Shaking Up the Synapses: How Repeated Mild Traumatic Brain Injury Disrupts the fine balance of Synaptic Plasticity in the Juvenile Dentate Gyrus

by

Allyson An Honger (宏儿) Gross
B.Sc., University of Victoria, 2020

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We acknowledge and respect the lək̓ʷəŋən peoples on whose traditional territory the university stands and the Songhees, Esquimalt and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day.

Supervisory Committee

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Abstract

In Canada, the occurrence of traumatic brain injury is on the rise, with over 165,000 people affected every year. This condition can arise from a variety of causes, including motor vehicle collisions, falls, sports-related incidents, or assaults. Adolescents are particularly susceptible to multiple head injuries due to increased participation in sports and high-risk activities. Repeated mild traumatic injury (r-mTBI) can exacerbate symptom severity and impede neuropsychological recovery, with cognitive and psychiatric changes such as memory impairment that may persist for months or even years. It is thought that the hippocampus, which is vulnerable to injury may be responsible for impairments in memory after r-mTBI. To investigate the influence of memory impairment following r-mTBI, we employed the awake closed head injury (ACHI) model, which entailed delivering eight impacts throughout the day to male juvenile rats (PND 25 – 29). Hippocampal slices were prepared one or seven days after the last injury for in vitro electrophysiological recordings, and we examined the potential for long-term potentiation (LTP) and two distinct long-term depression pathways in the medial perforant path (MPP) of the dentate gyrus (DG). These findings demonstrated that r-mTBI did not disrupt 1 Hz - LTD but did result in significant impairment in long-term potentiation (LTP) and 10 Hz - long-term depression (LTD) in the juvenile male dentate gyrus (DG). These data are the first to describe the adverse impact of r-mTBI on e-CB-dependent LTD in the male DG, which could help link a novel pathway impairment to the memory deficits observed in people who have suffered concussions.

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2- AG	2-arachidonoyl-glycerol	LTD	Long term depression
AC	Adenylyl cyclase	LTP	Long-Term Potentiation
ACHI	Awake closed head injury	mACHR	muscarinic acetylcholine receptors
aCSF	Artificial cerebral spinal fluid	MAGL	monoacylglycerol lipase
AEA	Anandamide	MF	Mossy fibres
AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazone-3-carboxamide	Mg	Magnesium
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid	mGLUR	Metabotropic glutamate receptor
AMPAR	a-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid receptor	ML	Molecular layer
APV	D (-) – aminophosphonovalerate	MML	Middle molecular layer
BBB	Blood brain barrier	MPEP	2-Methyl-6-(phenylethynyl) pyridine
CA	cornu ammonis	MPP	Medial perforant path
Ca	Calcium	MR	mineralocorticoid receptors
CaMKII	calcium-calmodulin dependent protein kinase II	mTBI	mild traumatic brain injury
CB1	cannabinoid type 1	Na	Sodium
CB2	cannabinoid type 2	NAP	Neurological assessment protocol
CCI	Cortical controlled impact	NMDA	N – methyl – D – aspartate
CHI	Closed head injury	NMDAR	N – methyl – D – aspartate receptor
CNS	Central nervous system	NT	Neurotransmitter
COX - 2	Prostaglandin-endoperoxide synthase 2	OML	Outer molecular layer
CTE	Chronic traumatic encephalopathy	PCS	Post-concussion syndrome
DAG	diacylglycerol	PI	Phosphatidylinositol
DG	dentate gyrus	PID	Post injury day
DGL	diacylglycerol lipase	PIP2	phosphatidylinositol 4,5-bisphosphate
DL - APV	DL-2-Amino-5-phosphonopentanoid acid	PKA	Protein kinase A
DSM - IV	Diagnostic and Statistical Manual of Mental Disorders	PKC	Protein kinase C
EC	Entorhinal cortex	PLC	Phosphoinositide-specific phospholipase C
ECB	Endocannabinoid	PML	Polymorphic layer
ECS	Endocannabinoid system	PND	Post-natal day
EPSP	excitatory post synaptic potential	PP	Paired pulse

FPI	Fluid percussion injury	PP1	Protein phosphatase 1
GABA	γ-Aminobutyric acid	PP2B	Calcineurin
GCL	Granule cell layer	PPD	Paired pulse depression
GCS	Glasgow Coma Scale	PPF	Paired pulse facilitation
GPCRs	G-protein coupled receptors	PPR	Paired pulse ratio
GR	glucocorticoid receptors	PTP	Post tetanic potentiation
HFS	High frequency stimulus	PTX	Picrotoxin
I/O	Input-output	ROS	Reactive oxygen species
ICD	International classification of diseases	SCAT5	Sideline concussion assessment tool 5
IML	Inner molecular layer	SEM	Standard error of the mean
IP3	Inositol triphosphate	SGZ	Subgranular zone
IPV	Intimate partner violence	SIS	Second impact syndrome
K	Potassium	STD	Short term depression
LE	Long-Evans	TBI	Traumatic brain injury
LFS	Low-frequency stimulus	VGCC	Voltage-gated Ca^{+2} channels
LOC	Loss of consciousness	Δ9-THC	Delta – 9 – tetrahydrocannabinol
LPP	Lateral perforant path		

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All figures are made with BioRender.com.

Dedication

I dedicate this thesis to my parents, Heather and Steve Gross as well as my loving partner Owen Hübner for their endless support.

1. Chapter 1 - Introduction

1.1. Traumatic Brain Injury

1.1.1. Epidemiology

Traumatic brain injury (TBI) has become a leading cause of death and disability in Canada (J. Z. Song et al., 2023). Globally over 50 million people a year are afflicted and these injuries incur costs upward of 540 billion Canadian dollars annually (Bernard et al., 2020). In Canada, 165,000 people are inflicted with TBI per year which is equivalent to 466 injuries occurring daily (*Statistics on Brain Injury | Brain Injury Canada*, 2023). TBI can be classified as mild, moderate or severe and out of this spectrum 70-90% are mild; this incidence is likely to be underestimated due to underreporting of injury in the clinical setting (Cassidy et al., 2004; Fehily & Fitzgerald, 2017). Severe TBIs incur 90% of hospital costs, however, it is important to note that mild traumatic brain injuries (mTBI) are also correlated with significant long-term costs due to disability, loss of work or neuropsychiatric complications (Nguyen et al., 2016). The leading causes of TBI consist of road accidents, falls, war-related injuries, violence and sports injuries (Hyder et al., 2007). Intimate partner violence (IPV) was previously under-represented when looking at the epidemiology of TBI, however, research suggests that the prevalence of TBI amongst cases of IPV is estimated to be 11 – 12 times greater than the published incidence of TBI from the previously listed primary causes of TBI (Lifshitz et al., 2019). While the majority of survivors of IPV are women, both cisgender and transgender (Toccalino et al., 2022), men are also at risk. TBI is primarily secondary to domestic violence and is reported mainly in women, however, men can also be victims of IPV and studies have shown 14% of men in their lifetime experience IPV (Costello & Greenwald, 2022). Studies have recounted that men with disabilities are at a higher risk of IPV compared to women with disabilities and able-bodied men and women (Ballan et al., 2017).

1.1.2. Traumatic Brain Injury

TBI can be considered an umbrella term, as what is included after an injury is thought to be implicit rather than explicit, however, a clearly accepted definition for TBI is that it is the result of an injury that alters brain function or changes in brain pathology caused by an external force (Menon et al., 2010). Symptoms of TBI include behavioural abnormalities, headaches, neurological deficits and much more (Galgano et al., 2017; Ng & Lee, 2019). Injuries to the brain can occur from a closed or open-head injury. Open head injuries involve a foreign body that penetrates the skull and travels through the dura and into the brain parenchyma; this type of injury is focal. Closed head injuries are more diffuse, are more common and are typically induced through a blunt impact that occurs from falls, sports activities and vehicle accidents (Ng & Lee, 2019). Despite the lack of fracturing of the skull, closed-head injuries can cause the brain to be severely injured. Coup-contrecoup injuries (coup referring to the injury at the point of impact and countercoup injury occurs on the opposing side of impact) can cause brain trauma and can be seen in instances where the head is jerked violently, such as boxers being punched or collisions occurring during the chase for a ball in soccer (Toma & Nguyen, 2019). TBI severity is determined using the Glasgow Coma Scale (GCS) and is the most widely used assessment for assessing the severity of brain injury (Matis & Birbilis, 2014). The GCS assesses eye-opening and verbal and motor responses and the score value is between 3 (lowest) and 15 (highest) (Jain & Iverson, 2022). Severity can be indicated by the given score where: 13-15 is minor, 9-12 is moderate, 5-8 is severe and 3-4 is very severe (Matis & Birbilis, 2014). Diagnosis can also be confirmed by imaging (i.e. CT), which is able to observe lesions, contusions and diffuse axonal injury (Parikh et al., 2007).

