Mild Traumatic Brain Injury Produces More Immediate and Prolonged Synaptic Plasticity Deficits in the Juvenile Female Hippocampus

by

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BSc, University of Victoria, 2011

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Supervisory Committee

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Abstract

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Traumatic brain injury (TBI) is the leading cause of disability in individuals under 45 years of age, with mild TBI (mTBI) accounting for the majority of cases. The juvenile brain is in a period of robust synaptic reorganization and myelination, making adolescence a particularly vulnerable time to incur a TBI. Learning and memory deficits that involve the hippocampal formation are often observed following mTBI in adults. To examine this issue in the juvenile brain, we assessed changes in hippocampal synaptic plasticity following closed-head mTBI in male and female Long-Evans rats (25-28 days of age). Synaptic plasticity of field excitatory post-synaptic potentials (fEPSPs) was assessed using *in vitro* electrophysiology at either one hour, one day, seven days, or 28 days following mTBI in the dentate gyrus (DG) and the *cornu ammonis* area 1 (CA1) regions of the hippocampus. In female rats, the CA1 region ipsilateral to the impact showed a significant reduction in long-term potentiation (LTP) as early as one hour following mTBI. Similar LTP deficits were apparent at one day in the DG, and persisted to 28 days following injury. In male rats, a deficit in both DG- and CA1-LTP was maximal in the ipsilateral hemisphere by seven days following injury, but these deficits did not persist to 28 days post-injury. These data suggest that the juvenile brain is susceptible to mTBI-induced impairments in plasticity, and sex and regional differences are apparent in the expression and recovery of synaptic plasticity following mTBI.

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AMPA/GluA – α-amino-3-hydroxy-5 methyl-4-isoxazole propionate
AP – action potential
AP5 – 2*R*-amino-5-phosphovaleric acid
BDNF – brain derived neurotrophic factor
BMI – bicuculline methiodide
CA – *cornu ammonis*
Ca^{2+} – calcium
CaM – calmodulin
CaMKII – calmodulin-dependent protein kinase II
cAMP – cyclic adenosine monophasphate
CB – calbindin
CCI – controlled cortical impact
Cl^- – chloride
CN – calcineurin
CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione
CNS – central nervous system
CRE – cAMP response element
CREB – cAMP response element binding protein
CS – conditioning stimulus
DG – dentate gyrus
DCG-IV – 2-(2,3-dicarboxycyclopropyl) glycine
DHPG – 3,5-dihydroxyphenylglycine
E-LTP – early long-term potentiation
EC – entorhinal cortex
EPSP – excitatory post-synaptic potential
ER – estrogen receptor
ERK – extracellular regulated kinase
fEPSP – field excitatory post-synaptic potential
FJC – fluoro-jade C
FP – fluid percussion
GABA – γ-aminobutyric acid
GFP – green fluorescent protein
GPCR – G-protein coupled receptor
HFS – high frequency stimulation
I1 – inhibitor 1
IEG – immediate early genes
I/O – input-output
IPSP – inhibitory post-synaptic potential
K^+ – potassium
L-AP4 – 2-amino-4-phosphobutyrate
L-LTP – long-lasting long-term potentiation
LPP – lateral perforant pathway
LTD – long-term depression
LTP – long-term potentiation
MAPK – mitogen activated protein kinase
Mg^{2+} – magnesium
mGluR – metabotropic glutamate receptor
MOPP – molecular layer perforant path-associated
MPP – medial perforant pathway
MS-DB – medial septum and diagonal band
mTBI – mild traumatic brain injury
MWM – Morris water maze
Na^+ – sodium
nACSF – normal artificial cerebrospinal fluid
NMDA/GluN – *N*-methyl-*D*-aspartate
PAP – Papanicolaou
PFA – paraformaldehyde
PKC – protein kinase C
PND – postnatal day
PP1 – protein phosphatase 1
PPD – paired-pulse depression
PPF – paired-pulse facilitation
PSD – post-synaptic density
PTD – post-tetanic depression
PTP – post-tetanic potentiation
Sb – subiculum
STP – short-term potentiation
TA – temporo-ammonic
TBI – traumatic brain injury
TBS – tris-buffered saline

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Dedication

This thesis is dedicated to my family, friends, and to caffeine.

1. Introduction

1.1 The Concussion Epidemic

Traumatic brain injury (TBI) is becoming recognized as a worldwide epidemic, occurring more frequently than AIDS, breast cancer, multiple sclerosis, and spinal cord injury combined (Brain Injury Association of America, 2002). TBI is believed to be the leading cause of death and disability in individuals under 45 years of age (Basso et al., 2001), resulting in approximately 18,000 hospitalizations in Canada annually, of which 30% are children and youth, and 8% result in fatality (Canadian Institute for Health Information, 2006). The most prevalent causes of TBI include falls, motor vehicle accidents, and being struck by or against objects (National Center for Injury Prevention and Control, 2003).

TBI in the child and youth population is also a significant and growing health concern. Each year, approximately 180 per 100,000 children 15 years and younger will suffer a mild to severe TBI episode (Kraus, 1995). While motor vehicle accidents and falls account for the majority of mild TBIs (mTBIs), sports injuries are the most prevalent causes of mTBI in children and youth between the ages of 10 and 19, with the number of incidents rising 60% over the past decade (National Center for Injury Prevention and Control, 2003). Although participation in sports remains dominated by males, young females are more likely to suffer sports-related mTBIs (Covassin et al., 2003; Gessel et al., 2007; Hootman et al., 2007; Powell & Barber-Foss, 1999), and often take longer to recover than males (Broshek et al., 2005; Colvin et al., 2009; Covassin et al., 2012). The majority of research to date has focused on the effects of mTBI and TBI in the male brain. Additionally, the juvenile brain is still undergoing development, which makes this age group especially vulnerable to injury and a target population for research.

Mild forms of TBI account for nearly 80% of all head injuries (Faul et al., 2010; Management of Concussion/mTBI Working Group, 2009), and are universally defined or classified as an injury to the head resulting in loss of consciousness for less than 30 minutes and/or confusion and disorientation for less than 24 hours, with a Glasgow Coma Score of 13 or greater (Teasdale & Jennett, 1974). However, the indicators of mTBI are

so varied and inconsistent that it is all too often overlooked and ignored by patients, parents, and clinicians alike due to the lack of a robust and consistent set of symptoms, and absence of significant neuromorphological alterations. Modern imaging techniques are primarily used to rule out a diagnosis of severe TBI (i.e. bleeding in the brain), and mTBI diagnoses are primarily based on patients reporting any one of a set of non-specific symptoms that can include: headache, dizziness, confusion, nausea, memory deficits, fatigue, balance problems, attention and concentration deficits, and sleep disturbances (Prins et al., 2010). Most clinical cases of mTBI usually resolve within 1-3 weeks following injury, but many patients continue to manifest prolonged, or even permanent neurocognitive dysfunctions. This "post-concussive syndrome" occurs in approximately 10-20% of mTBI patients (Iverson, 2005).

1.1.1 Cognitive Dysfunction Following TBI - Evidence from Clinical Studies

Cognitive impairment is often the most prominent feature following a closed head brain injury of any severity (Ylvisaker & Feeney, 1998; Ylvisaker, 1997). The most commonly reported cognitive deficiencies following TBI are learning and memory deficits (Baddeley et al., 1987; Dikmen et al., 1987; Tabaddor et al., 1984; Ylvisaker & Szekeres, 2002). Classically, memory dysfunction is most apparent during the acute stage of injury, in which the individual may experience retrograde amnesia and posttraumatic amnesia. However, even in the post-acute stage of injury, persistent learning and memory deficits remain the most common complaint (King et al., 1995; van Zomeren & van den Burg, 1985). A longitudinal study of individuals who suffered moderate to severe TBIs found that 50% of the subjects reported substantial memory impairments at 5 years post-injury (Millis et al., 2001). Interestingly, the age of the individual upon sustaining a TBI is directly correlated with cognitive outcome: the younger the individual upon injury, the more long-term the deficits are. Children sustaining a TBI before the age of 8 still presented with impaired cognitive outcome at 6 years post-trauma (Verger et al., 2000).

Clinical findings indicate that even those injuries that are categorized as mTBI result in neurophysiological disruptions (Xia et al., 2012). The majority of individuals with impaired scores on cognitive and formal memory tests experienced a progressive

decrease of deficits within 1-3 months following mTBI (Dikmen et al., 1986; Ruff et al., 1989). However, in 10-20% of individuals afflicted with an mTBI, these symptoms persist for over a year, leading to post-concussive syndrome (Iverson, 2005). The most common and reliable indication or deficit incurred by an individual with post-concussive syndrome is persistent cognitive symptoms in the domains of memory, attention, and processing speed (Dikmen et al., 1986a; Dikmen et al., 1986b; Gentilini et al., 1989; Gronwell, 1989; Bohnen et al., 1993; Ruff et al., 1989). Additionally, individuals who have survived a TBI are at increased risk for developing a psychiatric disorder. Major depression, anxiety, and post-traumatic stress disorder are frequent complications following brain injury, including mTBI (Bombardier et al., 2010; Jorge et al., 2004). This evidence clearly indicates that TBI, regardless of severity, affects learning and memory processes, as well as psychiatric and emotional well-being.

1.1.2 Structural Alterations Following TBI - Evidence from Clinical Studies

Neuroimaging studies have documented significant long-term structural alterations in the brain following TBI, characterized as widespread volume changes in both white and gray matter (Levine et al., 2008). Widespread brain atrophy has been observed in pediatric and adult brain injury victims (Bigler, 1999; Levine et al., 2008; Wilde et al., 2005), with reductions in white and gray matter (Berryhill et al., 1995; Wilde et al., 2005) and enlarged lateral ventricles (Verger et al., 2001). In adults who have suffered TBI, reductions in the size of the corpus callosum and hippocampal volume can be detected months to years after injury (Kim et al., 2008; Tomaiuolo et al., 2004); whereas pediatric patients demonstrate gray and white matter loss in prefrontal, orbitofrontal and temporal brain regions (Tasker et al., 2005; Wilde et al., 2005). Additionally, longitudinal studies have demonstrated that children who experienced mild to severe TBIs display significantly smaller hippocampal volumes 10-years post-injury, regardless of the severity of the initial injury (Beauchamp et al., 2011). The significant reductions in hippocampal size that are observed following TBI (Tasker et al., 2005; Wilde et al., 2007) suggest that this region is particularly susceptible to injury, particularly in the juvenile brain. Indeed, due to the nature of most TBIs, of which the

region of primary impact often occurs on the frontal and temporal lobes, it is not surprising that the hippocampus is frequently identified as a region of increased susceptibility (Bigler, 1999).

1.1.3 Postnatal Brain Development

At birth, the human brain, in contrast to other body tissues, is immature and undergoes substantial changes in structure and function during postnatal development that continues well into adulthood. There is particular concern that sustaining a TBI during this period of robust neurodevelopment can negatively impact the normal developmental trajectory of the brain. Imaging studies have shown that the overall volume of the human brain increases during childhood and adolescence (Giedd et al. 1999; Sowell et al. 2002; Gilmore et al. 2007), with the most substantial increases in volume occurring in the cerebellum, followed by subcortical areas and then the cerebral cortex (Knickmeyer et al, 2008). Specifically, cortical gray matter volume shows a transient increase that peaks during childhood, while white matter volume continues to expand into adulthood (Giedd et al. 1999; Sowell et al. 2002). It has been proposed that these age-related increases in white matter volume and net reduction in gray matter volume are predominantly due to intracortical myelination and increased axonal caliber (Giorgio et al., 2010, Paus et al., 2008 and Perrin et al., 2008). Alternately, changes in gray matter volume may reflect synaptic reorganization that occurs during adolescence (Huttenlocher, 1979 and Petanjek et al., 2011). The development of the CNS involves both progressive and regressive events (Cowan et al. 1984; Low and Cheng 2006). Progressive events such as neural proliferation, neurite outgrowth, and synaptogenesis contribute to the formation of neuronal networks. However, during brain development about 50% of neurons do not survive into adulthood (Cowan 2001; Cowan et al. 1984; Yuan et al. 2003). Later in development, regressive events such as apoptosis and synaptic pruning are necessary to refine the networks to a more precise and mature circuitry, and can be crucial for proper brain development and function (Giedd et al., 1999, Gogtay et al., 2004 and Sowell et al., 2001). Therefore sustaining a TBI during critical points of postnatal brain development has the potential to upset the normal balance of progressive

and regressive events, leading to aberrant and conceivably detrimental neuronal connectivity and signalling.

1.2 Modeling Traumatic Brain Injury in the Rodent Brain

Several animal models of experimental TBI are currently available and have been used to study the neuropathology of brain injury. Each model has its strengths and weaknesses with regards to injury type (focal or diffuse), severity, reliability and reproducibility, and the aspects of the injury that can be studied. The most commonly used models of experimental TBI include: (1) the fluid percussion (FP) model, which employs a rapid injection of fluid through a sealed port into the intracranial space, directly deforming the brain (Bagal et al., 2005; Dixon et al., 1991; Gennarelli, 1994; McIntosh et al., 1989); (2) the controlled cortical impact (CCI) model, in which a pneumatic piston drives an impactor tip that can deform the brain tissue directly through a craniotomy, or impact onto the intact skull, thus avoiding a skull fracture (Dixon et al., 1991; Dixon et al., 1994; Hamm et al., 1992; Lighthall, 1988; Lindner et al., 1998; Quigley et al., 2009). Both methods require surgical procedures (craniotomy or stereotaxic surgery) to induce an open-head injury and produce a focal contusion since the head of the animal is rigidly fixed in a stereotaxic frame. These techniques may be adjusted to provide a range of reproducible injury severities (Gennarelli, 1994). However, since the majority of concussive head injuries are closed head injuries (Faul et al., 2010), very mild, have a low rate of mortality, and lack gross neuropathology in the presence of neurological deficits (Prins et al., 2010), a third model was developed to more accurately replicate these criteria. The weight drop model employs a freely falling brass weight through a guide tube to strike a cone placed on the exposed skull (Adelson et al., 1996; Feeney et al., 1981; Flierl et al., 2009; Foda & Marmarou, 1994; Marmarou et al., 1994; Shapira et al., 1988). This procedure is less invasive and only requires limited surgical manipulations, allowing for high throughput and a shorter period of exposure to anesthesia. Additionally, the weight drop model can be easily manipulated to produce a very mild and more diffuse injury with widespread diffuse axonal injury, as in the case of human mTBI (Adams et al., 1989; Benson et al., 2007; Capruso & Levin, 1992; Evans,

1992; Levin et al., 1987). Finally, the lack of a stereotaxic frame allows for rotational acceleration, as often occurs in the case of human injuries. Given these characteristics, we chose to use the weight drop model to mimic mild, closed head concussions in the juvenile brain.

1.2.1 Cognitive Dysfunction following TBI – Evidence from Rodent Models

Importantly, both cognitive dysfunction and hippocampal atrophy have been observed in rodents following experimental brain injury (Bramlett & Dietrich, 2002; Creed et al., 2011; Milman et al., 2005; Smith et al., 1997; Tate & Bigler, 2000; Zohar et al., 2003; Zohar et al., 2011). Indeed, experimental brain injuries in animals produce measurable cognitive deficits, including impairments in hippocampal-dependent learning and memory tasks. Various studies have shown that significant deficits in the ability to perform the Morris water maze (MWM) test (Chen et al., 1996; Creed et al., 2011; Yamaki et al., 1997), novel object recognition test (Prins et al., 2010), and the metric change and temporal order tasks (Gurkoff et al., 2013) can be observed following TBI, indicating that hippocampal impairment is similar to that which occurs in humans. Even following mTBI using the weight drop model, deficits in the ability to perform in the MWM, the swim T-maze, and the passive avoidance tests were observed up to 90 days post-injury (Milman et al., 2005; Zohar et al., 2003). Additionally, the forced swimming test also revealed the occurrence of depression-like behaviours following this type of injury (Milman et al., 2005).

Of note, the hippocampus has been implicated in several cognitive and emotional processes, with the dorsal hippocampus being more involved in learning and memory (particularly spatial navigation), and the ventral hippocampus playing a role in affect-related functions such as depression and anxiety through its connections with the amygdala and other parts of the limbic system (Bannerman et al., 2004; McNaughton & Gray, 2000; Moser & Moser, 1998; Segal et al., 2010). Thus, it is reasonable to speculate that damage to the hippocampal structure following mTBI might underlie some of the cognitive and affective deficits that are characteristic of mTBI clinical presentation.

1.3 The Hippocampus

The hippocampus is located within the medial temporal lobe of the brain (**Figure 1**), and consists of the *Cornu ammonis* areas 1 through 3 (CA1-3). The hippocampus proper (CA1-3), together with the dentate gyrus (DG), entorhinal cortex (EC), presubiculum, subiculum, and parasubiculum, collectively form the hippocampal formation (Amaral & Lavenex, 2007). The basic layout of cells and fiber pathways in the hippocampus, which are generally conserved in most mammals, make it an ideal model for comparative neural studies.

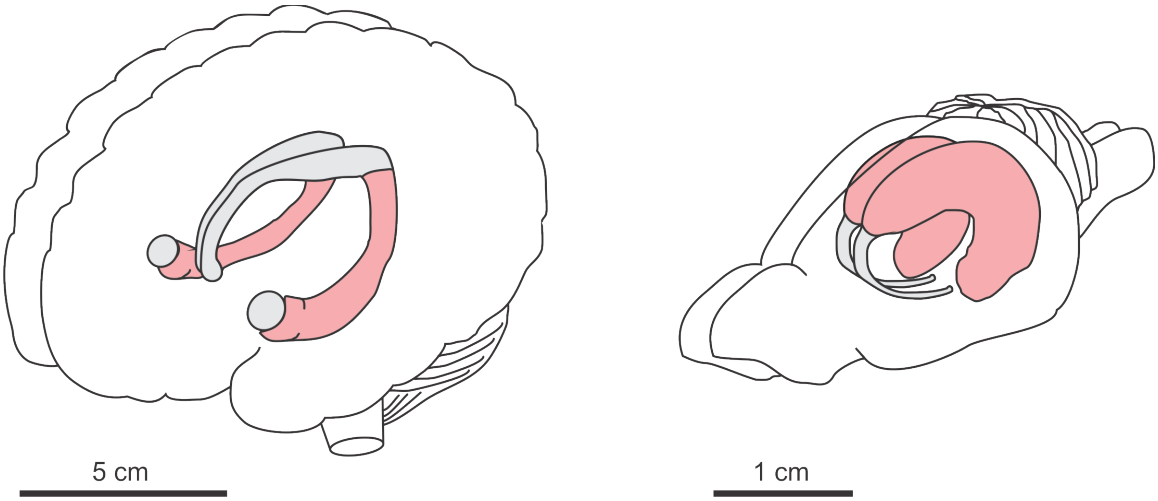

Figure 1. Location of the hippocampus in the human and rat brain. The hippocampal formation *(pink)* is located beneath the cerebral cortex, in the medial temporal lobe of the brain. In the human brain *(left)*, the hippocampus is linear in shape and horizontally oriented, whereas in the rat brain *(right)* it is characterized by its c-shape and vertical orientation. However, the general structure and connectivity of the hippocampus remains conserved in the mammalian brain.

1.3.1 Hippocampal Circuitry

In the hippocampal formation, unidirectional progression of excitatory pathways linking the DG, CA1 and CA3 subregions form the trisynaptic circuit (Amaral, 1993; Andersen et al., 1971). Unidirectional signal propagation originates from the medial and lateral EC axonal projections in layer II and passes to the granule cell dendrites of the DG molecular layer in an excitatory manner, via the medial and lateral perforant pathways (MPP and LPP, respectively). The granule cells extend unmyelinated axons termed mossy fibers; these fibers project onto proximal dendrites of pyramidal cells located in the stratum lucidum of the CA3 subfield. Schaffer collateral axons from the CA3 synapse onto CA1 pyramidal cells, which in turn project excitatory output to the subiculum (Sb), where it re-enters the EC at layer V/VI (Amaral & Lavenex, 2007) (**Figure 2**).

