Repetitive Mild Traumatic Brain Injury Induces Ventriculomegaly and Cortical Thinning in Juvenile Rats

by

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A Thesis Presented in Partial Fulfillment
of the Requirements For the Degree
Master of Science

Approved November 2014 by the
Graduate Supervisory Committee:

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ARIZONA STATE UNIVERSITY

December 2014

ABSTRACT

Traumatic brain injury (TBI) most frequently occurs in pediatric patients and remains a leading cause of childhood death and disability. Mild TBI (mTBI) accounts for 70-90% of all TBI cases, yet its neuropathophysiology is still poorly understood. While a single mTBI injury can lead to persistent deficits, repeat injuries increase the severity and duration of both acute symptoms and long term deficits. In this study, to model pediatric repetitive mTBI (rmTBI) we subjected unrestrained juvenile animals (post-natal day 20) to repeat weight drop impact. Animals were anesthetized and subjected to sham or rmTBI once per day for 5 days. At 14 days post injury (PID), magnetic resonance imaging (MRI) revealed that rmTBI animals displayed marked cortical atrophy and ventriculomegaly. Specifically, the thickness of the cortex was reduced up to 46% beneath and the ventricles increased up to 970% beneath the impact zone. Immunostaining with the neuron specific marker NeuN revealed an overall loss of neurons within the motor cortex but no change in neuronal density. Examination of intrinsic and synaptic properties of layer II/III pyramidal neurons revealed no significant difference between sham and rmTBI animals at rest or under convulsant challenge with the potassium channel blocker, 4-Aminophyridine. Overall, our findings indicate that the neuropathological changes reported after pediatric rmTBI can be effectively modeled by repeat weight drop in juvenile animals. Developing a better understanding of how rmTBI alters the pediatric brain may help improve patient care and direct “return to game” decision making in adolescents.

ACKNOWLEDGEMENTS

A profound thank you to my advisor, Dr. Trent Anderson. Without your mentorship, this accomplishment would not have been possible. Your passion for science done right inspires me to become as stalwart an investigator of the world around me as you are.

My lab mates, helping hands, coffee partners, late night, early morning, sounding board comrades in science: Josh Nichols and Chen Wu. I would not have gotten through this without your help, thank you for everything.

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CHAPTER 1

INTRODUCTION

Traumatic Brain Injury/Mild Traumatic Brain Injury

Traumatic brain injury (TBI) is described as an alteration in brain function, or other evidence of brain pathology, caused by an external force (Menon, Schwab, Wright, and Maas 2010). Rapid acceleration/deceleration of the head, blast wave overpressure, penetration of a foreign object, and blunt impact to the head are a few of the ways a TBI can occur. As the causation of TBI is broad, its incidence among the population is quite high. An estimated 1.7 million people per year sustain a TBI in the United States. Children aged 0-4, adolescents aged 15-19, and adults aged 65+ are the most likely to receive a TBI. Males have higher incidence rates than females across all age groups (Faul, Xu, Wald, and Coronado 2010).

TBI is an umbrella term that encompasses a spectrum of injuries commonly rated as mild, moderate, or severe. In clinical situations the Glasgow coma scale (GCS) is often utilized to quickly evaluate an incoming head injury patient. It assesses the patient's fundamental functions like their ability to open their eyes, speak coherently, and move on command. A GCS of 8 or less is considered comatose and a 3 or below is totally unresponsive. The duration of loss of consciousness (LOC), the duration of alteration of consciousness (AOC) such as confusion, the duration of post-traumatic amnesia (PTA), and presence of penetrating injury all play a role in defining the severity of TBI. A definition of mTBI widely used by medical and health organizations around the world is as follows: normal structural imaging, LOC less than 30 minutes, AOC less than 24hours,

PTA less than 24 hours, and a GCS of 13-15 (ACRM 1993) (Carrol et al 2004) (NCIPC 2003).

Mild traumatic brain injury (mTBI) accounts for approximately 80% of all treated cases of TBI in the United States (1.375 million hospital visits). However, it has been estimated that this number is only a fraction of the actual incidence of mTBI. Injuries without loss of consciousness are highly underreported, especially in sports-related environments (Yuh, Hawryluk, & Manely 2014).

Clinical symptoms of mTBI can include cognitive, behavioral, and physical features including: loss of consciousness, cognitive deficits, emotional liability, impaired coordination, motor deficits, sleep disturbance, memory impairments, and photophobia (Jordan 2013). While the term mild is used to describe the acute injury, the effects of mTBI can be significant and far reaching. Approximately 50% of patients with mTBI experience resolution of their symptoms by 3 months post-injury, another 40% see symptoms persist up to 6 months post injury, while others experience one or more symptoms indefinitely (Arciniegas, Anderson, Topkoff, & McAllister 2005). Use of neuroimaging, specifically computed Tomography (CT), is recommended by several professional societies/academies when: LOC is 30 seconds or longer, there is a prolonged altered or deteriorating state of consciousness, severe headache, focal neurological deficit or seizure, or worsening symptoms (Yuh, Hawryluk, & Manely 2014). Studies involving CT imaging have shown that 5% of patients with a GCS of 15 and 30% of patients with a GCS of 13 had an acute intracranial hemorrhage (Borg et al 2004). While MRI is not generally recommended for routine management of acute mTBI, in cases of chronic or malingering symptoms MRI has been shown to be a have prognostic value. A recent

study showed abnormal MRI findings in 10 of 34 patients diagnosed with “grade I” or mildest grade of concussion (Yuh et al 2013).

Currently there are no effective treatments for the symptoms of mTBI. Escalating factors include intracranial bleeding, skull fracture, and intracranial lesions. Aside from those factors, no effective markers of functional outcome have been established (Borg, Holm, & Cassidy 2004).

Repeated Mild Traumatic Brain Injury

A single mild traumatic brain injury (smTBI) can result in an array of acute cognitive, behavioral, and physical symptoms. However, the majority of patients (90%) see these symptoms resolve over the course of 6 months (Arciniegas, Anderson, Topkoff, and McAllister 2005). Studies of cognitive function after a smTBI have shown that deficits recover to the level of control groups with no history of mTBI within 3 months (Levin & Robertson 2013).

However, when there is a history of previous mTBI the acute symptomology, recovery times, and long-term outcomes are all significantly altered. A study of collegiate and high school athletes showed that players who had been diagnosed with a mTBI within the previous year were more likely to experience loss of consciousness (LOC) and post-traumatic amnesia (PTA) when injured again. Players with recurrent injuries also reported a greater number of symptoms at time of injury when compared to players with a non-recurrent injury (Guskiewicz, Weaver, & Padua 2000). Not only do the acute symptoms present more severely in cases of repeated mTBI (rmTBI), they also resolve more slowly. When compared to players experiencing their first mTBI, players with a history of rmTBI are twice as likely to have clinical symptoms last for greater than 1

week (Guskiewicz et al 2003). The effects of rmTBI extend past evident clinical symptoms. Covassin et al compiled several studies that show acute cognitive, attention, working memory, and visual memory deficits (Covassin, Stearne, & Robert 2008). The long-term effects of rmTBI and repeated sub-threshold concussions have long been hypothesized. The recent characterization of a neurodegenerative disease termed chronic traumatic encephalopathy (CTE) and its possible links to rmTBI has spurred a flurry of research into the topic. One study of retired athletes showed that 80% of individuals with a history of rmTBI had evidence of CTE upon autopsy (McKay et al 2013).

After an initial mTBI, the risk for another mTBI or other accident is greatly increased. A study of male high school football players showed that individuals with a history of mTBI have a 5.8 times greater risk of incurring another mTBI compared to individuals with no history of mTBI (Zemper 2003). An NCAA study showed that college athletes who have sustained an mTBI have a 1 in 15 chance of sustaining another mTBI within the same season. Of those that have same season repeat injuries 91.7% occur within a 10 day period and 75% occur within a 7 day window (Guskiewicz et al 2003). This risk is not limited to sports-related mTBI. A study of military personal who received 2 or more concussions showed that 20% received the second injury within 2 weeks and 87% received the second injury within 3 months (MacGregor, Dougherty, Morrison, Quinn, & Garlarneau 2011). Risk for injury outside of mTBI is also increased after an initial mTBI. A study of U.S. airmen who received a non-combat related mTBI were found to be at increased risk of having another “mishap” when compared to a control group with no history of mTBI. The “mishaps” included such incidents as car

accidents, sports injuries, and industrial related injuries. The increased risk was still evident over one month post-injury (Whitehead, Webb, Wells, & Hunter 2014).

The close temporal proximity seen with cases of rmTBI is of particular concern when considered in light of recent animal research that suggests there is a "window of vulnerability" after an initial mTBI. This window that is correlated with worse long term outcomes and altered physiological response to the subsequent injury. Mannix et al showed that consecutive injuries one week apart showed cognitive deficits up to a year after the last injury (Mannix et al 2013). The same cognitive deficits were not seen if the injuries occurred biweekly or monthly. Other studies have demonstrated that the physiological response is altered and the severity of neurodegeneration was increased when multiple injuries occurred within 3 days or 24 hours respectively (Weil, Gaier, & Karelina 2014) (Bolton & Saatman 2014).

The effect of rmTBI amongst the pediatric population is of particular concern. Not only do initial injury rates of high school football players exceed that of division I college athletes (5.6 vs. 4.4%), but rates of multiple injury also outpace division I athletes (17 vs. 9.8%) (Guskiewicz et al 2000). This trend continues to an even earlier age point, and in some comparison areas are considerably worse. Kontos et al found that the mTBI incidence rate during practices amongst 8-12 year old males was similar to high school players (Kontos et al 2013). However, the mTBI incidence rate for 8-12 year olds during games was 3 times higher than their high school counterparts and nearly twice as high as their collegiate counterparts (Kontos et al 2013). While the majority of pediatric mTBI studies focus on male football players, the epidemic is not limited to that population alone. Lincoln et al showed that in similar sports (baseball/softball, basketball, soccer)

girls had roughly twice the risk of mTBI as boys and that mTBI occurred in all 12 of the sports they studied (Lincoln et al 2011). Again, while most studies focus on sports-related injuries, mTBI is a threat that does not heed the boundaries of the sports field. When considering mTBI from all sources, children under the age of 5 have more than double the annual incidence rate compared to the general population. Children aged 5-14 and 15-24 also have elevated incidence rates compared to the general population (1.45 times and 1.37 times respectively). Sports-related injuries were the 5th most common behind falls, motor vehicle trauma, accidentally struck, and assault (Bazarian et al 2005). It is abundantly clear that rmTBI is a major concern across all epidemiological groups. However, it is of particular concern among males under the age of 24 who are involved in contact sports or are enlisted in the military.

Cortex

The human cortex is a highly convoluted sheet of tissue that measures between 2 to 3 millimeters in depth and consists of 6 layers. Layers I through VI are characterized by the type and distribution of their resident cell bodies as well as the connections they make with other cortical layers and subcortical structures. From lateral to medial, the outermost layer is I and the innermost layer is VI.

Layer I's (the molecular layer) most noticeable feature is its near complete lack of neuronal cell bodies. It is mostly comprised of incoming axonal ramifications, branching apical dendrites extending from pyramidal neurons located in the deeper layers, and glial cells (Shipp 2007). The connectivity of layer I primarily consists of a dense matrix of apical dendrites from the pyramidal cells from all the other layers (Staiger 2010).

Layers II (external granular layer) and III (external pyramidal layer) are closely related from both a cellular standpoint and their connectivity. Both layers are dominated small to medium sized pyramidal neurons. Layers II/III primarily project intralaminarly to layers V and VI, though the projections to layer VI are comparably weaker. The main intralaminar projections into layers II/III come from layer IV (Shipp 2007). In addition to vertical communication, layers II/III are unique in their extension of large arborizations within the same layer that play a large role in interlaminar communication (Douglas & Martin 2004). The unique nature of layers II/III pyramidal neurons allows them to issue and receive cortico-cortico projections both within their home hemisphere as well as the contralateral hemisphere (Staiger 2010). This establishes layers II/III as the primary area for collaborating input from disparate regions of the cortex.

Layer IV (internal granular layer) is a very dense-appearing layer that consists mainly of spiny stellate and star pyramidal neurons (Staiger 2010). Connectively, it is the primary target for incoming sensory signals from the thalamus which it then relays mostly to layers II/III (Shipp 2007). Layer IV also has lateral connections to layer III of adjacent cortical regions which also get relayed to layers II/III (Douglas & Martin 2004). Layer IV also sends projections down into layer VI which forms part of the thalamic-cortical loop that runs from the thalamus through layers IV, II/III, and VI and closes when layer VI projects back into the thalamus. The direct layer IV to VI projections are likely modulatory as they predominantly project upon inhibitory neurons. Unlike the other layers, layer IV is not ubiquitous across the cortex. The motor cortex, for one, notably lacks a layer IV (Shipp 2007).