1.1.3. Mild Traumatic Brain Injury

Mild traumatic brain injuries (mTBI) are often characterized by a disturbance in brain function, which is often accompanied by neurological symptoms such as headache, confusion and dizziness (Fehily & Fitzgerald, 2017). Many individuals who acquire an mTBI recover in a few weeks or months, but about 10-40% develop post-concussive syndrome (Mittenberg et al.,

2001; Prince & Bruhns, 2017). While mTBIs can cause impairment after injury, there are often no signs of structural injury and thus, no abnormalities are seen in standard structural neuroimaging techniques (McCrory et al., 2001). This renders clinicians to rely upon examination of signs and symptoms and patients' self-reporting, without the use of diagnostic tests or biological markers (Anzalone et al., 2023). Self-reporting as the main method of diagnosis is tough as most difficulties and symptoms reported after mTBI are not specific to brain injury, but can occur in the general population (Carroll et al., 2004). The Sideline Concussion Assessment Tool 5 (SCAT5) is a standardized tool that prompts medical professionals to review red flags, memory, GCS…etc. in response to a suspected concussion in athletes immediately after their injury, however, this tool is only appropriate in the sports setting and there is no comprehensive or validated tool that can be used in a clinical office (Katz et al., 2020).

1.1.4. Repeated Mild Traumatic Brain Injury

While mTBI used to be considered a benign phenomenon, it is has now become recognized as an injury with a diverse spectrum and a major cause of morbidity (Howlett et al., 2022). The concern regarding repeated mild traumatic brain injury (r-mTBI) has also grown considerably, especially among athletes and military veterans (Omalu, Bailes, et al., 2011; Omalu, Hammers, et al., 2011). While r-mTBIs are considered “mild”, this type of injury has been associated with greater severity of symptoms and difficulties in regard to the neuropsychological recovery (Luo et al., 2014). Increased severity of cognitive and psychiatric alterations, such as impaired memory and mood disorders are observed and the increased risk of neurodegenerative diseases such as chronic traumatic encephalopathy (CTE) has been shown through epidemiological studies (Aungst et al., 2014; Mouzon et al., 2014). As mentioned previously, the majority of TBIs are considered mild and the peak incidence occurs amongst young adults and adolescents (age 15-24); this is due to the fact this age group is most likely to participate in highly physical or sports-related activities and these activities confer 50% of all r-mTBIs (Ferguson et al., 2020). Despite the literature available, there is a gap in knowledge in terms of what high school athletes know and what they should know about concussions related to

sports (Wallace et al., 2017). While increased knowledge of concussion symptoms and concussion itself increases the likelihood of reporting a suspected concussion, changes in behaviours do not (Register-Mihalik et al., 2013). Common factors contributing to the absence of behavioural change include a lack of recognition of the severity of the injury, a desire to continue playing, and insufficient awareness of the possibility of a concussion (McCrea et al., 2004). It is pertinent that we keep pushing for concussion education as r-mTBIs can be deadly.

1.1.4.1. Rowan's Law

A paper written by Tator et al., 2019 recounts the case of Rowan Stringer a teenage rugby player who had lost her life after being tackled and landing on her head. Experts were puzzled, as there was no striking explanation for Rowan's death; she received immediate and quick expert care and the left subdural hematoma Rowan had could not explain the massive amount of brain swelling she experienced. A piece of evidence in the form of text messages led experts to believe that Rowan died from second impact syndrome (SIS). Texts indicated that Rowan complained of headaches, fatigue, and tinnitus after head injuries she received 5 and 2 days before the game that ultimately took her life. She had also googled concussion and confided to her friends that she "probably had a concussion" (Tator et al., 2019). Mccradden et al., explain that Rowan's law was instated on March 6th, 2018, and mandates that an athlete who is suspected of having a concussion be removed from the game and for concussion education be given to people involved in youth sports. However, despite the legislation being in place, athletes are finding loopholes. Terms like "concussion-like symptoms" and "upper-body injury" are used as euphemisms to evade the diagnosis of concussion. It is important that everyone involved in sports makes themselves responsible to prioritize brain health and to protect those vulnerable to concussion and in doing so we can hopefully create a future that can protect all athletes (Mccradden & Cusimano, 1997).

1.1.4.1.1. Long-term consequences of Repeated Mild Traumatic Brain Injury

As mentioned previously, while many with an mTBI recover from their symptoms in a few weeks or months, 10-40% develop post-concussion syndrome (PCS) (Mittenberg et al., 2001; Prince & Bruhns, 2017). Permenter et al., define PCS as a term that describes the constellation of symptoms that occur following an mTBI. These symptoms are not only limited to physical but can be cognitive, behavioural, or emotional. Symptoms that persist for longer than 3 months are related to PCS and these symptoms can have long-lasting effects on cognition, memory, learning and executive function (Permenter et al., 2022). Individuals who experience post-concussion symptoms have been reported to have a lower level of satisfaction with life, lower health-related quality of life and a slower recovery overall (Polinder et al., 2018). One in every 200 Canadians reported that a brain injury was the most disabling injury in 2014 (Bonn et al., 2022). PCS is diagnosed according to the International Classification of Diseases (ICD)-10, or by following the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV (Andelic et al., 2018). The problem, however, is symptoms related to PCS are not just subject to mTBI, but to many other injuries or health concerns; this is why knowing the impact on health outcomes after injury could help to optimize recovery time and raise awareness so that we can reduce the occurrence of these symptoms (Van Der Vlegel et al., 2021).

R-mTBI has also been shown to be a risk factor for a neurodegenerative disease known as CTE. CTE is a degenerative condition which is caused by an accumulation of abnormal tau protein, characterized by a unique clinical and pathological profile. The symptoms associated with this disease do not often appear until many years after an individual has sustained repeated concussions or sub-concussive head trauma. The tell-tale signs of CTE include widespread deposits of tau protein in the form of neurofibrillary tangles and astrocytic tangles, located primarily in the frontal and temporal cortices and distributed unevenly on the surface. Symptoms of CTE appear mid-life and include disordered cognition, mood disorders and Parkinson-like signs. CTE does not affect only one type of person with injury, such as an athlete, but can appear in many types of individuals such as those who experience a fall, accident, assault…etc. To date, CTE has only been identified through neuropathological analysis in

individuals with a history of repeated closed-head injuries (Gavett et al., 2010; McKee et al., 2009).

1.1.5. TBI pathophysiology

1.1.5.1. Primary Injury

The initial blast, penetration or blunt impact to the brain is considered the primary injury. The cause of primary injury varies greatly, and it is what is responsible for direct damage to neuronal tissues (Nolan, 2005). The damage caused by direct impacts causes the neurons and glial cells to be damaged which leads to axonal dysfunction. Laceration is considered the most severe type of primary brain injury and it can lead to the formation of intracranial hemorrhages and hematomas which can compress the brain resulting in severe neurological problems. Damage caused by primary brain injuries is beyond the control of clinicians and so the focus of clinical care should be on prevention measures, identifying and treating secondary brain injuries (Sande & West, 2010).

1.1.5.2. Secondary Injury

Not all neurological damage occurs immediately at the time of impact (primary injury), but rather develops later and this is called a secondary injury. Most TBI deaths in hospitals are caused by secondary brain injuries (Ghajar, 2000). The cause of secondary injury is due to brain swelling, with an increase in intracranial pressure and a restriction in cerebral perfusion which often leads to ischemia (Kinoshita, 2016). Intracranial injury can also cause an increase in the activity of excitatory neurotransmitters, the formation of reactive oxygen species (ROS), and the production of proinflammatory cytokines. These biochemical reactions due to injury can lead to neuronal cell damage, disruption in the blood-brain barrier (BBB), changes in cerebrovascular reactivity and potentially even cell death (Sande & West, 2010).

The reason behind neuronal cell damage and death can be linked to excitotoxic mechanisms that are a central consequence of secondary injuries (Donat et al., 2010). After TBI, excitotoxicity in the brain is initiated by the release of excitatory amino acids, mainly glutamate. What is accompanied by excitotoxicity is the persistent depolarization of neuronal cells and the imbalance in the flux of ions across the neuronal cell membranes (Faden et al., 1989). Injured cells try to regulate the abnormal ionic gradient by increasing the production of ATP to fuel the ATPases that are responsible for restoring ionic homeostasis. This increase in ATP in the cell is however disabled due to a lack of cerebral blood flow, the reduction in oxygenation in the tissues and the compromise of mitochondrial function following the TBI (Park et al., 2008). Due to the reasons described, the excitotoxic-induced ionic imbalance persists in the injured neural tissue (Mustafa & Al-Shboul, 2013).

Specific ions that have devastating events on neuronal cells due to excitotoxicity include Ca^{+2} and Na^{+}. Loss of Ca^{+2} homeostasis due to a massive influx is the central mechanism by which downstream pathological events due to secondary injury of TBI occur such as oxidative stress, mitochondrial dysfunction, and cytoskeletal degradation (Greve & Zink, 2009). On top of this, Na^{+} influx contributes to damage through the mediation of passive diffusion of water into neuronal cells that can cause cytotoxic edema (Choi, 1987). For a fantastic in-depth review of secondary injury cascades see (Mustafa & Al-Shboul, 2013).

