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The Effects of Repeated Concussive Traumatic Brain Injury at Acute and Chronic Time-Points

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The Effects of Repeated Concussive Traumatic Brain Injury at Acute and Chronic Time-Points

Abstract
In the past few decades, the rate of traumatic brain injuries (TBI), particularly concussions from sports or combat, has nearly doubled. TBI is thought to increase the likelihood of neurodegenerative diseases, such as chronic traumatic encephalopathy. The sequence of degenerative and regenerative responses underlying these disorders has been unclear and was the focus of this study. We utilized a closed-head model of the controlled cortical impactor to deliver injury to rats. Our results indicate a progressive increase in ventricular size and a corresponding progressive decrease in hippocampal, cortical, and corpus callosal volume. Stereological estimates indicate neuronal cell loss in region CA1 of the hippocampus, which progressed with time. Additionally, we identified an astrocytic response but neither a response from microglia nor oligodendrocyte progenitor cells. Overall, we characterized the subtle pathological changes that occur following repeat, mild traumatic brain injury using a clinically relevant model of concussions.

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LAKE FOREST COLLEGE
Senior Thesis

The Effects of Repeated Concussive Traumatic Brain Injury
At Acute and Chronic Time-Points

by

Sarah G. Chiren

April 7, 2016

The report of the investigation undertaken as a
Senior Thesis, to carry two courses of credit in
the Neuroscience Program

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Abstract

In the past few decades, the rate of traumatic brain injuries (TBI), particularly concussions from sports or combat, has nearly doubled. TBI is thought to increase the likelihood of neurodegenerative diseases, such as chronic traumatic encephalopathy. The sequence of degenerative and regenerative responses underlying these disorders has been unclear and was the focus of this study. We utilized a closed-head model of the controlled cortical impactor to deliver injury to rats. Our results indicate a progressive increase in ventricular size and a corresponding progressive decrease in hippocampal, cortical, and corpus callosal volume. Stereological estimates indicate neuronal cell loss in region CA1 of the hippocampus, which progressed with time. Additionally, we identified an astrocytic response but neither a response from microglia nor oligodendrocyte progenitor cells. Overall, we characterized the subtle pathological changes that occur following repeat, mild traumatic brain injury using a clinically relevant model of concussions.
Dedicated to my Daddy, in loving memory:

I discovered my love for learning and science while watching surgery shows with you on the kitchen television every Monday night (while sharing a family-sized bag of Doritos), and I stay motivated everyday hoping to make you proud of me.

“You already know I love you.”
Acknowledgments

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my intellectual curiosity, drive to seek improvement in myself and the world, and humble confidence from the years I had the wonderful opportunity to work in your lab, classes, and alongside you in projects. I am so grateful to have been guided every step of the way by such an incredible mentor and for the way you helped shape my path for college and beyond.

I’m a little biased, but my lab is truly awesome. Emily, I’m so grateful to have learned techniques from you and your meticulously perfect methods. I would lose my sanity if you weren’t there to chat with while making microscope slides. Rose, I’m so glad someone else works in lab who’s awake during all inordinate hours of the night to answer my frantic “please-fix-the-microscope-it-hates-me” texts. Your work ethic makes me want to work harder, and the way you help me problem-solve (even though you’re busy solving all the infinite mysteries of BMP signaling) has been beyond beneficial, but more importantly, kind.

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Daniel, you bring so much happiness to even the most horrifically mediocre activities (including 18-hour study marathons). I’m so thankful to learn more from you daily, for all the wonderful five-minute dance/karaoke parties, and for the time we cheated on our healthy-Whole-Foods-and-yoga-couple lifestyle and ate doughnuts past midnight.

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With endless love to everyone who has supported me,

Sarah
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**Abbreviations**

**AD**: Alzheimer’s disease  
**APP**: amyloid precursor protein  
**BBB**: blood brain barrier  
**CCI**: controlled cortical impact [model]  
**CSF**: cerebrospinal fluid  
**CNS**: central nervous system  
**CTE**: Chronic Traumatic Encephalopathy  
**GCS**: Glasgow Coma Scale  
**FP**: fluid-percussion [model]  
**MRI**: magnetic resonance imaging  
**mTBI**: mild traumatic brain injury  
**NFT**: neurofibrillary tangle  
**OPC**: oligodendrocyte progenitor cell  
**PBBI**: penetrating ballistic-like brain injury [model]  
**rmTBI**: repeat mild traumatic brain injury  
**TBI**: traumatic brain injury
Introduction

The brain, which is part of the central nervous system, is one of the most complex and least understood organs in the body. Due to its delicacy and susceptibility to injury, the brain requires several layers of protection. As a result of protective evolutionary advancements, the brain is encased in three layers (called meninges): the dura mater, the arachnoid mater, and the pia mater (Kandel, Schwartz, & Jessell, 2012). The brain and its meninges are further protected by an intricate network of vessels known as the blood brain barrier (BBB), which helps prevent toxins from reaching the brain (Kandel et al., 2012). The brain, its coverings, and its vascular system are all encased inside of the thick skull.

Despite these levels of protection, the brain is still vulnerable to harm from some toxins, disease, and degeneration. Direct trauma to the head, broadly classified as traumatic brain injury (TBI), can also lead to intracranial damage. The resulting TBI may be classified as severe or mild through the Glasgow Coma Scale (GCS) in which lower scores are indicative of mild injuries, and higher scores indicate more severe injuries (Xiong, Mahmood, & Chopp, 2014). Severe TBI involves impact that may include penetrating injuries or skull fractures, such as from gunshot, that produces loss of consciousness and danger of elevated intracranial pressure. Left untreated, severe TBI can lead to coma and eventual death. Falling, motor vehicle accidents, and sports accidents can result in closed-head injuries, which range in severity. In a closed-head injury, there is no penetration into the skull or brain, as occurs from gunshot. Instead, injury is caused by direct blow to the head. Concussions, for example, account for a subset of closed-head mild TBIs (mTBIs) and, although seemingly less severe in pathological damage, and although GCS scores are lower, still result in minor swelling and bruising in the brain (Smayda, 1999).

Although severe TBIs have gained considerable attention over time, mTBIs, such as concussions from falls, sporting accidents, vehicle accidents, or military injuries, also
present a notable public health issue, as the rate of the diagnosis of mTBI has nearly
doubled over the past few decades (CDC, 2003). An mTBI is an immediate paralysis of
the nervous function in the brain caused by a blow or strike to the head (Smayda, 1999).
Contrary to popular belief, mTBIs do not always result in a loss of consciousness (CDC, 2003).
Following an mTBI, a patient may become confused, be slow to respond, have
difficulty concentrating, become disoriented, and have some short-term memory deficits
(CDC, 2003). In a coup injury, the brain hits the interior of the skull, resulting in bruising
and swelling in the brain at the site of impact. In addition, the coup injury can result in a
contrecoup injury if the initial collision with the skull at the site of impact causes the brain
to ricochet to the opposite side of the skull. Therefore, contrecoup injuries exhibit a similar
pattern of bruising and swelling at the opposite side of the brain (Smayda, 1999; Figure 1).
Although the impact may lead to edema (swelling), a contusion (slight bleeding into tissue),
and/or a hematoma (bruising), these signs of damage are minor and, in basic magnetic
resonance imaging (MRI), would not be noticeable in the cortex, or the outer layer of the
cerebrum, the anterior portion of the brain (D. Peterson, personal communication).

![Coup and Contrecoup TBI](https://en.wikipedia.org/wiki/Human_brain#/media/File:Skull_and_brain_normal_human.svg)

Figure 1. Coup and Contrecoup TBI. In an mTBI, upon impact, such as that from helmet-
to-helmet contact in football (A), the brain hits the interior of the skull at the site of impact
(B), known as a coup injury, and as a result, can ricochet to the opposite side of the skull
(C), a contrecoup injury. This impact results in bruising and/or swelling, but no contusion,
bleeding, or overt damage. Images obtained from
Prevalence and symptoms of mild traumatic brain injury (mTBI)

Over time, the rate of diagnosed concussions (a subtype of mTBI) has been steadily increasing (CDC, 2003). In particular, mTBIs seem to occur more frequently in females than in males, but males are less likely to report a concussion (Baugh et al., 2012). Although this general increase in reported mTBIs may be due to improvements in detection, the true number of concussions per year may be genuinely increasing as a result of environmental factors. For example, as athletic training grows more competitive, an athlete’s muscle mass may increase, which likely results in higher impact collisions. There currently exists headgear, such as helmets, that can track the magnitude of a concussion, but no headgear that can prevent a concussion (D. Kozlowski, personal communication).

The National Institute of Health estimates that in a single year, 1.5 million concussions are reported in the United States at a rate of 618 per 100,000 persons, resulting in an estimated 200,000 emergency room visits (CDC, 2003). Athletes and military soldiers are particularly susceptible due to the highly physical nature of their lifestyles. Although concussions have traditionally been considered short-term function impairments, evidence has shown that long-term neurophysiological dysfunction can occur as well (Gaetz & Weinberg, 2000).

