Acute Astrogliosis and Neurological Deficits Following Repeated Mild Traumatic Brain Injury

by

Melissa A. Clarkson
BSc, University of British Columbia, 2012

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in the Division of Medical Sciences (Neuroscience)

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University of Victoria

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Supervisory Committee

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Supervisory Committee

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Abstract

Mild traumatic brain injury (mTBI), often referred to as concussion, has become increasingly recognized as a serious health issue in the general population. The prevalence of mTBI in athletes, particularly repeated injuries in young athletes, is of great concern as injuries to the developing brain can have long-term detrimental effects. In this study we used a novel awake closed-head injury (ACHI) model in rodents to examine repeated mTBI (rmTBI), to determine if repeated injuries produced the neurological and molecular changes evident with human concussion. Animals were administered 4, 8, and 16 rmTBIs and acute neurological assessments were performed after the injuries. Changes in glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1) levels were assessed using Western blot analysis at one day following rmTBI in the ipsilateral dentate gyrus (DG) and the cornu ammonis (CA) regions of the hippocampus and the cortex (CX) indicative of astrocyte and microglial cell reactivity. Results indicated that the ACHI model produces neurological deficits immediately after the injuries, with the most deficits arising in the rmTBI16 group. Despite deficits in all injury groups, histological staining with cresyl violet revealed no significant morphological tissue damage to the brain. Western blot analysis, however, showed a significant increase in DG and CX GFAP expression in the rmTBI16 group with no changes in Iba-1 levels. This suggests an acute activation of astrocytes in response to injury, with a delay or absence of microglial activation. Our findings show that with repetitive concussions, we are able to detect acute neurological and molecular changes in the juvenile female brain. However, further investigation is necessary to determine if these are transient changes.
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<th>Definition</th>
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<tbody>
<tr>
<td>ACHI</td>
<td>awake closed head injury</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CA</td>
<td>cornu ammonis</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CC</td>
<td>corpus callosum</td>
</tr>
<tr>
<td>CCI</td>
<td>controlled cortical impact</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CTE</td>
<td>chronic traumatic encephalopathy</td>
</tr>
<tr>
<td>CX</td>
<td>cortex</td>
</tr>
<tr>
<td>DAI</td>
<td>diffuse axonal injury</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DTI</td>
<td>diffusion tensor imaging</td>
</tr>
<tr>
<td>DTI</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FPI</td>
<td>fluid percussion injury</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GCS</td>
<td>Glasgow coma scale</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>HP</td>
<td>Hippocampus</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HSD</td>
<td>honest significance difference</td>
</tr>
<tr>
<td>Iba-1</td>
<td>ionized binding calcium adapter molecule-1</td>
</tr>
<tr>
<td>IF</td>
<td>intermediate filament</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>IL-1</td>
<td>interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>potassium</td>
</tr>
<tr>
<td>LFP1</td>
<td>lateral FPI</td>
</tr>
<tr>
<td>LOC</td>
<td>loss of consciousness</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>mCCI</td>
<td>modified CCI</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mTBI</td>
<td>mild traumatic brain injury</td>
</tr>
<tr>
<td>mWD</td>
<td>modified WD</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>sodium</td>
</tr>
<tr>
<td>NAP</td>
<td>neurological assessment protocol</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMAD</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>OF</td>
<td>open-field</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCS</td>
<td>post-concussive syndrome</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>PID</td>
<td>post injury day</td>
</tr>
<tr>
<td>PnC</td>
<td>caudal pontine reticular nucleus</td>
</tr>
<tr>
<td>PND</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>rmTBI</td>
<td>repeated mTBI</td>
</tr>
<tr>
<td>rmTBI</td>
<td>repeat mild traumatic brain injury</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered-Saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>TBS-Tween</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethlenediamine</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WD</td>
<td>weight drop</td>
</tr>
</tbody>
</table>
Acknowledgments

I would like to thank Dr. Brian Christie and Dr. Patrick Nahirney for giving me the chance to attend another university in the province and providing me with the guidance and support that I needed to create this thesis. Thank you Pat for sharing and trusting your lab space with me, not everyone gets a seat in the most spick-and-span area of the lab. Thanks to Brian for occasionally bringing in his furry friend Koda and talking bikes with me. Lab stuff is great but we all know puppies always win.

To my Lab gals (insert microscope and brain emoji)…and Juan, thank you for everything you do. Inside and outside of the lab, you guys are truly amazing! I’m basically fluent in Spanish now, I know how to organize my spreadsheets to the T, and I have learned that if I come into the lab at 6am I won’t be the first one there…all thanks to you guys (real valuable stuff, I know). Christine, thanks for having so many minions throughout the last couple years, my jokes have never been funnier.

Thanks to my tall friend Sara, who I hope moves on to become a professional gel-maker. Keep up the hard work girl.

Then there’s my family - Mom and Dad (Abby), Stef and Emma (Roxy, Riley, Cooper, Jake, Hudson, Madi, Daisy, Pekoe and Styne) and my partner in crime, Shafiq (Boof). Thank you putting up with me and being there for me every single day. I am forever indebted to you (literally).

Last and most importantly, my Rover family for getting me outside and active every single day – Dipsy, Jazz, Scully, Coco, Seamus, Buddy, Dandy, Hank, Beau, Lilly, Rolo, Jasper, Indy, Jaxx, Dexter, Mocha, Luna, Posey, Max, Moe, Finn, Benny, Harlie, Kiwi, Kearny, Tuvok, Sophie, Dixie, Miko, Peanut, Max, Dug and of course my three faves Penny, Koppa and Boof. Everything I know, I learned from dogs….sort of.
Dedication

This thesis is dedicated to my mom and dad. Enough said.
1. Introduction

1.1 Traumatic Brain Injury

1.1.1 Epidemiology

A head injury can refer to an external injury to the face, scalp or skull, however, a traumatic brain injury (TBI) is more specific and results from an external force being transmitted to the head, which further results in brain dysfunction (i.e. confusion, loss of consciousness, seizure, coma and neurological deficits) (Bruns and Hauser, 2003). In addition to being the major cause of death and disability throughout the world, TBI is a personal and financial burden to society with an estimated incidence of 500 per 100,000 population (Langlois et al., 2005). With that being said, individuals who did not seek medical attention could not be accounted for, giving an underestimation of the actual numbers of those sustaining a TBI (Schouten, 2007). External forces that elicit a TBI can include: sport injuries, motor vehicle accidents, falls, assaults and gunshots wounds (Brain Injury Canada Fact Sheet 2014). The age populations most likely affected by TBI fall into three main categories: 0 to 4 yrs, when developing motor skills and susceptibility to trauma are major contributors; 15 to 19 yrs, when youth tend to engage in more risk taking activities; and 65 yrs+, when mobility and vestibular issues contribute to an increase in accidents and falls (Faul et al., 2010). The younger populations are of great concern as their brains are still in the developmental stages, thus increasing their vulnerability to injury.

1.1.2 TBI Severity

A TBI can either be a penetrating injury or a closed-head injury. With the
penetrating injury, there is damage to the skull, dura and brain parenchyma, while these areas remain intact with a closed-head injury (Cassidy et al., 2004). There is a spectrum of TBI severity from mild to moderate to severe (Table 1). This classification is based on level of consciousness (duration and severity, if lost), memory and neurological deficits, and brain imaging such as computed tomography (CT) or magnetic resonance imaging (MRI) (Management of Concussion, 2009).

**Table 1. Classification of TBIs**

<table>
<thead>
<tr>
<th></th>
<th>Mild TBI</th>
<th>Moderate TBI</th>
<th>Severe TBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural brain imaging</td>
<td>Normal</td>
<td>Normal or abnormal</td>
<td>Normal or abnormal</td>
</tr>
<tr>
<td>Loss of consciousness</td>
<td>0–30 min</td>
<td>30 min to 24 hrs</td>
<td>&gt;24 hrs</td>
</tr>
<tr>
<td>Altered mental state</td>
<td>≤24 hrs</td>
<td>&gt;24 hrs</td>
<td>&gt;24 hrs</td>
</tr>
<tr>
<td>Post-trauma amnesia</td>
<td>≤1 day</td>
<td>1–7 days</td>
<td>&gt;7 days</td>
</tr>
<tr>
<td>Glasgow Coma Scale score</td>
<td>13–15*</td>
<td>9–12*</td>
<td>&lt;9*</td>
</tr>
</tbody>
</table>

Adapted from Traumatic Brain Injuries Review (Blennow et al., 2016). *Best score obtained in the first 24 hours following the injury.

**1.1.3 Glasgow Coma Scale**

Since 1974, the Glasgow Coma Scale (GCS) has been used as an assessment tool to clinically evaluate a patient’s level of consciousness following a traumatic brain injury. It is still a widely utilized scale in both the clinical and research worlds today. This neurological scale is used to independently examine motor response, verbal response and eye opening. Based on the responses to various stimuli, the sum of these individual elements gives a GCS score within the range of 3-15 and, as outlined in Table 1, a higher GCS score indicates a higher level of consciousness. This score can be correlated with the patient’s outcome and disability (Teasdale and Jennett, 1974; Teasdale et al., 2014),
however, since its development, people have had reservations about the GCS and critical comments towards it. First and foremost, confounding factors that could render some of the GSC components untestable include paralysis, intoxication, sedation or intubation (Middleton, 2012; Zuercher et al., 2009). Consequently, the GCS is not the only assessment used in TBI diagnosis (Table 1).

1.2 Mild Traumatic Brain Injury

1.2.1 Post-Concussive Syndrome

The most prevalent form of TBI, mild TBI (mTBI), is interchangeably used with the term concussion in the literature and represents 80-90% of TBI cases (McCrory et al., 2013). A concussed brain may or may not lead to a loss of consciousness with normal neuroimaging results (Table 1). Symptoms may include headache, dizziness, reduced attention, sleep disturbances, amnesia, fatigue, irritability, anxiety and depression. For a majority of patients, these subjective symptoms resolve on their own, however, if they persist in an individual for more than three months, this is termed post-concussive syndrome (PCS) (Hall and Chapman, 2005; Ling et al., 2015; Voormolen et al., 2018). It is thought that most of the disability suffered by mTBI patients is caused from PCS. Researchers have sought out specific factors contributing to the development of long term PCS: age, female gender, prior head injury, lower education, personality disorder and cognitive dysfunction pre-injury (Bazarian and Atabaki, 2001; Hall and Chapman, 2005; Nelson et al., 2016; Scopaz and Hatzenbuehler, 2013; Voormolen et al., 2018).

