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The Effects of Repeat Concussive Injury on Hippocampal Neurogenesis in Rats

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The Effects of Repeat Concussive Injury on Hippocampal Neurogenesis in Rats

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ABBREVIATIONS:

- 1°Ab = Primary Antibody
- 2°Ab = Secondary Antibody
- ^3_1H = *Tritiated* Thymidine
- Ab = Antibody
- ApoE = Apolipoprotein E
- BI = Blast Injury
- BrdU = Bromodeoxyuridine
- CA = Cornu Ammonis
- CBF = Cerebral Blood Flow
- CCI = Controlled Cortical Impact
- CNS = Central Nervous System
- CTE = Chronic Traumatic Encephalopathy
- DAI = Diffuse axonal injury
- DCX = Doublecortin
- DI = Diffuse Injury
- DG = Dentate Gyrus
- DNA = Deoxyribonucleic Acid
- DSU = Disk-Spinning Unit
- FI = Focal Injury
- FPI = Fluid Percussion Injury
- GFAP = Glial Fibrillary Acidic Protein
- LE = Long-Evans
- mTBI = Mild Traumatic Brain Injury
- NeuN = Neuronal Nuclei
- NSC = Neural Stem Cell
- PBS = Phosphate-Buffered Saline
- PNS = Peripheral Nervous System
- QNP = Quiescent Neural Progenitors
- RC = Repeat Concussion
- rTBI = Repeated Traumatic Brain Injury
- ROS = Reactive Oxygen Species
- SI = Stereo Investigator

- SGZ = Subgranular Zone
- SVZ = Subventricular Zone
- TAC = Transit-Amplifying Cell
- TBI = Traumatic Brain Injury
- TBS = Tris-Buffered Saline
- WDI = Weight-Drop Injury

INTRODUCTION:

The nervous system is a complex body system because of the abundance of cells required to perform a function and how the central nervous system (CNS) induces specificity during neural cell development. The activation of specific genes through transcription factors allows a neural cell to differentiate and mature into an appropriate cell type to function and influence behavior. This introduces a field of study in neuroscience, neurogenesis, which focuses on developing new neuronal cells in the CNS.

Many factors affect neurogenesis – Intrinsic factors include transcription factors such as Pax6, which aid in the proliferation of neural progenitor cells (Maekawa et al., 2005), and SOX2, responsible for differentiating adult neural stem cells (NSCs) into astrocytes and neurons (Suh et al., 2007). In addition, extrinsic factors such as exercise increase neurogenesis (vanPraag et al., 1999), while traumatic brain injury (TBI) can either increase (Chirumamilla et al., 2002; Rice et al., 2003; Ramaswamy et al., 2005) or decrease neurogenesis (Rola et al., 2006) depending on severity and location of the injury.

Impacts to the head are frequent, especially in sports and violence, and the number of reported cases has increased due to an increase in awareness of TBI in sports. Nevertheless, the number of reported instances still underrepresent the actual total amount of cases because some effects are mild and require medical attention for diagnosis. Mild traumatic brain injury (mTBI), also known as a concussion, affects 1.5 million individuals per year (Thurman et al., 1999; CDC, 2016). Unfortunately, there are no effective TBI treatments; current treatments deal with TBI symptoms such as headaches and dizziness. One therapeutic possibility would be to explore the neurogenic niche within the hippocampus in a region known as the subgranular zone (SGZ) of the dentate gyrus (DG). The adult SGZ has been observed to contain proliferating NSCs with self-renewal and multipotentiality properties (Altman, 1962; Gage et al., 1995). In adults, studies have also shown the multipotent NSCs can be reprogrammed into pluripotent cells (Kim et al., 2008). The adult NSCs can differentiate into many types of cells, including neurons, which can be integrated into the existing neural circuitry to play a functional and behavioral role. This neurogenic niche is sensitive to diseases, strokes, and injuries, and many studies are exploring its therapeutic potential (Goncalves et al., 2016; Pfisterer and Khodosevich et al., 2017).

In our laboratory, a closed head rat model of repeat concussion (RC) resulted in a decrease in cortical (Jamnia et al., 2017) and hippocampal neuron density (Peterson, unpublished results) compared to rats with a single concussion and sham. In these same rats, preliminary qualitative data has shown that RC also decreases the number of doublecortin positive (DCX+) cells, a microtubule-associated protein expressed by immature migrating neurons in the hippocampal DG 30 days, post-injury. The decrease of cortical and hippocampal neuron populations might be related to the reduction of DCX+ cells. Under normal conditions, DCX+ and neuronal nuclei positive (NeuN+), a transcription factor expressed by neurons, show a negative correlation; as newly generated neurons mature, DCX+ decreases as NeuN+ increases. Thus, a decrease in both DCX+ and NeuN+ cells suggests a problem with processes within the neurogenic niche.

The current study explored the effects of RC on the hippocampal neurogenic niche 40 days post-injury by examining two cell-type population measures. The first type of cell birth dated is the proliferating cells (BrdU-labeled) in the subgranular zone (SGZ) of the dentate gyrus to examine the extent of proliferation. The second type of cells is the number of DCX+ cells, which reveals the number of recently generated cells committed to a neuronal lineage but have not matured.

Determining if neurogenic proliferation and/or neuronal lineage commitment are altered can help us understand the extent to which repeated TBI (rTBI) affects the hippocampus. The study also considered sex differences in neurogenesis to observe if BrdU-labeled and DCX+ populations differ between male and female rats.

By understanding the extent to which adult neurogenesis is vulnerable to TBI, future studies can consider prevention and therapies to replace cells that have been lost due to head injuries. In addition, findings from this study may also potentially be used to treat neurodegenerative diseases.

LITERATURE REVIEW:

Adult Neurogenesis

What is Neurogenesis:

Neurogenesis can be defined as the neural stem cells' ability (NSCs) of the central nervous system (CNS) to differentiate into neurons. When neuronal cells fully develop, they integrate into the CNS to serve a functional and behavioral role. Neurogenesis occurs during embryonic development and throughout adulthood; however, differences are present. In embryonic neurogenesis, pluripotent cells' direct progression occurs during gastrulation, where the neuroectoderm generates the CNS. In adult neurogenesis, remnants of NSCs are limited with multipotency to generate either neurons or glia.

The areas of adult neurogenesis, also referred to as a neurogenic niche, are known to be in two regions of the brain: subgranular zone (SGZ; Altman, 1963) and subventricular zone (SVZ; Kaplan and Hinds, 1977), as observed in rodents. Using a thymidine analog, these areas were identified, which incorporates into the dividing cells specifically during the S-phase of mitotic division, to track where the NSCs proliferates (Altman, 1963; Kaplan and Hinds, 1977). Thus, the thymidine analog is the critical marker used in neurogenesis studies as it can be used to track the progress or development of progenitor cells as they differentiate and mature into different cells.

It is observed that neurogenesis in SVZ is not as prominent in humans as in rodents since humans lack migrating pathways from the SVZ to the olfactory bulb; the proliferating capabilities of SVZ in rodents are much higher than in humans; and SVZ houses more neurons in rodents than in humans (Sanai et al., 2004). This may be due to rodents' behavior; rodents highly depend on their sense of smell to survive, which could have been evolutionarily favorable for them. Given this difference between humans and rats in the SVZ, the current study will focus on adult hippocampal neurogenesis as it is observed to be active in humans and in our preliminary data showing that this region is affected by repeated concussions.

***Neurogenesis can occur in an Adult Mammalian Brain*:**

The science community did not openly accept early research in neurogenesis with the adult mammalian brain. Due to a popular decree by Cajal and early neuroscientists, the matured brain does not give rise to new neurons (Cajal and May, 1928). The first evidence of neurogenesis in an adult mammalian brain was observed by Joseph Altman and Gopal Das in 1960s (Altman, 1962; Altman, 1963; Altman and Das, 1967). Their collaboration in 1967 compared the brains of immature and mature guinea pigs to find the proliferating progenitor cells developing into neurons within the same region of the brain in immature and mature models. However, they were heavily criticized, and their work was not recognized. It was not until the late 20th century, studies by Kempermann et al. (1997), Eriksson et al. (1998), and Gould et al. (1999) supported the pioneering work of Altman and Das (1967), demonstrating that neurogenesis can occur within an adult mammalian brain. Kempermann et al. (1997) showed neurogenesis could be increased by putting mice in an enriched environment with a wheel, tunnels, and toys. Eriksson et al. (1998) were the first to show neurogenesis to occur in humans. Gould et al. (1999) conducted their research in mature primates and used a thymidine analog and antibodies against neuronal markers to observe newly formed neurons in the hippocampus.

***Stages of Neurogenesis*:**

Adult neurogenesis is divided into four phases: proliferation phase, early-survival phase, early-maturation phase, and late-maturation and survival phase (See Figure 1). Based on the morphology and presence of known transcription factors, the sequence of neuronal lineage commitment can be divided into five types of cells (See Figure 2). The initial proliferation of cells occurs in the subgranular zone (SGZ) of the dentate gyrus (Parent et al., 1997). The quiescent neural progenitors (QNPs), also known as Type-1 cells, are multipotent and appear glial in nature rather than as neurons. The QNPs rarely divide, but when division occurs, these cells divide asymmetrically, giving rise to one QNP and one transit-amplifying cell (TAC), also known as Type-2 cells. The TAC also divides asymmetrically. One cell can give rise to glioblast (immature glial cell) or neuroblast (immature neuron). The other TAC would reenter the cell cycle to divide again. The TACs do not continuously self-renew, unlike QNPs; TACs' mitotic ability can be exhausted (Ihrie and Alvarez-Buylla, 2008). Thus, new TACs need to be derived from the SGZ through QNPs.
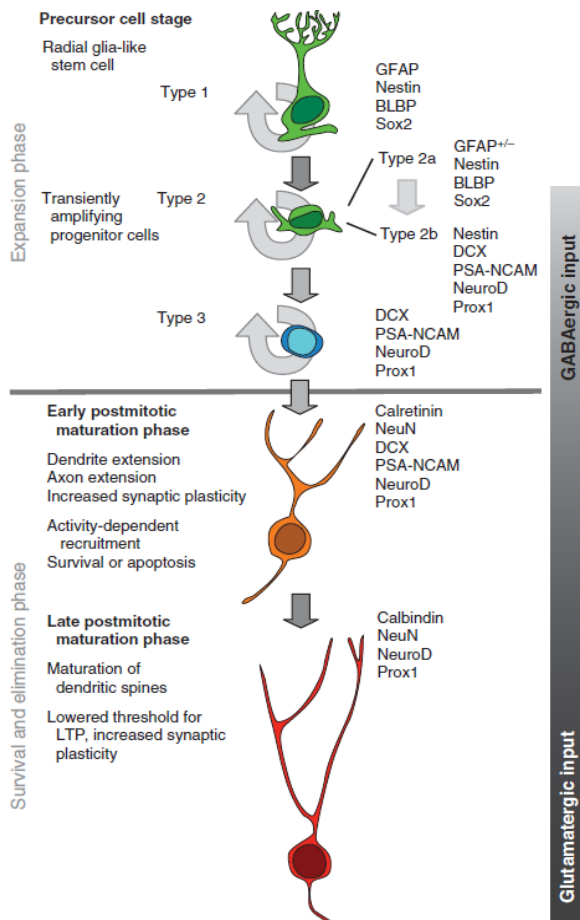
Figure 1. Adult Neurogenesis Cell Identity.
(Taken from Kempermann et al., 2015)

The proliferation and early-survival phase serve as a reservoir of new cells that might differentiate into neurons. The precursor cells, QNPs (Type-1), must exit the cell cycle and survive into intermediate precursor cells, TACs (Type-2 and -3), and continue through the differentiation and maturation process. In the early-maturation phase, Type-3 cells from the SGZ migrate to the dentate gyrus, where the immature neuron (Type-4) begins to form dendritic connections with neurons from the entorhinal cortex by sending its projections to Cornu Ammonis 3 (CA3) region through the mossy fiber tract. In the last phase, the late-maturation and survival phase, the Type-5 cells must successfully integrate into the central nervous system (CNS). Thus, it becomes indistinguishable from other mature neurons from an electrophysiological perspective. This whole process takes about seven weeks (Kempermann et al., 2015).
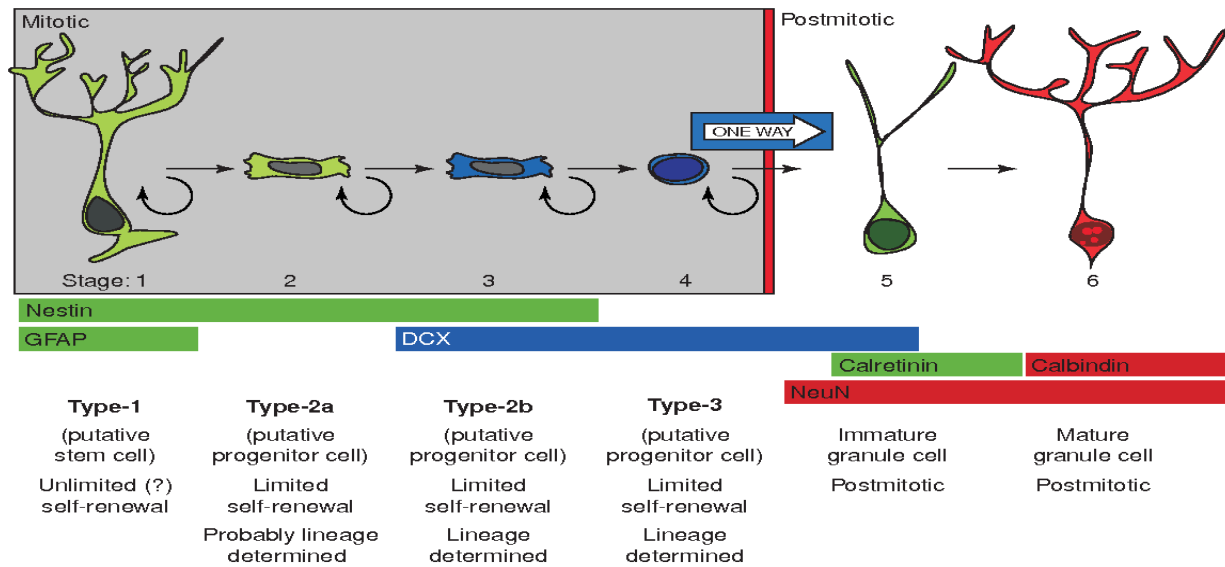
Figure 2. The neuronal development stages in the adult hippocampus. **(Figure taken from Kempermann et al., 2004).**

Traumatic Brain Injury

Traumatic brain injury (TBI) involves a blow or a jolt to the head with or without penetration through the skull. The type of TBI focused in this study is a closed head injury, also known as mild traumatic brain injury (mTBI) or concussion. This injury involves a physical insult to the head, resulting in a force that transmits through the cranium into the brain tissue without causing a skull fracture or significant overt pathology. However, the injury can cause the brain to undergo neuroinflammation (Xu et al., 2016) and tissue tearing from the forces, leading to changes in the brain's cellular physiology (Yang et al., 2015). If severe, these changes can cause neuronal death through apoptosis and necrosis, affecting brain-behavioral functions such as cognition, emotions, language, and sensation. Depending on the severity and location, this can create a spectrum of disabilities dependent on the frequency of the impact and/or injury. TBI cases have received more attention in recent years due to their prevalence in sports and following combat (Gessel et al., 2007; Champion et al., 2009; McKee et al., 2009). The pathophysiology observed in TBI brain tissues has shown to be a collection of a complicated disease process that adapts and constantly changes rather than be a stationary event (Masel and DeWitt, 2010).