In one of the largest and most recent concussion studies, Marshall et al. (2015) found a total of 375 concussions reported among 8,905 high school and college student athletes, who are at high risk for concussions. Compared to non-athletes, hockey and soccer players were at a significantly higher risk of concussion, but football players accounted for two-thirds of the reported concussions. The most frequently reported symptoms following a concussion included headaches, balancing difficulties, dizziness, “fogginess,” and difficulty concentrating, but the prevalence and severity of these symptoms seemingly decreased in a given patient over time. Although sensory sensitivity to light and
sound increased in prevalence and severity with time before eventually returning to baseline (Marshall et al., 2015), most symptoms, such as cognitive dysfunction and postural instability, have been shown to generally recover after several days (McCrea et al., 2003). The mechanism of this recovery has not been not clearly elucidated, but it may involve the reorganization of connectivity, similar to that which is seen in stroke recovery (Watila & Balarabe, 2015).

A survey of 2,714 US Army soldiers taken 2 to 4 months after a yearlong deployment, Hoge et al. (2008) found similar symptoms and incidence to Marshall et al.’s (2015) study of athletes who had sustained mTBI. The majority of these injuries were acquired in young male soldiers from a blast mechanism of injury during high intensity combat. About 15% of soldiers reported injuries that resulted in altered mental status, as well as symptoms of confusion, dizziness, and difficulty concentrating. Additionally, the soldiers with mTBI reported higher rates of other physical symptoms, such as shortness of breath, stomach and back pain, and mental health disorders, such as post traumatic stress disorder and depression, as compared to those soldiers without mTBI (as well as soldiers who had sustained similar types of blast injuries, but not mTBI, while in combat). While the underlying mechanisms behind these comorbidities are not clearly understood, they are likely the result of stress responses in the brain. For example, both PTSD and mTBI have been shown to involve activation of the hypothalamic-pituitary-adrenal axis, cell-mediated immune responses, cellular distress, and other biological processes (Hoge et al., 2008).

Despite the aforementioned reports describing the cognitive deficits that result from mTBI, Gentilini et al. (1985) did not find conclusive evidence suggesting that cognitive issues, such as word recognition deficits, shortened attention, and working memory deficits, persist following a single mTBI. Such research implies that the cognitive impairments
associated with mTBIs are likely temporary. Levin et al. (1987) studied the neurocognitive effects of mTBIs at different time-points. Although the study found significant acute disturbances of attention, memory, and information processing efficiency within the first few days after a single mTBI, they affirmed a study by Gentilini et al. (1985) in concluding there is little evidence to suggest a significant residual effect of mTBI following a single injury in more than a minority of patients.

However, De Beaumont, Lassonde, Leclerc, & Theoret (2007) and De Beaumont et al. (2011) found that concussions result in long-term residual motor dysfunction, such as impairment of postural control. Their findings support the idea that mTBIs can result in persistent alterations in motor function, as opposed to the previous notion that post-concussive symptoms were exclusively temporary. It should be noted that many of the athletes in the study sustained multiple concussions. To study the effects of repeat injuries (as opposed to a single mTBI) Wall et al. (2006) assessed neurological function in athletes who had sustained repeat injuries as compared to athletes who had sustained only a single injury. Repeat mTBIs (rmTBIs) were associated with persistent decreases in cognitive performance, namely attention difficulty and a deficit in response inhibition. Such findings necessitate not only the study of the long-term effects of single mTBI, but also the long-term effects of rmTBI (Table 1).
<table>
<thead>
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<th>Table 1. Symptoms following mTBI</th>
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<td><strong>Altered mental status</strong></td>
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<tr>
<td><strong>Altered mental health</strong></td>
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<td><strong>Temporary cognitive deficits</strong></td>
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<td><strong>Long-term motor dysfunction</strong></td>
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In addition to the cognitive deficits, headaches, and other short-term effects of mTBIs, there exist additional concerns regarding the long-term pathological effects that can result from rmTBI. For instance, individuals who have sustained rmTBI show accelerated accumulation of intracellular toxic protein that drives neurodegenerative pathology (McKee et al., 2010). This phenomenon is particularly prevalent in boxers and football players diagnosed with chronic traumatic encephalopathy (CTE) (Corsellis, Bruton, & Freeman-Browne 1973; Uryu et al., 2002). The way in which injuries are considered “repeat” varies, however. For example, repeat injuries can occur across a large span of time at various points throughout an individual’s life, or during a shorter span of time, such as an athlete receiving multiple concussions during his or her athletic career. In the present study, we define repeat as three successive injuries at 48-hour intervals. This paradigm is consistent with the repeat injuries that may occur in a shorter span of time, such as during an athletic career (D. Peterson, D. Kozlowski, personal communication). Human instances and animal models of mTBI allow characterization of the pathological progression that follows injury.

Mechanisms of injury and subsequent pathology in mTBI

In addition to the observed behavioral deficits that follow mTBI (whether single or repeat), molecular brain pathology shows signs of damage. Guskiewicz et al. (2003) found that football players who had suffered a concussion were significantly more likely to obtain a subsequent concussive injury, and a second injury has a slower recovery of neuronal function, such as firing rate, than the recovery of the initial injury. A single mTBI lends the brain vulnerable to sustain a subsequent injury during a window of susceptibility following the initial injury. For example, rats that sustained rmTBIs during the window of susceptibility showed an exacerbated breakdown of the BBB, reduction of
immunoreactivity, increase in β-app (an indicator of axonal degeneration), and neurobehavioral deficits compared to animals that sustained a single injury (Lauer et al., 2001). These pathological hallmarks of rmTBI contribute to the window of increased vulnerability of obtaining a second mTBI following an initial mTBI. Additionally, rmTBIs show longer-lasting deficits than single mTBIs, indicating that the physiological dysfunction may be multiplicative.

Humans who have sustained mTBI also demonstrate changes in brain volume in subsequent MRI scans (Mackenzie et al., 2002; Monti et al., 2013; Ross et al., 2012; Tate & Bigler, 2000; Zhou et al., 2012). A study by Ross et al. (2012) identified increased cerebrospinal fluid (CSF) volume following mTBI, which is potentially indicative of increased ventricle size. Dilated ventricles are a well-known sign of volume contraction elsewhere in the brain, which may indicate a potential neuronal loss (Kandel et al., 2012). In an effort to study volume changes throughout the brain, Tate and Bigler (2000) found that the fornix, which carries signals from the hippocampus to the hypothalamus (Kandel et al., 2012), was significantly smaller in injured individuals. Mackenzie et al. (2002) found atrophy in the brain parenchyma of patients who had received either mild or moderate TBI, and this atrophy was greater in patients who had lost consciousness. By analyzing the after effects of mTBI at different time-points, Ross et al. (2012) found progressive brain atrophy in the parenchyma, cerebral white matter, and the cerebellum. Zhou et al. (2013) reported that patients who received mTBI had global atrophy, but more specifically in the white matter of the anterior and left cingulate gyrus one-year post injury. Additionally, Monti et al. (2013) identified a decrease in the size of the posterior parietal lobe and the prefrontal cortex.
Many volume studies have addressed atrophy of the hippocampus in particular (Hicks, Smith, Lowenstein, Saint Marie, & McIntosh, 1993; Monti et al., 2013; Tate & Bigler, 2000). Using the GCS to objectify patients’ neurological states following injury, Tate and Bigler (2000) identified an overall correlation between severity of injury and hippocampal atrophy. Similarly, Monti et al. (2013) found a decrease in hippocampal volume following mTBI, which correlated with a decrease in performance during memory tasks. Such studies may provide insight to gain better understanding of the mechanisms underlying cognitive deficits that follow mTBI, particularly those regarding learning and memory. While the MRI data provide initial evidence for decreases in brain volume, rodent models of brain injury can afford a more detailed and less variable understanding of the specific structures affected by injury.

*Experimental animal models utilized to investigate TBI*

While human studies can provide initial, correlational evidence of pathological damage, rodent studies allow researchers to induce TBIs in a more precise and reproducible manner. Specifically, rats may provide greater insight than the more commonly used mice because of their more complex brains, more human-like pathology, and tamer behaviors. For instance, causational data regarding the effects of mTBI can be obtained by adapting models of injury to inflict mild injury. Numerous methods have been utilized to model the pathophysiological effects of TBI in rodents. McIntosh et al. (1989) utilized a fluid-percussion (FP) model, which is frequently used in TBI research, to demonstrate that injury is induced by rapidly injecting saline into the cranial cavity. Despite its ability to produce an injury absent of skull fractures (Cernak, 2005) and cognitive deficits, FP models have high rates of animal mortality compared to other models (Xiong et al., 2013), which may be due to a disproportionate impact to the brain stem (Cernak,
To model the effects of a penetrating TBI caused by gunshot wounds, Williams et al. (2005) described a penetrating ballistic-like brain injury (PBBI) method, in which a high-energy bullet-like projectile produces a cavity in the brain. However, PBBI can cause excessive hemorrhaging in the cerebrum (Xiong et al., 2013). In a blast model of injury, which can demonstrate the effects of most battlefield wounds, a compressed air shock tube produces a blast injury (Long et al., 2009). In the weight drop method, an impact is delivered to the cranium of an unrestrained animal. The method allows for the head and torso to move, as occurs in the human mTBI (Kane et al., 2012), but variability of injury among animals is still high (Xiong et al., 2013). There remains great variation between models and in methodology of inducing impact across laboratories, which has made meaningful comparisons of results and conclusions difficult (Xiong et al., 2013).

