.18$, $p = 0.005$) and sex and side ($F(1,20) = 5.379$, $p = 0.031$). Post hoc analysis revealed an increase in BrdU$^+$ cells in the ipsilateral and contralateral SGZ of male rmTBI animals compared to shams. Male rmTBI animals exhibited significantly higher numbers of Ki-67$^+$ cells in both hemispheres compared to female rmTBI animals. There were also more Ki-67$^+$ cells in the ipsilateral SGZ of male rmTBI animals compared to the contralateral SGZ. There were no significant differences between female rmTBI animals and female shams in either hemisphere. **B.** A two-way mixed effects ANOVA revealed a main effect of side ($F(1,11) = 26.86$, $p = 0.0003$) and a main effect of sex ($F(1,11) = 55.30$, $p < 0.0001$) as well as a significant interaction between side and sex ($F(1,11) = 14.72$, $p = 0.003$). As in the dorsal SGZ, post hoc analysis revealed greater fold change from sham in male rmTBI animals compared to females and a greater fold change in the ipsilateral SGZ compared to the contralateral SGZ. **C.** Representative images taken at 10X and 40X: scale bars are 50 μm and 25 μm respectively. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

Table 3.11: Statistical Analysis for Ki-67 Profile Counts PID 3

Superscript in Text	Figure	Test	Factors	Result
ad	3.13A	Three factor mixed ANOVA	i-Main effect Group	F (1, 22) = 13.69, p = 0.001
			ii-Main effect Sex	F (1, 22) = 25.19, p < 0.0001
			iii-Main effect Side	F (1, 22) = 0.311, p = 0.583
			iv-Group*Sex	F (1, 22) = 22.50, p < 0.0001
			v-Group*Side	F (1, 22) = 4.935, p = 0.037
			vi-Sex*Side	F (1, 22) = 0.079, p = 0.782
			vii-Group*Sex*Side	F (1, 22) = 3.439, p = 0.077
ae	3.13B	Two factor mixed ANOVA	i-Main effect Side	F (1, 11) = 9.732, p = 0.010
			ii-Main effect Sex	F (1, 11) = 28.65, p = 0.0002
			iii-Side*Sex	F (1, 11) = 7.528, p = 0.019
af	3.14A	Three factor mixed ANOVA	i-Main effect Group	F (1, 21) = 33.31, p < 0.0001
			ii-Main effect Sex	F (1, 21) = 21.83, p = 0.0001
			iii-Main effect Side	F (1, 20) = 0.305, p = 0.587
			iv-Group*Sex	F (1, 21) = 38.80, p < 0.0001
			v-Group*Side	F (1, 20) = 10.18, p = 0.005
			vi-Sex*Side	F (1, 20) = 5.379, p = 0.031
			vii-Group*Sex*Side	F (1, 20) = 3.601, p = 0.072
ag	3.14B	Two factor mixed ANOVA	i-Main effect Side	F (1, 11) = 26.86, p = 0.0003
			ii-Main effect Sex	F (1, 11) = 55.30, p < 0.0001
			iii-Side*Sex	F (1, 11) = 14.72, p = 0.003

Table 3.12: Mean Values Ki-67 Profile Counts PID 3

Sex	SGZ	Dorsal Mean ± SEM	Ventral Mean ± SEM
Male	Sham Contra	3402 ± 184.7	3240 ± 313.3
	TBI Contra	5926 ± 434.3	6578 ± 698.8
	Sham Ipsi	2812 ± 171.5	2641 ± 363.5
	TBI Ipsi	6822 ± 676.7	8807 ± 700
Female	Sham Contra	3008 ± 400.2	3976 ± 298.7
	TBI Contra	2538 ± 601.4	3432 ± 340.9
	Sham Ipsi	2992 ± 237.8	3119 ± 387
	TBI Ipsi	2655 ± 316.4	3299 ± 235.5
Fold Change	Male Contra	1.742 ± 0.128	2.03 ± 0.216
	Female Contra	0.844 ± 0.2	0.863 ± 0.086
	Male Ipsi	2.426 ± 0.241	3.334 ± 0.265
	Female Ipsi	0.888 ± 0.106	1.058 ± 0.076

3.10 Cell proliferation is not restricted to the DG at PID 1

The inital aim of this study was to investigate changes in cellular proliferation in the neurogenic niche after rmTBI. However, a robust and widespread proliferative response, identified with both Ki-67 and BrdU staining, was observed at PID 1; not restricted to the SGZ or even the hippocampus (see Appendix Figure 5.1 and Figure 5.2 for whole tissue slices). Though widespread proliferation was observed, the response was most robust in the hippocampus. Therefore the following analysis was focused on this region. To quantify this response, 10X images of the whole hippocampus were taken of sections stained with BrdU. The region was carefully traced and a particle analysis protocol was applied to the overlay in imageJ.

At PID 1, a three-way mixed effects ANOVA with group, sex and side as factors revealed main effects of group[ah-i], sex[ah-ii] and side[ah-iii]. The same test applied to the ventral hippocampus revealed main effects of group[ai-i], sex[ai-ii] and side[ai-iii] as well (Figure 3.15A, Figure 3.15B). In both the dorsal and ventral hippocampus, all two- and three-way interactions between group, sex and side factors were significant (dorsal hippocampus[ah]; ventral hippocampus[ai]). In the dorsal hippocampus, post hoc analysis revealed a significant increase in $BrdU^+$ cells per mm^2 in the contralateral and ipsilateral hippocampus in male rmTBI animals compared to shams (contralateral $p = 0.033$; ipsilateral $p < 0.0001$).There was a significantly higher number of $BrdU^+$ cells per mm^2 in the ipsilateral hippocampus in male rmTBI animals compared to the contralateral hippocampus ($p < 0.0001$). In the ventral hippocampus, post hoc tests revealed an increased number of $BrdU^+$ cells per mm^2 in the ipsilateral hippocampus of male rmTBI animals compared to shams ($p = 0.0002$) as well as a significantly higher number of $BrdU^+$ cells per mm^2 in the ipsilateral hippocampus as compared to the contralateral hippocampus in male rmTBI animals ($p = 0.0002$).

The effect of rmTBI on $BrdU^+$ cells per mm^2 at PID 3 followed that of $BrdU^+$ cell profile

counts in the SGZ; there was overall a return to sham levels in male rmTBI animals (Figure 3.16A, Figure 3.16B). A three-way mixed effects ANOVA for the effects of group, sex and side on $BrdU^+$ cells per mm^2 in the dorsal hippocampus revealed a significant main effect of group[aj-i]. Analysis in the ventral hippocampus revealed a significant main effect of group[ak-i] and a main effect of side[ak-iii].

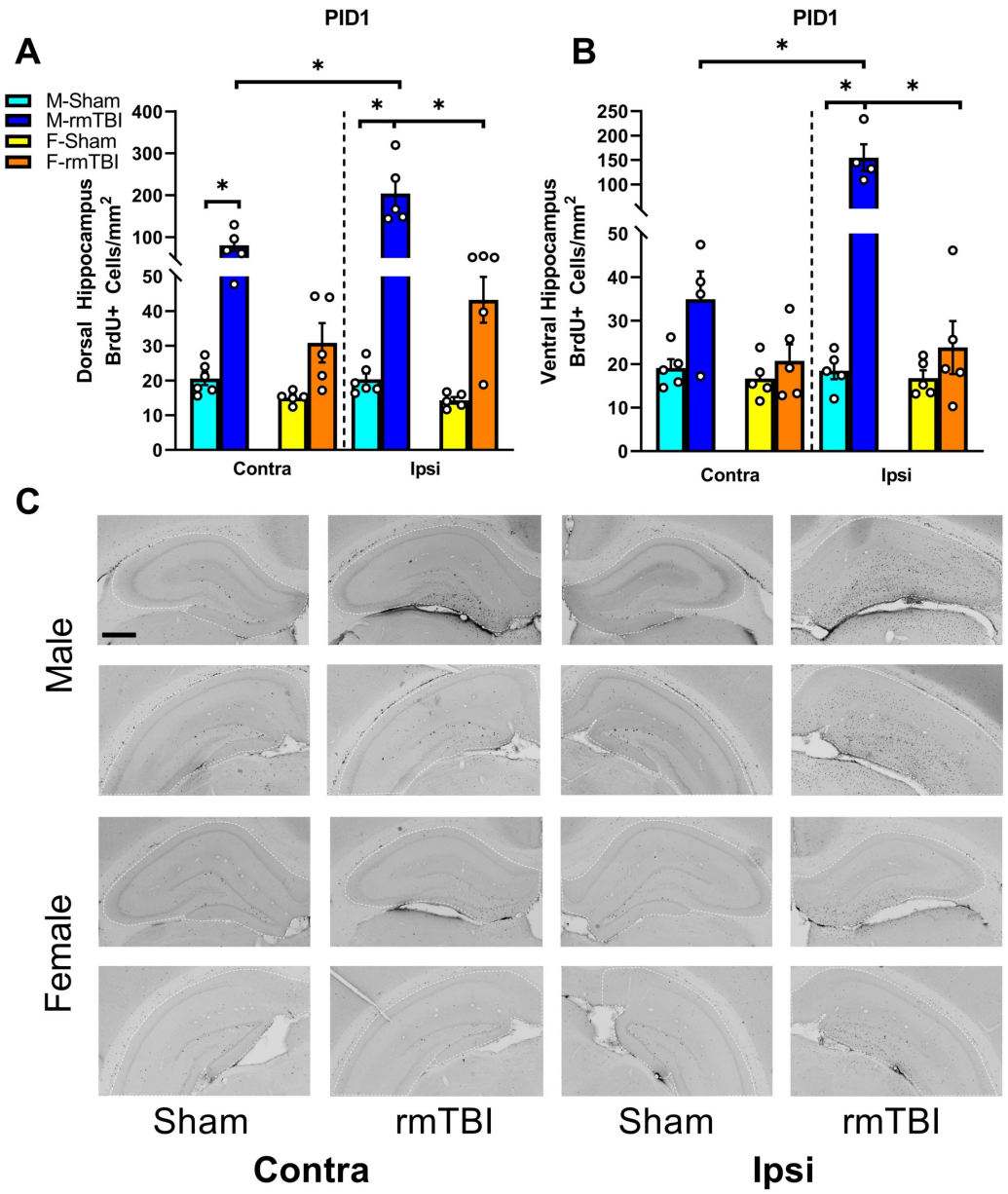


Figure 3.15: Proliferation is diffuse at PID 1, especially in males. A. $BrdU^+$ cells per mm^2 in the dorsal hippocampus at PID 1: A three-way mixed effects ANOVA to investigate the diffuse response of $BrdU^+$ cells in the dorsal hippocampus of rmTBI animals revealed main effects of group ($F(1,17) = 37.54$, $p < 0.0001$), sex ($F(1,17) = 22.21$, $p = 0.0002$), and side ($F(1,17) = 46.83$, $p < 0.0001$). All interactions analyzed between group, side and sex factors were significant. Post hoc analysis revealed significantly more $BrdU^+$ cells per mm^2 in rmTBI males compared to shams in the contralateral and ipsilateral hippocampus. Male rmTBI animals had significantly more $BrdU^+$ cells per mm^2 in the ipsilateral hippocampus compared to female rmTBI animals and compared to male rmTBI animals in the contralateral hippocampus. **B.** $BrdU^+$ cells per mm^2 in the ventral hippocampus at PID 1: A three-way mixed effects ANOVA to investigate the diffuse response of $BrdU^+$ cells in the dorsal hippocampus of rmTBI animals revealed main effects of group ($F(1,16) = 7.296$, $p = 0.016$), sex ($F(1,16) = 5.765$, $p = 0.029$), and side ($F(1,16) = 10.28$, $p = 0.005$). All interactions analyzed between group, side and sex factors were significant. Post hoc analysis revealed significantly more $BrdU^+$ cells per mm^2 in rmTBI males compared to shams in the ipsilateral hippocampus. Male rmTBI animals had significantly more $BrdU^+$ cells per mm^2 in the ipsilateral hippocampus compared to female rmTBI animals and compared to male rmTBI animals in the contralateral hippocampus. **C.** Representative images taken at 10X, scale bar is 500 μm. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

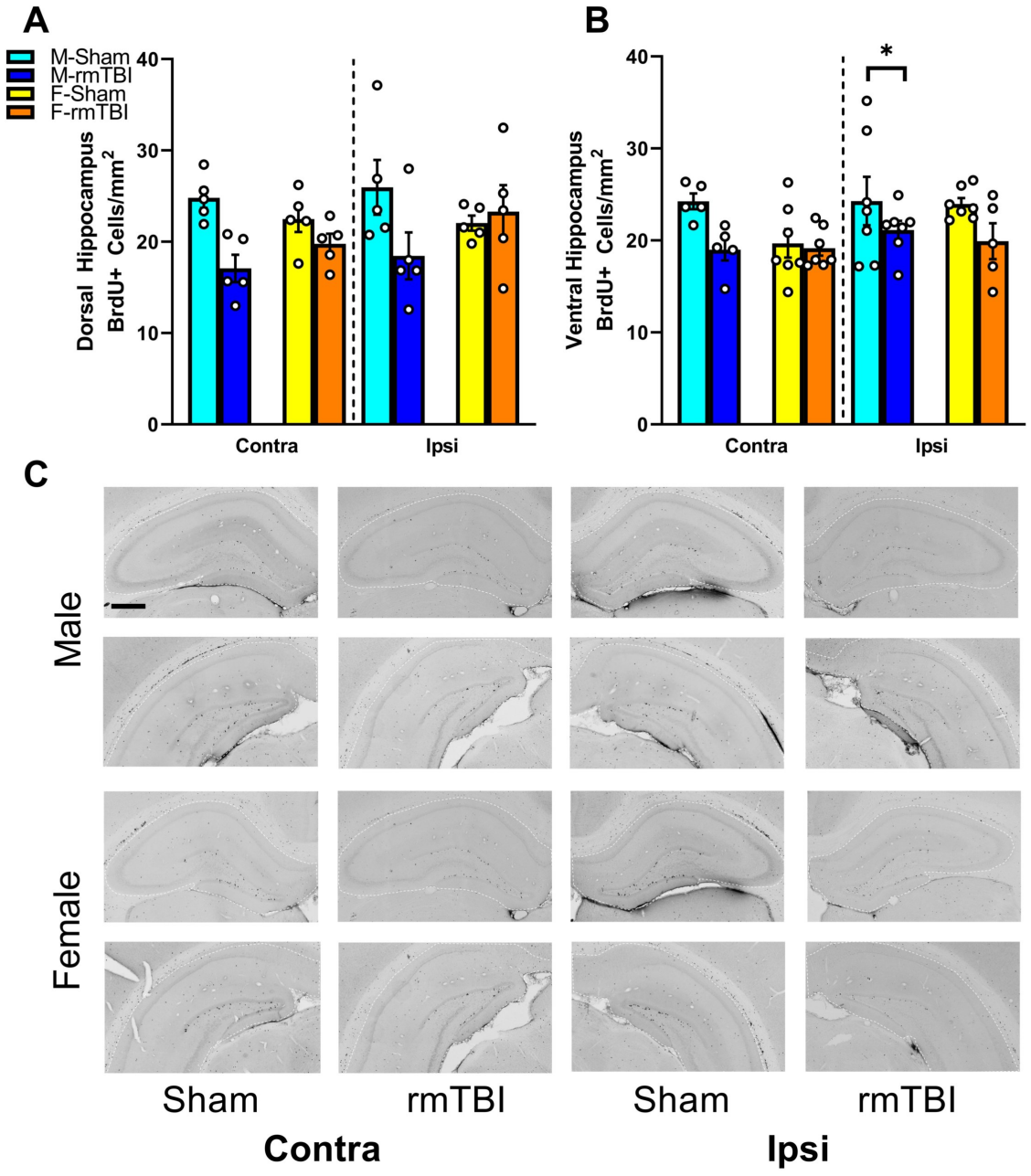


Figure 3.16: BrdU$^+$ cells in the dorsal and ventral hippocampus are comparable to sham level at PID 3. A. A three-way mixed effects ANOVA revealed a main effects of group ($F(1,16) = 16.42$, $p = 0.001$) on the number of BrdU$^+$ cells per mm^2 in the dorsal hippocampus at PID 3. **B.** There was a main effect of group ($F(1,16) = 12.68$, $p = 0.003$) and side ($F(1,16) = 5.282$, $p = 0.035$) on BrdU$^+$ cell per mm^2 in the ventral hippocampus and post hoc analysis revealed a trend towards lower numbers of BrdU$^+$ cells in the ipsilateral hippocampus of rmTBI animals compared to shams ($p = 0.067$). **C.** Representative images taken at 10X, scale bar is 500 μm. Data is represented as mean ± SEM and individual points represent 1 animal.