Layers V (internal pyramidal layer) and VI (multiform layer) both consist primarily of pyramidal neurons that project to subcortical structures. Layer V is populated by the largest pyramidal neurons amongst the six layers. Layer VI is populated by a morphologically variable mix of excitatory neurons (Staiger 2010). Layer V is the sole output center for the motor cortex to spinal motor neurons and it is at its densest in that region. Beyond spinal innervation, it serves as the 'motor' output center for the entire cortex. Nearly all signals directing behavior emanate from layer V, including projections to superior colliculus which governs head and eye movements. Primary inputs to layer V come from layers II/III (Shipp 2007). Layer VI also receives input from layers II/III as well as layer IV. Layer VI however, solely projects to the thalamus and is the outgoing component of the cortico-thalamo-cortical loop. In a general sense layer V is regarded as the source for driving output, while layer VI provides modulatory output (Shipp 2007).

Due to their primacy regarding cortico-cortico communication, layers II/III were the targets of investigation for the electrophysiological portion of this study. The clinical and neurobehavioral symptoms associated with mTBI and rmTBI involve integration of several different primary cortical regions such as motor and visual as well as more diffuse functions such as learning, memory, and attention (Kamper et al 2013). Furthermore, there have been demonstrated links between white matter disruption and rmTBI (Donovan et al 2014) (Mouzan et al 2012). Considering the extensive white matter involvement associated with layer II/III, it was hypothesized that damage to the white matter seen in rmTBI may result in abnormal electrophysiological activity within layers II/III.

Models of repetitive mild traumatic brain injury

Animal studies have long been used to replicate and intricately study human injuries. Their usefulness in elucidating mechanisms of injury, intervention strategies, and pathophysiology is evident throughout the history of science. However, the effectiveness of animal studies is intimately tied to the model used to replicate the injury being studied. In the field of repeated mild traumatic brain injury there are 4 major injury models: fluid percussion, controlled cortical impact, blast, and weight drop. While each of these models is usually modified to suit the needs of any given study, the fundamental characteristics of each can be discussed.

Fluid percussion (FP) translates energy through several mediums before it finally imparts an impact force upon the animal. First, a window craniotomy is performed to expose the intact dura of the animal. Then, either a metal pendulum or pneumatic device is used to impact a tube containing saline solution. This generates a fluid pressure pulse that moves along the tube, through a small exit nozzle, and strikes the exposed dura mater of the animal (Xiong, Mahmood, & Chopp 2013). Two main craniotomy locations are used to produce different effects. Midline FP (MFP) utilizes a craniotomy placed on the dorsal midline between lambda and bregma. MFP has been shown to produce bilateral cortical damage, axial brainstem movement, diffuse axonal injury, and death depending on the severity of force imparted. Lateral FP (LFP) places the craniotomy between lambda and bregma as well, but is moved lateral of the midline so that it exposes the parietal cortex. LFP can produce focal lesions, edema, tissue shearing, and blood brain barrier (BBB) disruption depending on the severity of the force used (Petraglia et al 2014). When scaled to a lower severity, this method has been shown to produce several

of the neurobehavioral symptoms associated with mild traumatic brain injury (mTBI). It has also been used to investigate repeated mTBI (rmTBI) (Petraglia et al 2014).

Controlled cortical impact (CCI) uses a pneumatic or electromagnetic device to impart its impact force to the animal. Like the FP model, a craniotomy is performed over the desired location to expose a target region. The animal is then placed in a stereotaxic frame and held rigidly in place. The impacter is then positioned over the craniotomy window and is programmed for a specific velocity, impact depth, and impact duration (Xiong, Mahmood, & Chopp 2013). This method provides a highly controlled impact at a precise location. The type of injury produced is generally focal in nature and has been associated with cortical tissue loss, hematoma, axonal injury, contusion, and BBB disruption. The resulting pathophysiological and behavioral effects are greatly associated with the depth and velocity of the impact. By lowering the impact severity, many of the cognitive, motor, and behavioral symptoms of mTBI can be replicated. However the need for a craniotomy restricts the ability to perform repeated injuries. In order to adapt this method to be used in modeling rmTBI, several modifications have been made to avoid the use of a craniotomy. Two common modifications are the use of a rubberized impact tip or the application of a protective helmet (Petraglia et al 2014).

Blast injury models utilize a shock tube to direct an overpressure wave at the animals head without any penetration or impact by a solid object. Most models use compressed air as the source of the overpressure wave. The resultant blast wave is recorded and used to produce a pressure-time curve. Current studies reveal that injury severity is tied to the area underneath this curve (Petraglia et al 2014). This model has been shown to produce prolonged behavioral, cognitive, and motor deficits at even mild

levels. Various pathophysiological effects including tissue damage, hemorrhage, neuroinflammation, neurodegeneration, and phosphorylated tauopathy have also been associated with blast injury (Xiong, Mahmood, & Chopp 2013).

Weight drop injury (WDI) utilizes a free-falling weight guided by a tube to impact the head of the animal. The height and mass of the falling object determines the force of impact (Petraglia 2014). Beyond the severity of impact force, this method is highly modifiable and has the most variations of any of the previous methods. However, a few core features stand out: the head is allowed to move freely after impact and the skull remains intact. Because of those two features, this method most authentically reproduces injuries seen in real life. Studies utilizing this method have shown a variety of cognitive, behavioral, motor, and pathophysiological effects (Mychasiuk, Farran, & Esser 2014) (Kane et al 2012) (Angoa-Perez et al 2014). When scaled to a mild impact force, many of the symptoms seen in human mTBI can be produced (Petraglia 2014) (Kane et al 2012). Multiple impacts are tolerated and this method has been used to investigate the effects of rmTBI (Kane et al 2012).

For the purposes of this study I wanted to impart a mild injury directly to an intact, unmodified skull of a juvenile rat. Furthermore, I wanted to allow for unrestricted head movement after the impact, acceleration/deceleration forces, and rotational forces. The impacts had to be tolerable enough to repeat up to 5 times with 24 hours in between impacts. For these reasons I utilized a modified weight drop technique characterized by Kane et al in adult mice and I scaled the method to be used in juvenile rats. The technique is described in depth in chapter 2. This method fulfilled all of my requirements and was

shown by Kane et al to produce cognitive, motor, and behavioral deficits similar to those seen after human mTBI.

CHAPTER 2

REPETITIVE MILD TRAUMATIC BRAIN INJURY INDUCES VENTRICULOMEGALY AND CORTICAL THINNING IN JUVENILE RATS

Traumatic brain injury (TBI) most frequently occurs in pediatric patients and remains a leading cause of childhood death and disability. Mild TBI (mTBI) accounts for 70-90% of all TBI cases, yet its neuropathophysiology is still poorly understood. While a single mTBI injury can lead to persistent deficits, repeat injuries increase the severity and duration of both acute symptoms and long term deficits. In this study, to model pediatric repetitive mTBI (rmTBI) we subjected unrestrained juvenile animals (post-natal day 20) to repeat weight drop impact. Animals were anesthetized and subjected to sham or rmTBI once per day for 5 days. At 14 days post injury (PID), magnetic resonance imaging (MRI) revealed that rmTBI animals displayed marked cortical atrophy and ventriculomegaly. Specifically, the thickness of the cortex was reduced up to 46% and the ventricles increased up to 970% beneath the impact zone. Immunostaining with the neuron specific marker NeuN revealed an overall loss of neurons within the motor cortex but no change in neuronal density. Examination of intrinsic and synaptic properties of layer II/III pyramidal neurons revealed no significant difference between sham and rmTBI animals at rest or under convulsant challenge with the potassium channel blocker, 4-Aminophyridine. Overall, our findings indicate that the neuropathological changes reported after pediatric rmTBI can be effectively modeled by repeat weight drop in juvenile animals. Developing a better understanding of how rmTBI alters the pediatric brain may help improve patient care and direct “return to game” decision making in adolescents.

Introduction

Traumatic brain injury (TBI) is a significant health concern that affects more than 1.5 million Americans each year(Langlois, Rutland-Brown, and Wald 2006)(Rutland-Brown et al. 2006)(Faul et al. 2010). At present, no single classification system has been developed that encompasses the host of clinical, pathological, behavioral and cellular changes that occur as a result of TBI. In general, TBI is categorized into mild, moderate and severe. Mild traumatic brain injury (mTBI), including concussions, accounts for nearly 75% of all TBI cases(Langlois, Rutland-Brown, and Thomas 2005)(Miniño et al. 2006)(Cassidy, Carroll, Peloso, et al. 2004)(Cassidy, Carroll, Côté, et al. 2004)(Elder and Cristian 2009)(Langlois, Rutland-Brown, and Wald 2006). Mild TBI (mTBI) is often called an "invisible wound" as it results in a minimal loss of consciousness (<30 minutes) and minimal acute neuropathological findings(Carroll et al. 2004)(Smith, Johnson, and Stewart 2013)(Morey et al. 2013). Consequently, mTBI is often difficult to detect and diagnose in the early acute stages after injury and may result in the incidence being underreported. Following mTBI patients may experience cognitive and behavioral impairments including confusion, cognitive deficits, and headaches (Barkhoudarian, Hovda, and Giza 2011). These symptoms usually resolve completely within 2–3 weeks after a single mTBI(Lovell et al. 2003)(McCrea et al. 2003). However, especially with repeat injuries these symptoms may persist for extended periods of time. This condition is known as post-concussion syndrome(Halstead, Walter, and Council on Sports Medicine and Fitness 2010)(Pellman et al. 2003)(Arciniegas et al. 2005).

Repetitive mTBI (rmTBI) significantly increases symptom severity(Collins et al. 2002), leads to longer term cognitive and motor deficits(Omalu, Hamilton, et al.

2010)(Kevin M. Guskiewicz 2011)(De Beaumont et al. 2007), and increases the risk for developing dementia(Kevin M. Guskiewicz et al. 2005) and neurodegenerative disorders(McKee et al. 2009)(McKee et al. 2010)(Masel and DeWitt 2010)(Plassman et al. 2000). Contrasting with a single mTBI event, repetitive mild traumatic brain injury (rmTBI) induces significant long term structural changes to the brain including brain atrophy and enlargement of the ventricles(Smith, Johnson, and Stewart 2013)(Huh, Widing, and Raghupathi 2007)(Wang et al. 2014)(Maxwell 2012)(Giza 2006). Currently no effective treatments are available to prevent the adverse complications associated with rmTBI. Development of new therapeutic strategies is contingent on an improved understanding of the underlying pathophysiological processes induced by rmTBI.

Recent public and research attention has focused on understanding rmTBI that occurs in adult athletes and military personnel. However, recent reports indicate that children may be particularly susceptible and sensitive to the effects of TBI(K. M. Guskiewicz et al. 2000)(Kontos et al. 2013)(Barlow et al. 2010)(Eisenberg et al. 2013)(Field et al. 2003). In children, traumatic brain injury (TBI) remains a leading cause of death and disability(Faul et al. 2010) with over 10% experiencing a mTBI by the age of 10(Barlow et al. 2010)(Bruns and Hauser 2003). As with adults, the source of the TBI varies greatly in children, but may occur from a combination of events including accidents, abuse (shaken baby syndrome) or adolescent sport concussions. The pediatric brain is unique from the adult brain owing to a host of on-going neurodevelopmental processes including cortical hypertrophy, synaptogenesis, use-dependent pruning, enhanced glucose metabolism, increased neurotrophic factors and altered excitatory amino acid receptors(Adelson 1999)(Chugani, Phelps, and Mazziotta 1987)(Prins et al.

2010) (Friedman, Olson, and Persson 1991)(Insel, Miller, and Gelhard 1990)(Giza 2006). These processes have often been thought to confer children with an advantage in coping with brain injury, but recent evidence suggests they may be particularly sensitive to the effects of repetitive mTBI(Eisenberg et al. 2013)(Field et al. 2003).

Several models of TBI have been developed(Cernak 2005)(Xiong, Mahmood, and Chopp 2013) but many are intended to induce a more severe TBI and do not effectively model mild TBI. In this study, we modified a recently published adult weight-drop rmTBI model(Kane et al. 2012a) for use in juvenile rats. This method of inducing rmTBI recapitulates the nature of the injury (closed head injury, unrestrained animal, linear and rotational acceleration forces) and was recently shown to produce several of the cognitive and behavioral outcomes associated with clinical mTBI(Kane et al. 2012b)(Viano, Casson, and Pellman 2007)(Meaney and Smith 2011). Using this method in juvenile rats, a single mTBI was sufficient to induce clinically relevant impairments to executive, motor and balance functions(Mychasiuk, Farran, and Esser 2014). The duration of these impairments are variable, but have been reported to persist for up to months following mTBI(Petraglia et al. 2014)(McCrea et al. 2003). The purpose of this study was to examine the neuropathological and neurophysiological changes induced early after rmTBI in juvenile rats using MRI, immunohistochemical and electrophysiological approaches. The results validate the use of this repetitive weight-drop method to effectively model pediatric rmTBI. Similar to what has been reported in humans(McCrea et al. 2003)(Halstead, Walter, and Council on Sports Medicine and Fitness 2010)(Smith, Johnson, and Stewart 2013), rmTBI in juvenile rats induced marked cortical atrophy (i.e. decreased cortical thickness) and enlarged ventricles that were most pronounced beneath

the impact site. Despite significant cortical atrophy no intrinsic or synaptic electrophysiological changes were evident in layer II/III neurons recorded from rmTBI animals. These findings are in contrast to the recent report from adult rodents(Kane et al. 2012a) and after single mTBI (Mychasiuk, Farran, and Esser 2014) and suggest impact number, severity and age are critical determinants to the pathophysiological changes following rmTBI.