A final model, the controlled cortical impact (CCI), was first described by Lighthall (1988). The CCI uses a cylinder to deliver an impact to intact dura at a known constant velocity and depth, which aids in minimizing impact variation. Rats can move in the impactor, which is consistent with the movement during a true concussive injury, but researchers can still maintain control over the stereotactic position and severity of impact, further minimizing variation of the cranial location of injury between trials. The CCI has recently been adapted to induce mild injuries. Lighthall (1988) found that adapting the apparatus to deliver mild injuries produces no obvious contusions but enough sensitivity to be used to model repeat injuries in rats. Furthermore, recent adaptation has allowed injuries to be delivered without performing a craniotomy, the removal of a portion of the skull, which is necessary to induce injury in many models of mTBI. Because a true concussive injury occurs closed-headed, the adapted CCI is more clinically relevant than other models (Prins, Hales, Reger, Giza, & Hovda, 2010). Although the apparatus can be
costly and difficult to operate, it presents greater advantages than other models for providing consistent injuries as a result of its reduced variation (Xiong et al., 2013).

Adapting these methods of injury to model the effects of an mTBI allows for the study of the pathophysiological effects of mTBI, such as changes in volume, changes in cell populations, and effects on the cell. These parameters provide insight into the neurological deficits that have been discussed. In addition, rat brains are easier to acquire than human brains, and by ensuring each injury is delivered to a similar location with a similar force, there is less variation between injuries, which aids in accurately identifying pathological characteristics of injury.

Rodent models of mTBI and subsequent pathology

Because injury cannot be manipulated in human studies, researchers must draw conclusions based on different degrees of severity of injury, locations of injury, and number of injuries. Using rodents as a model of mTBI can provide more detailed, causational data for the impact of mTBI on the brain. Researchers can manipulate injuries in rodent studies to reduce variability, for example, by ensuring that the delivery of injuries is of the same force, location, and at the same time-points. This precision allows for a mechanistic elucidation of the sequence of degenerative and regenerative responses and cellular response mechanisms, which is not possible using human studies. Furthermore, methods of intervention to repair damage can be tested.

Using the weight drop method in mice, Talbot (2014) showed an increase in the lateral ventricle volume, the region in which cerebrospinal fluid is produced, suggesting a potential neuronal loss (Kandel et al., 2012). In addition to the memory deficits that can occur following mild injury, studies have shown neuronal loss in hippocampal areas CA3 (Hicks et al., 1993; Tang, Noda, Hasegawa, Nabeshima, 1997), CA2 (Tang et al., 1997),

and in the dentate gyrus (Rachmany et al., 2013) of the hippocampus, which is involved in memory and a site of neurogenesis (Kandel et al., 2012). Because the hippocampus plays a central role in learning and memory, the implications of such cell loss to these areas must be further characterized.

A glial response to injury, including microglia and astrocytes, has been established (Mouson et al., 2013; Ojo et al., 2013; Shitaka et al., 2011; Singh et al., 2015) in response to mTBI. Following a repetitive closed-head model of mTBI in mice, in addition to cognitive deficits, IBA1, a marker for activated microglia, which act as macrophages in the brain, stained positively in the cortex (Ojo et al., 2013; Shitaka et al., 2011), dentate gyrus, corpus callosum, thalamus (Shitaka et al., 2011), and areas CA1 (Chohan et al., 2015; Ojo et al., 2013) and CA3 of the hippocampus (Ojo et al., 2013). Additionally, both single TBIs (Ojo et al., 2013; Singh, Trivedi, Devi, Tripathi, & Khushu, 2015) and rmTBIs (Mouson et al., 2013) result in increased GFAP staining, the marker for astrocytes, cells which provide nutrients and aid in the brain's repair processes, in the cortex and areas CA1 and CA3 of the hippocampus. Hippocampal neurons, especially in subregion CA1, are particularly susceptible to excitotoxicity after ischemic or hypoxic events, which may account for their cell death (Chohan et al., 2015).

Another type of glial cell, oligodendrocytes, which form the myelin sheath that coats neurons, play a role in the injury response. Oligodendrocytes originate from oligodendrocyte progenitor cells (OPCs) (Rafalski et al., 2013). OPCs actively divide and are held in reserve to allow for regeneration of oligodendrocytes. Furthermore, they proliferate in response to injury (D. Peterson, personal communication) and migrate toward focal injuries, such as stroke (Burda and Sofroniew, 2014). Similar to stroke, mTBI-mediated cell death may result from ischemic BBB breakdown (Burda and Sofroniew,
Regardless, the response of OPCs to mTBI remains poorly understand (Burda and Sofroniew, 2014).

The inflammatory response of the brain, which results from the glial response, has also been analyzed in response to mTBI. For instance, astrocytic and microglial responses, transcription factors that are associated with inflammation, phagocytosis (the elimination of debris), stress response, and cytoskeletal function were more active in the hippocampus and cortex of mice that had sustained a single injury compared to control animals that had not been injured (Israelsson et al., 2009).

The glial response to injury and the decrease in neurons in subregions of the hippocampus indicate a perturbation in the brain following injury. Following repeated injuries in a mouse model of injury, Slemmer, Matser, De Zeeuw, & Weber (2002) measured two common markers of central nervous system (CNS) damage: neuron-specific enolase and S-100β. Both markers were significantly elevated in mice that had sustained multiple injuries compared to mice who had received single injuries. The elevation in neuron-specific enolase is correlated with psychological deficits, and the elevation in S-100β likely initiates the glial response and indicates a disruption in the BBB (Slemmer et al., 2012). This observation is consistent with the thought that injury to the hippocampus following rmTBI is multiplicative and results in the longer-lasting cognitive side effects. In addition, following rmTBI, mice showed impaired cell membranes and fewer viable cells than control mice. More severe injuries additionally resulted in higher release of markers (Slemmer et al., 2002).

Moreover, mTBI is associated with irregular signaling that can lead to impaired energy metabolism. For example, extracellular glutamate levels are generally regulated by astrocytes to prevent calcium influx into neurons and resultant excitotoxicity (Kandel et al.,
Following rmTBIs, the impaired astrocytic response results in elevated extracellular glutamate regulation, resulting in calcium influx and cell death (Kandel et al., 2012). However, Ahmed et al. (2013) showed that increased calcium levels could be attenuated by utilizing the NMDA receptor antagonist MK-801. Furthermore, Ahmed et al. (2002) found MK-801 could attenuate the resulting decrease in mitochondrial membrane potential, which implies that NMDA-initiated increase in calcium results in a decrease in mitochondrial membrane potential following rmTBI. The attenuation of the decrease in mitochondrial membrane potential is substantial in that it could be a potential therapeutic. Almeida-Suhett et al. (2015) demonstrated a decrease in GABA interneurons and receptor subunits. Because GABA is important in modulating synaptic plasticity, there was a decrease in long-term potentiation, which is essential to the synaptic plasticity that underlies learning and memory (Almeida-Suhett et al., 2015). Vagnozzi et al. (2007) found altered levels of ATP products, such as decreased acetyl CoA and decreased overall ATP production. These alterations are indicative of mitochondrial dysfunction (Ahmed et al., 2013; Vagnozzi et al., 2007), which can also contribute to cell death.

Neurodegenerative pathology following mTBI

The observed volume and cellular effects on neurons following mTBI (specifically, rmTBI) have been discussed earlier, as well as the mechanisms behind neuronal death that can occur. Until recently, it was unknown that CTE can occur as a result of rmTBIs (Figures 2 & 3). CTE is an early-onset neurodegenerative disorder clinically characterized by headaches, attention difficulty, muscle weakness, muscle atrophy, spasticity, sleep disturbance, diffuse fasciculation, depression-like symptoms, cognitive impairments and/or dementia (Guskiewicz et al., 2007; Mckee et al., 2010; McKee et al., 2013; Petraglia et al., 2014).
The behavioral abnormalities that result from CTE have been pathologically characterized and are similar to Alzheimer’s Disease (AD). For instance, studies have shown that rmTBI may trigger pathways that increase the production and aggregation of proteins that are prone to insoluble accumulation, such as α-synuclein, tau, and amyloid-β (McKee et al., 2013; Uryu et al., 2002). Others have shown that an increase in protein accumulation can trigger the pathology of neurodegeneration (Golstein et al., 2013; Figure 2). Furthermore, the ventricles in patients with CTE increase, indicative of general atrophy throughout the rest of the brain (Stern et al., 2011; Figure 3).