Epidemiology of Traumatic Brain Injury:

TBI is a leading cause of death and disability. According to the Center for Disease Control and Prevention (CDC), TBI is defined as a blow or jolt to the head that can result in changes or interferences to the brain's normal functioning. In 2013, TBI affected around 2.8 million individuals, 2.5 million visits to the emergency department, 282,000 hospitalizations, and 56,000 deaths in the United States (Taylor et al., 2017). TBI cases have been noted to increase by comparing data collected from 2007 and 2013. The primary cause of TBI includes motor vehicle accidents, violence, work-related injuries, blast-trauma in wars, contact sports, and falls. The prevalence of TBI in contact sports and war have caused TBI to receive more attention in recent years (Gessel et al., 2007; Champion et al., 2009; McKee et al., 2009). The differences in the rate of injury observed in different ages and sex also play a role in TBI susceptibility. TBI is more prevalent in individuals 0–4 years, 15-24 years, and >75years (CDC, 2010). Males are more prone to TBI than females (Taylor et al., 2017). TBI susceptibility has also been observable in age-related health issues due to musculoskeletal and bone density that can cause an increase in the likelihood of an injury. In sex difference, it may be due to contact sports or war being dominated by males. The number of events recorded from emergency department visits, hospitalization, and deaths is still not entirely accurate because there are currently no effective methods that are foolproof in diagnosing the mild effects of TBI. This can lead to early TBI symptoms going unnoticed, and therefore, undiagnosed until the effects have accumulated and may become difficult to tolerate for the individual.

Types of Traumatic Brain Injury:

The current study focused on the closed-headed form of mTBI injuries, an injury-induced when a head accelerates and suddenly comes to a stop or decelerates by colliding with another object without experiencing a skull fracture. The exchange of biomechanical forces transmits from the object, through the skull, onto the brain, resulting in shearing and tearing of the brain tissue. Each TBI event can differ from another depending on the method and location of the injury, and the severity of the injury can range from mild (temporary loss of consciousness or no loss of consciousness) to severe (an extended period of loss in unconsciousness), which can result in different pathologies. Other factors that can also account for differences when observing TBI in patients arise from epigenetic differences, including age, sex, and past TBI-related experiences, as mentioned.

Concussion

The terms concussion and mTBI are used interchangeably, although the field is beginning to recognize that not all concussions are mild. According to the International Consensus Conferences on Concussion in Sport, a concussion is defined as "a complex pathophysiological process affecting the brain, induced by the biomechanical forces" (McCrory et al., 2013). A concussion results from an impact at a low velocity on the head that causes the brain to shake within the skull. The injury can occur either through the direct impact on the superior region of the head, the face, or any other body region that can lead to the biomechanical forces being transmitted to the brain. The effects of concussion are sudden, impairing neurological functioning by causing an acute disturbance in neuronal functioning rather than structural injury; thus, neurological abnormalities are not observable through neuroimaging. Symptoms related to a concussion may not always cause loss of consciousness (McCrory et al., 2013). Symptoms of a concussion usually last 7-10 days but longer for adolescents and children. When the symptoms transition from short-term to long-term, it is known as a post-concussion syndrome that can take weeks to recover or more than a year (Hall et al., 2005).

Repeat concussion occurs when an individual experiences multiple concussions over time and is associated with neurodegenerative disease, chronic traumatic encephalopathy (CTE) (McKee et al., 2009). It was initially observed in boxers and termed punch drunk syndrome (Martland, 1928). Dr. Omalu was the first to attend CTE in a professional football player (Omalu et al., 2005), which led McKee to examine more professional football players' brains (McKee et al., 2009), observing the similar pathology in Dr. Omalu's study. In CTE, the primary pathology is phosphorylated tau which is irregularly spread in focal patches that tend to be noticeable in superficial cortical layers. The phosphorylated tau pathology is also present in other neurodegenerative diseases such as Parkinson's or Alzheimer's disease (McKee et al., 2009) but does not share the same dispersal pattern of the protein as in CTE. Individuals with CTE experience changes in their memory, behavior, emotions and show parkinsonism (Stern et al., 2011). The mechanisms of CTE are still not well understood, but it is associated with the common mechanisms of TBI that involve inflammatory response, ischemia, and irregular release of neurotransmitters that lead to cell death (McKee et al., 2009). There is also evidence that suggests apolipoprotein E (ApoE) involvement in CTE. ApoE has been associated with depositing beta-amyloid proteins that may influence CTE development as seen in AD, but the direct association between ApoE and CTE remains unclear (Hartman et al., 2002; Omalu B. et al., 2011).

Brain Contusion

A more severe form of TBI that can result in observable degeneration in the brain is called a contusion. A contusion forms due to hemorrhages in a localized area. This can lead to ruptures in the brain's microvessels, termed hemorrhagic progression of a contusion. Contusion deprives certain regions of the brain of blood, resulting in tissue death that leads to loss of function, and the direct exposure of the blood to the brain is toxic (Kurland et al., 2012). The hemorrhagic progression of a contusion occurs after TBI, which requires monitoring to prevent it from happening.

Coup-Contrecoup Brain Injury

Coup-contrecoup brain injury is an injury on both sides of the brain due to one impact on the head. The impact on the initial side (coup) generates enough force to push the brain to the opposite side of the skull (contrecoup). Both types of injuries can lead to hemorrhages. However, due to the difference in the force exerted on the brain between primary and secondary impact, both sites experience a different pathology. (Bhateja et al., 2009). Mild forms of coup-contrecoup brain injury can be observed following a concussion as well.

Injury Mechanisms Following Traumatic Brain Injury:

The physiological effects of TBI are induced through primary and secondary mechanisms. The primary mechanisms are initiated by the rapid acceleration and deceleration of the brain due to an impact from either a linear or a rotational force. A linear force causes a direct impact onto the head, causing the head and the brain to move in an anterior-posterior direction, leading to gliding contusion on the cortex and axonal injury in the brain stem (Jordan, 1987). The rotational force on the head is caused by a sudden turn within the vertical or horizontal plane, as seen in boxing resulting in "twisting" the brain (Blennow et al., 2012). The brain's physical properties are more sensitive to the rotational forces that cause it to easily shear and cause concussions than linear forces (Meaney and Smith, 2011; Blennow et al., 2012). Thus, the primary mechanism induces mechanical disruption, leading to contusion, hemorrhages, and axonal shearing (Cernak, 2005). The secondary mechanism is the aftermath of the primary mechanism; the secondary mechanisms' effects are delayed by a few hours or days. It can change the brain's metabolic, cellular and chemical mechanisms of the brain, and can cause damage to the blood vessels, resulting in a hypoxic-ischemic event and, thus, cell death (Bramlett and Dietrich, 2007). The secondary mechanism is also associated with a buildup of the intracranial pressure due to the brain's expansion caused by swelling (Gennarelli, T.A., 1993). The swelling can be prevented and treated by regulating the cranial pressure (Mendelow and Crawford, 1997).

Injuries in TBI can be categorized into focal injury (FI) and diffuse injury (DI). The focal injury is generated by an injury focused at a single location, leading to a skull fracture. The focal injury is commonly seen in moderate and severe TBI. This type of injury can cause contusions, lacerations, and hemorrhages. In addition, the sudden acceleration and deacceleration of the head can cause DI. DI cannot be identified or associated with one injury site on the brain but in multiple regions. The event of DI can cause a temporary concussion, and in prolonged cases, it leads to diffuse axonal injury.

Diffuse Axonal Injury

Diffuse axonal injury (DAI) is a micro event that damages the axons of the nerve fibers, corpus callosum, and brain stem. The severity of the damage depends on the force of the impact on the head. Assessing DAI after TBI is essential in determining morbidity and mortality, leading to a disability or a neurovegetative state (Gennarelli, 1987). The DAI causes changes in cognition and behavior, which comprises the ability to interact socially and be productive. These effects are chronic and impair normal functions but do not destroy the function; thus, neural plasticity can remodel the brain to regain some functions. The acute phase of DAI is challenging to diagnose, but with computerized tomography and clinical observation, some early signs of DAI can be observed. In severe TBI cases, DAI can lead to hemorrhages in both the brain's ventricles (intraventricular), between the meninges, and the subarachnoid space (Vieira et al., 2017). DAI can also result in necrosis leading to neuroinflammation.

Pathophysiology of Traumatic Brain Injury:
Neurochemical changes

Regular activity of neuron communication is regulated by neurotransmitter release from the presynaptic neuron onto the post-synaptic neuron. The neurotransmitter release is dependent on changes in membrane potential by voltage-gated channels in an axon. Electrochemical changes are then equilibrated by sodium-potassium (Na^+/K^+) ATPase pump. When TBI is induced, this causes a disruption of ions in the presynaptic neuron, resulting in an increase in extracellular K^+ and increasing in intracellular Ca^{2+}. These changes cause the presynaptic neuron to release neurotransmitters and cause oxidative stress in its mitochondria due to the intracellular accumulation of Ca^{2+}. Within the first hour of TBI, the disruption of ions cause an unregulated and constant release of glutamate from the presynaptic neuron to the post-synaptic neuron thus, overexciting it and allowing excessive Ca^{2+} to also accumulate (Prins et al., 2013; Osteen et al., 2001). This intracellular accumulation leads to oxidative stress on the mitochondria due to mitochondrial Ca^{2+} reuptake and can initiate apoptosis (Xiong et al., 1997).

Glucose Metabolism

Glucose metabolism has been observed to change post-TBI. Initially, there is a great increase in glucose metabolism after an injury, which is proposed to be necessary, given the high amount of energy required to restore ionic imbalance after an acute event (Hovda et al., 1990). After the increase in glucose metabolism, there is a decrease in glucose metabolism (Andersen and Marmarou, 1992). The duration of glucose metabolism depression is associated with the severity of the injury and correlated with behavioral dysfunction, but it does not affect consciousness (Bergsneider et al., 2000). The mechanisms for glucose metabolism depression are still not clear. There are three explanations for the glucose metabolism depression: 1) the decrease in cerebral blood flow (CBF) (Yamakami and McIntosh, 1991); 2) impaired glucose transporter from CBF to the brain (Balabanov et al., 2001); 3) decrease in cerebral glucose level due to glycolysis disruption (Bartnik et al., 2005). Nevertheless, glucose disruption leads to an energy crisis in the brain, leading to further dysfunction.

Free radicals

"Free radicals are molecules with unpaired electrons" (Prins et al., 2013). They are highly reactive by taking electrons from the surrounding molecules, resulting in damage to proteins in the cell membrane, DNA, etc. There are two free radicals within our body system, which are highly regulated to prevent them from damaging the surrounding tissue: reactive oxygen species (ROS) and reactive nitrogen species (RNS). Under normal conditions, ROS is produced through metabolism and "deactivated" by the mitochondria's antioxidative reaction. In TBI events, ROS level production is increased or accumulated (O'Connell and Littleton-Kearney, 2013). Reactive nitrogen species have also been observed to increase after TBI (Clark et al., 1996). The accumulation of free radicals observed in TBI can induce an apoptotic event within a cell.

Treatment of TBI:

Survivors of TBI, depending on the severity and frequency of injury, can experience chronic and neurodegenerative pathology that interferes with cognition, motor, and personality. There are no drug treatments against the secondary effects of the injury. Drugs that are currently offered are only used to treat the symptoms such as headaches. Some treatments also involve rehabilitation to help patients regain some behavioral functions that have been hindered, such as locomotor movement. Therefore, the current goal of TBI studies is to understand the pathophysiology changes due to injury. Thus, specific treatments can be targeted towards these changes that occur post-injury.

*Animal Models***:**

Animal models are needed to study traumatic brain injury (TBI) to better understand the cascade of events and test potential therapies. Unfortunately, current treatments that have been introduced through animal models have continued to fail in clinical trials because there are currently no effective animal models that can replicate the biomechanics of the impact on a human compared to an animal. Therefore, animal models of TBI are constantly revisited and refined.

Current studies use smaller animals such as mice and rats because they are easily housed, cost-efficient, and many studies are conducted using these rodents. Animals that are larger and whose brain size is like humans are optimal but require extensive care compared to rodents and involve complicated ethical issues. There are currently four injury models commonly used in TBI research: fluid percussion injury, cortical impact injury, weight drop, and blast injury (Xiong et al., 2013). Figure 3 provides a summary of all types of TBI.
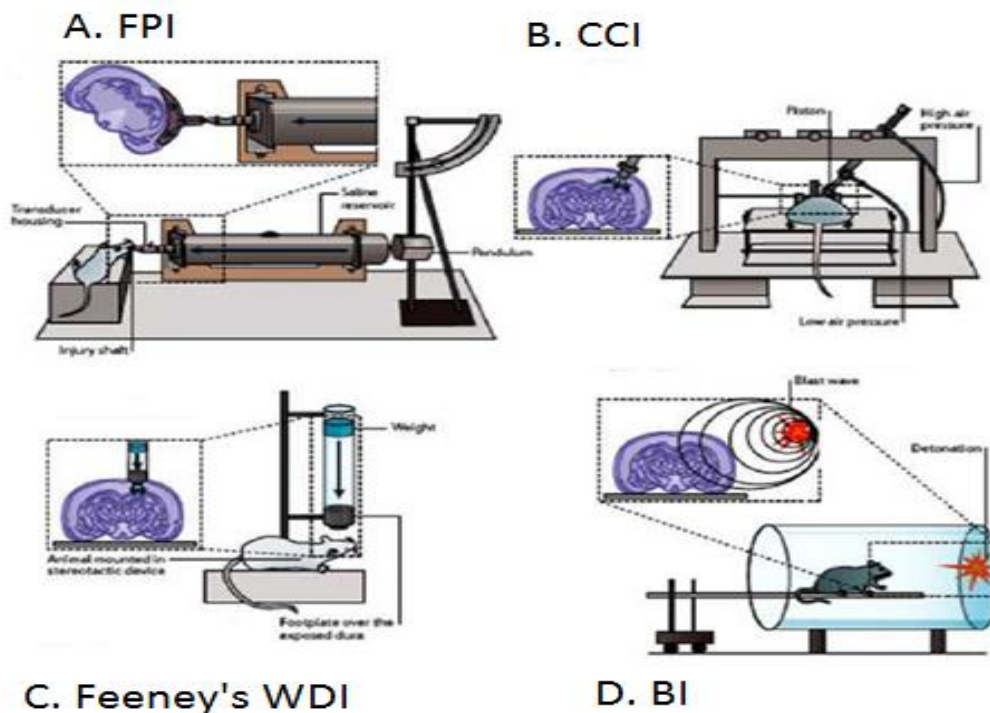
Figure 3. Animal TBI models. **A) FPI induced using fluid pressure; B) CCI-induced using electromagnetic forces at fixed parameters; C) Feeney's WDI-craniotomy; D) BI induced through a compressed shock tube (Figure modified from Xiong et al., 2013).**

Fluid Percussion Injury

The fluid percussion injury (FPI) requires the brain to be exposed through a craniotomy while the meninges are intact (See Figure 3A). The device contains a piston with fluid that is used to generate a fluid pressure force when a pendulum is released. The force's impact causes the brain to move within the animal's skull, as in a TBI. The injury does not involve fractures since the skull is already exposed, and the severity of the injury can be adjusted. Fluid percussion injury causes hemorrhages and swelling of the brain and can create a contusion at higher severities (Sanders et al., 1999; Graham et al., 2000).