Materials and Methods

All procedures were performed under protocols approved by University of Arizona Institutional Animal Care and Use Committee.

Repetitive Mild Traumatic Brain Injury

To experimentally model repetitive mild traumatic brain injury (rmTBI) we modified a recently developed model by Kane et al., 2012. This model replicates many of the clinical characteristics and mechanics of a mTBI injury including low impact force, low incidence of skull fracture and subdural hematoma, no immediate or early seizures and no gross cavitation at the impact site. This model has been shown to effectively induce and model rmTBI in adult mice and was herein modified to model pediatric rmTBI using juvenile rats. In brief, 20 day old (P20) male Sprague-Dawley rats were subjected to a single mTBI once per day for 5 consecutive days (Fig. 1). Rats were lightly sedated via isoflurane inhalation and immediately placed ventral side down on a tightly stretched Kimwipe© secured to a plexiglass stage (Fig. 2A). The animal's head was then carefully centered under the vertical aluminum guide tube. As the animal's skull and skin remain completely intact the animal's position was adjusted using external anatomical land marks (i.e. ear canals, eyes) so that its approximated impact was between the bregma and lambda sutures. The impact weight (92 grams, 9mm diameter) was then positioned at the top of the aluminum tube so that the bottom of the weight was precisely 865 millimeters above the animals head and was allowed to fall freely down the aluminum guide tube. The guide tube is threaded to allow for careful adjustment between animals to ensure accurate positioning of the guide tube and impact location (Fig 2A). The force of the impact caused the animal to break through the stretched Kimwipe©, rotate 180

degrees and land dorsally on the foam pad below. The rat falls away from the impact weight and no secondary impacts were observed. The animal's movement after impact is not mechanically constrained allowing simulation of the rotational and linear acceleration and deceleration forces most often associated with this type of injury. The animal is then monitored for righting reflex time, defined as the animal's ability to right itself from a supine to prone position. Once righted and ambulatory the animal was placed back into its home cage and monitored daily. In the rmTBI animal group, this procedure was repeated once per day for a total of 5 impacts. Age matched sham animals were given anesthesia, underwent mock impacts and were placed in a supine position to test righting reflex times. After the 5th sham or rmTBI animals were again monitored daily but left for 14 days to recover before further experimentation. All "post-injury day" (PID) descriptions were calculated as days between last impact and day of sacrifice.

Brain Fixation and Tissue Processing

At 14 days post-injury (PID 14) the animals were deeply anesthetized with isoflurane and perfused transcardially with cold 0.9% saline followed by a fixative containing 4% paraformaldehyde. The brains were then removed and fixed in paraformaldehyde overnight. The following day brains were cryoprotected in 2 stages: 15% sucrose for 24 hours followed by 30% sucrose for 24 hours. Brains selected for MRI imaging were then washed in PBS for 48 hours. For immunofluorescence, thirty-micrometer-thick sections were cut serially using a cryostat (Leica Biosytems, IL, USA) and stored at -20°C. Sections were then stained with the mature neuronal marker NeuN (Abcam, Cambridge, MA). In brief, the sections were first washed in PBS (2 x 15 minutes) before being permeabilized with 0.3% Triton-X for 1hr (Abcam, MA, USA).

Non-specific binding was blocked with CAS-Block (Life Technologies, NY, USA). Finally, the sections were moved into the primary NeuN antibody diluted to 1:2000 and incubated overnight on an orbital shaker at 4°C. The sections were then washed repeatedly in PBS and incubated with a Cy3 secondary antibody (1:1000, Jackson Immunoresearch, PA, USA) in the dark at 4°C overnight. Images were taken of both control and rmTBI animals using an epifluorescent or confocal microscope. For analysis measurements, a region of interest (ROI) of the motor cortex was created using ImageJ software (National Institute of Health) and NeuN positive cells within the ROI were manually counted. Cell count and density values are presented as the average cell count for 3 serial sections from each animal normalized to the area of the ROI.

Magnetic Resonance Imaging (MRI)

Magnetic resonance imaging (MRI) was performed on post-injury day 14 brains that had been previously perfusion fixed. Imaging was performed on a Bruker Biospec 7.0T small animal MR scanner (Bruker Medizintechnik, Karlsruhe, Germany) with 72mm transmitter coil and a rat brain surface receiver coil. 3D RARE sequence was used to acquire coronal T2-weighted image (TE 60ms, TR: 3000ms, RARE factor: 8, resolution 0.1mmx0.1mmx0.1mm, matrix 192x192x192, FOV 19.2mm x19.2mm x19.2mm, total acquisition time 3h50m) covering the posterior cerebellum to the frontal lobe. MRI data was analyzed using ImageJ software.

Electrophysiolgical Slice Preparation

Coronal brain slices were made as described previously(Anderson, Huguenard, and Prince 2010)(Anderson et al. 2005)(Iremonger et al. 2006). In brief, male Sprague-Dawley rats aged 38-45 days (PID 14-21) were deeply sedated via isoflurane inhalation

and decapitated. Brains were quickly removed and placed in ice cold (4°C) carboxygenated (95% O_2, 5% CO_2) high sucrose solution composed of (in mM): 234 sucrose, 11 glucose, 26 $NaHCO_3$, 2.5 KCl, 1.25 $NaH_2PO_4 \cdot H_20$, 10 $MgSO_4 \cdot 7\ H_20$, 0.5 $CaCl_2 \cdot 2\ H_20$. The tissue was kept in this solution while 350um thick coronal slices were taken using a vibratome (VT 1200; Leica, Nussloch, Germany). Brain slices were harvested from beneath the site of impact in rmTBI animals or from the corresponding area in sham animals. Slices were incubated for 1 hour in a water bath-warmed (32°C) container filled with carboxygenated artificial cerebral spinal fluid (aCSF) composed of (in mM): 126 NaCl, 26 $NaHCO_3$, 2.5 KCl, 10 Glucose, 1.25 $NaH_2PO_4 \cdot H_20$, 1 $MgSO_4 \cdot 7\ H_20$, 2 $CaCl_2 \cdot 2\ H_20$, pH 7.4. After the 1hr incubation, the slices were returned to room temperature before the tissue was moved to a recording chamber for whole-cell patch clamp recording.

Whole-Cell Patch Clamp Recording

Coronal brains slices prepared from rmTBI and sham animals was placed into the recording chamber and immersed in carboxygenated aCSF maintained at a temperature of 32°C. Initial visualization and identification of cortical layers was done under 4x brightfield magnification. Recordings were made from layer II/III neurons of motor cortex within the impact zone for rmTBI animals or the corresponding region in sham animals. An upright microscope (Axioexaminer, Carl-Zeiss, NY, USA) equipped with infrared differential interference contrast optics was used to acquire whole-cell patch clamp recordings from regular spiking cortical pyramidal neurons. Current-clamp firing behavior was used to identify regular spiking (RS) pyramidal neurons as previously described(Guatteo et al. 1994)(Connors, Gutnick, and Prince 1982). Electrode

capacitance and bridge circuit were appropriately adjusted. Neurons chosen for analysis had a stable membrane resistance (R_m) that was less than 20% of the input resistance (R_I), a resting membrane potential less than -55mV and over-shooting action potentials. All current and voltage clamp recordings were obtained utilizing a Multiclamp 700A patch-clamp amplifier (Axon Instruments, Union City, CA, USA). Borosilicate glass microelectrodes (tip resistance, 2.5-3.5 MΩ) were produced by a SutterP-97 automated pipette puller (Sutter instruments, Novato, CA, USA) and used for patch clamp recordings. For recording excitatory events, pipettes were filled with intracellular solution (in mM): 135 KGluconate, 4 KCl, 2 NaCl, 10 HEPES, 4 EGTA, 4 Mg ATP, 0.3Na TRIS. For recording inhibitory events, pipettes were filled with intracellular solution (in mM): 70 KGluconate, 70 KCl, 2 NaCl, 10 HEPES, 4 EGTA, 4 Mg ATP, 0.3 GTP with a calculated E_{Cl-} of -16mV resulting in inward $GABA_A$ currents at a holding potential of -70mV. This internal solution has been previously demonstrated(Anderson, Huguenard, and Prince 2010)(Sun, Huguenard, and Prince 2006) to facilitate detection of inhibitory events.

Data Analysis

Date was analyzed using pClamp (Axon Instruments), Prism (GraphPad), ImageJ and MiniAnalysis (Synaptosoft) software and is presented as means ± s.e.m. Spontaneous events were detected as previously described using automated threshold detection and manual verification(Nichols et al. in press). Input resistance was calculated from the voltage response to the input of a current step (1s, 50mV). The adaptation index was calculated based on the ratio of the last interspike interval (F_{Last}) divided by the second F_2 as per the equation 100 x (1-F_{Last}/F2). Pyramidal neurons often displayed a highly

variable first interspike interval and consequently F_2 was chosen for analysis. Firing frequency was calculated as the number of action potentials induced by a 1 second, 250pA current step. Rheobase current was determined as the minimum current step (50msec duration) that produced an action potential. Action potential threshold was calculated as the voltage at the maximum slope of the rheobase voltage recording(Nichols et al. in press). Statistical significance was determined using an unpaired t-test, one-way ANOVA or K-S test and differences determined to be significant if $P<0.05$.

Results

RmTBI is effectively modeled by repetitive weight drop

To model repetitive mild traumatic brain injury (rmTBI) in pediatric patients, we modified the weight drop method recently published by Kane et al.2012 to be used with juvenile rats (Fig 2A). To determine the reproducibility of the rmTBI method, we tested the consistency of the impact force across 20 trials. A force meter (Chatillon DFM-10, Ametek Instruments) was placed at the base of the guide tube and the peak impact force measured across 20 trials. We found the average impact force with a 92g weight to be highly consistent across trials with an average force of 7.890 ± 0.06 N and a maximum variation of less than 1N (Fig 2C. The rmTBI procedure resulted in no incidence of scalp lacerations and no immediate or late seizures were observed. At post-injury day 14 to 21 (PID 14-21) rat brains were removed for further experimentation. As previously reported, the incidence of skull fractures or intracranial bleeding was low and any animals displaying either where removed from further study(Kane et al. 2012a). Additionally no gross morphological changes, identifiable surface deformations, or tissue loss at the site of the impact were noted (Figure 2B). However, acute slices prepared from rmTBI brains revealed marked cortical thinning and ventriculomegaly (Fig 2B).

In humans, the duration of loss of consciousness (LOC) is an important criterion in assessing the severity of a brain injury. While brain injury may occur in the absence of LOC it is generally accepted that “mild” traumatic brain injuries induce LOC between a few seconds and less than 30 minutes(Carroll et al. 2004)(Smith, Johnson, and Stewart 2013). Assessing LOC in rats is difficult, but can be indirectly evaluated by measuring the righting reflex time as an indicator of neurologic restoration(Kane et al.

2012a)(Zecharia et al. 2012). Righting reflex time is defined as the time taken for an animal to right itself from a supine to prone position. Although both rmTBI and sham animals were lightly anesthetized we noted a significant increase in the righting reflex time between sham (92.31 ± 4.0s) and rmTBI animals (193.1 ± 6.7s)(Figure 2C). There was no significant change in the righting reflex time between day 1 and day 5 in sham (Day 1: 200.60 ± 18.2s vs. Day 5: 199.30 ± 14.6s) or rmTBI (Day 1: 86.92 ± 8.8s vs. Day 5: 88.59 ± 6.9s) animals ($P>0.05$ for both).

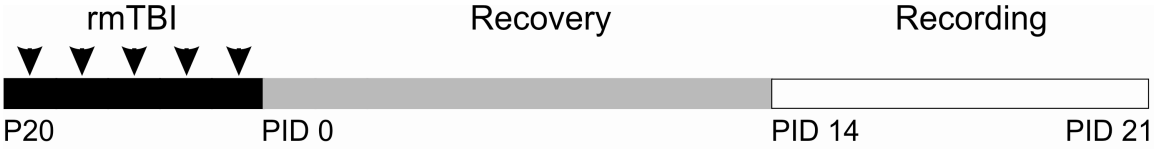

Figure 1. Experimental Timeline. Overview of the timeline used to model repetitive mild traumatic brain injury (rmTBI). Arrowheads represent time of single impact repeated once daily for 5 days. Control animals were given anesthesia only. Post-injury day (PID) indicates number of days following the 5th rmTBI injury.