Table 3.13: Statistical Analysis for BrdU Threshold on PID 1 and PID 3

Superscript in Text	Figure	Test	Factors	Result
ah	3.15A	Three factor mixed ANOVA	i-Main effect Group	$F(1, 17) = 37.54$, $p < 0.0001$
			ii-Main effect Sex	$F(1, 17) = 22.21$, $p = 0.0002$
			iii-Main effect Side	$F(1, 17) = 46.83$, $p < 0.0001$
			iv-Group*Sex	$F(1, 17) = 17.82$ $p = 0.0006$
			v-Group*Side	$F(1, 17) = 48.28$, $p < 0.0001$
			vi-Sex*Side	$F(1, 17) = 32.09$, $p < 0.0001$
			vii-Group*Sex*Side	$F(1, 17) = 31.46$, $p < 0.0001$
ai	3.15B	Three factor mixed ANOVA	i-Main effect Group	$F(1, 16) = 7.296$, $p = 0.016$
			ii-Main effect Sex	$F(1, 16) = 5.765$, $p = 0.029$
			iii-Main effect Side	$F(1, 16) = 10.28$, $p = 0.005$
			iv-Group*Sex	$F(1, 16) = 4.948$, $p = 0.041$
			v-Group*Side	$F(1, 16) = 10.49$, $p = 0.005$
			vi-Sex*Side	$F(1, 16) = 9.003$, $p = 0.009$
			vii-Group*Sex*Side	$F(1, 16) = 9.278$, $p = 0.008$
aj	3.16A	Three factor mixed ANOVA	i-Main effect Group	$F(1, 16) = 16.42$, $p = 0.001$
			ii-Main effect Sex	$F(1, 16) = 0.038$, $p = 0.848$
			iii-Main effect Side	$F(1, 16) = 1.867$, $p = 0.191$
			iv-Group*Sex	$F(1, 16) = 4.192$, $p = 0.057$
			v-Group*Side	$F(1, 16) = 1.033$, $p = 0.325$
			vi-Sex*Side	$F(1, 16) = 0.007$, $p = 0.936$
			vii-Group*Sex*Side	$F(1, 16) = 0.308$, $p = 0.587$
ak	3.16B	Three factor mixed ANOVA	i-Main effect Group	$F(1, 16) = 12.68$, $p = 0.003$
			ii-Main effect Sex	$F(1, 16) = 1.267$, $p = 0.277$
			iii-Main effect Side	$F(1, 16) = 5.282$, $p = 0.035$
			iv-Group*Sex	$F(1, 16) = 1.250$, $p = 0.280$
			v-Group*Side	$F(1, 16) = 1.451$, $p = 0.246$
			vi-Sex*Side	$F(1, 16) = 0.012$, $p = 0.914$
			vii-Group*Sex*Side	$F(1, 16) = 0.081$, $p = 0.780$

Table 3.14: Mean Values BrdU Threshold PID 1

Sex	SGZ	Dorsal Mean ± SEM	Ventral Mean ± SEM
Male	Sham Contra	20.55 ± 1.843	19.1 ± 2.032
	TBI Contra	80.49 ± 14.55	34.93 ± 6.386
	Sham Ipsi	20.31 ± 1.758	18.5 ± 1.959
	TBI Ipsi	203.7 ± 33.79	155 ± 27.36
Female	Sham Contra	15.05 ± 0.7936	16.7 ± 3.808
	TBI Contra	30.87 ± 5.656	20.75 ± 1.959
	Sham Ipsi	14.26 ± 0.938	16.8 ± 1.796
	TBI Ipsi	43.24 ± 6.624	23.82 ± 6.082

Table 3.15: Mean Values BrdU Threshold PID 3

Sex	SGZ	Dorsal Mean ± SEM	Ventral Mean ± SEM
Male	Sham Contra	24.8 ± 1.104	24.24 ± 0.8594
	TBI Contra	17.08 ± 1.507	18.99 ± 1.168
	Sham Ipsi	25.96 ± 2.989	27.11 ± 2.731
	TBI Ipsi	18.45 ± 2.56	20.27 ± 1.088
Female	Sham Contra	22.48 ± 1.407	21.2 ± 1.663
	TBI Contra	19.78 ± 1.086	19.32 ± 1.129
	Sham Ipsi	22.04 ± 0.818	24.36 ± 0.8249
	TBI Ipsi	23.3 ± 2.894	19.93 ± 1.949

3.11 Characterizing cell types in the SGZ

Because the SGZ of the DG is the site of cell proliferation and neurogenesis, it has been shown in normal rat brains that the majority of these cells will co-label with early neuronal markers or immature neuron markers (Cameron & Mckay, 2001). Other cell types that may be proliferating in the DG include activated radial glial cells, progenitor cells (*i.e.* cells that do not yet express early neuronal or immature neuronal markers) or microglia and oligodendrocytes. Few, if any astrocytes are normally dividing (Cameron & Mckay, 2001). With a disruption to the brain, like rmTBI, the increased number of dividing cells may be comprised more heavily of glial cells. Alternatively, progenitor cells may experience an accelerated cell cycle, or more progenitors may be actively proliferating (Hayes et al., 2018). To investigate the cell type of dividing cells in male rmTBI animals at PID 1, a triple stain analysis with BrdU, Iba1 and GFAP was performed to identify dividing microglia and macrophages and/or dividing astrocytes or radial glial cells (Figure 3.17). To this end, proliferating cells that are part of an inflammatory rather than neurogenic (*i.e.* proliferation of progenitor cells) response could be identified.

1 dorsal and 1 ventral slice (approx. Bregma -3.36 mm and Bregma -5.28 mm respectively) were chosen for imaging from n = 3 animals per group. Data is presented with dorsal and ventral slices averaged and presented as an n of 1. All $BrdU^+$ cells were identified in the DG from 20X stacks obtained via confocal microscopy. Exhaustive sampling was chosen due to the rarity of $BrdU^+$ cells in sham animals. $BrdU^+$ cells were then characterized as $BrdU^+/Iba1^-/GFAP^-$ (not a dividing glial cell), $BrdU^+/Iba1^+/GFAP^-$ (dividing microglia or macrophage) or $BrdU^+/Iba1^-/GFAP^+$ (dividing astrocyte or radial glial cell). Very few $BrdU^+/Iba1^-/GFAP^+$ cells were identified, however many cells at PID 1 were $BrdU^+/Iba1^+/GFAP^-$ indicating an inflammatory profile rather than neurogenic proliferative response (Figure 3.18, Figure 3.19). While this triple labeling experiment provides

information as to whether the majority of these cells are glial in nature, it cannot provide further information about neuronal fate or neuronal subtype. Further triple stain analysis with progenitor cell or immature neuron markers such as Sox2 or DCX would enable a more definitive conclusion as to the subtype of $BrdU^+/Iba1^-/GFAP^-$ cells.

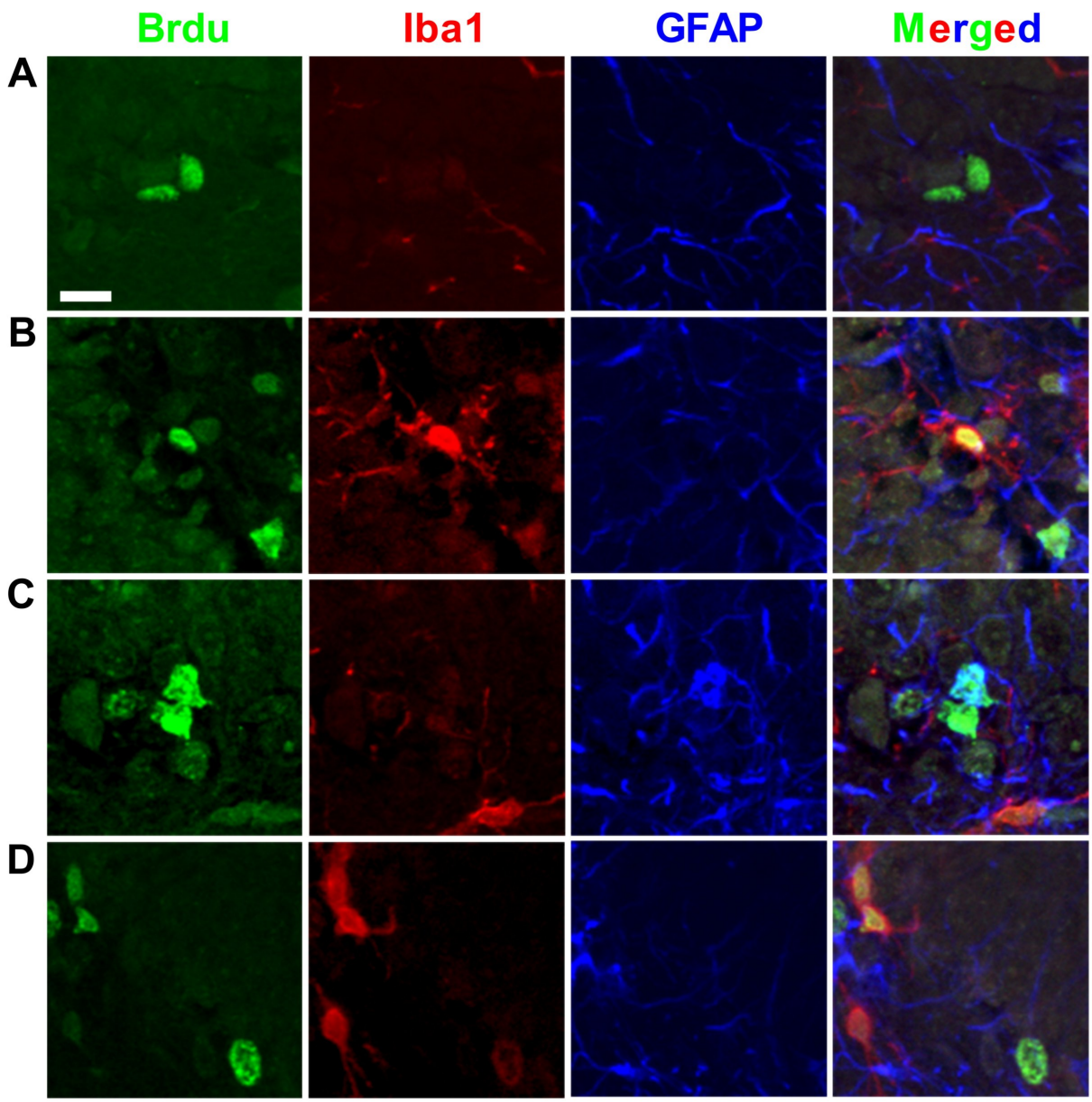


Figure 3.17: Representative images of cell types in the SGZ. A. $BrdU^+$ cells **B.** $BrdU^+$ and $Iba1^+$ cell **C.** $BrdU^+$ and $GFAP^+$ cell **D.** Two $BrdU^+/Iba1^+$ cells and one $BrdU^+$ cell. Scale bar is 10 μm

3.12 There is a greater proportion of BrdU$^+$/Iba1$^+$ cells in rmTBI animals at PID 1 compared to shams

At PID 1, a three-way mixed effects ANOVA with group, sex and side as factors revealed significant main effects of group[al-i], sex[al-ii] and side[al-iii] on percentage of BrdU$^+$/Iba1$^+$ cells in the SGZ relative to total BrdU$^+$ cells (Figure 3.18A). There were also significant interactions between group and sex[al-iv] and group and side[al-v] on the percentage of BrdU$^+$/Iba1$^+$ cells in the SGZ. Post hoc analysis revealed a significantly higher percentage of BrdU$^+$/Iba1$^+$ cells in the SGZ of male rmTBI animals in both contralateral and ipsilateral hemispheres compared to shams ($p = 0.0004$ and $p < 0.0001$ respectively) . Additionally, the ipsilateral SGZ of male rmTBI animals had a significantly higher percentage of BrdU$^+$/Iba1$^+$ cells as compared to the contralateral SGZ ($p = 0.006$). Interestingly, the ipsilateral SGZ in female rmTBI animals had a significantly higher percentage of BrdU$^+$/Iba1$^+$ cells as compared to shams ($p = 0.0001$) despite a non significant increase in BrdU$^+$ cells as shown with the DAB staining experiments.