Weight-drop Injury

The weight-drop injury (WDI) can be performed with or without craniotomy by using a free-falling weight (Morales et al., 2002). The severity of the injury can be adjusted by using appropriate weights and height for the falling object. In one model, Feeney's WDI exposes the brain to cause a cortical contusion and hemorrhage (Figure 3C). In Shohami's WDI, the injury is induced directly onto the head's surface (closed-head), which led to neurological impairment and loss of blood-brain barrier integrity. Further analysis of Shohami's WDI, magnetic resonance imaging showed that rodents' conditions resembled clinical conditions in humans (Albert-Weißenberger et al., 2012). These previous WDI models have not been able to replicate the rotational injuries as seen in motor vehicle and sports accidents; a newer model, the Maryland model, can generate a rotational acceleration causing the head to move anterior-posterior and sagittal direction, but more studies are currently needed to observe the pathology of Maryland model (Kilbourne et al., 2009).

Blast Injury

Blast injury (BI) uses a "compression-driven shock tube to simulate blast effects" that would be observed in explosions (Xiong et al., 2013) (Figure 3D). One study has shown mild BI effects causing deficits in recognition, memory, and motor coordination in mice (Koliatsos et al., 2011). Many studies have also studied BI's effects, but pathology varies between studies (Xiong et al., 2013).

Controlled Cortical Impact Injury

Controlled cortical impact (CCI) induces injury by exposing the brain through a craniotomy and injuring the brain directly by using an impactor tip to deliver a force directly onto the surface of the brain's outer covering (Figure 3B). The impactor uses electromagnetic energy that can be adjusted based on severity and aimed at a specific cortical region to induce the injury (Romine et al., 2014). This can produce a focal injury and cause cortical tissue loss and formation of a contusion along with acute hematoma, axonal damage, concussion, and loss in the blood-brain barrier integrity (Dixon et al., 1991). The effects of the CCI have also been seen in the hippocampus, and the thalamus, causing degeneration or dysfunction in these regions (Hall et al., 2005).

Our lab has used CCI to create a closed head injury model, where the impact tip is used to hit the head's surface with the skull intact instead of directly onto the brain's outer covering (See Figure 7). This provides a more clinical relevance than compared to models above because it mimics more closely what occurs in human TBI. This model also does not lock the rodent's head in place, thus allowing the head's movement as the force of the impact transmits throughout the rodent's head naturally. The rodent is anesthetized to prevent it from moving while lies on a foam bed; thus, CCI injuries can be repeated with high accuracy (Jamnia et al., 2017).

Hippocampus, TBI, and Memory

Memory formation within the hippocampus is conducted by a well-described tri-synaptic loop involving three essential structures: dentate gyrus (DG), Cornu Ammonis (CA), and entorhinal cortex. These structures are in both hemispheres of the brain within the temporal lobe.

A single network of neurons can be responsible for multiple memories simply by changing the synaptic pattern. Activation of a single memory can lead to the activation of various neurons. These memories are fixed into the neural system as long-term memories initiated by the hippocampus. The inputs for new memory formations enter from the entorhinal cortex. Neurons in the entorhinal cortex extend their axons to neurons in the granule layer of the DG. Neurons from the DG then send their projections through the mossy fiber tract to the neurons in the CA3. Neurons in the CA3 sends their projection through Schaffer collaterals to neurons in the CA1. Neurons in the CA1 then send their input to the entorhinal cortex. Thus, completing the synaptic transmission between the neurons in the DG, CA3, and CA1, also referred to as the trisynaptic loop (See Figure 4).
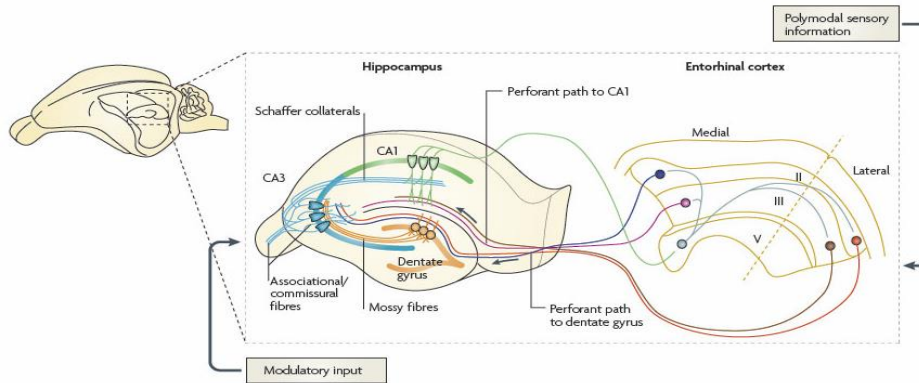
Effects Traumatic Brain Injury on Hippocampus:

One of the most typical symptoms following TBI is learning and memory dysfunction. Disorder in memory and learning can be due to pathological changes in the hippocampus and the medial temporal lobe resulting from TBI. The accelerating-deaccelerating forces on the temporal lobe make it easily susceptible to contusion and diffusional axonal injury due to its position on the middle cranial fossa (Tate and Bigler, 2000). As mentioned previously, TBI also causes secondary effects such as excitotoxicity thus, further influencing neuronal behavior by inducing apoptosis.

In moderate and severe TBI cases, observable neuronal loss occurs throughout the hippocampus (Tate and Bigler, 2000; Serra-Grabulosa et al., 2005). In concussion, no observable changes in morphology occur, but subtle changes in synaptic activity can be present. A study in cell morphology by Raghupathi (2004) showed no significance in hippocampal neuronal and glial cell death in concussion models, but as the severity of TBI increased, more neuronal and glial cell death was present. To observe neuronal cells' behavioral activity under the effects of concussion, Yu and Morrison (2010) collected and cultured sliced hippocampal sections from neonatal rat pups and induced *in vitro* TBI. They used microelectrode arrays to measure cellular behavior by observing the electrophysiological activity of cells within the hippocampal region, which showed neuronal network dysfunction compared to non-injured animals. This suggests that measuring neuronal function might effectively determine the effects of TBI rather than quantifying neuronal death.

Along with observable behaviors, Scheff et al. (1997) used a CCI model to test spatial learning (acquisition) and memory (retention) by inducing mild and moderate TBI. Rats with mild and moderate TBI scored poorly compared to sham in learning. Memory tasks were determined by the time it took the rat to find the target location and how frequently it visited the target location in a Morris water maze. Moderate TBI rats also scored poorly in the Morris water maze and exhibited observable histopathology compared to mild TBI rats with no observable histopathology. Another study also tested spatial learning memory by using a T-maze and a different injury model (lateral fluid percussion) to perform mild TBI. They observed no histological differences, no neuronal cell loss, and no changes in electrophysiology activity when testing working memory between sham and injured animals, but the injured animals did have fewer cells related to spatiotemporal activity, which correlated with poor working memory tasks (Eakin and Miller, 2012). This suggests mild TBI can cause disorganization in hippocampal activity without having cell loss.

In repeated mild TBI cases, one study used a lateral fluid percussion model to induced concussion three times and observed hippocampal and behavioral changes long-term (28 days). They observed the repeated mild TBI animals had significant neuronal cell loss and increased microglia activity. The electrophysiological test was also done on hippocampal sections for long-term potentiation, which was not present in repeated mild TBI animals but present in single mild TBI animals. They also further investigated behavioral responses for memory by using the Morris water maze and novel object recognition. For both behavioral tests, repeatedly injured animals performed poorly compared to sham and single injured animals (Aungst et al., 2014). Other studies have also investigated outcomes of behavioral response due to sex differences after repeated mild TBI. One study used mice in the Morris water maze (Velosky et al., 2017). The other study used rats in novel object recognition (Wright et al., 2017), which both shared the same conclusion—females were reported to perform better than males after injury.

Our laboratory's previous study used a clinically relevant model for concussion; three groups were introduced: sham, single concussion, and repeated concussion. The repeat concussion group showed a significant decrease in cortical (Jamnia et al., 2017) and hippocampal neuron density (Peterson, unpublished results); when compared to sham and single concussion, no difference was observed between single concussion and sham. The two markers were used to assess neuronal density: DCX+ (immature neuronal cell) and NeuN+ (mature neuronal cell).

In an *in vitro* repeated concussion study, the injury was induced using a stretch-induced model (an injury model that disrupts cell integrity by introducing strong physical forces such as high atmospheric positive pressure, high centripetal force, which varies depending on the method of injury, Ellis, E. et al., 1995) to measure hippocampal cellular pathology with propidium iodide (propidium iodide enters damaged cells) and fluorescein diacetate (poor retention of fluorescein diacetate when cells were damaged). The concussive injury was introduced twice, 1 hr. apart to mimic repeated cases, and cellular pathology was measured 24 hrs., post-injury. The study compared the repeated concussion group with the single concussion group and observed that the repeated concussion group received the worst outcome; glial cells were noted to have increased propidium iodide uptake. The neuron processes' damage was present in immature neurons due to the release of neuron-specific enolase and S-100 β protein, common clinical markers used for brain injury (Slemmer et al., 2002).

Thymidine Analogs

Thymidine analogs have served as one of the most critical markers in studying neurogenesis. The marker is used to detect proliferating cells in neurogenesis. It can be incorporated into the DNA when the DNA is replicating during the S-phase. The first type of thymidine analog to be used is tritiated thymidine ($^{3}_{1}H$) which helped Altman in discovering the hippocampal neurogenic niche. The use of $^{3}_{1}H$ is a time-consuming process, which took longer for the cells to incorporate $^{3}_{1}H$ into its DNA, and it is challenging to observe $^{3}_{1}H$-stained cells in thick tissues (West et al., 1991).

Bromodeoxyuridine (BrdU) was introduced in 1989 by Hoshino as means to detect proliferating cancer cells without having any induced radioactive effects like in $^{3}_{1}H$. BrdU was much more beneficial than $^{3}_{1}H$ since it is not toxic to the researcher, not time-consuming, and thick-stained tissues could be easily observed. Regardless of all the benefits of using BrdU, it also had limitations. The replacement of a halogen with a methyl group on the nucleotide does provide instability for the DNA, which could lead to mutations, and at a high dosage, could be toxic to the animal and cells (Taupin, 2007). Another limitation of BrdU is that it is used to target cells that are undergoing DNA synthesis. DNA replication is semiconservative; if the cells were to replicate again, the expression of BrdU might be reduced to half. Thus, the resolution in detecting cells that initially proliferated becomes reduced (Taupin, 2007). BrdU works well with precursor cells in neurogenesis because they do not replicate as much as true stem cells, or embryonic stem cells; precursor cells eventually differentiate rather than constantly multiply.

Other types of halogen-based thymidine analogs have also been introduced that replaces the Bromo-group with either a chloro- (CldU) or iodo- (IdU) group. Using different halogens allows for different antibodies for detection and expression at different time points to be observed (Vega, C. and Peterson, D., 2005). For example, if other types of halogen-based thymidine can be incorporated into the same tissue at different times, events such as initial-proliferation and late-proliferation can be observed by injecting IdU and CldU.; coupling the halogen-based thymidine with TBI, the initial effects, and the late effects of TBI on neurogenesis can be observed.

Traumatic Brain Injury and Neurogenesis

The effects of traumatic brain injury (TBI) on neurogenesis remain mixed, and the purpose it serves is still controversial. Some studies have shown an increase, and other studies have shown a decrease in neurogenesis. This may be due to many factors, including different models of injury that impact various regions of the brain at different severities and different time frames of injections with varying analogs of thymidine, which mark neurogenesis at different stages - thymidine analogs are essential in this research field that allows researchers to track proliferating progenitor cells, their development, and their displacement in the brain.

Fluid Percussion Injury (FPI) Induced Neurogenesis:

Chirumamilla et al. (2002) used rats and induced lateral FPI to study early proliferation post-injury. The animals were injected with *tritiated* thymidine ($^3_1$H) twice, 4 hrs. apart, 48 hrs. post-surgery. The animals were sacrificed 48h after the last $^3_1$H injection. Other rats were injected with bromodeoxyuridine (BrdU), which had a longer survival period of 24 hrs. They observed two neurogenic sites, the subventricular zone (SVZ) and dentate gyrus (DG), to find that most proliferating cells were cell types related to microglia and astrocytes. Interestingly, they found other cells in the SVZ but could not identify those cells because those cells might not have had enough time to differentiate.

Rice et al. (2003) performed lateral FPI on rats and injected BrdU four times, 2 hrs. apart on day 1, followed by injections on 4, 7, 9, and 14, post-injury. The animals were sacrificed 24 hrs. after the last injection. They observed new proliferating cells in both areas of SVZ and the subgranular zone (SGZ). They noticed an increase in proliferation at SGZ on day 1 and day 7, but they could not track those cells' further progress since BrdU was no longer visible; these cells could have also degraded. They found an increase in new neurons ten days after injury, but these neurons were derived from SVZ.

Weight-Drop Injury (WDI) Induced Neurogenesis:

Dash et al. (2001) performed weight drop injury on rats and injected them with BrdU six times (4, 6, 18, 20, 22, and 24 hrs.) and perfused 24 hrs. after the final injection. They performed a short-term study to observe the effects of neurogenesis due to WDI 3 days post-injury. They observed the BrdU co-labeled with the neuronal marker, calbindin, cells to have increased within the granule cell layer of the DG.