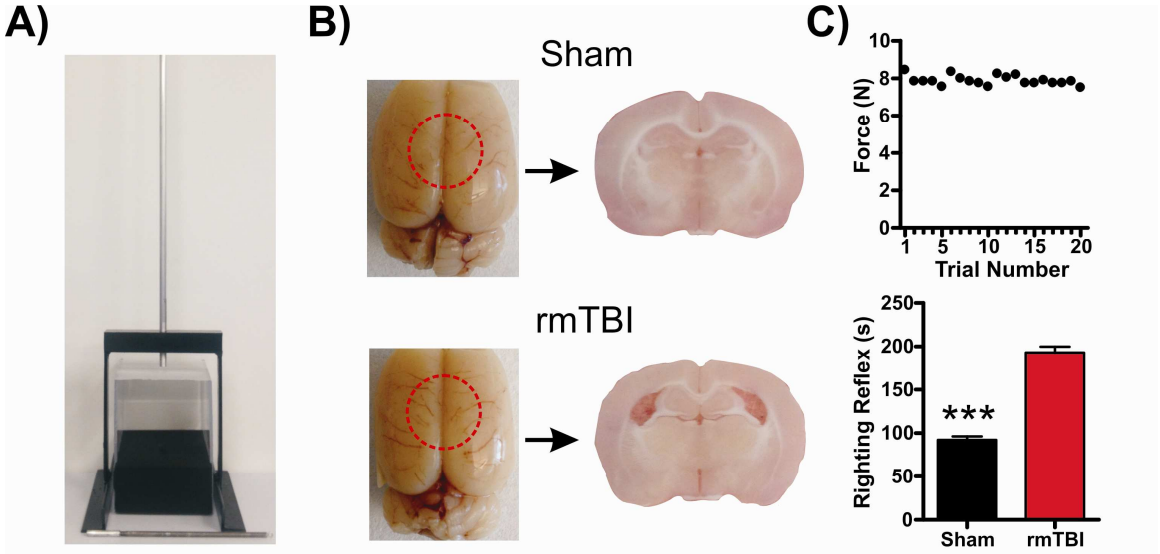

Figure 2. Experimental Model of rmTBI. **A)** Photograph of repetitive mild traumatic brain injury device and impact weight. **B)** (Left) Photographs of brains acutely prepared 14 days after sham injury or rmTBI (1 impact/day for 5 days) in juvenile (P20) rats. Red dotted circle indicates approximate site of impact. (Right) Photograph of coronal brain slices taken from respective sham or rmTBI brains. Note the presence of enlarged ventricles and cortical thinning after rmTBI. **C)** (Top) Scatter plot of impact force measurements taken across 20 trials (92g weight, 865mm drop height). (Bottom) Bar graph of average righting reflex time between sham (n=38) and rmTBI (n=42) animals. *** P<0.0001.

MRI of rmTBI reveals significant ventriculomegaly and cortical thinning

To better assess the anatomical and structural changes to the brain following rmTBI, we performed T2-weighted magnetic resonance imaging (MRI). Brains were perfusion fixed on post-injury day 14 and ex-vivo MRI imaging performed on control (n=4) or rmTBI (n=3) brains. MRI imaging was performed from the frontal cortex to posterior cerebellum. To determine changes in cortical thinning, we measured the depth of the motor, somatosensory and insular cortex across 3 brain regions – one region outside (bregma +2.3) and two regions within the direct impact zone (bregma -0.6 and -3.5). The rmTBI was delivered by a 9mm impact rod that spanned the region between bregma and lambda sutures in the rat. As imaging was performed ex-vivo, we utilized anatomical landmarks to approximate the image location relative to the impact zone and published stereotaxic co-ordinates (i.e. bregma +2.3mm, -0.6mm or –3.5mm respectively)(Paxinos and Watson 2007). In this way, we assessed changes in cortical depth across brain regions in the anterior-posterior as well as medial-lateral directions in both control and rmTBI animals. Substantial cortical thinning was observed in the motor cortex in all three brain regions with up to a 46% decrease in cortical depth within the impact zone (Fig. 4). Similarly the depth of the somatosensory cortex was significantly reduced by over 25%, but this reduction was restricted to directly within the impact zone (i.e. bregma -0.6mm and bregma -3.5mm). Measurement of the depth of the insular cortex revealed no significant difference across all three brains regions examined ($P>0.05$). We next performed similar measurements on the area of the third and lateral ventricles. While no significant difference in the area of the third ventricle was observed ($P>0.05$) the lateral ventricles area increased up to 970% after rmTBI (Fig. 5). Within the

impact zone (bregma -0.6 and -3.3), the lateral ventricle maximally increased from 1.37 ± 0.2 mm^2 to 13.30 ± 1.0 mm^2 ($P<0.0001$). Outside of the direct impact zone (bregma +2.3), the lateral ventricles were again significantly increased from 0.84 ± 0.1 mm^2 to 4.40 ± 0.4 mm^2 ($P<0.0001$). Collectively the data reveal that rmTBI induces rapid and significant reduction in the depth of the cortex and ventriculomegaly that is most substantial at the site of impact.

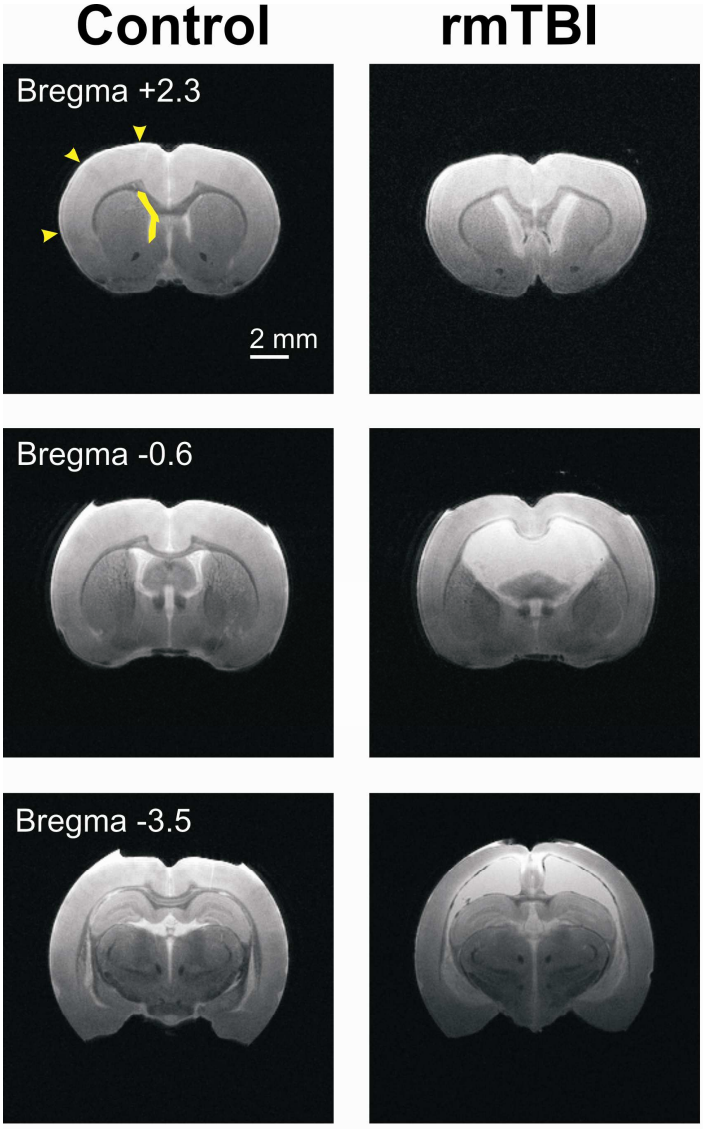

Figure 3. Magnetic Resonance Imaging (MRI) reveals significant structural changes following rmTBI. Coronal T2 weighted MRI images were obtained with a 7T MRI scanner from perfusion fixed brains 14 days after sham or rmTBI. Representative images from sham (left) or rmTBI (right) are presented. Approximate anatomical position of images are referenced relative to bregma. Scale bar represents 2mm. Yellow arrowheads and box represent regions where cortical depth and lateral ventricle area measurements where taken. Similar respective measurements where made across all sham and rmTBI images. In T2 weighted images water and edema are bright while gray and white matter appear darker. Note the significant cortical thinning and ventriculomegaly evident in rmTBI brains.

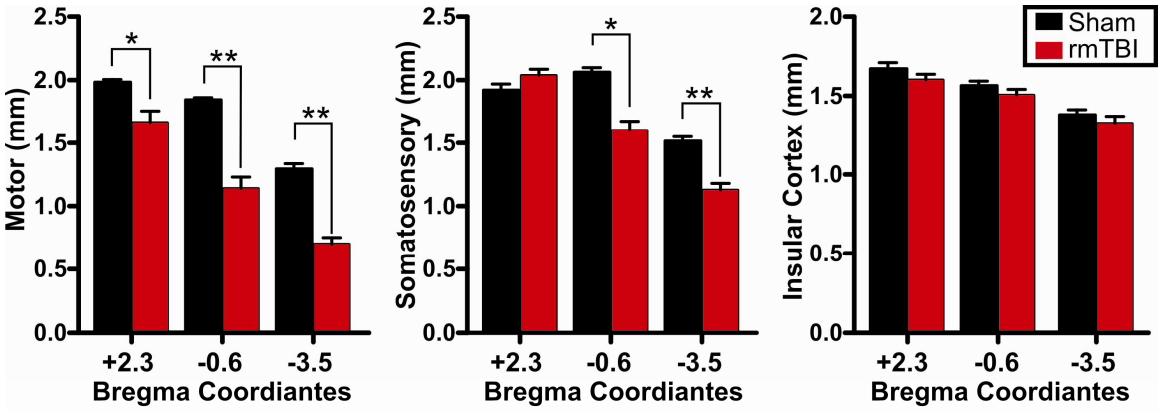

Figure 4. rmTBI induces cortical thinning. Bar charts of average cortical depth measured in MRI images from motor (left), somatosensory (middle) or insular (right) cortex. Corresponding sham (black) and rmTBI (red) bars are presented for regions in the anterior, middle and posterior positions in the brain. Positions in the brain are identified relative to approximated stereotaxic coordinates from bregma (i.e. +2.3mm, -0.6mm and -3.5mm). No statistical difference was observed between sham and rmTBI for somatosensory cortex at +2.3 ($P>0.05$), or for any insular cortex measurement ($P>0.05$). *$P<0.05$, **$P<0.01$.

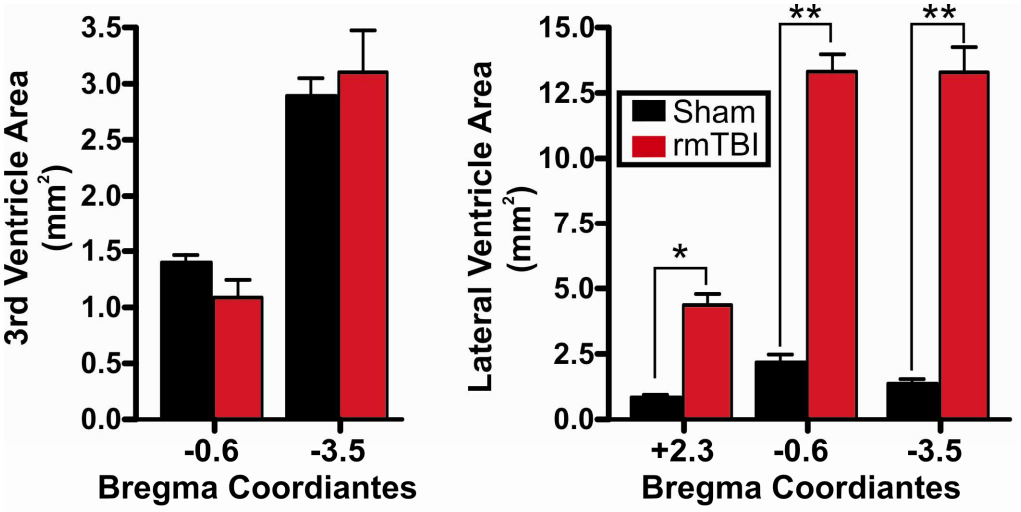

Figure 5. rmTBI induces lateral ventriculomegaly. Bar charts of average ventricle area measured in MRI images from the 3rd ventricle (left) or lateral ventricle (right). Corresponding sham (black) and rmTBI (red) bars are presented for regions in the anterior, middle and posterior positions in the brain. Positions in the brain are identified relative to approximated stereotaxic coordinates from bregma (i.e. +2.3mm, -0.6mm and -3.5mm). No statistical difference was observed in the area of the 3rd ventricle between sham and rmTBI. *P<0.05, **P<0.01.

RmTBI induces no change in neuronal density or gross tissue damage

To determine if rmTBI altered the total number or density of neurons within the cortex, we performed immunohistochemical analysis with the neuron specific marker NeuN (Fig 6A). As the amount of rmTBI-induced cortical thinning was most pronounced in the motor cortex, we focused the analysis to this region. The decrease in cortical thickness resulted in an overall decrease in total NeuN positive cells between sham (476.1 ± 17) and rmTBI (296.1 ± 24) animals ($P<0001$). However, analysis of the density of neurons (i.e. total neurons/area) revealed no significant change between shams or rmTBI animals ($P=0.21$)(Fig. 6B). Therefore, the data suggest that rmTBI induces a significant reduction in the volume of the cortex, but the cortex that remains is of similar neuronal density as sham animals.