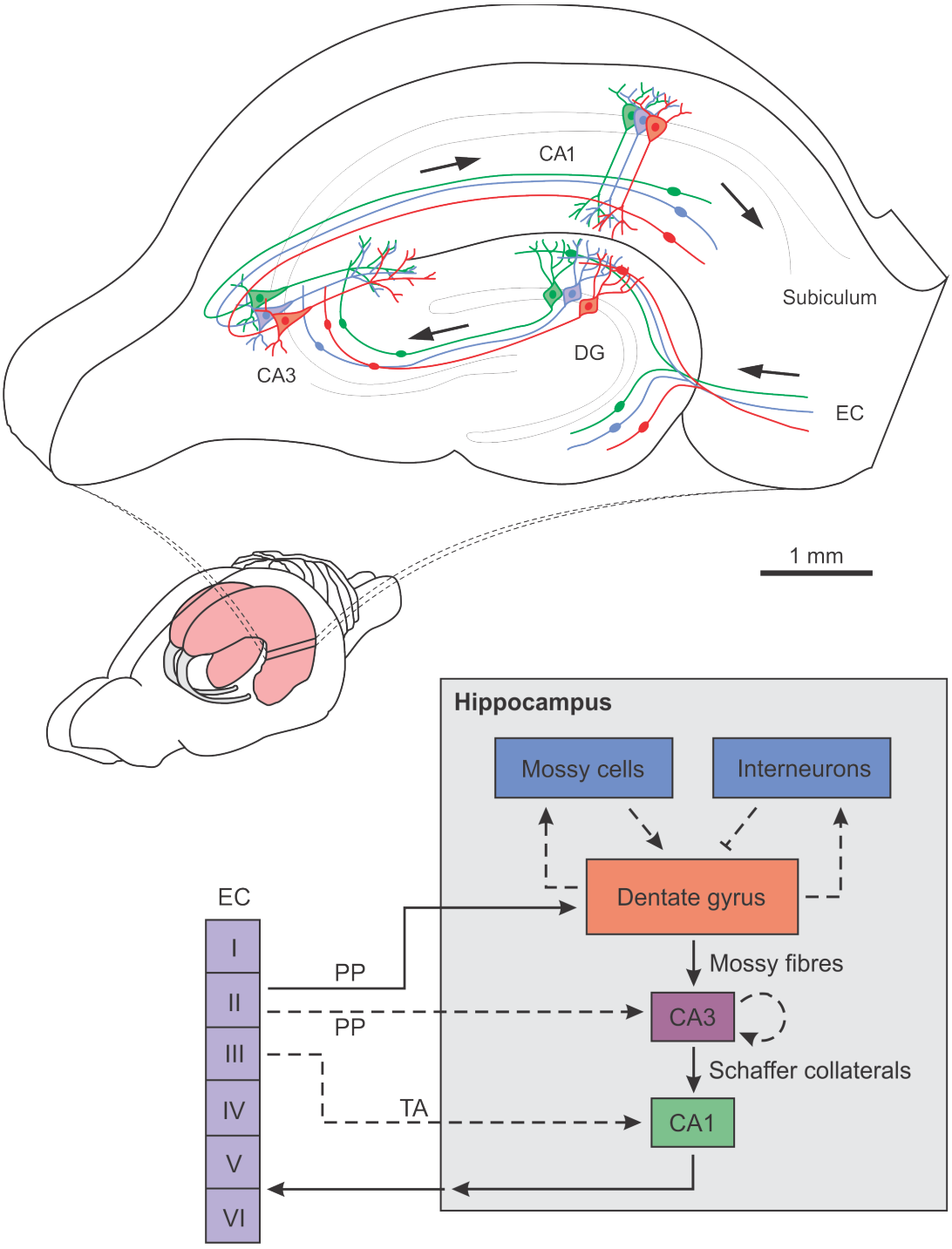

Figure 2. The hippocampal circuit. The hippocampal formation is organized into a unidirectional network with excitatory input from the EC passing along the medial and lateral perforant paths (PP) to the DG and CA3. The mossy fibers pass additional input from the DG to the CA3 pyramidal neurons. CA3 axons synapse with CA1 pyramidal neurons via the Schaffer collaterals, and are subsequently directed to the subiculum and EC. Neurons from the subiculum send axons back to the EC, completing the hippocampal circuit.

1.3.2 Anatomy and Connectivity of the Dentate Gyrus

Within the hippocampus, the DG region is comprised of two opposing blade structures, the suprapyramidal blade and infrapyramidal blade, which are joined by the crest to form a visually distinct V/U-shape. The DG possesses three distinct cellular layers, known as the molecular layer, granule cell layer, and polymorphic layer (also referred to as the hilus). These layers can be characterized by the distinct type(s) of neurons they contain, and their connectivity.

The primary cell type in the DG is the granule cell. Granule cells are distinct in that they possess elliptical cell bodies with no glial sheath between the cells, which allows them to pack together very densely (Rapp & Gallagher, 1996). A characteristic cone-shaped tree of spiny apical dendrites arises from the granule cell body (Amaral & Lavenex, 2007), and extends its projections up into the molecular layer (Desmond & Levy, 1985). It is through these granule cell dendrites in the molecular layer that that the DG receives its chief excitatory cortical input via the perforant pathway. Additionally, the granule cells are the only type of cell in the DG to extend axons that innervate another hippocampal field (Amaral & Lavenex, 2007).

The perforant pathway can be anatomically divided into the medial and lateral perforant pathways, corresponding to their distinct layer of origin in the EC and point of termination in the molecular layer of the DG. The molecular layer is divided into the lateral, medial, and inner layers, and innervation via the MPP and LPP follows a laminar fashion. The MPP afferents arise from the medial EC and innervate the middle one-third of the molecular layer. Conversely, LPP afferents originate from the lateral EC and project to the lateral one-third of the molecular layer (Hjorth-Simonsen & Jeune, 1972; Hjorth-Simonsen, 1972; McNaughton, 1980; Nafstad, 1967; Steward & Scoville, 1976).

In addition to receiving primary excitatory input from the perforant pathway, the DG also receives excitatory input from the mossy fiber pathway. Dentate granule cell axons form excitatory synapses with mossy cells in the hilus, which then target other granule cells in the molecular layer of the hippocampus as a form of feedback excitation (Scharfman, 2007; Seress et al., 1989). The mossy fiber pathway eventually projects to the CA region where mossy cell axons synapse with the pyramidal neurons of the CA3. Inhibitory basket cells in the molecular layer and hilus modulate this mossy fiber

pathway. Upon stimulation of granule cells, basket cell interneurons release the inhibitory neurotransmitter γ-aminobutyric acid (GABA) as a form of feedback inhibition (Scharfman, 2007). Additional inhibitory interneurons which reside in the outer edge of the molecular layer, known as molecular layer perforant path-associated (MOPP) cells (Han et al., 1993), receive excitatory input from the perforant pathway and inhibit the activity of granule cells as a form of feedfoward inhibition (Mott et al., 1997) (**Figure 2**).

1.3.3 Anatomy and Connectivity of the *Cornu Ammonis*

The CA region of the hippocampus is composed of pyramidal neurons, and many electrophysiological studies focus on the CA rather than the DG, due to the easily activated Schaffer collaterals that project from the pyramidal neuron axons of the CA3 subfield to the CA1 (Spruston & McBain, 2007). Pyramidal neurons have a distinct, triangular shaped soma for which they were named. Additional structural features include a single axon, a large apical dendrite arising from the soma apex, and multiple basal dendrites that arise from the base of the soma (Megías et al., 2001). The apical and basal dendrites are highly branched, and can reach several centimeters in length. Dendritic spines covering the surface of pyramidal cell dendrites display a wide array of morphological complexities, of which no preferential localization to a particular region of the CA1 dendritic tree is demonstrated (Spruston & McBain, 2007). Throughout the life of a pyramidal neuron, spine structure does not remain static, but instead changes in response to environmental factors, hormonal signals, or neurotransmitter receptor activation (Bonhoeffer & Yuste, 2002; Hering & Sheng, 2001; Nikonenko et al., 2002; Nimchinsky et al., 2002).

Excitatory impulses that enter a CA1 pyramidal neuron are targeted to dendritic spines, where glutamate receptors are clustered in the post-synaptic density (PSD). The CA1 receives its major extrinsic input connections from CA3 pyramidal neurons via the Schaffer collaterals and the commissural fibers of the contralateral hippocampus (Amaral & Witter, 1989). Additional input comes from the temporo-ammonic (TA) pathway from EC layer III excitatory cells (Steward & Scoville, 1976) and the medial septum and diagonal band (MS-DB) area (Freund & Antal, 1988; Gulyás et al., 1990) (**Figure 2**).

The CA1 is structured in clearly defined strata, and input into the CA1 is layer-specific. The stratum lacunosum-moleculare and radiatum are the most superficial layers of the CA1 and contain the apical dendritic trees of the pyramidal cells where EC afferents synapse. The commissural fibers and Schaffer collaterals project from the CA3 into the stratum radiatum, and the cell bodies of pyramidal neurons and axo-axonic, bistratified, and radial trilaminar interneurons are found in the stratum pyramidale. The stratum oriens contains the cell bodies of inhibitory basket cells and horizontal trilaminar cells, in addition to the basal dendrites of pyramidal neurons, which are innervated by other pyramidal cells, septal fibers, and recurrent collaterals. The alveus contains the axons from pyramidal neurons that propagate to the fimbria-fornix; it is the deepest layer of the CA1 and one of the major outputs of the hippocampus (Andersen et al., 2007). Pyramidal neurons project excitatory output to the subiculum, where it re-enters the EC at layer V/VI (Amaral & Lavenex, 2007).

1.4 Synaptic Plasticity

Synaptic plasticity is the ability of a synapse between two neurons to change in strength as a function of modifications in synaptic activity through excitatory and inhibitory networks. According to postulations by Canadian psychologist Donald Hebb in 1949, continuous firing of one neuron upon another induces lasting cellular changes that increase synaptic efficiency between the two neurons (Hebb & Konzett, 1949). Decades later, Bliss and Lomo discovered the phenomenon of long-term potentiation (LTP) in the hippocampus (Bliss & Lomo, 1973), a process characterized by the qualities of synaptic plasticity as defined in Hebb's rule. Further experimentation into LTP (Collingridge et al., 1988a, 1988b) revealed that it was one of the major putative cellular mechanisms behind learning and memory (Bliss & Collingridge, 1993).

1.4.1 Glutamatergic Transmission

The amino acid glutamate is the major excitatory neurotransmitter in the brain (Cotman & Monaghan, 1986) and essential to hippocampal synaptic plasticity. Release of glutamate sequestered in presynaptic vesicles can activate pre- and postsynaptic receptors

upon binding. One such class of receptor is the ionotropic glutamate receptor, which contains ion channels composed of four transmembrane regions. Upon agonistic binding of glutamate, ionotropic glutamate receptors conduct sodium (Na^+), potassium (K^+), and sometimes calcium (Ca^{2+}) cations to produce excitatory postsynaptic potentials (EPSPs) (Purves et al., 2004). The three classes of ionotropic glutamate receptor include *N*-methyl-*D*-aspartate (NMDA or GluN) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA or GluA) receptors, and kainate receptors (Ozawa et al., 1998).

1.4.1.1 NMDA Receptors

NMDA receptors (NMDARs) exist as heterotetramers composed of two mandatory NR1 subunits and two NR2 subunits (NR2A-D), which confer functional NMDAR characteristics (Benveniste & Mayer, 1991; Clements & Westbrook, 1991; Cull-Candy & Leszkiewicz, 2004). The subunits adopt a dimer of dimers arrangement to form the overall tetramer composition of the receptor. NMDARs are ligand-gated channels which require co-activation by binding of both glutamate and glycine; within each dimer, the NR1 subunit binds the co-agonist glycine while the NR2 subunit binds the neurotransmitter glutamate in their extracellular domains (Furukawa et al., 2005; Rambhadran et al., 2010). In addition to its ligand-gated properties of activation, the NMDAR is also a voltage-dependent channel. At hyperpolarized or resting membrane potentials, a voltage-dependent magnesium ion (Mg^{2+}) block exists in the channel pore, preventing the passage of cations through the receptor channel. Therefore, in order for activation and subsequent opening of NMDARs to create an EPSP, glutamate and glycine must bind the extracellular receptor domain (Johnson & Ascher, 1987), and sufficient depolarization of the postsynaptic membrane is needed to expel the Mg^{2+} block from the channel pore (Mayer et al., 1984; Nowak et al., 1984), which allows the flow of cations in and out of the cell. The NR2A and NR2B subtypes of the NMDAR are differentially expressed during development; during early postnatal development, the NR2B-type NMDAR predominates. However, at PD28 the rodent brain expresses an approximately equivalent ratio of NR2A/NR2B subtypes (Sans et al., 2000). This switch in NR2 subunit composition ultimately results in the predominant expression of the NR2A-type NMDAR

in the adult brain (Neyton & Paoletti, 2006). Therefore, mechanisms of NMDA-dependent synaptic plasticity vary according to age and stage of brain development according to NR2 subunit composition.

1.4.1.2 AMPA Receptors

AMPA receptors (AMPARs) are ionotropic glutamate-activated transmembrane receptors very similar to NMDARs in structure and overall subunit organization. Composed as a dimer of dimers of four subunits (GluR1-4) to form a heterotetramer, the AMPAR is permeable to select cations (K^+, Na^+, and sometimes Ca^{2+}) through a central channel in the tetramer of subunits (Kullman, 2007). In the hippocampus of the adult rodent brain, most AMPARs are GluR1-2 or GluR2-3 tetramers (Wenthold, Petralia, Blahos J, & Niedzielski, 1996). Post-transcriptional mRNA editing of the GluR2 subunit substitutes out the uncharged amino acid glutamine (Q) in the receptor's ion channel, for the positively charged arginine (R) residue (Sommer et al.,1991). Therefore, the presence of the GluR2 subunit governs AMPAR permeability; due to electrostatic repulsion between the bulky positively charged side chain of the arginine residue in the receptor's ion channel, the GluR2 subunit is rendered energetically unfavourable to Ca^{2+} transmission (Kullman, 2007). However, in spite of the lack of Ca^{2+} conduction in the hippocampus through AMPARs, they are responsible for mediating the majority of EPSPs and are thus prominent targets in the modulation of synaptic plasticity. Phosphorylation of specific serine (S) and threonine (T) residues within AMPAR subunits by protein kinases modulates the localization, open probability, and conductance of AMPARs (Banke et al., 2000; Derkach et al., 1999).

1.4.1.3 Kainate Receptors

Kainate receptors demonstrate 40% and 20% homology to AMPA and NMDA receptors, respectively (Bettler et al., 1990; Ozawa et al., 1998; Seeburg, 1993; Werner et al., 1991). Although abundant in hippocampal interneurons, kainate receptors play a minor role in synaptic plasticity by influencing the amount of neurotransmitter released from the presynaptic cell (Huettner, 2003).

1.4.1.4 Metabotropic Glutamate Receptors

Another receptor family implicated in synaptic plasticity and activated by glutamate ions is the metabotropic glutamate receptors (mGluRs), a class of group C family G-protein coupled receptors (GPCRs) responsible for the modulation of excitatory synaptic events. Depending on the receptor subtype composition, mGluRs may act through excitation or inhibition of synaptic transmission (De Blasi et al., 2001). A total of eight different types of mGluRs exist, divided into three groups (I, II, and III). Group classification is a function of physiological activity and slight variations in receptor structure. Group I mGluRs (mGluR1 and mGluR5) enhance excitability through activation by 3,5-dihydroxyphenylglycine (DHPG), while group II and III mGluRs reduce the activity of excitatory and inhibitory postsynaptic potentials through the selective agonists 2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) and 2-amino-4-phosphobutyrate (L-AP4), respectively (Shigemoto et al., 1997). As a modulator of synaptic activity, mGluRs have been implicated in the regulation of NMDA receptor activity (Lea et al., 2002; Skeberdis et al., 2001) and synaptic plasticity (Endoh, 2004).

1.4.2 GABAergic Transmission

Inhibitory synaptic transmission must also exist in order to reduce the excitation induced by glutamatergic transmission. γ-aminobutyric acid (GABA) is the most prominent inhibitory neurotransmitter in the brain (Roberts, 1980). GABA receptors exist in two classes: ligand gated ion channel receptors ($GABA_A$), or metabotropic G-protein coupled receptors ($GABA_B$) (Bormann, 2000). The $GABA_A$ receptor exists as a multimeric transmembrane receptor, with five subunits encircling a central ion-conducting pore. In the hippocampus, the composition of the $GABA_A$ receptor pentamer must include two α subunits and two β subunits, with the identity of the fifth subunit being γ or δ (Chang et al., 1996). The binding of two GABA molecules between the α and β subunit interface causes the receptor protein to shift to an open conformation, thus allowing chloride anions (Cl^-) to flow down their electrochemical gradient (Colquhoun & Sivilotti, 2004). Therefore, $GABA_A$ receptors rely on the chloride reversal potential, which is more hyperpolarized than the resting membrane potential of mature neurons.

Production of hyperpolarizing inhibitory chloride currents counteract the depolarization of the membrane by excitatory neurotransmitters, typically leading to an inhibitory postsynaptic potential (IPSP) that results in an inhibition of further action potentials (APs) by the postsynaptic neuron and a net reduction in neuronal activity (Purves et al., 2004). Thus GABAergic transmission plays a significant role in synaptic plasticity.

1.4.3 Short-term Synaptic Plasticity

Activity-dependent synaptic modification can originate from the presynaptic terminal, impacting neurotransmitter release and leading to changes in short-term plasticity (Mochida et al., 2008). Following a conditioning stimulus (CS) in the hippocampal formation, synapses may show a transient increase or decrease in synaptic efficacy over a time course of milliseconds (facilitation/depression), seconds (augmentation), or minutes (post-tetanic potentiation/depression).

1.4.3.1 Paired Pulse Facilitation & Depression

Paired pulse plasticity is a short-term form of synaptic plasticity. When two stimuli are delivered in rapid succession (5-100 msec inter-pulse interval), the magnitude of the synaptic response following the second stimulation is modified by the prior stimulation event. An increase or decrease in the paired pulse ratios between the second and first stimuli would indicate an alteration in the amount of the presynaptically released excitatory neurotransmitter glutamate. Most major synaptic inputs in the hippocampus – such as the Schaffer collaterals that project from CA3 pyramidal neurons to the CA1 subfield – exhibit paired pulse facilitation (PPF), which is defined as an increase in the size of the second synaptic response in comparison to the first. In contrast, the MPP of the DG normally exhibits paired-pulse depression (PPD), in which the size of the second synaptic response is reduced with respect to the first paired pulse stimulus (Bortolotto et al., 2011). Facilitation or depression of the second response largely depends on the neurotransmitter release probability of the presynaptic cell, and its initial resting state (Dobrunz & Stevens, 1997).

PPF is characterized by an increase in the probability of vesicle release containing neurotransmitters from the presynaptic vesicle pool via the presence of intraterminal Ca^{2+}. In the residual calcium hypothesis, Ca^{2+} released from the first pulse builds up in the presynaptic terminal. Thus, during subsequent APs no more than 100 ms apart, increased amounts of presynaptic Ca^{2+} (due to residual Ca^{2+}) signal more glutamate to be released, leading to a stronger EPSP (del Castillo & Katz, 1954; Katz & Miledi, 1968). More recently, however, it has been hypothesized that facilitation is the result of Ca^{2+} buffer saturation. Fast endogenous Ca^{2+} buffers, such as the presynaptic Ca^{2+} binding proteins calbindin (CB), calretinin, and parvalbumin, effectively bind Ca^{2+} and thus compete with Ca^{2+} sensors during an AP. When the second AP occurs, the fast Ca^{2+} buffers are still partially saturated with Ca^{2+}, therefore leaving more free Ca^{2+} to reach the sensor and increase the probability of vesicle fusion and neurotransmitter release (Blatow et al., 2003; Matveev et al., 2004).

When synapses have a low probability of neurotransmitter release, this results in PPF upon subsequent stimulation. However, at synapses with a high probability of vesicle release, the readily-releasable pool of docked vesicles containing neurotransmitter is temporarily depleted following the first pulse, and PPD occurs, as there are few vesicles left to release during successive stimulation (Liley & North, 1953) or the release probability of the reusable vesicle pool is temporarily decreased (Wu & Borst, 1999). Additionally, modulatory substances released by the presynapse or postsynapse can negatively feedback to inhibit subsequent release of neurotransmitter, or postsynaptic ligand-gated receptors could be desensitized to subsequent activity, all contributing to depression of the second response with respect to the first (Zucker & Regehr, 2002).