Blast Injury (BI) Induced Neurogenesis:

Adult mice were subjected to a short-term (5 days) and long-term (21 days) study. Before the injury was induced, the mice were injected once every day for 5 days with BrdU. The mice then experienced BI the next day after the 5th injection. The mice were subjected to six pulses of the blast at 194 dB and sacrificed accordingly to the short-term and long-term study. They noted a decrease in BrdU cells in both SVZ and DG in the long-term study when compared to the short-term study but observed a decline in DCX cells in both SVZ and DG in the short-term study when compared to a long-term study (Nair, 2015).

Controlled Cortical Impact (CCI) Injury Induced Neurogenesis:

Gao et al. (2009) performed moderate opened-head CCI on mice. The mice received BrdU injection once a day for a week and perfused 24 hrs. after the last injection. They observed two different precursor cells: quiescent neural progenitors (QNPs) and transit-amplifying cells (TACs) within the DG. They observed QNPs respond to injury by increasing its proliferating capabilities, whereas TACs did not respond to the CCI. This suggests that different types of cells respond to CCI, derived from other precursor cells that can aid in recovery

Ramaswamy et al. (2005) performed a moderate unilateral CCI on adult mice. The mice were injected with BrdU immediately post-injury and three times every 24 hrs. The animals were sacrificed 3 days post-injury. There was a proliferation of cells observed in the SVZ only ipsilateral to the injury, but not contralaterally. There was also migration of cells from the ipsilateral SVZ to the area of cortical injury.

Rola et al. (2006) performed a craniotomy to induce injury on mice (2-month-old). Animals were sacrificed at 6, 12, 24, and 48 hrs. and 7 and 14 days post-injury. The researchers were interested in neuronal death at SGZ due to apoptosis and necrosis. They used Ki-67 to identify proliferating cells and immature neurons. After a week post-injury, DCX+ cells were noted to have decreased on the contralateral side, which returned to normal levels within after another week. In addition to the study, the researcher also used 7 days of post-injured mice to observe the long-term effects of TBI. They introduce a single injection of BrdU on day 7 post-injury, followed by sacrificing the animal three weeks after the injection. They observed long-term effects of TBI to have reduced BrdU+ cells and new neurons (BrdU/NeuN) on the ipsilateral side but no changes on the contralateral side. Astrocytes were also noted to have increased on the ipsilateral side and activated microglial on both sides. The study here demonstrates TBI effects on neurogenesis and glial cells, which may influence cognition.

Summary of Traumatic Brain Injury and Neurogenesis:

Neurogenesis gives rise to both glia and neurons from NSCs. The injuries induced to observe neurogenesis following TBI provided contradictory results of the type of proliferating cells and whether TBI causes an increase or decrease in those proliferating types of cells. Table 1 summarizes the results of research that is relevant to the current study.

Table 1: Summary of TBI Effects on Neurogenesis. **Results are shown to be incomplete and contradictory on the effects of traumatic brain injury on hippocampal neurogenesis. Filled black boxes suggest researchers did not further investigate the cell's identity (+ = increase; -- = decrease).**

Researcher	Method of TBI	SGZ	Astrocytes	Neurons
Chirumamilla et al. (2002)	FPI	+	+	■
Rice et al. (2003)	FPI	+	+	■
Rola et al. (2006)	CCI	-	+	–
Gao et al. (2009)	CCI	+	■	–
Dash et al. (2001)	WDI	+	■	+
Nair (2015)	BI	–	■	■

Repeat Traumatic Brain Injury:

To the best of our knowledge, there are currently no observations of the effects of repeat TBI on neurogenesis. As mentioned, repeated events of concussions have resulted in the development of CTE in professional football players. Understanding the long-term effects of repeated TBI on neurogenesis can provide us insights into the functions and limitations of neurogenesis in head injuries.

Our laboratory has begun to examine this in a closed head model of rTBI that showed a decrease in cortical (Jamnia et al., 2017) and hippocampal neuron density (Peterson, unpublished results) compared to rats with single concussion and sham. Furthermore, in these same rats, preliminary data from our lab has shown through qualitative analysis that repeated concussion decreases the expression of migrating neuroblasts (DCX+) and neuronal (NeuN+) cells in the hippocampal DG 30 days, post-injury (See Figure 5).
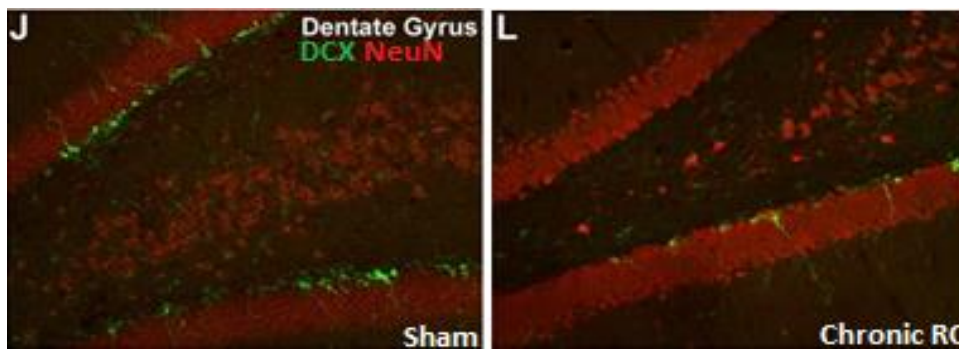

Figure 5. The decrease in DCX expression in the hippocampal DG. **Preliminary data collected from our lab has shown chronic RC decreases DCX+ (green) and NeuN+ (red) cells in the hippocampal DG (Right) when compared to sham (Left) (Figure taken from Dr. Peterson's Lab).**

Sex Differences in TBI and Neurogenesis

Studies have suggested that sex differences are observed in adult neurogenesis between male and female rats due to sex hormones playing a role in regulating adult neurogenesis (Duarte-Guterman et al., 2015). Another study has investigated sex with cell genesis, a rise of any new cell in the CNS. They had conducted a short-term (3 days) and a long-term (7 days) study and compared male and female rats. They induced hypoxia-ischemia and treated the animals with estradiol to serve as a neuroprotectant. They found cell genesis to be greater in females than males in the short-term study, but no observable differences in cell genesis in the long-term study (Waddell et al., 2016). Tanapat, P. et al. (1999) observed cell proliferation in the subgranular zone (SGZ) of the dentate gyrus (DG) to be more neuronal than glia in nature in both male and female rats. Their study suggests there is a correlation between the rodent estrous cycle and cell proliferation in the SGZ. The estrous cycle seems to also play a role in influencing cell survivability. The total number of cells that proliferated during high estrogen levels (proestrus) remained elevated even after 14 days. On the 12th day, the number of new neurons was the same in proestrus as in estrus (low estrogen levels). The cells that were generated in either proestrus or estrus phase expressed a mature neuronal marker, calbindin. The total number of granule cell numbers or the granule cell layer volume did not differ due to sex differences.

Sex differences are also prevalent in TBI. One study found male rats to be better at spatial learning than female rats post-injury; they observed that neurogenesis differed due to sex and concluded that male rats had an increase in neurogenesis than in female rats – outperforming females rats in spatial learning tasks. Learning and neurogenesis were associated with gonadal hormones as well (Mahmoud et al., 2016). Another study tested spatial memory by performing RC in male and female mice. They tested the mice's spatial memory using a Morris water maze and found females to be more active than the males—the females swam faster and a greater distance. Their results contradict the previous study, Mahmoud et al. (2016), but they both suggest hormones' role in TBI, which can also potentially affect neurogenesis (Velosky et al., 2017). To the best of our knowledge, sex differences in neurogenesis following TBI have not been examined.

Clinical Difference in Male vs. Female due to TBI:

In adults, epidemiological investigations show females were more likely to experience symptoms due to TBI along with surgical interventions and falls, but injury incidence is generally lesser in the female population: The concussion cases are observed to be higher in males, but females are more likely to experience greater severity of TBI, and morality (Munivenkatappa et al., 2016). It's been observed post-concussion syndrome and disability such as back pain are highly present in females compared to males due to concussion (Styrke et al., 2013). Female athletes were more likely to be injured with a concussion and have worsened outcomes than males (Dick, 2009). In long-term post-injury cases of moderate-severe TBI, males were reported to have difficulty in setting realistic goals, experiencing restlessness, increased sensitivity to noise, sleep disturbances, whereas compared to females who reported to experience headaches, dizziness, and loss of confidence, lack of initiation, and required supervision in daily tasks (Colantonio, 2010).

Bigler et al. 1996, observed changes in hippocampal volume using MRI and changes in memory assessed by behavioral test between non-injured and injured patients due to TBI. The effects of the structure-function relationship between hippocampal volume and memory became significant after 90 days post-injury—the reduction in hippocampal volume due to TBI correlated with a decrease in memory. A future study (Tate, D. and Bigler, E., 2000) investigated sex differences in hippocampal volume reduction due to TBI. There was no significant difference in hippocampal volume between male and female non-injured patients. There was also no significant difference in hippocampal reduction due to TBI between male and female patients.

The studies with animal models have suggested sex hormones play a role in TBI. Specifically, in animal models, estrogen is shown to act as a neuroprotectant, but in clinical studies, TBI in females had experienced more severe injury cases than in males. The discrepancy between animal studies and clinical studies rises from obvious physical differences amongst species' brains, such as brain geometry. Further discrepancies also arise depending on the rat strains used, which would yield different behavioral and histopathological results (Xiong et al., 2013). Further study is warranted.

Rationale

Neurogenesis continues throughout an animal's life, and it gradually decreases as the animal matures. Neurogenesis is found in the hippocampus's dentate gyrus, and it plays an important role in the animals' learning and memory. Repeated concussions have resulted in deteriorating effects on memory in rats, suggesting that repeat concussions may influence neurogenesis.

Study Objectives

The study measured the changes in BrdU-labeled and DCX+ populations within the hippocampus's DG following a repeat concussion in both male and female rats. Since our preliminary qualitative data has shown a decrease in DCX+ and NeuN+ population, post-injury, new qualitative and quantitative analysis will consider BrdU-labeled and DCX+ population to confirm these findings. We observed proliferating cells via BrdU-label to determine its neurogenic proliferative capabilities, followed by labeling neuronal committed cells with DCX+ to determine neuronal lineage commitment capabilities.

If sex differences play a role in neurogenesis and TBI, BrdU-labeled and DCX+ populations may differ between male and female rats. Female rats have been observed to have less neuronal loss due to estrogen playing a neuroprotective role.

Hypotheses

1. Repeated mTBI affects neurogenic proliferation in the DG post-injury by decreasing the BrdU-labelled population.
2. Repeated mTBI affects neuronal commitment in the DG post-injury by decreasing the DCX+ population.
3. Normal estrus-cycling female rats will not demonstrate reduced neuronal proliferation and commitment population due to repeat concussion.
4. There will be a relationship between the levels of DCX+, BrdU+, and behavioral measures of memory.

STATEMENT OF DESIGNED STUDY:

The current study used animals from previous repeated mTBI studies. Those animals underwent head injuries, behavioral tests (open field, novel object recognition, and foot fault), were injected with BrdU, euthanized, and perfused. The current project focused on immunostaining brains from these animals against BrdU+ and DCX+ cells using antibodies, using quantitative stereological methods to examine cell populations, and comparing neurogenesis with performance on novel object recognition test results done previously.

Experimental Design

The animals were initially acclimated to handling for one week before injuries were induced. The injuries were performed in male and female rats and compared to animals that were not injured (sham) to examine differences in adult neurogenesis. The sham animals also underwent anesthesia even though the animal group was not injured. The injures were aimed over the right forelimb sensorimotor cortex using closed head controlled cortical impact (CCI) as demonstrated by previous works in the lab (Jamnia et al., 2017). For repeat concussion (RC), three injuries were performed, each 48hrs. apart (See timeline below). After the last injury, animals were tested for behavioral and motor changes; on day 32, animals were tested using a novel object recognition test to measure memory after the last injury. Animals were injected with BrdU according to their weight the day before they were sacrificed, ~46 days since the initial mTBI. Animals were perfused, and the brain tissue collected, preserved with cryoprotectant, and prepared as sagittal sections (See Figure 6).

Primary antibodies (1°Ab) against BrdU and DCX were used to label and identify the proliferating and neuronal committed cells, respectively. Secondary antibodies (2°Ab) conjugated with fluorophores permitted the detection of co-localization to determine the cell's phenotype. These cell populations were assessed using confocal microscopy and quantified using design-based stereology, Stereo Investigator (SI) system.
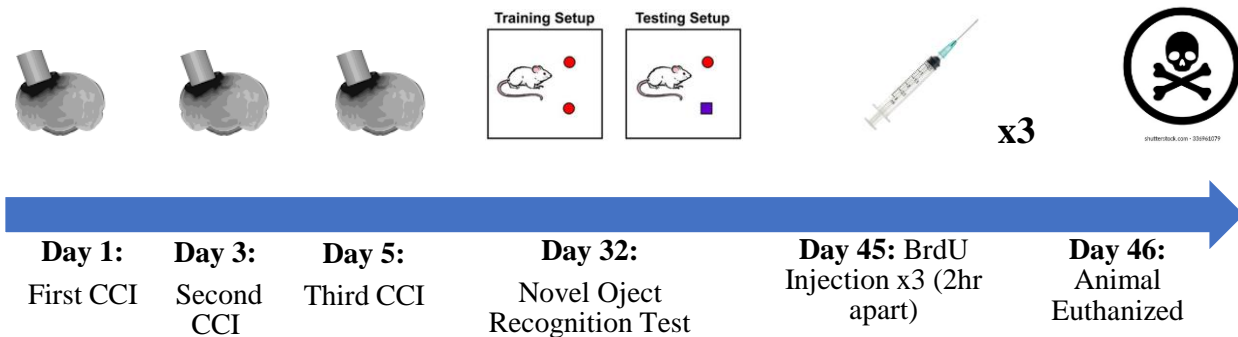
Figure 6. Experimental design timeline. **Animals were injured once every 48hrs, followed by a novel object test on day 32. Animals were injected with BrdU once every 2hr interval three times, then euthanized in 24hrs.**

Methods

Animals:

For this study, 13 male (450-600g) and 10 female (200-300g) Long-Evans adult rats were used. Adult rats are the leading animal model in behavioral studies since they share many similar behavioral deficits post-TBI with humans. One group of rats referred to as a sham received no injuries and another group referred to as chronic RC received three concussions. Sham is meant to be used as a negative control to observe experimental post-changes in neurogenesis are due to TBI. There were six sham males, five sham females, seven injured males, and five injured females.