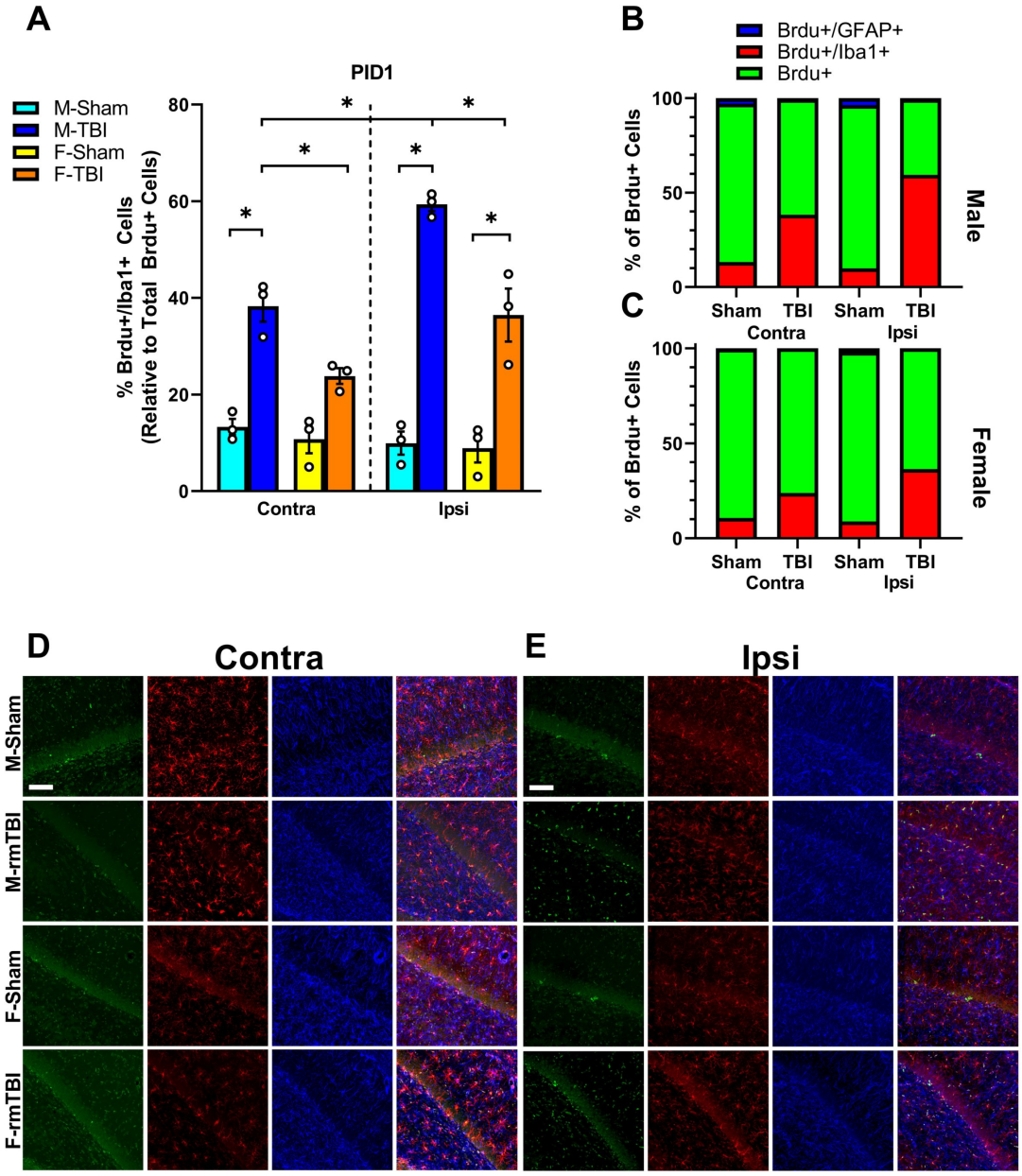


Figure 3.18: Increased proportion of BrdU$^+$/Iba1$^+$ cells at PID 1 in the SGZ of male and female rmTBI animals compared to shams. A. Quantification of proportion of BrdU$^+$ cells that were colocalized with Iba1: Three-way mixed effects ANOVA revealed a main effect of group ($F(1,8) = 152.4$, $p < 0.0001$), sex ($F(1,8) = 19.35$, $p = 0.002$) and side ($F(1, 8) = 14.64$, $p = 0.005$).There were significant interactions between group and sex ($F(1,8) = 13.16$, $p = 0.007$) and group and side ($F(1,8) = 27.32$, $p = 0.001$). Post hoc analysis revealed an increased proportion of BrdU$^+$/Iba1$^+$ cells in male rmTBI animals in the contralateral and ipsilateral SGZ compared to shams. Female rmTBI animals also showed a greater proportion of BrdU$^+$/Iba1$^+$ cells in the ipsilateral SGZ compared to shams. There was a greater proportion of BrdU$^+$/Iba1$^+$ cells in the ipsilateral SGZ of male rmTBI animals compared to the contralateral SGZ of male rmTBI animals. Additionally, the proportion of BrdU$^+$/Iba1$^+$ cells was greater in the contralateral and ipsilateral SGZ of male rmTBI animals compared to female rmTBI animals. **B,C.**% of each cell type in males **(B)** and females **(C)** at PID 1. There were very few BrdU$^+$/GFAP$^+$ cells and the majority were BrdU$^+$ only cells. **D,E.** Representative images of cell types in the contralateral **(D)** and ipsilateral **(E)** SGZ. Scale bar is 100 μm. Data is represented as mean ± SEM and individual points represent 1 animal.

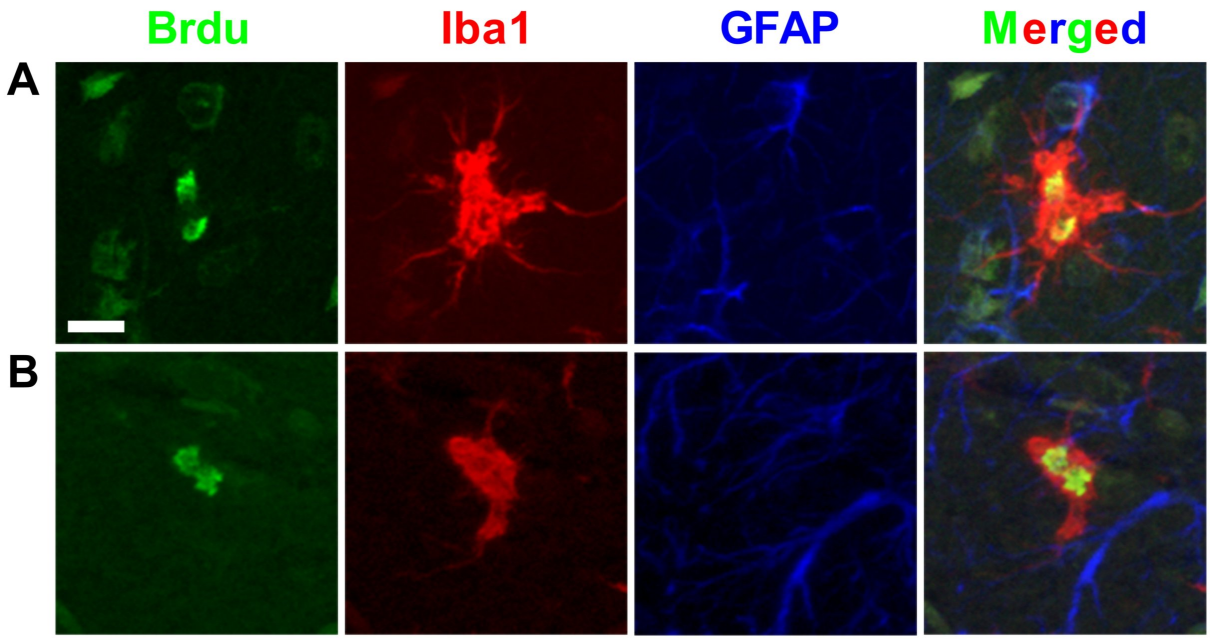


Figure 3.19: Dividing Iba1$^+$ cells after rmTBI. Actively dividing Iba1$^+$ cells were observed in both male **(A)** and female **(B)** rmTBI animals. Scale bar is 10 μm.

3.13 There is no difference in proportion of BrdU$^+$/Iba1$^+$ cells in rmTBI animals at PID 3 compared to shams

This proportion was also found to have returned to sham levels by PID 3. No significant main effects or significant interactions were observed^{am} (Figure 3.20A); in male and female animals, the majority of cells were BrdU$^+$/Iba1$^-$/GFAP$^-$ (Figure 3.20B, Figure 3.20C).

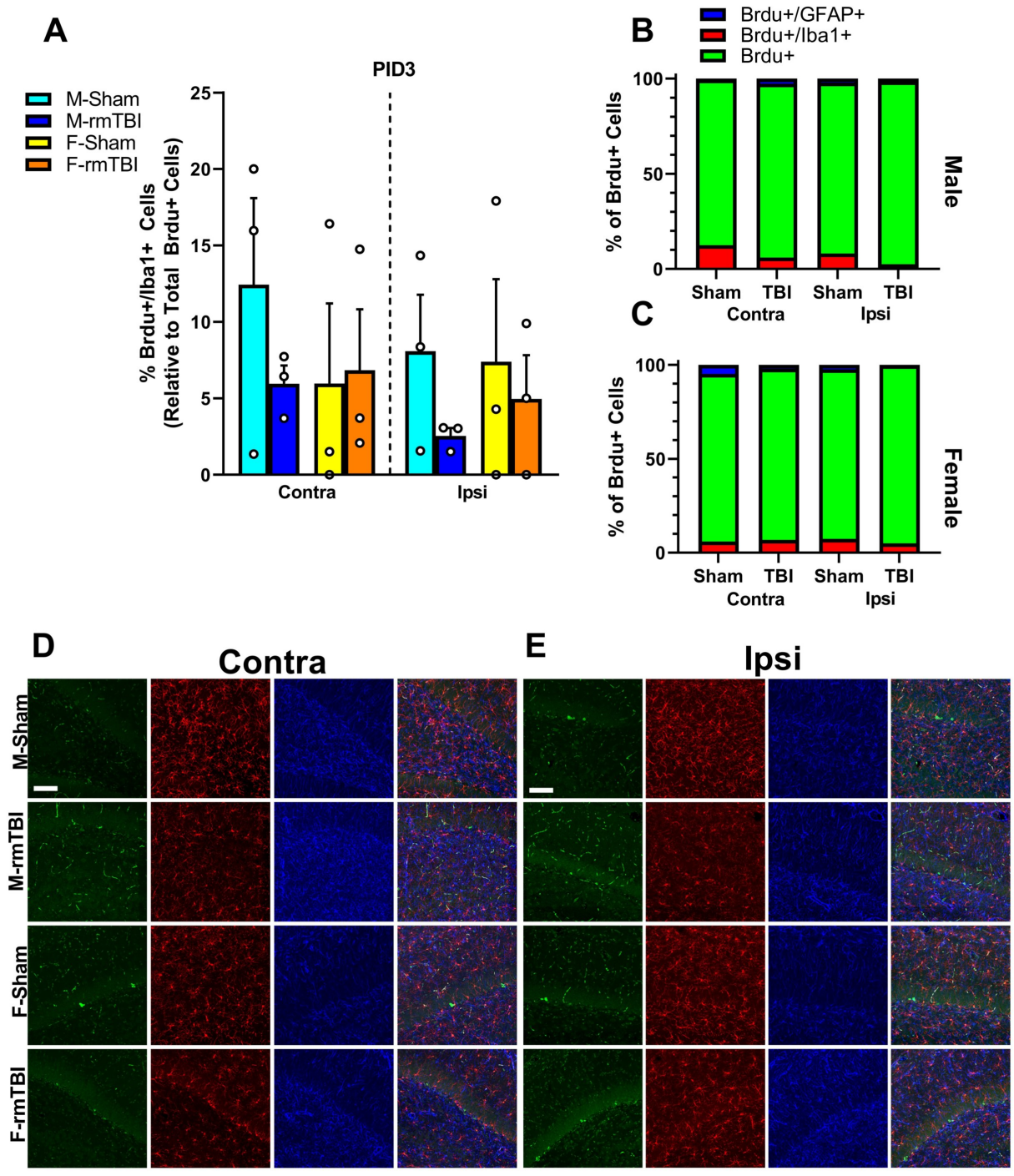


Figure 3.20: There was no difference in proportion of BrdU$^+$/Iba1$^+$ cells in the SGZ of rmTBI animals at PID 3 compared to shams. A. A three-way mixed effects ANOVA revealed no main effects of group, side or sex in the proportion of BrdU$^+$ cells that were BrdU$^+$/Iba1$^+$ in the SGZ at PID 3. **B,C.** The majority of BrdU$^+$ cells did not colocalize with GFAP or Iba1 in males **(B)** or females **(C)**. **(D, E).** Representative images of cell types in the contralateral **(D)** and ipsilateral **(E)** SGZ. Scale bar is 100 μm. Data is represented as mean $\pm$ SEM and individual points represent 1 animal.

Table 3.16: Statistical Analysis for SGZ Colocalization

Superscript in Text	Figure	Test	Factors	Result
al	3.18A	Three factor mixed ANOVA	i-Main effect Group	$F(1, 8) = 152.4$, $p < 0.0001$
			ii-Main effect Sex	$F(1, 8) = 19.35$, $p = 0.002$
			iii-Main effect Side	$F(1, 8) = 14.64$, $p = 0.005$
			iv-Group*Sex	$F(1, 8) = 13.16$, $p = 0.007$
			v-Group*Side	$F(1, 8) = 27.32$, $p = 0.001$
			vi-Sex*Side	$F(1, 8) = 0.864$, $p = 0.380$
			vii-Group*Sex*Side	$F(1, 8) = 1.794$, $p = 0.217$
am	3.20A	Three factor mixed ANOVA	i-Main effect Group	$F(1, 8) = 0.757$, $p = 0.410$
			ii-Main effect Sex	$F(1, 8) = 0.060$, $p = 0.812$
			iii-Main effect Side	$F(1, 8) = 5.621$, $p = 0.045$
			iv-Group*Sex	$F(1, 8) = 0.449$, $p = 0.521$
			v-Group*Side	$F(1, 8) = 0.466$, $p = 0.514$
			vi-Sex*Side	$F(1, 8) = 4.445$, $p = 0.068$
			vii-Group*Sex*Side	$F(1, 8) = 1.497$, $p = 0.256$

Table 3.17: Mean Values SGZ Colocalization

Sex	SGZ	Dorsal Mean ± SEM	Ventral Mean ± SEM
Male	Sham Contra	13.309 ± 1.674	12.422 ± 5.666
	TBI Contra	38.295 ± 3.239	5.957 ± 1.19
	Sham Ipsi	9.938 ± 2.39	8.091 ± 3.693
	TBI Ipsi	59.37 ± 1.4	2.542 ± 0.514
Female	Sham Contra	10.751 ± 2.911	5.977 ± 5.238
	TBI Contra	23.821 ± 1.628	6.85 ± 3.984
	Sham Ipsi	8.906 ± 2.973	7.402 ± 5.4
	TBI Ipsi	36.448 ± 5.495	4.969 ± 2.859

3.14 Iba1$^+$ cells exhibit various morphologies in the SGZ after rmTBI

After TBI microglia respond and become activated, going from a ramified morphology ("surveying" microglia) to an amoeboid morphology (Donat et al., 2017). Although morphometric analysis was not performed in this study, varying microglial/macrophage morphology was observed in this model of rmTBI. rmTBI animals appeared to have more bushy microglia/macophages or ramified with rough prolongations (Figure 3.21A, Figure 3.21B), compared to the ramified morphology of microglial/macrophages in sham animals (Figure 3.21C). Amoeboid round morphology was also seen in some male rmTBI animals (Figure 3.21D). Future studies using this model may wish to investigate and quantify potential sex differences in the microglial/macrophage response, including morphology and activation of microglia. Indeed, some animal models of TBI have shown sex differences in the morphology and activation of microglia (Villapol et al., 2017).