1.2.2 Types of Forces in Mild Traumatic Brain Injury

Many TBIs are caused by a combination of biomechanical forces acting on the
brain. When the head moves anteriorly to posteriorly, this is a result of linear acceleration. Rotational acceleration on the other hand, is when the head rotates sideways. Furthermore, if the head decelerates (i.e. hits the ground) this is the result of deceleration forces (Blennow et al., 2016). These forces contribute to a complex set of events described later in detail.

1.2.3 Brain Regions at Risk

Every injury is unique, however certain brain regions tend to be more vulnerable to damage, specifically, the hippocampus. This susceptible brain region within the medial temporal lobe is composed of the dentate gyrus (DG) and the *cornu ammonis* (CA) regions (Figure 1). The CA is further subdivided into 4 regions, CA1-4 (Bayer, 1980). A trisynaptic unidirectional loop exists with projections from the entorhinal cortex to the DG, to the CA3, to the CA1 and finally back to the entorhinal cortex (Knierim, 2015). The hippocampus has been implicated in learning and memory (dorsal) and affective behaviours such as depression and anxiety (ventral) (Bannerman et al., 2004). Concussions are commonly a result of an impact to the frontal or temporal lobes, making the hippocampus a region of susceptibility to mTBI (Geddes et al., 2003; McCarthy, 2003). Studies of severe TBI have shown decreased hippocampal volume in both adults (Kim et al., 2008) and juveniles (Tasker et al., 2005). Therefore, with the cognitive and emotional impairments that go along with TBI, it is reasonable to infer that hippocampal damage has occurred.
Figure 1. A Schematic Sagittal View of Human and Rat Brains Comparing the Hippocampus Location.

The hippocampus in both rats and humans can be described as extending along both a dorsoventral axis and rostrocaudal axis. The human anterior hippocampus is comparable to the rat ventral hippocampus and the human posterior hippocampus is comparable to that of the rat dorsal hippocampus. The dorsal hippocampus plays a role in spatial learning, memory, and navigation, while the ventral hippocampus has been implicated with reward processing, anxiety, and motivation. This figure was modified from Bizon and Gallagher, 2005 and O’Leary and Cryan, 2014.

1.2.4 Axonal Injury

The forces implicated in mTBI (1.2.2 Types of Forces in Mild Traumatic Brain Injury) generate intracranial pressure gradients, which lead to shearing and strain on the neurons, glial cells, and blood vessels in the brain (Blennow et al., 2016). Furthermore, axons span great distances within the brain and thus become more susceptible to this stretching, which leads to a condition called diffuse axonal injury (DAI), and the severity
of the injury is known to be related to the force of impact (King, 2000; Ling et al., 2015; McKee et al., 2009).

Despite the many unknowns regarding DAI, advances have been made with respect to identifying axonal damage using clinical imaging tools such as diffusion tensor imaging (DTI), since conventionally used CT and MRI imaging often appears normal. DTI is useful for detecting white matter damage, which is evident in TBI patients following acceleration/deceleration forces acting on the brain (Blennow et al., 2016; Smith et al., 2013). At the time of injury, the microtubules running down the length of the axon become disrupted and damaged, causing the axons to swell. Following this axonal swelling, the axons become disconnected and form axonal swellings or blebbings, which in turn disrupt transport (Barkhoudarian et al., 2016). A consistent finding among DAI research is the accumulation of amyloid precursor protein (APP) at the sites of axonal injury as a result of the impaired axonal transport (Blumbergs et al., 1994). Furthermore, with these advancements, researchers have been able to correlate DAI with cognitive disorders (Bazarian et al., 2007; Sugiyama et al., 2007)

1.2.5 Primary Pathophysiological Changes Post mTBI

Following the primary insult to the brain via acceleration and deceleration forces acting on the cellular components, a series of neurochemical and neurometabolic events can occur. As previously mentioned, these forces lead to axonal stretching and disruption of the cell membranes via traumatically induced mechanical poration (Farkas et al., 2006) (Figure 2A). This disruption causes an unregulated amount of ion flux, specifically potassium (K⁺) efflux and sodium (Na⁺) influx at the cellular level. The ionic flux and subsequent depolarization leads to an abundant release of neurotransmitters, particularly
the excitatory amino acid glutamate. Glutamate then binds to N-methyl-D-aspartate (NMDA), D-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), and kainate receptors on the post-synaptic neuron causing additional K⁺ efflux and further regional depolarization (Faden et al., 1992). Consequently, calcium (Ca²⁺) enters the cell via the NMDA receptors and acts as a second messenger triggering numerous pathways discussed in detail later on. In order to restore this ionic imbalance, there is an increase in activity of the Na⁺/K⁺ adenosine-triphosphate (ATP) dependent pumps that results in a depletion of the energy stores creating a metabolic crisis (Figure 2A). This cascade of events following the initial insult is believed to be the cause of the acute post-injury deficits (Barkhoudarian et al., 2016; Giza and Hovda, 2014). In order to replenish the energy stores, intracellular glucose is used up to generate more ATP causing hyperglycolysis. Experimental studies with rats have shown an increase in glucose metabolism as early as 5 min post-TBI and lasting up to 4 hrs (Yoshino et al., 1991), and this is followed by a period of hypometabolism of variable duration dependent upon injury severity (Peskind et al., 2011). Following this trauma-induced hyperglycolysis, there is an accumulation of lactate, resulting in acidosis, increased membrane permeability and cerebral edema (Kalimo et al., 1981). In addition to these energy perturbations, there is a large influx of Ca²⁺ via NMDA receptors which accumulates in the mitochondria resulting in impaired oxidative metabolism (Xiong et al, 1997). This mitochondrial dysfunction leads to decreased production of ATP, thereby worsening the energy predicament (Vagnozzi et al., 2007; Xiong et al., 1997). Furthermore, increased Ca²⁺ following an insult may lead to calpain activation and eventually cell death (Raghupathi, 2004).
1.2.6 Secondary Neuroinflammatory Response

The previously mentioned neurometabolic cascade, initiated by the mechanical forces of the injury, precedes the delayed secondary biochemical events which ultimately leads to neuronal dysfunction and sometimes even cell death (Patterson and Holahan, 2012). In response to injury, the central nervous system (CNS) recruits neutrophils and monocytes, which secrete cytokines and other signaling molecules to assist with tissue recovery (Wang and Feurstein, 2000). In addition to these recruited cells, the brain itself contains cells that are capable of initiating an inflammatory response as well (Figure 2B). Microglia and astrocytes, two types of glial cell residents in the brain become activated in response to injury and locally secrete inflammatory cytokines (see Section 1.3 Role of Glial Cells in mTBI) (Csuka et al., 2000; Singh et al., 2011). The specific cytokines and growth factors implicated in this secondary response include interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), nerve growth factor (NGF), IL-6, IL-10 and transforming growth factor-β (TGF-β). IL-1 is a family of 11 cytokines involved in the regulation of inflammatory and immune responses. In healthy brain tissue, IL-1β is capable of triggering the release of NGF from astrocytes, which promotes neuron growth and survival (Gadient at al., 1990). In both human and animal studies, IL-1, specifically IL-1α and IL-1β, has been shown to increase in response to both mild and severe TBI cases (Fan et al., 1995; Patterson and Holahan, 2012). An increase in IL-1β, predominantly released from microglia and astrocytes, has been linked to glutamate excitotoxicity and generation of free radicals, both of which can be detrimental to the cell (Clausen et al., 2011; Lucas et al., 2006). Animal studies have even shown that inhibition of this inflammatory cytokine following concussion reduces cerebral edema as well as improves
cognitive outcome (Clausen et al., 2011). Thus, IL-1β provides neuroprotection with NGF release, but is also associated with neurotoxic effects following mTBI (Clausen et al., 2011).

In addition to initiating the release of NGF, IL-1β is also capable of stimulating the release of another inflammatory cytokine, TNF-α. Similar to IL-1β in response to brain injury, there is a rapid release of TNF-α from microglia. This cytokine binds one of two receptors; TNF receptor (TNFR) 1 and TNFR2. Furthermore, it also appears to have both neuroprotective as well as neurotoxic effects which are dependent on the differential binding of TNF-α to these receptors; TNFR1 associated with pathological effects and TNFR2 to be neuroprotective (Perry et al., 2001; Ziebell and Morganti-Kossmann, 2010).

Both TNF-α and IL-1β have also been known to have overlapping synergistic effects on the brain (Chao et al., 1995; Shojo et al., 2010). Thus, it is believed that the presence of these pro-inflammatory cytokines mediates the post-traumatic inflammation and ultimately the secondary damage following mTBI (Allan and Rothwell, 2001).

In addition to the aforementioned pro-inflammatory cytokines, IL-6, which is stimulated by TNF-α, can act as both a pro- and anti-inflammatory cytokine (Lenzlinger et al., 2001). It is not yet known how IL-6 plays a role in the secondary neurodegeneration following concussion (Patterson and Holahan, 2012). The role of the anti-inflammatory cytokine IL-10 post-mTBI in the literature is controversial. Some studies show that it decreases reactive oxygen species (ROS) and the previously mentioned pro-inflammatory cytokines (Csuka et al., 1999), while others fail to report any beneficial effects of IL-10 following brain injury (Lyng et al., 2005). Finally, the function of TGF-β, an anti-inflammatory cytokine, post-mTBI is also poorly understood.
TGF-β is induced by the presence of inflammatory cytokines (IL-1, IL-6 and TNF-α) and through a negative feedback loop, it in turn suppresses their production (Benveniste et al., 1995). Both human and animal studies of mTBI show that TGF-β expression peaks 24 hrs post-injury (Csuka et al., 1999; Morganti-Kossmann, 2001). Overall, there is a complex relationship between pro- and anti-inflammatory cytokines following mTBI, which leads to an inflammatory response that has the potential to be both beneficial as well as detrimental to the CNS.

**Figure 2. Primary and Secondary Injury Following mTBI.**

(A) Immediately following mTBI, the brain experiences cellular changes, disrupting cellular homeostasis and leading to a cascade of neurochemical and neurometabolic events. (B) The delayed secondary response following the initial insult that can have both neuroprotective as well as neurotoxic effects on the brain. Figure created in collaboration Katie Neale. ATP = adenosine triphosphate, NMDA = N-methyl-D-aspartate, IL-1 = interleukin-1, IL-6 = interleukin-6, TNF-α = tumor necrosis factor- α, p-tau = phosphorylated tau.
1.3. Role of Glial Cells in mTBI

1.3.1 Microglia

Microglia are the smallest of the glial cell population and are considered to be the resident innate immune cells of the CNS, phagocytizing debris and secreting cytokines (Witcher et al., 2016). It was long believed that they developed from bone-marrow derived monocytes, but more recent studies have shown that microglia originate from myeloid progenitor cells in the yolk sac (Alliot and Pessac, 1999; Kierdof et al., 2013; Prinz and Priller, 2011). They represent 10-12% of all cells in the CNS and using their long ramified processes, they are able to constantly scan their surrounding microenvironment in what is deemed to be their “resting state” (Nimmerjahn et al., 2009). In response to signs of CNS injury, such as an increase in extracellular Ca$^{2+}$ or release of ATP from neighbouring cells, microglial cells become activated, proliferate, and undergo both a functional and morphological shift. This pathological state is termed microgliosis. These cells transform from a surveying ramified state to a phagocytic amoeboid-like state upon activation (Davalos et al., 2005; Kreutzberg, 1999; Sieger et al., 2012).

