Traumatic Brain Injury Induces Rapid Enhancement of Cortical Excitability in Juvenile

Rats

by

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ABSTRACT

Following a traumatic brain injury (TBI) 5-50% of patients will develop post traumatic epilepsy (PTE). Pediatric patients are most susceptible with the highest incidence of PTE. Currently, we cannot prevent the development of PTE and knowledge of basic mechanisms are unknown. This has led to several shortcomings to the treatment of PTE, one of which is the use of anticonvulsant medication to the population of TBI patients that are not likely to develop PTE. The complication of identifying the two populations has been hindered by the ability to find a marker to the pathogenesis of PTE. The central hypothesis of this dissertation is that following TBI, the cortex undergoes distinct cellular and synaptic reorganization that facilitates cortical excitability and promotes seizure development. Chapter 2 of this dissertation details excitatory and inhibitory changes in the rat cortex after severe TBI. This dissertation aims to identify cortical changes to a single cell level after severe TBI using whole cell patch clamp and electroencephalography (EEG). The work of this dissertation concluded that excitatory and inhibitory synaptic activity in cortical controlled impact (CCI) animals showed the development of distinct burst discharges that were not present in control animals. The results suggest that CCI induces early "silent" seizures that are detectable on EEG and correlate with changes to the synaptic excitability in the cortex. The synaptic changes and development of burst discharges may play an important role in synchronizing the network and promoting the development of PTE.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

Epilepsy

Epilepsy is defined as the risk of recurrent seizures which can vary in length and severity (Fisher et al., 2005). It cannot be cured, but in 70% of cases seizures can be controlled by medication (Eadie, 2012). Epilepsy goes back as far as the first medical records (*Atlas epilepsy care in the world.*, 2005). The first recorded account of epilepsy was made by the Babylonians who believed that seizures were the result of an evil spirit invading the body. This supernatural view was not challenged until the 17th century B.C. by Hippocrates (*Atlas epilepsy care in the world.*, 2005). Hippocrates postulated that epilepsy should be treated like any other natural disease, with diet and drugs before it becomes chronic. He believed that once a disease becomes chronic it is ultimately incurable (Temkin, 1994). Despite Hippocrates' proposal that heredity was a likely cause or his description of the physical characteristics and social stigma caused by it (Magiorkinis, Sidiropoulou, & Diamantis, 2010), it was still assumed that epilepsy was caused by evil spirits until the 17th century (*Atlas epilepsy care in the world.*, 2005). It was not until the mid 1800s that the first antiepileptic drug was developed, bromide (Perucca & Gilliam, 2012).

Sixty-five million people worldwide are known to have epilepsy (Thurman et al., 2011). The ways in which people develop the disease can be due to genetics or be a result of other conditions. However, in most cases for patients diagnosed with epilepsy, the cause is unknown (Fisher et al., 2005)

Epilepsy can happen in a variety of ways. Genetic factors can be a cause of epilepsy because certain genes can affect the likelihood of susceptibility as well as the interaction of multiple genes (Pandolfo, 2011). However, idiopathic generalized epilepsy is the most common type of genetically determined epilepsy, but how it is inherited is still unknown (*Atlas epilepsy care in the world.*, 2005). The other prominent mode of the development of epilepsy is symptomatic. Symptomatic epilepsies can occur as the result of brain tumors, cerebral anoxia, brain infections, birth trauma, cortical malformations, and head trauma (*Atlas epilepsy care in the world.*, 2005).

Compared to those that have not suffered seizures after a head injury, PTE patients are known to have a shorter life expectancy (Corkin, Sullivan, & Carr, 1984). In addition to shorter life expectancies, those with PTE recover from injuries slower and have more cognitive and motor issues (Camfield & Camfield, 2014).

Traumatic Brain Injury

Traumatic brain injury is the result of an external mechanical force that causes damage to the brain. The mechanical force can be the result of a rapid change in acceleration, blast waves, penetrating injury, or impact (Maas, Stocchetti, & Bullock, 2008). Classification of TBI is based on severity of the injury, anatomical features resulting from the injury, and how the injury was caused (Saatman et al., 2008).

Although TBI affects all ages, it remains a leading cause of death and disability in children. According to the National Center for Injury Prevention and Control (2009), in children 0- 14 years of age, TBI annually results in 435,000 trips to the emergency room and nearly 2700 deaths.

Symptoms of TBI largely depend upon which area of the brain was injured. Severe TBI will often result in a relentless headache, convulsions, nausea, aphasia, slurred speech, dysarthria, loss of coordination, weakness in limbs, restlessness, or agitation (Kim, 2002). When the primary damage to the brain has occurred there is frequently multiple secondary injury events. These events can include damage to the blood-brain barrier, inflammation, excitotoxicity, influx of calcium and sodium ions into neurons, and mitochondrial dysfunction (Park, Bell, & Baker, 2008). An increase in intracranial pressure may rise from swelling or from a hemorrhage. Brain death or herniation can occur when the pressure within the skull becomes too great (Werner & Engelhard, 2007). Also, ischemia can result from a decrease in cerebral perfusion pressure (Ghajar, 2000).

Researchers have constructed a various assortment of models such as fluid percussion injury, controlled cortical impact, blast models, and undercut models to better understand the implications and consequences of TBI.

Post Traumatic Epilepsy

Post traumatic epilepsy is a possible outcome as a result of traumatic brain injury. A sufferer of a traumatic brain injury can experience post traumatic seizures as quickly as one week after initial insult (Pagni & Zenga, 2005). PTE and symptomatic epilepsy accounts for 5% and 20% of epilepsy cases respectively (Garga & Lowenstein, 2006). A large problem to those that suffer TBI is the unknown likelihood of developing PTE (Pitkänen et al., 2007). Post traumatic seizures may occur after insult, but this does not mean that the patient will go on to develop post traumatic epilepsy (PTE). PTE is characterized as a chronic condition, where post traumatic seizures might only occur once

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or twice in a patient (Frey, 2003a). The likelihood of a person developing PTE is linked to the severity of the injury (Iudice & Murri, 2000). Mild TBI increases the risk, compared to that of an uninjured group, of PTE by one and a half fold (Annegers, Hauser, Coan, & Rocca, 1998). However, some estimates show that as many half of severe TBI sufferers will develop PTE (Iudice & Murri, 2000). A study done to understand the likelihood of the development of PTE in relation to severity showed that 2.1% of mild TBI sufferers will go on to develop PTE compared to 16.7% of severe TBI sufferers (Annegers et al., 1998; Pitkänen & McIntosh, 2006).

Currently, it is unknown what changes to the brain occur after trauma (Garga & Lowenstein, 2006; Mazarati, 2006). Researchers have proposed several possible mechanisms that can lead to PTE, however, multiple mechanisms may be found in an individual with PTE (Agrawal, Timothy, Pandit, & Manju, 2006). Several of these proposed mechanisms can range from formation of new synapses and axons, cells undergoing apoptosis or necrosis, and altered gene expression (Herman, 2002). A particular area that is believed to give rise to PTE is the hippocampus. This is due to decreased connectivity between the parietal cortex and hippocampus (Mishra et al., 2014). This reorganization of neural networks may make neurons hyperexcitable (Elaine Wyllie MD & Deepak K. Lachhwani, 2005). Neurons that become hyperexcitable can create an epileptic focus that leads to seizures (Gill, Chang, Prenosil, Deane, & McKinney, 2013). Furthermore, an increase in neuronal hyperexcitability in conjunction with a loss of inhibitory neurons can produce PTE (Elaine Wyllie MD & Deepak K. Lachhwani, 2005).

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Cortex

The human cortex has six cortical layers and each layer is characterized by the distribution of neural cell types and connections to other layers and subcortical regions. Each layer is roughly 2-3mm thick in humans. Layer I is the shallowest layer of the cortex. It is mostly free of neuronal cell bodies and is largely composed of branching apical dendrites of pyramidal neurons. Layer I, the molecular layer, receives excitatory input from other areas of the cortex (Douglas & Martin, 2007), but it has also been shown that a large number of thalamocortical neurons converge there as well (Rubio-Garrido, Pérez-de-Manzo, Porrero, Galazo, & Clascá, 2009). Layer II and III, the external granular layer and external pyramidal layer respectively; consist of small to medium sized pyramidal neurons that output to layer V/VI. However, the output to layer VI is weak despite layer V/VI being interconnected (Shipp, 2007). Layer IV, the internal granular layer, serves to relay signals to layers II and III and also include some inhibitory granule cells. Layer IV also directly outputs to layer VI, prominently in primary cortices. This IV to VI circuit loop is reasoned to serve as a modulatory loop as it mainly terminates upon inhibitory neurons. However, areas of the brain like the motor cortex is agranular, lacking a layer IV (Shipp, 2007). Layer V, the internal pyramidal layer, is comprised of pyramidal neurons that project to subcortical regions (Jones, 1998). Layer V is the primary output layer of the entire cortex and is densest in the motor region. This layer outputs to a variety of regions such as the superior colliculus, brainstem oculomotor centres, cerebellum, striatum and the thalamus (Shipp, 2007). Layer VI connects to the thalamus and is an outgoing component of a cortico-thalamo-cortical loop.

Considering layer V pyramidal neurons serve as the primary output for the brain, this led us to investigate if the pathogenesis of seizure generation after traumatic brain injury could uncover intracellular markers. It has been shown that deep layer cortical neurons initiate spike and wave discharges, in seizure models (Polack et al., 2007). Cortical layer V has been suggested as an important pathogenic synchronizing mechanism, as well as a contributor to the initiation of epileptiform events (Hoffman et al., 1994). Layer V neurons have shown spontaneous ictal-like epileptiform discharges after controlled cortical impact (CCI) (Yang et al., 2010). Furthermore, acute injury models that have undercut layer V, have shown to cause a decrease in synaptic inhibition and an increase in synaptic excitation as a result of reorganization of synaptic circuits (Jin, Huguenard, & Prince, 2011).