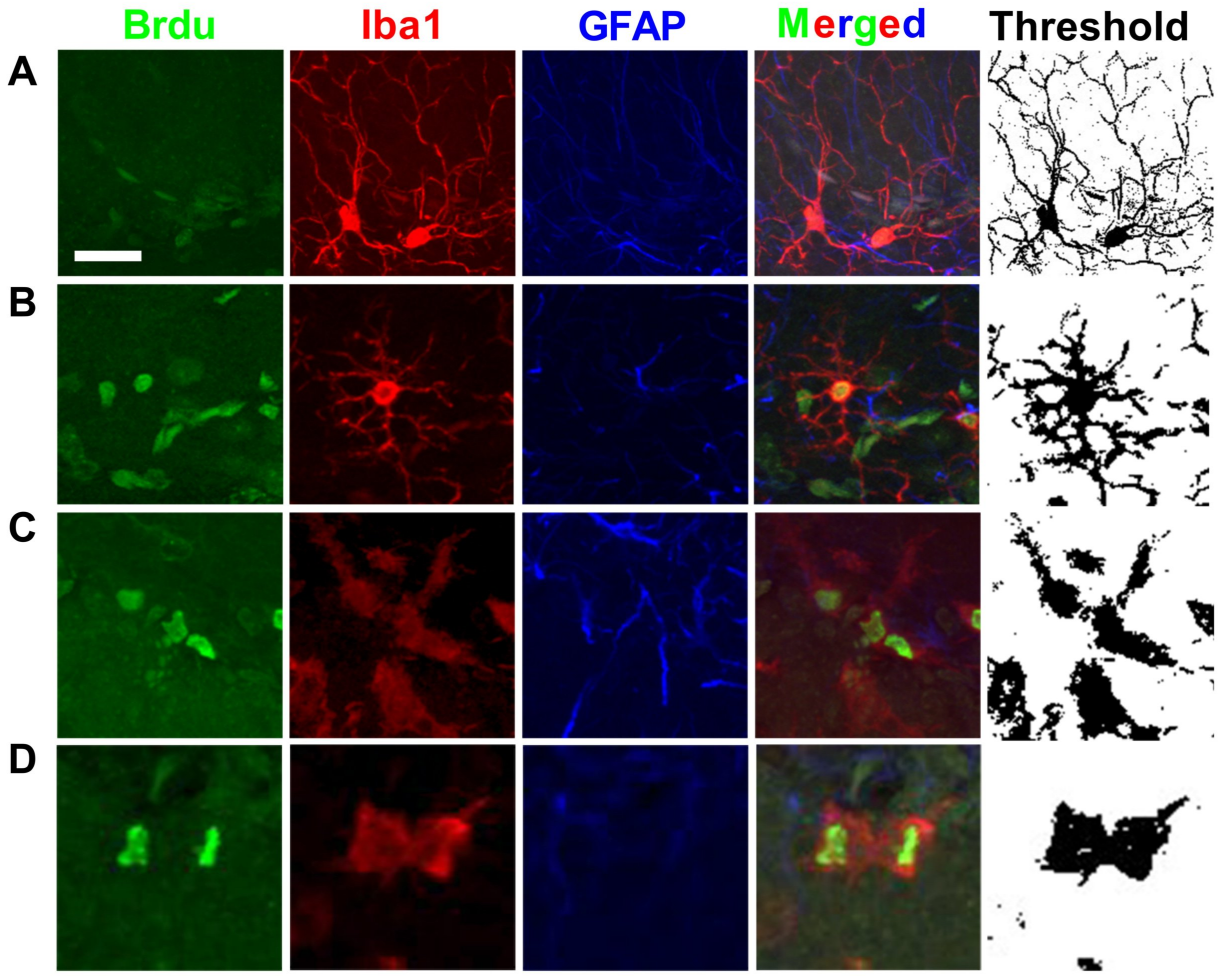


Figure 3.21: Representative images of different microglia/macrophage morphology in sham and rmTBI animals. A. A ramified Iba1$^+$ cell taken from a sham animal. **B.** Ramified Iba1$^+$ cell with rough prolongations imaged in a female rmTBI animal. **C.** Hypertrophied/bushy Iba1$^+$ cell with pseudopodia imaged in a male rmTBI animal. **D.** Amoeboid round Iba1$^+$ cell imaged in a male rmTBI animal. Scale bar is 10 μm.

CHAPTER 4

Discussion

4.1 Summary of Major Findings

The purpose of this study was to investigate the acute effects of repeat mild traumatic brain injury on cell proliferation in the adolescent rodent brain. This study also aimed to elucidate sex differences in this response. Previous studies have shown increases in cell proliferation after experimental TBI; however, none have investigated the effects of sex differences in adolescents. This is also the first time this response has been characterized in the ACHI model. The novel findings presented in this work reveal a robust proliferative response in the male rats after rmTBI. The response in female rmTBI animals was not as pronounced and analysis did not reveal significant differences in proliferation. The response in males was diffuse and heightened in several regions including the cortex, lateral thalamic nuclei, lateral ventricles, and hippocampus. Of note, the dorsal, compared to ventral hippocampus, and the ipsilateral compared to the contralateral hemisphere showed increased proliferation. Quantification was focused on the SGZ to investigate the effect of rmTBI on cell proliferation in the neurogenic niche. In the non-injured rat SGZ, proliferating cells are primarily ANPs – the rapidly dividing cell type involved in the first phase of neurogenesis. Some of these ANPs will continue to mature and become fully functional granule cells within approximately 5 weeks. However, it was found that microglia/macrophages comprise up to 60% or 40% of the cells involved in the response in the SGZ at PID 1 in both male and females, respectively. These cell types displayed activated morphologies that have been observed in the inflammatory response of other models. This study observed that injury-induced proliferation differed between males and females; thus, innate repair mechanisms or a neuroprotective response may exist to a greater extent

in the adolescent brain of one sex. Future investigation using the ACHI model will help to inform the long-term consequences of these sex differences in the acute response to rmTBI and identify what is beneficial and what is detrimental.

4.1.1 Repeat ACHI causes loss of consciousness

Male and female adolescent Long Evans rats were subjected to 8 ACHI procedures, 2 hours apart. ACHI was performed on awake animals to remove the neuroprotective effect of anesthetics. After each sham or ACHI, LOC and neurological impairment were recorded using the NAP. ACHI resulted in LOC and immediate acute neurological impairment in the absence of overt hippocampal structural damage. Apnea was not observed in any animals, but loss of toe pinch reflex and righting reflex occurred in both male and female rmTBI animals. Similar to Pinar et al. (2020), no sex differences in toe pinch reflex or righting reflex were found.

The percent of subjects that experienced LOC did not depend on the number of ACHIs administered. Therefore, in this preclinical model, the number of injuries (when spaced by at least 2 hours) do not increase injury severity as measured by LOC. In the clinical population, diagnosis of mTBI is based on a GCS score of 13-15, normal structural imaging and magnitude of LOC and amnesia dysfunction (loss of consciousness < 30 min, alteration of consciousness < 24h, post-traumatic amnesia < 1) (DeWitt et al., 2013).

Most preclinical models use latency to recover righting reflex as an analog to human LOC. However, the type and duration of anesthesia, which is administered prior to injury in most models, can influence the duration of righting reflex and LOC events (DeWitt et al., 2013; Hallam et al., 2004). Thus, a major translational benefit of the ACHI model is the ability to immediately assess LOC without the confound of anesthesia. This can enable a more clinically relevant categorization of mTBI. The extent of LOC and how it relates to outcome measures in these preclinical models

can be compared to what is actually observed in the clinical population. Currently, there is no clinical correlation between LOC and neuropsychological or functional outcome, but being able to study more subtle pathophysiological outcomes in preclinical models may uncover this further.

4.1.2 Repeat ACHI does not result in hippocampal structural damage, but can produce subdural hematoma

No hippocampal structural damage was observed in male or female rmTBI animals. Images of whole sections stained with cresyl violet appear normal, and there is no tissue loss under the injury site. In open head CCI models, there is often substantial tissue loss where the brain is impacted, and the resulting injury is moderate to severe (DeWitt et al., 2013). While conventional brain imaging techniques do not usually detect abnormalities in mTBI patients, subdural hematomas have been observed in the clinical population (Orlando et al., 2019; Wu et al., 2016). Subdural hematomas are caused by breaking of blood vessels and blood accumulation between the dura mater and arachnoid mater. In the clinical population, it is the most common type of intracranial hemorrhage observed after mTBI (Orlando et al., 2019). Subdural hematoma can be detected with CT or MRI scans, and mTBI patients with evidence of intracranial lesions like subdural hematoma are labelled as complicated mTBI (Borgaro et al., 2009; DeWitt et al., 2013). Subdural hematomas were present in 4 male and 2 female rmTBI animals. When the average total NAP score of rmTBI animals with and without bleeds were compared, no significant impairment was found to be associated with these bleeds. However, a larger sample of rmTBI animals with hematoma would need to be studied before drawing further conclusions about the correlation of subdural hematoma with loss of neurological impairment.

4.1.3 Repeat ACHI results in acute neurological deficits

The ACHI model also makes immediate assessment of neurological impairment possible. The NAP corresponds clinically to sideline testing in sports-related concussions (eg. Sport concussion assessment tool (SCAT)). The NAP measured basic reflexes and sensorimotor impairment in 4 simple tasks: startle response, limb extension, beam walk and rotating beam. Animals were scored on a scale of 0-3 with 3 being a perfect score on each task and 12 being a perfect overall score. Male and female rmTBI animals were significantly impaired compared to shams from the first ACHI to the last. Interestingly, animals did not become more impaired after each subsequent hit. This may be due to a lack of sensitivity in the NAP, or it could indicate that animals quickly plateau after reaching a certain degree of neurologic impairment. Increased injury severity or decreased intervals between each injury may be needed to elicit further impairment. The 8 ACHI in one day paradigm revealed impairment in male and female rmTBI animals compared to sex-matched shams in all NAP tasks except rotating beam: female rmTBI animals were not significantly impaired compared to sham animals. Previous studies using this model have observed similar acute neurological deficits using the NAP (Christie et al., 2019; Meconi et al., 2018; Wortman et al., 2018). Differences in each specific NAP task were also analyzed in Meconi et al. (2018), but without including sex as a factor and using a binary (pass/fail) scoring method. Similar to the results in this work, Meconi et al. (2018) showed rmTBI animals were impaired on each task.

4.1.4 rmTBI injury paradigm allows for more accurate PID 1

The injury paradigm used in this study is unique to the ACHI injury paradigms previously reported in Wortman et al. (2018) (4 ACHI, 2 injuries per day), Meconi et al. (2018) (4 ACHI, 2 injuries per day) and Pinar et al. (2020) (8 ACHI, 2 injuries per day). In each of these previous studies, injuries were administered over the course of 2-4 days. While the ACHI model and other

repeat injury models allow for flexibility in the injury paradigm, there are also new confounding variables introduced. For example, PID 1 becomes difficult to distinguish, as it technically corresponds to injuries administered on the last day but could also refer to PID 2 or PID 4 in the above paradigms. Many studies have shown that the time course of mTBI pathophysiology is very dynamic in the first few days, with inflammation reaching a peak between 48 -72 hours post injury. By administering injuries on one day, PID 1 becomes an easily defined and focal timepoint; the pathophysiological consequences of repeated injuries can be described with less temporal ambiguity. However, questions regarding injury interval - and how long after a first injury will subsequent injuries continue to worsen secondary damage - remains a critical issue. If an athlete returns to their sport before secondary damage has subsided, the consequences following another injury are intensified. Understanding the effects of a rmTBI sustained over 1 day and being able to compare outcomes to different injury paradigms can help to inform how injury frequency affects recovery.

4.1.5 Cell proliferation was increased in male rmTBI animals at PID 1

Cell proliferation was assessed in rmTBI and sham animals at PID1 and PID 3 using BrdU and Ki-67, two widely used markers of proliferation. Cells were counted in the dorsal and ventral SGZ, which were classified based on bregma coordinates along the septotemporal axis. An increase in $BrdU^+$ and $Ki\text{-}67^+$ cells in the dorsal SGZ of rmTBI males compared to shams at PID1 was observed. This increase was not observed in female rmTBI animals compared to shams, however there was a non-significant increase in proliferating cells in rmTBI females. The increased cell proliferation in rmTBI males was found in both the contralateral and ipsilateral dorsal hemispheres.

In the ventral SGZ of male rmTBI animals, there was a pronounced increase in the ipsilateral, but not contralateral, SGZ in $BrdU^+$ cells. The converse was true for $Ki\text{-}67^+$ cells: there was a significant increase in $Ki\text{-}67^+$ cells in the contralateral but not ipsilateral SGZ. There was a

substantial amount of variability in male rmTBI animals, and further analysis may characterize this response further. Taken together, this indicates a robust and diffuse response in the ventral SGZ of male rmTBI animals. Neither proliferation marker showed a change in injury-induced proliferation in the ventral SGZ of female rmTBI animals compared to shams.

Studies implicate the dorsal hippocampus in learning and spatial memory and the ventral hippocampus in emotional behaviour (Fanselow & Dong, 2010). In males, a more robust and diffuse response was observed in the dorsal hippocampus. If this acute response proves to have detrimental effects on long-term outcomes, the susceptibility of the dorsal hippocampus may bias learning and spatial memory rather than emotional deficits in male rmTBI animals. Indeed, in the clinical population, males tend to experience more cognitive and fewer emotional behavioural deficits. Cell proliferation in the SGZ is the first step in the process of neurogenesis. Studies have shown that increased neurogenesis improves learning and memory and DG-dependent tasks such as pattern separation (Sahay et al., 2011; Van Praag et al., 1999). However, high levels of proliferation, including seizure-induced division of NSCs, can cause a depletion of the neural stem cell pool, which leads to decreased neurogenesis and impairments in spatial learning (Fu et al., 2019). Aberrant migration of newborn neurons, and changes in dendritic morphology and complexity have been observed following increased proliferation after TBI (S. Ibrahim et al., 2016b; Villasana et al., 2015). Taken together, injury-induced changes in cell proliferation may lead to altered maturation and integration of adult born neurons, and in turn play a role in cognitive and/or emotional impairment.

4.1.6 Cell proliferation persists after PID 1, but occurs at a decreased rate in rmTBI animals on PID 3

According to BrdU quantification, proliferation on PID 3 had returned to normal levels: there was no difference in $BrdU^+$ cells in rmTBI animals compared to shams. In the dorsal and ventral SGZ there was a trend towards a decrease in cell proliferation, with the exception of rmTBI males who exhibited a significant decrease in $BrdU^+$ cells in the ipsilateral ventral SGZ. Indeed, fold change from sham was below 1 in males and females at PID 3. Although no significant differences were found in the total number of $BrdU^+$ cells in the dorsal and ventral SGZ, further investigation may reveal decreased proliferation after immediate, robust increases. In a model of FPI, Neuberger et al. (2017) showed that proliferating progenitor cells were decreased in TBI animals at later time points (PID 30 and PID 90) compared to shams. This decrease came after an acute increase at PID 3 and the authors suggest that the injury induced proliferation caused an exhaustion of the neural stem cell pool. This depletion of progenitor cells could have long-term consequences on neurogenesis and subsequently learning and memory.

While $BrdU^+$ cells showed a return to sham levels of proliferation on PID 3, Ki-67^+ cells remained elevated in rmTBI males compared to shams in the dorsal and ventral SGZ. In fact, the fold change from sham increased from 1.31 ± 0.138 and 1.47 ± 0.161 to 1.74 ± 0.128 and 2.43 ± 0.241 in contralateral and ipsilateral dorsal SGZs respectively. The ventral SGZ also showed marked increased fold change from sham at PID 3 compared to PID 1. This is, however, not entirely unexpected — Ki-67 is a marker for cells in all active stages of the cell cycle while BrdU only identifies cells undergoing DNA synthesis (ie. only in S phase of the cell cycle). Furthermore, because BrdU was injected 2 hours before perfusion on PID 3, it is only identifying dividing cells in a specific window of time on that day. The cell cycle is approximately 24 hours, so Ki-67 will

identify any cells that have been dividing during the 24 hour period prior to perfusion. By using both BrdU and Ki-67 as markers of proliferation in this study, novel information regarding the timecourse of the proliferative response of rmTBI in adolescent rats was revealed and is discussed below.