Calcium is a very important cation that acts as a signal mediator in the CNS through its binding to specific proteins such as ionized calcium-binding adapter molecule 1 (Iba-1). Iba-1 protein as its name depicts, is an adapter molecule that functions to mediate Ca$^{2+}$ signals in microglia. Confirmed both in vivo and in vitro, this 17 kDa protein was not present in neurons, astrocytes or oligodendrocytes (Ito et al., 1998). The detection of activated microglia (microgliosis) experimentally has been shown with a facial nerve axotomy experiment (Streit and Kreutzberg, 1988) and using this
experiment, others have been able to examine changes in Iba-1 expression in microglia (Ito et al., 1998). Through both immunohistochemistry (IHC) and Western blot analysis, Iba-1 expression was upregulated from CNS injury with peak levels at post-injury day 7 (PID7) which gradually declined after 28 days (Ito et al., 1998). Additionally, microglial activation in the retina, visible as an increase in Iba-1^+ cells, was shown with an optic nerve crush experiment (Davis et al., 2017). These findings suggest that Iba-1 is an applicable marker for the identification of microglia in the brain.

1.3.2 Astrocytes

The cortex (CX) is a complex structure in the brain that has distinct layers with neurons arranged throughout, all communicating with one another. This neuronal network aids communication within and between these layers and also extends into subcortical areas. (Rakic and Lombroso, 1998) A neuronal synapse is the junction between two neurons, and it was discovered that astrocyte processes make contact here as well (Gray, 1959). Astrocytes, named after their stellate shape, are the most numerous glial cells in the CNS representing 20-40% of the total cells (Verkhratsky and Butt, 2013). They are known to have multiple roles in the brain, from supportive functions, to maintaining homeostasis, to roles in plasticity. For instance, perivascular astrocytes contain endfeet that wrap around the vasculature and are a major component of the blood-brain barrier (BBB), regulating the passage between the blood and the brain (Kettenmann and Ransom, 2005). This neurovascular unit can also modulate synaptic transmission and plasticity by controlling glutamate levels in the extracellular space between pre- and post-synaptic neurons and adjust its concentration via glutamate reuptake (Oliet et al., 2001).

It is known that glutamate reuptake by astrocytes is coupled with glucose reuptake, since
Aerobic glycolysis in astrocytes is inhibited when the glutamate transporter is blocked (Zimmer et al., 2017). In addition, astrocytes supply energy to neurons in the form of lactate (converted from glycogen) and interestingly enough, this has been shown to be necessary for long-term potentiation and memory formation (Suzuki et al., 2011). But as previously mentioned, astrocytes play a role in the secondary neuroinflammatory cascade and similar to microglia in response to CNS injury; they become hypertrophied, proliferate, and upregulate the expression of intermediate filament (IF) proteins and cytokines. This increase in astrocyte numbers is often referred to as astrogliosis (Sofroniew and Vinters 2010). IF proteins such as glial fibrillary acidic protein (GFAP) and vimentin are the integral cytoskeletal proteins in astrocytes (Cekanaviciute and Buckwalter, 2016). In the CNS, GFAP is expressed in astrocytes and ependymal cells (Eng et al., 2000; Roessmann et al., 1980). Outside of the CNS, GFAP has been found in the kidneys, testis, pancreas, and liver (Apte and Haber, 1998; Buniatian et al., 1998; Davidoff et al., 2002). Being the first molecular biomarker for the identification of astrocytes, GFAP is commonly used in mTBI evaluation for investigating gliosis (Eng et al., 2000) (Table 2). Increased levels of astrocytic intermediate filaments appear to coincide with injury severity. If the injury is severe enough, the activated astrocytes can aggregate near the tissue damage and form scar borders to quarantine the damaged cells from healthy tissue to prevent further injury. However, with more mild diffuse injuries, these reactive astrocytes do not usually form glial scars, and not long after they return to their diverse functions prior to the injury (Wanner et al., 2013). Although not specific to astrocytes, S100 calcium-binding protein B (S100B) is another notable marker used for their identification (Olsson et al., 2011). This astrocytic Ca$^{2+}$-binding protein has been
reported to increase alongside GFAP in the serum of patients with TBI (Kovesdi et al., 2010; Mondello et al., 2011). However, S100B is also expressed in oligodendrocytes and extracerebral cells such as chondrocytes and adipocytes, raising skepticism among researchers (Olsson et al., 2011).

### 1.3.3 Synergism of Microglia and Astrocytes

Microglia and astrocytes begin to communicate with one another not long after they populate the brain parenchyma. This crosstalk is important for the function and development of the brain and for diseases affecting the CNS (Jha et al., 2018). During and throughout the activation of these two glial cells, bidirectional communication between them is present (Chen et al., 2015; Clarke et al., 2018). This intercellular conversation occurs through the release of ATP, cytokines, chemokines and growth factors (Jha et al., 2018). For example, following TBI, activated microglia release inflammatory molecules and this response is modulated by astrocytes, which decrease microglial levels of ROS (Min et al., 2006). Trauma can also trigger a calcium-induced release of ATP from astrocytes, which can then signal and recruit other astrocytes and microglia in the surrounding area (Burda et al., 2016). This synergism exerts a neuroprotective effect in the brain. It is when these cells become over-activated that the pathogenesis of neuroinflammation and neurodegeneration occurs (Min et al., 2006).

### 1.4 Experimental TBI

#### 1.4.1 Mimicking TBI with Animal Models

In order to examine the pathophysiology and biomechanics underlying TBI, animal models replicating this injury are necessary. Many factors must be considered
when developing this type of model and it must control for injury type (focal or diffuse), severity, reproducibility, sex, age and genetics (Margulies and Hicks, 2009). Rodents are most commonly used in TBI research simply due to their affordability, size and consistent outcomes post-injury. The most well known and characterized models of TBI include the weight drop (WD) model (Feeny et al., 1981; Marmarou et al., 1994), the fluid percussion injury (FPI) model (Dixon et al., 1987; Gennarelli, 1994), the controlled cortical impact (CCI) model (Dixon et al., 1991; Lighthall, 1988; Lindner et al., 1998) and the blast injury model (Cernak et al., 1996; Leung et al., 2008). All of the above models may also be modified to inflict a mTBI and will be discussed further (Albert-Weissenberger and Siren, 2010). Another mentionable model is the cryogenic injury model, where a cold rod is applied to the exposed dura to produce a focal injury. This model is primarily used to examine BBB changes after TBI, since it lacks the pathophysiological characteristic of TBI (axonal injury) (Pappius 1981).

1.4.2 Weight Drop Models

With WD models of TBI, a guided, free falling weight impacts the exposed skull. By increasing the mass of the weight and its height, the severity of the injury increases. In Feeney’s focal WD model, a craniotomy exposes the intact dura upon which the weight directly falls on (Feeney et al., 1981) (Figure 3A). Marmarou and colleagues on the other hand, developed an impact acceleration model to mimic diffuse TBI commonly seen in falls or car accidents (Marmarou et al., 1994) (Figure 3B). With this model, the skull is exposed and a steel disc is glued to the skull to prevent skull fracture. Both models involve a craniotomy, however Marmarou’s WD model is considered a closed-head injury since the skull remains intact, which more closely resembles the clinical condition.
Every model has its caveats and with the WD models, there is high variability in the severity of the injury sustained, as well as high mortality rates (Xiong et al., 2013).

### 1.4.3 Fluid Percussion Injury Model

The FPI model induces the injury using a pendulum, which hits a piston containing liquid, and sends a fluid pressure pulse to the exposed dura (Figure 3C). By varying the pressure pulse strength, the injury severity can vary. This injury model causes a direct deformation of the brain (Dixon et al., 1987; Gennarelli, 1994). The most commonly used variation of this model, the lateral FPI (LFPI) model, produces both a diffuse and focal injury (Thompson et al., 2005). This injury model is popular for examining neuronal cell death mechanisms, but some disadvantages include high mortality and the use of surgery (Xiong et al., 2013).

### 1.4.4 Controlled Cortical Impact Model

The CCI TBI model uses an electromagnetic impact device to drive an impactor onto the exposed dura, creating a deformation of the brain (Figure 3D). Therefore, a craniotomy is done and the resulting damage caused is usually widespread from the cortex, to the hippocampus, and to the thalamus (Hall et al., 2005). This model is deemed more useful than FPI models for biomechanical studies of TBI since the time, velocity and depth of impact can be easily controlled with CCI models (Wang and Ma, 2010).

### 1.4.5 Blast Injury Model

Finally, there is the blast injury model which was designed to represent a TBI experienced by military personnel, where they do not have any direct impact external injuries, yet have been diagnosed with TBI (Wang et al., 2011) (Figure 3E). To induce
the primary blast waves on the brain, studies have used a pressure-driven shock tube with some subjects wearing a Kevlar vest to reduce mortality and cause more widespread axonal damage (Cheng et al., 2010; DeWitt and Prough, 2009). The injury severity depends on the subject’s location within the shock tube. Some limitations include differences between the blasts wave from the shock tube and that experienced on the battlefield, reproducibility issues and inconsistent results between studies (Xiong et al., 2013).