1.1.6. Semantics: Mild Traumatic Brain Injury vs. Concussion

The terms mTBI and concussion are often used interchangeably in both research and clinical settings, despite having different definitions in the literature (Sussman et al., 2018). According to the American Congress of Rehabilitation Medicine in 1993, the most widely accepted definition of mTBI states that following injury, there is a period of loss of consciousness, loss of memory prior to or post-injury, alteration of mental state and a focal neurological deficit that could be transient or not ("Definition of Mild Traumatic Brain Injury," 1993). However, even with this definition, the identification of mTBI is difficult as there is wide variability in the criteria used for diagnosis and there is a lack of identifying spontaneous and

rapidly resolving symptoms (Pozzato et al., 2020). The term concussion also has some controversy. At the first international symposium on concussion in sports in Vienna, Austria, a group of experts defined concussion as a complex pathophysiological process that is induced by biochemical forces that results in neurological dysfunction and may or may not include loss of consciousness (Aubry et al., 2002). Carney et al., who wrote a systematic review in 2014, attempted to collate data to more precisely define concussion, as there was "currently no definition for concussion that [was] being uniformly applied in clinical and research settings" (Carney et al., 2014). With no clear pathological definition to distinguish concussion from other types of TBI, and due to the high overlap of symptoms and injury, there is no distinguishable reason to believe that concussion and mTBI could be distinguished pathologically (Sharp & Jenkins, 2015). For this study, reference to animal research will use the term mTBI and when referring to clinical settings, the term concussion will be used.

1.2. Pre-Clinical TBI models

1.2.1. Animals used to model TBI

There are many ethical implications and logistical issues when it comes to human research studies concerning TBI and so animal models are a necessity in order to study the changes occurring after brain injury (Petersen et al., 2021). In the 1980s several animal models were developed which mainly included non–human primates, cats, dogs, and pigs (Ackermans et al., 2021; Petersen et al., 2021). Large animal models can be more relevant in research that is translational because of their size, white-to-gray matter ratio and gyrencephalic brain (Vink, 2018). Pigs are popular as models for TBI not only because of their similarities with human anatomy in terms of brain size but also for their brain's composition, organization, vascular, development and inflammatory response to injury (Kinder et al., 2019). Pigs' hippocampi also lie deep within the temporal lobe and ventral in the brain, which is like humans, whereas rodent hippocampi are superior in the brain and more susceptible to injury due to their small size (Holm & West, 1994). Despite the fact that small animals have limitations (i.e., secondary axonal injury occurs more rapidly in rodents than in humans) rats and mice are the most used

animal model for TBI (Ackermans et al., 2021; Finnie, 2001). Rodents are the most ideal for modelling TBI because of their quick breeding time, ease to lodge, modest cost, small size, and ability to manipulate their genome (Ackermans et al., 2021; Petersen et al., 2021; Xiong et al., 2013b).

1.2.2. TBI Models

Experimental models for TBI in animals vary widely and each model has been designed to emulate and stimulate a certain type of TBI (Sempere et al., 2019). While early models of TBI focused on the biomechanical aspects of TBI, present models have been able to enhance our understanding of the complex and harmful molecular cascades that occur after this type of injury (Xiong et al., 2013b). Some of the injury models for TBI include the weight drop model (Feeney et al., 1981), controlled cortical impact (CCI) model (Edward Dixon et al., 1991), fluid percussion injury (FPI) model (Edward Dixon et al., 1991; McIntosh et al., 1989), the blast injury model (Aravind et al., 2020) and the awake closed head injury (ACHI) model (Christie et al., 2023; Meconi et al., 2018), which is the model used in this thesis (see (Ma et al., 2019; Xiong et al., 2013b) for in-depth reviews on the different models of TBI). The ACHI model is unique compared to other models, as there is no craniotomy surgery or skull exposure performed and the impacts are given to animals conscious and without the need for anesthesia, which may interfere with histopathological and functional outcomes (i.e., neuroprotection) (Gotts et al., 2000; Rowe et al., 2013). These factors may make the ACHI model more representative of clinical r-mTBI as compared to other experimental models.

1.3. Hippocampus

1.3.1. The hippocampus

In humans, the hippocampus is an elongated structure that is bilateral in nature and found deep within the medial temporal lobe (Knierim, 2015) and is made up of the Cornu ammonis (CA) 1, CA2, CA3 and CA4 regions and the dentate gyrus (Anand & Dhikav, 2012).

Italian anatomist and surgeon, Gulio Cesare Aranzio, is undisputedly credited with being the first to describe the hippocampus, naming it for its shape resembling a seahorse (Engelhardt, 2016). Since then, the hippocampus has been thoroughly investigated. The famous H.M case study in 1957 was the first to show how surgical bilateral medial temporal-lobe resection, in an effort to cure severe epilepsy, caused severe memory deficits to occur (Scoviille & Milner, 1957). It was this research that put the hippocampus to the forefront of neuroscience and allowed for the discovery of the hippocampus' role in long-term potentiation (LTP) which is now considered to be the leading model for understanding the cellular basis of memory (Knierim, 2015). The hippocampus is a unique region of the brain in that neurogenesis is ongoing (Anand & Dhikav, 2012). Neurogenesis is a vital process that contributes to the brain's adaptability, stability, and preservation of cognitive abilities. It also aids in repairing damaged brain cells caused by aging and neurological disorders (Poulose et al., 2017). For a detailed review of the hippocampus, see (Per Anderson et al., 2007)

1.3.2. Hippocampal circuitry

The hippocampus' circuitry is unique as it is mainly unidirectional, and the main circuit is called the trisynaptic loop (**Figure 1**). The main output pathway of the entorhinal cortex (EC) is the perforant path. Pyramidal cells in EC layer II send their axons through the subiculum to primarily connect with the granular layer in the dentate gyrus (DG). Some axons can project to the CA3 and few that project to CA1. Granule cell axons called mossy fibres in the DG pass information to the dendrites of CA3 pyramidal cells and this is considered the second synaptic connection. Following this connection, CA3 axons called Schaffer collaterals exit the deep part of the cell body and loop up to apical dendrites and extend to CA1. Axons from the CA1 region then project back to the EC which is the completion of the circuit. While the trisynaptic loop is the main flow of information in the hippocampus, several other connections play important roles in hippocampal functioning, however, for the purpose of this thesis we will be focusing on the DG. For a more in-depth review of hippocampal circuitry, see (David G. Amaral, 1993; Basu & Siegelbaum, 2015).

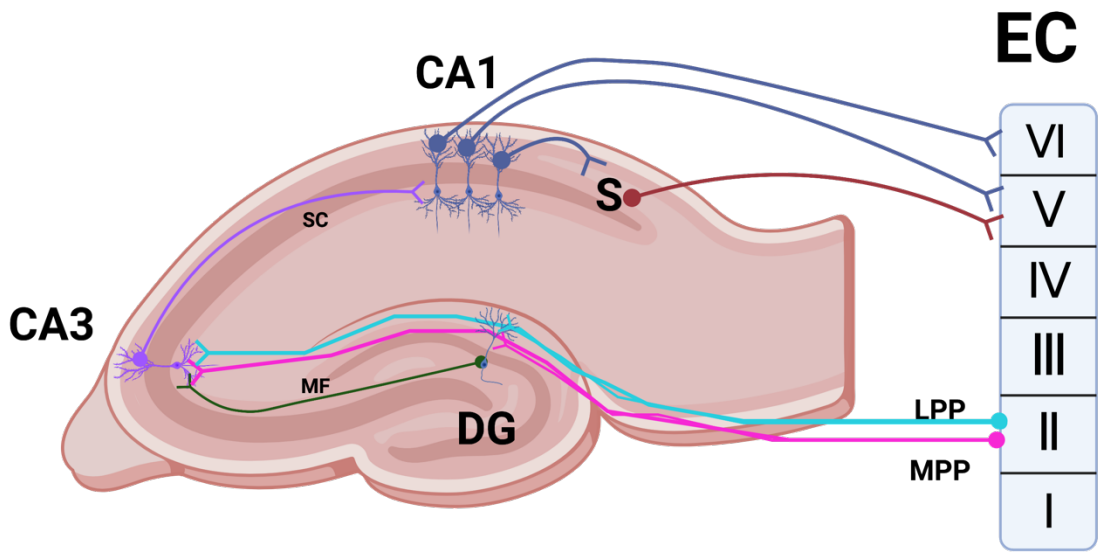


Figure 1. Trisynaptic loop in the hippocampus

Fibres that output from layer II of the entorhinal cortex (EC) make up what is known as the lateral perforant path (LPP - shown in teal) and the medial perforant path (MPP - shown in pink) and they provide input to the dentate gyrus (DG) and straight to the CA3 region of the hippocampus. Granule cell axons called mossy fibres (MF), shown as dark green, in the dentate gyrus pass information from the DG to the cornu ammonis (CA) 3 pyramidal cells. CA3 axons called Schaffer collaterals (purple) can loop upward and project to CA1 pyramidal cells, which then in turn project to the subiculum then back to the EC or can project directly back to the EC (blue). To note, the medial entorhinal input can travel directly to CA3 (Amaral and Witter, 1983, Liu et al., 2020). Both the LPP and the MPP can also project directly to the stratum moleculare lacunosum layer of the CA1 (Ito and Schuman, 2013, Marsukar 2017).