Controlled cortical impact traumatic brain injury:

To observe the clinical effects of NSCs proliferation and neuronal commitment in rat models, closed head TBI was performed. The rat's head was free to move around to allow the force of the impact to be transmitted throughout the animal's head naturally, as observed in a clinical event (See Figure 7).
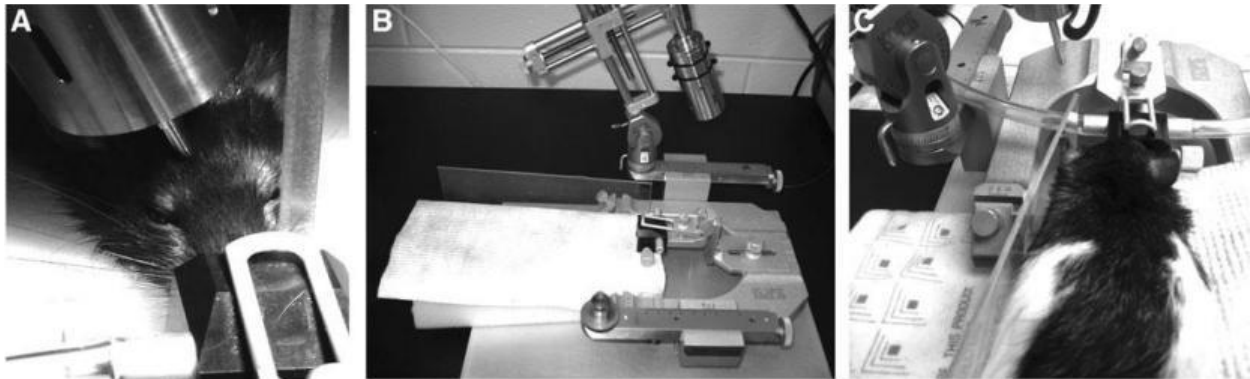
Figure 7. Rat model closed head CCI. **Rats are anesthetized and left on a foam bed. The head is adjusted to allow the device to create a concussion (Figure taken from Jamnia et al., 2017).**

Isofluorane anesthesia (2.0-3.0 mL/min) was administered through a Kopf stereotaxic apparatus fixed to the animal's nose (See Figure 7). The area of impact on the animal's head was shaved to aid in the impact's accuracy. Leica Impact One (Leica Microsystems Inc.) was used to aim at the bregma and adjusted to cause an impact on the forelimb sensorimotor cortex. The impact was performed at a 20° angle (medial/lateral) using a 5-mm flat tip at 6.5 m/s at a depth of 10.0 mm from the surface of the skin for 300 ms to mimic a concussion. After the injury, the rat was left on a heating pad to aid in recovery and regulate its body temperature around 36-37 °C. Since the study focuses on repeat concussion, rats had a total of three injuries, 48hrs. apart. The protocol was replicated from Jamnia et al. (2017) to produce the qualitative data, which showed the decrease in DCX+ and NeuN+ populations.

Novel object recognition test:

The novel object recognition test is a common test used to assess learning and memory in rodents. It can be completed within three days: habituation, training, and testing day. After the initial injury or anesthesia, the animals were given 24 hrs. to rest. The animals were introduced to the open-field arena; they were given 10 min to acclimate – habituation day. The habitation day serves as a day for the animal to explore within the open-field arena. This also serves for the animal to get comfortable within its surrounding. Twenty-four hours after habituation, the animals were then introduced to the same open-field arena with two same objects and were allowed to interact with the objects for 5 min – training day. Twenty-four hours later, the animal was again introduced to the same open-field arena with one of the objects swapped and allowed the animal to interact with novel and "familiar" objects for 5 min. Each trial was recorded, and the time was measured for the interaction with each object. As long the animal touched, sniffed, or interacted with the object within a few centimeters, it is observed as direct interaction with the object. The data were converted to percent of the novel object's total time (Jamnia, N. et al., 2017). The animal typically should spend more time on the novel object than the familiar object during the testing day. The novel object recognition test does not reward nor influence the animal's behavior to choose or spend more time on the novel object; this test requires little training and can be done within a short period (Antunes, M. and Biala, G. 2012; Lueptow, L., 2017). This behavioral test was already conducted in the previous study by Dr. Urban. In the current study, this data will assess the correlation between memory function and neurogenesis measures.

Thymidine analog injection:

Bromodeoxyuridine (BrdU) targets proliferating cells and binds to the cells' DNA during the S-phase (Gratzner, 1982). Bromodeoxyuridine was injected the day before the animals were sacrificed (sacrificed on 42-47 days). The animals were given three injections of BrdU (50 mg/kg) 2hrs. apart (Kuhn et al., 2016). The purpose of BrdU is to provide snapshots of neurogenic proliferation and lineage commitment at a chronic time point post-injury. The animals were sacrificed the following day after the injection.

Tissue processing:

Animals were euthanized 42-47 days after the first injury to assess long-term changes in neurogenesis. The rats were anesthetized and transcardially perfused to drain all the blood and maintain the brain's integrity. The transcardial perfusion takes advantage of the circulatory system by inserting the needle into the left ventricle, where the blood is pumped away from the heart. Isotonic saline was used for flushing out the blood and its proteins to prevent crosslinking with the fixative. The isotonic saline also does not affect the tissue's pH; if the pH changes, this can result in denaturing proteins present in the tissues. Another reason for using isotonic saline, it does not affect the osmolarity. Thus, water will not be withdrawn from the tissue causing the brain to shrink. Anticoagulants such as heparin may have been included to prevent blood clotting during the perfusion, which could interfere with even perfusion throughout the tissue. Subsequent perfusion with paraformaldehyde (4%) crosslinks proteins to maintain the tissue's integrity and fix the tissue in preparation for sectioning. If too much is used, Paraformaldehyde (4%) could affect the antibodies' permeability to penetrate the tissue; thus, hindering the antibody's ability to bind to its epitope on both the superficial and deeper layers of the section.

Some factors should be considered to obtain the uniform quality of fixation throughout the brain, include: ensuring the perfusion apparatus does not contain any air bubbles, the pressure of the perfusion is even or consistent, keeping the rat's head straight during perfusion can aid in even fixation, paying attention to the liver clearing, and perfusing at low temperatures (4 °C) hinders enzyme activity thus, preventing proteins from denaturing. Evenly perfused tissues allow for even fixation thus, allowing antibodies to penetrate evenly throughout the tissue.

After extraction, the brain was cryoprotected with 30% sucrose and stored at 4°C before making sagittal cryosections in preparation for immunostaining. The sagittal plane was chosen to maximize DG's amount within any given section for imaging and quantification.

Sectioning:

The freezing slide microtome was used to section the brain (1:6) into thin sections for staining and analysis. It has a freezing stage that maintains constant low temperature and thus prevents unequal section thickness. The sectioning was optimized for confocal microscopy by using thick sections (~40 μm), which made it easier to handle than the more fragile thin sections (<20 μm). In addition, the thicker sections can be used to observe multiple focal planes with better resolution, allowing for more information to be collected from one thick section rather than through multiple thin sections. Lastly, with thicker sections, protocols for immunostaining were adjusted, such as increasing incubation periods, allowing the antibodies to penetrate deeper into the tissue, and reducing nonspecific staining.

Immunofluorescence:

Primary antibodies against a protein of interest are generated from different species to allow specific secondary antibodies to bind to the primary antibody. Secondary antibodies are generated from a different species against the species of the 1°Ab and were conjugated with distinct fluorophores for later detection by confocal microscopy (See Figure 8). The 2°Ab used was raised in a donkey because none of the primary antibodies were raised in the donkey.

This study used commercially available primary antibodies specific to the antigen of interest, specifically mouse-anti-BrdU and goat-anti-DCX (See Table 2). This study also used commercially available secondary antibodies, donkey-anti-mouse Alexa488, and donkey-anti-goat Cy3. The choice of fluorophores conjugated to the secondary antibodies allows them to be imaged separately without overlap based on each fluorophore's emission spectrum. Secondary antibodies are specific for the 1°Ab of the species and the isoform of the antibody.
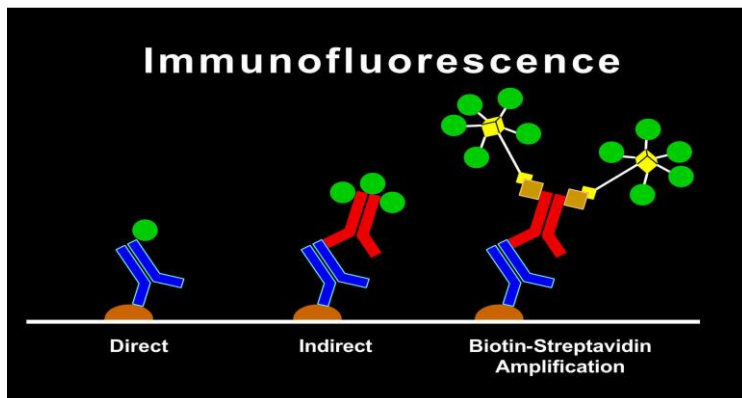

Figure 8. Immunofluorescence. **The primary antibody binds to an antigen of interest. The secondary antibody, conjugated with a fluorophore, binds to the primary antibody (Figure is taken from Stereology class by Dr. Peterson).**

To aid with the binding of the antibody to the antigen, some steps were taken to optimize specific-antigen binding, which included rinsing sectioned tissue with Tris-Buffered Saline (TBS) mixed with Triton-X to increase the cell membrane's permeability, which allowed the antibody to penetrate through the cell membrane and stain into the section's deeper layers; donkey serum was used as protein blocking that helps 2°Ab to have specific binding to the 1°Ab, while readily binding to non-specific sites thus, lowering background noises/nonspecific staining; antibodies were diluted without affecting the quality of the data, which helps to reduce non-specific staining by allowing high-affinity antigen-antibody binding and reducing free-floating antibodies, but to compensate for lower antibody concentration, the incubation period was increased (72 hrs.) to allow deeper penetration into the section; lastly, immunostaining at 4°C helps to reduce non-specific staining by allowing high-affinity antigen-antibody binding.

When the tissue was not used, it was stored at 4°C in the dark to preserve the fluorescence. Tissues were mounted onto a slide with PVA-Dabco, which acts as an anti-bleaching agent and covered with a coverslip.

Table 2: List of Antibodies. **Summary of commercial antibodies used for the study.**

Primary Antibody	Species	Anti-Species	Concentration	Manufacturer	Fluorophore
BrdU	Mouse	--	1:500	Becton Dickinson	--
DCX	Goat	--	1:500	Santa Cruz	--
Secondary Antibody					
Anti-BrdU	Donkey	Mouse	1:500	Jackson	Alexa488
Anti-DCX	Donkey	Goat	1:500	Jackson	Cy3

Microscopy and Stereology:

Tissues labeled with BrdU or DCX were quantified using quantitative stereology. Quantitative stereology is a method of studying an object using measurements from two-dimensional (2D) sections through an object to geometrically quantity the object within a three-dimensional (3D) setting. The 2D measurements depend on the geometry of interest of an object, such as the volume of the object within the environment, volume of the environment (the object itself), the object's surface area, or the length of the object. Depending on the interest of an object's geometry, an appropriate measurement tool called a probe can be used to collect the 2D measurements.

The probe is a non-physical structure that sets itself within the environment of the object of interest, and it uses countable events such as how often the object of interest has interacted with it and the location of the interaction, which are used to infer to the measurements of an object of interest. For example, if studying the feature, the length of a blood vessel, the blood vessel might interact with the probe in one area and interact again with the probe at a different area. The number of interactions and the location of each interaction can be used to estimate the length of a blood vessel.

For counting cells, the volume of an object within an environment, the probe is complimented with systematic random sampling (SRS), ensuring all cells have an equal chance to interact with the probe at random locations. This allows any location within a fixed area to be selected randomly without considering the whole region. Randomization also removes the bias of selecting sites by choice, which increases efficiency in data reproducibility.

The goal of the SRS is to ensure the sites on DG are counted with equal probability. However, the sampling density for counting was determined by examining several sections and undertaking preliminary counts to estimate the amount of error associated with sampling by determining the coefficient of error (CE). If CE ≤ 0.10, the count is appropriate, but if greater than 0.10%, the count would require a higher density with more sections or counting sites on the sections to achieve an acceptable sampling density. By conducting the Optical Fractionator sampling with optimized sampling density, it will provide a reliable estimate of the total number of each cell type in the hippocampus.

For this study, the focus is on both quantitative and qualitative data. Confocal microscopy is used to eliminate fluorescence emitted in the background or away from the focal plane of interest. The conventional fluorescence microscopy would bathe the entire specimen in light that would then capture all the light. In contrast, confocal microscopy would only capture light at one focal plane at a time due to its aperture that eliminates other light that is out of the focal plane of interest. This is advantageous in using confocal microscopy because it provides a higher resolution in defining fluorescence location in thick sections. This also aids in the usage of multiple labels in tissue to determine true co-localization. A disk-spinning unit (DSU) confocal microscope was used to collect different focal planes in 2D that were then stacked to provide a 3D image of thick sections. The 3D image aids in the determination of colocalization of more than one fluorophore from the same cell. The advantage of using the DSU is that it collects images faster due to its spinning aperture, optimal for a quantitative study.

Using confocal microscopy, 23 rat brains were imaged for the study (See Supplemental Data Table 1). The rat brain was sectioned using a systematic distribution (every 240 μm), resulting in approximately 15 sections per hippocampus selected for observations, 1 in 6 series. To prepare for image collection, a section was placed on an automated DSU stage followed by selecting the option "Acquiring SRS Image Stacks" on Stereo Investigator (SI) program (MBF Biosciences, Inc.). A virtual contour was created by tracing the region of interest to provide SI with the region's area and location on the automated DSU stage. The SI was systematically sampled by randomly placing an array of sampling sites at a determined spacing within the contour, thus generating the region's subsampling. The program then directed the stage to move towards the random site and manually adjusted the DSU focus to collect a 3D z-stack. The image is then saved on a hard drive.