1.4.3.3 Post-tetanic Potentiation & Depression

In the hippocampal formation, other forms of activity-dependent short-term plasticity that follow a longer time course than PPF and PPD can also be observed. Following repetitive pulses, synapses demonstrate post-tetanic potentiation (PTP) or depression (PTD) of the EPSP on a time scale of tens of seconds to minutes. PTP results from a slow accumulation of intracellular Ca^{2+} at the presynaptic axon terminal following the CS. The residual Ca^{2+} that lingers in the presynapse increases the number of vesicles

containing neurotransmitter released during each subsequent AP (Atluri & Regehr, 1996; Brain & Bennett, 1995; Delaney & Tank, 1994; Delaney et al., 1989; Kamiya & Zucker, 1994; Regehr et al., 1994). Mitochondria are integral to the regulation of intracellular Ca^{2+} concentrations, and experimental evidence indicates that mitochondria may play a critical role in the generation of PTP by sequestering Ca^{2+} during the CS and subsequently releasing the ions into the cytoplasm post-CS (Tang & Zucker, 1997). Oppositely, PTD is thought to result from inactivation of Ca^{2+} channels or depletion of the readily-releasable pool of docked vesicles containing neurotransmitter (Zucker & Regehr, 2002).

Since short-term plasticity operates on a milliseconds-to-minutes timescale, it is thought to serve as an important mechanism in modifying synaptic function during information processing. Experimental evidence suggests that short-term plasticity is crucial to the transmission of information through modification of release probability (Zador, 1998), and contributes to the optimization of information transfer for specific firing pattern of corresponding neurons (Rotman et al., 2011). Most notably, short-term plasticity is likely to contribute to the stability and duration of working memories (Mongillo et al., 2008).

1.4.4 Long-term Synaptic Plasticity

In addition to short-term synaptic plasticity, other, longer forms of synaptic plasticity also exist. Rather than modification occurring through neurotransmitter release at the presynaptic terminal, these changes originate from postsynaptic modulation and depend on protein synthesis (Mochida et al., 2008).

1.4.4.1 Long-term Potentiation

LTP can be characterized as a prolonged increase in synaptic efficacy between neurons in the central nervous system (CNS). In the hippocampal formation, LTP results mainly from an increase in postsynaptic receptor density via AMPAR externalization, and is triggered by glutamate-dependent NMDAR activation (Harris et al., 1984; Reymann et al., 1989). Following repetitive presynaptic activation, glutamate released

from the presynaptic membrane into the synaptic cleft binds to the extracellular domain of postsynaptic AMPA and NMDA receptors. Sodium ions enter the postsynaptic cell via open AMPARs, causing depolarization. Once the postsynaptic cell is sufficiently depolarized, the NMDAR Mg^{2+} ion channel block is expelled, allowing for the inward flow of Na^{+} and Ca^{2+} cations through the NMDAR (Frank et al., 1989; Wigström & Gustafsson, 1986). Accumulation of intracellular Ca^{2+} is thought to mediate the induction of LTP through enhancement of AMPAR currents (Derkach et al., 1999) and insertion of additional receptors into the postsynaptic membrane (Lu et al., 2001; Shi et al., 1999) via activation of intracellular kinase cascades.

Induction and maintenance of LTP can be divided into three main phases **(Figure 3)**, which are not mutually exclusive to one another and may overlap (Sweatt, 2009). Short-term potentiation (STP) occurs during the first 5-20 minutes of EPSP potentiation, as the result of enhanced NMDAR activation and Ca^{2+} influx in the postsynaptic cell (Malenka, 1994). STP is dependent on NMDARs, rather than activation of protein kinases (Sweatt, 2009).

At 20-30 minutes post-induction, accumulation of Ca^{2+} in the postsynapse triggers activation of various protein kinases which mediate the initiation of the early phase of LTP (E-LTP). This phase can last 2-3 hours. Calcium ions entering the postsynaptic cell bind to calmodulin (CaM), eventually triggering the autophosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Even after Ca^{2+} levels have subsided, CaMKII activity can persist (Malenka et al., 1989). CaMKII is subsequently translocated to the PSD, where it serves to phosphorylate membrane-bound AMPARs on the Serine-831 residue, ultimately enhancing AMPAR-mediated currents and resulting in the trafficking and insertion of additional AMPARs into the postsynaptic membrane (Malenka & Bear, 2004; Zamanillo et al., 1999). Additionally, activation of protein kinase C (PKC) by Ca^{2+} also leads to phosphorylation of Serine-831 on the AMPAR, suggesting PKC plays a similar role to CaMKII in E-LTP (Malenka et al., 1989; Malinow et al., 1988) (**Figure 4**).

Long-lasting LTP (L-LTP) can persist for hours to years (Lynch, 2004), and is dependent on gene transcription and protein synthesis at the synapse or dendrite (Winder et al., 1999). During L-LTP, the extracellular regulated kinase 1/2 (ERK1/2), an active

player in the mitogen activated protein kinase (MAPK) signalling pathway, is phosphorylated and translocates to the nucleus, leading to downstream activation of the transcription factor cAMP response element-binding protein (CREB) (Abraham & Williams, 2008; Davis et al., 2000; Lynch, 2004; Silva, 2003). Activation of the CREB signalling cascade and cAMP responsive element (CRE)-mediated gene expression regulates nuclear gene transcription and protein synthesis (Kandel, 2001). Although a few key mRNAs and proteins have been identified in the induction and maintenance of L-LTP, the identities of most of the proteins synthesized during L-LTP remain unknown (Vickers & Wyllie, 2007). However, experimental evidence does indicate that the same stimulation paradigms responsible for the induction of L-LTP also contribute to the induction of immediate early genes (IEGs) – including brain-derived neurotrophic factor (BDNF), Arc, and c-fos – strongly suggesting that these IEGs are crucial to the maintenance of L-LTP (Castrén et al., 1993; Cole et al., 1989; Lyford et al., 1995).

Modifications in gene expression and protein synthesis during L-LTP are not exclusive to the postsynapse. Experimental evidence implicates changes in presynaptic neurotransmitter release probability necessary for induction and maintenance of L-LTP occur via retrograde messengers, including nitric oxide release from the postsynapse (Lu et al., 1999) and the involvement of cell adhesion proteins (Malenka & Bear, 2004).

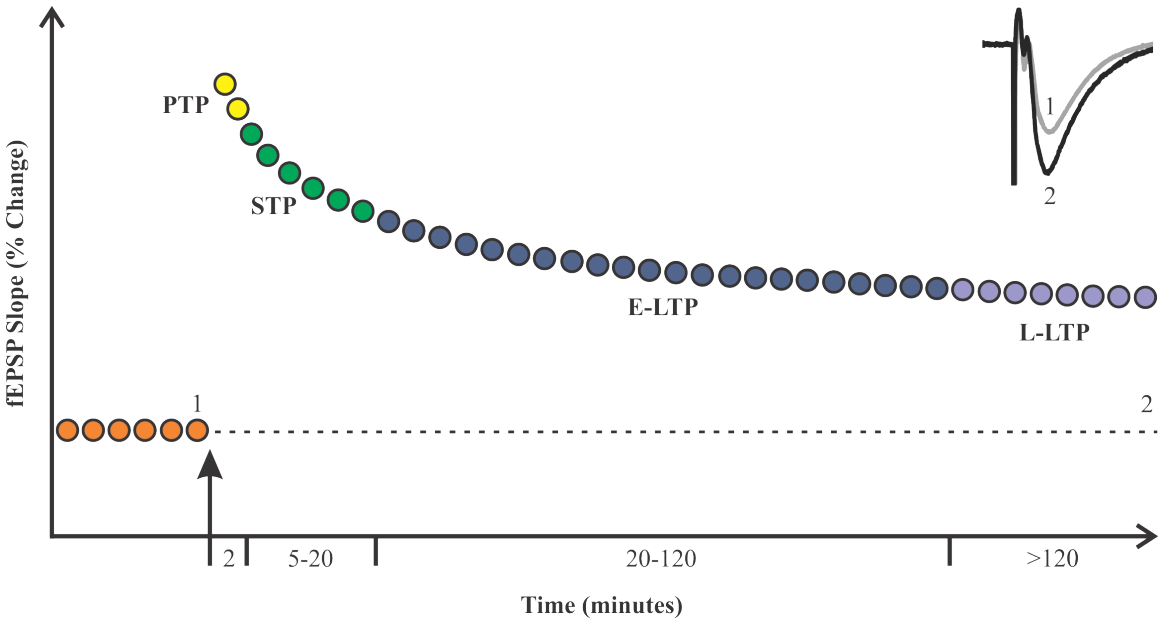

Figure 3. Stages of long-term potentiation. Schematic of the induction and maintenance phases of LTP. The slope of evoked EPSPs is measured to achieve a stable baseline **(1)**. A robust CS is applied (arrow) to induce LTP. PTP is observed immediately following the CS, and lasts for up to 2 minutes. Enhanced NMDAR activation and Ca^{2+} influx lead to the induction of STP. The decay of STP depends on the CS, but usually lasts for the first 5-20 minutes of EPSP potentiation. Approximately 20 minutes following the CS, the onset of E-LTP is apparent; this phase relies on activation of protein kinases and can last for 2-3 hours. L-LTP is protein synthesis-dependent and can persist for hours to years. Representative traces taken at the time of the baseline **(1)** and at 120 minutes post-CS **(2)** illustrate the change in EPSP slope during LTP.

1.4.4.2 Long-term Depression

In order to continuously strengthen synaptic connections and maintain the capacity to learn, receptor turnover must exist in a process opposite to LTP – thus preventing maximal saturation of the postsynaptic membrane by weakening specific synaptic connections. Long-term depression (LTD), another form of synaptic plasticity, can be characterized as a persistent weakening in synaptic efficacy between neurons in the CNS and opposes the synaptic strengthening put forth by LTP (Purves et al., 2004). In the hippocampal formation, LTD results mainly from a decrease in postsynaptic receptor density, via AMPAR internalization (Purves et al., 2004) and is triggered by the activation of NMDARs, group 1 mGluRs, or endocannabinoids (Paradiso et al., 2007). LTD induction results from the release of glutamate from the presynaptic membrane into

the synaptic cleft, where it is bound to the extracellular domain of the postsynaptic AMPA and NMDA receptors. As with the induction of LTP, Na^{2+} enters the postsynaptic cell via open AMPARs, causing depolarization and expulsion of the NMDAR Mg^{2+} ion channel block. Once again, the removal of Mg^{2+} allows for the inward flow of Na^{+} and Ca^{2+} cations through the NMDAR. However, in LTD, the Ca^{2+} activates protein phosphatases instead of protein kinases. The determinant between LTP and LTD lies in the amount of Ca^{2+} in the postsynaptic cell. Protein phosphatases possess an increased affinity for Ca^{2+}; therefore it is proposed that a small rise in postsynaptic Ca^{2+} would activate protein phosphatases (resulting in LTD), whereas a large influx of Ca^{2+} leads to activation of protein kinases (CaMKII) as in LTP (Lisman, 1989). In fact, low levels of Ca^{2+} activate the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (CN) (formerly known as protein phosphatase 2B), which in turn activates a cascade of phosphorylation and dephosphylation events in the postsynaptic cell (Mulkey et al., 1994). Activated CN leads to the dephosphorylation of inhibitor 1 (I1), which in its active, phosphorylated form, inhibits the activity of protein phosphatase 1 (PP1). Thus, removal of PP1 inhibition allows the protein phosphatase to dephosphorylate its cellular target, CaMKII (Blitzer et al., 1998; Lisman, 1989; Mulkey et al., 1994; Mulkey et al., 1993), resulting in AMPAR internalization via clathrin-coated endocytosis (Beattie et al., 2000). The decrease of synaptic AMPARs in the postsynaptic membrane imparts a reduced sensitivity to future glutamate released into the synaptic cleft (Purves et al., 2004), thereby producing LTD (**Figure 4**).

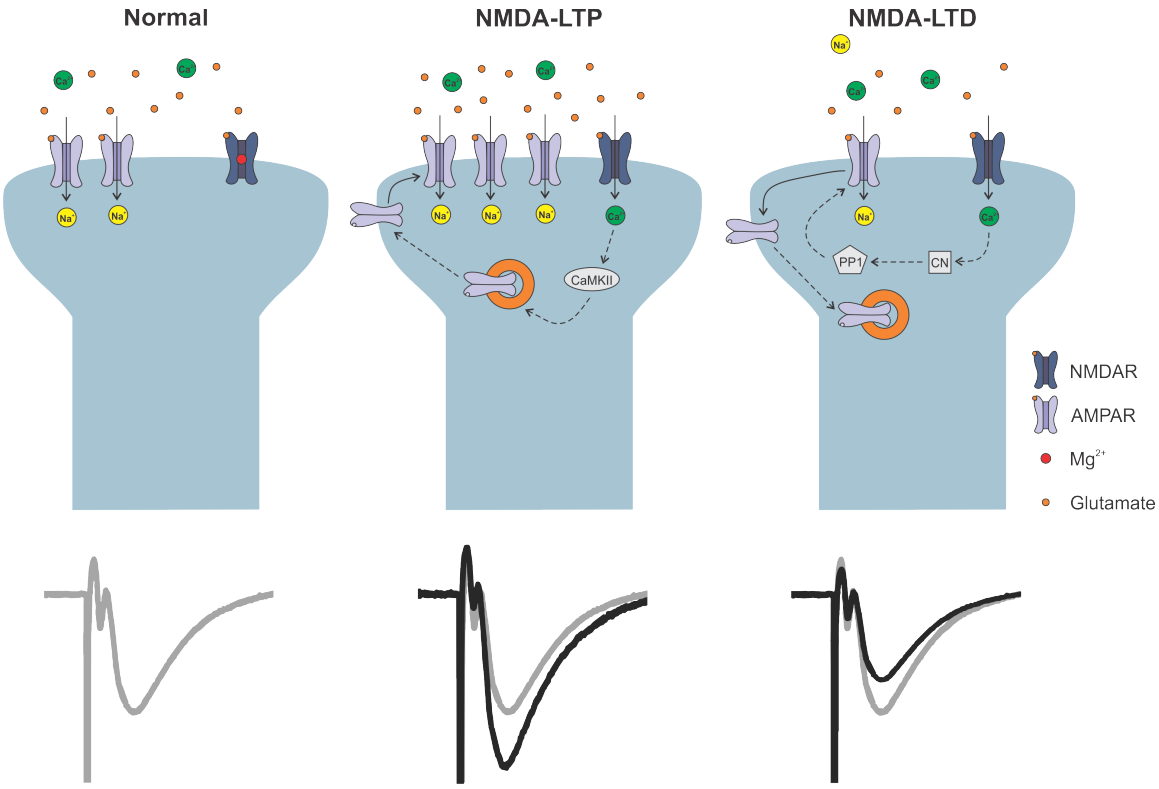

Figure 4. Basic cellular mechanism of NMDA receptor-dependent LTP and LTD. Schematic of the post-synaptic mechanisms associated with an increase or decrease in synaptic efficacy. Representative traces are displayed below each model; change in the slope or amplitude of the EPSP represents change in synaptic transmission. *Left:* During baseline synaptic transmission, glutamate (orange circles) binds to postsynaptic AMPA and NMDA receptors. Na^+ ions (yellow circles) enter the postsynaptic cell via open AMPARs, while the NMDAR is blocked by Mg^{2+} (red circle). *Middle:* Robust stimulation induces LTP by sufficiently depolarizing the postsynaptic cell, expelling the Mg^{2+} ion channel block, and allowing for the inward flow of Na^+ and Ca^{2+} (green circles) cations through the NMDAR. Accumulation of intracellular Ca^{2+} activates CaMKII and mediates the insertion of additional AMPARs into the postsynaptic membrane (*top*) resulting in an increase in synaptic transmission (*bottom*). *Right:* During LTD, a small rise in postsynaptic Ca^{2+} activates CN, which subsequently activates PP1 and leads to the internalization of AMPARs (*top*). Induction of LTD results in a decrease in synaptic transmission, and apparent reduction in EPSP slope and amplitude (*bottom*).

1.4.4.3 Candidate Mechanism for Learning and Memory Formation

It is widely hypothesized that altering the strength of connections between neurons – as occurs during activity-dependent bidirectional synaptic plasticity – is the mechanism by which memory traces are encoded and stored in the brain (Bliss & Collingridge, 1993). Formation and long-term storage of memories requires a rapidly inducible mechanism that gives rise to changes in synaptic excitability and efficacy that will outlast the physical event that triggered them. Several properties of NMDAR-dependent LTP make it a suitable model for hippocampal-dependent memory encoding and storage, including rapid induction, synapse specificity, associative interactions, persistence, and dependence on correlated synaptic activity (Escobar & Derrick, 2007).

Rodent studies have documented evidence linking impaired performance on hippocampal-dependent behavioural tasks to corresponding declines in evoked LTP. Research examining the impact of aging on learning and memory processes statistically correlated the persistence of LTP to the rate of learning and the ability to retain spatial memories over time (Barnes 1979; Barnes & McNaughton 1985). More recently, in a murine model of Alzheimer's disease overexpressing mutant amyloid precursor protein, an age-related decline in performance of a delayed spatial alternation task was correlated with attenuation of LTP both *in vivo* and *in vitro* (Chapman et al., 1999). Exploration of novel environments, a type of spatial learning, induces an increase in field EPSPs evoked in the perforant pathway of the DG (Green et al., 1990; Sharp et al., 1989). Additionally, pharmacological blockade of NMDAR through application of NMDAR specific antagonists abolishes induction of LTP and impairs some types of hippocampal-dependent learning (Bannerman et al., 1995; Morris, 1989). An alternative way of exploring the relationship between synaptic plasticity and memory involves genetic manipulation: specific genes targeted for modification can alter both LTP induction and behavioural performance on hippocampal-dependent tasks (Grant et al., 1992; Hinds et al., 1998; Migaud et al., 1998). These correlational deficits support a functional requirement of LTP in spatial learning and memory formation.

1.4.5 Dysregulation of Structural and Synaptic Plasticity following TBI

Several studies have suggested that alterations in structural plasticity occur in various experimental models of TBI, including changes in the rate of neurogenesis (Emery et al., 2005; Richardson et al., 2007; Rola et al., 2006; Urrea et al., 2007), dendritic arborization (Kaindl et al., 2007), spine density (Campbell et al., 2012), and expression of synaptic proteins (Ansari et al., 2008a, 2008b; Campbell, et al., 2012; Gobbel et al., 2007; Wakade et al., 2010). Importantly, these structural and morphological changes are often associated with alterations in synaptic plasticity (Bruel-Jungerman et al., 2007a, 2007b).

It has also been demonstrated that experimental TBI causes impairments in hippocampal synaptic plasticity (See **Tables 1 and 2** for a literature summary). Indeed, basal excitatory synaptic transmission, as evaluated by measuring the changing slope of the EPSP in response to increasing stimulus intensities, is depressed in the hippocampal CA1 for hours to days following a TBI (D'Ambrosio et al., 1998; Miyazaki et al., 1992; Norris & Scheff, 2009; Reeves et al., 2000; Witgen et al., 2005; Zhang et al., 2011a, 2011b), but usually resolves within 1 to 2 weeks post-TBI (Norris & Scheff, 2009; Reeves et al., 2000). This is most likely due to alterations in the balance of inhibitory and excitatory basal synaptic transmission; previous studies have reported an increase in population spike amplitude, while the population spike threshold is decreased (Akasu et al., 2002; Miyazaki et al., 1992; Reeves et al., 1997; Reeves et al., 1995; Witgen et al., 2005). However, in the perforant pathway of the DG, the opposite has been observed: following TBI, basal EPSPs are enhanced, while basal inhibitory postsynaptic currents are depressed (Bonislawski et al., 2007; Golarai et al., 2001; Hunt et al., 2010, 2011; Lowenstein et al., 1992; Mtchedlishvili et al., 2010; Witgen et al., 2005).

A mild to severe single-impact FP-induced TBI has been repeatedly shown to result in impaired LTP (but not LTD) in the Schaffer collaterals of the CA1 hippocampal subfield in adult rats (D'Ambrosio et al., 1998; Miyazaki et al., 1992; Reeves et al., 1995; Sanders et al., 2000; Schwarzbach et al., 2006; Sick et al., 1998; Zhang et al., 2011). These deficits can be detected for hours up to 8 weeks following injury, although most previous studies only examine up to 2 weeks post-injury (D'Ambrosio et al., 1998; Miyazaki et al., 1992; Reeves et al., 1995; Sanders et al., 2000; Schwarzbach et al., 2006;

Sick et al., 1998; Zhang et al., 2011). However, only D'Ambrosio and colleagues (1998) have examined synaptic plasticity deficits in the juvenile population, using a model of severe TBI. They found that CA1-LTP is significantly attenuated 24 to 48 hours following severe TBI, but it is currently unknown how long the LTP deficit can last or even whether the impairment is in fact permanent and not reversible. Furthermore, it is also unclear whether such deficits in LTP are also present in the adjacent DG hippocampal subfield (Yamashita et al., 2011; Zhang et al., 2011).