Cortical Development

The development of the cortex begins as progenitor cells transfer inside-out along radial glia (Noctor et al., 2001). The first pyramidal neurons migrate out of the ventricular and subventricular zones from the preplate. The preplate will eventually become layer I and the subplate will form a middle layer, or cortical plate, that will go on to develop into layers V and VI. Neurons that come later will migrate radially through the deep layers and become layers II to IV (Rakic, 1988). Pyramidal neurons, the brain's primary excitatory unit, begin to increase in soma size, apical dendrite length, and basal dendrite length in rats between postnatal day 3 and 21 (Zhang, 2004). GABAergic interneurons serve as the brain's primary inhibitory units. During development, GABA is primarily excitatory because the gradient of chloride is reversed in immature neurons,

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meaning the reversal potential is higher than the resting membrane potential of the cell (Ben-Ari et al., 2007; Li & Xu, 2008). As a result of GABAergic interneurons maturing faster and the GABA signaling mechanisms occurring earlier than glutamatergic transmission, GABA is the major excitatory neurotransmitter in the brain before the maturation of glutamatergic synapses (Rheims et al., 2009).

The development of excitatory and inhibitory synaptic circuits occurs during the generation of cells and their movement before reaching their final position in the cortex. This occurs during the first two postnatal weeks of a rat's development (Shatz, 1990). Neurons begin to extend their axons and dendrites to proper synaptic partners. As the synapses are constructed and mature within neural circuits, they undergo continuous refinement and reformation. The refinement and pruning period is dependent upon interactive mechanisms and patterned neuronal activity, and in rats occurs during the second and third postnatal week of rats (Katz, 1993; Shatz, 1990). The reduction of axons, dendrites, and synapses, and death of neurons, by apoptosis, is a very important counter process to the excessive growth of axons, dendrites, and synapses (Cowan., 1984). The pruning and refinement process begins in late gestation and dramatically increases postnatally. Although synaptogenesis differs across brain regions, the sensory and motor cortices experience the most refinement after birth, and regions that handle cognitive functions are done later (Levitt, 2003).

Summary

Studies have shown that young children have a 42.5% chance of developing early posttraumatic seizures (EPTS) after TBI. Which is almost double that of adult sufferers (Arndt et al., 2013). Due to this large discrepancy, we find that a juvenile model provides

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the best option for having a substantial number of animals to develop PTE. Using a juvenile severe TBI model allowed us to identify a possible marker during the pathogenesis of posttraumatic TBI. We can identify a marker by looking for synaptic and intrinsic changes during whole-cell patch clamp recordings. We hypothesized that if TBIs were known to result in PTE, we would see a synaptic or intrinsic change in layer V pyramidal neurons intracellularly.

In chapter 2, we study changes in the synaptic and intrinsic properties of layer V pyramidal neurons. We identify that there are no synaptic or intrinsic changes, but we do find that 80% of animals that suffered a severe TBI showed cellular bursting. We find that animals that suffered a severe TBI are 60% more likely to show cellular bursting.

CHAPTER 2

SPONTANEOUS SYNAPTIC BURST ACTIVITY IN JUVENILE RATS AFTER

CONTROLLED CORTICAL IMPACT

Following a traumatic brain injury 5-50% of patients will develop post traumatic epilepsy. Pediatric patients are particularly susceptible with the highest incidence of PTE. Currently we cannot prevent the development of PTE and we have a limited understanding of the basic epileptogenic mechanisms that are initiated by TBI.   We hypothesize that early on following injury the cortex undergoes distinct cellular and synaptic reorganization that facilitates cortical excitability and promotes the development of seizures. To induce traumatic brain injury, we performed controlled cortical impact (CCI) in juvenile rats (post-natal day 17). Controlled cortical impact has been shown to induce the development of cellular and synaptic changes that are thought to promote increased cortical excitability. In–vivo we performed EEG epidural recordings for 14 days following CCI. All animals initially displayed electrographic seizures that terminated within the first week and were presumed to be injury induced. Following a quiescent period 40% (6 of 15) animals had the reemergence of recurrent electrographic seizures with an average event duration of 15.5s. These seizures were primarily "silent" with no overt behavioral seizure phenotype but demonstrated sustained changes in cortical excitability. To further study these changes, we performed in-vitro whole cell patch clamp recording on layer V pyramidal neurons in the peri injury zone from CCI or sham (craniotomy only) animals. Pyramidal neurons represent the major source of excitatory output from neocortical layer V, a lamina that has been implicated in both acute and chronic models of neocortical epileptogenesis. First we examined for changes

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in intrinsic excitability and found no significant difference in input resistance, action potential threshold, firing rate or resting membrane potential. Next, we examined for changes in excitatory synaptic function by recording spontaneous excitatory post-synaptic currents (sEPSCs) and found enhanced excitatory activity evidence by a decrease in the inter-event interval of CCI vs control animals with no change in the amplitude of events. This increased in excitatory activity was not accompanied by a change in inhibitory drive suggesting CCI alters the E-I balance. Specifically, we observed no change in the amplitude or IEI of spontaneous inhibitory synaptic currents (sIPSCs). In addition, both excitatory and inhibitory synaptic activity in CCI animals showed the development of distinct burst discharges that were not present in control animals. The results suggest that CCI induces early "silent" seizures that are detectable on EEG and correlate with distinct changes to the synaptic excitability in the cortex. The synaptic changes and development of burst discharges may play an important role in synchronizing the network and promoting the development of PTE.

Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability in children (Faul,. et al, 2010) and often leads to the development of post-traumatic epilepsy (PTE) (Annegers et al., 1998; Caveness et al., 1979; Englander et al., 2003; Iudice & Murri, 2000). PTE develops in up to 20% of children and depends on several factors including the severity of injury, age of the patient, and injury site (Barlow, Spowart, & Minns, 2000)(Appleton & Demellweek, 2002) .   The underlying pathophysiology of PTE is poorly understood, but develops in the wake of injury and leads to spontaneous recurrent seizures. Over the long term these post-traumatic seizures (PTS) may cause secondary brain damage through mechanisms including increased metabolic requirements, hypoxia, increased intracranial pressure, and/or excessive release of neurotransmitters (Medelow and Crawford, 1997; Teasdale and Bannan, 1997 and Graham et al., 2006 for review). Exacerbating the clinical management of PTE is that the seizures are often refractory to anti-epileptic drugs (Bauer & Burr, 2001) and are ineffective at reducing the risk of developing PTE (Adelson et al., 2003; Arango et al., 2012; Kochanek et al., 2012). However, evidence suggests that there may be a critical window following TBI for clinical intervention (Graber & Prince, 2004). Development of new therapeutic strategies in children requires an improved understanding of the processes and timing of events that occur early after injury in the genesis stages of PTE.

In humans, PTE develops slowly over months and even years. The slow development of PTE provides a unique temporal window to study and identify the epileptic changes as they occur "on the road" to PTE. We hypothesize that understanding

the early changes that occur following TBI may help to define the critical period for intervention and potentially identify unique therapeutic targets. The pediatric brain is in the midst of neurodevelopment and is undergoing a host of age-dependent physiological changes including synaptogenesis, use-dependent pruning, enhanced glucose metabolism(Chugani, Phelps, & Mazziotta, 1987), increased neurotrophic factors (Friedman, Olson, & Persson, 1991), and increased excitatory amino acid receptors (Insel, Miller, & Gelhard, 1990). These changes may confer unique advantages and disadvantages to the outcome of a TBI event and shape the development of PTE.

Injuries ranging from mild (concussion) to severe penetrating wounds and skull fractures may fall under the broad term of TBI. The incidence of PTE is significantly higher following severe TBI which is effectively modeled in animals by controlled cortical impact (CCI). CCI has been used extensively as a model of head injury (Lighthall et al., 1989; Liu et al., 2013; Mannix et al., 2011) and more recently as an effective means to model severe TBI (Cantu et al., 2014; Hunt, Scheff, & Smith, 2009)(Yang et al., 2010). Following CCI, studies have shown significant cavitation and neuronal cell loss at the site of injury (K. J. Anderson, Miller, Fugaccia, & Scheff, 2005; Fox, Fan, Levasseur, & Faden, 1998; Goodman, Cherian, Bryan, & Robertson, 1994; Hall et al., 2005; D. H. Smith et al., 1995), hippocampal neurogenesis, and synaptogenesis in the hippocampus (Rola et al., 2006; Scheff et al., 2005). Direct injury induced seizures have been reported to occur within the first 48 hours following CCI (Nilsson et al., 1994) but the development ofspontaneous recurrent post-traumatic seizures (PTS) occurs in 12.5 to 36% of animals following a latent period of weeks to months (Statler et al., 2009)(Hunt et al., 2009). In juvenile animals we have previously

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shown that CCI induces necrotic loss of cortex, damage to the underlying corpus callosum and hippocampus, synaptic reorganization (Card, Santone, Gluhovsky, & Adelson, 2005)(Jenkins et al., 2002) and deficits in spatial learning and memory (Adelson, Fellows-Mayle, Kochanek, & Dixon, 2013). In this study, we examined the underlying mechanisms that may contribute to cortical hyperexcitability and epileptogenesis in juvenile animals following CCI.

Pyramidal (PYR) neurons are the major source of excitatory output from layer V, a lamina that has been implicated as the site of origin of interictal epileptiform discharge in both acute and chronic models of neocortical epileptogenesis (Hoffman et al., 1994; Prince and Tseng, 1993). A recent preliminary report by Yang and colleagues has shown that CCI performed in juvenile rats rapidly induces spontaneous epileptiform activity and burst firing in layer V cortical pyramidal neurons (Yang et al., 2010). Burst firing is known to increase the fidelity of synaptic information transfer (Izhikevich, Desai, Walcott, & Hoppensteadt, 2003; Lisman, 1997) and may help to promote epilepsy by facilitating the propagation of local areas of hyperexcitability and synchrony. In the present study we examined the underlying mechanisms that may contribute to the development of epileptiform activity in juvenile rats following CCI. Utilizing electrophysiolgical approaches, we determine that CCI in juvenile rats induces the rapid development of *in-vivo* epileptiform activity and the preferential enhancement of *in-vitro* excitatory pre-synaptic burst discharges. These synaptic bursts occurred in the absence of significant changes in intrinsic excitability of layer V pyramidal neurons and may be

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driven by altered afferent cortical synaptic input. Our findings suggest that juvenile

animals undergo unique pathophysiological changes early after TBI that may be involved

in the pathogenesis of PTE.