This study's feature is to sample a known subvolume of the structure, which was used to infer the number of objects within the structure by using the Optical Fractionator. Hence, to count the number of BrdU+ and DCX+ cells within the DG of the hippocampus. The Optical Fractionator is a volumetric (cubic) probe, which allows systematic and equal interaction with objects of interest (BrdU+, and DCX+) cells within the volume of the environment (DG). The cubic probe also provides inclusion (green lines) and exclusion (red lines) sites to aid in counting cells and prevent repetitive counting (See Figure 9).
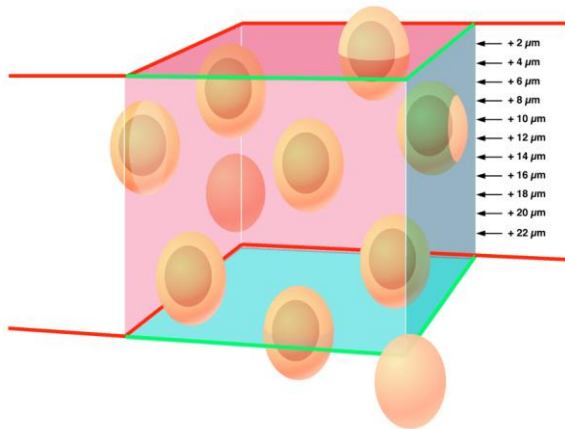
Figure 9. Design-based stereology. **A two-dimensional square has been stacked to create a three-dimensional cube. The red borders are restricted sites where cells should not be counted, and the green borders are sites where cells should be included in the count (Figure is taken from Stereology class by Dr. Peterson).**

The region of interest for each histological section was defined by drawing a contour around the DG. Parameters for SRS grid size (X: 150 μm; Y: 250 μm) were set, and the counting frame size (X:100 μm; Y: 100 μm) within the SRS was defined (See Figure 10). The SRS grid size allows even distribution throughout the DG, and the counting frame size determines the area fraction within the SRS grid to be collected and counted. This can be adjusted according to preference. If a smaller the counting frame is used, the more accurately cells can be counted, but it will also result in more sites to count, and if the counting frame is large, the more cells can be counted at a site, but with fewer sites to count, however, each site will require more attention to count and may be prone to user error.
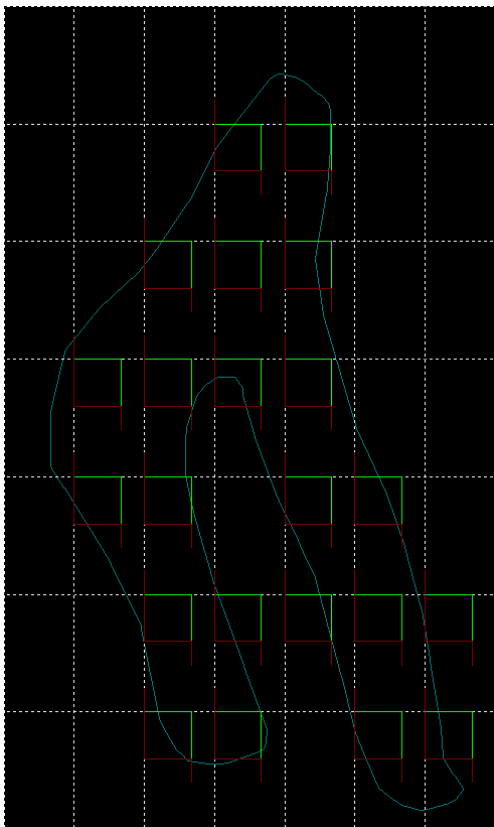
Figure 10. StereoInvestigator parameters. **The contour is outlining the dentate gyrus (teal line) of the hippocampus. The dentate gyrus is displayed on the grid (white rectangular boxes) with randomly generated sampling sites (red and green square boxes).**

The mounted section thickness was determined by sectioning at 40 μm, and image acquisition was set to collect a focal plane image at every 2 μm. This was to ensure images can be collected faster without obstructing the identity of the whole cell. An additional 5 μm was added to collect images at the top of the section to ensure all the section thickness was collected.

When counting the cells using SI, other parameters to include for counting are the guard zones and probe height. The guard zones were set to 3 μm this prevented cells at the top and bottom of the section from being part of the count. The probe height was defined by the mounted thickness subtracted by 4 μm to ensure all sections from one animal are counted and covered evenly throughout the z-axis.

Random sites were generated within the DG. Each cell was identified and manually counted according to its fluorescence (See Figure 11).
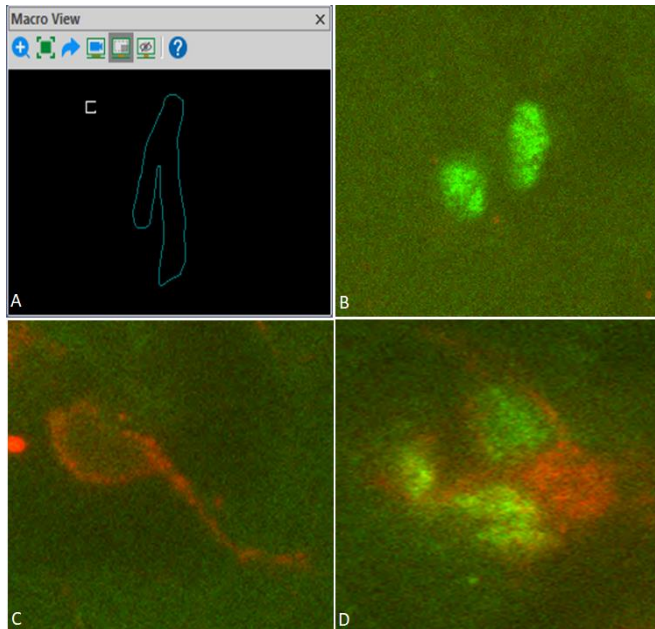
Figure 11. Stereo Investigator. Confocal microscopy was used to create a z-stack. **A) A contour is drawn around the DG, where Stereo Investigator randomly selects sites for counting cells. B) Proliferating cells were BrdU+; Alexa488. C) Early-neuronal committed cells were DCX+; Cy3. D) Cells that were actively proliferating and have differentiated into early-neuronal cells can be observed due to colocalization of BrdU and DCX antibodies.**

Calculations:

The Optical Fractionator (3D) was used to count the number of cells present within each section of an individual animal. The final cell count represents a fraction of the total number of cells within each animal since sectioning was prepared in the 1:6 series. Also, only the right hemisphere was injured and assessed. The total number of cells within an animal depends not only on the count of cells per animal but also on each animal's probe height and mounted thickness. Table 3 below shows the equations used to estimate the number of cells within the whole animal.

Table 3: Optical Fractionator (3D) Equations. **The ASF and SSF are constant values for all animals within the study.**

Settings	Parameters	Equations
Counting Frame (CF)	x: 100 μm; y; 100 μm	CF = x*y
Sampling Area (SA)	x: 150 μm; y; 250 μm	SA = x*y
Area Sampling Fraction (ASF)	CF; SA	ASF = CF/SA
Section Sampling Fraction (SSF)	1:6	SSF = 1/6
Height Sampling Fraction (HSF)	Probe Height (PH) and Average Mounted Thickness (AMT) per Animal	HSF = PH/AMT
Fraction (F)	ASF; SSF; HSF	F = ASF*SSF*HSF
Raw Count (RC)	Number of Cells per Animal	RC = Number of Cells in Section 1 + Section 2 +…
Total Number of Cells (Q)	RC; F	Q = RC/F

Statistical analysis:

To assess differences between groups, statistical analyses were performed using GraphPad Prism and RStudio. The approaches used were as follows:

1. Power analysis: To determine the minimum sample size for sections and animals to observe BrdU-labeled and DCX+ populations.

2. Student's t-test: For hypotheses 1 and 2, which examined BrdU-labelled and DCX+ population, male and female rats were pooled together, and comparisons were made between sham and injured with a significance level set at $\alpha = 0.05$.

3. ANOVA: For hypothesis 3, male and female rats receiving either a sham or injury were compared using two-way ANOVA (effect of injury and sex). If significance ($p \leq 0.05$) is reported, the test was followed by Tukey HSD posthoc to determine where a significant difference ($p \leq 0.05$) existed between the four groups.

4. CORRELATION: For hypothesis 4, the correlation between the BrdU+, DCX+, and BrdU+/DCX+ cell count from different groups (sham and injured; male and female) and novel object data was determined to examine the relationship between neurogenesis and memory function.

Limitations

The purpose of knowing the limitations of the experiment serves to fine-tune protocols accordingly and, more importantly, to demonstrate our understanding of the experiments and their conclusions and implications.

Animals:

There are currently no perfect animal models when studying TBI because the biomechanics and physical forces on the brain of a human cannot be replicated in a rodent. There are obvious physical differences between humans and rats, which also causes variations regarding how those physical forces act on the body. The current study uses a rat injury model by Jamnia et al. (2017), which serves as a clinically relevant model due to inducing impacts on the head's surface that transmit naturally throughout the animal, as seen in humans. The model also results in behavioral deficits such as memory loss, decreased motor coordination, and anxiety, also seen in humans.

Both rats and mice can be used for behavioral studies, but rats are preferred due to their ability to be trained more readily. Another thing to take into consideration is the different strains of rats. For this study, the Long-Evans rat serves as a good model for studying cognitive behaviors. Long-Evans rats also tend to show similar behavioral deficits as humans in multiple injuries and neurological disease models.

Thymidine analog injection:

Thymidine analog may be toxic if administered at high concentrations to the animal due to substituting a thymidine with an analog that has a bromide attached (BrdU). This occurs during DNA transcription when DNA polymerase substitutes excessive thymidine analog, altering neuronal function resulting in an unexpected confound for the study. Thus, this can affect our ability to observe all proliferating cells and BrdU intensity evenly throughout the section. To compensate for this, pilot studies have been generated to find a balance between the quality of the data observed and cytotoxicity induced on the animal. Bromodeoxyuridine is administered at 50 mg/kg (with saline) three times, two hours apart. The injection timing is to provide snapshots of neurogenesis before the animal is sacrificed the following day within 24 hrs. to minimize the potential disruption of DNA transcription due to the thymidine substitution.

Some reports suggest that thymidine analogs have the potential to be incorporated in cells that are undergoing apoptosis or necrosis. This might result in false positives of neurogenic proliferative capabilities. However, this concern is mitigated due to the long-term post-injury study when the BrdU was injected more than 40 days after the last TBI so that any active cell death would already have occurred.

Tissue processing and Sectioning:

The goal of tissue processing is to maintain the integrity of tissue, prevent proteins from denaturing, and aid antibodies in penetrating the deeper layers of the tissue. Poor perfusion can leave auto-fluorescing red blood cells in the tissue, appearing to be proliferating cells. Poor fixation can cause uneven immunostaining or no observable staining on the tissue. By using confocal microscopy, fragile, thin sections can be avoided. Even section prevents adjusting immunostaining protocols to compensate for the different thicknesses that can interfere with antibody penetration. However, there is still a risk of incomplete penetration leaving a zone without staining in the center of the section, but this can be easily monitored

Immunofluorescence:

When creating an antibody against an antigen, it must be certain that the antibody is binding to the antigen. To test if the antibody is binding to the antigen of interest, a knock-out animal can be used to see if the antibody still binds to protein when it is supposedly not present. Furthermore, western blot can be used to separate and identify proteins based on molecular weight. The isolated protein then can be stained with an antibody to see if it has an affinity for it. Also, pre-adsorb secondary antibodies help to determine if the secondary antibody is binding to the intended primary antibody when multiple primary antibodies are used. This ensures the secondary antibody is not cross-reacting with another species' unintended primary antibody; it aids against the non-specific binding.

Western blot analysis by the manufacturer validated the antibodies used in this study. Reports from many laboratories had established them as reliable for the detection of BrdU and DCX.

One must be cautious when using polyclonal antibodies as they are generated in different lots, which may arise due to the antibody being harvested from different animals. It is also good practice to generate an image stack throughout the entire section thickness and construct a three-dimensional image to ensure the antibodies have fully penetrated.

Microscopy and Stereology:

When observing two different fluorescent probes, it must be validated if the two fluorescent signals are either coming from the same cell or another cell, affecting the cell's identity. To prevent this, the SI software has the option to render the two-dimensional image stacks into a three-dimensional image. Thus, being able to identify the cell accurately. This allows the observation of true colocalization because each fluorophore may mix to show a false colocalization (such as red and green mixing to appear yellow) when the fluorophore may not be in the same three-dimensional space. This can be due to adjacent cells or different cell compartments. Each fluorophore's signal intensity should be appropriately adjusted to stay within the dynamic range, and the three-dimensional location of each signal should be determined before consulting there is a true colocalization.

Determining the cell's identity can also be aided by choosing fluorophores with two emission wavelengths that greatly differ from each other. This prevents the lower wavelength signal from bleeding through into the detection of the longer wavelength fluorophore. This results in two different fluorophores being observed within the same channel/filter.

In the SI system, it shows a histogram that helps to determine signal distribution. This helps obtain images that are entirely within the detector's dynamic range and that no signal data is lost to oversaturation.

Subsequent adjustments of the image in software can be made using the histogram as a reference to avoid distorting the image data.

In stereology, the CE might not always be close or lower than 0.10%, no matter how many sites are counted. This is because the CE is largely a statistical representation of how homogenous the tissue is and how its population is distributed. Rare and clustered cell populations will inherently have a higher CE, no matter how dense the sampling. The goal is to maximize the sampling so that the resulting CE goes as low as possible.

When creating the region of interest, here the DG, by drawing a contour, the entire structure of DG must be covered in all sections in which it appears to ensure that the estimate reflects the actual structure of interest and not just some part of it.

RESULTS:

<u>Animals:</u>

The study started with 25 animals that included both sexes (male and female) and treatments (sham and injured). The animals 16-002-31 and 16-003-52 were initially part of the study but were omitted as statistical outliers. Animals 16-003-49 and 16-003-55 were excluded from correlation analysis since novel object interaction for these animals is currently not present. Supplemental Table 1 (Under Supplemental Materials) summarizes all the animals considered for the statistical analysis. After removing four animals, two new animals were added to support DCX+ population statistical analysis – only 23 animals were part of the final statistical analysis.