**Figure 3. Experimental Animal Models of TBI**

(A) A guided free weight is released directly on to exposed dura in Feeney’s weight drop model. (B) In Marmarou’s weight drop model, the subject has a metal disk attached to its skull to prevent skull fracture and disperse the damage. (C) The fluid percussion injury model utilizes fluid pressure to directly impact the brain. (D) An electromagnetically driven piston is used in the controlled cortical impact model to accurately control for the biomechanics of the injury. (E) Blast injury models use a compressed air-driven shock tube to indirectly illicit a TBI. This figure was modified from Xiong et al., 2013.
1.4.6 Animal Model Limitations

The WD (Deford et al., 2002), FPI (Creeley et al., 2004), blast (Wang et al., 2011) and CCI (Meconi et al., 2018) models of TBI have all been modified in attempts to mimic the clinical conditions of mTBI. However, there are some caveats to these models with regards to simulating a true closed-head injury. Most models of mTBI involve anaesthesia, accompanied with a scalp incision and/or craniotomy, yet neither of these mimic true closed-head concussive injuries in humans. Furthermore, anaesthesia has been known to be neuroprotective (Flower and Hellings, 2012; Statler et al., 2006a; Statler et al., 2006b) and surgical procedures can trigger an inflammatory response which may confound the data (Cole et al., 2011). In addition, the number of concussions sustained and the inter-injury interval can be varied dramatically, which may result in variable neurological impairments (Table 1).
Table 2. Rodent Models of mTBI Investigating Gliosis

<table>
<thead>
<tr>
<th>Injury Model</th>
<th>Anaesthesia</th>
<th>Species/Sex/Age</th>
<th># of mTBI</th>
<th>Post-Injuy Time Point</th>
<th>Analysis</th>
<th>Region of Interest</th>
<th>GFAP</th>
<th>Iba-1</th>
<th>Authors</th>
</tr>
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<tbody>
<tr>
<td>mCCI</td>
<td>yes</td>
<td>Mice/♂/7 wks old</td>
<td>5 @ 24 hrs apart</td>
<td>2 hrs, 1, 7, and 42 days</td>
<td>IHC</td>
<td>CC</td>
<td>↑ PID 7 only</td>
<td>n/a</td>
<td>Fengshan et al., 2017</td>
</tr>
<tr>
<td>mCCI</td>
<td>yes</td>
<td>Mice/♀/8-10 wks, 11-12 mo old</td>
<td>5 @ 48 hrs apart</td>
<td>15 days</td>
<td>IHC</td>
<td>CC</td>
<td>↑ PID 15</td>
<td>↑ PID 15</td>
<td>Ferguson et al., 2017</td>
</tr>
<tr>
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<td>yes</td>
<td>Rats/♂/Juvenile</td>
<td>2 @ 3 days apart</td>
<td>14 days</td>
<td>IHC</td>
<td>CX</td>
<td>↑ PID 14</td>
<td>↑ PID 14</td>
<td>Huang et al., 2016</td>
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<tr>
<td>mWD</td>
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<td>Rat/♂/7 wks old</td>
<td>1</td>
<td>1 day</td>
<td>IHC</td>
<td>CX</td>
<td>↑ PID 1</td>
<td>n/a</td>
<td>Kim &amp; Han 2017</td>
</tr>
<tr>
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<td>Mice/♂/3 mo old</td>
<td>3 @ 24 hrs apart</td>
<td>180 days</td>
<td>IHC</td>
<td>CX, CA3</td>
<td>↑ PID 180</td>
<td>n/a</td>
<td>Luo et al., 2014</td>
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<td>yes</td>
<td>Mice/♀/8-12 wks old</td>
<td>1</td>
<td>3 days and 35 days</td>
<td>WB</td>
<td>HP</td>
<td>↑ PID 1</td>
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<td>Mice/♀/12 wks old</td>
<td>2/week for 3 or 4 mo</td>
<td>6 months</td>
<td>IHC</td>
<td>CC</td>
<td>↑ PID 1</td>
<td>↑ PID 1</td>
<td>Ojo et al., 2016</td>
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<td>1, 10 days</td>
<td>IHC</td>
<td>DG, CA</td>
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<td>30 @ 3/day</td>
<td>15 days</td>
<td>IHC</td>
<td>HP</td>
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<td>1, 7 days</td>
<td>IHC</td>
<td>HP</td>
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<td>1</td>
<td>60 days</td>
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<td>PFC</td>
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<td>1</td>
<td>1 and 35 days</td>
<td>WB</td>
<td>Not specified</td>
<td>↑ PID 1</td>
<td>n/a</td>
<td>Schultz et al., 2015</td>
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<td>Rats/♀/Adult</td>
<td>1</td>
<td>4 hr, 1, 3, 5 days</td>
<td>IHC</td>
<td>CX, HP, CC</td>
<td>↑ PID 3,5, no change</td>
<td>n/a</td>
<td>Singh et al., 2017</td>
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<td>2 @ 15 min apart</td>
<td>1, 3 and 14 days</td>
<td>IHC</td>
<td>PC</td>
<td>↑ PID 3,14</td>
<td>↑ PID 3</td>
<td>Tagge et al., 2018</td>
</tr>
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<tr>
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<td>Mice♂/Adult</td>
<td>1</td>
<td>1, 8, 14, and 28 days</td>
<td>IHC</td>
<td>CC</td>
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<td>↑ PID 1,8,14</td>
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<td>CC</td>
<td>↑ PID 90</td>
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<td>Zhang et al., 2015</td>
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</table>

mWD = modified weight drop  
mCCI = modified controlled cortical impact  
PID = post-injury day  
CC = corpus callosum  
PC = perirhinal cortex  
DG = dentate gyrus  
CA = cornu ammonis  
CA1 =  
CA3 =  
RT-PCR = real-time polymerase chain reaction  
PnC = caudal pontine reticular nucleus  
PFC = prefrontal cortex  
HP = hippocampus  
CX = cortex

### 1.5 Repeated Concussions

There has been an increase in evidence showing that repeated concussions lead to cumulative and long-term neurological effects, and the aforementioned models of mTBI allow researchers to examine these effects (Daneshvar et al., 2011a; Daneshvar et al., 2011b). A single mTBI or concussion may be considered minor, however, the magnitude and duration of its symptoms can become amplified with repeated mTBI (rmTBI) (Guskiewicz et al., 2003). Patients with repetitive concussions have slower motor deficit recovery, increased learning disabilities and memory impairments, slower processing speeds and increased headaches (Slobounov et al., 2007; Gronwall and Wrightson, 1975; Collins et al., 1999; Gaetz et al., 2000). These symptoms seem to be inversely related to age, so the younger they are, the worse off they are (Collins et al.,
2006; Prins et al., 2010). The likelihood of sustaining a rmTBI may not be that common in the general population, but it is certainly an issue with athletes participating in contact sports (Vagnozzi et al., 2007).

Following injury, the brain is experiencing a cellular disturbance and metabolic crisis, and during this time it is very vulnerable. If a second mTBI occurs in this window, the result is thought to be a more severe brain injury and has been described as second impact syndrome (Barkhoudarian et al., 2016; Cantu, 1998). These disturbances, such as increased metabolism, can lead to the further production of free radicals and neuronal damage (Baker and Patel, 2000).

This phenomenon is heavily debated in the literature and controversy arises surrounding a couple of main issues. Firstly, whether the cerebral edema is actually caused by the second hit and not just a progression of the initial injury. Secondly, how far apart the concussions may be (McLendon et al., 2016). Vagnozzi and colleagues investigated this temporal window of vulnerability with a rat WD model and found that the effects were most significant when they spaced the mTBIs 3 days apart (2007). Similar results were found with a mouse model of CCI, with the vulnerable window lasting 3 to 5 days (Longhi et al., 2005). Further research on this must be conducted, but a longer window between the first and second impact might decrease risk.

More recently, there have been studies on the development of chronic traumatic encephalopathy (CTE) as a consequence of repetitive concussions (Goldstein et al., 2012; MacGregor et al., 2011; McKee et al., 2013; Petraglia et al., 2014; Tagge et al., 2017). Clinically, this disease presents itself as a condition of behavioural, mental and cognitive
impairments, usually in the absence of sensorimotor deficits (Stern et al., 2013). This late onset progressive neurodegenerative disease has only been clinically diagnosed post-mortem and further research must be done to answer some unknowns, such as how many injuries are required to develop this disease, how can it be diagnosed prior to death, and what is the current incidence of CTE.

1.6 The Understudied, At-Risk Populations

Mild TBI, specifically rmTBI is very common among young individuals, and within this population it is the major cause of hospitalizations and emergency room visits (Langlois et al., 2005). This group is particularly susceptible considering the fact that their brains are still developing and exacerbated damage can arise from repeated injuries (Shrey et al., 2011). Multiple concussions in young adulthood have raised concern of potential early onset of cognitive and behavioural deficits (Guskiewicz et al., 2005).

With more and more females participating in contact sports, a greater understanding of the gender-specific outcomes following rmTBI is necessary (Broshek et al., 2005). A study looking at men and women participating in soccer, lacrosse, basketball, softball and baseball showed that female athletes sustain more concussions per game than male athletes (Covassin et al., 2003). Overall, in clinical studies, females are likely to sustain more injuries and they can take longer to recover than males (Broshek et al., 2005; Covassin et al., 2012; Farace and Alves, 2000). Meanwhile in controlled experimental animal models of mTBI, females had improved survival and cognitive function in comparison to males, which supports the neuroprotective effects of estrogen. (Bramlett and Dietrich, 2001; Wagner et al., 2004). Resolving the discrepancy between studies will aid with future clinical trials in humans.
In summary, most studies focus on single injuries with adult males to avoid any confounds with respect to hormone differences. However, the juvenile female population, which is more at risk, deserves just as much attention.

1.7 Summary and Objectives

Mild TBI and its associated neurological sequelae have developed to become this silent epidemic worldwide (Dashnaw et al., 2012; Bailes et al., 2013). Additionally, those with prior mTBI(s) and the juvenile population are at greater risk for worsened outcome following rmTBI.

rmTBI has been associated with cognitive and behavioural deficits and to understand and address its consequences, a model of mild closed-head injury closely mimicking human concussion is essential. In this study, we will use our model of awake closed-head injury to simulate this clinical condition. We first want to determine if this model produces acute neurological deficits. Secondly, we will look for signs of overt morphological tissue damage. Thirdly, we will investigate behavioural changes following rmTBI. Lastly, we will investigate the effects of this injury on gliosis at an acute time point.
2. Materials and Methods

2.2 Animals

All animal procedures carried out were approved by the University of Victoria Animal Care Committee and the Canadian Council for Animal Care. Long Evans rats were purchased from (Charles River Laboratories, St. Constant, PQ) and bred at the University of Victoria. At postnatal day (PND) 21, the offspring were weaned and housed in same-sex groups of two to three. Only female animals were used in these studies, and animals were randomly assigned to one of five experimental groups (control, sham, rmTBI4, rmTBI8, rmTBI16) at weaning. At PND 25-28, the female rats received their first mTBI or sham procedure. Control animals were left in their cages and not exposed to any procedures, while sham animals followed the same procedure as the rmTBI4 without actually receiving the impacts. All animals were housed under standard laboratory conditions with a 12 hr light/dark cycle, *ad libitum* access to food and water, and room temperature was maintained at 22.5°C ± 2.5°C. Animals were examined by animal care staff daily for signs of injury or illness.