Materials and Methods

Protocols used for all experiments were approved by the University of Arizona

Institutional Animal Care and Use Committee.

CCI Injury

To experimentally model TBI, a controlled cortical impact (CCI) was performed

on 29 post-natal day 17 (P17) Sprague-Dawley rats as previously described (Adelson et

al., 2013) (Card et al., 2005) (Jenkins et al., 2002). In brief, male Sprague Dawley rats

were sedated with isoflurane and injected interperitoneal (IP) with a mixture of ketamine

(50mg/kg) and xylazine (5mg/kg) at 0.01mL per 10g of rat weight.   Surgery site was

shaved and animals were fixed into a stereotaxic frame. A midline scalp incision was

then performed to expose the skull and a 6-mm craniotomy over the right somatosensory

region was performed. The bone flap from the craniotomy was removed and placed in

saline solution. Precaution was taken during the craniotomy to avoid damaging the

underlying dura and inducing significant bleeding. A frontoparietal controlled cortical

impact (CCI) (5mm tip, 4m/s, 2.0 mm depth) was performed using a pneumatic impactor

(Amscien Instruments, Richmond, VA).   After the CCI, the bone flap, that was removed

during the craniotomy, was placed over the injury site and secured with dental cement.

During this time, electroencephalography (EEG) leads were mounted and also secured

with dental cement. The skin was then sutured closed and the incision area swabbed with

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betadine. Animal temperature was maintained with an electric heating pad and monitored post-surgery until ambulatory (< 3 hours). Following the initial recovery, animals were returned to standard housing and monitored daily. Animals that were to be connected to EEG were given 24 hours post-injury to recover prior to EEG monitoring. All other animals were allowed to recover until further experimentation began on post-injury day (PID) 14.

Seizure Monitoring with Electroencephalograpy (EEG)

Rats subjected to CCI or age-matched controls were implanted with epidural recording electrodes. Experimental evidence indicates craniotomy may induce alterations to the cortex (Cole et al., 2011; Olesen, 1987). As such, we considered the craniotomy a component of the injury process and used appropriate naïve age-matched control animals. Epidural recording electrodes were made from #0-80 x 1/8 inch stainless steel screws at the following stereotaxic co-ordinates: AP: 2.0mm, Lateral: ± 3.0, Depth: 1mm; AP: -4.0mm, Lateral: -3.0mm, Depth: 1mm; AP: -8.0mm, Lateral: +3.0, Depth: 1mm (Fig. 1). After recovery from surgery, animals were placed in acrylic cages where they could move freely and were connected through commutators to the recording system. Animals were singly housed during this period. EEG signals were recorded continuously for 13 days post-injury using an Xltek 128 channel Neurolink IP amplifier (1.0Hz and 70Hz cutoffs, 512Hz sampling rate) . Two independent, blinded, and trained personnel analyzed the digital EEG files and their results were compared for consistency and averaged. As previously described, epileptiform activity was defined by the presence of epileptiform discharges or seizure-like events (Ziyatdinova et al., 2011). Epileptiform discharges (ED) were defined by rhythmic transients containing spikes and uniform sharp

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waves that lasted between 1 and 5 seconds. High-amplitude rhythmic discharges that were clearly distinguishable from background and lasted for greater than 5 seconds have been considered seizures (Horita, Uchida, & Maekawa, 1991). As simultaneous behavioral seizure activity was not monitored, this activity has been classified as seizure-like and most likely represents subclinical electrographic seizures.

Preparation and maintenance of brain slices

Coronal brain slices were prepared as previously described (T. R. Anderson, Huguenard, & Prince, 2010; T. R. Anderson, Jarvis, Biedermann, Molnar, & Andrew, 2005) from CCI or age-matched control animals. Slices were prepared from the somatosensory cortex beneath the injury site in CCI animals or from corresponding control cortex. Male Sprague-Dawley rats aged 31-35 days (PID 14-19) were deeply anaesthetized with inhalation of isoflurane and decapitated. The brain was rapidly removed and and coronal slices (350um thick) of the somatosensory cortex taken using a vibratome (VT 1200; Leica, Nussloch, Germany). Harvesting of slices was performed beneath the site of CCI or in corresponding control cortex. The site of CCI was readily identifiable in slices as significant cavitation and tissue loss. Initial harvesting was performed in an ice cold (4°C) carboxygenated (95% O_2, 5% CO_2) high sucrose solution containing the following (in mM): 234 sucrose, 11 glucose, 26 $NaHCO_3$, 2.5 KCl, 1.25 $NaH_2PO_4H_2O$, 10 MgS_47H_2O, 0.5 $CaCl_22H_2O$. Slices were then incubated for 1h at 32°C in carboxygenated artificial CSF (aCSF) containing (in mM): 126 NaCl, 26 $NaHCO_3$, 2.5 KCl, 10 Glucose, 1.25 $Na_2H_2PO_4H_2O$, 1 $MgSO_47H_2O$, 2 $CaCl_2H_2O$, pH 7.4 and then returned to room temperature before being moved to the recording chamber for whole-cell patch clamp recording.

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Electrophysiological recording

Slices prepared from CCI or control animals were submerged in flowing carboxygenated aCSF heated to 32°C. Submerged slices were first visualized under 4x brightfield for identification of layer V cortex. For slices from CCI rats, recordings were made in the peri-injury zone within 2 mm of the injury induced cavitation. Recordings from control slices were made in the recorded in the corresponding cortex. Whole-cell recordings were obtained from regular spiking cortical pyramidal neurons using an upright microscope (Axioexaminer, Carl-Zeiss, Thornwood, NY, USA) fitted with infrared differential interference contrast optics. Regular spiking (RS) pyramidal neurons were distinguished based on their current-clamp firing behavior (Guatteo, Bacci, Franceschetti, Avanzini, & Wanke, 1994). The electrode capacitance and bridge circuit were appropriately adjusted. The series resistance (R_s) of neurons chosen for analysis was less than 20% of membrane input resistance and monitored for stability. Membrane potential was not corrected for a calculated 10 mV liquid junction potential. A Multiclamp 700A patch-clamp amplifier (Axon Instruments, Union City, CA, USA) was used for both current and voltage-clamp mode. Recordings were obtained at 32°C using borosilicate glass microelectrodes (tip resistance, 2.5-3.5 MΩ) filled with intracellular solution (in mM): 135 KGluconate, 4 KCl, 2 NaCl, 10 HEPES, 4 EGTA, 4 Mg ATP, 0.3 Na TRIS for excitatory recording resulting in a calculated ECl- of -16 mV. For recording of inhibitory events, an intracellular solution containing the following was used (in mM): 70 KGluconate, 70 KCl, 2 NaCl, 10 HEPES, 4 EGTA, 4 Mg ATP, 0.3 GTP. This internal solution has been used previously (T. R. Anderson et al., 2010)(Sun, Huguenard, & Prince, 2006) and facilitates detection of inhibitory events. The calculated E_{Cl} was

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approximately −16 mV, resulting in inward $GABA_A$ currents at a holding potential of −70 mV. Inhibitory events were pharmacologically isolated by bath application of 2-Amino-5-phosphonopentanoic acid (d-APV; 50 μm) and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 20 μm) purchased from Ascent Scientific (Abcam Biochemicals, Cambridge, MA).

Data Analysis

Data were analysed using pCLAMP (Axon Instruments), Prism (GraphPad) and MiniAnalysis (Synaptosoft) software and are presented as means ± s.e.m. For detection of spontaneous synaptic events automated threshold detection was employed through MiniAnalysis and detected events were subsequently manually verified. Synaptic bursts events were detected based on previously published characteristics (Prince and Connors, 1986; Prince and Tseng, 1993) and were defined by a minimum of 3 synaptic events occurring in 250 milliseconds that temporally summated. Input resistance was calculated from the voltage response to the input of a current step (1s, 50mV). Adaptation index was calculated as $100 \times (1 - F_{Last}/F_2)$, where F_{Last} corresponds to the firing rate of the last interspike interval and F_2 the second interspike interval. Many of the pyramidal neurons had a high variability in the first interspike interval, so the second interspike interval was chosen for analysis. Intrinsic burst index was calculated as the inter-event interval between the first set of action potentials divided by the second. Statistical significance was tested using an unpaired *t* test and differences were determined to be significant if $P < 0.05$.

Results

To model severe TBI in pediatric patients, we subjected 17-day-old rats to controlled cortical impact (CCI (n=13) and compared them to age-matched controls (n=9). The CCI procedure results in a significant cavity in the cortex at the site of the injury and extensive necrosis (Adelson et al., 2013; Yang et al., 2010). In the weeks and months that follow after CCI, up to 36% of adult animals will develop spontaneous behavioral seizures (Hunt et al., 2009) and over 85% have been shown to develop epileptiform activity (Statler et al., 2009). In humans, PTE develops following a latent period that can last from months to years (Agrawal et al., 2006). At the point in which seizures are clinically identifiable, the underlying neural activity and networks have undergone significant change. We believe this activity begins early after the injury, and leads to hyperexcitability and subclinical electrographic changes well in advance of PTE. To investigate the changes that occur in juvenile rats early after CCI, we examined for electrophysiological changes and mechanisms that may promote epileptogenesis.

Epileptiform Activity is rapidly induced In-Vivo following Traumatic Brain Injury

To monitor for the development of epileptiform activity, we performed electroencephalograpy (EEG) recordings of CCI animals (n=16) or age-matched controls (n=9). Following recovery from the CCI surgery, EEG activity was continuously recorded for the first two weeks. Epileptiform activity was detected post-recording based on previously published characteristics (Ziyatdinova et al., 2011) and as detailed in the methods. Two trained personnel where blinded to the animal's experimental condition and averages values taken from the independent grading of the EEG recordings. Within the first 24 hours of recording, 87.5% of CCI animals developed epileptiform activity that

was absent in control animals. This epileptiform activity was considered to be injury-induced and akin to "early" post-traumatic seizures. Animals had a variable latent period (2-7 days) following this initial early stage, but all CCI animals subsequently developed recurrent epileptiform activity that was synchronized across all EEG leads (Fig. 1). On average, 16.4±3 epileptiform events were detected over the post-latency recording period. In 7 of 16 CCI animals, prolonged seizure-like events were also detected. The development of epileptiform activity within 14 days after CCI, and in advance of PTE, suggests the presence of on-going epileptogenic activity. Post-injury day 14 was chosen for further *in-vitro* experimentation as it was the earliest time point that all animals reliably displayed *in-vivo* epileptiform activity.