Adult Neurogenesis Following Repeat Concussion:

The concussion did not result in any changes in cell populations in the dentate gyrus. No statistical significance was observed for any of the cell populations – BrdU+ ($F_{(1,17)} = 0.9768$; $p = 0.3369$), DCX+ ($F_{(1,19)} = 0.7593$; $p = 0.3944$), and BrdU+/DCX+ ($F_{(1,17)} = 0.2297$; $p = 0.6378$). This lack of effect was not due to tissue damage. From a histological perspective, no observable macroscopic and microscopic damage to the tissue was observed. The hippocampal DG sites were selected randomly, and using stereology, consistency in cell count was maintained (See Figure 12).
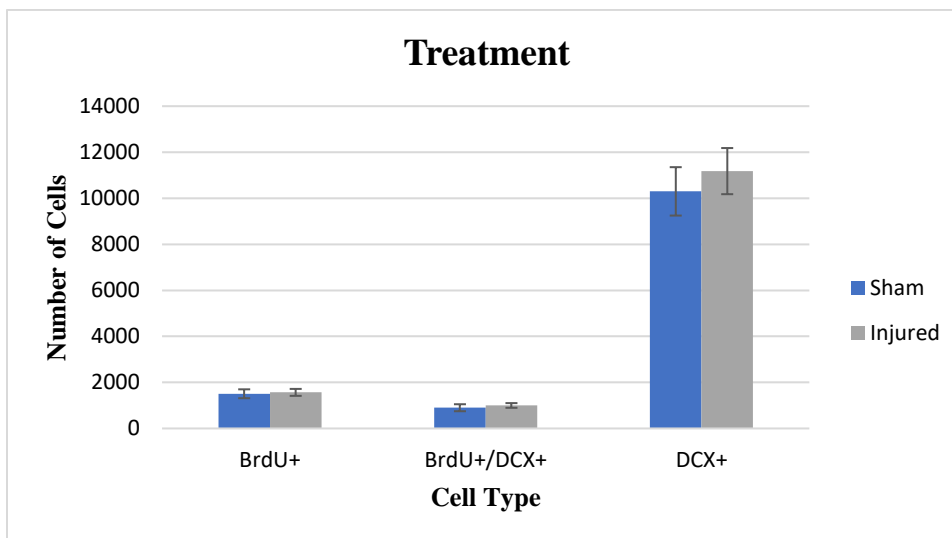
Figure 12. <u>Doublecortin positive cells</u> **were more plentiful than BrdU+ cells in the DG. All sham animals from both sexes were added together and compared to all injured animals; no significance was observed due to treatment.**

Sex Differences in Adult Neurogenesis Following Repeat Concussion:

No statistical significance in the BrdU+ and BrdU+/DCX+ populations were reported due to sex differences between male and female rats (BrdU+ ($F_{(1,17)} = 0.05945$; $p = 0.8103$) and BrdU+/DCX+ ($F_{(1,17)} = 6.949e\text{-}0.05$; p= 0.9934). The interaction between sex and injury was not significant (p>0.05) for any cell groups – BrdU+ ($F_{(1,17)} = 0.08229$; p= 0.7777), DCX+ ($F_{(1, 19)} = 0.1822$, p= 0.6743), and BrdU+/DCX+ ($F_{(1,17)} = 0.01364$; p= 0.9084) (See Figure 13).

There was no significant effect of injury on DCX+ expression ($F_{(1, 19)}=0.7593$, p=0.3944) and no significant interaction ($F_{(1, 19)}=0.1822$, p=0.6743). However, there was a significant effect reported due to sex in DCX+ population – $F_{(1, 19)} = 9.322$, p = 0.0065 (See Figure 13). Overall, the females showed a greater DCX+ expression than the male population (See Figure 14). Although there was no significant interaction effect with injury, a t-test was conducted to examine whether there was a difference between injured males. There was significant difference between injured male (AVG = 9358, SD = 2977) and injured female population with injured females having more DCX+ expression than injured males (AVG = 13727, SD = 2432); t(10) = 2.6915, p-value = 0.0226 (See Supplemental Table 2 and 3 under Supplemental Materials)). Given this statistic was not warranted due to the lack of significance in the ANOVA, we cannot treat it as an official finding of the study. However, it is an interesting trend that could be further explored.
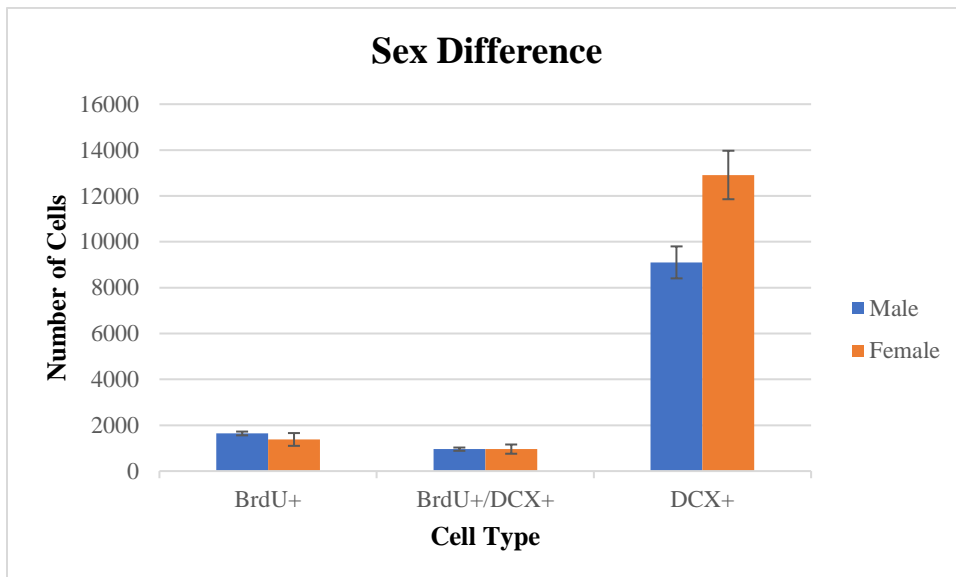
Figure 13. Sex Differences in Cell Populations. **The DCX+ are represented in higher counts than other cell types. Female shown to have more DCX+ than males *p<0.05**
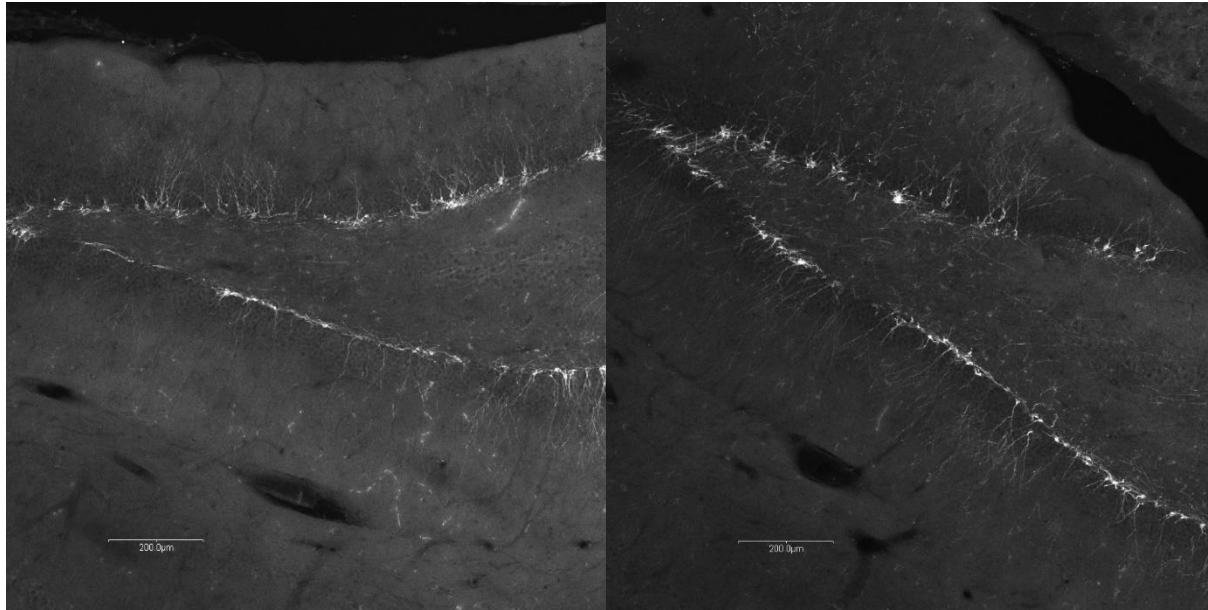
Figure 14. Male and Female injured DCX+ (100x). **The left image represents the male injured rat model, and the right image represents the female injured rat model. On a low magnification, we observe more fluorophores in female rats.**

Novel Object Study and Correlation Analysis Between Cell Type:

The novel object test was performed to assess memory function as mentioned above. The comparisons were made between injury and sex (See Figure 15) using multiple unpaired t-test. When comparing all sham and all injured, no statistical significance was observed. When comparing sham males to injured males, no statistical significance was observed. When comparing sham females to injured females, no statistical significance was observed. When comparing sham males to females, no statistical significance was observed. When comparing injured male to female a statistical significance was observed (p-value < 0.05; p-value $= 0.0447$).
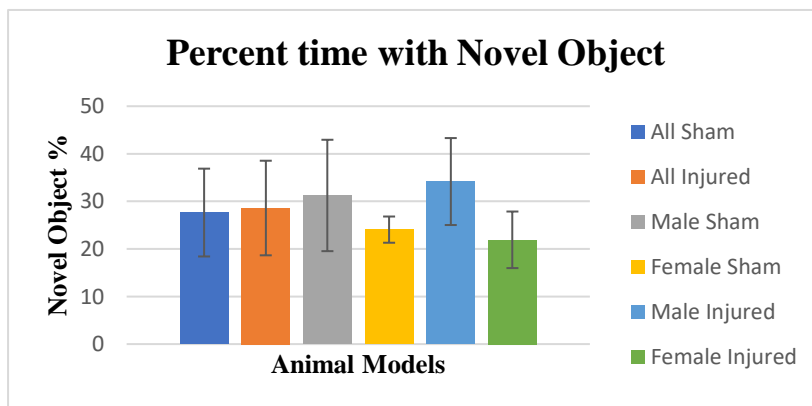
Figure 15. **Percent Time Spent with the Novel object.**

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The correlation analysis was used to determine if a relationship was observable between BrdU+, DCX+, and BrdU+/DCX+ and hippocampal function in memory (novel object test). For each cell population, a correlation was conducted by comparing novel object interaction (%) with sex or treatment.

No significant correlations were observed when all conditions were compared with novel object interaction (%). The highest R-value was reported in DCX+ in female injured (R = 0.702) with a p-value = 0.186 (See Table 4).

Table 4. Correlation Analysis. **The table summarizes the R-value used to examine the correlation between cell type and the novel object under different conditions and the p-value used to determine the correlation's significance. No correlations were statistically significant.**

	BrdU+	DCX+	BrdU+/DCX+
All Sham			
Multiple R	0.104	0.157	0.329
P-Value	0.664	0.664	0.806
All Injured			
Multiple R	0.126	0.458	0.017
P-Value	0.712	0.156	0.961
Male Sham			
Multiple R	0.166	0.290	0.412
P-Value	0.789	0.636	0.490
Male Injured			
Multiple R	0.335	0.623	0.533
P-Value	0.517	0.186	0.276
Female Sham			
Multiple R	0.657	0.467	0.733
P-Value	0.544	0.428	0.476
Female Injured			
Multiple R	0.187	0.702	0.387
P-Value	0.764	0.186	0.520

DISCUSSION:

Mild traumatic brain injury is more prevalent than other severe forms of TBI. Many mTBI cases are underreported due to missed diagnosis that comes from a lack of sensitive measures. Many studies have shown the effects of TBI inducing neuropathology, yet there are no treatments available to limit the neuropathology and significantly affect behavioral outcomes. Recent evidence demonstrates an increase in adult neurogenesis following certain types of injury and stroke. It provides a potential solution to explore brain repair for TBI and neurodegenerative disorders by recruiting new cells to the site(s) of injury to replace damaged cells. Our current study examined whether neurogenesis occurs following a repeat concussion and whether a sex difference was present in the response. The study has demonstrated that repeat mTBI does not lead to changes in adult neurogenesis (BrdU+, DCX+, and BrdU+/DCX+) when comparing sham vs. injured rats, and there is also no overall effect of sex in adult neurogenesis (BrdU+, BrdU+/DCX+, and DCX+) following repeat mTBI. However, further analysis revealed that DCX+ was higher in female injured rats than in male injured rats, suggesting a potential for sex differences post-injury that can be further explored.

Several studies have shown that TBI can induce neurogenesis changes, but these studies have concluded with mixed and contradictory results. This is most likely attributed to the fact that different studies use different injury models (FPI, CCI, WDI, and BI), different severity of injury (moderate and severe), different labeling of proliferating cells (BrdU, Ki67, and tritiated thymidine), and observe labeled cells at different time intervals post-injury. In addition, different injury induction methods and cellular assessment, such as BrdU injection time points and time of sacrifice post-injection, can significantly impact results. This suggests neurogenesis have highly regulated control points for proliferation, survival, differentiation, migration, and integration that are influenced by the surrounding extracellular environment through neurotrophic and transcription factors (Faigle and Song, 2013), neuroinflammation by microglia (Belarbi and Rosi, 2013), metabolism (Cavallucci, V. et al., 2016), and glutamatergic neurons (Chancey, J. et al., 2014). To date, this study is the first to examine changes in neurogenesis following a repeat concussion model.

Our study using closed-head CCI has shown repeat mTBI leads to no changes in BrdU+, BrdU+/DCX+, and DCX+ populations in the SGZ of the DG when examined around 40 days post-injury. A recent study by Wang, X. et al. (2016) performed opened-head CCI at different severity types. Mild TBI resulted in no significant changes in mature neuronal loss and minor alterations (non-significant) to changes in new proliferative cells and new immature neuronal cells in short (24hrs. post-injury) and long-term (4 weeks post-injury) observations. Moderate TBI showed a reduction in immature neurons within 24 hrs., but no changes to the mature neuronal population. They found only an increase in proliferative cells at four weeks but no changes in immature neuronal cells. Severe TBI resulted mainly in the loss of mature neurons after 24 hrs. In the long-term, the SGZ responded with a greater increase of proliferative cells than in moderate TBI and increased mature neuronal cell production. These studies suggest that neurogenesis is more likely to respond with more robust cellular proliferation as the severity of the injury becomes greater. Therefore, it is possible that our repeat concussion model did not produce a severe enough injury to result in significant effects on neurogenesis.

No significance between TBI and neurogenesis in our study could also be explained by the timing of our labeling of proliferation, and when post-labeling, the rats were sacrificed. Our study labeled neurogenesis later in post-injury (46 days) than most previous studies that examined later time points around 30 days or examined proliferation early post-injury. Furthermore, our study also injected BrdU 24 hrs before animals were sacrificed after 40+ days from their initial injury. In contrast, other studies have perfused the animal at different time points after BrdU injection. For example, Gao et al. (2009) introduced BrdU for one week before euthanizing the animal after an acute time point, demonstrating a decrease of cell proliferation in the SGZ, and Rola et al. (2006) introduced BrdU injections 7 days after injury followed by euthanizing the animal three weeks later also demonstrating decreases. The majority of the studies have also reported the effects of TBI on neurogenesis after injecting BrdU multiple times post-injury. Studies by Rice et al. (2003) injected BrdU every 2 hrs for four injections on days 1, 4, 7, 9, 14 post-injury, then euthanizing the animal 24hrs after, while Nair (2015) injected one dosage of BrdU every 24 hrs for 5 days before the injury was introduced and then again on day 6 and day 21, and showed a decrease of cells in the SGZ. The time point of BrdU injection is an essential factor in neurogenesis investigation since it serves as an observable marker for events occurring at the time BrdU is introduced; thus, providing a snapshot of cellular events. In our current study, it is possible that the lack of significant results at day 46 was due to injecting BrdU at a more chronic time point, potentially after events of TBI had resolved, or when any neurogenic changes were too small to detect. Future studies can introduce BrdU injection at different times post-injury, perhaps on days 1, 7, and every other 7-day interval to capture neurogenesis changes throughout the post-injury period.