Table 1. Literature summary of TBI and hippocampal synaptic plasticity *in vitro*.

Hippocampal Subregion	TBI Model	Severity	Rodent Model	Test Age or Weight	Post-injury Time Point	Long-term Plasticity Results	Reference
CA1	FPI	Mild-Moderate (1.4-2.1 atm)	C57BL/6J mice	5-7 wks 20-25g	7 days	LTP ↓[1] LTD –	Schwarzbach *et al*., 2006
		Mild-Moderate (1.7-2.1 atm)	Male Sprague Dawley	Adult 250-300g	4 hours 48 hours	LTP ↓[2]	Sick *et al*., 1998
		Mild (1.5±0.1 atm) Moderate (1.9±0.2 atm)	Male Fischer	3 months 19 months	2 weeks	LTP ↓	Titus *et al*., 2013
		Mild (1.1-1.4 atm) Moderate (1.8-2.1 atm) Severe (2.2-2.7 atm)	Male Sprague Dawley	Unknown	6 days 8 weeks	6 days: LTP – 8 wks: LTP ↓[2]	Sanders *et al*., 2000
		Severe (2.4 atm)	Male Sprague Dawley	P24-31	24-48 hours	LTP ↓ LTD –	D'Ambrosio *et al*., 1998
	CCI	Moderate (1.5mm at 3.5m/s)	Male Sprague Dawley	Adult 250-275g	2 days 7 days 14 days	LTP –	Norris *et al*., 2009
		Moderate	Male Sprague Dawley	Adult 250-300g	48 hours	LTP ↓ LTD ↑[3]	Albensi et al., 2000
	CHI (WD)	Moderate-Severe (75g at 18cm)	Male Sabra mice	8-10 wks	16 days	LTP ↓	Yaka *et al*., 2007
DG	FPI	Moderate (4.2 atm)	Male Wistar	Adult 230-260g	7 days	LTP ↓[4]	Yamashita *et al*., 2011

[1] LTP in contralateral hemisphere attenuated.

[2] LTP attenuated at 30 and 60 minutes post-tetanus, but failure to maintain LTP.

[3] Unable to induce LTD in sham or control animals.

[4] No differences between LTP levels in ipsilateral and contralateral hemispheres.

Table 2. Literature summary of TBI and hippocampal synaptic plasticity *in vivo*.

Hippocampal Subregion	TBI Model	Severity	Rodent Model	Test Age or Weight	Post-injury Time Point	Long-term Plasticity Results	Reference
CA1	FPI	Mild (1.5 atm)	Male Sprague Dawley	Adult 280-345g	2-3 hours	LTP ↓	Miyazaki *et al.*, 1992
		Moderate (1.8-2.0 atm)	Male Wistar	Adult 280-320g	7 days 11 days	LTP ↓	Zhang *et al.*, 2011
		Moderate (2.0±0.05 atm)	Male Sprague Dawley	Adult 250-300g	2 days 7 days 15 days	LTP ↓	Reeves *et al.*, 1995
		Moderate-Severe (200-233 kPa)	Male Wistar	20 months 500-550g	21 days	LTP ↓	Wang *et al.*, 2012
DG	FPI	Moderate (1.8-2.0 atm)	Male Wistar	Adult 280-320g	7 days 11 days	LTP ↑	Zhang *et al.*, 2011
	CHI (WD)	Severe (200g at 2m)	Male Wistar	Adult 250±20g	48 hours	LTP ↓	Farbood *et al.*, 2015

1.5 Summary and Objectives

A history of mTBI is associated with lasting impairments in cognition and behaviour, including increased learning and memory deficits. These learning and memory processes are believed to be reliant on hippocampal synaptic plasticity. Clinical studies have found reduced hippocampal volumes following TBI. Experimental rodent models of adult TBI have found, in addition to hippocampal atrophy, that LTP is impaired in the hippocampal CA1 subregion in males following mild to severe TBI. However, currently very little is known about how TBI impacts synaptic plasticity in the hippocampal DG and if the female brain is differentially impacted.

There is particular concern for mTBI in children, as the juvenile brain is still undergoing significant structural development, making this age group particularly vulnerable to injury that can negatively impact the normal developmental trajectory of the brain. Our goal was to characterize the short- and long-term deficits that mTBI incurs on hippocampal synaptic plasticity in the juvenile male and female brain using an animal model of mTBI. This research will help elucidate the mechanisms underlying cognitive impairments that are observed in young individuals who have experienced a single episode of mTBI.

2. Materials and Methods

2.1 Animals

All animal procedures were approved by the Animal Care Committee at the University of Victoria and the Canadian Council for Animal Care. Experimenters were blinded to the group identity of all animals during the course of electrophysiological and histological experimentation and data analysis.

2.1.1 Generation of Animals

Long-Evans female rats (Charles River Laboratories, St. Constant, PQ, Canada) were paired with proven male breeders of approximately 70 days of age (250-275 grams). Dam and pups were not disturbed for the first 24-36 hours post-partum to facilitate bonding. Pups were counted and monitored to ensure all were thriving. All litters were culled to 10 pups, 5 males and 5 females, on postnatal day (PND) 2.

2.1.2 Weaning

Pups were weighed and weaned at PND 22 and housed in groups of 2-3. Male and female offspring were separated at weaning, and assigned to one of two experimental groups: (1) mTBI, or (2) sham. No more than two males and two females from the same litter were assigned to the same experimental group. The first group of animals received an mTBI in the form of a weight drop injury (see section 2.2) at PND 25-28, while the second group was subjected to a sham surgery with no weight drop. Experiments were conducted 1 hour, 1 day, 7 days, or 28 days following mTBI or sham surgery.

2.1.3 Estrous Cycle

During the estrous cycle, levels of gonadal hormones present in the blood of female mammals fluctuate, and may influence structural and synaptic plasticity in the brain (Foy et al., 1999; Woolley, 1999). Female rats reach sexual maturity at PND 40-60

(Kohn & Clifford, 2002; Long & Evans, 1920; Sengupta, 2013), so to ensure all female rats used in these experiments were at the same stage in their estrous cycle, all females sacrificed at the 28 day timepoint (PND 53-56) were subjected to vaginal lavage immediately prior to decapitation, followed by Papanicolaou (PAP) histochemistry. Vaginal lavages were performed with cotton-tipped swabs dipped in 0.9% saline, and samples smeared on superfrost plus microscope slides (Fisher Scientific, Ottawa, Ontario, Canada). Slides were fixed in 95% ethanol for 3 seconds and allowed to air-dry overnight. Once dry, slides were rinsed in a gentle stream of tepid tap water for 30-60 seconds, and then immersed in Gill's hematoxylin solution No. 1 (Sigma-Aldrich, Oakville, ON, Canada) for 3 minutes. Following a second rinse with tap water, the slides were immersed in Scott's tap water substitute (0.3% $NaHCO_3$ and 2% $MgSO_4$ anhydrous in distilled water) for 20 seconds, and transferred to PAP stain (1:1 Orange G6 and Eosin-azure 50; Sigma-Aldrich, Oakville, Ontario, Canada) for 3 minutes. The slides were then rinsed through 2 changes of 100% ethanol for 30 seconds each, cleared in a xylene substitute (Citrisolv; Fisher Scientific, Ottawa, Ontario, Canada) for two 5 minute incubations, and coverslipped with permount mounting medium (Electron Microscopy Sciences, Burlington, Ontario, Canada).

All PAP stained slides were analyzed on an Olympus conventional light microscope (Model BX51TF; Olympus Corporation, Center Valley, PA, USA), and samples examined using a 20X objective lens to identify the stage of estrous cycle. Images were obtained with a Q-color 3 camera (Olympus Corporation, Center Valley, PA, USA) and Image Pro Plus software (MediaCybernetics Inc., Bethesda, Maryland, USA). Any female rats in the proestrus stage of their cycle were omitted from the experimental data, as proestrus has been associated with alterations in structural and functional plasticity (Gould et al., 1990; Ooishi et al., 2012; Warren et al., 1995; Wong & Moss, 1992; Woolley & McEwen, 1992).

2.2 Mild TBI Weight Drop Model

For the purpose of this thesis, we chose to operationally define mTBI as a non-penetrating external force to the head that causes functional cognitive deficits in the

absence of gross structural abnormalities and cell death. A single mTBI was administered using a weight-drop model, based upon a device developed by Prof. Esther Shohami (Chen et al., 1996; Yatsiv et al., 2005). On the day of the surgery, animals were anesthetized using an induction chamber and a vaporizer with 2-4% isoflurane in oxygen (0.8-1 L/min). Once the animal had lost the ability to right itself, anesthesia was maintained at 1-3% isoflurane in oxygen (0.8-1 L/min) delivered via nose cone, with anesthesia maintenance periodically checked through loss of toe-pinch reflex. Lubricating eye gel was applied to both eyes, and body temperature (37±1ºC) was maintained using a heating pad placed under the animal. Additionally, room temperature sterile saline solution (1 mL/100 g) was injected subcutaneously for hydration. An electric shaver was used to remove fur on top of head, and the skin overlaying the head of the animal was surgically scrubbed with chlorhexidine (2%) solution and 70% ethanol. The skull was exposed by making a 3-4 cm long incision along the midline of the scalp, and the local anesthetic lidocaine hydrochloride (2%) applied directly to the open incision to further minimize pain. After waiting 60 seconds, the skin overlying the skull was gently removed with a sterile Q-tip. Sterile cotton gauze folded over, was placed on the skull covering the area of the parietal bone between bregma and lambda. The gauze minimizes skull fractures as well as avoids direct contact of the Teflon-tipped cone of the injury device with the animal's skull. Between animals the Teflon-tipped cone was wiped down with 70% ethanol.

Prior to the mTBI procedure, toe and eye reflexes were checked to confirm the animal was deeply anesthetized. The animal was moved under the weight-drop device, and the head of the animal manually positioned and held in place by the researchers fingers so that the Teflon-tipped cone (2 mm diameter) of the device rested on the top of the gauze in the left mid-parietal area. In order to mimic a mTBI, a 200 g brass weight was dropped from a height of 10 cm. Following weight drop, the animal was immediately removed from the injury device, and, as soon as adequate respiration was observed following injury, the gauze was removed and the skull observed for fractures. If no skull fracture was evident, the scalp incision was closed using suture (silk 5/0 black 18” 68sG with 3/8 reverse cutting needle; AVP) and the animal returned to a recovery cage under a heat lamp, until waking from anesthesia, whereupon the animal was returned to its home

cage. Sham controls underwent the same procedure, minus the weight-drop event. All animals were singly housed following surgery, and monitored every 15 minutes until they were awake and moving. Animals suffering from skull fracture after injury were immediately euthanized.

2.3 Linear Acceleration of Impact

These experiments were performed in collaboration with Jay Leung and Paul Leslie from Dr. Kerry Delaney's laboratory to characterize the linear acceleration of impact for our mTBI weight drop device. Animals were anesthetized with an intraperitoneal injection of urethane (6 mL/kg), and an electric shaver was used to remove fur on top of the head. The skull was exposed by making a 3-4 cm long incision along the midline of the scalp, and the skin overlying the skull was gently removed with a Q-tip. The inertial measurement unit (IMU) device (developed by Paul Leslie) was adhered to the interparietal bone with dental cement (Jet tooth shade powder 65/D3, fast curing acrylic resin liquid ref #1404; Lang Dental). The IMU device was equipped with a 3-axis accelerometer (±200g sampling range; ADXL377; Analog Devices) that measures linear acceleration (m/s^2). As outlined in Section 2.2, a 200 g brass weight was dropped from a height of 10 cm onto the left mid-parietal area. Signals from all of the transducers were acquired at a sampling rate of 10 kHz, downloaded through the USB port, and analyzed with IgorPro by Jay Leung. A linear acceleration of 655.6 ± 51.4 m/s^2, or 66.8 ± 5.2 g's (n=15) in the *z*-plane was recorded.

2.4 Electrophysiology

2.4.1 Slice Preparation

At 1 hour, 1 day, 7 days, or 28 days following TBI or sham surgery, male and female Long-Evans rats were anesthetized with isoflurane (Isoflo; Abbott Laboratories, North Chicago, IL, USA), decapitated, and the brain immediately excised and submerged in ice-cold normal artificial cerebrospinal fluid (nACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.25 $NaHPO_4$, 25 $NaHCO_4$, 2 $CaCl_2$, 1.3 $MgCl_2$, and 10 dextrose (300±10

mOsm; pH 7.2-7.4), and continuously oxygenated with 95% O_2–5% CO_2. The brain was manually sectioned on a vibratome 1500 (Ted Pella Inc., Redding, CA, USA) at a thickness of 350 μm. The slicing chamber contained oxygenated nACSF that was maintained at a constant chilled temperature of 2.5±1˚C. Transverse hippocampal slices were incubated in 35±1˚C oxygenated nACSF for a minimum of one hour prior to recording.

2.4.2 Field Recordings

The hippocampal slices were transferred to recording chambers where they were continuously bathed in 30±1˚C oxygenated nACSF at a rate of approximately 2 mL/min. Motorized micromanipulators (Siskyou Design, OR, USA) were used to place electrodes in the medial molecular cell layer of the DG, or the stratum radiatum layer of the CA1. Stimulating and recording electrodes were placed approximately 200 μm apart. The medial perforant pathway (MPP) of the DG or the Schaffer collaterals of the CA1 were stimulated using a concentric bipolar stimulating electrode (FHC, Bowdoin, ME, USA) and a digital stimulus isolation unit (Getting Instruments, CA, USA) delivering a 0.12 ms (10-40 μA; 15 s inter-stimulus interval) current pulse. Field excitatory postsynaptic potentials (fEPSPs) from the DG or CA1 of hippocampal slices were recorded using glass microelectrodes (0.5-1.5 MΩ) containing nACSF, and a MultiClamp 700B microelectrode amplifier (Molecular Devices, CA, USA). The current was adjusted to produce a stimulus strength of 40-50% the maximum fEPSP amplitude. A paired pulse (PP) experiment was conducted at this new stimulus strength using an inter-pulse interval of 50 ms (5x; 15 s between pairings). Subsequently, a steady baseline was maintained for 20 minutes in nACSF including the $GABA_A$ antagonist bicuculline methiodide (BMI; 10 μM; Sigma-Aldrich, Oakville, ON, Canada) before inducing short- and long-term plasticity. Post-tetanic potentiation (PTP), and short- and long-term potentiation (STP; LTP) were induced with a high frequency stimulus (HFS) protocol (50 pulses at 100 Hz, 4x with a 30 s inter-train interval); decay rate was observed for 60 minutes post-HFS in nACSF lacking BMI. Additionally, post-experimental input-output (I/O) protocols were performed with increasing stimulation magnitude (30-300 μs pulse width; 15 s intervals).

2.4.3 Analysis of Electrophysiological Recordings

Electrophysiological data was recorded using pClamp 10.2 (Axon Instruments, CA, USA), and analyzed with Clampfit 10.2 (MDS Analytical Technologies, Toronto, ON, Canada). The initial slope of the fEPSP waveform was measured for all electrophysiology experiments. PP ratios were assessed by dividing the slope of the second fEPSP by the first, expressed as a percent change (average of all 5 traces). To examine if PTP or STP were present, a comparison between the slope of the fEPSP for the first minute (average of first 4 traces; PTP) or first 5 minutes (average of first 20 traces; STP) post-HFS was made with the mean slope of the baseline fEPSP recorded in the 20 minutes prior to the CS and reported as percent change from the baseline. To quantify the changes in synaptic plasticity (LTP), the mean fEPSP slope for 55-60 minutes (average of last 20 traces) post-LTP induction was compared to the mean slope of the fEPSP prior to the onset of the HFS protocol and reported as percent change from the baseline. I/O curves were assessed by normalizing traces to the slope of the fifth pulse, and reported as a percent change, with the fifth pulse represented as 100%. All data are expressed as mean ± standard error of the mean (SEM).

2.4.4 Statistical Analysis

Statistical significance ($P < 0.05$) of means ± SEM from PP, PTP, STP, and LTP experiments were determined by a one-way analysis of variance (ANOVA; Statistica, Statsoft, Inc., Tulsa, OK, USA) between condition (sham, contralateral, ipsilateral) for each sex (female, male), hippocampal subregion (DG, CA1), and time point (1 hour, 1 day, 7 days, 28 days) examined. Post hoc analyses tests were conducted using Tukey HSD test to further analyze any significance when appropriate. I/O profiles were assessed with repeated measures ANOVA, followed by post hoc Tukey HSD tests where necessary.

2.5 Histology

2.5.1 Tissue Processing

A separate cohort of mTBI and sham animals were sacrificed at 1 day and 7 days following surgery. Animals were deeply anesthetized with an intraperitoneal injection of urethane (250 mg/mL in water; 1.5 g/kg of body weight) and transcardially perfused with 0.9% sodium chloride (NaCl) followed by 4% paraformaldehyde (PFA). The brains were excised and left in 4% PFA at 4ºC for 24 hours, and then transferred to 30% sucrose. Upon sucrose saturation, serial coronal sections at 30 µm thickness were collected on a vibratome (Leica VT1000S, Nussloch, Germany) at a 1/6 section-sampling fraction, and stored in cryoprotectant (0.04 M tris-buffered saline (TBS), 30% ethylene glycol, 30% glycerol) at 4ºC. Prior to slicing, the right cortex of each brain was notched to differentiate ipsilateral from contralateral hemisphere.

2.5.2 Fluoro-Jade C Histology

Prior to staining, free-floating brain sections were washed 3 times for 10 minutes in TBS (0.1M) and mounted from TBS onto gelatin-coated slides and air-dried overnight. Gelatin-coated slides were pre-prepared by immersion of superfrost plus microscope slides (Fisher Scientific, Ottawa, ON, Canada) in 60-80ºC gelatin solution (Type B, 100 Bloom; Fisher Scientific, Ottawa, ON, Canada) and air-dried overnight. Once dry, slides bearing PFA-fixed cut tissue sections were dipped twice in distilled water, and then immersed in 100% ethanol for 5 minutes and 70% ethanol for 3 minutes. They were then rinsed by dipping twice in distilled water, and incubated in 0.06% potassium permanganate solution for 15 minutes on a shaker board. Following 2 rinses of 2 minutes each in distilled water, the slides were incubated in a 0.001% solution of Fluoro-Jade® C (FJC; excitation: 485 nm; emission: 525 nm; Cat. # MAB3402C3, Lot # 2366581; EMD Millipore Corporation, Temecula, CA, USA) for 30 minutes on a shaker board, keeping covered to protect from the light. The working concentration of FJC was accomplished by first making a 0.01% stock solution of 10 mg dye in 100 mL distilled water, and diluting it to 0.001% by adding 10 mL FJC stock to 90 mL of 0.1% acetic acid in distilled water. The slides were then rinsed through two changes of distilled water for 5 minutes

each and dried on a slide warmer at 60ºC for 15 minutes. Once dry, the slides were cleared in a xylene substitute (Citrisolv; Fisher Scientific, Ottawa, ON, Canada) for 5 minutes and coverslipped with permount mounting medium (Electron Microscopy Sciences, Burlington, ON, Canada).

2.5.4 Cell Quantification

All FJC stained slides were analyzed on an Olympus fluorescence microscope (Model BX51; Olympus Corporation, Center Valley, PA, USA) equipped with a filter system suitable for visualizing green fluorescent protein (GFP; excitation: 395 nm (major), 475 nm (minor); emission: 509 nm). Pictures were obtained with a Q-color 3 camera (Olympus Corporation, Center Valley, PA, USA) and Image Pro Plus software (MediaCybernetics Inc., Bethesda, Maryland, USA). FJC positive cells were identified by a blind observer using a 40X objective lens and counted in the left and right cortex, CA, and DG regions of the brain. Counting in the cortex started at the midline of the hemisphere and ended at the line parallel to the DG. Only the portion of the cortex superior to the hippocampus in coronal sections was quantified. Counting in the CA region included the CA1 and CA3 regions, and counting in the DG was limited to the granule cell layer. Positive cells were identified based on location, size, and shape of the fluorescent cell. Counts were logged into Excel (Microsoft, 2011). Total approximate number of FJC positive cells per brain region was calculated by summing up the number of cells positively labeled for each section multiplied by the distance between each section counted (1/6 section-sampling fraction; 150 μm), and expressed as mean ± SEM. Representative images were obtained using a 20X or 40X objective lens on an Olympus laser scanning confocal microscope (Model BX61 WI; Olympus Corporation, Center Valley, PA, USA) equipped with a laser system suitable for visualizing GFP and Olympus FluoView software (Version FV10-ASW; Olympus Corporation, Center Valley, PA, USA). Image stacks were collected using a Kalman filter (average of 2 images) in 8 μm z-steps at 1024 × 1024 pixels (0.828 μm/pixel) with a pixel dwell time of 8 μs. Average intensity z-projections of each image stack were produced and stitched together in Image J software (NIH, version 1.44d).