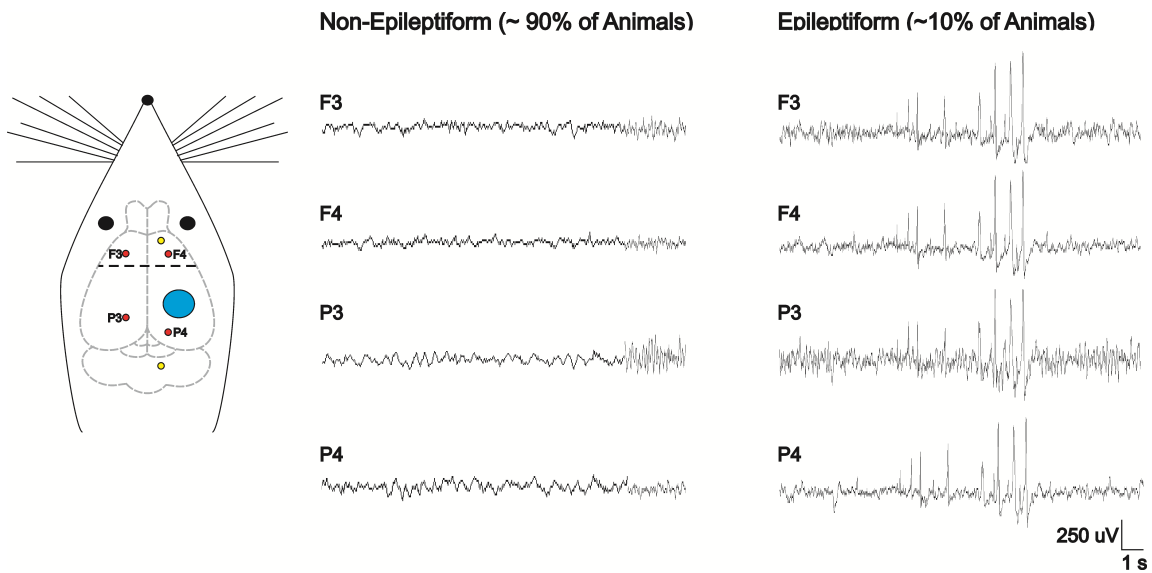Figure 1. CCI Induces Epileptiform Activity. Left: Schematic representation of rat brain indicating site of CCI injury (blue circle) and EEG recording electrodes (red circles). Middle and Right: Epidural EEG recordings from rats made 14 days after CCI. Middle panel is from a control animal without observable epileptiform activity and right panel from a CCI animal that displayed spontaneous epileptiform discharges.

Epileptiform synaptic bursting is induced in-vitro following TBI

Epileptogenesis has been extensively studied in numerous animal models and is generally thought to occur as the result of disruption to intrinsic excitability, synaptic inhibition and/or synaptic excitation (Prince and Connors, 1986 for review). To investigate the contribution of these mechanisms to CCI induced epileptiform activity, we examined for electrophysiological changes in cortical brain slices. Epileptiform activity detected by EEG was widespread and synchronous within and across cortical hemispheres. Pyramidal neurons in layer V are the major output pathway of the cortex and have been implicated in network synchronization (Telfeian & Connors, 1998). As such, whole-cell patch clamp recordings of physiologically-identified layer V pyramidal neurons were made 14 days after CCI or in age-matched control. All recordings were made in the peri-injury zone (i.e. within 2 mm of injury site) or corresponding control cortex.

Intrinsic Excitability

The intrinsic membrane properties of a neuron have been repeatedly shown to be altered in various models of epilepsy (Willmore, 1990; Yang, Benardo, Valsamis, & Ling, 2007). Neurons that are predisposed or have pathological enhancements to intrinsic excitability may be spontaneous generators of epileptic activity. To examine this possibility, we first recorded for changes in the intrinsic membrane properties from CCI and control pyramidal neurons. Recording under current-clamp, we found no statistical difference between control and CCI resting membrane potential (-67.5 ± 1.0 mV (control); -67.7 ± 0.9 mV (CCI), $P<0.92$) and input resistance (198.5 ± 16.1 MΩ(control); 192.3 ± 12.9 MΩ (CCI), $P<0.76$). Next, we evaluated the firing-current (f-I) relationship

in control and CCI animals. A series of current steps (-150pA to 300pa, 50pA steps, 1 second) were injected through the patch pipette and the membrane voltage response was recorded (Fig 2A). We examined for changes in the firing frequency and adaptation index but found no statistical difference (Fig 2B). Finally, using a rheobase protocol (50 msec, 5 pA steps) we examined for changes in membrane excitability. We found no statistical difference in rheobase current or action potential properties (threshold, amplitude, and half-width)(Fig 3). Overall, these results suggest that changes in the intrinsic membrane excitability of layer V pyramidal neurons does not significantly contribute to the early development of epileptiform activity following CCI.

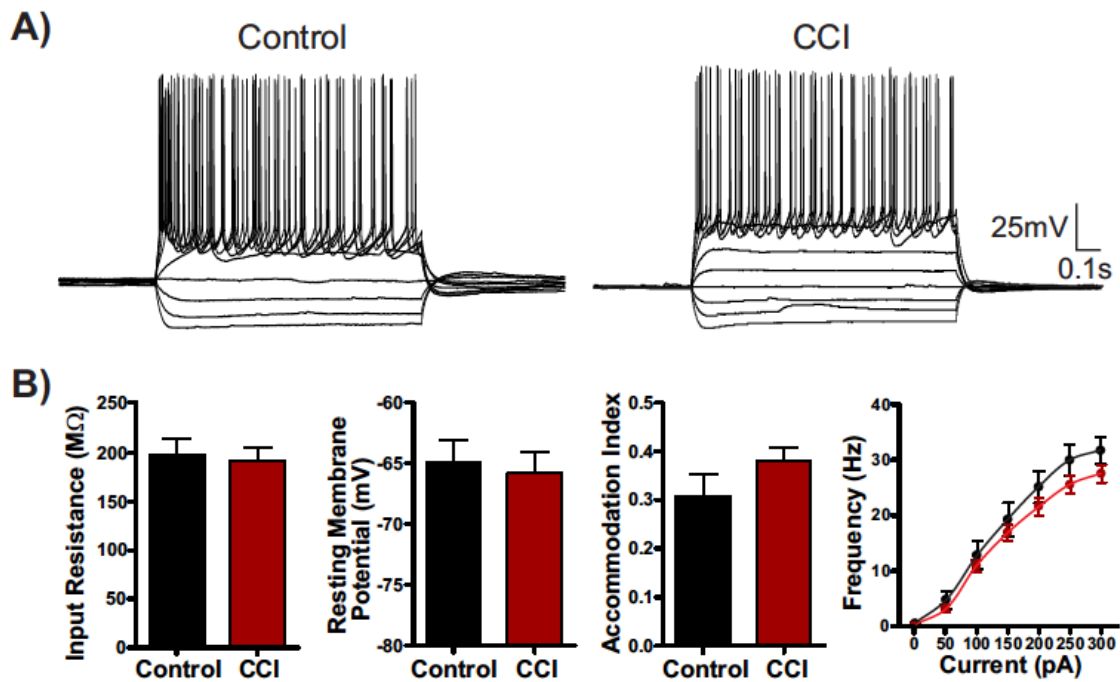Figure 2. Intrinsic excitability is not altered by CCI. A) Current Clamp recordings in response to intracellular current steps (-150pA to 250pA, 1s) in pyramidal neurons from control or CCI injured animals. Note the similarity in the intrinsic cellular response. B) Bar charts of average response values of various intrinsic membrane properties from (control n=14, CCI n=41). n=38)n=38).n=38).animals (n=41).
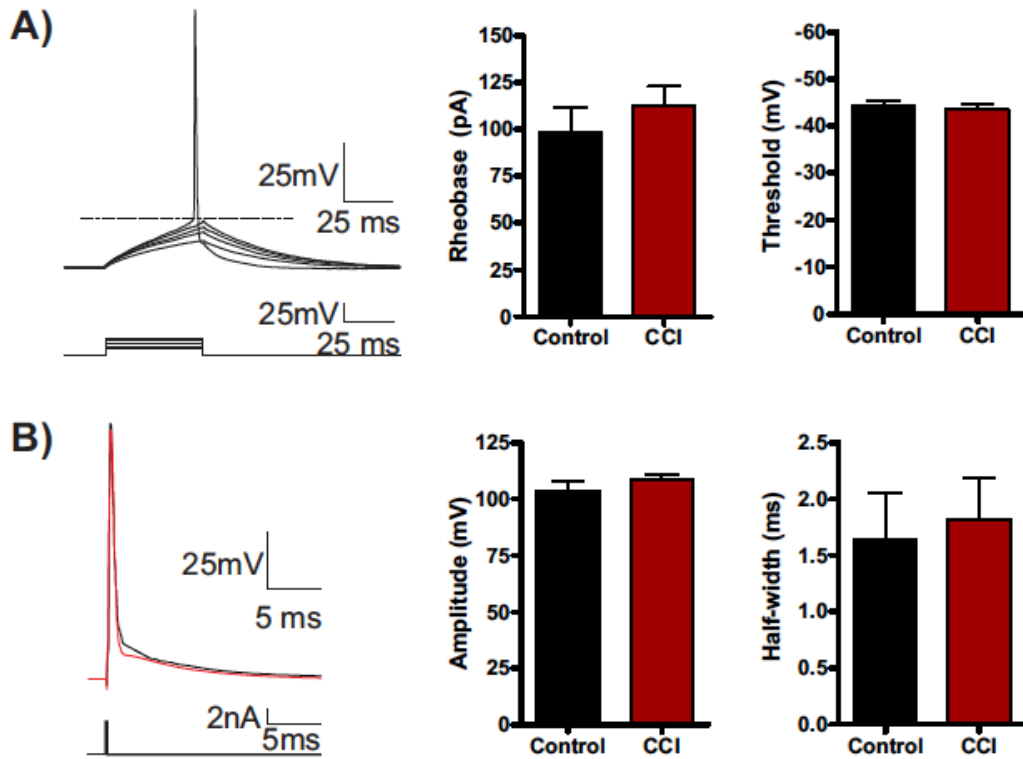
Figure 3. Action Potential Firing is not altered by CCI. A) Reprentative whole-cell current clamp recording in response to a series of 50msec injection (5pA steps). Bar charts of average values for control or CCI. Rheobase was calculated as the minimum current which produced an action potential. Threshold was measured at the greatest change in calculated slope. B) Current clamp single action potential step (2nA, 0.5ms) was injected to measure action potential properties.