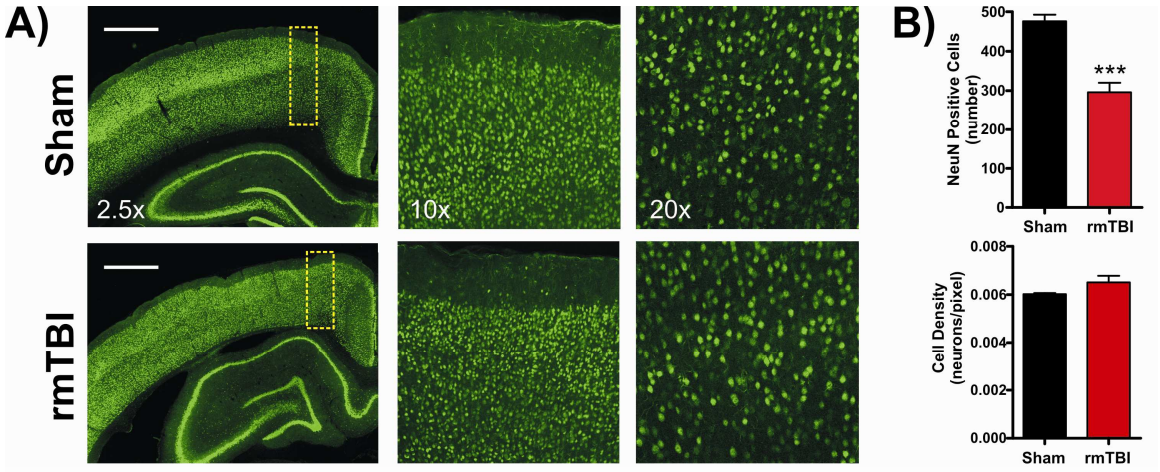

Figure 6. Effect of repetitive mild traumatic brain injury (rmTBI) on NeuN staining. A) Representative epilfluorescence and confocal images taken from sham (n=8) or rmTBI (n=6) stained with the neuron specific marker NeuN (green). White scale bar is 1mm. Images are at 2.5, 10 and 20x magnification. **B)** Bar graphs of average neuronal number (top) and density (bottom) within the motor cortex. Cell counts were made of NeuN positive cells within standardized regions of interest (yellow dotted box). Note the substantial reduction of NeuN positive cells following rmTBI but absence of neuronal density changes. ***P<0.0001.

RmTBI does not significantly alter the electrophysiological properties of layer II/III motor neurons

In humans, the consequence of rmTBI includes a host of cognitive, sensory, motor and behavioral complications(Barkhoudarian, Hovda, and Giza 2011)(Halstead, Walter, and Council on Sports Medicine and Fitness 2010)(Pellman et al. 2003). Structurally, this study has revealed a significant reduction in the depth of the cortex that is the most widespread and profound within the region of the motor cortex. Layer II/III cortical pyramidal neurons are involved in intracortical communication that is known to play a significant role in the sensory and motor processing adversely affected by rmTBI(Douglas and Martin 2004)(Kamper et al. 2013). Recently, pyramidal neurons in layer II/III of the cortex have also been reported to undergo extensive dendrite degeneration and synapse reduction following TBI(Gao and Chen 2011). As such, to determine if functional changes to the intrinsic and synaptic properties of neurons within the cortex have been altered we performed electrophysiological experiments in layer II/III pyramidal neurons 14 days after animals had received rmTBI or in age-matched shams.

Intrinsic Excitability

Intrinsic excitability refers to the propensity of a neuron to fire an action potential and is governed by the membrane properties, currents and channels expressed by a neuron. Alterations to intrinsic excitability have been shown in numerous models of CNS disorders(Willmore 1990)(Yang et al. 2007) and may contribute to the pathophysiology of rmTBI. To examine for changes in intrinsic excitability induced by rmTBI, we recorded under current clamp the response of sham (n=10) or rmTBI (n=14)

neurons to a series of hyperpolarizing and depolarizing steps (-100pA to 350pA, 50pA steps). Analysis revealed no statistical difference in the input resistance (P=0.38), resting membrane potential (P=0.77), firing frequency or accommodation index (P=0.82) between sham and rmTBI neurons (Fig. 7). Using a rheobase protocol (50msec, 5pA steps) we performed a more detailed analysis of action potential properties, but again found no statistical difference in rheobase current (P=0.73), action potential threshold (P=0.52), or amplitude (P=0.31)(Fig. 8).

Spontaneous Activity

The frequency of activity and strength of synaptic connections between neurons are fundamental to the way the brain processes and relays information. To investigate if rmTBI disrupts or alters cortical synaptic excitability we again recorded from layer II/III pyramidal neurons in the motor cortex of sham or rmTBI animals. First, under voltage clamp (V_{hold}=-70mV), we examined for rmTBI-induced changes to spontaneous excitatory post-synaptic currents (sEPSCs). To minimize detection of inhibitory events, neurons were held near and positive of the reversal potential of chloride (V_{hold}=-70mV, calculated E_{Cl-}=-80mV) and only inward synaptic events were detected. Pharmacological isolation of glutamatergic events was avoided as the resultant synaptic disinhibition may mask rmTBI induced changes to network excitability. In neurons from rmTBI animals, there were no significant changes in the average inter-event interval (P=0.77), amplitude (P=0.94), decay time (P=0.82) or charge transfer (0.34) of sEPSCs (Fig. 9). Next, we similarly examined for changes in spontaneous inhibitory post-synaptic currents (sIPSCs). Inhibitory events were pharmacologically isolated with bath application of the glutamate receptor antagonist Kyuranate. To enhance detection fidelity of inhibitory

synaptic events, a modified high intracellular chloride internal solution was used as previously described(Anderson, Huguenard, and Prince 2010)(Sun, Huguenard, and Prince 2006). Again, no significant change was observed in sIPSCs properties including inter-event interval (P=0.90), amplitude (P=0.74), decay time (P=0.33) or charge transfer (P=0.46). Representative traces and summary of these results are detailed in figure 10.

Finally, the effects of rmTBI in humans are often subtle and may not be reflected in changes to baseline synaptic activity, but only become evident during periods of high activity or demand. The reduction of cortical volume and neuronal number in rmTBI animals relative to control suggested that the peak level of network activity may be reduced in rmTBI animals. To test these possibilities we challenged pyramidal neurons from shams (n=15) or rmTBI (n=18) with the convulsant 4-Aminopyridine (4-AP, 100uM). Bath application of 4-AP for 15 minutes induced a rapid decrease in inter-event interval of sEPSCs recorded in neurons from both sham (53.59 ± 5.6s) and rmTBI animals (81.21 ± 20.0s). The amplitude of sEPSCs was similarly increased by 4-AP in neurons from both sham (22.47 ± 0.6 pA) and rmTBI (22.40 ± 1.1 pA) animals. However, neither the inter-event interval nor amplitude during application of 4-AP were statistically different between neurons recorded from sham and rmTBI animal (P>0.05). Overall, this suggests that despite a significant loss of the depth of the motor cortex in rmTBI animals, the injury fails to alter excitatory or inhibitory synaptic properties or the potential peak state of synaptic excitability.

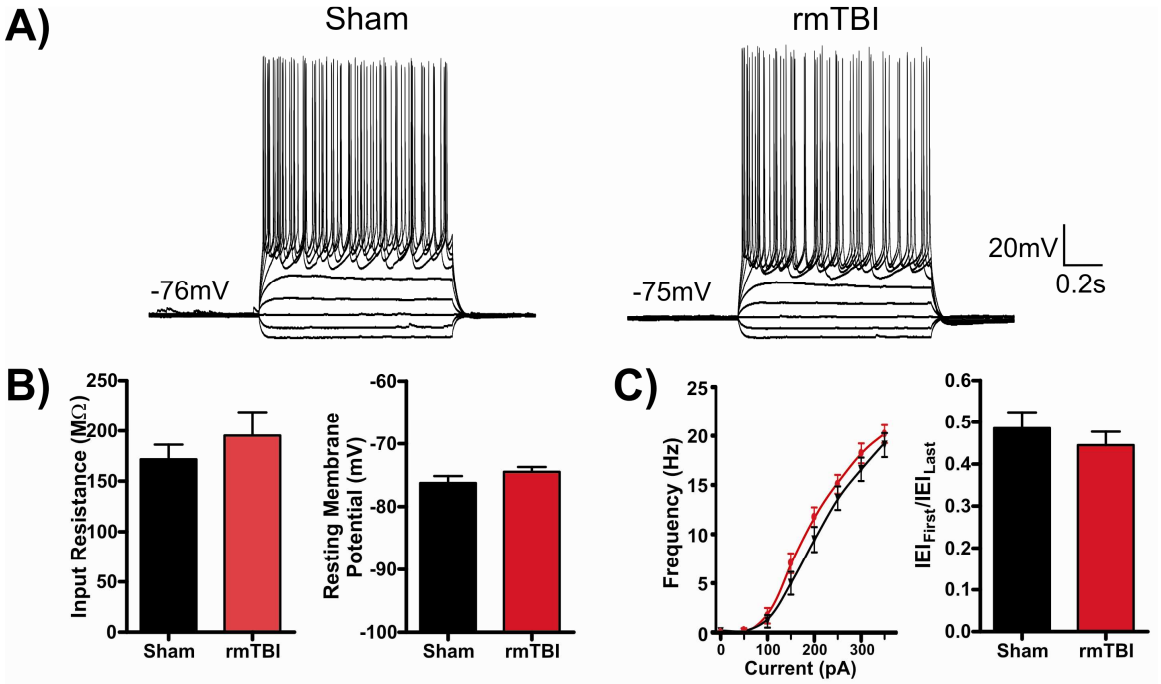

Figure 7. Intrinsic membrane properties are not altered by repetitive mild traumatic brain injury. A) Representative current clamp recordings in response to intracellular current steps (-100pA to 350pA, 1 second) in layer II/III pyramidal neurons from sham (n=10) or rmTBI (n=14) animals. Note the similarity in the intrinsic cellular response. **B)** Bar charts of average intrinsic membrane properties. No significant difference was found for input resistance (P=0.38) or resting membrane potential (P=0.77). **C)** Comparison of firing properties of a sham and rmTBI animal. (Left) Plot of average firing frequency versus current (*f-I* curve). (Right) Bar chart of adaptation index (first interevent interval between action potentials/last interevent interval). *P<0.05.

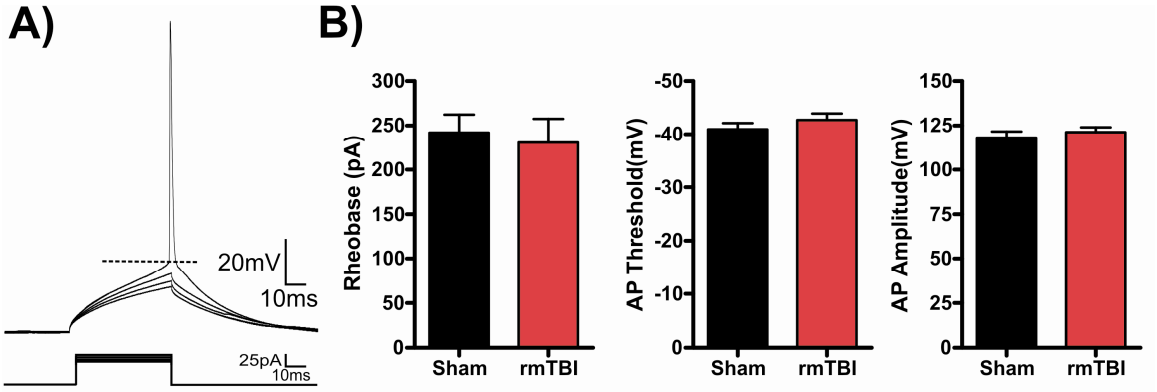

Figure 8. Action potential properties are not altered by repetitive mild traumatic brain injury (rmTBI). A) Representative whole-cell current clamp recording in response to a series of 50msec injection (5pA steps). **B)** Bar graphs of average values for sham (n=15) or rmTBI (n=18). Rheobase was calculated as the minimum current which produced an action potential. Threshold was measured at the greatest change in calculated slope. Amplitude was measured as the difference between threshold and the peak of the action potential. No statistically significant differences were found between control and rmTBI animals for rheobase (P=0.73), action potential amplitude (P=0.52) or threshold (P=0.31).