2.5.5 Statistical Analysis

Statistical significance ($P < 0.05$) of total FJC positive cells was determined by a two-way factorial ANOVA (Statistica, Statsoft, Inc., Tulsa, OK, USA) between time point (1 day, 7 days) and condition (sham, contralateral, ipsilateral) for each brain region (Cortex, CA, DG) and sex (male, female) examined. Post hoc analyses were conducted when appropriate using the Tukey HSD test.

3. Results

3.1 Synaptic Plasticity is Significantly Impacted by mTBI

3.1.1 Early-onset Impairment of Hippocampal Long-term Potentiation Persists into Adulthood following mTBI in Juvenile Females

3.1.1.1 DG-LTP in Juvenile Females

To determine whether female animals that have experienced an mTBI have the same capacity to undergo synaptic plasticity as sham animals, LTP was induced in the MPP of the DG using an HFS protocol. PTP and STP were measured by averaging fEPSP responses over the first minute, and five minutes, respectively. No significant differences in evoked levels of PTP and STP were found with respect to condition (**Figure 5A1**). HFS produced robust LTP in sham animals 1 hour post-surgery, when measured 55 to 60 minutes following conditioning stimulus (70.3 ± 13.0 %; n = 8; **Figure 5A**). The same conditioning stimulus produced similar levels of LTP in the contralateral (60.1 ± 14.3 %; n = 9) and ipsilateral (47.1 ± 7.8 %; n = 12) hemispheres ($F_{(2,26)} = 1.095$, $P = 0.350$) of mTBI animals.

At 1 day following mTBI, LTP was once again robustly evoked in the DG of sham animals (61.7 ± 10.8 %; n = 7; **Figure 5B**). In the contralateral hemisphere of mTBI animals, levels of LTP were similar to those of sham animals (49.3 ± 6.7 %; n = 11). However, a significant difference was found between the three groups ($F_{(2,23)} = 3.628$, $P = 0.043$). The ipsilateral hemisphere of mTBI animals revealed significant attenuation of LTP from sham levels (27.1 ± 10.2 %; n = 8; $P = 0.039$). No significant differences in PTP and STP were found (**Figure 5B1**).

At 7 days post-injury, no significant effect of injury was found on PTP. However, levels of evoked STP were significantly different between conditions ($F_{(2,34)} = 4.104$, $P = 0.025$; **Figure 5C1**), with the ipsilateral hemisphere of mTBI animals showing a 47.1% reduction in STP from sham levels ($P = 0.019$). A significant effect of condition on LTP was found ($F_{(2,34)} = 4.563$, $P = 0.018$). LTP evoked in the ipsilateral hemisphere of mTBI animals (27.0 ± 5.2 %; n = 21; **Figure 5C**) remained significantly decreased from sham

levels at 7 days post-mTBI (59.1 ± 13.2 %; n = 8; $P = 0.023$). There were no significant differences between LTP in sham animals and the contralateral hemisphere of mTBI animals (48.6 ± 10.3 %; n = 8; $F_{(2,24)} = 1.308$, $P = 0.289$).

Twenty-eight days following mTBI, both PTP and STP were significantly different between conditions (PTP: $F_{(2,26)} = 4.873$, $P = 0.016$; STP: $F_{(2,24)} = 4.942$, $P = 0.015$; **Figure 5D1**), with the ipsilateral injured hemisphere of mTBI animals displaying significantly reduced levels of PTP and STP from the contralateral hemisphere of the same animals (PTP: 62.3% reduction, $P = 0.012$; STP: 65.1% reduction, $P = 0.011$). Robust LTP could be elicited in sham animals (51.8 ± 12.8 %; n = 7; **Figure 5D**) and in contralateral slices taken from mTBI animals (59.1 ± 8.7 %; n = 9), although a significant effect of condition was found ($F_{(2,31)} = 5.010$, $P = 0.013$). Post hoc analysis revealed that although levels of LTP in the ipsilateral hemisphere of mTBI animals (20.7 ± 6.9 %; n = 13) were not significantly decreased from those of sham levels, LTP in the ipsilateral hemisphere was markedly attenuated from levels elicited in the contralateral hemisphere of mTBI animals ($P = 0.010$).

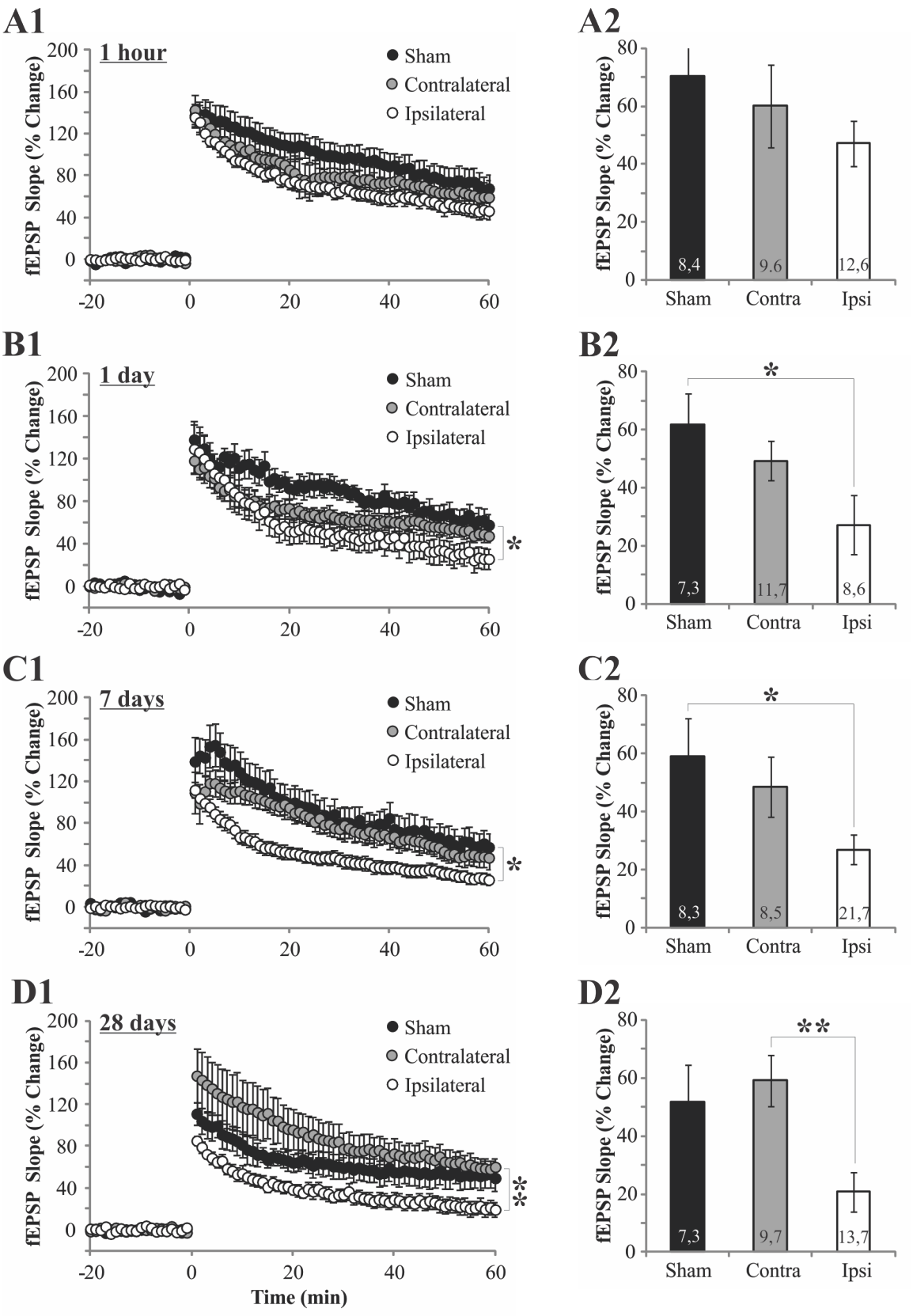
Female DG
A1
1 hour
Sham
Contralateral
Ipsilateral
fEPSP Slope (% Change)
A2
8,4
9.6
12,6
Sham
Contra
Ipsi
B1
1 day
*
B2
7,3
11,7
8,6
C1
7 days
C2
8,3
8,5
21,7
D1
28 days
**
D2
7,3
9,7
13,7
Time (min)

Figure 5. Effect of mTBI on long-term potentiation in the female DG. HFS (50 pulses at 100 Hz, 4x with a 30 s interval) at time zero elicited significant LTP in sham rats and both hemispheres of mTBI rats at all time points, as measured at 55-60 minutes post-HFS. **(A)** LTP was not significantly altered at 1 hour following mTBI. **(B)** However, mTBI significantly attenuated LTP in the ipsilateral hemisphere of mTBI animals at 1 day post-injury as compared to sham animals. **(C)** In the ipsilateral hemisphere, LTP deficits persisted following 7 days post-injury. **(D)** Conversely, the ipsilateral injured hemisphere showed significantly less LTP than the contralateral hemisphere at 28 days post-mTBI. However, neither hemisphere significantly differed from sham levels of LTP. Data presented as mean fEPSP slope (% change) ± SEM. *P<0.05, **P<0.01.

3.1.1.2 CA1-LTP in Juvenile Females

LTP was also induced in the Schaffer collaterals of the CA1 using an HFS protocol. HFS produced robust PTP and STP in injured hemispheres and sham animals that were not significantly different (**Figure 6A1**). Additionally, the CS induced significant LTP in sham animals 1 hour post-surgery, when measured 55 to 60 minutes following HFS (sham = 55.8 ± 4.3 %; n = 7; **Figure 6A**). A significant difference was found between the three groups ($F_{(2,25)}$ = 5.343, P = 0.012). Post-hoc analysis revealed that the same CS produced a significant decrease in levels of LTP observed in the ipsilateral hemisphere of mTBI animals (27.3 ± 5.8 %; n = 11; P = 0.037), and the contralateral hemisphere (21.4 ± 9.7 %; n = 10; P = 0.012) when compared to sham animals.

By 1 day post-injury, LTP evoked in the contralateral hemisphere of mTBI animals (55.9 ± 14.5 %; n = 7; **Figure 6B**) had recovered to levels similar to that of sham (51.4 ± 11.2 %; n = 10) and the ipsilateral hemisphere of mTBI animals (49.3 ± 15.1 %; n = 8; $F_{(2,22)}$ = 0.055, P = 0.946). There was no significant effect of condition on evoked levels of PTP and STP (**Figure 6B1**).

At 7 days following mTBI, LTP was once again evoked in the CA1 of sham animals (34.3 ± 4.8 %; n = 7; **Figure 6C**). In the contralateral hemisphere of mTBI animals, levels of LTP were similar to those of sham animals (45.0 ± 8.4 %; n = 8), as was LTP in the ipsilateral hemisphere of mTBI animals (35.7 ± 11.8 ; n = 7; $F_{(2,19)}$ = 0.473, P = 0.631). Levels of PTP and STP remained statistically unaffected by injury (**Figure 6C1**).

Twenty-eight days following mTBI, levels of PTP and STP were not significantly affected by condition (**Figure 6D1**), and robust LTP could be elicited in sham animals (48.8 ± 10.3 %; n = 7; **Figure 6D**), as well as in the contralateral (41.2 ± 7.0 %; n = 9), and ipsilateral (42.6 ± 9.2 %; n = 7) hemispheres of mTBI animals, with no significant differences between the groups ($F_{(2,20)} = 0.228$, $P = 0.798$).

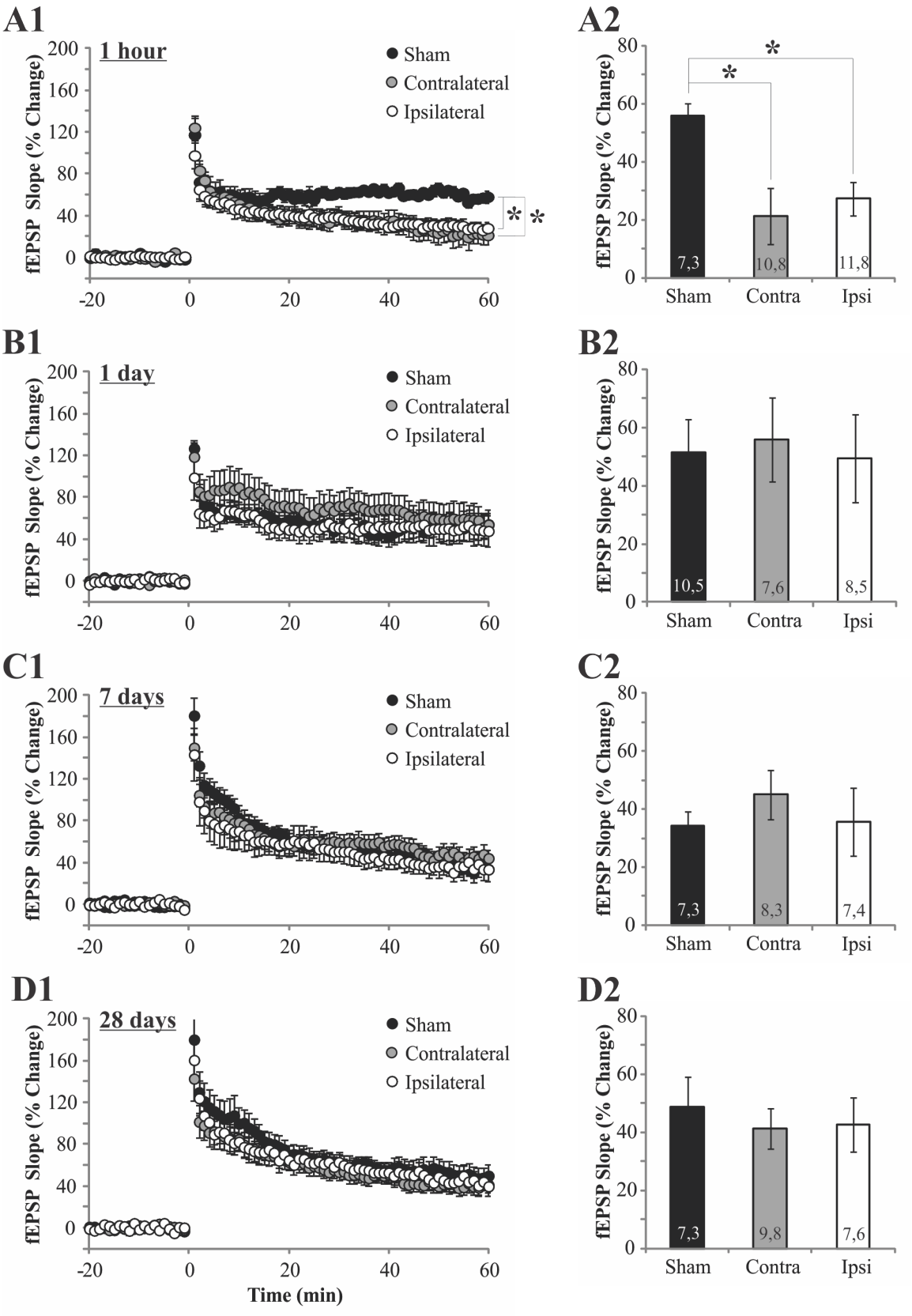
Female CA1
A1
1 hour
Sham
Contralateral
Ipsilateral
fEPSP Slope (% Change)
A2
fEPSP Slope (% Change)
7,3
10,8
11,8
Sham
Contra
Ipsi
B1
1 day
B2
10,5
7,6
8,5
C1
7 days
C2
7,3
8,3
7,4
D1
28 days
D2
7,3
9,8
7,6
Time (min)

Figure 6. Effect of mTBI on long-term potentiation in the female CA1. HFS (50 pulses at 100 Hz, 4x with a 30 s interval) at time zero elicited significant LTP in sham rats and both hemispheres of mTBI rats at all time points, as measured at 55-60 minutes post-HFS. **(A)** LTP was significantly attenuated in the contralateral hemisphere at 1 hour following mTBI compared to sham animals. **(B)** LTP deficits were fully recovered by 1 day post-injury, and remained unchanged at **(C)** 7 days and **(D)** 28 days following injury. Data presented as mean fEPSP slope (% change) ± SEM. **P*<0.05.

3.1.2 Acute and Delayed Impairment of Hippocampal Long-term Potentiation following mTBI in Juvenile Males

3.1.2.1 DG-LTP in Juvenile Males

To determine whether male animals that have experienced an mTBI have the same capacity to exhibit LTP as sham animals, LTP was induced in the MPP of the DG using an HFS protocol. PTP and STP were measured by averaging fEPSP responses over the first minute, and five minutes, respectively. No significant differences in evoked levels of PTP and STP were found with respect to condition (**Figure 7A1**). HFS produced robust LTP in sham animals 1 hour post-surgery, when measured 55 to 60 minutes following conditioning stimulus (44.6 ± 8.1 %; n = 9; **Figure 7A**). The same conditioning stimulus produced similar levels of LTP in the contralateral (46.6 ± 9.5 %; n = 8) and ipsilateral (33.0 ± 11.7 %; n = 10) hemispheres of mTBI animals ($F_{(2,24)} = 0.564$, $P = 0.576$).

At 1 day following mTBI, LTP was once again evoked in the DG of sham animals (41.5 ± 5.3 %; n = 8; **Figure 7B**). In the contralateral hemisphere of mTBI animals, levels of LTP were similar to those of sham animals (44.2 ± 10.7 %; n = 7), as was LTP in the ipsilateral hemisphere (40.8 ± 8.4 %; n = 10; $F_{(2,22)} = 0.044$, $P = 0.957$). No significant differences in PTP and STP were found (**Figure 7B1**).

Seven days following mTBI, no significant effect of injury was found on PTP. However, levels of evoked STP was significantly different between conditions ($F_{(2,27)} = 3.473$, $P = 0.045$; **Figure 7C1**), with the ipsilateral hemisphere of mTBI animals showing a 34.9% reduction in STP from sham levels ($P = 0.046$). Robust LTP was once again evoked in the sham DG at this time point (42.5 ± 6.2 %; n = 12; **Figure 7C**). In the contralateral hemisphere of mTBI animals, levels of LTP were similar to those of sham

animals (33.8 ± 8.2 %; n = 8). However, a significant difference was found between the three groups ($F_{(2,27)}$ = 8.506, P = 0.001). The ipsilateral hemisphere of mTBI animals revealed significant attenuation of LTP from sham levels (5.5 ± 6.7 %; n = 10; P = 0.001) and levels of LTP evoked in the contralateral injured hemisphere (P = 0.026).

By 28 days post-injury, both PTP and STP were not significantly different between conditions (**Figure 7D1**). LTP evoked in the ipsilateral hemisphere of mTBI animals (37.8 ± 10.1 %; n = 12; **Figure 7D**) had recovered to levels similar to that of sham (43.8 ± 8.2 %; n = 7) and that seen in the contralateral hemisphere of mTBI animals (29.8 ± 8.2 %; n = 9; $F_{(2,25)}$ = 0.483, P = 0.622).