Spontaneous Synaptic Activity

The generation of epileptiform activity and seizures is thought to occur through altered network activity and increased neuronal recruitment that may involve changes to synaptic properties and efficacy (Prince and Connors, 1986 for review). We tested this possibility by examining post-synaptic currents received by layer V pyramidal neurons.

Excitation

First, under voltage clamp (V_{hold}=-70mV) we examined for changes in spontaneous post-synaptic currents. For these experiments pharmacological isolation of excitatory glutamatergic post-synaptic currents (ePSCs) was not possible as GABA antagonists are known to disinhibit the slice and promote epileptiform activity and thereby mask CCI induced changes. To minimize the detection of inhibitory events, neurons were held near and positive of the reversal potential of chloride (V_{hold} = -70mV, calculated E_{Cl^-} = -16mV). This allowed detection of only excitatory positive-directed (inward current) events in isolation from any small inhibitory outward events. First, we examined for changes in the inter-event interval (i.e. frequency) of excitatory sPSCs and found no statistical difference between control (295.6 ± 42.9 ms) and CCI (243.2 ± 26.7)(P=0.28) Similarly we found no statistical difference between control and CCI in the amplitude of excitatory sPSCs (16.8 ± 1.2 pA (control) vs 17.4 ± 1.4 pA (CCI), charge transfer (69.5 ± 7.0 fC (control) vs 73.1 ± 5.4 fC (CCI)(P=0.70) or decay (3.6 ± 0.2 ms (control) vs 3.5 ± 0.2 ms)(CCI)(P=0.77)(Fig 4). The data suggest that CCI does not alter overall excitatory synaptic activity.
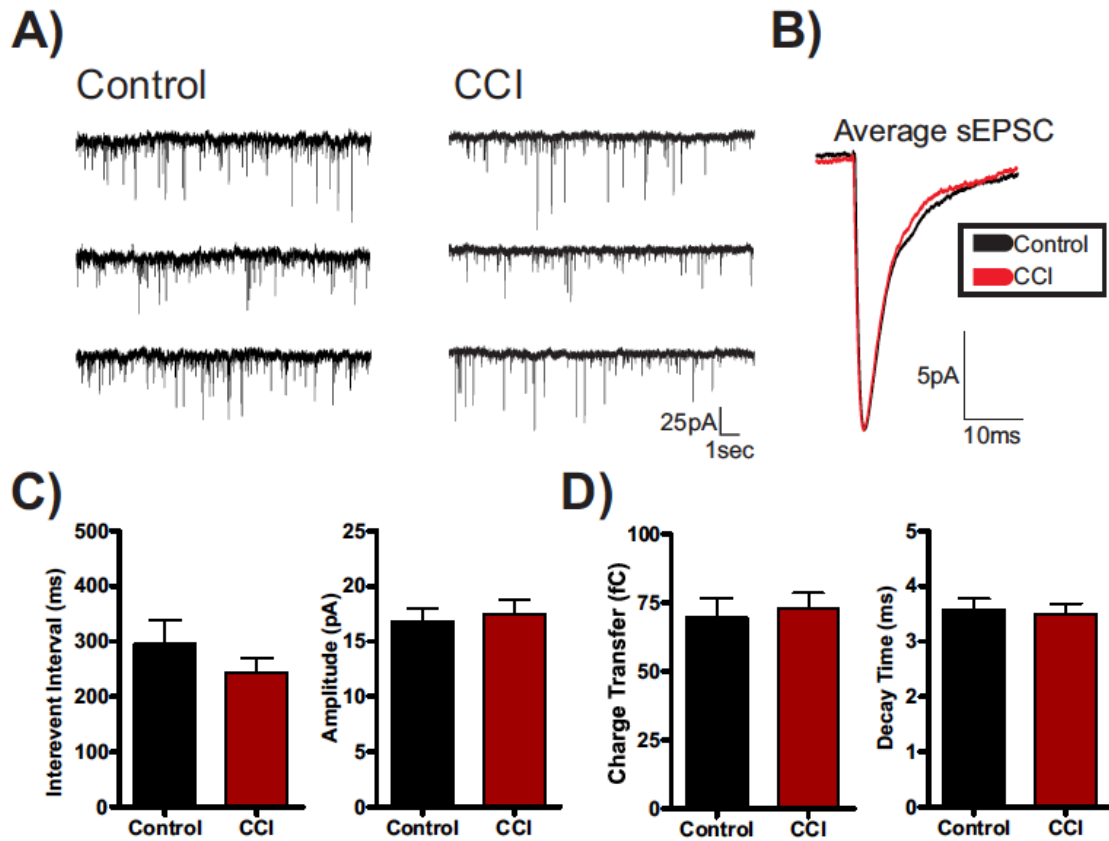
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Figure 4. CCI fails to alter excitatory post-synaptic currents. A) Voltage clamp recordings of spontaneous post-synaptic current (sPSC) in control or CCI injured animals. B) Overylayed and amplitude scaled average sPSC recorded from either control (black) or CCI (red) animals. C) and D) Average sPSC properties are plotted for control (n=13) or CCI (n=38). Vhold = -70mV.

Inhibition

Second, to directly examine for changes in inhibition we recorded spontaneous inhibitory post-synaptic currents (sIPSCs) that were pharmacologically isolated by bath application of d-AP-V (50uM) and DNQX (20uM). We also utilized a modified internal patch solution with an elevated chloride concentration. This internal has been extensively used(T. R. Anderson et al., 2010)(Bacci & Huguenard, 2006) and increases the signal to noise and detection fidelity of inhibitory synaptic events. Voltage clamp recordings were made at -70mV from CCI or control animals. We found no significant change in amplitude (24.6 ± 2.4 pA (control) vs 24.9 ± 3.3 pA (CCI)(P=0.95) or inter-event interval (385.1 ± 85.9 ms (control) vs 328.2 ± 50.4 ms (CCI)(P=0.55). However in contrast to excitatory synaptic activity, a significant increase in the decay time of inhibitory sIPSCs was observed (5.2 ± 0.56 ms (control) vs 7.5 ± 0.70 ms (CCI))(P<0.03)(Fig 5). A similar trend was observed in the charge transfer but it failed to reach statistical significance area (126.1 ± 17.6 fC (control); 185.1 ± 28.2 fC (CCI), P=0.15)(Figure 5B). The net effect of these changes would be to increase the efficacy of inhibition following CCI by increasing the temporal window over which inhibition acts.
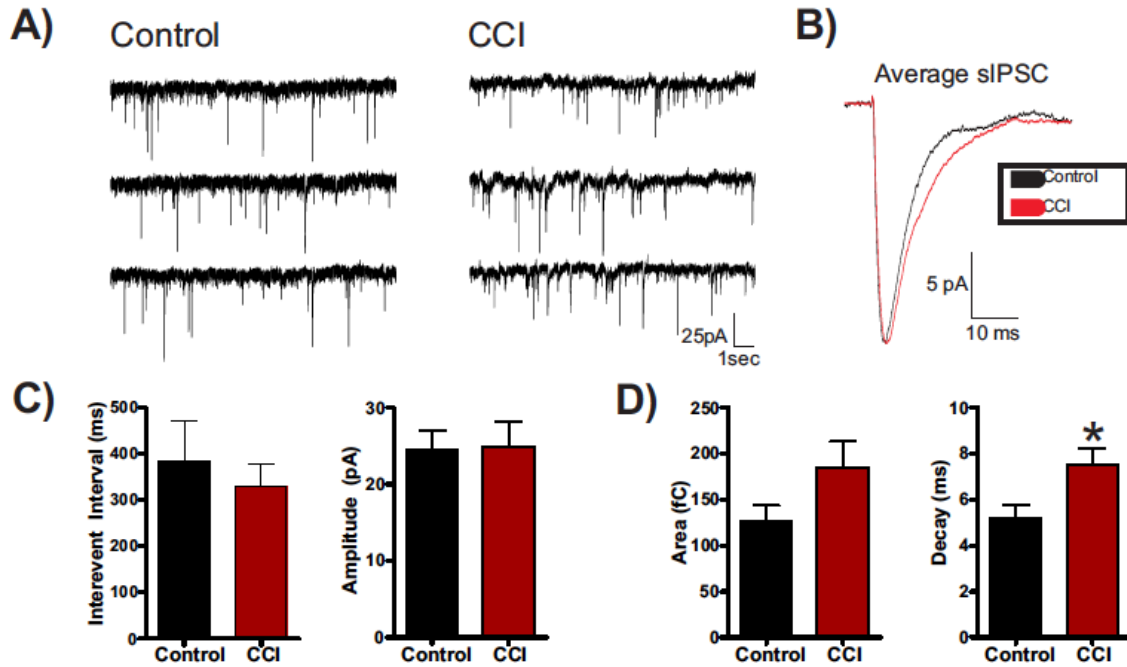
Figure 5. CCI increases inhibitory synaptic decay. A) Voltage clamp recordings of spontaneous inhibiotry post-synaptic current (sIPSC) in control or CCI injured animals. For inhibitory recording glutamate receptor antagonists (APV/DNQX or kynurenate) were applied. B) Overlayed and amplitude scaled average sIPSC recorded from either control (black) or CCI (red) animals. C) and D) Average sIPSC properties for control (n=9) or CCI (n=16). Vhold = -70mV. * P<0.05.