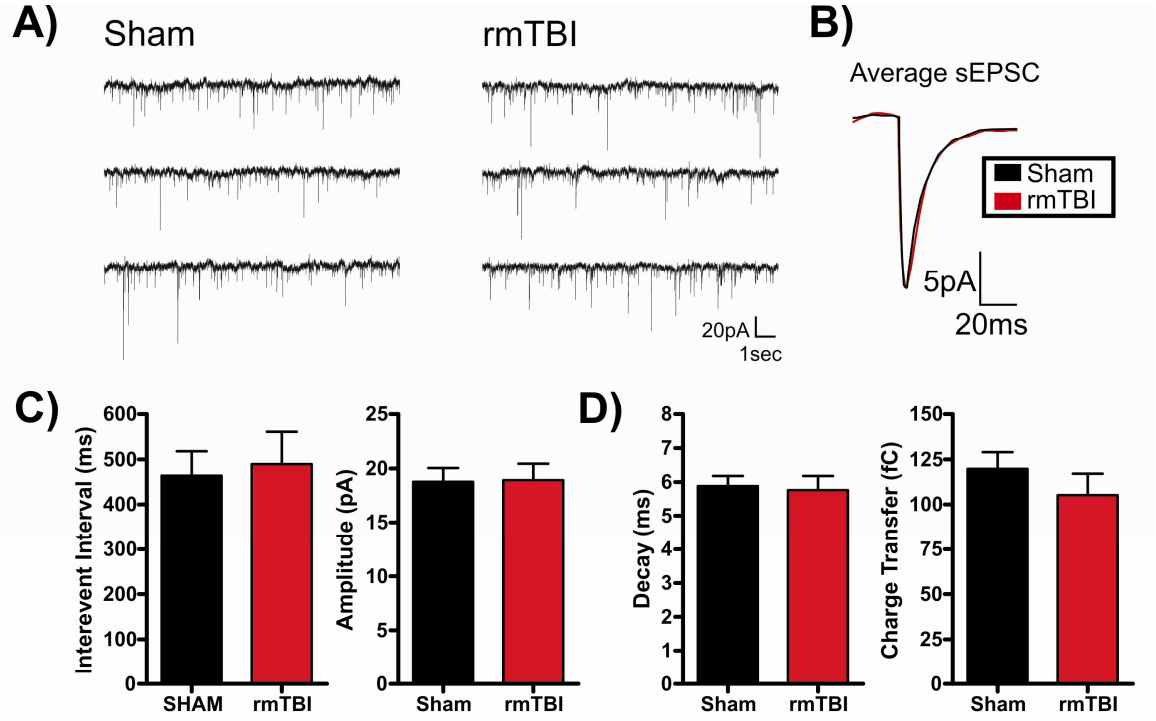

Figure 9. Excitatory spontaneous synaptic activity is not altered by repetitive mild traumatic brain injury (rmTBI). A) Voltage clamp recordings of spontaneous excitatory post-synaptic currents (sEPSC) in sham (n=19) or rmTBI (n=14) animals. **B)** Overlay of sham and rmTBI scaled average sEPSC. **C)** Bar charts of average sEPSC inter-event interval (IEI) and amplitude for sham (black) and rmTBI (red). No significant difference was determined for IEI (P=0.77) or amplitude (P=0.94). **D)** Bar graphs of average sEPSC kinetic properties. No significant difference was detected between sham and rmTBI for sEPSC decay time (P=0.82) or charge transfer (P=0.34). Vhold = -70mV.

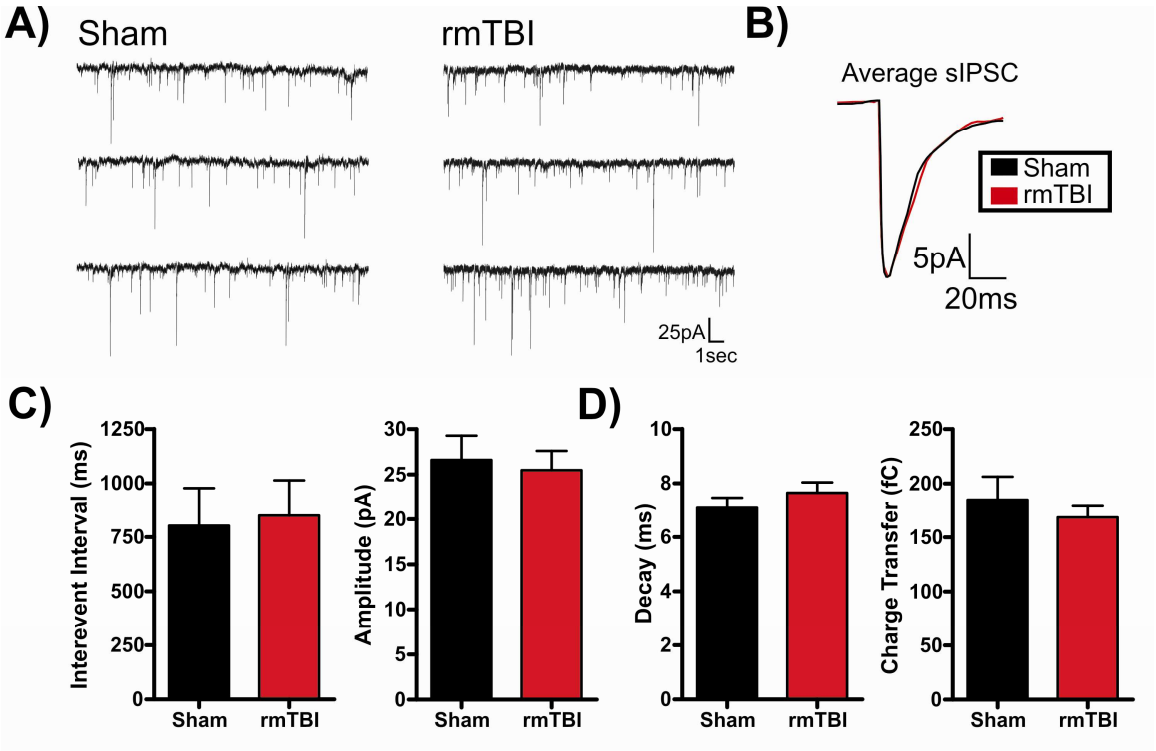

Figure 10. Inhibitory spontaneous synaptic activity is not altered by repetitive mild traumatic brain injury (rmTBI). A) Voltage clamp recordings of spontaneous inhibitory post-synaptic currents (sIPSC) in sham (n=15) or rmTBI (n=18) animals. **B)** Overlay of sham and rmTBI scaled average sIPSC. **C)** Bar charts of average sIPSC inter-event interval (IEI) and amplitude for sham (black) and rmTBI (red). No significant difference was determined for IEI (P=0.90) or amplitude (P=0.74). **D)** Bar graphs of average sIPSC kinetic properties. No significant difference was detected between sham and rmTBI for sEPSC decay time (P=0.33) or charge transfer (P=0.46). Vhold = -70mV.

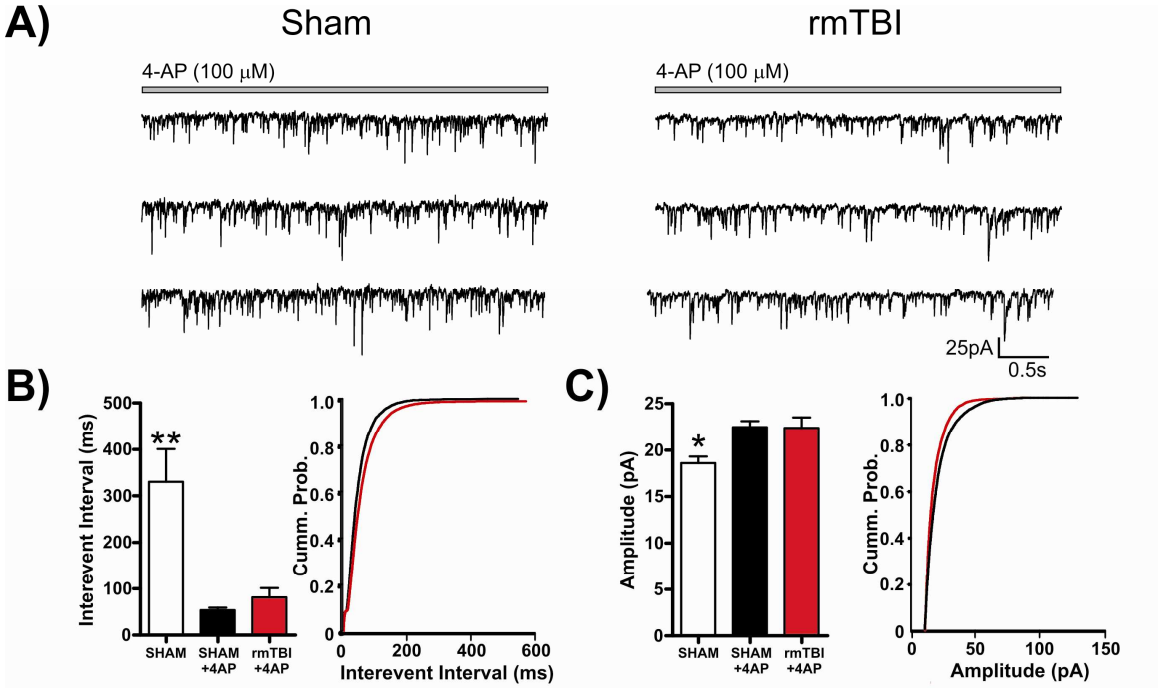

Figure 11. Repetitive mild traumatic brain injury (rmTBI) does not enhance the response to the convulsant 4-AP. A) Voltage clamp recordings of spontaneous excitatory post-synaptic currents (sEPSC) from sham (n=14) or rmTBI (n=12) animals during bath application of 4-AP (100uM). **B)** Bar chart and cumulative probability curves of sham (white), sham during 4-AP (black) and rmTBI during 4-AP (red) for inter-event interval **(B)** or amplitude **(C)**. Bath application of 4-AP induced a significant decrease in IEI and amplitude of sEPSCs. However, the effects of 4-AP on sEPSC IEI and amplitude where not statistically different between sham and rmTBI. Vhold = -70mV. *P<0.05. **P<0.01.

Discussion

In the pediatric population, traumatic brain injury (TBI) remains a significant health concern that is known to place patients at risk for adverse long-term cognitive and behavioral changes. TBI may vary in severity, but over 75% of all TBI is classified as mild(Langlois, Rutland-Brown, and Thomas 2005)(Miniño et al. 2006)(Cassidy, Carroll, Côté, et al. 2004)(Cassidy, Carroll, Peloso, et al. 2004)(Elder and Cristian 2009). In this study, we sought to determine how repetitive mild traumatic brain injury (rmTBI) affects the pediatric brain. To effectively model human rmTBI, we modified a recently developed method for inducing rmTBI(Kane et al. 2012a) in adult animals for use in juveniles. This rmTBI weight-drop method produced highly consistent impact forces across trials. The impacts occurred in a non-restrained animal and have been shown to effectively model the direct, acceleration and deceleration forces determined to be important to human TBI(Kane et al. 2012a) (Panzer, Wood, and Bass 2014)(Holbourn 1943)(Gennarelli and Thibault 1982)(Ommaya, Yarnell, and Hirsch 1967). Following mTBI, animals exhibited a significant increase in righting reflex time that suggests a brief injury induced period of "loss of consciousness". In contrast to what has generally been reported following single mTBI(Mychasiuk, Farran, and Esser 2014) or rmTBI in adult animals(Kane et al. 2012a), rmTBI in juvenile animals induced significant structural changes to the brain including cortical atrophy and ventriculomegaly. This is supported by recent evidence that indicates children may be more prone to the effects of repeat concussions(Eisenberg et al. 2013)(Field et al. 2003). Neuronal specific immunostaining revealed that the cortical atrophy was accompanied by a loss of total cortical neurons. However, this overall neuronal loss was not due to a specific reduction in cortical density.

The cortical atrophy was most pronounced in the motor cortex with up to a 46% decrease in cortical thickness beneath the site of injury in rmTBI animals. Despite the significant structural changes to the motor cortex, we observed no significant changes in the intrinsic or synaptic properties of layer II/III pyramidal neurons at rest or under convulsant challenge. Overall our results indicate the effectiveness of this new weight-drop method for reliably inducing a clinically relevant rmTBI. The select changes induced by rmTBI in juvenile rats suggest a potentially unique pathophysiological response to TBI in children.

Modeling repetitive mild traumatic brain injury (rmTBI)

Recent attention by patients, families, researchers and the media has highlighted the significant short and long term consequences of rmTBI (Allen, Gerami, and Esser 2000)(Creeley et al. 2004)(Longhi et al. 2005)(Shitaka et al. 2011)(Friess et al. 2009). Critical to understanding the pathophysiological mechanisms that drive rmTBI has been the development of new, clinically relevant models. Effective modeling of rmTBI requires an induced injury that reflects the type of impact and forces known to occur in mTBI and that results in neuropathological and clinically relevant outcomes. Mild TBI is characterized as occurring in a closed skull with minimal skull fractures and minimal tissue loss after a single mTBI. The impact of the mild TBI induces direct force to the skull that translates into acceleration, deceleration and shearing forces in the brain that are thought to be important to the injury process(Duhaime et al. 2012). Several models of TBI exist, including controlled cortical impact and fluid percussion(Xiong, Mahmood, and Chopp 2013), but these require a craniotomy and/or a fixed skull that inadequately models these forces. Limited data exists on the exact biomechanical forces

that would be classified as “mild” or concussion inducing, but the most comprehensive data has been obtained from head impact telemetry devices placed within athlete’s helmets. An in depth review of the combined telemetry impact studies revealed that concussion is correlated with g-forces above 100g(Beckwith et al. 2013). In our study, calculated impact forces were on average 26.8g and well within the “mild” range (i.e. G-Force = (F=ma)/9.8m/s^2; F=7.89N, m= 30g (P20-25rat). The method used in this study overcomes these limitations and effectively models both the biomechanical forces of the impact as well as shown to induce clinically relevant cognitive and behavioral clinical changes(Kane et al. 2012a)(Panzer, Wood, and Bass 2014)(Gennarelli and Thibault 1982)(Meaney and Smith 2011)(Mychasiuk, Farran, and Esser 2014).