1.3.3. Dentate Gyrus

The DG is an important cortical region that makes up a portion of the larger brain structure called the hippocampus (or hippocampal formation). This portion of the hippocampus is a model system for study due to the regular organization of its principal cell layers that have a highly ordered laminar distribution (D. G. Amaral & Witter, 1989). The EC is a source of cortical sensory information, and it provides a major input to the dentate gyrus which is why it is believed that the DG is the primary step in the processing of information that leads to the production of episodic memories. The DG consists of three layers: the molecular layer (ML), the granule cell layer (GCL) and the polymorphic layer (PML, found at the hilus of the DG) (David G. Amaral et al., 2007). For a more detailed review of the DG, see (David G. Amaral et al., 2007; Kesner, 2013).

The ML of the DG is divided into three sections, namely the outer molecular layer (OML), middle molecular layer (MML) and inner molecular layer (IML) (**Figure 2**) (Witter, 2007). While this layer contains dendrites of the dentate granule cells, it itself is devoid of cells. The perforant path fibres, the major input from the cortex that originates in the EC, are also found in the ML (David G. Amaral et al., 2007). The lateral perforant path (LPP) is formed by fibres from the lateral part of the EC that run through the OML, while the medial perforant path (MPP) consists of fibres from the medial EC that pass through the MML (Hjorth‐Simonsen & Jeune, 1972). These outer two layers (OML and MML) are able to receive both excitatory glutamatergic input from the ipsilateral EC (Scheff & Price, 1998). The inner portion of the molecular layer is a poorly understood region, it is known that the IML can receive projections from the PML (David G. Amaral et al., 2007) and contains what Ramón y Cajal described as spiny interneurons and densely spiny granule–like cells (Ramón y Cajal, 1995). Since then, still little is known about these cells, however, they have been found through patch clamp recording experiments that IML spiny neurons are unique from granule cells, and they exhibit greater activation upon stimulation of the hilus region (Williams et al., 2007).

The main cell layer of the DG is the GCL which is packed full of densely packed granule cells, which are glutamatergic in nature. While granule cells are the primary type of cell present in this layer, there are some other neurons found at the boundary of the granule and polymorphic layers that include cell bodies of dentate pyramidal basket cells (inhibitory interneurons). Granule cell dendrites project into the ML and can receive glutamatergic inputs from the MPP and LPP (David G. Amaral et al., 2007). Granule cells give rise to mossy fibres, which are able to terminate onto many cell types in the polymorphic layer of the DG and in another region of the hippocampus called the CA3 (Ribak et al., 1985). These fibres are known to innervate a greater number of inhibitory cells than excitatory cells (Acsády et al., 1998).

The PML contains mainly mossy cells, and these cells were named so because, after Golgi staining, these cells looked ‘mossy’ in appearance with clusters of complex spines. Mossy cells are a type of glutamatergic principal cells, and while their dendrites usually remain in this

layer, there are occurrences where the dendrites can penetrate the GCL and enter the ML. Exclusively, the IML is the region in the DG that receives projections that originate from the PML. Mossy cells are able to excite granule cells and also indirectly inhibit them by activating GABAergic interneurons (David G. Amaral et al., 2007; Scharfman, 2016). It should also be of note that there is a region located between the GCL and the PML which is called the subgranular zone (SGZ) which is the region in the DG where neurogenesis occurs (Zaverucha-do-Valle et al., 2013).

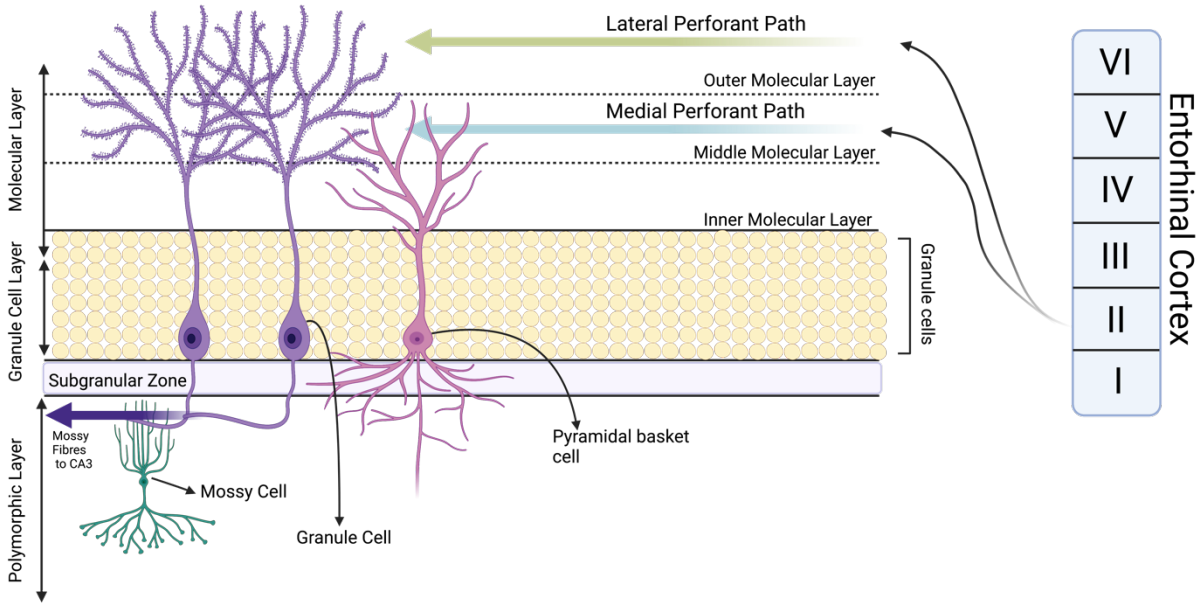


Figure 2. Cellular makeup and connections in the dentate gyrus

Layer II of the entorhinal cortex projects fibres to the outer molecular layer and the middle molecular layer which make up the lateral and medial perforant path (LPP and MPP) respectively (light green and blue arrows). Granule cells are densely packed in the granule cell layer (light yellow). Larger representative granule cells (purple) in the dentate gyrus can project up into the molecular layer and can receive inputs from both the LPP and MPP. These two paths can also provide input to inhibitory pyramidal basket cells. Mossy cells (green)in the polymorphic layer can excite granule cells.

1.4. Hippocampal Synaptic Plasticity

1.4.1. Synaptic plasticity and its implications in learning and memory

The delicate equilibrium between our capacity to remember and our capacity to forget exemplifies a characteristic of what makes us human. Before we were able to understand the complexities of the brain, there were many theories and descriptions of what the brain could

do. The Greek philosopher Plato, in an effort to explain cognitive variability from a structural standpoint used a wax block analogy in that, "[an] individual may be larger or smaller and composed of wax that is comparatively pure and muddy, and harder in some, softer in others and sometimes just the right consistency. A man has in his mind a good thick slab of wax smooth and kneaded to the right consistency and the impressions that come to the senses are stamped on these tables… then the imprints are clear and deep enough to last a long time. When the block is muddy or made of impure wax or over soft or hard, the people with soft wax are quick to learn but forgetful, those with hard wax the reverse" (Mann, 2016). Ramirez & Arbuckle explain that this was Plato's way of describing changes occurring in the brain and in the present, the vernacular used to describe these changes during learning and memory is not as different as they may seem (Ramirez & Arbuckle, 2016). Now it is more common for us to describe changes in the brain through the concept of plasticity which is defined by the capability of the nervous system to alter its functioning in response to internal or external stimuli through the reorganization of its structure, connections or functions (Mateos-Aparicio & Rodríguez-Moreno, 2019).

Synaptic plasticity is defined as the changes in neuronal connections and is considered the primary mechanism for learning and memory (Ramirez & Arbuckle, 2016). This phenomenon was first coined in 1894 by Ramon y Cajal who proposed that memories are created through the strengthening of existing neuronal connections, based on the belief that the number of neurons in the brain remains stable through life (Jones, 1994). Donald Hebb (1949) also had an influential voice on how learning-related changes occur in the brain whereby he postulated that if cell A "repeatedly or persistently takes part" in the firing of cell B, then the strength of their connection should increase (Magee & Grienberger, 2020). Since his time, there has been strong support for his theory and the most important discovery is that of long–term potentiation (LTP) and long-term depression (LTD) in the mammalian hippocampus (T. V.P. Bliss & Lømo, 1973; Howland & Wang, 2008; Lynch et al., 1977). The ability of neurons to undergo further synaptic remodelling is maintained by the balanced distribution of LTP and LTD and this balance is crucial for proper learning and memory (Zhong & Gerges, 2010). This being said, as a whole the

processes underlying learning and memory have not been definitely resolved (Timothy V P Bliss & Cooke, 2011).

1.4.2. Long-Term Potentiation

Bliss and Lomo are considered the founders of LTP as they were the first to demonstrate that the strength of excitatory synapses in the hippocampus of anesthetized and awake rabbits could be rapidly and significantly increased through induction of high-frequency stimulation (tetanus) (T. V.P. Bliss & Gardner‐Medwin, 1973; T. V.P. Bliss & Lømo, 1973). Since then, LTP has been extensively studied and many reviews outline the importance and mechanisms underlying this phenomenon (Blitzer, 2005; Madison et al., 1991; Nicoll, 2017).