4.1.7 Cell proliferation paradigms inform the timecourse of injury-induced proliferation

While both BrdU and Ki-67 are widely used markers of cell proliferation, the injection paradigm for BrdU is important to consider. On PID 1, BrdU was a measure of cells undergoing DNA synthesis between 16-18 hours after rmTBI and prior to perfusion while Ki-67 was used as a measure of cells proliferating since the time of injury. Because the length of a full cell cycle is 24 hours, Ki-67 staining will identify cells that have been actively dividing up to 24 hours prior to perfusion (Cameron & Mckay, 2001). Both $BrdU^+$ and $Ki\text{-}67^+$ cells were increased in male rmTBI, but not female rmTBI animals at PID 1. On PID 3, BrdU quantified cells undergoing DNA synthesis within a specific 2-hour window, 72 hours after rmTBI. Ki-67 staining on PID 3 tissue captured all cells proliferating in the 24 hours prior to perfusion. It was apparent that these are important differences in staining paradigms; BrdU and Ki-67 used in combination allows for a more comprehensive analysis of cell proliferation. While there was a transient increase in $BrdU^+$ cells at PID 1 in male rmTBI animals compared to shams, Ki-67 staining showed a prolonged increase in injury-induced proliferation. Thus, BrdU staining, coupled with Ki-67 staining, indicated that cells continue to proliferate transiently after rmTBI for at least 48 hours, but begin to decrease by PID3. Barha et al. (2011) also looked at BrdU and Ki-67, and found that when BrdU was injected at 48 hours post injury, there was no change in the number of $BrdU^+$ cells, but there was an increase in $Ki\text{-}67^+$ cells at PID 8. While the experimental endpoint was different for this study, it also demonstrates how BrdU paradigms assess a specific temporal snapshot of proliferating cells while Ki-67 captures a more general picture of proliferation.

An increase in proliferation following TBI is consistent with what is seen in the literature. However, the peak of proliferation tends to differ. Cope et al. (2016) found an increase in BrdU after CCI at 24 hours post injury, but no difference 1 week later (BrdU was injected 1 hour and 5 hours after injury). Others have shown a sustained increase at PID 7, but no change by PID 14 (Rola et al., 2006; Sun, 2005). Following CCI in rats, Dash et al. (2001) found that 6 injections within 24 hours prior to perfusion showed an increase at 24 hours, which peaked at PID 3 in ipsilateral SGZ of TBI animals. Gao et al. (2009) found that perfusion following injection of BrdU at 4 hours after injury and 72 hours after injury resulted in a greatest increase in proliferation in TBI animals at 72 hours. Ye et al. (2014) also showed a peak in proliferation at PID 3 compared to PID 1. Taken together, proliferation appears to peak later than PID 1 and the response subsides in most models by PID 14. Few studies have investigated sex differences in proliferation; although Hong et al. (2016) and Peters et al. (2018) both included females as part of a non sex-separated cohort. In a mouse WD model of closed head injury, Villasana et al. (2014) did not find sex differences in the number of newborn neurons. While injury paradigms, injection paradigms and experimental endpoints differ between studies, there is overall consensus for increased cell proliferation after TBI. The work done in this thesis is consistent with these reports, and contributes to the paucity of information regarding sex differences in cell proliferation after rmTBI.

4.1.8 Cell proliferation was diffuse in male rmTBI animals at PID 1

The initial objective of the cell proliferation experiment was to examine the response in the SGZ, as it may relate to changes in neurogenesis. However, it immediately became apparent that at PID 1 in both BrdU and Ki-67 stained sections, there is a widespread proliferative response. Rather than having immunopositive cells be constrained to the neurogenic niche, there was increased staining present in all regions of the brain. Quantification of $BrdU^+$ cells in the hippocampus was performed from images of whole tissue sections (analysis was not performed on Ki-67 stained sec-

tions). The greatest amount of staining occurred in the hippocampus, though substantial numbers of immunopositive cells were visible in the cortex, along the lateral ventricles, and in the thalamus. This widespread proliferative response is the first evidence of a diffuse injury using this model in adolescent rats. Although the left parietal cortex was impacted, the majority of proliferating cells are not seen under the injury site.

The proliferative response was increased in the dorsal hippocampus in both hemispheres of male rmTBI animals compared to shams according to profile counting in the SGZ. Female rmTBI did not show an increase in proliferation in the hippocampus; however, qualitative analysis shows that this response may be constrained to smaller, more specific regions. The border between the molecular layer of the hippocampus and the thalamus (lateral nuclei) as well as the auditory cortex appear to be regions where more robust proliferation is observed in females. Whole section analysis of the dorsal hippocampus showed rmTBI males experienced a more robust response compared to rmTBI females not only in the neurogenic niche, but in the whole hippocampus. In the ventral hippocampus however, only the ipsilateral hemisphere showed a significant increase in proliferation in rmTBI males compared to shams. Again, there was no increase in proliferation in females rmTBI animals compared to shams. There was a significantly greater proliferative response in the ipsilateral compared to contralateral hippocampus for dorsal and ventral sections.

Taken together, this is novel evidence to suggest that like closed head CCI models, the ACHI delivers a diffuse injury: the ipsilateral side sustains greater injury-induced proliferation, but the response reaches the contralateral side. Unlike open head CCI models, the region under the impact site is not identified by tissue loss, nor is it a site of excessive proliferation. The diffuse nature of the ACHI is more pronounced dorsally along the septotemporal axis, which may lead to region-dependent differences in functional impairment. However, given that the position of the hippocampus in rodents is vertical in orientation, it could be that proximity to the impact site

plays a role in the strength of the response. A diffuse proliferative response has been observed in models of CHI (Bye et al., 2011) and FPI (Rice et al., 2003; Urrea et al., 2007). However, none of these studies investigated functional outcomes, though Bye et al. (2011) did observe neurogenesis 8 weeks after the injury and found no change.

4.1.9 Cell proliferation in the hippocampus was slightly decreased in rmTBI animals at PID 3

By PID 3, there was no change in proliferation in rmTBI animals compared to shams in the dorsal or ventral hippocampus. This suggests that the robust and widespread proliferative response peaks prior to PID 3 before beginning to return to sham levels. This was observed qualitatively in the Ki-67 sections as well, indicating that although there is no longer a widespread proliferative response, there is still injury-induced proliferation in the neurogenic niche.

4.1.10 There is an increased proportion of BrdU$^+$/Iba1$^+$ cells at PID1 in rmTBI animals compared to shams

When using BrdU and Ki-67 as markers for cell proliferation, the localization to the SGZ and morphology aid in the classification of these cells as ANPs. However, following rmTBI in both males and females, BrdU$^+$ and Ki-67$^+$ were not restricted to the neurogenic niche. Neuroinflammation is part of the secondary cascade of events that leads to ongoing damage after a head injury. The hippocampus in particular has been identified as vulnerable to secondary damage and inflammation (Acabchuk et al., 2016). Thus, immunofluorescence was used to characterize the proliferating (BrdU$^+$) cells using markers of key cell types involved in the inflammatory response.

Triple staining revealed that in male rmTBI animals, there was an increased proportion of BrdU$^+$/Iba1$^+$ cells (proliferating microglia/macrophages) in the SGZ compared to shams. Interestingly, female rmTBI animals also had a greater proportion of proliferating microglia/macrophages in their ipsilateral SGZ. This suggests that although there are no significant differences in overall

number of BrdU$^+$ cells in females, rmTBI induced a different proportion of cell types compared to sham animals. This could indicate sex differences in the innate repair mechanism where females see a more controlled inflammatory response than males. Taken together, these results indicate that the injury-induced proliferative response in the neurogenic niche is not exclusively comprised of ANPs; it is also comprised of proliferating microglia/macrophages. This substantial inflammatory response in the SGZ may have important consequences on the micro-environment of the neurogenic niche.

4.1.11 BrdU$^+$ cells in the SGZ are not colocalized with inflammatory markers at PID 3

At PID 3, there was no longer an increased proportion of proliferating microglia/macrophages; BrdU was no longer significantly colocalized with Iba1 or GFAP. However, it cannot be determined whether these cell types have died, migrated, or continue to survey the injured environment after exiting the cell cycle. Quantification of Iba1$^+$ and GFAP$^+$ could address this last option as the cells may still be present in the hippocampus and SGZ, but are no longer actively dividing. They are likely progenitor cells, which could be confirmed with colocalization with markers like nestin or Sox2. Additionally, there was substantial variability in the numbers of BrdU$^+$/Iba1$^+$ cells, and quantification would benefit from additional animals and sections.

Several groups have observed widespread proliferation using BrdU, but characterization of these immunopositive cells has been inconsistent. Some studies identify BrdU$^+$ cells as reactive astrocytes, while others suggest they are microglia/macrophages (Bye et al., 2011; Gao et al., 2009). Urrea et al. (2007) used a BrdU injection paradigm before and after FPI to look at mitotically active cells from 3 hours to 14 days after TBI. They observed BrdU labeled cells in the ipsilateral; cerebral cortex, ipsilateral hippocampus, ipsilateral white matter structures and some contralateral regions and double labelling studies confirmed that the cells were predominantly glia. However,

these studies observed colocalization of BrdU with glial markers at both acute and later timepoints. In this study, BrdU cells no longer colocalized with Iba1 or GFAP by PID 3; this novel finding indicates that immunopositive cells in the SGZ at PID 3 are not glial cells following rmTBI in adolescent rats. Taking this study into account, the increase in Ki-67 that was observed in the SGZ in the work presented here is therefore unlikely to be a prolonged glial response not captured by the BrdU injection paradigm.

4.1.12 Iba1$^+$ cells display activated morphology in rmTBI animals

Several different morphologies of Iba1$^+$ cells were observed in rmTBI, but not sham animals. Under normal conditions, microglia survey the brain with ramified processes. When they detect signs of injury or pathological alterations, they increase production and secretion of chemokines and cytokines. They also morph into more spherical, amoeboid-like cells, with phagocytic abilities (Donat et al., 2017; Loane & Kumar, 2016). While microglia with ramified process were observed in the sham group, several activated morphologies were observed in rmTBI animals. Ramified Iba1$^+$ cells with rough prolongations were observed in female rmTBI animals. In male rmTBI animals, hypertrophied/bushy and amoeboid round Iba1$^+$ cells were identified. Although quantification of each Iba1$^+$ morphology was not performed, preliminary observations suggest sexual dimorphism in DG microglia/macrophage morphology similar to the findings reported by Villapol et al. (2017) in a mouse model of CCI. Specifically, they found that males have increased numbers of activated hypertrophied/bushy microglial cells compared to females at 4 hours and 1-day post TBI. Males also exhibited an increase in Iba1$^+$ cells with amoeboid morphology which peaked at PID 7. The hypertrophied/bushy and amoeboid morphologies were not observed in female TBI animals until PID 7. Thus, the latest time point in this study, PID 3, may not capture the delayed inflammatory response in female rmTBI animals.

4.2 Sex Differences after rmTBI

The majority of preclinical research has been conducted in male animals only; most preclinical and clinical studies have excluded females (Zakiniaeiz et al., 2016). Will et al. (2017) found that as of 2014, 40% of preclinical models in neuroscience literature report males only, and 19% fail to report sex. Articles reporting both males and females represented only 35% of the literature, and in many of those studies sex was not examined as a variable (Will et al., 2017). It is well established that sex differences exist in the non-injured brain that influence social behaviour, pattern separation and spatial learning (Choleris et al., 2018; McCarthy et al., 2015). Furthermore, sex differences have been reported in the prevalence of disorders, including depression, Alzheimer's disease and TBI (Koss & Frick, 2017). Evidence has shown that biological sex plays an important role in functional outcomes and the pathophysiological response to TBI (Caplan et al., 2017; Späni et al., 2018). The exclusion of female sex in the preclinical literature suggests that 50% of the population is not represented, which can have important consequences on the sucess of clinical trials (Beery & Zucker, 2011).

In this study, male and female animals were examined at PID 1 and PID 3 following rmTBI. Interestingly, there were differences in SGZ proliferation between male and female sham animals at PID 1, but not PID 3. BrdU and Ki-67 staining both indicated an increase in proliferating cells in male sham animals when compared to female sham animals. Previous studies have shown greater amounts of SGZ proliferation in males compared to females at PND 30, but not PND 45 (Siddiqui & Romeo, 2019). In this study, rats were aged PND 29 and PND 31 at PID 1 and PID 3 respectively, and showed increased proliferation in male sham animals compared to female sham animals at PND 29. By PND 31, Ki-67$^+$ and BrdU$^+$ proliferation in males was no longer statistically significant compared to females. In fact, female shams showed an increase in BrdU$^+$

cells at PID 3 compared to PID 1. While it seems unlikely that there would be such significant changes within a matter of days (from PND 29 to PND 31), it is possible that the onset of puberty could be influencing the dramatic change between PIDs. The onset of puberty in rats is PND 30 – 39 in females and PND 40-45 in males (Koss et al., 2015). In adult female rats, it has previously been shown that there is an increase in proliferation during proestrus — when a peak of estrogens occurs (Tanapat et al., 1999). It is possible that the female animals used in the work presented here were affected by changing levels of sex hormones due to the onset of puberty. Vaginal opening was observed in some but not all females at PID 3 and in no females at PID 1. If females were in proestrus at PID 3 during BrdU injections, this could explain an acute increase in one stain but not the other. Clearly, it is important to look for signs of the onset of puberty in preclinical adolescent studies.

Following ACHI, the proliferative response quantified in this study was more robust in rmTBI males compared to male shams than in female rmTBI animals relative to female shams. These sex differences were apparent in $BrdU^+$ cell numbers at PID 1, and Ki-67^+ cell numbers at PID 1 and PID 3. This could mean that males have a more robust innate repair mechanism or that neuroprotection plays a role in the female proliferative response. Neuroinflammation, if not excessive, can be beneficial to the injured brain and exert an anti-inflammatory response. However, if left uncontrolled it can lead to increased secondary injury and result in cell death. The response in males may produce these beneficial effects without becoming excessive and harmful while the response in females may not be robust enough. Conversely, the limited response in rmTBI females may be better controlled and the reduced inflammatory response could be neuroprotective. If the later is true, taken together with the analysis of sham animals, neuroprotection in females may be conferred due to the onset of puberty. Alternatively, females may exhibit a delayed physiological response to injury that was not apparent by PID 3.

There is substantial evidence to suggest that the developing male brain has a predisposition to injury-induced inflammatory responses. During development, males have higher levels of inflammatory mediators and microglia (Arambula et al., 2019). These innate sex differences suggest that the male brain may experience a greater degree of neuroinflammation after injury due to elevated activity of these systems at baseline. Indeed, in this rmTBI model there was an increase in widespread $BrdU^+$ cells in the hippocampus and an increase in $BrdU^+$ and Ki-67^+ cell proliferation in the SGZ of male rmTBI animals. Triple labeling in the SGZ confirmed that a large proportion of the response is due to inflammatory cell types (microglia/macrophages). Although female rmTBI animals also displayed an increase in $BrdU^+$/$Iba1^+$ cells, overall, they did not show as great a neuroinflammatory response. There is a paucity of data on sex differences in inflammation in both adult and adolescent animals; the inflammatory response after rmTBI in adults rats has been well characterized in males, but there are limited studies on this response in adolescent animals of either sex (Turner et al., 2015; Wu et al., 2018).