Neurodegenerative atrophy. Following rmTBI, the human brain shows signs of degeneration, namely an increase in ventricle size and general tissue atrophy (B) compared to a control human brain (A). Images obtained from http://www.bu.edu/cte/files/2011/11/Stern-et-al-2011-PMR-Long-term-Consequences-of-Repetitive-Brain-Trauma1.pdf

Neurofibrillary tangles (NFTs) are insoluble accumulations of highly phosphorylated tau protein that are observed in individuals with rmTBI (Geddes, Vowles, Nicoll, & Revesz, 1999). Tau protein aids in microtubule binding when adequately phosphorylated (Bancher et al., 1989), and can therefore indicate cytoskeletal cellular abnormalities in injured individuals (Geddes et al., 1999). When hyperphosphorylated, the protein is dysfunctional and accumulates in the deeper layers of the cortex in the form of NFTs, which are a pathological hallmark of Alzheimer’s Disease (AD) (Kandel et al., 2012). Hof was the first to identify that in CTE, NFTs are also seen in the superficial layers of the neocortex (Hof et al., 1992). Similarly, Goldstein et al. (2012) found phosphorylated tau pathology in a post-mortem examination of the brains of military veterans and athletes who had suffered rmTBIs. In addition, these brains showed evidence of cortical and hippocampal neurodegeneration, such as axonal swelling, particularly in subregions of the hippocampus CA1, CA3, and the dentate gyrus (Goldstein et al., 2012; Lowenstein, Thomas, Smith, & McIntosh 1992).
Amyloid-β proteins are also commonly observed in insoluble accumulation in CTE (Uryu et al., 2002). This accumulation was first found in the post-mortem brains of several boxers. As Roberts, Allsop, & Bruton (1990) found, the brains of retired boxers who had suffered repeat mild injury showed diffuse amyloid plaques and β protein deposits similar to the accumulation pathology seen in AD. Murakami et al. (1998) used an FP mouse model of severe TBI to find a similar increase in amyloid precursor protein (APP), the precursor to amyloid-β. The common pathology implies a common mechanism behind neurodegenerative diseases and the neurodegeneration that occurs following repeat mild injuries.

**Remaining gaps in knowledge underlying rmTBI**

Despite the emergence of research examining mTBI, particularly repeat injuries, the underlying neurodegenerative mechanisms remain poorly elucidated. Although evidence shows that rmTBIs can eventually lead to neurodegenerative pathology, such as is seen in CTE, the early neuropathology, including cell loss, glial responses, and volume changes, remains unclear.

In addition, pathology has not yet been evaluated in a closed-head CCI rat model. Additionally, to the best of our knowledge, this is the first study to deliver closed-head injury to rats using an adapted model of the CCI, which renders the model more clinically relevant to understanding the degenerative and regenerative mechanisms following mTBI than other preexisting models. Furthermore, this study contributes a better understanding of the progression of neuropathology that follows rmTBI, as it looks at both acute and chronic time-points. Finally, although many studies utilize design-based stereological methods to gain unbiased estimates of volume and cell populations, their methods often
present limitations that preclude nuanced characterization of the subtle changes following mTBI.

The present study sought to analyze the progression of the neuropathology that results from closed-head, CCI-induced rmTBI in a rat model, with a focus on volume change, change in neuronal populations, and glial responses in the hippocampal subregions. First, volume changes in the cortex, hippocampus, striatum, corpus callosum and ventricles were analyzed using the Cavalieri stereological probe. The neuronal response was analyzed using the Optical Fractionator probe in randomly selected areas in the region of interest, such as the hippocampus or cortex. Astrocytes, microglia, and OPCs were qualitatively evaluated as well. Based on preexisting and observable, qualitative data, I hypothesized that in response to rmTBI, we would observe a progressive decrease in neuronal populations in area CA1 of the hippocampus and progressive decrease in ipsilateral brain volume.
Methods

Animal care

Male Long-Evans rats (Charles River Laboratory, Chicago, IL) weighing between 300-450 g were housed in a 12:12 dark/light cycle at the DePaul University (Chicago, IL) Research Support Facility. Food and water were available to rats ad libitum. One week prior to injury, rats were handled daily by DePaul University collaborators. All experiments were performed in accordance to the National Institute of Health Guide for the Care and Use of Animals and were approved by the DePaul Institutional Animal Care and Use Committee.

Inducing injury

Rats were arbitrarily placed into the following experimental groups: an acute time-point sham, a chronic time-point sham, a repeat injury animal that was sacrificed at an acute time-point, and a repeat injury animal that was sacrificed at a chronic time-point. See Table 2 for conditions of each experimental group.

Closed-head Controlled Cortical Impactor

To inflict mTBI, we utilized a novel adaptation of the CCI that does not require a craniotomy. Prior to injury, rats were sufficiently anesthetized through a nose cone with 2.0-3.0 mL/min of isoflurane and then placed in a Kopf stereotactic apparatus on a 5-cm-thick foam bed in a Plexiglas frame. The lateral side of the head was placed lightly against the frame, which maintained stability, but allowed the rat to move. The tip of the impactor was centered on bregma and moved over the forelimb sensorimotor cortex 0.5 mm anterior and 4 mm lateral to the bregma.
<table>
<thead>
<tr>
<th>Condition</th>
<th># Injuries</th>
<th>Force of Impact</th>
<th>Sacrificed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Sham</td>
<td>0</td>
<td>NA</td>
<td>8 days post-impact (of experimental animals)</td>
</tr>
<tr>
<td>Chronic Sham</td>
<td>0</td>
<td>NA</td>
<td>30 days post-impact (of experimental animals)</td>
</tr>
<tr>
<td>Acute Repetitive</td>
<td>3</td>
<td>6.5 m/s</td>
<td>8 days post-impact</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10d/v</td>
<td>48 hours apart</td>
</tr>
<tr>
<td>Chronic Repetitive</td>
<td>3</td>
<td>6.5 m/s</td>
<td>30 days post-impact (of experimental animals)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10d/v</td>
<td>48 hours apart</td>
</tr>
</tbody>
</table>
Impact was delivered for 300 msec with Impact One (Leica Microsystems Inc., Buffalo Grove, IL) 20° from the vertical at a speed of 6.5 m/s, at a depth of 10 mm into the skin. After the impact, rats received topical antibiotics and analgesics and returned to their cages. After behavioral testing, which included novel object recognition foot fault, and open-field activity, animals were sacrificed following injury while anesthetized with Equithesin injected into the peritoneal cavity. Brains were perfused using phospho-ta-buffered saline with 0.005% heparin and 4% paraformaldehyde. Brains were fixed in cryoprotectant, stored at 4°C, and delivered to Rosalind Franklin University Medical School (North Chicago, IL) for sectioning.

**Tissue sectioning**

Brains were sectioned 40 μm thin on a freezing microtome (Leica Biosystems, Buffalo Grove, IL) in a coronal orientation. Brains were first rinsed from sucrose storage using 0.1 M phosphate buffer. Using a razor blade, the cerebellum was removed, and a notch was delivered to the hemisphere contralateral to the impact to distinguish between the hemispheres. The microtome stage was frozen using phosphate buffer at -30°C. Once brains were placed on the stage in parallel fashion to the microtome blade, additional 0.1 M phosphate buffer was used to create an ice well to hold the brains steadily on the stage. Sections were then stored in correct anatomical sequence in a 96-well plate, with each well containing 200 μL of cryoprotectant, and stored in a -20°C freezer. A 1 in 6 series (every sixth anatomical section) was randomly selected for histological staining and analysis (Figure 4).
Figure 4. **Serial storage of tissue.** Sections are stored in 96 well plates in proper anatomical sequence. At random, a 1 in 6 series (highlighted in yellow) is selected for histology, which allows for an equal distribution of sections for analysis.

**Histology**

Brain sections were randomly selected from a 1 in 6 series for immunofluorescence (Figure 5). For each stain, sections were first rinsed three times for ten minutes each with 3 mL of 0.1 M tris-buffered saline (TBS) followed by a 3-hour rinse using TBS++. Sections were incubated in primary antibodies (see Table 2) diluted in TBS+ for 72 hours in 4°C. Antibody dilution factors were determined based on serial dilutions trials. The complete protocols for all solutions used in histological processing are presented in Appendix A.

Following incubation, sections were rinsed twice, one hour per rinse, with TBS++. After the rinses, sections were incubated in secondary fluorophore antibodies (see Table 3) diluted in TBS and triton detergent in the dark for two hours followed by two, 15-minute rinses in 0.1 M TBS in the dark. Sections in the impact stain were finally counterstained with Sytox Green diluted in TBS, rinsed in 0.1 M TBS, and stored in 0.1 M TBS. Sections were mounted in phosphate buffer using PVA-Dabco (Figure 6). Special precautions were taken to allow for minimal drying before applying a cover-slip to prevent shrinkage.
Figure 5. *Immunofluorescence.* In fluorescence microscopy, antibodies are used to tag specific cells of interest. In indirect immunofluorescence, antibodies are raised in a species against the cell of interest, and a secondary antibody is raised against the primary antibody. The secondary antibody contains fluorescence that allows the cells of interest to be observed when excited with the appropriate wavelength of light.