Synaptic Burst Discharges

Excitatory burst discharges are thought to increase synaptic efficacy by increasing the probability of inducing the post-synaptic cell to fire an action potential. In our initial experiments, we examined the average sPSC properties and found that CCI had no impact on the average excitatory synaptic activity. However, during recording we observed distinct spontaneous synaptic burst discharges that resembled the epileptiform activity observed *in-vivo*. Based on previous reports detection of synaptic bursts was determined by the presence of a minimum of three simultaneous sPSCs within 250 ms that did not return to baseline. These detection parameters were highly sensitive and allowed for detection of a small number of synaptic bursts in control animals. Overall, the presence of excitatory burst discharges were significantly greater following CCI as 79.5% of recorded CCI neurons displayed synaptic bursting compared to 23.1% of control. However, the average number of synaptic bursts detected in a CCI animal (avg. of 7.7 ± 2) during a single recording session were dramatically increased over control (avg. of 0.38 ± 0.2) ($P<0.04$). The average excitatory sPSC burst in CCI animals consisted of 5.9 ± 1 synaptic events and lasted on average for 858.0 ± 240 milliseconds (Fig 6). Bath application of 1um tetrodoxin (TTX) eliminated excitatory burst discharges (n=4). On the inhibitory side, similar burst discharges were observed in 75% of CCI neurons compared with 22% in control. However, the average number of sIPSC bursts in CCI (3.6 ± 1.1 neurons remained significantly increased over control ($0.6 + 0.4$). The average inhibitory sIPSC burst in CCI animals consisted of 11.4 ± 2.6 synaptic events with a duration of 431.0 ± 71 milliseconds (Fig 7). Overall, following CCI, there was a

significant increase in excitatory and inhibitory burst discharges. In comparison, CCI induced greater excitatory bursting than inhibitory bursting by both frequency of total neurons bursting, and average number of burst per neuron. This suggests CCI induced synaptic bursting may be preferentially increasing excitatory synaptic coupling.
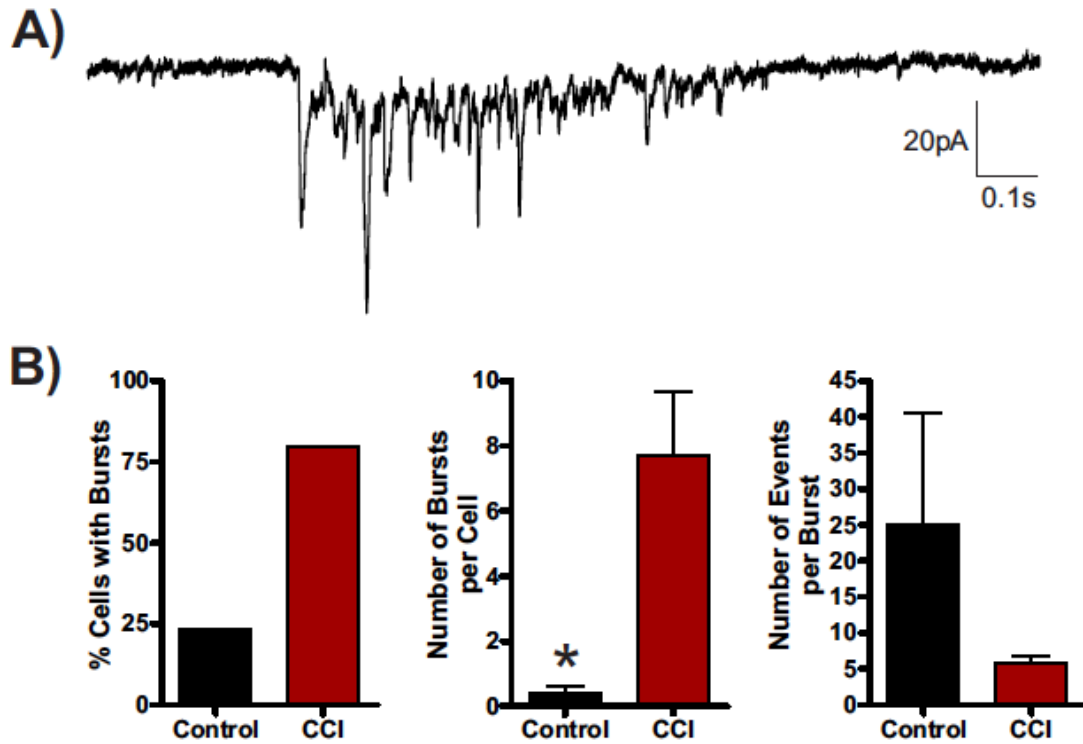
Figure 6. Excitatory Synaptic Bursts are Induced by CCI. A) Voltage clamp recording of spontaneous excitatory burst discharge observed in a CCI animal with an epileptiform EEG. Note the burst is comprised of compound sPSC and resembles paroxysmal discharges observed in epileptic animals. B) Bar charts of average values of various burst properties (control = 13, CCI = 38). *P<0.05.
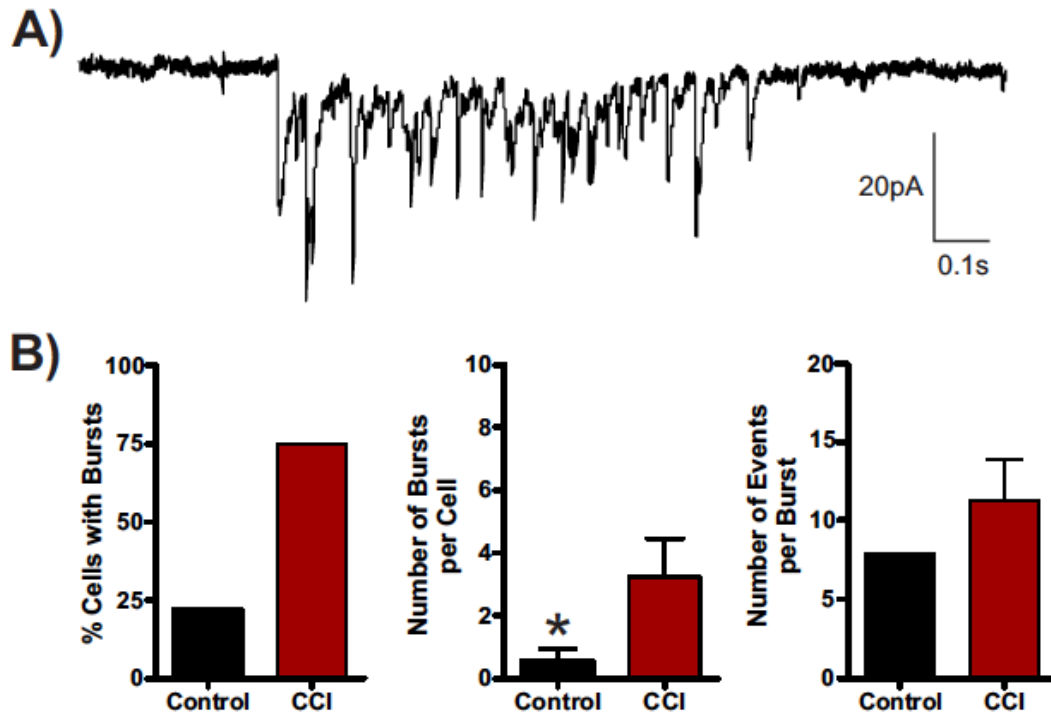
Figure 7. Inhibitory Synaptic Bursts are Induced by CCI. A) Voltage clamp recording of spontaneous inhibitory burst discharge observed in a CCI animal with an epileptiform EEG. For inhibitory recording glutamate receptor antagonists (APV/DNQX or kynurenate) were applied. Note the burst is comprised of compound sIPSC and resembles epileptiform discharges observed in epileptic animals. B) Bar charts of average values of various burst properties (control (n=9), CCI(n =16).

Discussion

This study was undertaken to better understand the early changes to cortical excitability induced by traumatic brain injury and to gain insight into how they may facilitate the development of post-traumatic epilepsy (PTE). Controlled cortical impact (CCI) in rodents has been effectively used to model traumatic brain injury (TBI)(Bolkvadze & Pitkänen, 2012; Cantu et al., 2014; Hunt et al., 2009). However, these studies have primarily focused on CCI performed in adult animals. The outcome, incidence and clinical management of TBI in children differ significantly from adults. In this study, we examined the development of epileptiform activity and the underlying pathophysiology that occurs in juvenile (PND 17) rats following CCI. The results of this study suggest that within 14 days of CCI injury epileptiform activity is induced that can be detected *in-vivo* by EEG as synchronous discharges across multiple cortical regions. At a cellular and synaptic level this epileptiform activity was accompanied by a lack of change in intrinsic membrane properties but a 44% increase in the decay of inhibitory synaptic input onto layer V pyramidal neurons. In addition, spontaneous epileptiform bursting was observed in both excitatory and inhibitory synaptic recordings. Synaptic bursting is thought to enhance synaptic coupling between neurons and may promote PTE through enhanced hyperexcitability and network synchrony.

Development of Epileptiform Activity Following CCI in Juvenile Rats

The hallmark of PTE is the development of spontaneous recurrent seizures. In humans, these seizures develop after several months to years following the initial injury(Agrawal et al., 2006). The progressive development of PTE suggests an evolving

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process that may begin early after injury. To directly examine the development of epileptiform activity early after injury we performed continuous EEG for the first 14 days post-injury. Epileptiform activity and electrographic seizures were observed in 87.5% of animals within the first 24 hours after CCI. These early seizures are thought to be injury induced and may be separate from the underlying epileptogenic processes that lead to PTE. However, children are also more prone to developing early seizures and the prevalence and contribution of these early seizures to development of PTE in pediatric TBI remains to be determined. Following a variable latent period all CCI animals proceeded to develop spontaneous recurrent epileptiform activity by 14 days post-injury. This activity was primarily characterized by high-amplitude rhythmic discharges that were routinely synchronized across all 4 cortical EEG leads. This activity resembles epileptiform discharges and inter-ictal spiking that has been previously shown in other epileptic animal models (Hunt et al., 2013). This epileptiform activity and late seizures that develop after the first week of injury are positive predictors of PTE (Frey, 2003 for review). Furthermore, the presence of similar inter-ictal EEG abnormalities are strong predictors of disease severity and outcome (Ramantani, 2013 for review). Further work recording EEG continuously for several months will be required to determine the prognostic value of the observed early epileptiform activity. To our knowledge, no other study has examined the development of early EEG changes after injury during the time period as animals transition from these presumed injury induced seizures to the development of the first recurrent spontaneous epileptiform activity. The study of PTE is

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complicated by the presence of multiple injury, repair and adaptive processed initiated by the TBI – only a portion of which are presumed to be epileptogenic. Examination of animals early after injury has a potential reductionist advantage while determining early pathophysiological changes that may define a critical window or targets for therapeutic intervention. We have now validated that 14 days after injury is the earliest time point after CCI where animals reliably display *in-vivo* and *in-vitro* epileptiform activity.