Two studies that have looked at sex differences in inflammation and cell proliferation after rmTBI in adolescent rats are Wright et al. (2017) and Yamakawa et al. (2017). However, both studies examined the longer-term (PID 14) impact of rmTBI on markers of proliferation. Wright et al. (2017) characterized GFAP expression in the corpus callosum and prefrontal cortex in a lateral impact rmTBI model (PND 30 rats; 3 injuries 4 days apart examined at PID 14). Average GFAP expression was found to be increased in the corpus callosum in female, but not male rmTBI animals compared to shams and increased in the PFC in both male and female rmTBI animals. At approximately PID 17, Yamakawa et al. (2017) found no change in Ki-67^+ cells in the DG of male and female rmTBI animals compared to shams. In the lateral hypothalamus, they found an increase in $Iba1^+$ cells in rmTBI males compared to shams, but no difference between female sham and rmTBI animals. This prolonged increased response in male rmTBI animals may indicate that

although the robust effect observed in the SGZ of rmTBI animals using the ACHI model recovers by PID 3, other regions of the brain could continue to exhibit an inflammatory response. The lack of changes in Ki-67^{+} cell numbers at the later time point indicates that in the ACHI model, if the female physiological response is delayed, it likely appears before PID 17. Taken together, these studies indicate sex differences in the inflammatory response may persist after rmTBI in adolescent rats, but the differences may be region-specific. While these studies provide insight into the pathophysiology of adolescent rmTBI, there is still a knowledge gap in the response seen immediately after injury. Because of the acute timepoint studied, it provides novel information to the adolescent rmTBI literature above. Future studies using the ACHI model could investigate other regions to determine whether sex differences differ by region.

There is conflicting evidence on whether sex hormones influence the microglial response after TBI. When treated with testosterone or estradiol acutely after a cortical stab wound injury, orchidectomized male rats showed decreased microglia in the hippocampus (Barreto et al., 2007). However, estrogen supplementation in another model did not affect microglial activation in the hippocampus (Bruce-Keller et al., 2007). Studies based on other CNS disease models show the anti-inflammatory effects of estrogens on microglial function: estradiol decreased microglial activation following lipopolysaccharide-induced inflammation (Vegeto et al., 2001). After middle cerebral artery occlusion, progesterone and estradiol suppressed microglia activation and downregulated proinflammatory cytokines in male animals (Dang et al., 2011). Progesterone in particular has been the subject of several preclinical trials, and was associated with reduction in several pathways of TBI pathophysiology including glutamate excitotoxicity, membrane lipid peroxidation and inflammation (Loane et al., 2015). So far, these promising preclinical results have not translated to success in clinical trials (Stein, 2015).

Overall, these studies indicate a role for sex hormones in the regulation of the inflammatory

response after TBI. However, there is a lack of data on their role in adolescents. During puberty in rodents, progesterone and estradiol in females and testosterone in males begin to increase (Bell, 2018); thus, the greater increase in proliferation and inflammation observed in males in this work could be due to sex hormones. Specifically, neuroprotection exerted by these sex hormones may be greater in females, leading to a decreased proliferative/inflammatory response. Manipulation of the response observed, by exogenous administration of sex hormones, sex hormone receptor antagonists or gonadectomy, have the potential to elucidate underlying mechanisms in this model.

In summary, this work found notable sex differences in both the inflammatory and proliferative response to rmTBI in the SGZ and hippocampal formation. The mechanisms behind the increased response in males may be due to higher baseline levels of microglia, and current literature points to a neuroprotective role of sex hormones, whose effect is greater in females (Caplan et al., 2017). At PND 28-31, the age of animals in this study, female rats are closer to the onset of puberty than males. Therefore, it is possible that the effects of sex hormones were greater still in females compared to males in this study. Future studies using this model and paradigm may further clarify the effect of puberty and associated changing profile of sex hormones on the proliferative and inflammatory response following rmTBI in adolescents.

4.3 Limitations

4.3.1 Consequences of a single injury vs repeated injuries:

The results discussed in this work describe what was observed after 8 repeated injuries were administered to adolescent rats. Because a single hit group was not included, it is unknown as to whether a single ACHI would also result in similar findings. As the ACHI technique is a novel preclinical injury model, future studies including a single hit group will better be able to identify what is significant and unique to repeated versus single injuries.

4.3.2 Cell counting method:

To quantify $BrdU^+$ and $Ki\text{-}67^+$ cells in the SGZ, a careful protocol for profile counting was followed. Profile counting has been widely used as a means of quantifying cell numbers in the SGZ (Gil-Mohapel et al., 2013; Hayes et al., 2018). However, cell estimation using unbiased stereology could add more rigor to cell quantification. Unbiased stereology makes quantitative estimates of geometrical features in objects of interest. It uses statistical sampling methods aimed to reduce sampling and systematic bias. To ensure sampling is uniform and random, every part of an object or specimen must have an equal chance at being quantified and sampled. To reduce systemic bias, the correct measurement tool should maximize precision (improved by more being sampled) and accuracy (using methods that are inherently unbiased). The amount of error introduced from the sampling method can be quantified in stereological methods using the coefficient of error (CE). Furthermore, because it uses a virtual 3D geometrical probe, stereology is able to resolve confounds of volumetric differences. In profile counting, it is not possible to provide a CE; the benefit of a CE value is that it provides an indication of whether the sampling method is able to resolve differences between two groups. It can indicate whether more tissue needs to be sampled. There is some debate as to whether heterogeneous cell populations like BrdU can be properly sampled using stereological techniques (Noori & Fornal, 2011). Others contend that there is no advantage to unbiased stereology over absolute cell counting (Crews et al., 2004). In this study, profile counts were taken with stereological best practices in mind. All tissue had an equal chance of being sampled (a random 1 in 6 series was chosen for each staining procedure). Strict counting rules were also applied: cells were counted by scrolling though all focal planes so that within one section, all cells had the same probability of being counted, made possible by counting at 100X. Objects at the very top and very bottom of each plane were not counted to avoid artifacts (West, 1999).

4.3.3 Defining Neural Progenitors

There are also limitations in how much can be concluded from the triple stain data. While the colocalization analysis of BrdU, Iba1 and GFAP provided insight into the inflammatory response in the SGZ, it is not appropriate to conclude that $BrdU^+$ only cells were neural progenitor cells. Further staining with Sox2 or nestin could estimate whether there is an increase in ANPs specifically. Furthermore, staining with GFAP could have provided an underestimation of the number of BrdU/GFAP positive cells; immunopositive staining of GFAP is mainly constrained to the processes, which makes colocalization analysis difficult.

4.3.4 BrdU

BrdU is widely used in studies of cell proliferation and neurogenesis. Double labeling of dividing ($BrdU^+$) cells has allowed for specific phenotypic analysis and its utility in lineage analysis has been harnessed to track cell survival. However, there are some disadvantages of using BrdU (see Taupin (2007), Encinas & Enikolopov (2008)). BrdU is toxic to cells at high doses and may be incorporated into the DNA of damaged cells or cells undergoing repair. However, at the dose used in this study, BrdU has not been shown to exert a toxic effect on dividing cells (Taupin, 2007). Furthermore, injection of BrdU especially in acute paradigms, may induce stress-related changes to proliferation; Ki-67 was used in this study to mitigate the potential confounds of stress.

4.4 Future Directions

4.4.1 Further Characterization of Stem Cells and Glial Response analysis

The data presented in this thesis investigates the proliferative and inflammatory responses in the hippocampus after rmTBI in adolescent male and female rats. Further examination of the neurogenic response will provide a better understanding of which specific cell types are affected and

are more susceptible to injury induced proliferation. Studies looking into the extent of this response outside of the hippocampus will help identify other regions of the brain that are also vulnerable to rmTBI.

Immunofluorescent labeling studies of multiple markers, similar to the triple staining performed in this study, could confirm the cell type of proliferating neural stem and progenitor cells. In this thesis, triple labeling confirmed that around 40-60% (males) and 20-40% (females) of $BrdU^+$ cells in the SGZ were $Iba1^+$, indicating a response to injury by microglia/macrophages. The remaining $BrdU^+$ cells are most likely neural stem or progenitor cells; triple labelling with a marker specific to these cells – Sox2 or nestin – would help to determine whether the neurogenic proliferative response specifically is increased due to injury. Furthermore, staining with Ki-67 indicated that the BrdU injection paradigm used in this study captured only a small portion of proliferating cells. While this was expected, characterization of the proliferative response from time of injury to PID 3 by injecting animals on PID 1 for the PID 3 endpoint would provide clarity to the type of response (neurogenic vs inflammatory) at PID 3. The current paradigm indicates that the dividing $BrdU^+$ cells are rarely inflammatory cells; very few $BrdU^+/Iba1^+$ cells were identified.

Because there were differences in the proliferative responses measured by BrdU relative to Ki-67, threshold analysis of the Ki-67 stain would reveal important insight into the diffuse nature of the injury. The widespread response of $BrdU^+$ cells observed at PID 1 in rmTBI animals had returned to normal levels by PID 3. A preliminary assessment of Ki-67 staining shows that it too has returned to control levels, and Ki-67^+ cells outside the SGZ appear to be present at a level comparable to shams. Quantification of this response could confirm this, and would suggest that the increase in proliferation at PID 3 measured by Ki-67^+ cells is neurogenic (constrained to the SGZ) rather than inflammatory in nature.

Tissue sections were stained with Iba1 and GFAP to identify whether the response in the SGZ was inflammatory in nature. However, because of the widespread response observed, further analysis of this inflammatory response in other regions of the brain would provide insight into sex- and region-specific differences in this model. The BrdU whole sections imaged in this thesis can be used as a road map to identify regions that show widespread proliferation. Cell counting or threshold analysis can be performed on each stain individually or in conjunction with BrdU to identify reactive gliosis. Preliminary analysis revealed various morphologies of activated microglia, and classification and quantification of these morphologies may uncover additional sex differences (Villapol et al., 2017). To date, the inflammatory response has not been characterized in the ACHI model except after a single hit in adult rats (Pham et al., 2019). Pham et al. (2019) found an increase in $Iba1^{+}$ labelling in the parietal cortex, corpus callosum but not the DG of single hit TBI animals. Time since injury appears to influence the response in the corpus callosum, but not the parietal cortex. This group did not look at sex differences, so a thorough investigation into sex differences in the region-specific inflammatory response would provide novel insight into the ACHI paradigm. Because anesthesia can influence brain pathology after TBI, including inflammation, characterizing the inflammatory response after rmTBI in awake animals provides preclinical paradigms that more accurately reflect the clinical population (Luh et al., 2011; Rowe et al., 2013).

4.4.2 Potential long term consequences

Learning and memory

Injury-induced cell proliferation may or may not lead to changes in neurogenesis, and there is currently no consensus in the preclinical literature. This work demonsrated that there is an increase in cell proliferation in adolescent rats following rmTBI; however, this response was greater in males than in females. Understanding the long-term consequences of these two responses will

reveal whether targeting the proliferative response can improve outcomes. To this end immunohistochemistry performed at 1-month post injury could determine whether cells proliferating at time of injury are able to survive, mature into granule cells, and integrate into existing neural networks. Multiple injections of BrdU could be administered after injury and markers of mature neurons – NeuN and Calbindin – could be analyzed for colocalization with BrdU. If rmTBI decreases cell survival, there will be fewer $BrdU^+$/NeuN+ cells in rmTBI animals. Conversely, if rmTBI increases cell survival there will be more $BrdU^+$/NeuN+ cells. Immunohistochemistry of immediate early genes (IEGs) can be coupled with behavioural tasks; because IEGs are markers for neural activity, colocalization with BrdU would indicate active newborn granule cells. If proliferation were to lead to greater numbers of fully functional newborn granule cells, there would be increased numbers of $BrdU^+$ colocalized with an IEG marker following hippocampal-dependent behavioural tasks such as pattern separation (Yagi et al., 2016). If BrdU does not colocalize with an IEG marker to the same extent as shams, especially if the rmTBI animal performs worse on a task, results would indicate a failure of the newborn granule cells to integrate and function properly. Immunohistochemistry and behavioural studies to examine the long-term outcome and the effect of the robust response in males compared to the reduced response in females will further our understanding of rmTBI on learning and memory.

CTE pathology

One main interest in preclinical models of rmTBI is the association of multiple injuries to the development of CTE. CTE has been investigated primarily in sports-related injuries and clinical pathophysiological evidence is primarily comprised of former NFL players (Baugh et al., 2014; Mckee et al., 2009). Repeated impacts sustained by these athletes has been associated with the development of CTE. CTE is characterized by tauopathy - abnormal accumulation of phosphorylated tau (ptau) in neurons, astrocytes and cell processes (McKee & Daneshavar, 2015). In a study

of deceased football players, Cherry et al. (2016) found that increased $CD68^+$ microglia and reactive microglial morphology was associated with tau accumulation and CTE. Given that increased numbers of microglia/macrophages were observed after rmTBI in this study, development of CTE could be studied in this model of rmTBI, especially if inflammation persists at chronic timepoints. Future studies may also consider manipulating the injury interval to determine whether there is a frequency at which the inflammatory response is damped. Looking at the effect of different recovery periods on acute and long-term outcomes may help inform return to play status; that is, how long might an athlete need to wait prior to returning to their sport.

4.4.3 Sex differences at onset of puberty: outcomes after rmTBI

This thesis showed that sex differences in outcomes following rmTBI exist in the adolescent brain; males exhibited a more robust proliferative and inflammatory response compared to females. Animals were impacted during adolescence, at a timepoint that corresponds to the onset of puberty in females. When proliferation in sham animals was analyzed, it became apparent that baseline sex differences exist. Examination of sex hormones was beyond the scope of this study, but these results indicate that further analysis into the onset of puberty, sex hormone levels, and their effect on rmTBI, is warranted. The human adolescent is going through many changes, and oftentimes symptoms of TBI – psychosocial changes, changes in emotional response, increased risk-taking - can be confused with those of a healthy adolescent (Koss & Frick, 2017). Thus, it is important to understand that sex differences exist prior to injury which likely influences the brain's response to injury. When considering therapeutic interventions, biological sex differences are critical to consider as females and males may respond differently to, for example, manipulation of the proliferative response.

4.5 Conclusions

The ACHI model used to induce rmTBI in adolescent rats produced an injury that is consistent with the clinical definition of sports related concussion. There was transient LOC, and short-lived impairment of neurological function in the absence of major structural damage. This demonstrated that rmTBI results in acute neurological impairment in both sexes without hippocampal structural damage. Subdural hematomas were not expected in this model, and future studies should take this observation into account as a standard procedure. Assessment of cell proliferation showed that rmTBI results in robust and widespread cellular proliferation in males. This response is greater in males than in females and persists until PID 3. While cells that are localized to the SGZ are generally progenitor cells, characterization of cell types in both male and females showed a heightened inflammatory response at PID 1. The sex differences in the acute proliferative and inflammatory response to rmTBI reported here provide novel information about the response of the adolescent hippocampus following rmTBI; future studies will help to inform whether these sex differences contribute to changes in functional outcomes in neurogenesis and learning and memory. Understanding what is neuroprotective and what is harmful regarding the heightened hippocampal response in males after rmTBI and the minimal response in females will provide exciting opportunities for intervention. These novel findings highlight a need for the inclusion of females in preclinical and clinical TBI research. Future studies will continue to inform how military personnel exposed to blast waves, the many women affected by IPV, and athletes engaged in contact sport may better recover from rmTBI.