Figure 6. *Histology Schematic.* A 1 in 6 series is randomly selected for immunostaining. During the first day (A), sections are rinsed in TBS, made permeable to antibody in TBS++, and are then left in primary antibody for 72 hours. After 72 hours (B), sections are rinsed in TBS++ followed by secondary antibody. They are then washed in TBS, counterstained in nuclear dye Sytox Green, and then stored in TBS until they are mounted.
Table 3. List of antibodies and their sources used for immunofluorescence staining

<table>
<thead>
<tr>
<th>Primary Dilution antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Secondary Antibody</th>
<th>Source</th>
</tr>
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<tr>
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<td></td>
<td></td>
</tr>
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<td>Dk ms Cy5</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Jackson</td>
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<tr>
<td>Gt anti DCX</td>
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<td>Dk gt Cy3</td>
<td>1:500</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Jackson</td>
</tr>
<tr>
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<td>Dk rb Cy3</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Jackson</td>
</tr>
<tr>
<td>Glial response</td>
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<tr>
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</tr>
<tr>
<td></td>
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<td></td>
<td>Jackson</td>
</tr>
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<td>Rb anti IBA1</td>
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<td>Dk Rb Cy3</td>
<td>1:500</td>
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<tr>
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<td>Jackson</td>
</tr>
<tr>
<td>Rb anti Olig2</td>
<td>Millipore</td>
<td>1:1000</td>
<td>Dk Rb Cy3</td>
<td>1:500</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>Jackson</td>
</tr>
</tbody>
</table>
**Microscopy and histological quantification: design-based stereology**

A darkfield stereoscope (Olympus SZ10, Olympus America, Inc., Valley Center, PA) was utilized to obtain gross pathology. Images were then used for stereological analysis of volume changes. Confocal imaging (Olympus FluoView 500, Olympus America, Inc., Valley Center, PA) was used for higher resolution imaging. Photoshop (CS3, Adobe Systems Inc., San Jose, CA) was used to apply minimal alteration of signals to balance the intensity of images.

Volume changes (16x magnification, 0.5x objective, total magnification 8x) were analyzed using darkfield stereoscope images on StereoInvestigator software (StereoInvestigator, MBF Bioscience, Williston, VT) utilizing the Cavalieri probe with a point probe size of 150 µm to obtain a coefficient of error below 0.07 (Figure 7). To estimate cell populations, the Optical Fractionator probe was used in the Systematic Random Sampling WorkFlow (StereoInvestigator, MBF Bioscience, Williston, VT). Images were obtained on the Disk-Scanning Unit (Olympus America Inc., Valley Center, PA). Because appropriate parameters are variable based on sampling density, and are therefore not *a priori* knowledge, parameters were established based on the sampling density of a test tissue and were analyzed via a statistical analysis of sampling error before being applied to test tissue (Table 4). A grid was randomly overlaid on the regions of interest at a size of 200x550µm and counting frames along the grid were of size 110x130µm. The depth of the frame was 5 µm, and the top and bottom guard zones were 5 µm and 3 µm, respectively.

Results were analyzed using Prism 6 (GraphPad Software Inc., La Jolla, CA). An independent samples with equal variance one tailed t-test was run to analyze significance in neuronal populations between sham and chronic animals, and a one-way ANOVA was
used for volume analyses to compare shams to acute rmTBI and chronic rmTBI followed by Tukey’s post-hoc test. Because acute and chronic time-point shams were not statistically different, their results were pooled. Analysis presented is in comparison to shams unless otherwise stated. Data is presented as mean ± SEM.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Size</th>
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<tr>
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<tr>
<td>Point Probe</td>
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<tr>
<td><strong>Optical Fractionator (CA1, CA3)</strong></td>
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<tr>
<td>Grid Size</td>
<td>200x550µm</td>
</tr>
<tr>
<td>Counting Frame</td>
<td>110x130µm</td>
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<tr>
<td>Top Guard Zone</td>
<td>5 µm</td>
</tr>
<tr>
<td>Bottom Guard Zone</td>
<td>3 µm</td>
</tr>
<tr>
<td>Probe Size</td>
<td>5 µm</td>
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</tbody>
</table>
Figure 7. **Design-Based Stereology: Optical Fractionator**

On the DSU microscope, using StereoInvestigator software, the number of sections, section cut thickness, and interval between sections were entered into the Optical Fractionator Workflow Program (A). Regions of interest are traced (B) in their real positions on the automated stage of the microscope (C). The counting frame, or the boundaries between which cells will be counted, were estimated based on a statistical analysis of the coefficient of error (D). To ensure cells suitable for counting are obtained, a guard zone above and below the probe (or the area that cells will be counted) was obtained as well (E). Finally, a focus map was created across all sections (F). The Systematic Randomized Sampling program was then used to obtain three-dimensional stacks of images to allow for quantification. Cavalieri: Regions of interest are contoured (G), and points are assigned in a systematic manner to distinguish regions of interest (H). Assigned points are used to calculate volume in regions of interest based on the size of the grid and depth of each section.
Results

Closed-head rmTBI utilizing the CCI does not produce obvious cortical damage

In an effort to ensure that the CCI produced only mild injury, which would not result in obvious contusions (or other signs of damage), images of the whole brains and coronal sections were taken on the darkfield stereoscope. Upon gross observation of the whole brains, the CCI was found to not produce any contusions, bruising, bleeding, or swelling at either the acute time-point or the chronic time-point compared to the sham animal (Figure 8). When sectioned in coronal orientation, it was evident that the ventricles appeared to increase over time in animals sacrificed at the chronic time-point, as compared to animals scarified at the acute time-point, and in both the acute and chronic animals compared to the sham animals (Figure 9).

Regional volume is progressively altered in response to closed-head rmTBI

To quantify volume changes in major regions of the brain, we utilized the Cavalieri method. We have found that following closed-head injury, rmTBI progressively reduces the volume of the corpus callosum, striatum, cortex, and hippocampus. Stereological measurement of volume in the hemisphere ipsilateral to injury was analyzed using a one-way ANOVA followed by Tukey’s post-hoc test. These tests reveal decreased volume in the following areas: in the corpus callosum for acute subjects (p<0.01 compared to sham); in the striatum in chronic subjects (p<0.001 compared to sham and p<0.01 compared to acute); in the cortex in chronic subjects (p<0.001 compared to sham) and in acute subjects (p<0.01 compared to sham); and in the hippocampus in chronic subjects (p<0.01 compared to sham). Although there was an increase in ventricular size in acute and chronic animals compared to sham, the increase was not statistically significant (p>.05).
Neuronal density in hippocampal sub-regions is progressively altered in response to rmTBI

To gain a better understanding of changes in neuronal populations following rmTBI, sections were stained for neurons (NeuN) and neuroblasts (DCX). Neuronal populations examined via NeuN immunoreactivity at low magnification (4x objective, 400x total magnification) showed subtle hippocampal disruption, particularly in area CA1 at the chronic time-point compared to acute and sham (Figure 11). At higher magnification (60x objective, 600x total magnification), chronic animals contained fewer neurons in area CA1 compared to acute and sham animals (Figure 11).

Neuronal populations examined via DCX immunoreactivity showed no obvious qualitative changes between sham animals and those sacrificed at acute time-points. However, in comparison to sham and acute time-point animals, animals sacrificed at the chronic time-point showed a distinct loss of neuroblasts (which stain positively for DCX) in the dentate gyrus. This alteration was confirmed at higher magnification (60x objective, 600x total magnification) (Figure 11). An independent samples t-test revealed neuronal populations in area CA1 were found to be significantly lower (p<.05) compared to sham (Figure 12).

Astrocytes respond to rmTBI, but OPCs and microglia do not

To further characterize the injury response, sections were stained for glial cells. Using GFAP, IBA1, and Olig2, we analyzed astrocyte, microglia, and OPC response, respectively. Astrocytic populations examined via GFAP immunoreactivity at low magnification (4x objective, 40x total magnification) revealed an immediate response in both the acute and chronic time-points. GFAP appeared to increase between the blades of the dentate gyrus as well as between area CA1 and the dorsal blade of the dentate gyrus (Figure 13). No detectable differences were observed in microglia, which were
immunoreactive to IBA1 (Figure 14), nor were differences observed in OPCs, which were immunoreactive to Olig2 (Figure 15).
Figure 8. *The CCI produces no obvious cortical damage.* The CCI did not produce any obvious surface damage, bleeding, bruising, or contusion following repeat injuries at either acute (B) or chronic (C) time-points compared to sham (A).
Figure 9. The CCI results in minimal cytoarchitectural changes. Darkfield stereoscope images reveal that the CCI did not result in gross cytoarchitectural changes at acute (B, E, H) or chronic (C, F, I) time-points compared to sham (A, D, G). However, a progressive ventricular enlargement was evident in chronic time-point animals (C, F, I) compared to acute time-point animals (B, E, H) and in acute time-point animals compared to sham animals (A, D, G). The left cortices (contralateral to impact) were notched to ensure orientation and images shown are representative rostral/caudal intervals (in which A, B, and C are more rostral, and G, H, and I are more caudal).
Figure 10. *rmTBI* progressively reduces volume of the corpus callosum, striatum, cortex, and hippocampus. Stereological measurement of volume in the hemisphere ipsilateral to injury reveals decreased volume in the following areas: in the corpus callosum (B) for acute
subjects (p<0.01 compared to sham); in the striatum (C) in chronic subjects (p<0.001 compared to sham and p<0.01 compared to acute); in the cortex (D) in chronic subjects (p<0.001 compared to sham) and in acute subjects (p<0.01 compared to sham); and in the hippocampus (E) in chronic subjects (p<0.01 compared to sham). Although there was an increase in ventricular (A) size in acute and chronic animals compared to sham, the increase was not statistically significant.