LTP has been defined as a form of activity-dependent plasticity that results in the persistent enhancement of synaptic transmission (Timothy V P Bliss & Cooke, 2011). The most common method through which LTP is studied is through recording the size of the excitatory postsynaptic potential (EPSP) in the hippocampus (Madison et al., 1991). While LTP occurs in other regions of the brain such as the amygdala, cortex, and cerebellum, the hippocampus is usually chosen as a site of study since the hippocampus is a highly laminated structure and allows for easy placement of stimulating and recording electrodes (Andersen et al., 1971). The tetanic stimulation used to induce LTP must meet two requirements: 1. the stimulus must reach a threshold, known as cooperativity, that reflects the need for a minimum number of presynaptic fibres to be simultaneously stimulated to induce LTP (McNaughton et al., 1978) and 2. the stimulus must be at a high-frequency (Dunwiddie & Lynch, 1978). The relationship between stimulus strength and frequency is such that increasing one is able to decrease the requirement for the other, for instance, a weak stimulus delivered at a low frequency will not result in LTP, but by increasing the frequency, the same weak stimulus can become effective in inducing LTP (T. V.P. Bliss & Lømo, 1973).

The most widely observed form of LTP in the hippocampus relies on the activity of α – amino – 3 – hydroxy – 5 – methyl – 4 – isoxazolepropoionic acid receptors (AMPARs) and

N – methyl – D – aspartate receptors (NMDARs). NMDARs are known as coincidence detectors, as it not only requires postsynaptic depolarization to function but also require glutamate release from the presynaptic side (Cooke & Bliss, 2006). When excitatory glutamatergic inputs are stimulated, such as in this study in the MPP, glutamate is released into the presynaptic cleft where it interacts with various postsynaptic receptors, including AMPARs, NMDARs, and mGluRs and this is the initiation of LTP. AMPARs are ionotropic receptors which allow for the influx of sodium (Na^+) and the efflux of potassium ions into the postsynaptic cell. The influx of Na^+ depolarizes the cell (positive charge accumulation at the postsynaptic terminal) and allows for the removal of the magnesium (Mg^{+2}) ion blocking the NMDAR pore. Alleviating this Mg^{+2} block allows the flow of calcium (Ca^{+2}) ions (in addition to Na^+ entering and K^+ exiting) in the postsynaptic cell through NMDARs. To read an in-depth review of LTP see (Isaac et al., 2007).

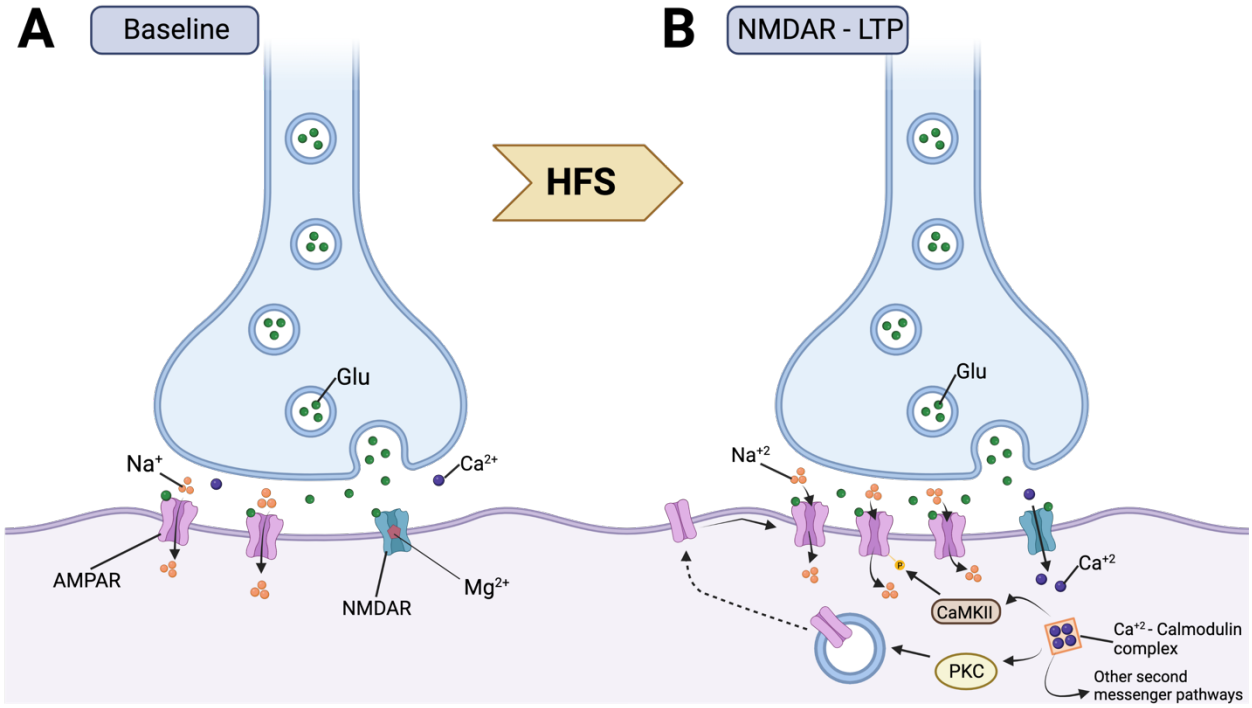


Figure 3. Simplified mechanism of NMDAR – LTP

(A) *Under baseline conditions at glutamatergic terminals, glutamate (Glu) is released from the presynaptic terminal and binds to a-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid receptor (AMPARs) and N-methyl-D-aspartate receptor (NMDARs). When Glu binds to AMPARs, they are opened and allow the flux of Na^+ into the post-synaptic cell, however, NMDARs cannot be opened with the binding of Glu alone; there must be sufficient depolarization of the cell to displace the magnesium (Mg^{2+}) block inside NMDARs to allow cations (Na^+) to flow through.* ***(B)*** *When a high-frequency stimulus is applied, Glu can bind to both AMPARs and NMDARs where the flux of sodium through AMPARs can now cause sufficient depolarization of the membrane to displace the Mg^{+2} from NMDARs and allow the passage of both Na^+ and Ca^{2+}. The increase in Ca^{2+} in the postsynaptic cell can activate calcium-calmodulin-dependent protein kinase II (CaMKII) and other secondary messenger pathways that can initiate the insertion of AMPARs into the post-synaptic membrane which ultimately aids in potentiating fEPSPs.*

The influx of Ca^{+2} is important as it is able to act as a secondary messenger which can trigger complex signalling cascades that are required for LTP and the dependency on kinases (as secondary messengers) is known as early LTP (Baltaci et al., 2018; Evans & Blackwell, 2015). Protein Kinase C (PKC) is activated by Ca^{+2} in the hippocampus and is thought to play a critical role in the maintenance of potentiation through its ability to phosphorylate GluA1 at Ser818 and subsequent incorporation of AMPARs at the activated synapse (Ren et al., 2013). For an in-depth review of AMPARs subunits see (Isaac et al., 2007). The activation of calcium-calmodulin-dependent protein kinase II (CaMKII) has also been shown to be important in both the induction and maintenance of LTP (John Lisman et al., 2014). This kinase can act as a protein switch (Madison et al., 1991) and when the calcium–calmodulin complex activates it, CaMKII is able to autophosphorylate which allows its activity to persist even after calcium falls to baseline levels (Miller & Kennedy, 1986). CaMKII can also phosphorylate AMPAR subunit GluR1 at site S831, which can increase channel conductance if stargazin (an AMPA receptor auxiliary subunit) is present and enhance the likelihood that high conductance states will be activated through intermediate glutamate concentrations. Exocytosis of AMPAR–containing vesicles into the plasma membrane of the post-synaptic cell may also be regulated through CaMKII, though this pathway remains unclear (John Lisman et al., 2014; Miller & Kennedy, 1986; Tomita et al., 2005). Following the action of kinases, late – LTP (lasting at least 24 hours) occurs which involves the activation of transcription factors, and changes in protein synthesis and its maintenance depends on CPEB3 which is a protein that promotes the translation of GRASP1, which is important for AMPAR recycling (Baltaci et al., 2018).

NMDA receptors and their role in the mechanism of LTP have also been extensively studied. As discussed earlier, NMDARs are important for the influx of Ca^{+2} into the postsynaptic cell, but the opening of this receptor requires strong depolarization to relieve the Mg^{+2} block (Isaac et al., 2007). The use of D (-) – aminophosphonovalerate (APV), a specific antagonist of NMDARs to block the induction of LTP without affecting synaptic transmission was first discovered by (Collingridge et al., 1983). Since then, others have studied this antagonist amongst others and have been able to further confirm NMDARs involvement in LTP (Harris et

al., 1986; Wigström et al., 1986). Errington et al., found through in vivo experiments that NMDA receptor antagonist APV blocks the induction of LTP in the MPP in the dentate gyrus and that APV does not affect previously established LTP. These researchers also found that APV in vitro but not in vivo was able to inhibit glutamate release at higher concentrations (50 uM & 100 uM). This finding suggests the existence of NMDARs on the presynaptic side that participates in a type of positive feedback (Errington et al., 1987).