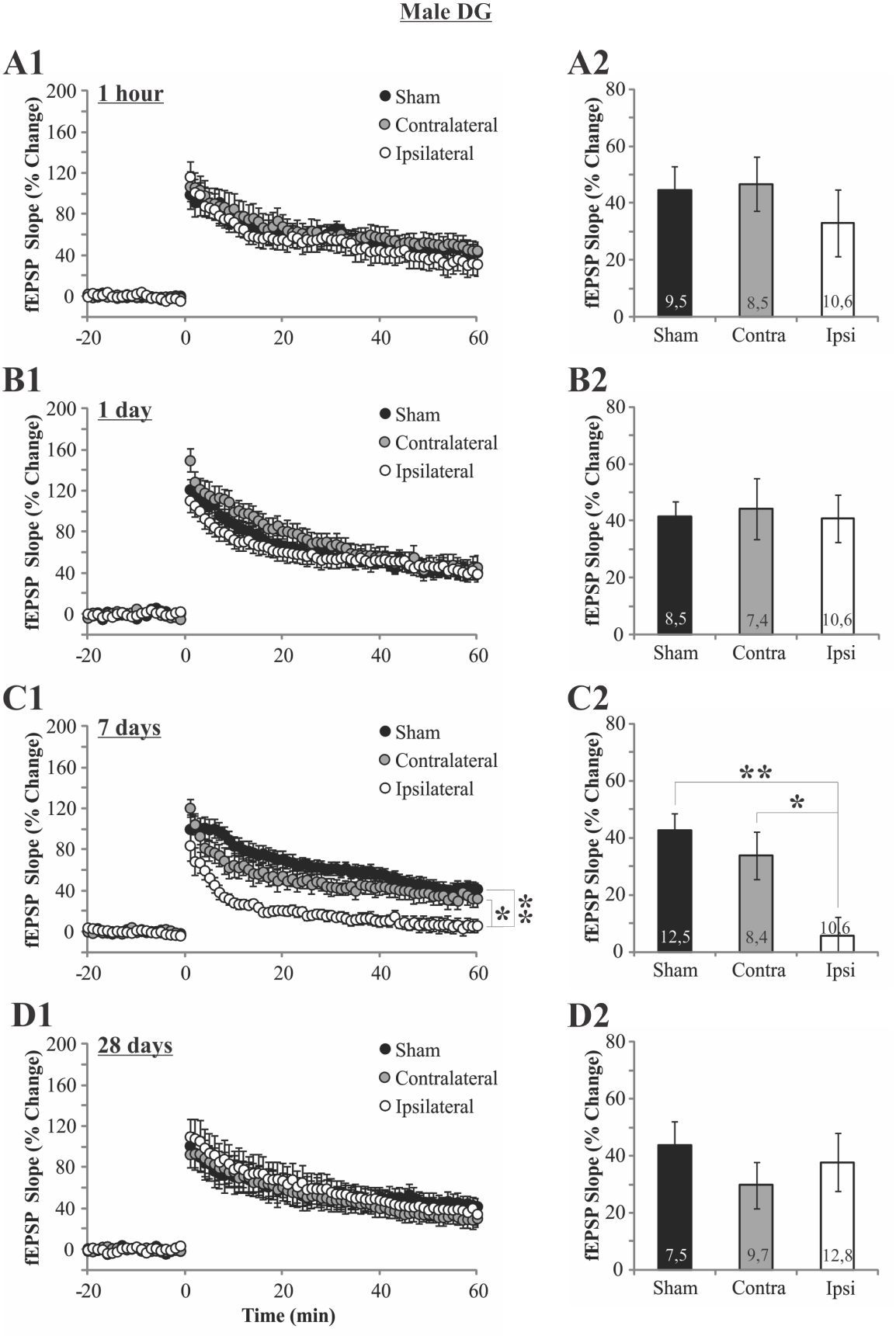
Male DG
A1
1 hour
Sham
Contralateral
Ipsilateral
fEPSP Slope (% Change)
A2
9,5
8,5
10,6
Sham
Contra
Ipsi
B1
1 day
B2
8,5
7,4
10,6
C1
7 days
C2
12,5
8,4
10,6
D1
28 days
D2
7,5
9,7
12,8
Time (min)

Figure 7. Effect of mTBI on long-term potentiation in the male DG. HFS (50 pulses at 100 Hz, 4x with a 30 s interval) at time zero elicited significant LTP in sham rats and both hemispheres of mTBI rats at all time points, as measured at 55-60 minutes post-HFS. LTP was not significantly altered at **(A)** 1 hour or **(B)** 1 day following mTBI. **(C)** However, mTBI significantly attenuated LTP in the ipsilateral hippocampal DG of mTBI animals at 7 days post-injury as compared to sham animals and the contralateral hemisphere. **(D)** LTP deficits were fully recovered following 28 days post-injury. Data presented as mean fEPSP slope (% change) ± SEM. **P*<0.05, ***P*<0.01.

3.1.2.2 CA1-LTP in Juvenile Males

LTP was induced in the Schaffer collaterals of the male CA1 using a robust HFS protocol. HFS produced robust PTP and STP in both hemispheres of mTBI animals and sham animals that were not significantly different (**Figure 8A1**). Additionally, the CS induced LTP in sham animals 1 hour post-surgery, when measured 55 to 60 minutes following HFS (38.9 ± 9.9 %; n = 8; **Figure 8A**). The same CS produced similar levels of LTP in contralateral (49.7 ± 9.8 %; n = 7) and ipsilateral (31.1 ± 9.5 %; n = 7) hemispheres of mTBI animals ($F_{(2,19)} = 0.891$, $P = 0.427$).

At 1 day following mTBI, robust LTP was once again evoked in the CA1 of sham animals (63.4 ± 12.9 %; n = 10; **Figure 8B**). In the contralateral hemisphere of mTBI animals, levels of LTP were similar to those of sham animals (52.9 ± 7.3 %; n = 8), as was the LTP elicited in the ipsilateral hemisphere of mTBI animals (59.7 ± 13.4 %; n = 7; $F_{(2,22)} = 0.228$, $P = 0.798$). There was no significant effect of condition on evoked levels of PTP and STP (**Figure 8B1**).

Seven days following mTBI, LTP was once again evoked in the CA1 of sham animals (59.3 ± 9.7 %; n = 10; **Figure 8C**). In the contralateral hemisphere of mTBI animals, levels of LTP were similar to those of sham animals (43.3 ± 6.0 %; n = 9). However, a significant difference was found between the three groups ($F_{(2,26)} = 3.835$, $P = 0.035$). LTP in the ipsilateral hemisphere of mTBI animals was significantly attenuated when compared to sham levels (27.6 ± 8.4 %; n = 10; $P = 0.027$). Levels of PTP and STP remained statistically unaffected by injury (**Figure 8C1**).

By 28 days post-injury, levels of PTP and STP were not significantly affected by condition (**Figure 8D1**), and LTP evoked in the ipsilateral hemisphere of mTBI animals (31.8 ± 11.1 %; n = 10; **Figure 8D**) had recovered to levels similar to that of sham (39.7

± 6.7 %; n = 7) and in the contralateral hemisphere of mTBI animals (40.0 ± 5.8 %; n = 12; $F_{(2,26)} = 0.339$, $P = 0.715$).

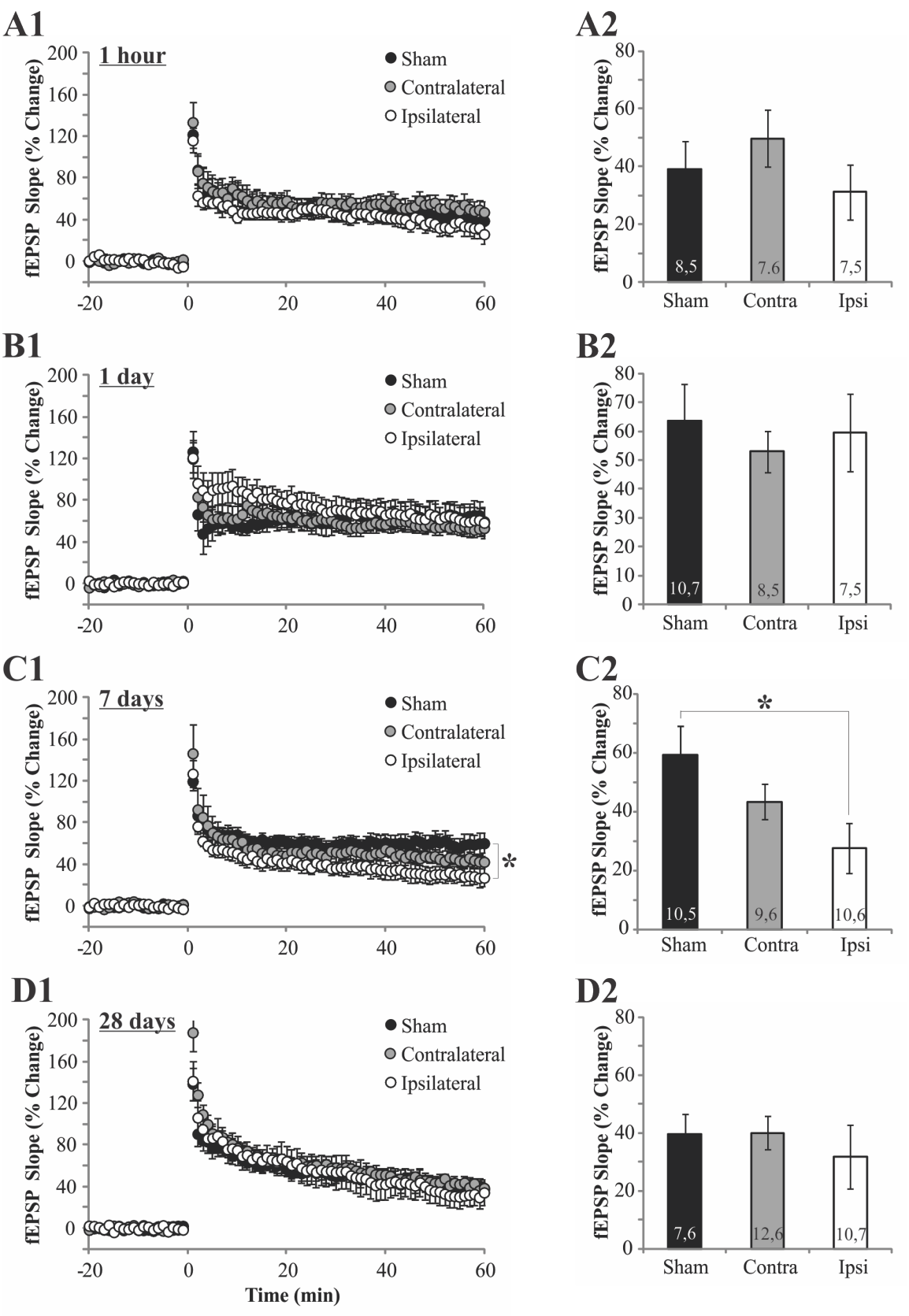
Male CA1
A1
1 hour
Sham
Contralateral
Ipsilateral
fEPSP Slope (% Change)
A2
fEPSP Slope (% Change)
8,5
7.6
7,5
Sham
Contra
Ipsi
B1
1 day
B2
10,7
8,5
7,5
C1
7 days
*
C2
10,5
9,6
10,6
D1
28 days
D2
7,6
12,6
10,7
Time (min)

Figure 8. Effect of mTBI on long-term potentiation in the male CA1. HFS (50 pulses at 100 Hz, 4x with a 30 s interval) at time zero elicited significant LTP in sham rats and both hemispheres of mTBI rats at all time points, as measured at 55-60 minutes post-HFS. LTP was not significantly altered at **(A)** 1 hour or **(B)** 1 day following mTBI. **(C)** However, mTBI significantly attenuated LTP in the ipsilateral hippocampal CA1 at 7 days post-injury as compared to sham animals. **(D)** LTP deficits were fully recovered following 28 days post-injury. Data presented as mean fEPSP slope (% change) ± SEM. **P*<0.05.

3.1.3 Summary of Sex-specific Long-term Potentiation Deficits

In the female DG, LTP was significantly impaired in the ipsilateral hemisphere at 1 day and 7 days post-mTBI (1 day: $P = 0.039$; 7 days: $P = 0.023$), and persisted to 28 days following injury ($P = 0.010$ from contra; **Figure 9C**). However, in the males the time course of LTP deficits was more delayed and acute; DG-LTP deficits in the ipsilateral hemisphere were only apparent 7 days following injury ($P = 0.001$ from sham; $P = 0.026$ from contra; **Figure 9C**). Representative traces taken at 7 days post-mTBI highlight the significantly diminished post-tetanic fEPSP slope in the ipsilateral hemisphere of females (32.1% reduction) and males (37.0% reduction) when compared to sham animals (**Figure 9A**).

In the CA1 region of the hippocampus, LTP was more immediately impaired in females than males. Females displayed significant deficits in CA1-LTP in the ipsilateral and contralateral injured hemispheres at 1 hour post-mTBI (ipsi: $P = 0.037$; contra: 0.012; **Figure 9D**), whereas the impairments in LTP were not apparent in males until 7 days following injury ($P = 0.027$; **Figure 9D**). Representative traces taken at 7 days post-mTBI illustrate significantly attenuated post-tetanic fEPSPs in the male ipsilateral CA1 of mTBI animals compared to the fEPSP slope of sham animals (31.7% reduction; **Figure 9B**).

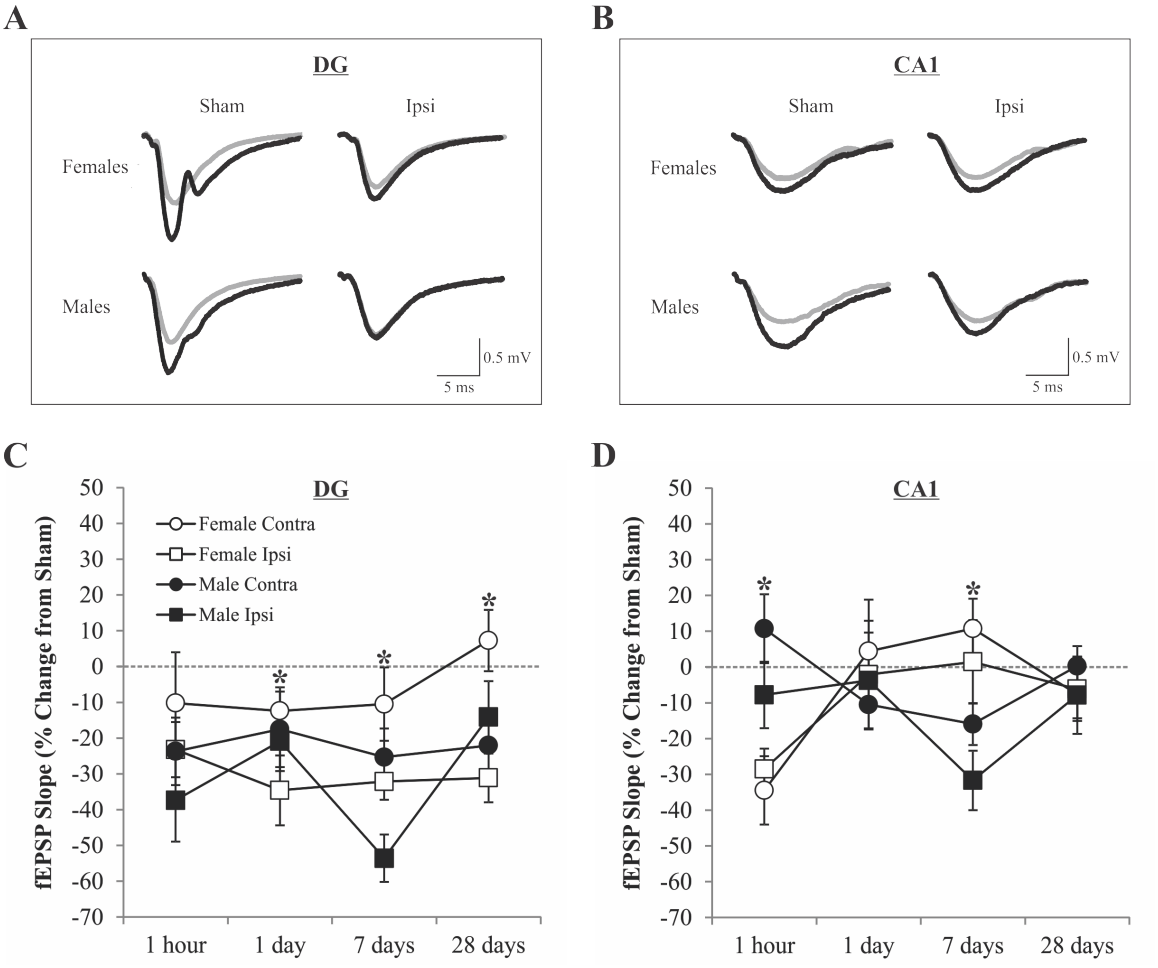

Figure 9. Mild TBI significantly impairs DG- and CA1-LTP in juvenile females and males. Representative fEPSP traces in the **(A)** DG and **(B)** CA1 at 7 days post-mTBI. LTP was most significantly impaired in females and males at 7 days post-mTBI. Pre-tetanic representative traces are indicated in grey, post-tetanic in black. **(C)** DG-LTP in females was significantly impaired in the ipsilateral hemisphere at 1, 7, and 28 days post-mTBI. In the males, DG-LTP in the ipsilateral hemisphere was impaired from sham and contralateral LTP at 7 days post-injury. **(D)** CA1-LTP in females was significantly impaired in the ipsilateral and contralateral hemispheres at 1 hour post-mTBI, whereas the males exhibited a deficit in ipsilateral CA1-LTP at 7 days. Data expressed as mean fEPSP slope (% change from sham) at 55-60 minutes post-HFS ± SEM. **P*<0.05.

3.2 Synaptic Transmission is Not Affected by mTBI

3.2.1 Evoked Synaptic Transmission is Not Altered by mTBI

To determine if an mTBI has implications on basal physiological parameters in the DG and CA1, I/O functions were used to assess synaptic transmission and slice health in slices from sham and mTBI animals. In all slices, the slope of the fEPSP increased

significantly in response to increasing stimulation from a pulse width of 30 to 300 μs (**Figure 10**). In the female DG, all time points examined following sham or mTBI surgery displayed no significant differences between groups (1 hour: $F_{(18,28)} = 1.055$, $P = 0.438$; 1 day: $F_{(18,34)} = 0.970$, $P = 0.512$; 7 days: $F_{(18,32)} = 0.499$, $P = 0.939$; 28 days: $F_{(18,42)} = 0.379$, $P = 0.985$; **Figure 10A**). In the female CA1, no significant differences were observed between groups at all time points examined following sham or mTBI surgery (1 hour: $F_{(18,24)} = 0.849$, $P = 0.635$; 1 day: $F_{(18,26)} = 0.730$, $P = 0.753$; 7 days: $F_{(18,24)} = 1.505$, $P = 0.173$; 28 days: $F_{(18,22)} = 0.788$, $P = 0.694$; **Figure 10B**). In all slices from the male DG, no significant differences were observed between groups at all time points examined (1 hour: $F_{(18,30)} = 1.042$, $P = 0.448$; 1 day: $F_{(18,30)} = 1.249$, $P = 0.287$; 7 days: $F_{(18,44)} = 1.064$, $P = 0.416$; 28 days: $F_{(18,32)} = 0.623$, $P = 0.856$; **Figure 10C**). These results indicate that sustaining an mTBI does not impact evoked synaptic transmission in the female DG or CA1, and in the male DG.

When the I/O in slices from the CA1 of male animals was examined, a significant difference was found between the three groups at 1 hour post-injury ($F_{(18,20)} = 3.656$, $P = 0.003$). However, post-hoc analyses did not reveal any significant differences. One day following mTBI, no significant differences in I/O function were observed between groups ($F_{(18,22)} = 0.618$, $P = 0.848$; **Figure 10D**). However, at 7 days post-injury, a significant difference was found between the three groups ($F_{(18,34)} = 2.585$, $P = 0.008$; **Figure 10D**). Post hoc analysis revealed that at a pulse width of 180 μs, slices from the contralateral hemisphere of mTBI animals had a significantly increased fEPSP slope when compared to slices from sham animals ($P = 0.032$), and at 210 μs, slices from the ipsilateral hemisphere of mTBI animals had a significantly increased fEPSP slope compared to slices from sham animals ($P = 0.028$). No significant differences were found between any groups at subsequently increasing pulse widths of 240-300 μs. By 28 days after sustaining an mTBI, no significant differences between fEPSP response to increasing stimulation were observed in I/O functions between the three groups ($F_{(18,34)} = 0.979$, $P = 0.503$; **Figure 10D**). While these results suggest that sustaining an mTBI may briefly impact synaptic transmission in the male CA1, it is quickly recovered. No differences were observed between groups in the female DG or CA1, the male DG, or at any other

time point in the male CA1, indicating that sustaining an mTBI has no detrimental effect on evoked synaptic transmission in the hippocampus.