To further understand neurogenesis following TBI, it may be necessary to further evaluate the different stages of neurogenesis and how they may respond differentially to the injury. Although BrdU will label all cells that have divided, further staining of these cells for which phase of neurogenesis they're experiencing may result in interesting findings. As mentioned previously and most relevant to our method of injury, Gao et al. (2009) and Rola et al. (2006) performed a moderate opened-head CCI and observed different neural cell populations. Gao et al. (2009) found differences in the activity of QNPs (multipotent cells which appear glial in nature) and TACs, (daughter cells of QNPs that can differentiate into glioblast or neuroblasts) when measured at different time intervals (4 or 72 hrs) within the hippocampal dentate gyrus (BrdU has injected either 4 or 72 hrs. after a single injury for its proliferative capabilities, and the animal was euthanized within 24 hrs. after the injection). The QNPs increased, but its progeny, TACs, showed no significant changes. The measured proliferative capabilities of these cells using BrdU observed in a moderate opened-head CCI suggests QNPs do respond in endogenous repair under TBI-induced acute changes. However, TACs' proliferative capabilities are limited. This suggests the potential increase in neuroblast cells, or future differentiated cells under acute injury are limited by the TAC population. Rola et al. (2006) suggested that both new proliferating cells and new immature neurons decreased under long-term effects of moderate TBI after a single injection of BrdU was introduced within a week post-injury animal euthanized three weeks later. In summary, from an acute perspective, Gao et al. (2009) showed proliferation of QNPs, but no changes in TACs. Rola et al. (2006), who showed a chronic perspective, demonstrated a decrease in proliferating cells and immature neurons. This implies some specific cells can be upregulated, such as QNPs, but when only using a proliferative marker without other cell-specific markers such as in the study Rola et al. (2006), it can obscure the identity of the cell and the developmental stage of the cell that are upregulated or downregulated. Together, these studies suggest that TBI affects the initial proliferative capabilities of the precursor cells and their ability to survive, differentiate, migrate, and integrate into the neuronal networks.

One limitation of our study was a small sample size, Sham Male; 6, Sham Female; 5, Injured Male; 7, and Injured Female; 5. It's very possible that increasing our sample size would have provided more conclusive results. Our sample size was limited by the animal tissues that were collected and prepared in this study. There was a suggested significance as noted above between DCX+ injured females when compared to males. According to power analysis, the statistical tool suggested adding 57 more females to reach a statistical significance. This was not feasible for this thesis.

Previous research has demonstrated that sex differences can result in observable changes in response to TBI (Mahmoud et al., 2016, Velosky et al., 2017), in neurogenesis (Tanapat, P. et al., 1999, Duarte-Guterman et al., 2015, Waddell et al., 2016, and Larson T. 2018), and in-memory (Mahmoud et al., 2016, Velosky et al., 2017). One study has investigated sex differences in neurogenesis due to TBI (Smith, D.H. et al., 1995). They performed moderate TBI and observed the effects under a chronic event (35 days post-injury) using Nissel staining to assess the neuronal loss and Gallyas silver staining for degenerating neurons. The study suggested neuronal cell loss in the DG without any variation implicated due to sex differences. In our current study, using a repeated mTBI model has shown no overall effect of sex on TBI-induced neurogenesis measured by BrdU and DCX. However, we demonstrated one finding where injured female rats showed a higher number of DCX+ compared to injured male rats. This can imply that a sex difference in neurogenesis is possible; however additional studies would need to be conducted to examine this further.

Since there is limited data examining sex differences in neurogenesis post-TBI to support or negate our findings, data from examining sex differences in neurogenic responses due to stress may provide additional insight. Acute stress has been shown to induce a decrease in the general cell population in the adult hippocampal dentate gyrus of male rats, but no changes in female rates (Falconer, E, and Galea, L., 2003). When the stress is chronic, neurogenesis is reduced in female rats but no effect in male rats (Hillerer, K. et al., 2013). The effects of stress can affect sex hormones. As stress increases, males and pre-menopausal and post-menopausal females have shown decreases in their respective sex hormones. It is unclear whether the reduction of sex hormones is due to a reduction in luteinizing hormone caused by stress or inadequate response by the hypothalamus onto the pituitary gland (Assad, S. et al., 2017). Since our rTBI model has been shown to increase cortisol levels (Jamnia et al., 2017), a differential effect on stress on neurogenesis may be possible. As previously stated, it may be that the time point examined and the intensity of the injury need to be considered in further examinations of sex differences in neurogenesis following TBI, along with a larger sample size to maximize statistical power.

It has been demonstrated that TBI can affect memory, which has been observed in our laboratory in the behavioral paradigm, the novel object test. Additionally, it is well-documented that the hippocampus and hippocampal neurogenesis play a role in memory formation. Our lab demonstrated that TBI could decrease hippocampal neuron density in rats with a single concussion and repeat concussion compared to sham, along with memory deficits (Jamnia et al., 2017). In these same rats, preliminary qualitative data has shown that repeat concussion injury decreases the number of doublecortin positive (DCX+) cells and results in memory deficits in the novel object task. Therefore, the current study sought to investigate whether there existed a correlation between performance on the novel object task and neurogenesis measures following repeat concussion. No significant correlations were found. The highest R⁻value was $R = 0.702$ with a p-value $= 0.186$when observing female injured rats. This can propose that RC injury can lead to an increase in DCX+ cells; thus, increasing in exploring in novel object longer, but further studies would be needed to confirm this relationship.

CONCLUSION:

The current study did not demonstrate that repeated mTBI affected neurogenic proliferation in the DG by observing BrdU+ cells and neuronal commitment in the DG by observing DCX+ cells. Our study also did not conclusively show an effect of sex on the neurogenic response to repeat concussion. As mentioned in the introduction, the neurogenic response is very dependent upon the type and severity of the injury, as well as the time at which it is observed. As such, future studies can revisit these hypotheses using repeat mTBI CCI models by increasing the sample size and exploring the neurogenic response at different time points post-injury by introducing more time points or with varying severities of injury. Additionally, the current study did not monitor the estrous state of the females. It has been shown that the stage of estrus is important to monitor as it can influence injury response. Therefore, future studies should also injure female rats during their estrus cycle's follicular phase, when estrogen levels are the highest

Neurogenesis is a linear path for neural stem cells to differentiate and mature into neuronal cells. Future investigations should explore the relationship between each cell type within the neuronal lineage (Refer to Figure 2). This would include identifying the cell type that responded the most post-CCI TBI in an acute event under different severity. Identifying those cell type(s) can then compare with previous and/or future generations within the neuronal lineage. This can potentially lead to another study regarding the dedifferentiation of another early lineage of cells that have started the neuronal lineage commitment – this is only implied if the measured future neuronal lineage population were greater than the previous neuronal lineage of cells. These suggestions can limit the observations when exploring cell induction effect on neurogenesis to just two cell generations.

Further investigation can explore cell induction factors that act in an autocrine and/or paracrine fashion to initiate and/or hinder cell proliferation, cell differentiation, and/or cell maturity. Identifying the endogenous factors that just promote proliferation, differentiation, and maturity is incomplete if the prospective neuronal cell does not integrate and is functional. Exploring important endogenous factors, typical or atypical exogenous factors can later be introduced to localize brain repair due to trauma and pathology.

Revisiting the current research with the above considerations in mind would provide better insight into understanding which specific cell populations are affected by the chronic effects of repeat concussion and if sex plays a role in determining changes in neurogenesis. This could provide a clear therapeutic target identifying which cell type needs to be further explored to optimize its proliferation, differentiation, and/or maturation. Additionally, transcriptional factors, neurotrophic factors, neurotransmitters, and hormones can then be explored between in-vivo and in-vitro models to observe if neurogenesis can be upregulated by affecting the significant cell type. Determining the target cell with the appropriate intrinsic factors to upregulate neurogenesis can then be utilized to develop a pharmacological or biotherapeutic approach which can be introduced in human trials to assess its effectiveness for traumatic brain injury. Furthermore, this also has implications for intervention in other brain pathologies, such as neurodegenerative disease, stroke, and transient ischemic attack, seizure disorders, and mental disorders, by replacing and/or recruiting novel cells to the area of interest.

SUPPLEMENTAL MATERIALS:

Supplemental Tables

Supplemental Table 1: rTBI Animal Models. **All rTBI animals were injured three times, 48h apart. They were euthanized 30-47 days after the first injury.**

Animal #	Sex	Est	Weight Rat (g)	CCI Condition Sham/ Single/ Multiple	Time Interval Acute/ Chronic	Date of first CCI	Date perfused	Euthanized day post first injury	Sectioned	BrdU Inj
16-001-03	F	est	254	Sham	Chronic	2/24/2016	3/25/2016	Day 30	Sagittal 40um	
16-001-05	F	di	276	Sham	Chronic	2/24/2016	3/25/2016	Day 30	Sagittal 40um	
16-002-19	F	met		rTBI	Chronic	3/31/2016	5/17/2016	Day 47	Sagittal 40um	5/16/16 (3x 2hours)
16-002-20	F	di		Sham	Chronic	4/1/2016	5/17/2016	Day 46	Sagittal 40um	5/16/16 (3x 2hours)
16-002-22	F	met		rTBI	Chronic	3/31/2016	5/17/2016	Day 47	Sagittal 40um	5/16/16 (3x 2hours)
16-002-23	F	est		Sham	Chronic	4/1/2016	5/17/2016	Day 46	Sagittal 40um	5/16/16 (3x 2hours)
16-002-28	M			Sham	Chronic	4/1/2016	5/17/2016	Day 46	Sagittal 40um	5/16/16 (3x 2hours)
16-002-29	M			Sham	Chronic	4/1/2016	5/17/2016	Day 46	Sagittal 40um	5/16/16 (3x 2hours)
16-002-30	M			Sham	Chronic	4/1/2016	5/17/2016	Day 46	Sagittal 40um	5/16/16 (3x 2hours)
16-002-34	M			rTBI	Chronic	3/31/2016	5/17/2016	Day 47	Sagittal 40um	5/16/16 (3x 2hours)
16-002-35	M			rTBI	Chronic	3/31/2016	5/17/2016	Day 47	Sagittal 40um	5/16/16 (3x 2hours)
16-002-36	M			rTBI	Chronic	3/31/2016	5/17/2016	Day 47	Sagittal 40um	5/16/16 (3x 2hours)
16-003-37	F	pro	281	rTBI	Chronic	5/4/2016	6/15/2016	Day 42	Sagittal 40um	6/14/16 (3x 2hours)
16-003-38	F	pro	275	rTBI	Chronic	5/4/2016	6/15/2016	Day 42	Sagittal 40um	6/14/16 (3x 2hours)
16-003-40	F	met	296	rTBI	Chronic	5/4/2016	6/15/2016	Day 42	Sagittal 40um	6/14/16 (3x 2hours)
16-003-45	F		284	sham	Chronic	5/5/2016	6/15/2016	Day 41	Sagittal 40um	6/14/16 (3x 2hours)
16-003-47	M		602	rTBI	Chronic	5/4/2016	6/15/2016	Day 42	Sagittal 40um	6/14/16 (3x 2hours)
16-003-48	M		538	rTBI	Chronic	5/4/2016	6/15/2016	Day 42	Sagittal 40um	6/14/16 (3x 2hours)
16-003-49	M		576	rTBI	Chronic	5/4/2016	6/15/2016	Day 42	Sagittal 40um	6/14/16 (3x 2hours)
16-003-50	M		597	rTBI	Chronic	5/4/2016	6/15/2016	Day 42	Sagittal 40um	6/14/16 (3x 2hours)
16-003-51	M		501	sham	Chronic	5/5/2016	6/15/2016	Day 41	Sagittal 40um	6/14/16 (3x 2hours)
16-003-53	M		476	sham	Chronic	5/5/2016	6/15/2016	Day 41	Sagittal 40um	6/14/16 (3x 2hours)
16-003-55	M		576	sham	Chronic	5/5/2016	6/15/2016	Day 41	Sagittal 40um	6/14/16 (3x 2hours)

Supplemental Table 2: Male Raw Data

SHAM - MALE						% Novel Object --
Animal	Sections	Sites	DCX+	BrdU+	BrdU+/DCX+	27 day
16-002-28	11	237	9986	1630	694	19.2
16-002-29	11	260	9085	1834	1209	26.5
16-002-30	10	222	6989	1278	679	21.2
16-003-51	13	285	8784	1291	882	50
16-003-53	13	297	11885	1808	1385	39.3
16-003-55	12	315	6073	1561	509	■
AVG.	12	269	8800	1567	893	31
STD.	1	36	2084	242	339	13
INJURED - MALE						% Novel Object --
Animal	Sections	Sites	DCX+	BrdU+	BrdU+/DCX+	27 day
16-002-34	10	244	7383	2070	1242	36
16-002-35	10	281	8614	1730	1072	38.1
16-002-36	11	266	9306	1722	1093	38.5
16-003-47	10	239	8206	1118	830	42.3
16-003-48	12	259	11343	1435	1128	35.8
16-003-49	12	330	14887	2090	953	■
16-003-50	10	253	5770	1759	741	14.3
AVG.	11	267	9358	1703	1008	34
STD.	1	31	2977	342	176	10

Supplemental Table 3: Female Raw Data

SHAM - FEMALE						% Novel Object --
Animal	Sections	Sites	DCX+	BrdU+	BrdU+/DCX+	27 day
16-001-3	11	237	11364	■	■	27.2
16-001-5	12	336	19373	■	■	26.5
16-002-20	10	233	11195	2624	1749	20.8
16-002-23	11	280	9421	868	643	25
16-003-45	12	298	9133	668	350	20.8
AVG.	11	277	12097	1387	914	22
STD.	1	43	4190	1076	738	2
INJURED - FEMALE						% Novel Object --
Animal	Sections	Sites	DCX+	BrdU+	BrdU+/DCX+	27 day
16-002-19	13	323	11892	2093	856	30
16-002-22	11	275	11391	612	398	23.5
16-003-37	10	287	13975	1344	1132	25.9
16-003-38	14	328	13806	737	705	15.4
16-003-40	12	287	17570	2092	1830	14.8
AVG.	12	300	13727	1376	984	22
STD.	2	24	2432	710	542	7

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