Epileptogenesis has been extensively studied in numerous animal models and may result from a variety of mechanisms. While no common epileptogenic mechanism has been found a combination of disruption to intrinsic cellular properties, synaptic inhibition and/or synaptic excitation has been frequently reported(Prince and Connors, 1986 for review). Recently, a preliminary report by Yang and colleagues has indicated the development of hyperexcitability and spontaneous epileptiform activity following CCI in juvenile animals(Yang et al., 2010). We extend these findings here to examine the underlying mechanism and determine the impact of CCI on known intrinsic and synaptic changes that are thought to be epileptogenic. Overall our results indicate that CCI fails to alter intrinsic membrane properties, neuronal firing or average excitatory synaptic activity while promoting burst discharges and enhanced inhibitory synaptic decay. Specifically, the intrinsic excitability of a neuron is determined in large part by its membrane properties and ion channels and enhanced intrinsic excitability may be epileptogenic. However, following CCI in juvenile animals layer V pyramidal neurons displayed no change in intrinsic excitability. This included resting membrane potential, input resistance, action potential threshold and rheobase. Similarly, there was no change

in the firing properties (frequency or accommodation), input-output relationship (f-I curve) or single action potential waveform. Together these results suggest that alterations to intrinsic excitability do not significantly contribute to the observed development of epileptiform activity following CCI.

At a synaptic level, our results indicate that overall excitatory synaptic input onto layer V pyramidal neurons was not altered. There was no change in the amplitude, inter-event interval or kinetics of excitatory spontaneous post-synaptic currents. Regulatory control over spontaneous synaptic activity is complex, but in general changes to amplitude and kinetics occur as a result of changes to the pre- or post-synaptic neuron including mechanisms such as quantal content or receptor subunit composition. Altered inter-event interval is thought to reflect changes to the pre-synaptic neuron (e.g. probability of release). The lack of change in sPSCs after CCI suggests that altered excitatory synaptic activity is not driving the development of epileptiform activity. In examining inhibition in the cortex, we similarly found no change in the amplitude or inter-event interval of spontaneous inhibitory post-synaptic currents. However, the decay of inhibitory responses was significantly increased following CCI. Alterations to the time course of synaptic GABAergic events will have a profound effect on the excitability of individual neurons and networks by altering the temporal integration window, the time over which a GABAergic event may reduce a coincident excitatory event. Our finding of an increase in sIPSC decay is consistent with increases observed in other models of epilepsy(Calcagnotto, Paredes, Tihan, Barbaro, & Baraban, 2005). The time course of inhibitory events is determined by both pre and post-synaptic factors including expression of synaptic GABA transporters, synchrony of GABA release and subunit

composition(Overstreet and Westbrook, 2003; Keros and Hablitz, 2005; Barberis et al., 2007). In general, an increase in synaptic decay is predicted to counteract the observed hyperexcitability but may also be impacted by neural trauma induced changes in the chloride reversal potential(van den Pol, Obrietan, & Chen, 1996). Altered intracellular chloride may also impact the kinetics of chloride dependent GABAergic inhibition(Houston, Bright, Sivilotti, Beato, & Smart, 2009) and would be in line with the observed CCI induced changes. Determining the role of increased synaptic decay in promoting or resisting epileptic changes following CCI remains an open question.

Development of synaptic bursting following CCI in juvenile rats

The development of epilepsy is commonly associated with the synchronous discharge of cortical neurons. Excitatory burst discharges are thought to increase synaptic coupling by increasing the probability of inducing the post-synaptic cell to fire an action potential. In this study we have identified unique epileptiform burst discharges following CCI in the absence of changes to intrinsic membrane, firing properties or global changes in excitatory synaptic currents. Taken together it suggests that layer V pyramidal neurons are not the initiator of the epileptiform discharges but are driven by afferent input. As no changes were observed in excitatory synaptic IEI it suggests the synaptic bursting is not due to altered pre-synaptic probability of release. As the bursts were sensitive to blockade with TTX it suggests they are being driven by action potential dependent afferent input. How the bursts directly impact synaptic coupling and the output of layer V pyramidal neurons remains to be determined. As synchronous spontaneous epileptiform activity was observed on EEG across cortical regions and hemispheres (Fig 1) it suggests network recruitment and widespread propagation of the

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epileptic activity. Layer V pyramidal neurons receives input from all other cortical layers as well as from thalamus and are implicated in synchronization of cortical activity(Peters & Jones, 1984; Telfeian & Connors, 1998; Wise, 1975). The increase in excitatory burst discharges may therefore promote epileptiform activity by increasing the excitability of layer V pyramidal neurons that are perfectly placed to increase cortical output and network synchrony. Determining if the epileptiform activity is specific to layer V pyramidal neurons, the location of the afferent driver of the epileptiform activity and the specific contribution of layer V changes to in-vivo epileptiform activity are areas of current investigation.

In addition to excitatory bursting, distinct inhibitory bursting was similarly observed.   To isolate inhibitory synaptic currents we routinely blocked glutamatergic neurotransmission with bath application of APV and DNQX.   As inhibitory bursting persisted in the presence of glutamatergic blockade this suggests inhibitory bursting is not mediated by afferent glutamatergic input. This appears is in contrast to our findings on excitatory bursting and may reflect intrinsic excitability changes and spontaneous burst discharges form inhibitory interneurons themselves. Inhibitory interneurons in the cortex are a diverse group of neurons that have distinct anatomical, morphological and cellular properties(Markram et al., 2004 for review). Based upon our results we cannot ascertain if changes to inhibition are confined to one class of interneuron and future work will be needed to determine its specific role in mediating CCI induced epileptiform activity and PTE.

Pediatric Traumatic Brain Injury

       Traumatic brain injury that occurs in children differs from adults with a decreased mortality rate(Luerssen, Klauber, & Marshall, 1988), increased incidence of skull fractures and epidural hematomas (Sarkar et al., 2014) and greater deficits in cognitive and behavioral functioning(Anderson et al., 2005c; McKinlay et al., 2002). In this study we have begun examining if the pathophysiology of TBI in children V pyramidal neurons. The development of epileptiform activity early after injury may be the first step "on the road" to PTE. Understanding how TBI alters cortical excitability early after injury may help define therapeutic targets and a critical window of intervention.

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Discussion

Using whole-cell patch clamp and EEG techniques in juvenile rats this study demonstrates that (a) all rats within fourteen days post CCI injury show epileptiform activity by EEG, (b) there were no changes to synaptic or intrinsic properties in layer V pyramidal neurons, (c) excitatory and inhibitory synaptic bursting is greater in CCI animals compared to control, and (d) there is greater synaptic bursting than inhibitory bursting, suggesting a hyperexcitable network develops post injury. Previous studies have shown an increase in postsynaptic currents after injury, but not while examining for changes in cell properties. Other CCI studies that have shown increased spontaneous events, have not presented findings of synaptic bursts, both inhibitory and excitatory.

Neuronal firing usually occurs in a single action potential in isolation in response to discrete input postsynaptic potentials combine and cause the membrane potential to depolarize. Neurons sometimes will have periods of rapid action potentials as opposed to the single firing event. Neuronal bursting is often seen as necessary to increase the reliability of neuronal communication (Izhikevich et al., 2003). Homeostatic synaptic plasticity has been implicated in an increase of network excitability after traumatic brain injury resulting in network burst activity (Houweling et al., 2005). It has been shown that bursting activity resulted from the upregulation of excitatory synapses between pyramidal neurons (Houweling et al., 2005).

Electrical brain activity is normally non synchronous and when an epileptic seizure occurs, several neurons begin firing unusually, excessively and in synchrony. When an excitatory neuron fires, the resistance to continue to fire or fire again is because of the effect of inhibitory neurons or intrinsic properties of the neuron itself (Somjen,

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2004). However, during epilepsy the resistance of the excitatory neuron to fire is

decreased due to changes in ion channels or irregular activity of inhibitory neurons

(Somjen, 2004). Individual neurons are capable of intrinsic bursting in response to input

(Traub & Wong, 1982). When several neurons begin to burst in synchrony, a functional

heterogeneity of cortical regions for seizure generation can lead to seizures (Timofeev &

Steriade, 2004). Previous studies have examined mechanisms responsible for post-

traumatic epileptogenesis in rodents models using techniques such as lateral fluid

percussion (Thompson et al., 2005), CCI in mice (Cantu et al., 2014; Hunt et al., 2009;

Hunt, Scheff, & Smith, 2011), and cortical undercutting (Jacobs, Graber, Kharazia,

Parada, & Prince, 2000; Yang et al., 2010). To our knowledge, only one study has found

bursting, but this was done during extracellular recordings after CCI injury (Yang et al.,

2010). Our findings provide evidence that excitatory intracellular bursting is more

apparent in neurons in a TBI brain. Furthermore, we show the increase in bursting in

conjunction with intrinsic and synaptic properties unchanged after TBI. This suggests that

pyramidal neurons of layer V are experiencing a change in input from other regions of

the brain. The surprisingly similar bursting patterns, intrinsic and synaptic properties

seen in CCI compared to control suggest that cortical network connectivity and function

is unchanged, but the manner in which neurons are transmitting information is different

following severe cortical injuries.

Epilepsy research has shown that epileptiform activity can result from a shift of

balance excitation and inhibition toward excitation (Dichter & Ayala, 1987; Galarreta &

Hestrin, 1998; Nelson & Turrigiano, 1998; Tasker & Dudek, 1991). This has been shown

by studies that have elicited experimental seizures by blocking inhibition (Matsumoto &

Marsan, 1964; Prince, 1978; Steriade, Amzica, Neckelmann, & Timofeev, 1998). Other

favorable conditions to generate seizures would be to increase inhibition and decrease

excitation (Timofeev & Steriade, 2004). How this balance is altered during TBI to

contribute to the development of PTE is still unclear. Our findings show that there is an

increase in excitation after TBI. This is consistent with other TBI studies. However, it is

our understanding that we are the first to show this shift in balance, toward excitation,

during two weeks post injury in juvenile rats intracellularly.