1.4.3. Long-Term Depression

For new information to be encoded, the selective weakening of synapses is required; this is because if synapses were to continuously strengthen indefinitely as a result of LTP, they will ultimately reach a maximum level of effectiveness which would hinder the encoding of new information (Purves et al., 2001). LTD is a form of synaptic plasticity whereby synaptic transmission is weakened in an activity-dependent manner (Wiegert & Oertner, 2013). Two major categories of LTD exist, namely heterosynaptic and homosynaptic. Heterosynaptic LTD takes place at synapses that are inactive during high-frequency stimulation (HFS) of a converging synaptic input whereas homosynaptic LTD, the type of LTD we are eliciting, can occur in synapses subjected to a low-frequency stimulus (LFS). Homosynaptic LTD shares similar induction criteria to that of LTP: postsynaptic depolarization and an increase in postsynaptic Ca^{+2} concentration, however, the quantitative difference in induction between these two is that LTP requires a much stronger postsynaptic depolarization than LTD (Bear & Abraham, 1996). LTD has received less attention compared to LTP in research, however, several mechanisms have been identified.

LTD is typically induced through repetitive low-frequency stimulation (900 stimuli at 1 Hz) which can partially unblock the Mg^{+2} block in NMDARs (Dudek & Bear, 1992; Selig et al., 1995). Like LTP, NMDAR-dependent LTD (**Figure 4**) requires an increase in postsynaptic Ca^{+2} to be induced, however in a much more modest amount (Cummings et al., 1996). The induction of LTD triggers a Ca^{+2}-dependent protein phosphatase cascade where calmodulin activates the protein phosphatase 2B (PP2B), otherwise known as calcineurin, which then can activate

protein phosphatase 1 (PP1). A protein called inhibitor – 1 blocks PP1 until it is dephosphorylated by calcineurin and LTD cannot occur until this has occurred (J. Lisman, 1989). The current prevailing theory is that the mechanism behind NMDAR-dependent LTD is due to AMPARs undergoing activity-dependent endocytosis at synapses, however, an exact mechanism has not been elucidated. Endocytosis of AMPARs is thought to be controlled through Ca^{+2}-dependent dephosphorylation where PP1 is able to dephosphorylate stargazin on AMPARs which is able to deactivate them and target them for endocytosis (Citri & Malenka, 2008).

Another form of LTD involves mGluRs (**Figure 4**) and this form of LTD's mechanism of expression is not as well understood as NMDAR-dependent LTD (Gladding et al., 2009). This type of LTD requires stimulation of moderate frequencies which can activate extrasynaptic group 1 mGLURs (mGluR1 and mGluR5). Upon activation, phosphoinositide-specific phospholipase C (PLC) can hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 can promote the release of Ca^{+2} from intracellular stores and DAG can activate various kinases such as PKC which can phosphorylate AMPAR subunit GluA2 at the ser-880 site which can allow for the lateral diffusion and internalization of AMPARs (Citri & Malenka, 2008; Lüscher & Huber, 2010).

It is important to note the induction of LTD through either mGLUR or NMDAR activation does not prevent the other from occurring, this means they can both take place simultaneously (Gladding et al., 2009).

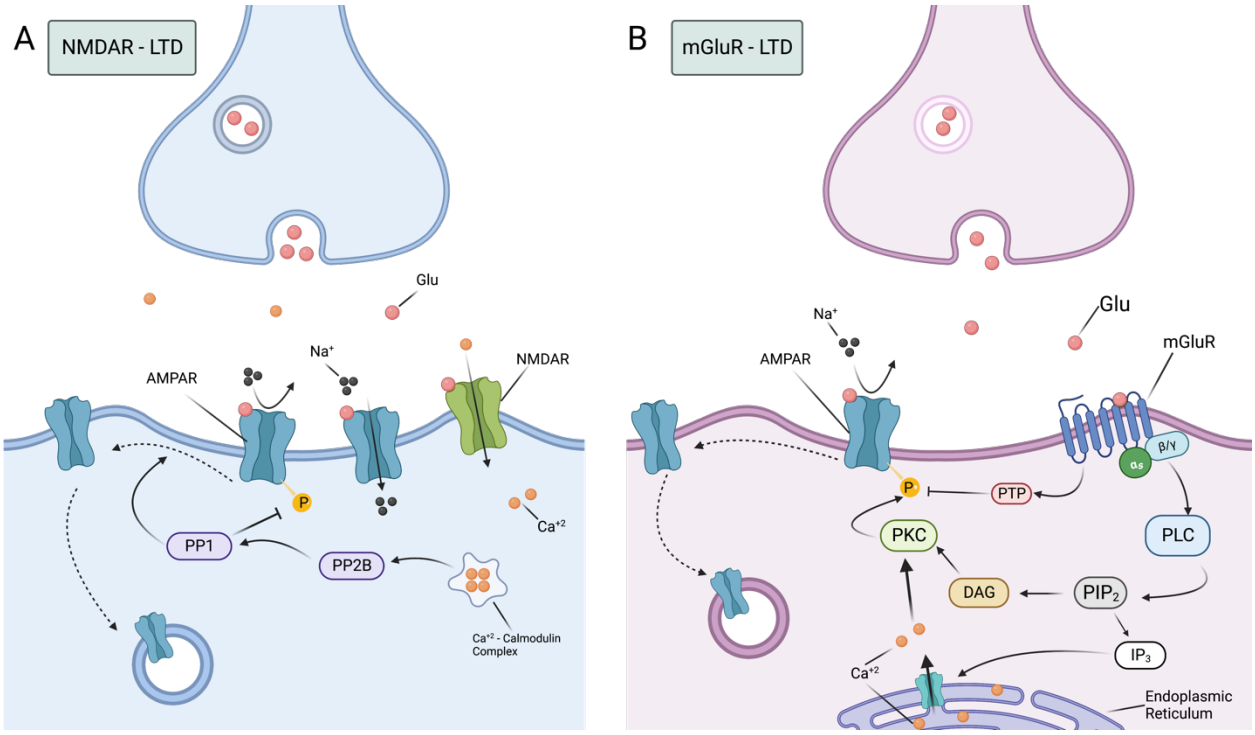


Figure 4. Simplified mechanism for NMDAR LTD and mGluR LTD

(A) *N-methyl-D-aspartate receptor (NMDAR) – dependent LTD occurs at glutamatergic terminals. When a low-frequency stimulus (LFS) is elicited, the magnesium (Mg^{+2}) ion blocking NMDARs is partially unblocked which allows for low calcium (Ca^{+2}) influx into the postsynaptic terminal. This Ca^{+} influx allows for the formation of the calcium-calmodulin complex which activates calcineurin (PP2B) which in turn activates PP1. PP1 is thought to dephosphorylate stargazin, a protein located on the a-amino-3-hydroxy-5-methyl-4-isoxazole propanoic acid receptor (AMPAR) which deactivates the receptor and targets them for endocytosis.* ***(B)*** *metabotropic glutamate receptor (mGluR)-dependent LTD is activated through LFS which initiates phosphoinositide-specific phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 promotes the release of Ca^{+2} from intracellular stores (such as in the endoplasmic reticulum) and DAG can activate many kinases including protein kinase C (PKC). PKC can phosphorylate AMPAR subunit GluA2 at the ser-880 site which allows for lateral diffusion and internalization of AMPARs.*

1.4.4. Paired Pulse and Input-output

When a synapse is stimulated in quick succession, the response of stimulation can be stronger or weaker than the first and this is referred to as paired-pulse (PP) facilitation (PPF) and paired-pulse depression (PPD). It has been noted that the paired-pulse ratio (PPR), which is the ratio of the amplitude or slope of EPSPs of the second response to that of the first, is a way to measure the probability of the release of neurotransmitters. Using this measure is important as it allows researchers to evaluate the possible presynaptic changes after other kinds of manipulations of the synapse (Hanse & Gustafsson, 2001; Manita et al., 2007). In this study,

PPR is used for this exact purpose: to determine whether r-mTBI impacts the neurotransmitter release from MPP inputs.

A simplified mechanism for PP is that during the first stimulation pulse, vesicles from pools that are readily able to be released in the presynaptic terminal are released into the synaptic cleft, which allows for measurement of the fEPSP on the post-synaptic neuron. The size of the second fEPSP is dependent on the amount of NT that was already released during the first pulse and thus in consequence can be either larger or smaller in amplitude than the first fEPSP measured. If a terminal has a high probability of release, this means that more vesicles were released in the first pulse, leaving fewer available for release during the second pulse, resulting in a smaller second fEPSP (PPD). On the other hand, if a terminal has a low release probability, that means fewer vesicles were released in the first pulse, with residual calcium facilitating vesicle release during the second pulse, leading to a larger second fEPSP (PPF) (Santschi & Stanton, 2003; Zucker & Regehr, 2003).

Input-output (I/O) curves are representative of the relationship between stimulus strength and the size of the response to neural stimulation. Typically, I/O curves reflect the excitability of a stimulation target which is affected by neuromodulators, incoming endogenous signals, the brain state and even a variety of disorders. This curve shows the behaviour from a weak stimulus (with minimal response) to a strong stimulus (where the response should be saturated). The slope of the I/O curve represents the general measure of cortical excitability and reflects the level of synaptic connectivity and plasticity (Mohammad Mahdi Alavi et al., 2021). In this study, the current intensity is constant, but the pulse width is gradually increased (30 to 300 μs pulse width; 15 s intervals) and I/O curves were used to compare between groups the ability of the slice to respond to increasing stimulation, which allows for the interpretation of slice health.