**NB:** **significant at p<0.01, *** significant at p<0.001**
Figure 11. rmTBI alters neuronal population in hippocampal subregions. Hippocampal regions CA1, CA3, and the dentate gyrus were evaluated for neurons (NeuN, red) and neuroblasts (DCX, green). Low power magnification (4x objective, 40x total magnification) (A, B, C) revealed no obvious signs of neuronal loss in any subregions. However, higher magnification (60x objective) showed neuronal loss in chronic time-point animals (I) compared to sham animals (G). Low power magnification appeared to show a decrease in neuroblasts at the chronic time-point animals (F) compared to the sham animals (D). This loss of neuroblasts was confirmed in the higher magnification imaging (J, L).
Figure 12. *rmTBI alters neuronal populations in area CA1 at the chronic time-point.* There were significantly fewer (\(p<.05\)) cells in area CA1 following rmTBI at the chronic time-point than there were in the control animal.
Figure 13. Astrocytes show response to repeat injury at the chronic time-point. Sections were stained with GFAP (red) and with Sytox green (green). Low power magnification (4x objective, 40x total magnification) revealed GFAP response in the region between CA1 and the dorsal blade of the dentate gyrus and between the blades of the dentate gyrus at both the acute (B) and chronic (C) time-points, compared to sham (A). High power magnification (60x objective, 600x total magnification) further confirmed that GFAP in area CA1 increased in both chronic (I) and acute (H) time-points compared to sham (G). GFAP in the dentate gyrus also increased in both chronic (L) and acute (K) time-points compared to sham (J).
Figure 14. **Microglia show no obvious response to rmTBI.** No detectable differences were observed in microglia (IBA1, red; counterstained with Sytox Green, green) in response to repeat injury at acute or chronic time-points. Low power magnification (4x objective, 40x total magnification) revealed no obvious change in microglia (A, B, C). This finding was further confirmed at high power magnification (60x objective, 600x magnification) in areas CA1 (G, H, D), the dentate gyrus (J, K, L), and CA3 (M, N, O) across all three experimental groups.
Figure 15. **OPCs show no obvious response to rmTBI.** No detectable differences were observed in OPCs (Olig 2, red; counterstained with Sytox Green, green) in response to repeat injury at acute or chronic time-points. Low power magnification (4x objective, 40x total magnification) revealed no obvious change in OPCs (A, B, C). This finding was further confirmed at high power magnification (60x objective, 600x magnification) in areas CA1 (G, H, I), the dentate gyrus (J, K, L), and CA3 (M, N, O) across all three experimental groups.
Discussion

With over 1.5 million concussions diagnosed per year (CDC, 2003), many from sports and combat, the diagnosis of mTBI has steadily increased over the past few years. Besides physical symptoms, individuals who have sustained mTBIs have demonstrated reduced brain volume, neuronal loss, and eventual neurodegenerative pathology, such as accumulation of amyloid-β and NFTs (Hof et al., 1992). To investigate the effects of rmTBI in humans, we utilized the CCI in a rat model to deliver three mild, closed-head injuries. The CCI was adapted to produce an mTBI without having to perform a craniotomy, which is clinically relevant in terms of modeling a true concussive injury. Three injuries were delivered at an interval of 48 hours between each impact. Consistent with the pathology of mild injuries (Prins et al., 2010), the closed-head injury model produced no detectable surface trauma to the brain, but it did produce some disruption of cortical cytoarchitecture at the location below the impact. We observed post-injury reactivity and disruption in the hippocampus, and for that reason, we focused subsequent analysis on injury-induced alterations in the hippocampus.

In addition to histological evaluation of hippocampal changes, we performed quantitative histology utilizing design-based stereology, namely the Cavalieri estimator and Optical Fractionator, to quantify volume and cell number, respectively. Using these methods, we identified an increase in ventricle volume and a corresponding reduction in the volume of the cerebral cortex, corpus callosum, and hippocampus, as well as neuronal loss in hippocampal subregion CA1, DCX loss in the dentate gyrus, and astrocyte response near the dentate gyrus, but no obvious change in microglia or OPC expression. While many of these results are consistent with findings in previous studies of direct open cortical impact, this study is the first to use the CCI in a rat model of a mild repetitive closed-head
injury, which can demonstrate the effects of a true concussive injury with greater precision than models used in the past. We report that a closed-head repetitive injury produces neuronal loss in CA1 and loss of DCX positive neuroblasts in the dentate gyrus as determined by design-based stereological quantitation. Our ability to demonstrate both short- and long-term effects of rmTBI in hippocampal cell populations is an additional contribution of this study.

Clinical Relevance of the CCI

Although the CCI is costly and challenging to operate, it holds several benefits over other methods of producing injury. In humans, a concussive injury does not necessarily produce a cortical contusion (Prins et al., 2010). We were able to faithfully reproduce this kind of injury in rats using the closed-head CCI, because it did not result in any visible contusions on the surface of the brain, and the pathology did not show any obvious signs of atrophy. Additionally, the CCI ensures all injuries are delivered to rats at identical stereotactic positioning with identical force, greatly increasing precision and reproducibility over human studies of rmTBI (reference). Most importantly, the CCI has been adapted to allow for a closed-head injury, which may be more clinically relevant than other methods that have attempted to model an mTBI. For example, in an effort to model rmTBI, Aungst, Kabadi, Thompson, Stoica, & Faden (2014) and Chohan et al. (2015) utilized CCI, but their methods required they perform a craniotomy on the rats prior to impact. Since human concussions do not result in open-head injury, models relying on a craniotomy are inappropriate. Indeed, because of the harm inflicted, studies that perform craniotomy are more likely to consistently model moderate/severe injuries than mild ones.

Furthermore, the CCI produces behavioral deficits consistent with previous findings of rmTBI (Prins et al., 2010). Prior to sacrifice but acutely post-injury, rats for this
study were assessed behaviorally by collaborators at DePaul University (Chicago, IL). These assessments showed that following repeat injuries, rats spent more time in the center of an open field and less time with a novel object, demonstrated hypoactivity and decreased locomotive capability (indicated by the foot fault test), and had higher resting corticosterone levels. These findings suggest that following repeat injury, rats produce an appropriate anxiety, recognition, stress, and motor activity response. Although some studies, such as Matthiasin and DiCamillo (2010), suggest novel object recognition can provide insight into hippocampal function, a Morris Water Maze spatial memory task would better evaluate a rat’s spatial memory performance, which could provide stronger evidence for hippocampal deficits that have been demonstrated at the molecular level. Regardless, the deficits in the novel object recognition test provide support to analyze the pathological changes in the hippocampus.

Microscopic Resolution of Volume Estimation

Utilizing the Cavalieri stereological probe, we identified a significant, progressive reduction in hippocampal, cortical, corpus colloidal, and striatal size following rmTBI. These findings have not been previously reported in a closed-head model of rmTBI. In previous research (Levine et al., 2008; Monti et al., 2013; Ross et al., 2012; Tate & Bigler, 2013; Zhou et al., 2013), volume studies have utilized quantified MRI data to develop an understanding of decreases in brain matter. For example, MRI data gathered from human patients who had received mTBIs showed increased ventricle size indicative of tissue volume loss elsewhere in the brain (Zhou et al., 2013). MRI studies have been enabled the

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1 This project is part of an ongoing collaboration between Drs. Peterson, Urban, Stutzman, and Marr from Rosalind Franklin University Medical School (North Chicago, IL) and Dr. Kozlowski from DePaul University. Reference to data that has arisen from the collaboration outside of work included in this thesis, mainly behavioral studies, will be referred to as “personal communication.”
detection of general atrophy in the brain parenchyma (Levine et al., 2008; Mackenzie et al., 2002) which progressed over time (Ross et al., 2012), as well as a reduction in cerebral white matter (Ross et al., 2012). In addition, MRI data has revealed decreased volume in the cerebellum (Ross et al., 2012), parietal lobe (Monti et al., 2013), fornix (Tate & Bigler, 2013), and hippocampus (Monti et al., 2013; Tate & Bigler, 2013).

Ross et al. (2012) and Levine et al. (2008) have utilized MRI quantification to characterize the effects of single mTBI. Ross et al. (2012) analyzed patients who had sustained a single mTBI and scored similarly on the GCS. MRI data were loaded into NeuroQuant (a computer program used for quantification) and analyzed according to brain regions. By scanning twice over a two-year period, progressive atrophy was seen in whole-brain parenchyma, cerebral white matter, and the cerebellum. Similarly, Levine et al. (2008) used MRI scanning and a semiautomated brain region estimator, which autotraced regions of interest, to identify a post-mTBI reduction in brain parenchyma that was less severe than the reduction identified following moderate injuries but more atrophied than uninjured brains. Our present study affirms Levine et al. (2008) and Ross et al. (2012) in our observation of brain tissue atrophy, as well as Ross et al. (2012) in further characterizing the atrophy as progressive. However, in order to better elucidate the neurodegenerative mechanisms, we chose to study repeat injuries exclusively.

Since the hippocampus plays an important role in learning and memory and learning and memory deficits are often seen following rmTBI, some researchers have sought to draw a link between mTBI and hippocampal changes. Monti et al. (2013) used MRI data of patients with mTBI to segment subcortical regions into regions of interest. They found smaller hippocampi in injured patients with a corresponding deficit in memory performance tasks. However, patients in the study may have had more than a single injury, which is problematic because single and repeat injuries can affect the brain differently,
evoking different pathological hallmarks. Thus, pooling these patients together may have confounded conclusions. In contrast, our study ensured each rodent had the same injuries at the same location resulting from the same force, which would not be possible with human subjects with a variety of histories.