CHAPTER 5

Appendix

5.1 ACHI Monitoring

5.1.1 Restraint Scores

Restraint scores were recorded for each animal during the ACHI procedure. A restraint score of 0 means the animal showed no response to restraint, while higher scores (max score of 6) reflect vocalization and squirming due to being put into restraint cones. The average restraint score total is for all 8 hits, so the maximum score would be 48. Overall, male and female animals did not show distress to being restrained.

Table 5.1: Restraint scores.

Group	Average Restraint Score Total	Average Restraint Score/ACHI	Frequency of Non Zero Score
Sham Male	2.07	0.28	2.36
rmTBI Male	6.50	0.87	5.50
Sham Female	2.93	0.40	3.29
rmTBI Female	6.64	0.88	6.64

5.1.2 Pain Scores

Prior to each impact, animals were assessed on a pain score to check for abnormal behaviour due to ACHI. Animals were scored based on locomotion, social behaviour and breathing, palpitation of the impact site and skin turgor. Scores of 0-1 reflect no abnormalities while higher scores (max score of 12) reflected need for support including extra monitoring or removal from the study. Animals showed low average pain scores and no animals required removal from the study.

Table 5.2: Pain Scores.

Group	Average Pain Score/ACHI	Average Pain Score Total
rmTBI Male	0.21	1.64
rmTBI Female	0.35	2.79

5.2 Whole tissue sections - BrdU

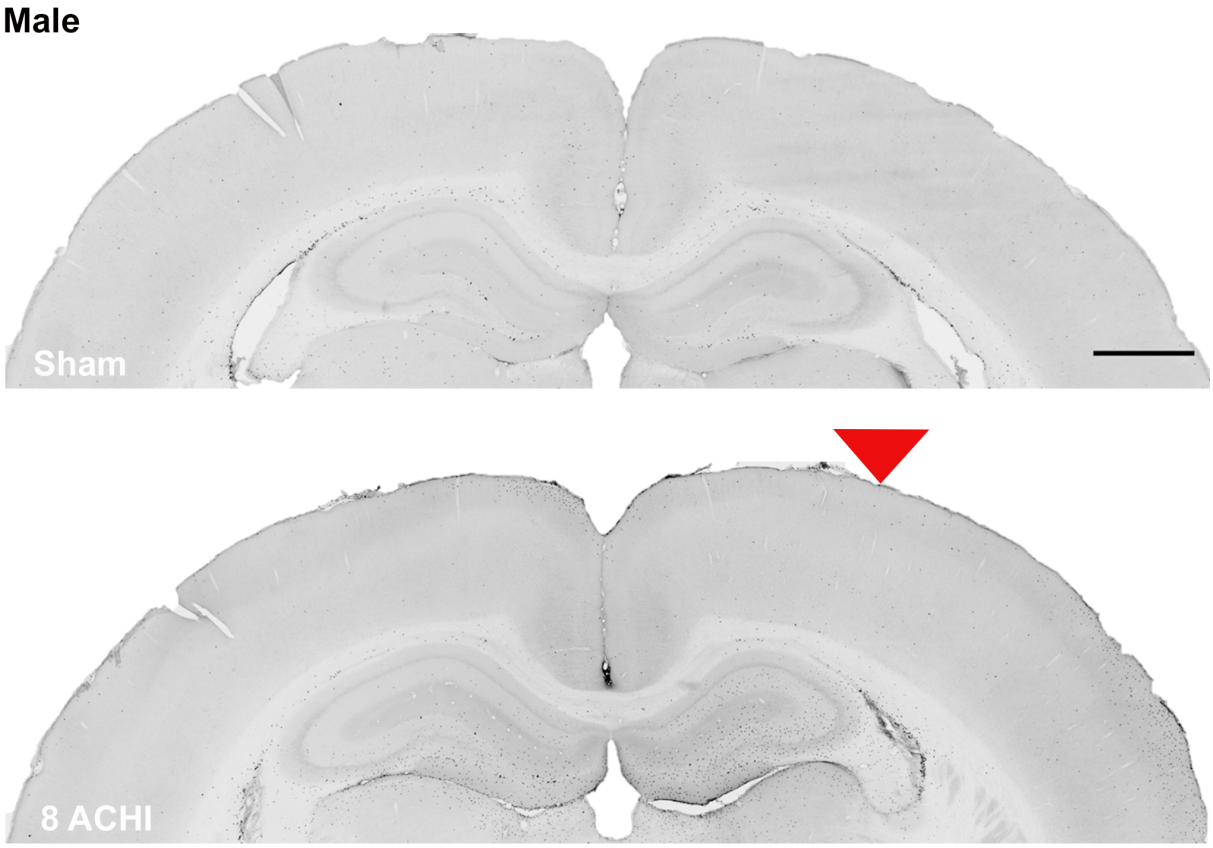


Figure 5.1: Widespread response of BrdU$^+$ cells in males at PID 1 after rmTBI Scale is 1 mm.

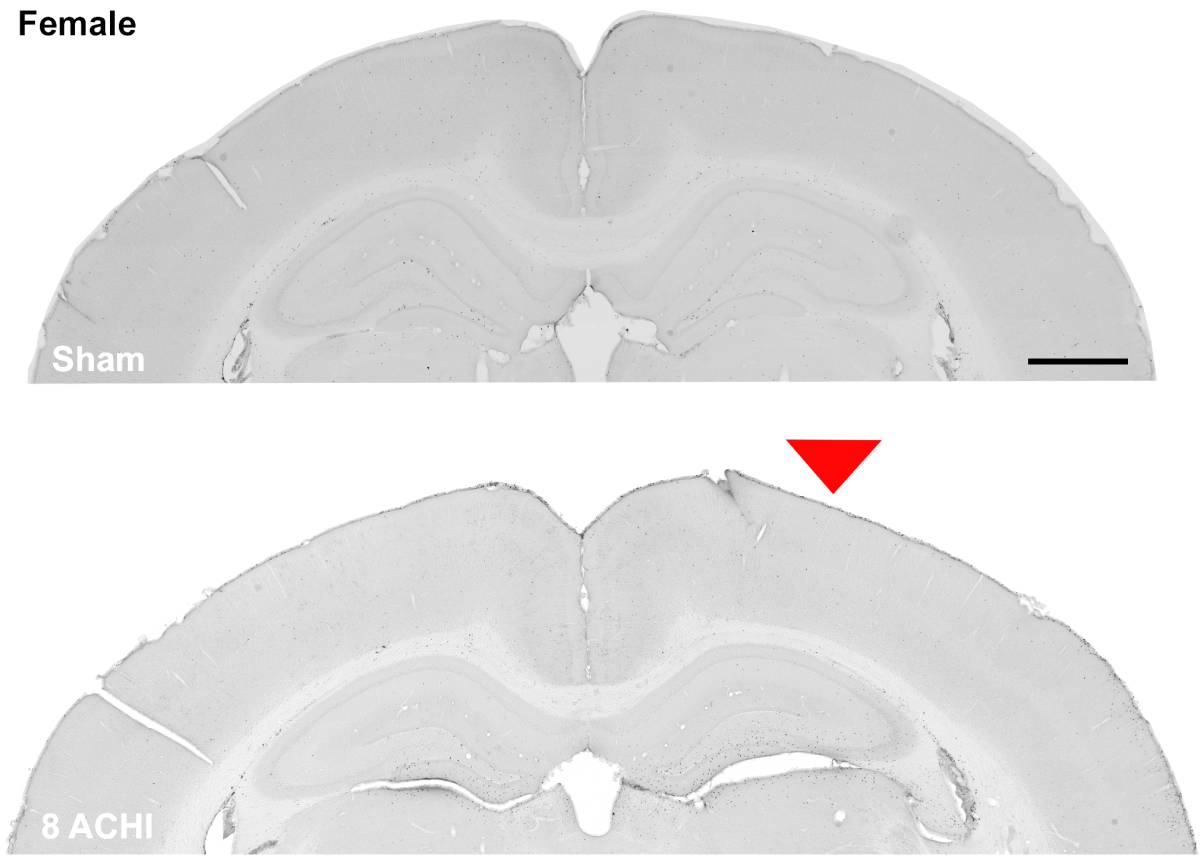


Figure 5.2: Proliferative response of BrdU$^+$ cells in females at PID 1 after rmTBI Scale is 1 mm.

5.3 BrdU PID 3 ventral SGZ pooled results

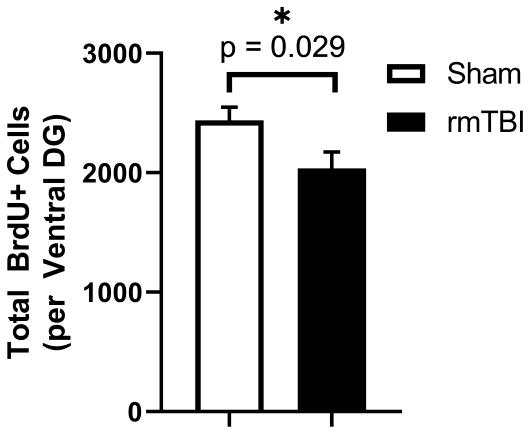


Figure 5.3: BrdU$^+$ cells in the vDG at PID 3: Sham vs rmTBI. When a three-way ANOVA was conducted with group, sex and side as factors, only a main effect of group was shown. Side and sex were pooled and an unpaired t-test was run. rmTBI animals had significantly decreased numbers of BrdU$^+$ cells compared to shams ($p = 0.029$).

5.4 Sex differences in male vs female shams at PID 1 and PID 3

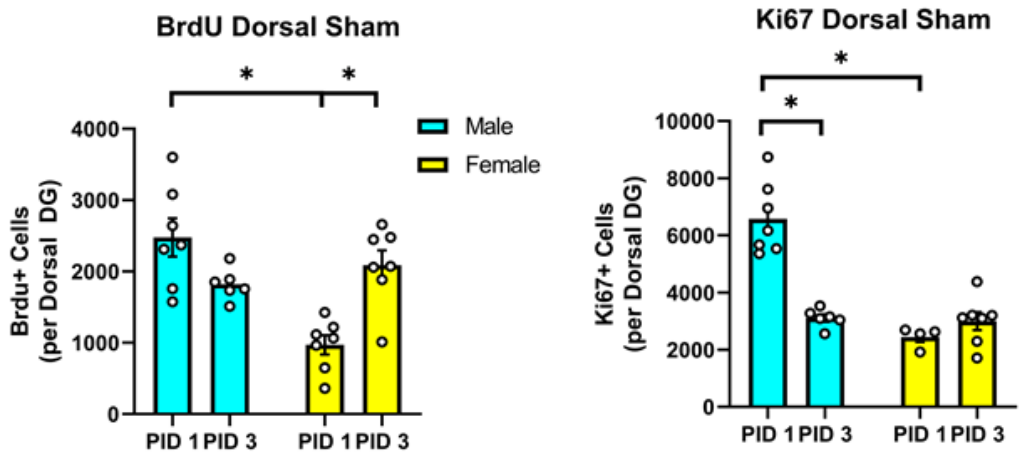


Figure 5.4: BrdU$^+$ and Ki-67$^+$ cells in the dorsal SGZ at PID 1 and PID 3: Sex differences in sham animals. Two-way ANOVA with PID and sex as factors revealed a main effect of sex ($F(1, 23) = 10.17$, $p = 0.0041$) and a significant interaction of PID and sex ($F(1, 23) = 20.78$, $p = 0.0001$) on BrdU$^+$ cells in the dorsal SGZ. Post hoc analysis revealed significantly more BrdU$^+$ cells in male shams at PID 1 compared to females ($p < 0.0001$). At PID 3, there were more BrdU$^+$ cells in the SGZ of female shams compared to PID 1 ($p = 0.002$).For Ki-67 stained sections, a two-way ANOVA revealed a main effect of Sex ($F(1, 20) = 28.33$, $p < 0.0001$), PID ($F(1, 20) = 12.77$, $p = 0.0019$ and a significant interaction between sex and PID ($F(1, 20) = 12.77$, $p = 0.0019$). Post hoc analysis revealed a significantly higher number of Ki-67$^+$ cells in the SGZ of male shams at PID 1 compared to females ($p < 0.0001$) and compared to males at PID 3 ($P < 0.0001$).

5.5 No differences in DG area

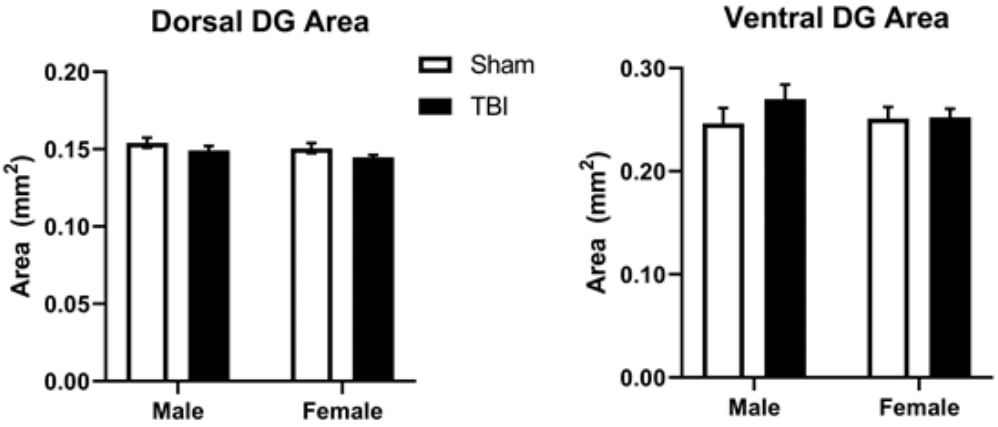


Figure 5.5: No sex differences or injury-induced changes in DG area. To test whether differences in DG area exist, area measurements were taken by tracing the granule cell layer of the DG using ImageJ. A three-way ANOVA using sex, group and PID as factors revealed no main effects or interactions, so PID 1 and PID 3 were pooled. Because no structural damage was observed in the hippocampus (cresyl staining), measurements for the contralateral and ipsilateral hemispheres were averaged per animal. A two-way ANOVA revealed no main effects or significant interactions in the dorsal or ventral hippocampus. Therefore, DG area is not a confounding factor in the profile counting performed in this study.