2.3 Awake Closed-Head Injury

In this study we used an awake closed-head injury (ACHI) model that was adapted from a model developed for adult mice (Petraglia et al., 2014). Animals were handled for two days prior to any experimental manipulations. Prior to an ACHI procedure, animals were gently guided into a clear plastic restraint cone (Model DC-200, Braintree Scientific, Braintree, MA) that immobilized them for the procedure. There is an opening at the anterior end to allow for proper ventilation, and posterior end was sealed with a plastic hair clip.
In order to dissipate the force of the impact, prevent skull fracture and ensure accurate delivery of the injury, a 3D printed (Replicator-2, MakerBot, Brooklyn, NY) helmet was used. The helmet was secured with double-sided tape and an elastic band, with the back of it positioned at the interaural line (Figure 4B). The top of the helmet is situated directly over the left parietal cortex, and has a 7 mm diameter circular surface that acts as the impact site. To allow for the acceleration-deceleration component of the injury, the restrained rats were placed on a piece of three-inch thick foam (Super-Cushioning Polyurethane Foam Sheet, McMaster-Carr, OH) that was situated right beneath the impactor.

A modified controlled cortical impact (CCI) device (Impact One, Leica Biosystems Inc., ON, Canada) was mounted on a stereotaxic frame to allow it to be used to induce the rapid movement of the head (Figure 4A). A 7 mm flat rubber tip was added to the impactor, and placed directly on the target of the helmet (Figure 4C). An electromagnetic piston was set to drive the impactor tip 10 mm beyond the contact point of the helmet at a velocity of 6 m/s (Figure 4A). To prevent rebound impact with the impactor itself, the impactor tip was set to quickly retract with a dwell time of 100 ms. Immediately after each impact, the rats were quickly removed from the restraint cones and subjected to a rapid neurological assessment protocol (NAP). Rats in the rmTBI4 group received four impacts in one day, with each impact separated by 2 hrs (Figure 6A). Those in the rmTBI8 group followed a similar pattern with four impacts in one day, each separated by 2 hrs over the course of 2 days. The impacts on the second day occurred at the same time as the hits administered on the initial day. Similarly, the rmTBI16 group was administered four impacts each day, each separated by 2 hrs, over 4 days. Lastly, the
sham group followed the same schedule as the rmTBI4 group, however, the impact tip
did not make contact with the helmet and was triggered directly beside the animal’s head
(Figure 6B).

Figure 4. Awake Closed Head Injury Model.
Images of the apparatus and subjects used in the ACHI model. (A) An electromagnetically
controlled piston (i) used to illicit the repeated injury. Using the control box, the velocity of the
impact is set to 6 m/s with a dwell time of 100 ms. (B) The unanaesthetized juvenile female rat
restrained in the plastic cone with a 3D printed helmet (ii) placed directly in front of the interaural
line and secured to the head. (C) Close-up image of the restrained subject placed on a foam
platform and directly under impactor tip.

2.4 Neurological Assessment Protocol (NAP)

Prior to the initial impact and immediately after each injury, a Neurological
Assessment Protocol (NAP) was performed. The NAP is comprised of two categories of
assessment: (1) level of consciousness and (2) a series of sensorimotor assessments
(Table 2).
Once the subject is removed from the restraint cone, their breathing is assessed. If the rat is not breathing, the duration of apnea is recorded. If there are no signs of apnea, the toe pinch reflex is assessed next. Here, the hind limb, which is contralateral to the impact site, is firmly pinched and its retraction is noted. If there is no retraction of the hind limb, the pinching procedure is repeated every 5 secs until a reflex is observed and this latency is recorded. Next, the righting reflex in assessed. The rat is placed on its back and it should immediately flip right-side up. If there is a delay to upright, this latency is recorded.

Table 3. Neurological Assessment Protocol Outline

<table>
<thead>
<tr>
<th>Assessment</th>
<th>Test</th>
<th>Description</th>
<th>Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level of Consciousness</td>
<td>1. Apnea</td>
<td>Suspension of breathing</td>
<td>Pass = 1 Fail = 0*</td>
</tr>
<tr>
<td></td>
<td>2. Toe pinch</td>
<td>Retraction of the hind limb in response to pain</td>
<td>Pass = 1 Fail = 0*</td>
</tr>
<tr>
<td></td>
<td>3. Righting  reflex</td>
<td>Reflexive righting of the body</td>
<td>Pass = 1 Fail = 0*</td>
</tr>
<tr>
<td>Sensorimotor</td>
<td>4. Startle</td>
<td>Reflexive response to a hand clap</td>
<td>Pass = 1 Fail = 0</td>
</tr>
<tr>
<td></td>
<td>5. Limb extension</td>
<td>Full extension of fore limbs</td>
<td>Pass = 1 Fail = 0</td>
</tr>
<tr>
<td></td>
<td>6. Beam walk</td>
<td>Walk across a 100cm long beam with zero foot slips</td>
<td>Pass = 1 Fail = 0</td>
</tr>
<tr>
<td></td>
<td>7. Rotating beam</td>
<td>Hold onto a beam while its rotated at 1 rotation per second</td>
<td>Pass = 1 Fail = 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>/7 Total Score</td>
</tr>
</tbody>
</table>

*If the subject fails the Apnea, Toe Pinch or Righting Reflex test, the time to recover is recorded.
Once the subject completes the level of consciousness assessment, they are then subjected to the series of sensorimotor tests. These four tests are scored in a pass (1) or fail (0) fashion (Table 3). The first test is the startle test, where the rat is placed in a clean, empty, standard housing cage and subjected to a loud hand-clap above the centre of the cage. If the rat startles in response to this acoustic stimulus, they receive a passing score, if there is no response, they receive a zero. Next is the limb extension test (Figure 5A), where the subject is suspended the base of its tail, approximately 50 cm in the air. If both forelimbs are fully extended, this constitutes a pass. If one or both of the forelimbs are contracted, this constitutes a failing score. Then there is the balance beam test (Figure 5B), where a 100 cm long x 2 cm wide x 0.75 cm thick beam is elevated 22 cm with an empty cage at one end, the subject’s home cage at the other, and a padded surface below. The rat is placed on the centre of the beam, facing its home cage. If it can successfully navigate across the beam using all four limbs, it receives a passing grade. The rat fails the balance beam test if it is immobile, unable to grasp the beam, or falls off of it. Lastly, the rat is again placed on the centre of the beam in the same scenario previously described, however, this time the beam is elevated approximately 75 cm above the padded surface and rotated once per second for a total of 4 rotations (Figure 5C). If the rat can remain on the beam the entire time, this is a pass, and if not, a fail.

If present, the duration and latency for the level of consciousness assessments were recorded for each animal. The sensorimotor tests, if all successfully completed, could result in a maximal score of 4 and ultimately be recorded as the animal’s NAP score. After each impact, the animal received a NAP score out of 7, and the NAP scores for that particular animal were averaged. For instance, an animal from the rmTBI4 group
had 4 NAP scores averaged, 8 for the rmTBI8 group and 16 for the rmTBI16 group. Between animals, the equipment used for the NAP was cleaned with 70% ethanol.

Figure 5. Neurological Assessment Protocol.
Representative images of the sensorimotor tests in the NAP (Startle test not imaged): (A) Limb Extension, (B) Beam Walk and (C) Rotating Beam. Images on the left of each group represent a pass with a score of 1, images on the right represent a fail scoring 0.

2.5 Behavioural Assessment

2.5.1 Open Field Test

At post-injury day 1 (PID1), locomotion and anxiety-like behaviour was evaluated with the open field test. The subjects were positioned in the centre of a novel, circular arena, which was located in a brightly lit room, and given 5 min to freely explore the area. Using a tracking software (EthoVision XT 11.5, Noldus, Netherlands), the exploration of the rats was recorded for later analysis. If the animals spent more time in the perimeter of the arena (thigmotaxis) or less time in the centre of the arena, this is considered to be a measure of anxiety-like behaviour (Prut and Belzung, 2003; Jones et al., 2008). In addition to duration spent in the centre versus perimeter of the arena, the average velocity and total distance moved were recorded.
Figure 6. Injury Timeline.

(A) rmTBI animals sustained 4 injuries per day with a 2 hrs interval between injuries. The NAP was performed before the initial mTBI and immediately after each subsequent injury. (B) rmTBI groups received either 4, 8 or 16 injuries over 1, 2 and 4 days, respectively. The Sham animals followed the same procedure and timeline as the rmTBI4 group, however, they did not receive an injury.

2.6 Histology

2.6.1 Tissue Processing

A separate cohort of control, rmTBI4, rmTBI8 and rmTBI16 animals were sacrificed 24 hours after their final impact. The rats were deeply anaesthetized with inhaled isoflurane (Abbott Laboratories, North Chicago, IL) and perfused transcardially with ~75 ml heparinized phosphate-buffered saline (0.1 M PBS, pH 7.4) and then administered 2% paraformaldehyde (PFA) in 0.1 M PBS. The brains were removed and immersion fixed in 2% PFA overnight at 4°C and then transferred to PBS. Coronal brain sections were cut at a thickness of 50 μm using a vibratome (VT1000, Leica Biosystems)
Inc, ON, Canada). Every sixth section was mounted onto positive charged glass slides (Fisher Scientific, Ottawa, Ontario, Canada) and left to air-dry.

### 2.6.2 Cresyl Violet Stain and Imaging

The slides containing the dried and mounted slices were dipped in water, followed by a gradient of ethanol (EtOH) dehydrating steps (70% EtOH for 1 min, 95% EtOH for 5 min and 100% EtOH for 10 min). The slides were then immersed in Citrosolv for 20 min and then rehydrated with 100% EtOH for 5 min, 95% EtOH for 1 min and 70% EtOH for 1 min. The sections were then stained with 0.5% aqueous Cresyl Violet (Sigma, St. Louis, MO) for 10 min. After staining, the sections were exposed to 100% EtOH for 5 min, followed by Citrosolv for 10 min. Lastly, they were cover-slipped with Permount mounting medium (Fisher Scientific, PA, USA) and allowed to air-dry overnight. Nine images of each slice were taken on an Olympus conventional light microscope (Model BX51TF; Olympus Corporation, Center Valley, PA) using a 2x objective. The images were then combined using Adobe Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA).

### 2.7 Protein Analysis

#### 2.7.1 Tissue Processing

The cohort of control, sham, rmTBI4, rmTBI8 and rmTBI16 animals that underwent the open field test at PID1 were subsequently anaesthetized with inhaled isoflurane (Abbott Laboratories, North Chicago, IL) and decapitated immediately. The brains were carefully dissected from the skull and placed in ice-cold 0.1 M PBS briefly before being bisected on ice. As previously described by Hagihara et al., the DG was
separated from the CA and the two regions were flash-frozen in liquid nitrogen. A region of the CX above the hippocampus was also dissected and frozen in the same manner. Prior to the sonication steps, the specimens were kept at -80°C.