Repetitive mild traumatic brain injury (rmTBI) induces significant neuropathology

A single mild traumatic brain injury (mTBI) often resolves quickly and has generally not been associated with any significant neuroimaging abnormalities (Petchprapai and Winkelman 2007)(Belanger et al. 2007)(Morey et al. 2013). As a result, mTBI is often referred to as an “invisible wound” and is difficult to diagnose. Whether a single mTBI induces long-term deficits is currently a source of significant debate(Vasterling et al. 2012)(Konrad et al. 2011)(Vanderploeg, Curtiss, and Belanger 2005)(Klein, Houx, and Jolles 1996)(Carroll et al. 2004)(Yuh, Hawryluk, and Manley 2014). It is clear, however, that when a patient receives multiple mTBI within a short period of time the result is more severe symptoms, a longer recovery period and increased risk for serious long term consequences(K. M. Guskiewicz et al. 2000)(Kevin M. Guskiewicz et al. 2003). In contrast to a single mTBI event, repetitive mild traumatic brain injury (rmTBI) patients show clear neuropathological findings including enlarged

ventricles (ventriculomegaly) and cortical atrophy(Smith, Johnson, and Stewart 2013)(Huh, Widing, and Raghupathi 2007). These findings are supported by the results of this study which indicate that following rmTBI, the lateral ventricles may be increased up to 970% while the thickness of the cortex may be reduced by up to 46%. These changes were not observed following rmTBI in adult animals(Kane et al. 2012b) suggesting a potentially unique response to TBI in juvenile animals. In humans, rmTBI can induce a neurodegenerative disease termed chronic traumatic encephalopathy (CTE)(Gavett, Stern, and McKee 2011)(Smith, Johnson, and Stewart 2013) that has been most commonly found in professional athletes(Omalu, Hamilton, et al. 2010)(Omalu, Fitzsimmons, et al. 2010)(McKee et al. 2009) or soldiers exposed to blast or concussive injury(Goldstein et al. 2012). CTE can currently only be diagnosed on autopsy, but results in degeneration of brain tissue (i.e. cortical atrophy) and ventriculomegaly similar to what was observed in this study. Additional characteristics of CTE include tau accumulation, cognitive impairments, memory loss, confusion and depression(H. Miller 1966)(McKee et al. 2009)(McKee et al. 2010). Further work examining these characteristics will be required to determine if rmTBI in this study induced CTE. CTE has normally been examined in older adult brains, but a recent autopsy by McKee et al., of an 18 year old high school football player with a history of repeat concussions confirmed the presence and ability of pediatric brains to experience CTE. It remains unknown how the impact severity, time between, and incidence of TBI events influence the development of CTE, but it is clear that repetitive TBI is a significant risk factor. In our study, the ventricular enlargement was isolated to the lateral ventricle and did not include any significant changes to the third ventricle. This is in contrast to findings from

studies of professional athletes who have been diagnosed post-mortem with chronic traumatic encephalopathy (CTE) and displayed both third and lateral ventriculomegaly(Mez, Stern, and McKee 2013). However, those studies were performed many years after the injuries were incurred, whereas our study only examined brains 14 days after injury. As CTE is thought to be a progressive neurodegeneration disease, analysis of MRI across a wider range of time points following injury (acute and long term) may reveal significant additional findings.

Cortical excitability is not altered by repetitive mild traumatic brain injury (rmTBI).

Structurally, this study revealed extensive thinning of the cortex that was most pronounced beneath the site of injury in the motor cortex. Immunohistochemical staining revealed that rmTBI reduced the total number of cortical neurons, but this was not accompanied by a decrease in neuronal density. The significant loss of motor cortex is supported by several studies which have indicated persistent motor dysfunction and abnormalities in the motor cortex following mTBI (De Beaumont et al. 2011)(De Beaumont et al. 2012)(Sara Tremblay et al. 2014). In addition, many of the behavioral deficits associated with rmTBI such as balance, reaction time, and visual memory involve high levels of integration across cortical regions(Slobounov et al. 2007) (Khurana and Kaye 2012)(Covassin, Stearne, and Elbin 2008) which are thought to be governed by input and output from layer II/III cortex(Douglas and Martin 2004)(Kamper et al. 2013). This is in line with a recent study that has found mTBI induces specific dendritic degeneration and synaptic reduction in cortical layer II/III pyramidal neurons(Gao and Chen 2011). As such, in this study we began by examining for rmTBI induced changes in

the intrinsic and synaptic properties of layer II/III pyramidal neurons within the motor cortex.

A neuron's intrinsic excitability determines the probability it will fire an action potential and the output pattern of that firing has been shown to contribute to the pathophysiology of several other neurological disorders(Prince and Connors 1986)(Bush, Prince, and Miller 1999)(Prinz et al. 2013)(van Zundert et al. 2012). However, we investigated several possible measures of intrinsic excitability and found no significant differences between our rmTBI and sham groups. This finding is supported by recent work from our lab where even severe TBI in juvenile rats failed to alter the intrinsic properties of cortical pyramidal neurons(Nichols et al. in press). At a synaptic level, again no significant changes were found in the strength, frequency, or kinetics of excitatory or inhibitory synaptic neurotransmission following rmTBI. To our knowledge this is the first study to investigate detailed intracellular electrophysiological changes following rmTBI. In humans, the use of transcranial magnetic stimulation from 72 hours to 2 months after mTBI has shown increases in intra-cortical inhibition(N. R. Miller et al. 2014). Young athletes who have sustained multiple concussions have also been reported to have abnormal intracortical inhibition(De Beaumont et al. 2007)(Sara Tremblay et al. 2011)(De Beaumont et al. 2011). While no change in inhibition onto pyramidal neurons was observed in this study, future examination of the impact of rmTBI directly on other cortical layers and inhibitory interneurons may reveal distinct changes. The lack of synaptic excitability changes observed following rmTBI in this study contrast with recent findings following severe TBI from our lab in juvenile rats (Nichols et al. in press), and

from previous reports in adult animals(Cantu et al. 2014). This supports the literature that suggests the pathophysiology of rmTBI is distinct from more severe TBI.

The lack of intrinsic and synaptic changes in layer II/III pyramidal neurons following rmTBI may be an important component to understanding the "silent" nature of the injuries. In fact, the effects of mTBI may often be subtle and only evident when the cortex is challenged with a high demand task(Abdel Baki et al. 2009). With the clear loss of mature neurons and significant cortical atrophy, we hypothesized that differences may arise only when the cortex was put under "stress". To test this, we examined the synaptic properties of sham and rmTBI animals during application of 4-Aminopyridine (4-AP), a potassium channel blocker and known convulsant. 4-AP has been shown to increase synaptic excitability(Boudkkazi, Fronzaroli-Molinieres, and Debanne 2011)(Buckle and Haas 1982) and affect cortical pyramidal neuron intrinsic excitability(Higgs and Spain 2011)(Shu et al. 2007). As expected, both the frequency and amplitude of spontaneous excitatory activity were increased from control periods by bath application of 4-AP. However, the effects of 4-AP were not statistically different between sham and rmTBI animals. Therefore, even when cortical excitability is pharmacologically increased, rmTBI animals remain equally responsive and able to enhance synaptic activity as compared with sham animals. In this study we only tested the response of a saturating dose of 4-AP that produces near maximal level of synaptic activity. The use of a dose-response protocol may reveal changes in the threshold to 4-AP following rmTBI.

In conclusion, rmTBI has been associated with serious clinical consequences including chronic traumatic encephalopathy and an increased risk for the development of dementia and neurodegenerative diseases(McKee et al. 2009)(McKee et al. 2010)(Omalu,

Fitzsimmons, et al. 2010). In this study, we found that rmTBI can be effectively modeled in young animals using a modified weight drop method. The impacts can be consistently delivered and replicate clinically relevant impact forces and structural changes including cortical atrophy and ventriculomegaly. This method of inducing mTBI has also recently been shown in juvenile(Mychasiuk, Farran, and Esser 2014) and adult(Kane et al. 2012a) animals to induce clinically relevant changes to cognition and behavior. At present, the findings from this study suggest the pathophysiology of rmTBI may be unique when occurring in pediatric patients. An improved understanding of how the pediatric brain responds to rmTBI may help identify novel therapeutic targets, influence pediatric treatment and improve "return to game" decision making in adolescents.

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CHAPTER 3

DISCUSSION

Mild traumatic brain injury (mTBI) is an epidemic throughout the United States. It affects nearly 1.4 million individuals every year, with children being particularly susceptible. The clinical symptoms of mTBI usually resolve rapidly with little intervention necessary. However, the underlying effects may be much more far-reaching. Repeated insults have been shown to exacerbate both the acute and long term consequences of mTBI, but the mechanisms underlying these observations are poorly understood. In order to reproduce as authentic of an injury as possible, we chose a weight drop technique that left the skull intact, did not restrain the head, and did not utilize any helmet or cap. This model was demonstrated by Kane et al to produce cognitive and behavioral outcomes in adult mice that were comparable to the symptoms reported after mTBI in humans. We modified this method to utilize pediatric-aged rats in order to investigate the unique effects mTBI have on this vulnerable age group. After repeated mTBI, we observed an increase in the size of the lateral ventricles that was clearly evident by upon dissection. To further investigate this phenomenon, we utilized magnetic resonance imaging (MRI). The scans confirmed our observations and allowed us to quantify this ventriculomegaly. In addition, the scan revealed an uneven thinning of the cortex in the brains of rmTBI animals. The level of cortical thinning corresponded to the relative proximity of the cortical region to the impact center. An immunohistochemical analysis of rmTBI animals revealed that the cortical thinning was not accompanied by any change in neuronal density. At a cellular level, we recorded electrical activity from layer II/III pyramidal neurons present in the motor cortex of rmTBI animals. These

neurons showed no significant change in their electrophysiological properties when compared to their sham counterparts. They also responded similarly to their sham counterparts when challenged with a known convulsant, 4-Aminopryidine.

Weight drop injuries consistently reproduce mild traumatic brain injury criteria

In order to successfully study the effects of repeated mild traumatic brain injury (rmTBI), we had to ensure that every injury we imparted was consistent and appropriate. Determining consistency was a straight-forward process of recording the impact force of 20 trial runs with a force meter. The results showed an average impact of 7.890 newtons (N) with a standard deviation of 0.06 N, yielding a coefficient of variation (CV) of 0.76%. When compared to a published value for impact velocity using a controlled cortical impact device (5.23 +/- 0.03 m/s, CV = 0.57%) (Brody et al 2008) we saw that our weight drop model was nearly as consistent as a computer controlled model. Therefore we could confidently proceed, knowing that every impact delivered a reliable and consistent force.

Assessing the appropriateness of our impacts was a more complex matter. Several studies have attempted to utilize force, lateral acceleration, rotational acceleration, or a combination of those measurements to create biomechanical criterion for mTBI (Duhaime et al 2012). However, these attempts have been far from precise and have only been performed in human subjects. Use of any criteria based on human studies would also have to be scaled to our animal model. Scaling between species is a very difficult proposition that involves a multitude of variables. Differences in physical morphology, tissue properties, and metabolism all play a role in how the brain will respond to an injury (Panzer, Wood, & Bass 2014). Scaling models using body mass or brain mass ratios have

been proposed, however none have been validated using a range of species (Panzer, Wood, & Bass 2014). Considering the lack of an established mTBI impact force criteria and the difficulties involved in human to animal scaling, we have chosen to gauge our models appropriateness by injury outcomes instead of by biomechanical properties.

Mild TBI is generally defined as any non-penetrating injury to the head that involves one or more of the following: a period of confusion or unconsciousness not to exceed 30 minutes, any period of amnesia around the time of injury not to exceed 24 hours, or any observed signs of neurological distress (NCIPC 2003). While some of those criteria are difficult to evaluate in an animal model, righting reflex time in rodents is commonly correlated to loss of consciousness in humans (Kane et al 2012) (Zecharia et al 2012) Righting reflex time is defined as the time it takes an animal to right itself from a supine to prone position under its own power. After a single impact from our model, injured animals showed a significant increase in their righting times when compared to sham animals (rmTBI: 200.6s versus sham: 86.92s). This effect was seen after each of the five injuries imparted and remained consistent between the first and last injuries (rmTBI day 1: 200.6s versus rmTBI day 5: 199.3). This shows an average increase in righting reflex time of approximately 100 seconds. This period of unconsciousness places the injury severity of our model well within the bounds of accepted mTBI criteria.