1.4.5. Short term plasticity

Two forms of short-term plasticity that are relevant to this thesis work are post-tetanic potentiation (PTP) and short-term depression (STD). PTP refers to an increase in the release of neurotransmitters on a brief minute time scale after HFS, which is likely caused by the accumulation of residual Ca^{+2} within the nerve terminal during periods of high-frequency firing (Korogod et al., 2007). The residual Ca^{+2} has been thought to not be the only reason for the triggering of neurotransmitter-containing vesicles, but this residual Ca^{+2} is able to activate secondary messenger pathways which in turn potentiate transmitter release (Brager et al., 2003). STD is the result of a brief period of enhanced synaptic depression that occurs after an LFS. This phenomenon is thought to be caused by the depletion of a pool of release-ready neurotransmitters due to the lengthier nature of LFS (Weis et al., 1999) or that LFS can cause a reduction in the amount of Ca^{+2} that can enter the presynaptic terminal which in turn results in a reduction in transmission (Kamiya & Zucker, 1994).

1.5. Endocannabinoids in the brain

The endocannabinoid system (ECS) is an important neuromodulatory system that has become a popular topic of study over the last 25 years (Fontaine et al., 2020; Lu & MacKie, 2016; Peñasco et al., 2019). This system has been found to play an important role in the functioning of neural networks, can contribute to neurodevelopment in the central nervous system and has a role in regulating synaptic plasticity (C. G. Song et al., 2021). The ECS includes endogenous cannabinoid ligands or endocannabinoids (eCBs) anandamide (AEA) and 2-arachidonylglycerol (2-AG), cannabinoid receptors 1 and 2 (CB1-R/ CB2-R) and their associated enzymes, transporters and agonists (Shohami et al., 2011). eCBs are unique in the way that unlike “typical” neurotransmitters, they are not stored in presynaptic vesicles, but are synthesized ‘on demand’ (Zou & Kumar, 2018). The synthesis of eCBs occurs from membrane phospholipids in response to a rise in intracellular Ca^{+2} in the postsynaptic terminal due to neuronal activation. This increase in Ca^{+2} can occur alone (through voltage gated Ca^{+2} channels) or in conjunction with the activation of mGluRs or M1/M3 muscarinic acetylcholine receptors

(mACHRs) (Alger & Kim, 2011). The ECS has been of particular interest in regard to TBI as some research has found neuroprotective effects against secondary injury mechanisms post TBI (Mechoulam & Shohami, 2017; Panikashvili et al., 2001). However, other research has found that CB1-Rs are involved in hippocampal spatial learning and memory impairment that is induced post-TBI (Xu et al., 2019). This research hopes to further investigate the ECS and its role in TBI.

1.5.1. CB1 and CB2 Receptors

CB1-R and CB2–Rs were both discovered about 3 decades ago. The first cannabinoid receptor (CB1) was first identified from the cDNA library of a rat cerebral cortex and was found to mediate the pharmacological effects of delta – 9 – tetrahydrocannabinol (Δ9-THC), the psychoactive component of cannabis (Matsuda et al., 1990). CB2 receptors were found 3 years later in the human promyelocytic leukemia cell line HL60 (Munro et al., 1993). Both these receptors are part of the superfamily of G protein-coupled receptors (GPCRs) and are inhibitory in nature through Gi/o proteins (Pertwee et al., 2010; Schurman & Lichtman, 2017). CB1 receptors are highly expressed in the central nervous system (CNS) and are found at terminals of central and peripheral neurons in the cortex, hippocampus, etc.… where they can mediate the release of excitatory and inhibitory neurotransmitters (Howlett et al., 2022; Schurman & Lichtman, 2017). Due to their widespread nature in the brain, CB1 receptors can alter the control of motor function, induce analgesic effects and affect memory and cognition processes (Pertwee et al., 2010). While CB2 receptors are expressed by some neurons, they are mainly expressed in immune cells to aid in modulating immune cell migration and cytokine release (Pertwee et al., 2010; Turcotte et al., 2016). The best-known endogenous cannabinoids include 2-AG which is an agonist for both cannabinoid receptors with moderate to low affinity and AEA which is inactive at CB2 – 2 but is a high-affinity partial agonist at CB1-Rs (Zou & Kumar, 2018). Both these eCBs are produced on demand in response to increased intracellular calcium, however, a unique feature of 2-AG is that it is able to participate in retrograde signalling (Castillo et al., 2012; Pacher et al., 2006).

1.5.2. Endocannabinoids and synaptic plasticity

ECB signalling in relation to LTD was first thought to have emerged in 2002 at excitatory synapses in the dorsal striatum (Gerdeman et al., 2002). Now, eCBs -LTD has been seen in other brain structures such as the amygdala, somatosensory cortex and hippocampus (Augustin & Lovinger, 2018). eCB – LTD induction can occur at inhibitory or excitatory synapses, and each has its own unique use in the brain. Inhibitory eCB LTD (I – LTD) works to inhibit the release of GABA and aids in facilitating the induction of LTP at excitatory inputs (Chevaleyre & Castillo, 2004). Excitatory eCB – LTD is found at glutamatergic synapses and contributes to the decrease of glutamate neurotransmitter release. eCB – LTD can occur through several mechanisms that include retrograde signalling, non-retrograde or autocrine signalling and signalling through astrocytes; for the purpose of this study, we will be focusing on the retrograde eCB – LTD (Castillo et al., 2012; Fontaine et al., 2020; Peñasco et al., 2019).

While 2-AG and AEA are both endogenous eCBs in the ECS, 2-AG is the principal eCB required for regulating synaptic function. 2-AG can be produced in one of two ways: 1. depolarization of the synapse which causes an increase in intracellular Ca^{+2} concentration through voltage-gated Ca^{+2} channels (VGCCs) or 2. through activation of postsynaptic group 1 metabotropic glutamate receptors (mGLUR-1) through the binding of glutamate (Glu). Regardless of whether Ca^{+2} influx or activation through mGLUR-1 is initiated, both these metabolic pathways result in the activation of enzyme phospholipase C (PLC), however, the duration of LTD is dependent on the type of initiation. Ca^{+2} influx is associated with short-term plasticity, whereas mGLUR-1 activation is associated with long-term plasticity. Once PLC is activated, this enzyme hydrolyzes phosphatidylinositol (PI) which is then converted to diacylglycerol (DAG). DAG then is converted to 2 -AG by a postsynaptic localized enzyme called diacylglycerol lipase (DGL). Once 2-AG is synthesized, this eCB is released back into the synaptic cleft where it travels across the synapse to bind to CB1-Rs. The process by which 2-AG can move retrograde across the synapse is still unknown although it has been stipulated that it requires an eCB membrane transporter. Once CB1-Rs is activated, a secondary messenger cascade is initiated to inhibit adenylyl cyclase (AC) which in consequence also inhibits protein

kinase A activity which ultimately decreases neurotransmitter release (Glu). The above mechanistic explanation is for excitatory eCB – LTD. I – LTD functions similarly to excitatory eCB - LTD, in the way that once 2-AG bind to CB1 receptor AC and PKA activity is reduced, however, in this pathway there is the activation of calcium-sensitive phosphatase calcineurin (CaN). CaN de-phosphorylates an unknown presynaptic target and this process leads to a decrease in the release of the neurotransmitter GABA. This research will focus solely on excitatory eCB – LTD (see (Castillo et al., 2012; Heifets et al., 2009) for review, Figure 5).