2.7.2 Preparation of Protein Lysates

The DG, CA and CX samples were weighed and for each sample, 10 mL of lysis buffer (Table 4) was added per gram of tissue. Each sample was then sonicated (Fisher Scientific, Pittsburgh, PA) on ice four times for 5 secs with 15 secs between each sonication. Using a microcentrifuge (Fisher Scientific) at 4°C, the lysates were centrifuged at 14,000 g for 15 min. The supernatant was collected and kept at -80°C until the protein assay could be completed.

2.7.3 Protein Quantification

In order to quantify the total protein concentration in each sample, a bicinchoninic acid (BCA) protein assay was carried out (BCA Protein Assay Kit, Pierce, Rockford, Illinois, USA). To detect the protein concentration in the samples, they were diluted 1:50 with 0.1 M PBS and added in triplicates to the sample wells of a 96 well microtitre plate. Using the kit, a working reagent was made by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B as well as a 2 mg/ml bovine serum albumin (BSA) reference standard stock. Dilutions of the BSA standard stock (0.03125 mg/ml – 2 mg/ml) were added to the standard wells of the same microtitre plate. Then 200 µL of the working reagent was added to all standard and sample wells. The microtitre plate was incubated for 30 min at 37°C and the absorbance was subsequently measured at 562 nm in the plate reader (VersaMAX, Molecular Devices, Sunnyvale, CA, USA). The results were
analyzed using Softmax Pro 5.2 (Molecular Devices) and the curve fit for the standard curve was a log-log fit as determined by the protein concentration (µg/ml).

**Table 4. Buffers and Solutions**

<table>
<thead>
<tr>
<th>Buffers and Solutions</th>
<th>Ingredients</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>20mM Tris pH8; 137mM NaCl; 0.1% (v/v) NP-40; 10% (v/v) glycerol; 2mM ethylenediaminetetraacetic acid (EDTA); 1X Halt™ phosphatase and protease inhibitor (100X)</td>
<td></td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS)</td>
<td>0.8% NaCl; 2.7mM KCl; 10mM Na₂HPO₄·H₂O; 1.8mM KH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>PBS-Tween</td>
<td>0.8% NaCl; 2.7mM KCl; 10mM Na₂HPO₄·H₂O; 1.8mM KH₂PO₄; 0.05% (v/v) Tween-20</td>
<td></td>
</tr>
<tr>
<td>Tris-buffered saline (TBS)</td>
<td>839mM Tris-HCl; 160mM Tris-Base; 1.54M NaCl</td>
<td></td>
</tr>
<tr>
<td>TBS-Tween (TBS-T) Wash Buffer</td>
<td>839mM Tris-HCl; 160mM Tris-Base; 1.54M NaCl; 0.05% (v/v) Tween-20</td>
<td></td>
</tr>
<tr>
<td>SDS-PAGE electrophoresis (running) buffer</td>
<td>1.92M Glycine; 0.25M Tris-base; 20% SDS; dH₂O</td>
<td></td>
</tr>
<tr>
<td>SDS-PAGE separating gel buffer (12%)</td>
<td>20% (w/v) SDS; 1.5M Tris (pH 8.8); 30% (w/v) acrylamide/bis-acrylamide; 10% (w/v) Ammonium persulfate solution (APS); 0.1% (v/v) tetramethylethylenediamine (TEMED); Milli Q H₂O</td>
<td></td>
</tr>
<tr>
<td>SDS-PAGE stacking gel buffer (5%)</td>
<td>20% (w/v) SDS; 1.0M Tris (pH 6.8); 30% (w/v) acrylamide/bis-acrylamide; 10% (w/v) Ammonium persulfate solution (APS); 0/1% (v/v) TEMED; dH₂O</td>
<td></td>
</tr>
<tr>
<td>Transfer Buffer</td>
<td>1.92M Glycine; 0.25M Tris-base; 10% (v/v) methanol; dH₂O</td>
<td></td>
</tr>
<tr>
<td>Reducing SDS-PAGE Sample buffer (5x)</td>
<td>0.2M Tris (pH 6.8); 5% (v/v) SDS; 1.5% (w/v) bromophenol blue; dH₂O; 37.5% (v/v) glycerol; 0.25M Dithiothreitol (DTT)</td>
<td></td>
</tr>
<tr>
<td>Coomassie Blue Stain</td>
<td>0.15% Coomassie Blue; 50% Methanol; 10% Acetic Acid</td>
<td></td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>5% Skim milk (Difco™), 0.05% Tween-20 in TBS</td>
<td></td>
</tr>
<tr>
<td>BSA antibody solution</td>
<td>5% BSA, 0.05% Tween-20 in TBS</td>
<td></td>
</tr>
<tr>
<td>Stripping Buffer</td>
<td>62.5mM Tris HCl pH 6.7, 2% (v/v) SDS and 1% (v/v) of β-mercaptoethanol</td>
<td></td>
</tr>
</tbody>
</table>
2.7.4 Western Blotting

A 5X Reducing Sample Buffer (Table 4) was added to the samples and the diluted samples were then heated for 5 min at 95°C. Following the heating step, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. A total of 15 µg of protein (as determined from the BCA Assay kit) from each sample was loaded into the wells of a 12% separating gel (Table 4), and at least one well contained 10 µL of a kaleidoscope molecular weight marker (Biorad). The gel was housed in an electrode assembly (Biorad), which was placed into an electrophoresis tank (Biorad) containing SDS-PAGE running buffer (Table 4). Using 130V at room temperature (23°C), the proteins were separated in the gel via SDS-PAGE and subsequently transferred overnight in Transfer buffer (Table 4) to a Polyvinylidene Fluoride (PVDF) membrane (Perkin Elmer, Boston, MA, USA) at 4°C using 40V. This transfer was confirmed by a Coomassie blue stained gel (Table 4).

The membranes were blocked for 1 hr at room temperature (23°C) using a skim milk blocking buffer (Table 4). Membrane blots were probed with polyclonal rabbit anti-GFAP (Abcam, Toronto, ON), polyclonal rabbit anti-Iba-1 (Wako, Richmond, VA) and monoclonal rabbit anti-glyceraldehyde 3-phoshate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA) all raised in rabbit (see Table 5 for concentrations and incubations times). The GAPDH signal was used as a loading control. Following incubation with the primary antibodies, the membranes were washed 3 x 5 min with TBS-Tween (TBS-T) (Table 4) and then incubated at room temperature with goat α-rabbit IgG (H+L) horseradish peroxidase (HRP)-conjugate (Millipore, Temecula, CA) for 1 hr. The membranes were then washed with TBS-T (3 x 5 min).
In order to image the bands, Clarity™ enhanced chemiluminescence (ECL) substrate (Bio-Rad, Hercules, CA) was applied to the membranes for 5 min and then imaged with a G:Box Chemi-XR5 and GENESys software (Syngene, Cambridge, UK). The immunolabeled protein bands were quantified using densitometry via the Java-based image analysis program, ImageJ (NIH). The band densities of the proteins of interest (GFAP and Iba-1) were measured and normalized to GAPDH.

Table 5. Table of Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Molecular Weight</th>
<th>Blocking Buffer</th>
<th>Dilution</th>
<th>Buffer</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1°</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyclonal Rabbit α-GFAP</td>
<td>~50kDa</td>
<td></td>
<td>1:10000</td>
<td>5% (w/v) BSA</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>Monoclonal Rabbit α-GAPDH</td>
<td>~37kDa</td>
<td>5% (w/v) Skim milk</td>
<td>1:10000</td>
<td>5% (w/v) BSA</td>
<td></td>
</tr>
<tr>
<td>Polyclonal Rabbit α-Iba-1</td>
<td>~17kDa</td>
<td></td>
<td>1:500</td>
<td>5% (w/v) Skim milk</td>
<td>Room temp for 1hr</td>
</tr>
<tr>
<td>2°</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat α-rabbit IgG HRP-conjugate</td>
<td>N/A</td>
<td>N/A</td>
<td>1:10000</td>
<td>5% (w/v) Skim milk</td>
<td></td>
</tr>
</tbody>
</table>

2.8 Statistical Analysis

Statistical analyses were performed using RStudio (RStudio, Boston, MA). All data are presented as the mean ± standard error of the mean (SEM). The scoring of the NAP is in a pass or fail fashion, therefore the data is non-parametric and analyzed accordingly. The Kruskal-Wallis test with Nemenyi post hoc analysis were used to compare the total average NAP scores between the four groups. This non-parametric...
statistical test was also used to compare the average score that each of the four groups had on the individual sensorimotor tests (Startle, Limb Extension, Beam Walk and Rotating Beam). The latency scores for the toe pinch and righting reflex tests as well as the open field test results were analyzed using a one-way analysis of variance (ANOVA) test. Post hoc analysis was conducted using the Bonferroni correction. Western blotting data was analyzed with a one-way ANOVA and Tukey’s Honest Significance Difference test (HSD) test was used for post-hoc analysis. A p value of <0.05 was considered to be statistically significant.
3. Results

3.1 rmTBI Causes Acute Neurological Deficits

Immediately following the sham or injury procedure, the NAP is performed. It consists of three tests to assess the animal’s level of consciousness and four tests to assess their sensorimotor function. The highest total NAP score that an individual subject can receive is 7, which indicates no neurological impairment, and the lowest being 0, which indicates severe deficits. The total NAP scores for each animal and group were then averaged; i.e. an rmTBI4 animal goes through the ACHI procedure a total of 4 different times, so that particular animal had 4 different NAP scores averaged and then all of the averaged scores for the rmTBI4 group are averaged to come up with a total average NAP score for that group (Figure 7A). The average NAP scores for the sham, rmTBI4, rmTBI8 and rmTBI16 groups were 6.7, 4.1, 4.0, and 3.4, respectively. Kruskal-Wallis analysis showed that the average NAP score was significantly affected by repeated injuries, $H(3) = 200.9, p = 2.2 \times 10^{-16}$. Post hoc Nemenyi analysis revealed that all three injury groups had significantly lower NAP scores in comparison to the sham animals (rmTBI4, $p = 2.0 \times 10^{-7}$; rmTBI8, $p = 2.0 \times 10^{-16}$, rmTBI16, $2.0 \times 10^{-16}$). When comparing injury groups, there was also a significant decrease in NAP score performance when comparing both rmTBI4 ($p = 0.016$) and rmTBI8 ($p = 0.019$) groups to rmTBI16.
3.2 rmTBI Affects Level of Consciousness

The total NAP score gives a general overview of the animal’s neurological outcome after the sham or injury procedure, however, looking at their performance on each of the individual tasks provided more information as to where the neurological deficits were occurring. As outlined in Table 3, there are three measures for the level of consciousness assessment; apnea, toe pinch and righting reflex. These measures make up the first three points of the NAP score and are scored immediately after the sham or injury procedure. Apnea was not apparent in any of the animals used in this study and is therefore not reported in Figures 7 and 8. The toe pinch response and righting reflex were not lost in any of the sham animals, therefore, they all received a score of 1 (Figure 8B and Figure 8C). However, following the ACHI procedure, some rmTBI animals in each group received a failing score of 0 for one or both of these tests. The toe pinch score was significantly affected by repeated injuries, H (3) = 76.538, p = 2.2 x 10^{-16}. Further analysis showed that the rmTBI16 group was the only injury group to perform significantly worse than the sham group (p = 2.2 x 10^{-12}) and no difference between injury groups (rmTBI4 p = 0.138, rmTBI8 p = 0.066). The righting reflex was also significantly affected by repeated injuries H (3) = 38.273, p = 2.474 x 10^{-8}. Again, post hoc analysis revealed that only the rmTBI16 animals had a significantly lower righting reflex score than the sham animals (p = 1.9 x 10^{-6}).