Table 5.3: Cell Proliferation following TBI

Reference	Animals	Age	Sex	Injury Model	Anesthesia	Single/Repeat	Markers	BrdU Injections	PID	Finding
Acosta et al., 2013	SD rats	10 wks	not stated	CCI - craniotomy	Yes	Single	Ki-67, DCX, OX6		8 wks	Ki-67 - decrease (SGZ) DCX - no change (SGZ) OX6 - increase in ipsi ctx, STR, TH, CC, cerebreal peduncle, fornix, no change cerebellum
Barha et al., 2011	SD rats	adult	male	CCI - craniotomy	Yes	Single	BrdU, Ki-67, DCX	48h after CCI, single injection (200mg/kg)	PID 8	BrdU - no change Ki-67 - increased in TBI compared to sham BrdU/DCX - increased in TBI compared to sham
Bregy et al., 2012	SD rats	adult (276-395g)	male	FPI	Yes	Single	BrdU, DCX	24h and 30min before injury; everyday until sac (50mg/kg)	PID 3 or PID 7	BrdU - increase PID 7 contra and ipsi, PID 3 data not shown DCX - no change PID 3 or PID 7
Bye et al., 2011	SD rats	12-16 wks	male	mild Acceleration-TAI	Yes	Single	BrdU, GFAP, Ox42, NG2, RECA1	24h after injury, 2 injections daily for 4 d (200mg/kg)	PID 7, 14, 4 wks, 8 wks	BrdU - non significant increase at PID 7 and PID 8 wks in SGZ compared to sham; 80% reduction between 1 to 2 wks sham and TBI. Proliferation concentrated in CA1-3 BrdU/GFAP - mostly in HIP and basal thalamic region at 1 wk BrdU/Ox42 - none in HIP 1 wk PI BrdU/NG2 - mostly in CC and TH, small amount in HIP at 1 wk RECA1 (endothelial cells) - small amount , mostly in CTX at 1 wk
Chen et al., 2016	SD rats	8 wks	male	FPI	Yes	Single	BrdU	Immediate or 1, 2, 3, 4, or 5 d after injury (10mg/100g)	PID 7 or 4 wks	BrdU - 1 wk: increase in ipsi SGZ in TBI; 4 wk: decrease in ipsi SGZ in TBI compared to 1 wk TBI
Chirumamilla et al., 2002	SD rats	adult	male	FPI	Yes	Single	BrdU, Vimentin, ED-1	48h after injury, 2 doses 4hr apart (50mg/kg)	PID 3 (24hr after last injection)	BrdU - increase in ipsi HIP BrdU/vimentin - increased ipsi HIP BrdU/ED-1 - increased ipsi HIP
Choi et al., 2014	SD rats	8 wks	male	Weight drop - open	Yes	Single	BrdU, Ki-67, DCX	24h after injury, twice daily for 4 d (50mg/kg)	PID 7	BrdU - increased in TBI animals Ki-67 - increased in TBI animals
Cope et al., 2016	SD rats	10 wks	not stated	CCI - craniotomy	Yes	Single	EdU, DCX	1h and 5h after injury (50mg/kg)	24 hrs and 1 wk	EdU - increased in TBI animals at 24h; no difference at 1 wk EdU/DCX - no difference at 1 wk
Dash et al., 2001	Long Evans rats	275-300g	male	CCI - craniec-tomy	Yes	Single	BrdU, TOAD-64, GFAP	Timecourse: 6 injections before sac (50mg/kg); Incorporation: daily injection for 9 consecutive d (200mg/kg)	24hr, 3d, 1wk, 2wk, 3wk	BrdU timecourse - increased in TBI animals at 24hrs, 3d, in ipsi and contra BrdU incorporation - increase in ipsi and contra at PID 9 BrdU/TOAD-64 or GFAP - no data shown; co-localized with TOAD-64 and infrequently with GFAP

Emery et al., 2005	SD rats	350-400g	male	FPI	Yes	Single	BrdU, PSA-NCAM	1 injection 1h prior to injury (200mg/kg)	2 wks	BrdU - increased in TBI animals contra and ipsi at PID 3 and 2 wks (fewer cells at 2 wks comapred to 3 d) BrdU/PSA-NCAM - was observed. No quantification shown
Gao, Enikolopov and Chen 2009	C57/BL6 or Nestin-GFP trans-genic mice	6-8 wks	male	CCI - craniotomy	Yes	Single	BrdU, GFAP, Nestin	a) once daily for 7 d after TBI; b) once 4h after TBI; c) one 72h after TBI (50mg/kg)	24h after last BrdU injection	BrdU - a) increase in ipsilateral ML, GCL and hilus in TBI animals, not SGZ (ie. Probably not neural stem/progenitor cells) BrdU/GFAP - BrdU+ cells in ML, GCL and hilus were mostly colocalized with GFAP = reactive astrocytes BrdU/Nestin - BrdU+ cells in SGZ were mostly colocalized with Nestin BrdU/GFP b) - 4h after tbi: increased percentage of proliferating QNPs in TBI aniamls, non significant increase in ANPs BrdU/GFP c) - 72h after tbi: further increase in proliferating QNPs, non significant increase in ANPs
Gao & Chen 2013	C57BL/6 or Nestin-GFP trans-genic mice	8 wks	male	CCI - craniotomy	Yes	Single	BrdU	Pulse: immediately, 20h, 44h, 68h or 1wk after TBI (100mg/kg)	pulse label - 4h after injection	BrdU pulse label - peak at 48h, increase in TBI animals at 48h and 72h, no increase at 1 wk. Cells not restricted to SGZ in TBI animals BrdU/GFAP and BrdU/Iba1 and BrdU/Nestin- only BrdU+ cells in SGZ colocalized with Nestin; most of the BrdU+ cells in the ML colocalized with GFAP or Iba1 (mostly Iba1) BrdU/GFP- most of the BrdU+ cells in ipsi SGZ are NSCs in sham and TBI
Ye et al., 2014	C57BL/6 mice	adult	male	CCI - craniec-tomy	Yes	Single	BrdU, SOX2	Immediately, 3 injections 8hr apart (0.01mL/g)		BrdU - increase in TBI compared to sham at PID 1, peak at PID 3, but still significantly increased at PID 7, 14 and 21 BrdU/SOX2 - majority of BrdU+ cells were NSCs
Ye et al., 2016	SD rats	250-300g	male	CCI - craniotomy	Yes	Single	BrdU, Sox2, NeuN	Proliferation: 3 injections (100mg/kg) 8 h apart on PID 6; Differentiation: 1 injection (100mg/kg) per day from PID 1-PID 7	BrdU pro-liferation - PID 7 (1 day after injections) BrdU dif-ferentiation - PID 28	BrdU/Sox2 - PID 7, increased in TBI animals (SGZ) BrdU/NeuN - PID 28, increased in TBI animals (SGZ)

Yu et al., 2008	Nestin e-GFP trans-genic mice	8 wks	male	CCI - craniec-tomy	Yes	Single	BrdU, GFP (Nestin), DCX	12 and 2h prior to perfusion (100mg/kg)	PID 1, PID 3, PID 7	DCX - ipsi decrease at 72h compared to sham, but rebound by 7 d compared to 72h. Contra: increase at 7 d compared to sham and other TBI groups (all in "subgranular layer") GFP - ipsi no changes in SGL. Contra: no changes in SGL GFP/BrdU+ (relative to all GFP+)- ipsi no changes in SGL. contra: increase at 7 d compared to sham and 24 and 72h in SGL DCX/BrdU+ (relative to all DCX+) - ipsi and contra: increase at 7 d compared to sham and 24h and 72h in SGL
Zhang et al., 2014	C57BL/6 mice	12 wks	male	FPI	Yes	Single	BrdU, DCX	4 injections (100mg/kg) 2.5 hours apart before sac	2 hours after last injection on PID 3, 7	BrdU - PID 3 two fold higher in TBI animals in ipsi SGZ DCX - PID 3 and PID 7 higher in TBI mice in ipsi SGZ
Zhang et al., 2015	C57BL/6 mice	12 wks	male	FPI	Yes	Single	BrdU, DCX	100mg/kg twice daily, PID 1 and PID 2, 2.5 hours apart	PID 3	BrdU - PID 3 3 fold higher in TBI animals ipsi SGZ DCX - PID 3 increased in TBI mice in DG
Hong et al., 2016	GFP with nestin promo-tor	8 wks	males and females	CCI - craniec-tomy	Yes	Single	BrdU	1 injection 2hr prior to perfusion 48h after TBI (100mg/kg)		BrdU - increased in ipsi only compared to sham (151% increase) GFP (Nestin) - 120% increase in ipsi SGZ, 89% increase contra SGZ at 48h in TBI BrdU/GFP -2 fold increase in TBI in ipsi DG
Neuberger et al., 2017	Wistar rats	23-25 d	male	FPI	Yes	Single	DCX, Ki-67, TBR2		PID 3, PID 7, PID 30, PID 90	DCX - ipsi SGZ : increase in TBI animals at PID 3 and PID 7 compared to sham. Decreased in TBI animals compared to sham at PID 30 and PID 90. Tbr2 and Tbr2/Ki-67: ipsi SGZ increase in TBI animals compared to sham at PID 3. Decreased compared to sham at PID 90 (decreased perentage of proliferating NPCs, exhaustion of stem cells)
Ng et al., 2012	C57BL/6 mice	12-14 wks	not stated	CHI skull fracture	Yes	Single	BrdU	4 injections (200mg/kg) daily for 4 d starting PID 1	PID 1 wk, PID 6 wks	BrdU - increased in ipsi SGZ at 1 wk and 6 wks, but not in contra SGZ DCX - no change in ipsi or contra at 1 wk or at 6 wks
Peters et al., 2018	C57BL/6 mice	8-12 wks	males and females	CCI - closed	Yes	Single	BrdU	a)- 3 injections per day (50mg/kg) 2 hrs apart on PID 2; b) 2 injections per day (300mg/kg) 4h apart on PID 2 and PID 3	a) PID 3 b) PID 14	BrdU PID 3 - no difference BrdU PID 14 - increased in TBI animals (Note GCL and SGZ) BrdU/DCX PID 14 - no difference BrdU/GFAP PID 3 - no difference BrdU/GFAP PID 14 - increased in TBI animals BrdU/Iba1 PID 3 - no difference

Potts et al., 2009	WT and GPx Tg mice	PND21	male	CCI - craniotomy	Yes	Single	Ki-67, DCX		PID 14	Ki-67 - PID 14 decrease in TBI animals compared to shams DCX - PID 13 no change
Rice et al., 2003	SD rats	300-350g	not stated	FPI	Yes	Single	BrdU	Proliferation: every 2h for 3 injections on PID 1, 4, 7, 9, or 14 (90mg/kg); Differentiation: 18 and 20h after injury (120mg/kg)	Proliferation - 24 hours after last injection on PID 2, 5, 8, 10, 15 Differentiation - PID 2, 5, 8, 10, 15	BrdU proliferation - increased in TBI Ipsi SGZ at PID 2, PID 8. Decreased in TBI ipsi SGZ at PID 5, PID 15. Increased in contra SGZ at PID 2, PID 5 BrdU differentiation - increased in TBI ipsi SGZ at PID 2, no difference otherwise; the cells do not survive or quickly migrate away BrdU/GFAP (data not shown) - no double labeling in DG at PID 10 and PID 15 BrdU/BIII Tubulin (differentiation protocol): no colocalization at PID 2-8, did see colocalization at PID 10 and 15
Rola et al., 2006	C57BL/6 mice	2 months	male	CCI - craniotomy	Yes	Single	Ki-67, DCX		6h, 12h, 24h, 48h, 7d, 14d post injury	Ki-67 - decrease in TBI animals at 24h, increase at 48h, normal at PID 14 (ipsi SGZ) DCX - decrease in TBI animals at PID 14
Shapiro et al., 2017	C57BL/6 mice	6 wks	male	FPI	Yes	Single	DCX		PID 1, PID 7	Increase in DG at PID 7, no change at PID 1
Sun et al., 2005	SD rats	PND28 and 3 months	male	FPI	Yes	Single	BrdU	Proliferation: 3 injections (50mg/kg) at PID 2, 7 or 14; Differentiation: 3 injections (50mg/kg) at PID 2	Proliferation - 24 hours after last injection Differentiation - 5, 12 or 26 d after last injection	Proliferation - 48hrs peak increase in ipsi DG in juveniles and adults compared to shams; cells in general restricted to SGZ and hilus Proliferation - 7 d significant but lower increase in ipsi SGZ injuveniles and adults compared to shams Prolifefation - 14 d no differences BrdU/NeuN - PID 7 increase in juvenile TBI animals compared to adults at PID 7, 14 and 28 BrdU/GFAP - increase in adult TBI animals compared to juveniles at PID 14 and PID 28
Sun et al., 2007	SD rats	3 months	male	FPI	Yes	Single	BrdU	Daily for 3 d starting at PID 2 (300mg/kg)	PID 5	BrdU - 4 fold greater than shams at PID 5
Sun et al., 2009	SD rats	3 months	male	FPI	Yes	Single	BrdU	Daily injections for 5 d starting at PID 2 (50mg/kg)	PID 7	BrdU - increased in ipsi and contra SGZ in TBI animals
Sun et al., 2015	SD rats	3-4 months	male	FPI	Yes	Single	BrdU, DCX	Daily (50mg/kg) for 5 d starting at PID 2	PID 7	BrdU - increase in ipsilateral and contralateral SGZ at PID 7

Urrea et al., 2007	SD rats	250-250g	male	FPI	Yes	Single	BrdU	24h and 30min before injury and daily until sacrifice (65mg/kg)	3h, PID 1, 2, 3, 7, 14	BrdU 3 hr - mostly in SVZ in TBI animals, BrdU - no comparison to sham animals, but peak appears at PID 3 in HIP BrdU/GFAP - mostly around contusion area
Villasana et al., 2014	C57BL/6 mice and POMC-EGFP mice	3 months	males and females	Weight drop - CHI	Yes	Single	BrdU, GFP, DCX, GFAP, Mac-2	Twice daily for 7 d starting PID 1 (300mg/kg)	PID 14	BrdU - in GCL: mild no difference, severe increased in ipsi compared to shams GFP(newborn neurons 2 wks post mitosis) - in GCL: mild no difference, severe increased in ipsi compared to shams and mild BrdU/DCX - no difference GFAP - increase in severe only (ipsi DG) no sex differences Mac-2 - increase in severe only (ipsi DG)
Wang et al., 2016	C57BL/6 mice	9 wks	male	CCI - craniotomy	Yes	Single	BrdU	1 injection (10mg/kg) 4 hours before perfusion	24h, 48h	BrdU - in SGZ increased at 48h compared to sham and 24h TBI group
Wang et al., 2016	C57BL/6 mice	9 wks	male	CCI - craniotomy	Yes	Single	BrdU	1 injection at 44h after TBI (100mg/kg)	PID 2 and PID 14	BrdU/Sox2 - PID 2 no change in mild; increase in moderate compared to mild and sham; increase in severe compared to sham, mild and moderate (SGZ) DCX - PID 14 increase in severe only (compared to all other groups)
Yang et al., 2019	SD rats	8-10 wks	male	CCI - craniotomy	Yes	Single	BrdU, Sox2	3 injections (100mg/kg) at 8hr intervals on PID 6	PID 7	BrdU/Sox2 - PID 7 increase in TBI (SGZ)

Note:
Abbreviations:
[1] BrdU: Bromodeoxyuridine; DNA synthesis;
[2] DCX: Doublecortin; immature neuron marker;
[3] ED-1: Microglia/macrophages;
[4] EdU: Ethynyldeoxyuridine; DNA synthesis;
[5] GFAP: Glial fibrillary acidic protein; astrocytes;
[6] Ki-67: Cell proliferation (cell cycle regulating protein);
[7] Mac-2: Galactin-3; microglia;
[8] Nestin: Neuroectodermal stem cell; stem cells, progenitor cells;
[9] NeuN: Neuronal nuclei; mature neurons;
[10] NG2: Neural/glial antigen 2; polydendrocytes;
[11] OX6: Activated microglia;
[12] PSA-NCAM: polysialylated neuronal cell adhesion moleculr; developing and migrating neurons;
[13] RECA1: Endothelial cells;
[14] SOX2: Sex determining region Y -box2; stem cells;
[15] TBR2: T-box brain protein 2; progenitor cells;
[16] TOAD-64: Turned on after division, 64 kDa; newborn cells;
[17] Vimentin: astrocytes;

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