In addition to mTBI, age can affect brain volume and may, therefore, confound the results of studies that do not control for age. Tate and Bigler (2000) performed MRI on patients two months post-injury. Regions of interest, particularly the fornix and the hippocampus, were manually traced from an MRI scan with a volume estimation program. Patients’ scans were divided into groups based on the severity of injury (mild, moderate, severe). In addition to reduced fornix volume, they affirmed Monti et al. (2013) in observing hippocampal atrophy, which was correlated with memory performance. However, patients ranged from teenagers to sexagenarians. Although the older patients were free of dementia and other age-related disorders, brain volume changes with age (Kandel et al., 2012) may have been a confounding factor in their study. For that reason, we ensured rats in the study were all of the same age.

Few studies have utilized rodent models to study volume changes following rmTBI, and those that have often encounter obstacles in data collection or analysis. For example, Mannix et al. (2013), studied rmTBI volume changes in rats utilizing the weight drop method. MRI performed six months post-injury demonstrated no evidence suggesting significant volume change. However, subregions appeared difficult to distinguish in MRI figures, which should have precluded volume analysis, which requires clear regional boundaries for proper analysis. Such issues call into question the utility of MRI in volume studies.

The aforementioned studies all used MRI data (quantified with computerized programming), and were, therefore, susceptible to the well-studied limitations of MRI
imaging in volume studies. Our study, on the other hand, used darkfield stereoscope imaging to provide clearer boundaries around regions of interest than an MRI. In darkfield stereoscope imaging, coronal sections are manually traced for superb clarity when volume is subsequently quantified in subregions of interest. This clarity affords precise determination of volume in regions of interest, which improves upon past MRI studies. Although potentially faster than darkfield stereoscope imaging, MRI data are limited in their ability to accurately evaluate the volume in a specific region of interest.

In addition, MRI data can only be used to gather volume data from human studies and even then is limited in its utility because of the reduced precision. For instance, MRI may not be sensitive enough to reveal the subtle volume changes that occur following a mild injury (Van Boven et al., 2009). In human studies, the brain is large enough such that a small magnetic field can be used to identify regions of the brain. However, this imaging is not sensitive enough to analyze subregions of the brain. If the magnetic field were to be increased, theoretically increasing the sensitivity of the imaging to allow for detection of smaller subregions of the brain, the magnetic field would then be large enough to adversely collect background noise (Denic et al., 2011). MRI presents the same catch-22 in rodent studies, in which the magnetic field must be large enough to detect the small volume of the rodent brain. This large magnetic field, when adapted to the rodent brain, collects background noise, making it difficult to precisely analyze changes in regional volume (Denic et al., 2011). For these reasons, MRI data are limited when analyzing subregional volume. Stereological methods, such as the Cavalieri method, have improved the quantification of volume changes.

The Cavalieri method provides a rigorous estimate of volume by mathematically multiplying the sum of all the points assigned to a particular area of interest across all sections in an animal by the area associated with each point by the depth associated with
each point: \( \text{Volume} = \sum P \times A_p \times t \) in which \( P \) is the sum of all points sampled across sections, \( A_p \) is the calibrated area associated with each point in the array, and \( t \) is the depth associated with each point. This equation yields an estimate of volume in a particular brain region in a precise, reproducible area with greater specificity than an MRI allows (Peterson, 2004). Aungst et al. (2014) used the Cavalieri method to assess volume and found a similar decrease in hippocampal volume in an open-head model of mTBI.

Although human volume studies are useful in identifying trends, one cannot gain the same causality as in rodent studies, in which brains are manipulated to have an identical number of injuries of identical force at the same sites of injuries as a result of the CCI’s reproducible impacts. MRI analyses of human rmTBI cannot guarantee that all individuals were subjected to similar impacts, which introduces avoidable variability. MRI data are also generally restricted to cortices and lobes and thus, unlike the Cavalieri probe, cannot be used to gather regional specificity. For these reasons, our novel use of the Cavalieri probe in a closed-head mTBI rodent model is advantageous in comparison to pre-existing MRI data.

**Design-based Estimation of Neuronal Loss**

Our findings demonstrated a progressive decrease in the number of neurons in area CA1, a region of the hippocampus which is heavily involved in memory encoding (Kandel et al., 2012). Other studies of mTBI have alluded to CA1 degeneration (Chohan et al., 2012; Meyer, Davies, Barr, Manzerra, & Forster, 2012), but this is the first time it was demonstrated using a closed-head model of rmTBI at both short- and long-term time-points. A decrease in CA1 neurons has been demonstrated following a single closed-head injury at a short-term time-point (Meyer et al., 2012) and in an open-head model of a
mild/moderate TBI at a chronic time-point (Chohan et al., 2015). Studies that demonstrate cell loss following injury, however, often use methods of quantification that can introduce methodological biases into their studies. For example, one result of injury and cell loss may be a change in hippocampal volume or in the volume of surviving cells. Studies that do not account for such shrinkage artifacts may be subject to a methodological bias, consistently skewing data as a result of cellular distortion (Evans et al., 2004). To avoid such pitfalls, we used a design-based approach that is sensitive to tissue shrinkage or distortion and can estimate cell populations without bias due to changes in cell size, shape, or distribution.

Although other studies have utilized stereology, their approaches often present methodological issues. Meyer et al. (2012) used a weight drop method that does not require a craniotomy to model a closed-head injury. A single injury was delivered and rats were sacrificed 4 or 9 days later. Similar to our study, stereology was used to count CA1 neurons, but they counted cresyl violet positive neurons at 20x objective (200x total magnification). Cresyl violet stains for Nissl substance, which can be positive in both neurons and glial cells. Although they concluded there was a decrease in neurons in CA1 9 days after injury, staining for Nissl may not be the most accurate way to count neurons because Nissl stains for protein synthesis, which is present in both glial cells and neurons. For that reason, equating Nissl positive staining with neurons is dependent upon an individual’s ability to morphologically classify a Nissl cell as neuronal or glial. Additionally, it is difficult to differentiate neurons from glial cells at a 20x objective. We used the NeuN antibody in our study because it specifically targets mature neurons. In our study, cells were counted using a 60x objective (600x total magnification) with oil immersion to increase the resolution and thus increase the ability to resolve ambiguous boundaries between cells.

In the aforementioned study by Chohan et al. (2014), a CCI was used in a mouse model with a craniotomy, which led to a visible contusion at the site of impact. Similar to
Meyer et al. (2012), Nissl positive neurons in the CA1 decreased following injury, which led to a corresponding decline in performance on the Morris Water Maze spatial memory task. This suggests that area CA1 contains place cells (Morris et al., 1982) involved in learning and spatial memory. Similarly, Aungst et al. (2014) found a progressive CA1 cell loss in repeat and single injuries, but their method also required a craniotomy and counted Nissl positive neurons. A study by Almeida-Suhett et al. (2015) was performed with similar parameters as Chohan et al. (2014), but they did not find a significant decrease in area CA1. This discrepancy could be explained by the higher velocity of impact in Chohan et al.’s study, which was closer to the velocity of impact in our study than in Almeida-Suhett et al.’s. The source of CA1 cell loss may be ambiguous, given that in previous studies that found CA1 cell loss, a craniotomy was performed, and the loss could therefore result from either the craniotomy or the mild injury. To clarify the source of CA1 neuronal loss, our study utilized a closed-head impactor, which eliminated the open-head aspect of past studies.

To quantify histological data, we utilized the Optical Fractionator, another stereological probe. This method of cell counting is useful because it provides less variability than other commonly used methods of cell counting that can lead to methodological errors (Peterson et al., 2004), and thus allows for reproducible results. For example, planar probes provide no indication concerning the thickness of a cell, and therefore may only identify larger and longer cells (Peterson et al., 2004). This bias leads to inaccurate sampling and, therefore, inaccurate counting.

The Optical Fractionator avoids these data quantification biases by tracing region of interest using stereology software. In our study, the program creates three-dimensional focal planes in random locations along a grid, which is overlaid on top of the region of interest in a random x/y position. The three-dimensional focal plane allows for equal
probability of counting a particular cell, regardless of size and orientation, throughout the tissue. The number of cells counted is then multiplied by the fraction of sites sampled to estimate the total number of cells in the tissue. The randomized placement of the grid, selection of the regions to count, and unbiased sampling yields reproducible data with fewer sampling errors and biases than other methods (Peterson et al., 2004).

**Alteration of Hippocampal Neurogenesis**

Despite the original dogma that the adult brain does not generate new neurons, neurogenesis in the dentate gyrus of the hippocampus occurs throughout adulthood (Villasana, Westbrook, & Schnell, 2014). DCX, a marker for neuroblasts, generally increases in response to TBI (Richardson, Sun, & Bullock, 2007), and this response can aid in restoration of impaired, post-injury cognitive function (Lu, Mahmood, Zhang, & Copp, 2003). Meyer et al. (2012) noticed that the post-mTBI dentate gyrus demonstrated apoptosis, but not cell loss. They suggested that this discrepancy may be because of the neurogenesis that follows injury in that region. In our study, however, the injury was mild enough that DCX staining in the dentate decreased, so it is unlikely that new neuroblasts are compensating for the absence of cell loss in the dentate gyrus.