If the animal failed any of the consciousness tests, the latency to recovery was recorded as the average latency per animal and per group and is shown in Figure 8. This allowed for further examination of their level of consciousness following repeat injuries.
None of the sham animals failed any of the latency tests and therefore the sham group is not shown in Figure 8. The average latencies for the Toe Pinch for the rmTBI4, rmTBI8 and rmTBI16 groups were 2.28 sec, 3.74 sec, and 17.5 sec, respectively. A one-way ANOVA revealed that this toe pinch latency was significantly affected by repeated injuries ($p = 1.43 \times 10^{-4}$) (Figure 8A). Post hoc analysis with Bonferroni correction showed that the toe pinch latency to recover was significantly different between the rmTBI4 and rmTBI16 ($p = 1.3 \times 10^{-4}$) and rmTBI8 and rmTBI16 ($p = 1.20 \times 10^{-4}$). There was no difference between the rmTBI4 and rmTBI8 groups ($p = 1.00$). There were similar findings with the righting reflex, in which the latency to recover was significantly affected by repeated injuries ($p = 2.26 \times 10^{-4}$) (Figure 8B). Again, these differences were found when both the rmTBI4 ($p = 1.4 \times 10^{-4}$) and rmTBI8 ($p = 0.0151$) animals were compared to the rmTBI16 animals, but not between one another ($p = 0.349$).

### 3.3 rmTBI Impairs Sensorimotor Function

The sensorimotor tests comprised of the startle, limb extension, beam walk and rotating beam make up the remaining four points of the total NAP score with each test receiving a maximum score of 1. The startle ($H(3), p = 2.2 \times 10^{-16}$), limb extension ($H(3), p = 2.2 \times 10^{-16}$), beam walk ($H(3), p = 9.27 \times 10^{-12}$), and rotating beam ($H(3), p = 2.2 \times 10^{-16}$) were all affected by repeated injuries (Figure 7D-G). None of the sham animals failed the startle, limb extension or beam walk and therefore all three tasks had an average score of 1, indicative of 100% performance. However, when averaging the sham score for the rotating beam task, it was 0.76, giving the sham group a total average score of 6.76 out of 7. Further statistical analysis of the startle test showed that both rmTBI8 ($p = 1.1 \times 10^{-9}$) and rmTBI16 ($p = 8.8 \times 10^{-15}$) animals had significantly lower
scores than the sham animals, but this was not the case for the rmTBI4 (p = 0.39) animals. There were also differences in the startle test when between the rmTBI4 and rmTBI8 groups (p = 3.3 x 10^{-10}) as well as between rmTBI4 and rmTBI16 (p = 2.8 x 10^{-10}) (Figure 7D). Post-hoc analysis with Nemenyi revealed that the limb extension scores for all three injury groups were significantly less than that of the sham group (rmTBI4, p = 0.0061; rmTBI8, p = 4.6 x 10^{-11}; rmTBI16, 2.0 x 10^{-16}). Additionally, there was a significant difference in limb extension performance between the rmTBI4 and rmTBI8 (p = 0.0036) as well as the rmTBI4 and rmTBI16 (p = 4.8 x 10^{-7}) (Figure 7E). Similarly, performance on the beam walk test showed that the rmTBI4 (p = 2 x 10^{-16}), rmTBI8 (p = 2 x 10^{-16}), and rmTBI16 (p = 2 x 10^{-16}), animals scored significantly lower than the sham animals. Again, the rmTBI4 group scored significantly higher than both the rmTBI8 (p = 0.0278) and rmTBI16 (p = 0.0018) groups (Figure 7F). Looking at the final sensorimotor test, the rotating beam task, post hoc analysis showed that the sham group outperformed rmTBI4 (p = 0.0025), rmTBI8 (p = 3.1 x 10^{-8}) and rmTBI16 (p = 1.6 x 10^{-10}) groups. The only other difference in rotating beam score was the rmTBI16 score being significantly worse than the rmTBI4 score (p = 0.0430) (Figure 7G).
Figure 7. Repeated Injuries Causes Acute Neurological Deficits.

(A) Compared to the sham animals, all rmTBI animals had a significantly decreased NAP score. (B, C) rmTBI16 group performed significantly worse than the sham group for both the toe pinch and righting reflex tests. For the startle response (D), rmTBI16 and rmTBI8 groups performed significantly worse than the sham group and the rmTBI4 group. (E) All injury groups had a significantly lower NAP score than the sham animals in the limb extension (E), beam walk (F), and rotating beam (G) tasks. However, between injury groups, with the limb extension and beam walk tasks, rmTBI8 and rmTBI16 performed worse off than rmTBI4 but with the rotating beam there was only a difference between rmTBI4 and rmTBI16. Data presented as Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001
Figure 8. Level of Consciousness is Greatly Affected by Repeated Injuries.

(A) The average latency to recover for the Toe Pinch result was greatest with the rmTBI16 group. There was no difference in latency between rmTBI4 and rmTBI8. (B) Similar pattern with the Righting Reflex test where the rmTBI16 had a significantly greater average latency than the other two injury groups. Again, there were no differences between the rmTBI4 and rmTBI8 groups. Note the different scaled axes. Data presented as mean ± SEM with the solid black horizontal bars representing the mean for each group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4 The ACHI Procedure Results in No Morphological Damage

Cresyl violet stained tissue from two different slices beneath the impact site revealed no serious morphological damage from individuals who were subjected to the ACHI procedure up to 16 times. These representative slices from rmTBI4, rmTBI8 and rmTBI16 animals were comparable to those of the control animals (Figure 9B). There is no obvious damage at the site of impact (Figure 9A) and the ventricles appear to be intact. However, in two instances, slices from the rmTBI16 group showed signs of possible hemosiderin, or a blood stain on the brain tissue. This tissue discoloration was not at the site of impact, but rather located more laterally. Multiple coronal-sections from a rmTBI16 brain, approximately 300µm apart illustrate the depth of this abnormality (Figure 10).
Figure 9. Cresyl Violet-Stained Brain Sections Showed No Significant Morphological Damage Following Repeated Injury.

(A) A representative perfused brain from the rmTBI4 group with the red dashed circle representing the impact location for the ACHI procedure. (B) Low magnification images (2x) of 50 µm thick sections reveal no significant structural damage in the rmTBI groups. The injury groups rmTBI4, rmTBI8, and rmTBI16 were sacrificed at 1 day post-ACHI with the red * indicating the impact location. Note that the cortical layers and ventricles beneath the injury site are intact with no obvious morphological abnormalities. Also note the possibility of hemosiderin in the ipsilateral cortex region of one section (indicated by the arrowhead).
Figure 10. Brain From 16 Repeated Injuries with Tissue Abnormalities PID1.
Coronal sections (50 µm) of a brain subjected to 16 injuries, perfused, and stained with Cresyl violet 1 day after the final injury. The asterisk indicates the site of impact from the ACHI procedure. Note, the light brown spot on the ipsilateral cortex in some of the sections.
3.5 rmTBI Leads to Anxiety-Like Behaviour in One of the Injury Groups at PID1

The open-field test was conducted at PID1 and using a tracking software, it was possible to record their behaviour over the 5 min trial. After examining the total distance moved (p = 0.101) (Figure 11A) and the velocity of travel (p = 0.0975) (Figure 11B) for each of the four groups, there were no differences found. This suggests that both the subjects did not experience motor impairments at PID1. When separating the distance moved in the centre (p = 0.282) and the perimeter (p = 0.14), there were also no differences found between groups (Figure 11C). Looking at the duration in the perimeter, the rmTBI8 group spent significantly more time there than the sham group, indicative of anxiety-like behaviour (p = 0.015) (Figure 11D). However, this was not the case for any of the other groups. Furthermore, the rmTBI8 group spent significantly less time in the centre of the novel arena in comparison to the sham animals (p = 0.53) (Figure 11D). Figure 11E shows representative tracking images for each of the four groups. The tracking image for the rmTBI8 group noticeably illustrates the thigmotaxic behaviour.
Figure 11. rmTBI8 Shows Signs of Anxiety-Like Behaviour PID1.

There were no differences in the total distance moved (A) or velocity (B) between any of the experimental groups. (C) Similarly, there were no differences between groups for the distance moved in the centre or the perimeter. (D) rmTBI8 group appeared to spend more time in the centre and less time in the perimeter in comparison to the sham animals. Data presented as Mean ± SEM. *p<0.05.
3.6 rmTBI Induces Astrogliosis in the Dentate Gyrus and Cortex

To investigate the possible change in specific protein expression levels, a quantitative GFAP Western blot analysis of brains from injured (rmTBI4, rmTBI8, rmTBI16) and non-injured (control, sham) rats was conducted. As previously mentioned, GFAP has become a prototypical marker for the identification of astrocytes and thus an increase in GFAP expression can be either due to astrocyte activation or astrogliosis. The brain tissue dissected for analysis came from the ipsilateral DG, CA and cortical regions. In comparison to the sham and control animals, there was a significant increase in total GFAP expression in the DG of the rmTBI16 group (sham, p =0.0000448; control, p = 0.00114) (Figure 12A2). Similarly, the GFAP expression level of the rmTBI16 animals was significantly greater than all of the other injury groups as well (rmTBI4, p = 0.0000317; rmTBI8, p = 0.0000543) (Figure 12A2). However, the other two injury groups; rmTBI4 and rmTBI8 did not differ from each other nor the sham and control groups. The CA did not appear to have any changes in GFAP following repeated injuries in comparison to the sham and control animals (p = 0.999) (Figure 12B2). There was also an increase in GFAP in the cortex of rmTBI16 animals when compared to the sham (p = 0.0110) (Figure 12C2) and control (p = 0.0526) (Figure 12C2) groups.