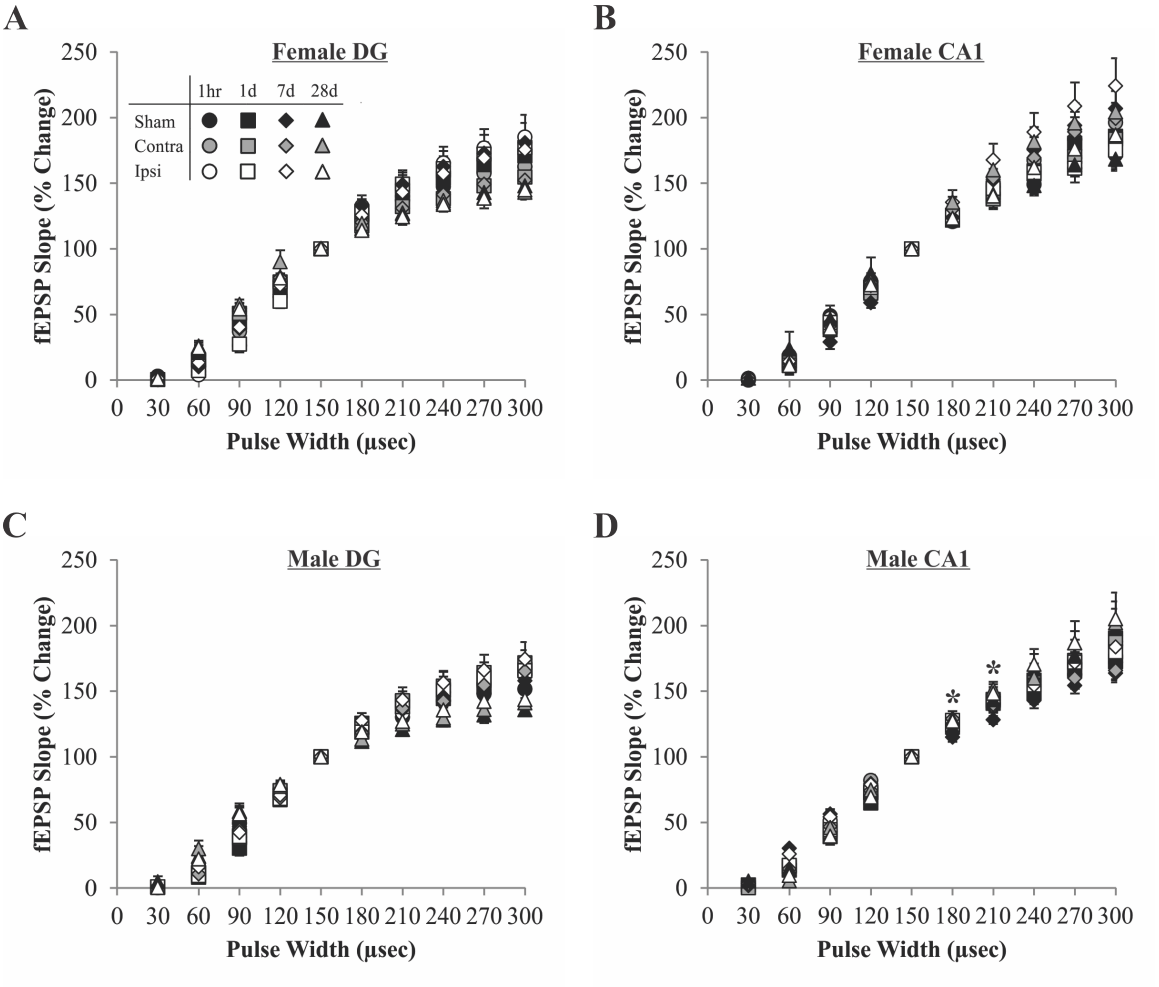

Figure 10. No change in stimulus input-output functions in the female and male DG and CA1 following mTBI. No significant differences in synaptic responses to a series of single pulse stimuli at incrementally increasing intensities (30-300 µs pulse width) were measured at 1 hour (circles) 1 day (squares), 7 days (diamonds), or 28 days (triangles) post-injury in the **(A)** DG and **(B)** CA1 of the female brain following mTBI. Additionally, there were no significant differences in synaptic responses between sham animals, and slices from the contralateral or ipsilateral hemispheres of mTBI animals at **(C)** all time points in the male DG and at **(D)** 1 hour, 1 day, and 28 days post-mTBI in the male CA1. However, at 7 days post-injury, the contralateral and ipsilateral hemispheres of mTBI animals demonstrated significantly altered fEPSP slopes from sham animals at pulse widths of 180 µs and 210 µs, respectively. Data presented as mean fEPSP slope (% change) ± SEM. *P<0.05.

3.2.2 Presynaptic Neurotransmitter Release is Not Altered by mTBI

Paired-pulse stimulation was used to examine if mTBI affects presynaptic neurotransmitter release. In the female DG, no significant differences in levels of paired-pulse plasticity were observed in contralateral and ipsilateral slices taken from mTBI animals when compared to slices from sham animals at 1 hour, 1 day, 7 days, or 28 days post-injury (1 hour: $F_{(2,22)} = 0.757$, $P = 0.481$; 1 day: $F_{(2,21)} = 0.505$, $P = 0.611$; 7 days: $F_{(2,23)} = 0.496$, $P = 0.616$; 28 days: $F_{(2,28)} = 1.145$, $P = 0.333$; **Figure 11A**). Additionally, in the female CA1, no significant differences in levels of paired-pulse plasticity were observed in contralateral and ipsilateral slices taken from mTBI animals when compared to slices from sham animals at all time points post-injury (1 hour: $F_{(2,20)} = 2.107$, $P = 0.148$; 1 day: $F_{(2,18)} = 3.296$, $P = 0.060$; 7 days: $F_{(2,20)} = 0.275$, $P = 0.763$; 28 days: $F_{(2,20)} = 2.453$, $P = 0.112$; **Figure 11B**). These results indicate that mTBI does not alter glutamate release in the female DG and CA1 following mTBI.

At 1 hour following injury in the male DG, significant differences in paired-pulse plasticity were observed ($F_{(2,22)} = 3.932$, $P = 0.035$; **Figure 11C**). The ipsilateral hemisphere of mTBI animals displayed significantly less paired-pulse facilitation than the contralateral hemisphere of the same animals ($P = 0.031$) but not slices from sham animals. However, no significant differences in levels of paired-pulse plasticity were observed in slices from the contralateral and ipsilateral hemispheres of mTBI animals at all subsequent time points post-injury (1 day: $F_{(2,25)} = 3.071$, $P = 0.064$; 7 days: $F_{(2,26)} = 3.226$, $P = 0.056$; 28 days: $F_{(2,24)} = 1.114$, $P = 0.345$; **Figure 11C**). In the male CA1, no significant differences in levels of paired-pulse plasticity were observed in slices from the contralateral or ipsilateral hemispheres of mTBI animals at all time points post-injury (1 hour: $F_{(2,19)} = 1.198$, $P = 0.324$; 1 day: $F_{(2,21)} = 0.208$, $P = 0.814$; 7 days: $F_{(2,26)} = 1.943$, $P = 0.163$; 28 days: $F_{(2,24)} = 0.210$, $P = 0.812$; **Figure 11D**). Considering the only significant difference in paired pulse plasticity was between contralateral and ipsilateral injured hemispheres of mTBI animals, and not sham animals, mTBI does not appear to alter presynaptic neurotransmitter release in the hippocampus.

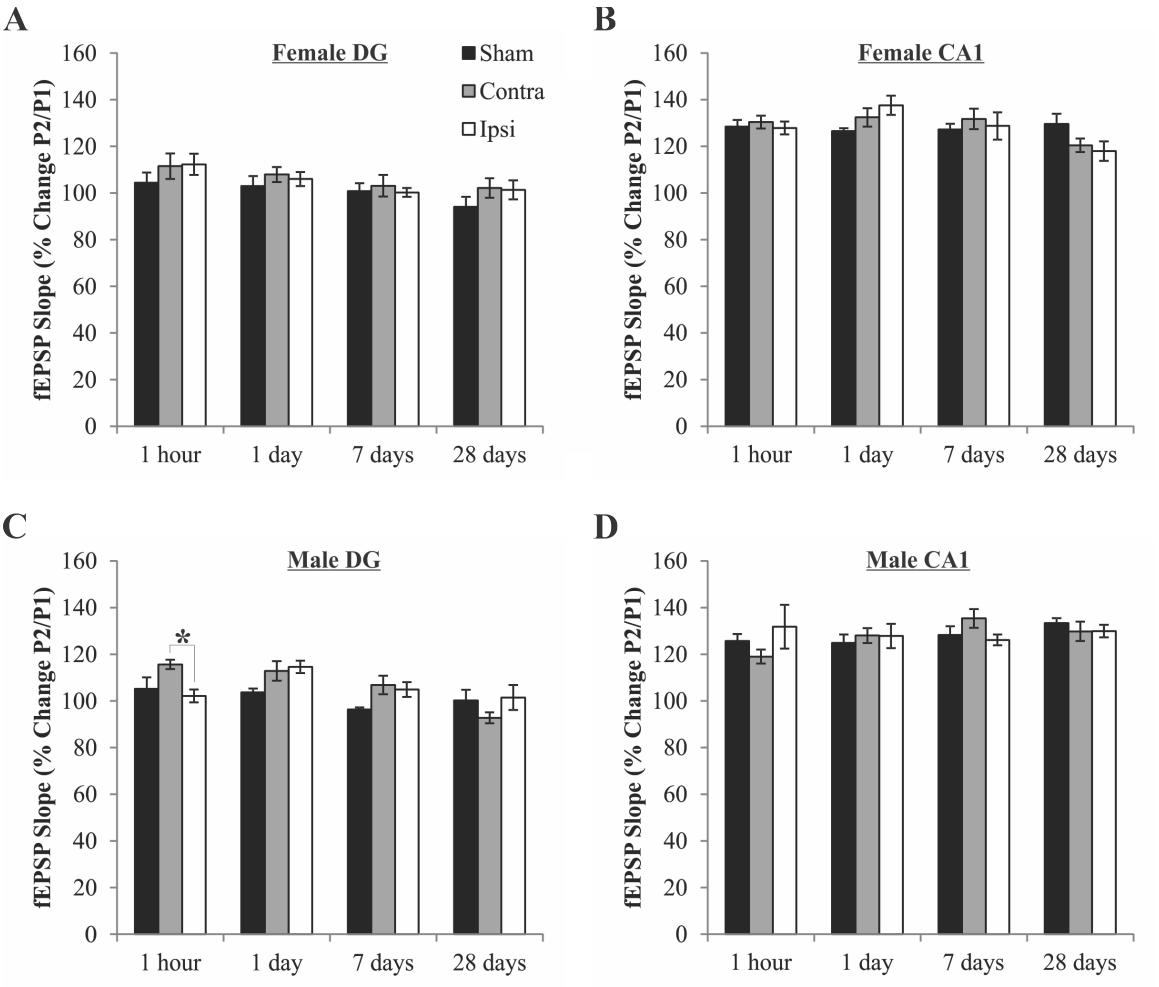

Figure 11. No change in paired-pulse plasticity in the female and male DG and CA1 following mTBI. Paired-pulse ratios obtained from the medial molecular layer of the DG or the stratum radiatum of the CA1. In the female **(A)** DG and **(B)** CA1 no significant differences were observed in PP plasticity at 1 hour, 1 day, 7 days, or 28 days following mTBI. Additionally, no significant differences in paired-pulse plasticity were observed in the male **(C)** DG or **(D)** CA1 at all time points following mTBI when compared to sham animals. Data presented as mean fEPSP slope (% change P2/P1) ± SEM. **P*<0.05.

3.3 Neuronal Degeneration in the Hippocampus is Not Significantly Affected by mTBI

To determine if an mTBI causes tissue damage in the brain, FJC histology was used to quantify neurodegeneration in the cortex, as well as the DG and CA regions of the hippocampus. In animals inflicted with mTBI, there was diffuse distribution of FJC+ cells throughout the CA, and DG regions. The cortex exhibited a focused concentration

of positive cells at the site of impact with greater contrast than other regions (**Figure 12A4**). A two-way ANOVA revealed significance between time points for the female cortex ($F_{(1,47)}$ = 14.238, P = 0.0005; **Figure 12B1**) and the female CA region ($F_{(1, 47)}$ = 4.323, P= 0.043; **Figure 12B2**). Post-hoc analysis revealed that there was a significant difference in the number of FJC+ cells between the 1 day and 7 day time points for the female cortex (P = 0.0006) and CA regions (P = 0.047), with the cortex and CA regions exhibiting a greater number of FJC+ neurons in animals sacrificed at the 1 day time point compared to those sacrificed at the 7 day time point. Additionally, a two-way ANOVA revealed a significant interaction between time point and condition in the female cortex ($F_{(2,47)}$ = 3.6901, P = 0.0325; **Figure 12B1**), and post hoc analysis revealed that a significantly greater number of FJC+ cells were found in the ipsilateral hemisphere of mTBI animals compared to sham animals at 1 day post-injury (P = 0.009). There were no significant differences in the number of FJC+ cells in the male brain, regardless of region, time point, or condition. Although there was more positive staining in the contralateral and ipsilateral hemispheres of mTBI animals compared to the sham group, apart from the female cortex, no significant differences were found between the conditions.

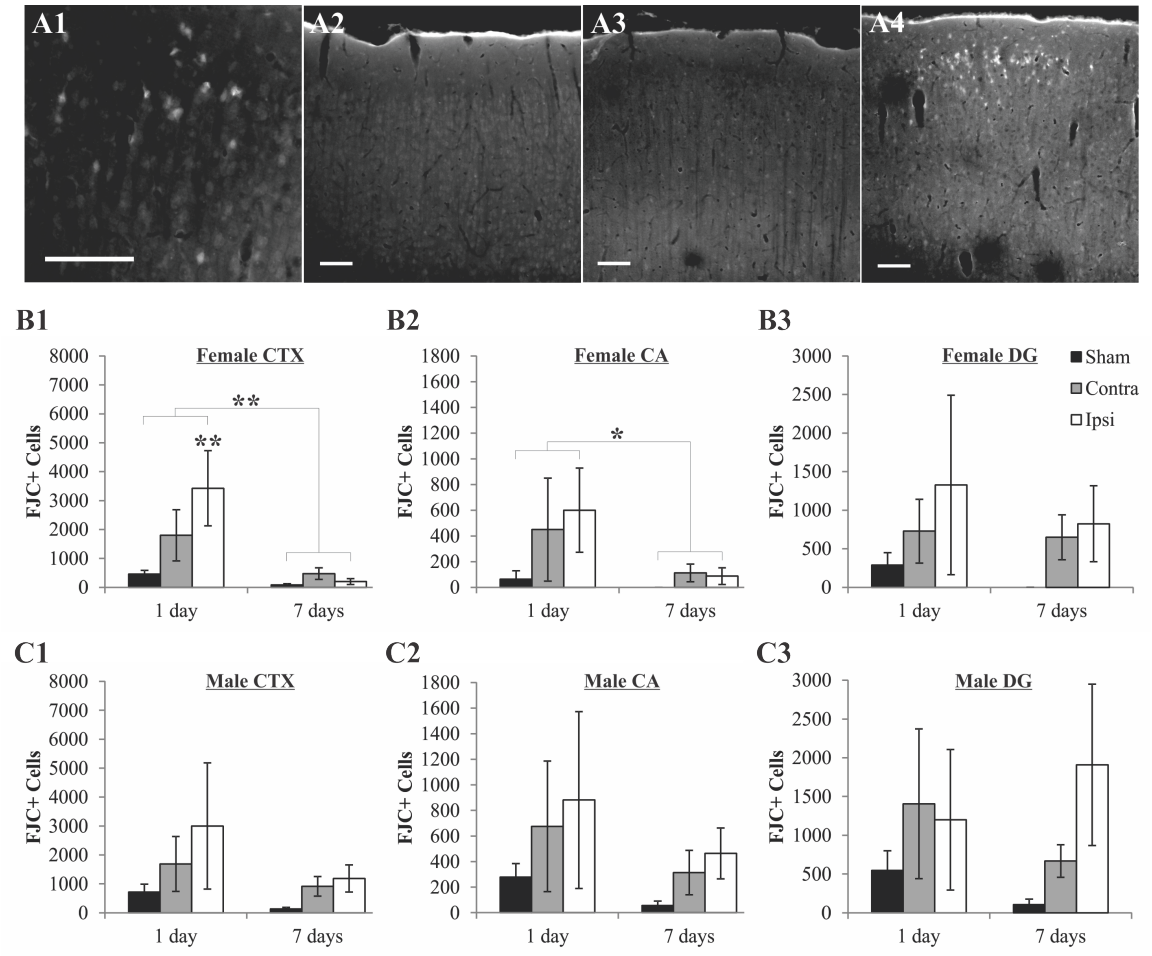

Figure 12. No change in number of FJC positive cells in the female and male CA and DG following mTBI. (A) Representative images of the cortex captured with a 20X or 40X objective of FJC+ neurons from female animals sacrificed at 1 day post-mTBI. Images are of **(A1)** FJC+ cells, **(A2)** sham cortex, **(A3)** contralateral cortex, and **(A4)** ipsilateral cortex. Scale bar = 100 µm. In the female brain, the **(B1)** cortex and **(B2)** CA regions exhibited significantly greater numbers of FJC+ cells at 1 day post-injury compared to 7 days. **(B1)** Additionally, more FJC+ cells were found in the cortex in the ipsilateral hemisphere of mTBI females compared to sham females at 1 day post-mTBI. **(C)** There were no significant differences in the number of FJC+ cells in the male brain, regardless of region, time point, or condition. Data presented as mean number of FJC+ cells ± SEM. **P*<0.05, ***P*<0.01.

4. Discussion

Here we show, for the first time, that hippocampal synaptic plasticity is differentially impacted in male and female juvenile brains following mTBI. While mTBI was not accompanied by significant changes in synaptic transmission or neurotransmitter release, there were significant regional and sex-specific changes in synaptic plasticity. These deficits had a more rapid onset in the female DG and CA1 regions of the hippocampus than in males. Moreover, these impairments in hippocampal synaptic plasticity persisted for longer in females than males. In contrast to the females, deficits in synaptic plasticity in the male hippocampus exhibited a delayed onset, and showed a more rapid return of function.

A unique feature of this work was the utilization of a closed-head model of mTBI. The majority of concussive head injuries are closed-head injuries (Faul et al., 2010), very mild, have a low rate of mortality, and lack gross neuropathology in the presence of neurological deficits (Prins et al., 2010). Therefore the use of a closed-head model circumvents some of the issues associated with the more invasive CCI and FP models, such as disruption of the blood brain barrier and the compromise of the cranial structure. In addition, the brain itself is directly impacted when these models are employed, producing significant physical damage that commonly extends into the hippocampus (Bramlett & Dietrich, 2002; Pierce et al., 1998). These studies may be better classified as either moderate or severe TBI, to reflect the invasive nature of the procedure, and the actual degree of damage produced (Namjoshi et al., 2013). In the present study there was no significant cell death in the hippocampus (**Figure 12**), similar to the extent of damage normally seen in clinical cases of mTBI (Tate & Bigler, 2000).

4.1 Deficits in Synaptic Plasticity following mTBI

Cognitive impairments are often the most prominent complaints following a closed head brain injury in humans (Ylvisaker & Feeney, 1998; Ylvisaker, 1997). Both the CCI and FP models also produce learning and memory deficits (Baddeley et al., 1987; Dikmen et al., 1987; Tabaddor et al., 1984; Ylvisaker & Szekeres, 2002). Learning and

memory is thought to rely on changes in the way neurons communicate with one another in specific areas of the brain such as the hippocampus. Following either CCI or FP, synaptic transmission has been shown to be depressed in the hippocampal CA1 for hours to days (D'Ambrosio et al., 1998; Miyazaki et al., 1992; Norris & Scheff, 2009; Reeves et al., 2000; Witgen et al., 2005; Zhang et al., 2011a, 2011b), which usually resolves within 1 to 2 weeks post-TBI (Norris & Scheff, 2009; Reeves et al., 2000). This is most likely due to alterations in the balance of inhibitory and excitatory evoked synaptic transmission, possibly due to changes in metabolic processes within the cell that can occur in these models (Giza & Hovda, 2014). Previous studies have reported an increase in population spike amplitude, while the population spike threshold is decreased, indicating that pyramidal cells in the CA1 region are more excitable (Cao et al., 2006; Miyazaki et al., 1992; Reeves et al., 1997, 1995; Witgen et al., 2005). However, in the DG, evoked EPSPs are enhanced, while inhibitory postsynaptic currents are depressed, indicating that the balance between excitation and inhibition is being altered (Golarai et al., 2001; Hunt et al., 2010, 2011; Lowenstein et al., 1992; Mtchedlishvili et al., 2010; Schwarzbach et al., 2006; Witgen et al., 2005). In the present study, we show that mTBI does not alter evoked excitatory transmission (**Figure 10**), suggesting that closed-head models may produce significantly less direct damage to the brain.