Further evaluation of human mTBI studies reveals more commonalities between clinical mTBI and the injury produced by our model. While they have yet to show significant prognostic use, neuroimaging can be useful in determining if an injury is truly mild or whether it should be escalated. In a review of neuroimaging studies, one study showed that only 5% of patients diagnosed showed signs of skull fracture. Another study

showed that only 3% of patients with mTBI showed any abnormality including hematomas, hemorrhaging, skull fractures, and intracranial lesions (Borg et al 2004). A statistical analysis of TBI in the U.S. showed a high incidence of mTBI, but only a 0.07% mortality rate (Bazarian et al 2005). These findings suggest that any model designed to replicate mTBI should have a very low incidence of any of these factors: skull fracture, intracranial bleeding, tissue cavitation/lesion, or death. The injuries produced by our model showed low to zero incidence in all of those areas.

We have demonstrated that the weight drop method described in chapter 2 of this thesis delivers a consistent and reliable impact force. Based on generally accepted inclusion criteria, the injury produced by our model can confidently be described as a mild traumatic brain injury. This description is further supported by its low incidence of mortality or escalating factors.

Magnetic resonance imaging reveals ventricular enlargement and atrophy of the cortex after repeated mild traumatic brain injury

A frequent finding in the study of mild traumatic brain injury is that a single injury often has transient clinical symptoms and minimal neuropathology (Weil, Gaier, & Karelina 2014) (Levin & Robertson 2013). Although there is emerging evidence that a single injury can have lasting cognitive effects (Dawish et al 2012). In contrast, repeated injuries result in more pronounced acute symptoms that take longer to resolve, often result in long term deficits, and demonstrate more pronounced neuropathology (Tremblay et al 2013) (Mannix et al 2013) (Slobounov et al 2007) (Covassin, Moran, & Wilhelm 2013). This is particularly true when the injuries occur within 24 hours of each other (Bolton & Saatman 2014) (Prins et al 2013) (Weil, Gaier, & Karelina 2014). Two

features that are clearly evident in cases of repeated mTBI (rmTBI) that are not generally seen after a single mTBI are cortical atrophy and enlargement of the ventricles (ventriculomegaly) (Smith, Johnson, & Stewart 2013). To further investigate this phenomenon, we performed magnetic resonance imaging (MRI) on animals that received a single mTBI, animals that received 5 mTBI at 24 hour intervals, and age matched sham animals that only received anesthesia. In the injury cohort, we performed the imaging 14 days after the last injury was imparted (PID 14). In the sham group, we performed the imaging at an age-matched time point to our injured cohorts. In line with previous imaging studies, our results showed no differences in ventricular size of cortical atrophy between the singly impacted and sham groups (Eierud et al 2014) (Yuh, Hawryluk, & Manley 2014). However, our rmTBI group showed significant changes in both lateral ventricle size and regions of pronounced cortical atrophy.

Interestingly, in our study, the ventricular enlargement was isolated to the lateral ventricle and did not include any significant changes to the third ventricle. This separates our findings from studies of professional athletes who have been diagnosed post-mortem with chronic traumatic encephalopathy (CTE). In those studies, both the lateral ventricle and the third ventricle showed signs of enlargement (Mez, Stern, & McKee 2013). However, those studies were performed many years after the injuries were incurred, whereas our study only included a time point of PID 14. Since CTE is thought to be a progressive neurodegeneration disease, involvement of the third ventricle may only be present as the disease progresses. This possibility could be tested by performing MRI analysis across a wider range of time points, spanning from the acute (days post injury) to the long term (months to years' post injury).

The MRI scanning procedure takes serial images from the posterior cerebellum to the end of the frontal cortex, thus producing a series of 2-dimensional coronal sections beginning caudally and ending rostrally. In order to better characterize our findings, we compared measurements across three distinct longitudinal areas with respect to the center of the impact zone. The impact zone stretched from the coronal sutures Bregma to Lambda (approx. 9mm) with its center at approximately 4.5mm caudal to Bregma. Utilizing anatomical features found on the MRI scans and comparing them to coordinates from *The Rat Brain in Stereotaxic Coordinates*, we selected three images from each series. The first area was approximately under the center of impact, the second was near the rostral limit of the impact zone, and the last area was several millimeters rostral of the impact zone. We determined the size of the lateral ventricles at each of these three areas. Within these three areas we also measured the cortical thickness (to determine cortical atrophy) of three different cortical regions. From dorsal-medial to lateral-ventral, we choose a region proximal to the impact, a region distal from the impact zone, and a region in between. Approximately these three regions correspond to the motor cortex, the insular cortex, and the somatosensory cortex respectively. By comparing measurements taken at each of these areas/regions, we were able to get an idea of how the changes in ventricular size and cortical atrophy correlated with proximity to the impact center both rostral-caudally and medial-laterally.

The lateral ventricular size was significantly increased at all three areas measured. However, the most pronounced increases were seen in the two areas within the impact zone. The area rostral to the impact zone saw an increase of approx. 5 fold while the area within the impact zone saw more drastic increases of approx. 9 fold. This may indicate

that the lateral ventricular increase is initiated under the impact zone and then extends to areas outside the injury zone. To my knowledge, no other study has compared ventricular size with the relative proximity to the impact site.

The cortical atrophy we observed also correlated with proximity to the impact zone. The most lateral-ventral region measured, the “insular cortex”, did not show any significant atrophy neither in the areas within the impact zone nor the area rostral to the impact zone. The “somatosensory” region showed significant cortical atrophy in the areas within the impact zone, but not the area rostral to the impact zone. The most dorsal-medial region, the “motor” cortex, showed significant atrophy in both the areas under the impact zone as well as the area rostral to the impact zone. While these findings point toward more diffuse cortical atrophy as proximity to the impact increases, the cause remains unknown. It may also be the case that the regions most grossly affected are simply more vulnerable to atrophy regardless of where the impact occurred. Human studies have shown that the frontal and temporal lobes are particularly susceptible to atrophy in cases of moderate and severe TBI (Bergson et al 2004). It could also be attributed to flexibility of the immature cranial vault deforming longitudinally. Future studies could utilize a weight with a smaller diameter and multiple impact sites (dorsal, lateral, frontal, caudal) to compare cortical atrophy in relation to impact proximity.

To better understand our cortical atrophy findings, we stained the tissue with the mature neuron marker NeuN. In line with previous mTBI studies, we showed no signs of gross tissue loss or cavitation at the site of impact (Petraglia et al 2014) (Kane et al 2012) (Mychasiuk, Farran, & Esser 2014). While the cortex was thinned, the density of the neurons remained unchanged compared to sham controls. Additionally, no differences in

the dispersion or composition of neurons within the cortical layers were evident between sham and rmTBI. This indicates a generalized loss of NeuN positive cells from all cortical layers accounting for the overall atrophy. As we did not perform cognitive or behavioral testing we do not know if this globalized neuronal loss correlated with any particular deficit. However, previous studies in both humans and animal models have shown correlations between degree of cortical atrophy and a variety of cognitive, behavioral, motor, and emotional deficits (Bergeson et al 2004) (Kamper et al 2013) (McKee et al 2009) (Tremblay et al 2012). In future studies we plan to test this possibility by pairing MRI imaging with various outcome testing.

Repeated Mild Traumatic Brain Injury Does Not Significantly Alter the Electrophysiological Properties of Layer II/III Motor Cortex Pyramidal Neurons

Structurally, this study revealed significant and extensive thinning of the motor cortex. Immunohistochemical staining revealed that the density and distribution of mature neurons was unchanged between rmTBI and sham groups. This led to the conclusion that generalized neuronal loss was one of the factors behind the observed cortical atrophy. A recent study of surviving layer II/III pyramidal neurons present after mTBI demonstrated extensive dendritic degeneration and synaptic reduction (Gao & Chen 2011). Furthermore, many of the behavioral deficits associated with rmTBI such as balance, reaction time, and visual memory involve high levels of collaboration across cortical regions (Slobounov et al 2007) (Khurana & Kaye 2012) (Covassin, Stearne, & Elbin 2008). As layer II/III is the main hub of cortico-cortico communication, there was strong evidence to support investigating pyramidal neurons within this layer of the motor cortex (Staiger 2010) (Douglas & Martin 2004). Therefore, we conducted

electrophysiological experiments within this population to determine if any abnormal functionality could be elucidated.

There are two main tests of a neuron's functionality; its intrinsic excitability and its spontaneous firing activity. A neuron's intrinsic excitability determines the likelihood that it will fire an action potential. Several neurological disorders have been linked to abnormal intrinsic excitability, and it may contribute to the pathophysiology observed in rmTBI (Prinz et al 2013) (Holth et al 2013) (Van Zundert et al 2012). We investigated several possible measures of intrinsic excitability and found no significant differences between our rmTBI and sham groups. Since our structural findings were present at 14 days post-injury, we used a time point of 14 to 21 days post-injury for our electrophysiological recordings as well. While we observed no abnormalities within this timeframe, it should be noted that they may be present at an earlier or later time point. They may only accompany the transient acute symptomology or they only may be evident once further neurodegeneration occurs. Furthermore, we only targeted pyramidal neurons present in a specific layer of a specific cortical region. Extensive further investigation is required to determine what role intrinsic firing properties play in the pathophysiology of rmTBI.

A neuron's spontaneous synaptic activity is determined by the activity and strength of its connections to other neurons. Changes in spontaneous activity reflect the state of the neural network that the recorded neuron is connected to. Altered spontaneous activity is a hallmark of neurological diseases that involve the abnormal recruitment and sustainment of network synchronicity such as epilepsy. A link between severe TBI and development of spontaneous epileptiform activity has recently been demonstrated our lab

(Nichols et al 2014). To investigate if any abnormal spontaneous activity was present, we once again recorded from layer II/III pyramidal neurons within the motor cortex. Our investigation timeframe was 14 to 21 days after the last injury occurred. We isolated both the excitatory and the inhibitory spontaneous activity, however, neither one demonstrated any significant change to their activity rate or kinetic profile. These findings have the same limitations as our observations concerning intrinsic firing properties. Additional time points, cell types, cortical layers, and cortical regions must be investigated before the complete picture regarding rmTBI and spontaneous activity can be determined.

The effects of mTBI can often be subtle and may only be evident when the cortex is placed under high demand (Baki et al 2009). With the clear loss of mature neurons and significant cortical atrophy, we hypothesized that difference may arise when the cortex was put under stress. To test this, we applied the known convulsant 4-Aminopyridine (4-AP) to our pyramidal neurons and recorded their ability to respond. In healthy pyramidal neurons, application of 4-AP results in a significant increase in the rate and amplitude of its spontaneous synaptic activity. Both our rmTBI and sham groups responded similarly to the application of 4-AP, with no significant differences observed. This indicates that despite the generalized loss of mature neurons, the surviving layer II/III pyramidal neurons retain their maximum excitability state. However, we only tested the response to a saturating dosage. Through the utilization of a dose-step protocol that incrementally increased the concentration of 4-AP, it may be possible to determine if rmTBI neurons differ in their excitability threshold. It is conceivable that rmTBI neurons could demonstrate a higher sensitivity to the convulsant. It is clear from our findings that extensive future research must be undertaking to elucidate the underlying

pathophysiological mechanisms behind the functional deficits associated with repeated mild traumatic brain injury.

Conclusion

Mild traumatic brain injury (mTBI) is an epidemic that affects millions of individuals every year. A history of multiple mTBI has been shown to be associated with an increased risk of long term cognitive, behavioral, and functional deficits (Jordan 2013) (Petraglia et al 2014) (Aungst et al 2014). Repetitive mTBI (rmTBI) is also a risk factor for the development of dementia and neurodegenerative diseases including chronic traumatic encephalopathy (Chen & D'Esposito 2010) (Masel & DeWitt 2010) (Plassman et al 2000). Unfortunately, many of those affected are young and otherwise healthy children participating in various sporting endeavors. Much of the current research focus regarding mTBI and the effects of repeated mild traumatic brain injury (rmTBI) has been on adult subjects and many of the current models are geared towards that age group. Pediatric and juvenile age groups have an array of unique physiologic attributes and their response to injury is often markedly different than their older counterparts. In this study, we modified an adult model of rmTBI for use with a younger cohort. This model has been shown to reproduce many of the functional outcomes associated with mTBI in adult (Kane et al 2012) and pediatric (Mychasiuk, Farran, & Essar 2014), while also maintaining an authentic injury mechanism. We further characterized the models consistency, showing that it produces a reliable and reproducible impact force. The ventriculomegaly, cortical atrophy, and generalized neuronal loss observed in this study have not been previously reported in older age groups at this time point. This could be an indication of an age-related pathophysiology that requires further investigation to fully

characterize. In order to best treat mTBI and to mitigate the long term effects of rmTBI, we need to understand not only the mechanisms of injury, but also how different age groups respond to them.

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