3.7 No Evidence of Microgliosis at PID1 Following rmTBI

In addition to GFAP, a quantitative Iba-1 Western blot analysis was done to determine the presence of microgliosis, or an increase in Iba-1 expression levels, following a repeated injury. In the DG, there were no significant differences in Iba-1 protein expression between any of the five groups, both injured and non-injured (p =
0.059) (Figure 12A3). Similar findings were found in the CA (p = 0.359) (Figure 12B3) and cortex (p = 0.679) (Figure 12C3) regions.

Figure 12. Acute Astrogliaosis Present in the Dentate Gyrus and Cortex Following 16 Injuries.

(A1, B1, C1) Representative Western blots of the three ipsilateral brain regions with the control, sham and injury groups. (A2) There was a significant increase in total GFAP expression in the rmTBI16 group in comparison to all other groups. (B2) There was no difference in GFAP expression between any of the groups in the CA region. (C2) In the cortex, the rmTBI16 group had significantly higher GFAP expression in comparison to Control and Sham groups. (A3, B3, C3) There were no differences in Iba-1 expression, in any of the regions, between all five groups. Data represented as Mean ± SEM. *p<0.05 **p<0.01 ***p<0.001.
4. Discussion

Using our model to produce mild close head-injuries in juvenile female rats, we were able to mimic the symptoms apparent in human concussion (Meconi et al., 2018). Additionally, the ACHI model allows us to use unanaesthetized subjects and without the use of surgery. Removing these confounds, allows us to better represent this clinical condition. It also gives us the opportunity to immediately conduct a neurological assessment following mTBI, which could not be done if anaesthesia was involved. With this suitable model of mTBI, we were able to address the consequences of concussion immediately after the injury, and with a highly susceptible age group.

4.1 Neurological Deficits Immediately After rmTBI

The significantly lower total NAP score for all three injury groups in comparison to sham animals, illustrated that our ACHI model was capable of inducing neurological impairment. 16 repeated injuries produced the greatest deficits including the lowest total NAP score and longest duration for the LOC measures. Sections 1.2.6 Secondary Neuroinflammatory Response and 1.5 Repeated Concussions state that the brain enters this volatile state after sustaining a single concussion and any additional injuries within this window of vulnerability can greatly affect the functioning of the brain. When we teased apart the NAP score into its 7 individual components, sensorimotor deficits were evident following 4, 8 and 16 rmTBI. LOC and neurological deficits immediately after the repeated injuries also coincides with the clinical population and results from other similar models of rmTBI (Erlanger, 2015; Marshall et al., 2015; Petraglia et al., 2014) and our initial publication (Meconi et al., 2018). A concussion, as defined by the
Consensus Statement on Concussion in Sport, is a brain injury that typically results in short-lived neurological impairment and may or may not result in LOC (McCrory et al., 2013). Thus, our ACHI model and its findings support the use of this model for studying rmTBI.

4.2 Behavioural Analysis

At one day post-injury, locomotor deficits and anxiety-like behaviour were tested for using the open field test. The total distance travelled and speed of travel throughout the test can used as a measure of locomotor activity. As shown in Figure 11A, B, regardless of how many injuries sustained, the total distance travelled and velocity did not vary between groups or compared to the sham animals, therefore illustrating that their mobility was not affected at PID1. To examine symptoms of anxiety post-injury, it is common to compare the time spent in the center of the novel maze versus the perimeter. Anxiety-like behaviour was only evident for the rmTBI8 group, where this injured group compared to the shams spent significantly more time in the periphery. This is termed thigmotaxis, where the rats avoid the exposed center and seek out the edges (Crawley, 1985). In the clinical population, behavioural impairments develop with time, so future studies using our ACHI model to investigate long-term behavioural consequences following rmTBI would help us gain insight the behavioural sequelae post-injury (McCrory et al., 2013).

4.3 Histology

Cresyl violet stain indicated that there was no serious morphological damage to the brain. Again, these results indicate the usefulness of our model for depicting human
concussion as the absence of overt physical damage to the brain is another defining
criterion of mTBI (McCrory et al., 2013). However, in a subset of our 16rmTBI group,
there appeared to be brown discolouration in some the tissue slices. This was not
observable in any of the other groups. Small bleeds into the parenchyma of the brain will
lead to a breakdown product of red blood cells known as hemosiderin (Benson et al.,
2012; Bigler, 2013). This by-product of blood degradation has been used as an indication
of shear-force injury. This makes sense with our model as shear forces are generated
from rotational acceleration which can occur in mTBI when the head motion is not
constrained. Further investigation into this theory must be done before any conclusions
about the abnormality are made.

4.4 Molecular Analysis

4.4.1 Astrogliosis

Western blotting experiments revealed an increased expression of GFAP in the
DG and CX of the rmTBI16 group at PID1, an indication of astrocyte activation or
astrogliosis. Astrogliosis is typically related to injuries of the CNS providing evidence
that our mild model of TBI can have not only neurological consequences but also
molecular ones. This finding was not apparent in the CA. Studies have shown increased
vulnerability of the DG to trauma which supports the findings of this study. This
susceptibility arises from the variable cell types and organization of inputs and outputs in
the hippocampus. The granule cell layer and neurons in the hilus region of the DG are
more likely to be affected by brain injury (Golarai et al., 2001; Lowenstein et al., 1992).
In a study of repeated mild head injury using the WD model, MRI analysis revealed
enlarged lateral ventricles (Qin et al., 2018). If the lateral ventricles are changing post-
injury, the DG is in closer proximity to the ventricles than the CA and is perhaps being affected as well. Another notable report is the affect of neurogenesis in the DG on neighboring cells. Perhaps the DG is tightly regulated as a result of neurogenesis and this regulation occurs with the help of astrocytes and the BBB. Therefore, the slightest disturbance here may lead to activated astrocytes and ultimately increased GFAP expression in the DG. Using the ACHI model to induce 16 repeated injuries with the impact tip hitting the same area on the rat’s head each time, it is not surprising that there was astrocyte activation in the cortex. The damage may be diffuse as a result of the brain moving within the skull after impact, since we see astrocyte activation in the hippocampus, but the cortex directly beneath the impact site is being repeatedly subject to the blow.

4.4.2 Microgliosis

Our experimental results indicate that after sustaining repeated mild head trauma with the ACHI model, there was no evidence of microgliosis at PID1 in any of the investigated regions. Interestingly, microglia activation is negative in the presence of an astrocytic response to injury. In recent years, rmTBI studies using the WD model have shown that microglial activation was present in invasive models but not in the closed-head models (Kane et al., 2012; Lafrenaye et al., 2015). Perhaps studies involving surgery activate microglia and trigger an inflammatory response. There are also multiple models of head injury classified as mild, yet inconsistency between results is apparent (Table 2). Our ACHI model induces symptoms that match the defining criteria of a mTBI, but our model of mTBI could potentially be more mild than others, and the recruitment and proliferation of microglia is not imperative for recovery. Another factor
to consider is the timeline of the study. At PID1, astrocytes appear to be activated in the DG and CX, but microglia may take longer, especially if they have to be recruited from other areas of the brain. Some researchers show evidence of microgliosis at PID 7, 10 and 14 (Table 2). Verderio and Matteoli (2001) showed that ATP release rapidly recruits microglia, therefore, mTBI-induced ATP release from astrocytes may trigger calcium signaling which then draws microglia to the area of damage.

4.5 Limitations and Future Directions

This study involved juvenile females, therefore the evidence of astrogliosis here should only pertain to this population as differing age and sex can produce variable results (Margulies and Hicks, 2009). A comparative study with juvenile males could reveal potential sex differences following rmTBI. In relation to this, post-injury time point is confounded by the duration of the injury induction groups. For example, the first rmTBI occurs between PND25-28 and there are impacts are 4 per day and subjects are sacrificed 1 day after their final injury. Thus, the rmTBI4 group is sacrificed at PND26-29, while the rmTBI8 at PND28-30 and rmTBI16 group at PND30-33. This was done in order to keep their age consistent among groups receiving their first mTBI, but may have potentially confounded the results. Furthermore, the timeline of the current study was short and could therefore only provides evidence for acute changes post-injury. Thus, this study highlights the importance of using a prolonged experimental timeline to report on the activation of glial cells.

In order to strengthen the findings of this study, it would be useful to provide other supportive evidence for changes in astrocyte reactivity. Vimentin, another IF protein found in astrocytes, have been shown to be unregulated after brain injury and
studies with mice lacking both GFAP and vimentin have shown impaired astrocyte reactivity and BBB dysfunction (Liu et al., 2014; Pekny et al., 1999). It would be interesting to note if vimentin expression levels were also increased following ACHI.

Another notable consideration is the validity of the loading control used. GAPDH is an important enzyme for energy metabolism and ATP production (Nicholls et al., 2011). As discussed in 1.2.5 Primary Pathophysiological Changes Post mTBI, following injury the brain is experiencing this metabolic crisis, so perhaps the GAPDH levels are varying between injured and non-injured animals and this would greatly confound my results. More investigation into the metabolic changes post-mTBI is needed.

Lastly, our model of ACHI is still undergoing characterization and I think it’s important to confirm the diffuse nature of this injury. In order to assure reproducibility of our mTBI, we impact the animals in the same location every time, but the helmet used is supposed to help disperse the forces of impact. In order to confirm this, it would helpful to conduct a comparative study looking at both the ipsilateral and contralateral sides of the brain.

### 4.6 Summary and Conclusions

Several animal models of TBI have been established, however, many of these models do not efficiently simulate mTBI due to their invasive nature. Our model removes any potential confounds of anaesthesia or surgery and allows for the delivery of an impact to unrestrained subject. This allows for acceleration-deceleration forces, an
important characteristic of human concussion. We are also able to gain insight into the
rmTBI's experienced by athletes.

Pathologically, we observed numerous changes following rmTBI with our ACHI
model: (1) neurological impairment after mTBI as indicated by the NAP score. (2) the
absence of gross morphological damage to the brain beneath the point of impacts. (3)
those subjected to 16 repetitive injuries presented with astrogliosis in the DG and CX, as
shown by increased GFAP expression. In conclusion, we demonstrated that a mild closed
head injury, which is the most common accidental injury, can elicit consequences in the
brain when it occurs at high frequency.
Bibliography


http://doi.org/10.1016/j.neuroscience.2010.10.018


http://doi.org/10.1038/jcbfm.2015.56


# Appendix A – Neurological Assessment Protocol Scoring Sheet

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Ap    Apnea  St    Startle Response
LOC   Loss of Consciousness LE   Limb Extension
RR    Righting Reflex    BW    Beam Walk
     Rotating Beam