Evaluating synaptic plasticity in the hippocampus provides a means to evaluate the dynamic capacity of the neuronal circuits that are involved in spatial memory processing in rodents. In adult rodents, both CCI and FP models reliably impair LTP of synaptic efficacy (Albensi et al., 2000; D'Ambrosio et al., 1998; Miyazaki et al., 1992; Reeves et al., 1995; Sanders et al., 2000; Schwarzbach et al., 2006; Sick et al., 1998; Witgen et al., 2005; Zhang et al., 2011). These deficits can be quite persistent, lasting up to 8 weeks in the CA1 hippocampal subfield (Sanders et al., 2000) and 7 days in the DG (Yamashita et al., 2011). Following closed-head mTBI, we found that LTP in the CA1 was impaired at one hour post-injury in females (**Figure 6A**), but was not significantly decreased from sham levels at one day following mTBI (**Figure 6B**). However, LTP deficits in the female DG presented themselves at one day following injury (**Figure 5B**), and persisted to 28 days (**Figure 5D**), where LTP in the ipsilateral hemisphere remained significantly diminished from the contralateral hemisphere. In the males, LTP in the DG

and CA1 was acutely impaired selectively at the 7 day time point (**Figure 7C, 8C**). Therefore, while mTBI has no significant effect on evoked synaptic transmission, our study demonstrated that both male and female juvenile brains exhibited LTP deficits in the hippocampal DG and CA1 subfields, and that these deficits were persistent in the female DG.

4.2 Hemispheric Differences in LTP Deficits

The model used to induce TBI may also influence the pattern of damage observed. Our model is a lateralized model of TBI, and the ipsilateral hemisphere receives a more direct and severe injury than the contralateral hemisphere (Thompson et al., 2005). While many studies focus on examining deficits in the ipsilateral hemisphere, the intact skull more evenly distributes the force of the impact across both hemispheres, causing a more diffuse injury (Namjoshi et al., 2013, 2014). This is more representative of the type of injury suffered by humans, where the skull most commonly remains intact (Faul et al., 2010). In the present study, LTP deficits were apparent in the ipsilateral hemisphere of the female and male DG and CA1. However, at one hour following mTBI in the female CA1, LTP in the contralateral hemisphere was also significantly impaired (**Figure 6A**). Normally, the brain is surrounded by a cushion of cerebral spinal fluid (CSF) that protects the surface of the brain from the bony processes inside the skull. However, in a head injury, rapid acceleration and deceleration of the brain inside the skull leads to a coup injury at the site of impact as the skull is temporarily bent inward and impacts the brain. The force of the initial impact of the skull with the brain, in combination with the rapid movement of CSF inside the skull, can contribute to a contrecoup injury as the brain collides with the opposite side of the skull to the initial impact (Gengenbach & Hyde, 2007; Gurdjian, 1976; Shaw, 2002). As a result, cellular damage and functional deficits are not always apparent solely at the original site of impact.

At 28 days following mTBI, DG-LTP in the ipsilateral hemisphere of injured animals was significantly decreased relative to the contralateral hemisphere, but not to that observed in sham animals (**Figure 5D**). This could suggest a compensatory

mechanism by the contralateral hippocampus following a persistent LTP deficit in the female DG. A similar effect has been seen following ischemic injury in humans and in rodents. In stroke patients, activation of the uninjured hemisphere was observed shortly after the ischemic event with fMRI (Binkofski & Seitz, 2004; Feydy et al., 2002). Furthermore, in a rodent model of photothrombotic stroke in the barrel cortex, Jablonka and Kossut (2006) found increased activation in the barrel cortex of the uninjured hemisphere up to 7 days following insult. Brain injury can trigger spontaneous plasticity, often resulting in considerable restoration of function in the peri-infarct zone, as well as through re-mapping to more distant sites (Brown et al., 2009). Although the mechanisms of a stroke and mTBI are vastly different, and both acquired brain injuries result in distinctive pathologies, it is possible that mTBI may also trigger similar neuroplastic events following injury, as the non-injured hemisphere attempts to compensate for the impaired ipsilateral function.

4.3 Subregion-specific Hippocampal Vulnerability to mTBI

Particular areas of the hippocampus may be more vulnerable to injury than others due to the susceptibility of subregion-specific cell types and the organization of synaptic inputs and outputs in the hippocampus. In the hippocampal formation, there is a unidirectional propagation of information in what is known as the trisynaptic circuit (**Figure 2**) (Amaral, 1993; Andersen et al., 1971). Granule cells in the DG receive excitatory input from layer II of the EC via the perforant pathway (Winson & Abzug, 1977). The dentate hilar neurons, and newborn neurons in the inner granule cell layer of the DG, are particularly vulnerable to injury and seem to be selectively lost after TBI (Gao et al., 2008; Golarai et al., 2001; Grady et al., 2003; Hicks et al., 1993; Lowenstein et al., 1992). Our results are in agreement with these findings; after sustaining an mTBI, LTP deficits persisted for longer in the female DG than CA1 region (**Figure 5, 6**). The mossy fibers of the DG pass input from the DG to the CA3 pyramidal neurons. CA3 axons synapse with CA1 pyramidal neurons via the Schaffer collaterals. The CA1 pyramidal cells provide the excitatory output from the trisynaptic loop; pyramidal cell axons project to layer V of the EC, and the subiculum (Amaral & Lavenex, 2007). In the

CA, the CA3 subregion of the hippocampus appears to be more susceptible to injury than the CA1 (Golarai et al., 2001; Grady et al., 2003; Mao et al., 2013). Our synaptic plasticity results are in support of these findings, as LTP is impaired for a significantly longer period of time in the DG than the CA1 following mTBI.

4.4 Sex-specific Differences in LTP Deficits

Synaptic plasticity was differentially impacted in the juvenile male and female brain following mTBI. Impairment of LTP in the female brain had a more rapid onset, and deficits persisted for longer than in the male brain (**Figure 9**). The majority of previous studies in this field examine the effects of mTBI solely in the male brain. The advantage to studying only the male brain is to control for the estrous cycle, which can impact neuronal morphology and function in the female brain (Fester & Rune, 2014; Foy et al., 1999; Kramár et al., 2009a, 2009b; Smith & McMahon, 2005; Spencer et al., 2008; Woolley et al., 1990). Gonadal steroids such as estrogen (17β-estradiol) can regulate synaptic profiles and morphology in the male and female hippocampus (Brake et al., 2001; Woolley, 1999, 2007). Specifically, proestrus (a period of high estrogen) has been associated with alterations in structural and functional plasticity, and can enhance LTP in the hippocampus (Foy et al., 1999; Warren et al., 1995). Female rats reach sexual maturity between PND 40-60 (Kohn & Clifford, 2002; Long & Evans, 1920; Sengupta, 2013). While the majority of time points examined post-mTBI in the present study were in prepubescent rats, females sacrificed 28 days post-injury were sexually mature (PND 53-56), although all females in the proestrus stage of their cycle were omitted from the experimental data.

Women often fare worse than males after suffering a TBI, and take longer to recover (Broshek et al., 2005; Colvin et al., 2009; Covassin et al., 2012). Our results are in agreement with these findings: LTP deficits in the hippocampal DG are more persistent in females than in males. Contrary to this, experimental evidence suggests that estrogen may be neuroprotective and a potent enhancer of neuroplasticity following TBI (Arevalo et al., 2010; Azcoitia et al., 2011; Foy et al., 1999). Enhanced NMDAR-mediated transmission and increased magnitude of LTP was elicited from hippocampal

slices of estrogen-injected ovariectomized female rats compared to vehicle-injected ovariectomized females (Smith & McMahon, 2006). However, recent clinical research demonstrates that following severe TBI, female subjects had lower endogenous CSF estrogen versus control subjects. CSF estrogen levels were not significantly different in males compared to females after severe TBI. Decreased CSF estrogen in females following injury was associated with mortality or poorer outcomes as measured with the Glasgow Coma Scale (Garringer et al., 2013), further indicative of the neuroprotective effects of estrogen.

There are two types of estrogen receptors (ERs) that are differentially distributed in the brain: ER-α and ER-β (Shughrue et al., 1997). Overall, a much greater concentration of ER-β than ER-α is found in the female cerebral cortex and hippocampus, suggesting that the β-type of the ER plays an important role in cognitive functioning. However, when total estrogen-binding cells were localized and quantified in the male and female rodent brain, it was found that female mice and rats express more ER-β in the hippocampus than the males (Koch & Ehret, 1989; Zhang et al., 2002). Specifically, ER-β labelling was more strongly concentrated in the DG than the CA1 hippocampal subregions in both the male and female brain (Zhang et al., 2002). This differential distribution of ER types between the male and female brain – specifically the increased concentration of ER-β in the female hippocampal DG, combined with decreased CSF estrogen following TBI in females – may contribute to the prolonged LTP deficits apparent in the female DG compared to males at 28 days post-mTBI.

The hippocampus is larger in males than in females, with the difference in size most apparent in the CA1 subregion (Allen & Gorski, 1990; Allen et al., 1989; Isgor & Sengelaub, 1998, 2003; Madeira & Lieberman, 1995; McCarthy & Konkle, 2005; Nuñez et al., 2003a, 2003b; Rabinowicz et al., 1999). This sex-specific difference in hippocampal volume is evident as early as PND 7 in rats, and corresponds to a greater number of neurons in the male hippocampus (Hilton et al., 2003; Nuñez et al., 2003a, 2003b). Specifically, male adult rats displayed greater CA1 and CA3 pyramidal cell field volume and soma size compared to females (Isgor & Sengelaub, 1998). In the DG, the rate of granule cell maturation is sex-specific, with granule cells developing more rapidly in the molecular layer of the DG in males than in females (Muramatsu et al., 2007). This

indicates that cell proliferation and perhaps rates of adult neurogenesis may be sex-specific which may account for the differences observed between males and females in this study. Additionally, prepubescent and adult male rats have a significantly wider and longer granule cell layer in the DG, and males exhibit asymmetric laterality (Roof & Havens, 1992; Roof, 1993). These results correlate to sex-differences in hippocampal-associated spatial learning strategies such as Morris water maze (MWM) performance (Isgor & Sengelaub, 1998; Roof, 1993). Our study suggests that these differences may also contribute to post-mTBI recovery, with females showing a more pronounced deficit and poorer rate of recovery than males.

In addition to the effects attributed to sex hormones, receptors, and hippocampal size, other non-hormonal sex differences exist in the brain prior to puberty as a result of genetic factors. Genes encoded on the X- or Y-chromosome have sex-specific effects on non-gonadal tissues such as the brain, sex-biased expression of genes expressed on other chromosomes, and selective gene inactivation (Arnold, 2009; Mank, 2009). In addition to the sex-specific LTP deficits we observed following mTBI in juveniles, sex differences in other models of neural injury have also been observed in prepubescent rodents. In a neonatal model of hypoxia-ischemia, different apoptotic mechanisms were activated in the male and female brain following injury. Females exhibited a caspase-3-mediated mechanism of apoptosis, whereas in males cell death was caspase-independent (Zhu et al., 2006). These findings support our hypothesis that even prior to puberty, the male and female brain responds differently to neural injury at the cellular level.

4.5 Possible Mechanisms of LTP Deficits

The cellular and molecular mechanisms underlying the mTBI-induced synaptic plasticity deficits remain under debate. Here, no significant neuronal death was observed in the hippocampus following mTBI (**Figure 12**), suggesting that the observed LTP deficits were not due to significant neuronal loss. Nevertheless, possible mechanisms that may contribute to the observed hippocampal dysfunction and attenuation of LTP include inflammation, decreases in blood-brain barrier integrity (Adelson et al., 1998; Thal & Neuhaus, 2014), dendritic spine remodelling (Campbell et al., 2012), and modification of

synaptic proteins including alterations in the expression of PSD-95 (Ansari et al., 2008a, 2008b; Wakade et al., 2010). Due to its important role in synaptic maturity and plasticity associated with NMDAR signalling, loss of the scaffolding protein PSD-95 in the hippocampus has been implicated in the cognitive deficits observed following TBI in both juvenile and adult rodents (Ding et al., 2009; Gao et al., 2011; Gobbel et al., 2007; Wakade et al., 2010; Walker & Tesco, 2013). In addition, dysregulation of metabolic pathways after TBI can influence plasticity in a number of ways (Babikian et al., 2010; Giza & Hovda, 2014), including neuronal damage via oxidative stress (Ansari et al., 2008a, 2008b) and changes in the expression level of the trophic factor BDNF (Kaplan et al., 2010), a key regulator of synaptic transmission and neuroplasticity known to promote the health of existing neurons (Hariri et al., 2003; Huang & Reichardt, 2001). Abnormalities in neurotransmitter systems, including glutamatergic (Biegon et al., 2004; Giza et al., 2006; Kumar et al., 2002; Miller et al., 1990; Osteen et al., 2004; Sihver et al., 2001), GABAergic (Lowenstein et al., 1992; Sihver et al., 2001), and cholinergic (Dixon et al., 1996, 1997, 1999; Gorman et al., 1996; Shao et al., 1999; Sihver et al., 2001) neurotransmission have also been reported in both juvenile and adult models of TBI.

One of the hallmarks of brain injury is the immediate and indiscriminate release of excitatory amino acids and subsequent activation of glutamate receptors (Faden et al., 1989; Katayama et al., 1990; Kawamata et al., 1992). This marked elevation in extracellular glutamate and aspartate adjacent to the trauma site is followed by an apparent downregulation in synaptic NMDA receptors hours to days post-injury (Biegon et al., 2004; Giza et al., 2006; Kumar et al., 2002; Miller et al., 1990; Osteen et al., 2004; Sihver et al., 2001). In PND 19 pups subjected to FP injury, hippocampal NR2A expression was reduced for up to 4 days post-injury (Giza et al., 2006). In uninjured animals, the NR2A and NR2B subtypes of the NMDAR are differentially expressed in the hippocampus during development, with NR2B prevailing in the immature brain, and NR2A expression increasing dramatically during maturation and ultimately predominating in the adult brain (Neyton & Paoletti, 2006; Sans et al., 2000; Tovar & Westbrook, 1999). Electrophysiological studies demonstrate significantly impaired DG-LTP in mice lacking the NR2A subunit (NR2A-/-), whereas only minor impairments were observed in the CA1 (Kannangara et al., 2014), suggesting that the NR2A subunit is

required for NMDA receptor-dependent LTP in the DG. Following an mTBI at PND 25-28, when NR2A:NR2B ratios are approximately equivalent, we found that LTP deficits persisted for longer in the DG than CA1 in juvenile females. This data suggests that impaired glutamatergic neurotransmission through downregulation of NR2A-containing NMDARs may underlie deficits in synaptic plasticity following mTBI.

Downregulation of NMDA receptors is linked to a decline in glutamatergic neurotransmission: NMDA and AMPA currents appear decreased at 7 days following injury (Schwarzbach et al., 2006). However, in our study we found no significant alterations in evoked synaptic transmission following mTBI as assessed with I/O functions (**Figure 10**). Under standard recording conditions in nACSF, CA1 pyramidal cell and DG granule cell EPSPs are composed of primarily AMPA and NMDA receptor-mediated components. Electrophysiological studies have shown that selective antagonism of NMDARs with 2*R*-amino-5-phosphovaleric acid (AP5; 50 μM) blocked the late portion of the EPSP, whereas bath application of the AMPAR-specific antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) selectively blocked the early portion of the EPSP, demonstrating that AMPAR-mediated currents dominate the initial slope of the EPSP (Dozmorov et al., 2006; Xiao et al., 1995). Therefore a lack of change in I/O curves suggests that mTBI did not alter AMPAR-mediated currents, but without isolating the NMDAR-mediated component of the EPSP we cannot speculate on whether mTBI causes NMDAR-mediated changes in glutamatergic transmission based on our I/O results.

Many biochemical pathways involved in the maintenance of LTP downstream of the NMDA receptor are also altered by TBI. For example, ERK1/2 is required for LTP maintenance and long-term memory formation by stimulating gene transcription via the downstream effector molecule CREB (Waltereit & Weller, 2003). Inhibition of ERK1/2 activation impairs the maintenance phase of LTP, similar to the LTP deficits observed following TBI (Atkins et al, 1998, 2011; English & Sweatt, 1997). Activation of the transcription factor CREB is also regulated by the upstream cAMP-PKA pathway. Cyclic AMP and protein kinase A (PKA) levels in the hippocampus are significantly decreased in young adult animals from 4-24 hours following FP injury, and return to basal levels after 3 days (Atkins et al., 2007). However, when hippocampal slices were stimulated

with glutamate or KCl depolarization, it was found that activation of ERK1/2 and CREB were significantly impaired up to 12 weeks post-injury compared to sham-stimulated slices, although basal levels of both proteins were not altered following TBI (Atkins et al., 2009). Additionally, recent evidence links hippocampal ER activation to increased activation of the ERK-CREB signalling pathway (Boulware et al., 2013), suggesting decreases in endogenous estrogen may downregulate ERK-CREB signalling. Together, these results suggest that sustaining a TBI results in chronic deficits in the ERK-CREB signalling pathway in the hippocampus, leading to long-term deficits in learning and memory that may be exacerbated in females.

4.6 Future Directions

Impaired hippocampal synaptic plasticity is associated with cognitive deficits in learning and memory capabilities, which are common symptoms after mTBI (Baddeley et al., 1987; Dikmen et al., 1987; Tabaddor et al., 1984; Ylvisaker & Szekeres, 2002). Future studies using the MWM behavioural task, which measures hippocampal-dependant spatial memory ability (Morris, 1981a; Redish & Touretzky, 1998; Vorhees & Williams, 2006), would be able to discern if the deficits in LTP observed in mTBI animals translate to impaired performance in the MWM, and impaired learning and memory. To detect any behavioural abnormalities that are specific to either DG or CA dysfunction, the DG-associated Metric Change and the CA1-associated Temporal Order tests may be used to identify such deficits (Hunsaker et al., 2008; Kesner, 2007; Lee et al., 2005; Mumby et al., 2002; Poucet, 1993).

As mTBI has been shown to impair LTP, attenuated synaptic plasticity may be associated with alterations in the composition, expression, or function of NMDA receptors. Western blotting or ELISAs may be used to determine the levels of the various NMDA receptor subunits (NR1, NR2A, and NR2B) in the DG and CA. Synaptosomal fractions should be used to determine whether numbers of NMDARs are altered at the synapse. Molecular analysis of NMDAR expression could be used in conjunction with whole-cell electrophysiology techniques to examine how mTBI may impact NMDAR currents in the hippocampus. Selective antagonism of NMDARs with AP5 could be used

to determine whether LTP was NMDA receptor-dependent at the various time points examined following mTBI, and additionally whether mTBI changes the amount of NMDA receptor-dependent LTP evoked. These studies could provide a means to evaluate the effects of mTBI on NMDAR concentration and subtypes present in the hippocampus, and whether changes in NMDAR currents mediates the observed deficits in LTP.

Sustaining a TBI has been linked to alterations in the expression and activation of the ERK-CREB signalling pathway in the hippocampus (Atkins et al., 2009; Hu et al., 2004). This pathway is crucial to the formation of long-term memories through the maintenance of LTP (Waltereit & Weller, 2003). Western blotting may be used to determine the levels of ERK and CREB proteins, and their phosphorylated counterparts, in the DG and CA. These studies could provide a means to evaluate the effects of mTBI on intracellular signalling protein expression and activation in the hippocampus, which may be linked to synaptic plasticity, and learning and memory.

4.7 Summary and Conclusions

A history of mTBI is associated with lasting impairments in cognition and behaviour, including increased learning and memory deficits. These learning and memory processes are believed to be reliant on hippocampal synaptic plasticity. Clinical studies have found reduced hippocampal volumes following TBI. Experimental rodent models of open-head TBI have found, in addition to hippocampal atrophy, that LTP is impaired in the hippocampal CA1 subregion following mild to severe TBI in males. Previously, very little was known about how a mild closed-head TBI impacts synaptic plasticity in juvenile animals, with regards to both sexes. Our research has now shown that an mTBI produces more immediate and prolonged LTP deficits in the juvenile female brain than male brain, and that the DG is more vulnerable to injury than the CA1. Further research is required to tease apart the exact cause of these LTP deficits and sex differences, but we can conclude that the weight drop model of mild closed-head injury is informative as it shows how an mTBI can impact synaptic plasticity at a mechanistic level.

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