Over time, rmTBI alters neurogenesis regardless of the distance of the dentate gyrus from the site of impact. Although severe injuries generally lead to an increase in DCX, a well known index of neurogenesis (Von Boven et al., 2007; Zhang et al., 2013), qualitative data suggest a clear decrease in neuroblasts with time as shown by a gradual decrease in DCX. Previous research has demonstrated that following a single mild closed-head injury, DCX decreases at a chronic time-point in the dentate gyrus (Zhang et al., 2013). A similar pattern of neuroblast increase occurs in models of moderate injuries (Xiang Gao et al., 2012). The loss in DCX has been implicated as a contributing factor in
learning and memory impairment (Xiang Gao et al., 2012; Zhang et al., 2013). A decrease in neuroblasts that should theoretically mature into neurons to aid in brain repair may be related to the lack of brain repair following rmTBI. Paradoxically, more severe TBIs can lead to an increase in DCX (Villasana et al., 2014). This discrepancy suggests that a different molecular cascade may result from mild injuries than results from more moderate contusions.

**Glial Response to Injury**

Glial staining appears consistent with other models of rmTBI (Ojo et al., 2013; Myer, Gurkoff, Lee, Hovda, & Sofroniew, 2013; Israelsson et al., 2009; Carbonell & Grady, 1999; Rice et al., 2015; Shitaka et al., 2011), which provides further validity of the CCI and its adaptation to produce a mild injury. For example, astrocytes increase in the hippocampus in response to repeated closed-head impacts (Ojo et al., 2013). However, the study by Ojo et al. (2013) used a model in which the rodent was immobile while receiving the mTBI, and in a true concussive injury, an individual is mobile. An increase in hippocampal astrocytes has also been demonstrated in moderate/severe injury models (Myer et al., 2006), a single closed-head injury (Israelsson et al., 2009), and an open-head model of rmTBI (Uryu et al., 2002).

In addition, microglial activation has been reported in studies of mTBI (Shitaka et al., 2011; Ojo et al., 2013) and severe TBI (Carbonell & Grady, 1999; Rice, 2015). However, the mTBI studies may have inflicted too severe of injuries to arrive at a conclusion on microglial activation following mTBI. For example, a study by Shitaka et al., (2011) reported that some animals showed hemorrhaging. Although such animals were excluded, hemorrhaging may imply too forceful of an impact. A study by Ojo et al. (2013) found a similar increase in microglial activation but delivered more impacts to rodents than
in our study, which also may have produced more severe deficits than typical mTBIs. The weight-drop model used by Israelsson et al. (2009), was difficult to adapt to mild injuries (Xiong et al., 2013). Microglia generally respond to a more severe injury (Kandel et al., 2012). Our lack of microglial activation may provide further validation for the closed-head CCI as a clinically relevant model of a concussive injury.

OPCs generally proliferate in response to injury and migrate toward focal injuries, such as stroke. The response of OPCs to mTBI remains poorly understood (Burda and Sofroniew, 2014). Through exclusively qualitative analysis, we did not observe an increase in OPCs following rmTBI at acute or chronic time-points. Similar to the lack of microglial response, this may provide further support that the closed-head CCI does produce a truly mild injury. OPCs are a prime candidate population for reprogramming because of their proliferative nature. Although historically it has been assumed that OPCs must actively migrate toward injury to be utilized in reprogramming, recent studies have shown otherwise (Bazarek, 2015).

**Future Studies**

Future studies will first continue to quantify neuronal populations in the subregions of the hippocampus using the Optical Fractionator method. Qualitative histology imaging has shown potential neuronal loss in the dentate gyrus, particularly in the dorsal blade. DCX will also be quantified to provide significance for the observed decrease in neuroblasts over time. By analyzing DCX, we can gain a better understanding of the lack of neurogenesis following mTBI and seek to identify other mechanisms of repair following mTBI. Furthermore, by understanding the brain’s mechanistic processes following injury, we can begin to research methods of intervention to repair observed pathological damages.
Because the loss of neurons has been demonstrated in a clinically relevant model of closed-head mild brain injury, attention should now be turned to replacing such molecular deficits in an effort to recover behavior. OPCs are a prime candidate population to target for reprogramming. OPCs actively divide and proliferate in response to injury and are kept in reservoir to replace oligodendrocytes (Gage, Kempermann, Palmer, Peterson, & Ray, 1998). OPCs have been used in reprogramming after injury, but until recently, it was believed that OPCs had to be actively proliferating to be utilized in reprogramming. Bazarek et al. (2015) found that inactive OPCs could also be targeted and, using neurotrophic transcription factors, reprogrammed into neurons to replace neuronal loss. Once a neuronal profile is attained electrophysiologically, morphologically, and genetically, its connections can be mapped using the retroviral rabies vector, which is able to trace neuronal connections transsynaptically (Kandel et al., 2012). By replacing cells that are lost following rmTBI, a subject could perhaps regain learning, memory, or behavioral function.

While creation of new CA1 neurons by reprogramming and stimulation of dentate gyrus neurogenesis are important therapeutic goals, it will also be beneficial for future studies to explore the mechanisms of cell loss to determine possible interventional strategies. Additional studies can continue characterizing the pathology underlying cell death in rmTBI. Oxidative stress, another potential mechanism of cell death, can be analyzed via a cytosolic glutathione assay, and apoptosis can be analyzed through TUNEL staining. Studies that seek to repair neurons before death may be difficult, as papers have shown immediate post-injury axonal shearing (and eventual death of the neuron). However, with proper understanding of cell death mechanisms, intervention before extensive neuronal death occurs could be possible, barring any need for reprogramming.

Changes in gene expression in the hippocampus associated with neuronal death can be further characterized as well. Using laser microdissection, our lab has shown the
complex changes that occur following a wire knife lesion (Bazarek et al., 2015). A similar microarray of the cytokines, interleukins, and other inflammatory elements that follow mTBI can be analyzed.

Although our chronic time-point does provide evidence for progressive atrophy, longer-term survival studies may eventually provide insight into the relationship between these hippocampal injuries and the eventual development of CTE-like pathology. By sacrificing animals at a time-point of chronicity longer than 30 days and staining for phosphorylated tau protein and amyloid-β, we can analyze the progression of protein accumulation, volume changes, and concurrent changes in neuronal and glial populations.
Conclusion

Overall, we have classified the molecular basis of a closed-head rmTBI in the hippocampus, which had not been well-characterized in any similar models. The injury did not require a craniotomy and produced no obvious bruising, bleeding, or sign of contusion, which suggests it is more clinically relevant than other methods of delivering concussive-like injuries. Through volume analysis, we have identified a progressive increase in ventricle size and a corresponding progressive decrease in cortical, hippocampal, striatal, and corpus callosal size. We have also identified a progressive decrease in CA1 neurons, an astrocytic response near the dentate gyrus, and an absence of hippocampal microglial and OPC responses, which is consistent with molecular profiles of mTBI. This is the first study to identify the subtle changes in a clinically relevant model of concussive-like brain injury.
References


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Appendix A: Solutions List

Cryoprotectant

124 ml of Glycerin, 150 ml of Ethylene Glycol, and 250 ml of 0.1M $PO_4$ are stirred slowly until mixture is homogenous. The solution is refrigerated at 4° and used within one month.

30% Sucrose

500 ml 0.1M $PO_4$ and 150 g of sucrose are stirred. Solution is refrigerated at 4°C and used within one month or until solution becomes cloudy (whichever comes first).

0.1M Tris Buffered Saline (TBS)

26.44 g of Trizma Hydrochloride, 3.88 g of Trizma Base, 18.00 g of Sodium Chloride, and 2 L of ddH$_2$O are stirred. Solution should be adjusted to pH 7.5. Solution is stored at room temperature and used within one month.

TBS+

500 ml of 0.1M TBS and 1.25 ml Triton X-100 detergent are stirred. Solution is stored at 4°C and used within one month.

0.2M $PO_4$

Part A: 2 L of dd H$_2$O and 48 g of Sodium Phosphate Monobasic

Part B: 2 L of dd H$_2$O and 56.8 g of Sodium Phosphate Dibasic

460 ml of part A and 1540 ml of part B are combined. Part A, Part B, and the resulting 0.2M $PO_4$ are stored at room temperature and can be used for up to one month.
0.2M Tris-Hydrochloric Acid (HCl)

250 ml ddH₂O and 7.88 g Tris-HCl, and 9 pellets of sodium hydroxide are mixed. pH is adjusted to 8.0-8.5 using 6N NaOH.

2.5% polyvinyl alcohol (PVA)- diazabicyclo[2.2.2]octane (DABCO)

6ml of glycerin and 2.4 g of polyvinyl alcohol (PVA) are mixed until PVA is coated in glycerin, at which point, 6ml of distilled water are added. The solution is mixed overnight at room temperature using a rotator. Once mixed, 12 ml of 0.2M Tris-HCl are added, and the solution is heated to 50°C in a water bath for 30 minutes, shaking the tubes every 5 minutes. Centrifuge at 5000 g for 15 minutes, place supernatant in a new tube, and add 0.625 g of DABCO and mix well. Centrifuge the new tube at 5000 g for 15 minutes, and aliquot the supernatant. PVA-DABCO is stored at -20°C and can be used for up to six months.