There are many possibilities that play a role in TBI pathology leading to PTE

including increased inflammation (Johnson et al., 2013; C. Smith et al., 2013), white

matter degeneration (Johnson et al., 2013), oxidative and nitrosative damage (Abdul-

Muneer et al., 2013), and mitochondrial changes (Balan et al., 2013; Cheng, Kong,

Zhang, & Zhang, 2012). Also, further work needs to be conducted to elucidate what

region of the brain is driving this rapid enhancement of excitability in layer V neurons.

Perhaps, as axon sprouting and enhanced excitatory synaptic connectivity onto layer V

pyramidal neurons has been shown in chronic models of posttraumatic epileptogenesis

(Jin, Prince, & Huguenard, 2006; Salin, Tseng, Hoffman, Parada, & Prince, 1995), using

biocytin on neurons in affected regions could provide visual data to under projection

changes after injury.

In conclusion, improvements in treatment and diagnosis of PTE are desperately

needed. Current treatment of children that suffer from TBI approaches the problem by

treating any child that suffers an injury. Providing anticonvulsants to a developing brain,

when there may be no risk of PTE, could have undesirable developmental and cognitive

ramifications. Here we have shown that, on a cellular level, those that suffer an injury are

experiencing a bursting phenomenon. This bursting phenomenon could lead to the area of the brain that may play role in driving a hyperexcitable cortex. By studying this TBI 14 days post injury in juvenile rats, we are observing the period in which PTE is developing. PTE could develop because of the modification of the network during the brain's healing process.

Conclusion

This dissertation uses common electrophysiology approaches to better understand the pathogenesis of PTE. In chapter 2, we explored possible changes in intrinsic and synaptic properties after TBI. We began this work by understanding how TBI patients are currently being treated and found that any individual that suffers TBI is prescribed anticonvulsants. This led us to investigate possible ways to distinguish who is going to develop PTE and who will not.

Our investigation led us to find an increase in excitatory cellular bursting, both inhibitory and excitatory, while observing no change in intrinsic or synaptic properties in juvenile rodents within fourteen days after sever injury. Bursting frequency is a unique characteristic in the brain that develops after an individual suffers from TBI. Our results are the first to show this unique phenomenon that could eventually lead to a better understanding of the pathogenesis of PTE epilepsy. A better understanding of the origin of this bursting phenomenon is only the beginning to understand the cortical circuit related to layer V and its role in the pathogenesis of PTE.

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APPENDIX A

CURRICULUM VITAE

EDUCATION

Aug. 2012 - Current   M.S. in Biology
                       Co-Chair: Trent Anderson, Ph.D. – *University of Arizona – Basic Medical Sciences*
                       Co-Chair: Konstantinos Tsakalis, Ph.D. – *Arizona State University - Department of Electrical Engineering*
                       Expected Graduation: August 2014

May 2010   B.A. with honors magna cum laude

                       Honor's Thesis Title: Monitoring the Change in 5HT2A in Response to Fear Conditioning
                       Chair: William J. Tyler, Ph.D. – *Arizona State University*

HONORS/AWARDS

2006-2010   Dean's List, Arizona State University, Tempe, AZ
2008-2010   Barrett Honor's Scholar

RESEARCH EXPERIENCE AND TRAINING

Aug 2012 – Current   **Arizona State University – School of Life Sciences**
                       Phoenix, AZ
                       Master of Science - Biology
                       Investigating cortical hyperexcitability as a result of traumatic
brain injury.

                       Responsibilities:
                       • Perform controlled cortical impacts on rats and implant recording EEG electrodes.
                       • Whole cell patch clamp recordings on cortical pyramidal neurons.
                       • Analyze signal data using Clampfit, Excel, and Prism software.
                       • Perform signal analysis of EEG data recorded from rats and humans suffering from traumatic brain injury using Matlab, Excel, and C.

                       Principle Investigators – Trent Anderson, Ph.D. & Konstantinos Tsakalis, Ph.D.

June 2011-Aug 2012   **University of Arizona-College of Medicine Phoenix**
                       Phoenix, AZ
                       Research Technician
                       Investigating the role of neurosteroids in regulating cortical
                   excitablity.
                       Responsibilities:

- Perform voltage and current clamp techniques to neurons in situ while pharmacologically inducing epileptiform activity.
- Test different anti-convulsing drugs concentrations *in situ* and evaluate intrinsic drug effect.
- Perform data analysis using MINI software and Excel.

Principle Investigator – Trent Anderson, Ph.D.

Aug 2011 – Aug 2012 **Arizona State University – School of Life Sciences**
Tempe, AZ
Research Technician
Understanding the development and function of motorneuron dendritic architecture.
Responsibilities:
- Write and perform protocols for patch clamp recordings of third-star larvae Drosophila.
- Perform technical functions for Drosophilia including: preparing food and vials, maintain and care for back-up fly lines.

Principal Investigator: Carsten Duch, Ph.D.

2008-2011 **Arizona State University – School of Life Sciences**
Tempe, AZ
Honors Research Scholar
Investigate ultrasonic neuromodulation, and homeostatic plasticity using the rodent olfactory system as prime model.
Responsibilities:
- Conduct *in vivo* behavioral assays including: Morris water maze, rot-a-rod, wire hang test, reward and aversive conditioning, etc.
- Model wave properties of acoustic pressure generated by ultrasound on brain fluids.
- Fluorescently label receptors by immunohistochemistry and image tissue slices with laser scanning confocal microscopy.
- Conduct *in vivo* imaging using two-photon microscopy and perform data analysis using Matlab.

Principal Investigator: William Jamie Tyler, Ph.D.

PROFESSIONAL MEMBERSHIP
Society for Neuroscience
2013-Present

PUBLICATIONS & PRESENTATIONS

Publications:

Joshua Nichols, Chen Wu, Roxy Perez, Lucy Treiman, and Trent Anderson (Submitting June 2014) Traumatic brain injury induces rapid enhancement of cortical excitability in juvenile rats.

Corey Goddeyne, **Joshua Nichols**, Chen Wu, and Trent Anderson (In Preparation) Repetitive mild brain injury selectively decreases cortical inhibition in juvenile rats.

Li MM, Tufail, Y., **Nichols, J.**, Cruz, Karina, and Tyler, WJ (In Preparation). Serotonin modulates sensory input gains in a context-dependent manner at olfactory glomeruli.

J.F. Georges*, X. Liu*, J. Eschbacher, **J. Nichols**, R. Spetzler, B.G. Feuerstein, M.C. Preul, K. Van Keuren-Jensen, T. Anderson, H. Yan*, P.Nakaji* (In Review) Rapid *ex vivo* identification of central nervous system lymphoma with conformational switching aptamer.

Joseph F. Georges, Nikolay L. Martirosyan, Jennifer Eschbacher, **Joshua Nichols**, Maya Tissot, Mark C. Preul, Burt Feuerstein, Trent Anderson, Robert F. Spetzler, Peter Nakaji (2014) Sulforhodamine 101 selectively labels human astrocytoma cells in an animal model of glioblastoma. *Journal of Clinical Neuroscience* 21 (5):

Joseph Georges, BS, Aqib Zehri, BS, Elizabeth Carlson, BS, **Joshua Nichols, BA**, Michael A. Mooney, MD, Nikolay L. Martirosyan, MD, Layla Ighaffari, M. Yashar S. Kalani, MD, PhD, Jennifer Eschbacher, MD, Burt Feuerstein, MD, PhD, Trent Anderson, PhD, Mark C. Preul, MD, Kendall Jensen, PhD, and Peter Nakaji, MD (2014) Contrast-free microscopic assessment of glioblastoma biospecimens prior to biobanking. *Neurosurgical Focus* 36(2): E8.

Abstracts:

Society for Neuroscience– **Joshua Nichols**, Chen Wu, Roxy Perez, Lucy Treiman, and Trent Anderson (2013) Traumatic Brain Injury Induces Rapid Enhancement of Cortical Excitability in Juvenile Rats

American Association of Neurological Surgeons - Joseph F. Georges, Nikolay L. Martirosyan, Jennifer Eschbacher, **Joshua Nichols**, Maya Tissot, Aqib H Zehri, George AC Mendes, Anna Joy, Ali Elhadi, Mark C Preul, Burt G Feuerstein, Robert F Spetzler, Trent Anderson, Peter Nakaji (2013) Ex Vivo Fluorescence Neuropathology: Immediate and Specific Diagnosis of Human Astrocytic Brain Tumors

Neurotrauma Symposium - Trent Anderson, Corey Goddeyne, **Joshua Nichols**, Anna Yoshihiro, Roxy Perez, Lucy Treiman, and P. David Adelson (2012)

Enhanced Cortical Excitability in the Peri-Injury Zone of Immature Rats after Experimental TBI

American Association of Neurological Surgeons - Joseph F. Georges, Nikolay L. Martirosyan, Jennifer Eschbacher, **Joshua Nichols**, Maya Tissot, Ali M. Elhadi, George Mendes, Michelle McQuilkin, Burt G. Feuerstein, Robert F. Spetzler, Trent Anderson, Mark C. Preul, Peter Nakaji (2012) Rapid and Specific Diagnosis of Human Astrocytic Brain Tumors by Immediate Imaging with Sulforhodamine 101

Society for Neuroscience - Monica Li Tauchmann, Yusuf Z. Tufail, **Joshua Nichols** and William J. Tyler (2010) Amplitude modulation by 5HT2A receptors at primary olfactory synapses underlies a learning-dependent peripheral sensory gate.

Presentations:
University of Arizona – College of Medicine Phoenix Basic Medical Seminar Series (June 2013) **Enhanced Cortical Excitability in the Peri-Injury Zone of Immature Rats After Experimental TBI**

Other Work History
Academic Mentor of Student Athletes | *Arizona State University* | Tempe, AZ
  6/11- Current
Student Service Assistant of University Housing| *Arizona State University* | Tempe, AZ
  10/10-6/11
Success Coach – Peer Mentor | *Arizona State University*| Tempe, AZ
  8/07-5/10