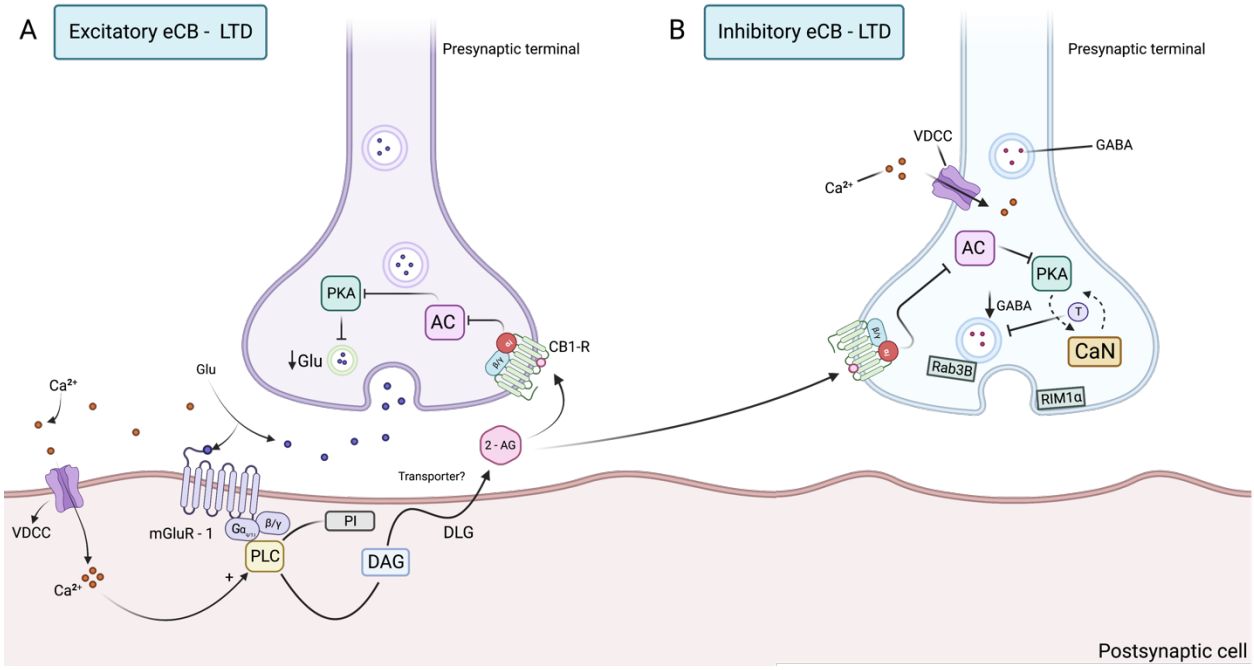


Figure 5. Simplified Mechanism of eCB – LTD

(A) Excitatory eCB – LTD begins through 1. depolarization of the synapse that causes an increased intracellular concentration of Ca^{+2} or 2. the activation of metabotropic glutamate receptor (mGLUR -1). Regardless of which initiation step is taken, both activate phospholipase C (PLC) which hydrolyzes phosphatidylinositol (PI) to create diacylglycerol (DAG). DAG is then converted to the eCB 2 – arachnidonylglycerol (2-AG) through the enzyme diacylglycerol lipase (DLG). 2 – AG is released back into the synaptic cleft through an unknown transporter and can move in a retrograde fashion to bind to cannabinoid 1 receptor (CB1). Once bound, secondary messenger cascades are initiated, and inhibition of adenylyl cyclase (AC) and protein kinase A (PKA) occurs which ultimately leads to a decrease in the neurotransmitter release of glutamate. (B) Inhibitory eCB – LTD functions similarly to excitatory eCB LTD, in that 2 – AG is produced through the same mechanism and moves retrograde to bind to CB1 receptors on the presynaptic terminal. AC and PKA also inhibited, however this then leads to the activation of calcium sensitive phosphatase calcineurin (CaN) which de-phosphorylates an unknown presynaptic target (T) that is able to inhibit the neurotransmitter release of GABA.RIMα (an active zone protein) and RAb3B (a vesicle associated protein) are also necessary for the induction of I – LTD.

While CB1-Rs are predominantly located at inhibitory terminals, CB1-Rs at excitatory glutamatergic terminals in the DG are able to actively participate in endocannabinoid LTP (eCB

– LTP) (W. Wang et al., 2016) and eCB - LTD (Peñasco et al., 2019). In this research, the induction of excitatory eCB – LTD through in vitro electrophysiological experiments was done through the application of a low-frequency stimulus (LFS) train of 6000 pulses at 10 Hz in the medial perforant path of the dentate gyrus. The Christie lab has previously researched the reliability of this protocol to induce eCB – LTD through the application of eCB and glutamate receptor antagonists. Fontaine *et al*. found that LTD induced by the 10 Hz LFS could be blocked through the CB1 inverse antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazone-3-carboxamide (AM251) and through a selective metabotropic glutamate receptor 5 ($mGluR_5$) antagonist 2-Methyl-6-(phenylethynyl) pyridine (MPEP). An NMDAR antagonist was also used (DL-2-Amino-5-phosphonopentanoid acid (DL-APV)) and it was found that it could not inhibit 600 x 10 Hz – LTD (Fontaine et al., 2020). This research confirmed work done by Peñasco *et al*., who had previously found that AM251 was able to inhibit synaptic depression via eCBs (Peñasco et al., 2019). While there is a growing plethora of research looking at hippocampal eCB-LTD, there is a disproportionate amount that studies the cornu ammonis 1 (CA1) (Chevaleyre & Castillo, 2004; Mato et al., 2004; Yasuda et al., 2008) compared to the DG. This research hopes to expand the understanding we currently have of eCB – LTD in the DG.

1.6. Consequences in Synaptic Plasticity in the hippocampus after TBI

Memory problems are seen commonly in brain injury survivors and these deficits can be a consequence of direct effects on memory encoding or secondary effects on concentration and attention (Serra-Grabulosa et al., 2005). Impairments in declarative memory, a type of long-term memory that allows us to store and retrieve personal information and general knowledge, can persist for up to three months post-mTBI and in worse cases for more than a year (Baddeley, 2004; Bedard et al., 2020). The hippocampus is critical for the formation of declarative memories and there has been evidence that atrophy in the hippocampus contributes to dysfunction in memory (Ross et al., 2012; Serra-Grabulosa et al., 2005).

To uncover other functional alterations in the hippocampus that could play a role in memory impairment, research began exploring neurotransmission and synaptic plasticity mechanisms (Atkins, 2011). A study using in vitro electrophysiology by White et al., found that LTP in the DG was significantly reduced 1-day post-injury that persisted to 28 days following injury in female juvenile rats after mTBI. In males, these researchers found that the deficit in LTP was significant to the impact site 7 days post-injury, but these injuries did not persist to 28 days post-injury (White et al., 2017). Many in vivo electrophysiological experiments have been used to study synaptic plasticity after TBI (models of TBI including fluid-percussion and cortical impact models) and found a resounding consistent attenuation in LTP responses (D'Ambrosio et al., 1998; Miyazaki et al., 1992; Reeves et al., 1995). While LTP has been extensively investigated, LTD has been only studied by a few groups and have yielded differing results. In the CA1, conflicting results have been reported regarding the effect of TBI on LTD, some have found that LTD is unaffected (D'Ambrosio et al., 1998), while others have demonstrated enhancement (Albensi et al., 2000). The variation in the results may be caused by differences in injury models used, which lead to distinct patterns of hippocampal damage (Atkins, 2011). Further research is needed to determine if TBI affects LTD in other parts of the hippocampus.

1.7. Summary and project aim

TBI is a major contributor to both death and disability, and its yearly accumulated occurrence is higher than the combined total of spinal cord injuries, HIV/AIDS and breast cancer (McGuire et al., 2017). Most TBIs are mild, and although a single mTBI may not always cause behavioural issues, multiple injuries can lead to cumulative effects, including increased susceptibility to future mTBIs and long-term functional deficits (Aungst et al., 2014; Belanger et al., 2010; Mouzon et al., 2014).

Brain injury survivors frequently report learning and memory difficulties, which more often than not resolve in weeks, but persistence in these deficits can last for months to even years (Cancelliere et al., 2023; Permenter et al., 2022). The hippocampus is a region in the brain that

has been implicated in learning and memory and the process by which it is able to do so is through a phenomenon known as synaptic plasticity (Leuner & Gould, 2009). Two major forms of synaptic plasticity include NMDA-dependent LTP and LTD, both of which are needed to have proper learning and memory functioning (T. V.P. Bliss & Lømo, 1973; Blitzer, 2005; Dong et al., 2015; Nabavi et al., 2014; Wiegert & Oertner, 2013). Now, however, a different type of LTD is also considered to contribute to memory formation and this is known as eCB-LTD, which is mediated through the endocannabinoid system (Augustin & Lovinger, 2018; Gerdeman et al., 2002; Heifets et al., 2009). While there has been much research looking at deficits in LTP after mTBI (D'Ambrosio et al., 1998; Miyazaki et al., 1992; Weston et al., 2021), there have been conflicting results regarding LTD (Albensi et al., 2000; Albensi & Janigro, 2009; D'Ambrosio et al., 1998) and very little research on eCB-LTD post-TBI.

The main purpose of this thesis is to investigate the effects of r-mTBI and its relationship to synaptic plasticity in the dentate gyrus of juvenile male Long Evans rats. To achieve this, I investigated LTP and both homosynaptic LTD (1 Hz - LTD) and eCB – LTD (10 Hz - LTD) through in vitro electrophysiology. The question of whether the ACHI model produces an injury bias was also investigated as well as whether there was a correlation between the severity of NAP scores and the amount of synaptic plasticity. Overall, I hypothesize that there will be deficits in synaptic plasticity at the MPP-DG connections in young rats and that this will be shown through a reduction in LTP and both types of LTD compared to their SHAM counterparts. This work is important as it may correlate to difficulties in learning and memory in human concussion survivors.

Aim 1: To determine if the ACHI model causes lateralization of injury.

Aim 2: To determine whether the ACHI model of r-mTBI affects synaptic plasticity over time.

Aim 3: To assess the strength of the correlation between neurological assessment scores and the amount of synaptic plasticity recorded.

2. Chapter 2 - Methodology

2.1. Materials and Methods

2.1.1. Animal Ethics

All animal procedures performed were approved by the Animal Care Committee at the University of Victoria and were done in accordance with the guidelines set by the Canadian Council for Animal Care. All animals used followed the animal protocol #2019-002 (Appendix E).

2.1.2. Animal Ordering and Generation

A total of 62 animals were used in this thesis and the age range for animals during experimental work was between PND 25-36. All animals were ordered from Charles River Laboratories (Kingston, NY, USA) and were allowed to acclimatize to their novel housing environment for 1 week. Rats were maintained in standard cage housing at 22 - 26°C and had access *ad libitum* to food and water on a 12-hour light-dark cycle. Precipitation in the room was kept to 40-60% and the room pressure was positive. The ordered dams came with 8-10 pups which were weaned when they reached PND 21 and then placed into new cages in groups of two or three. In the case of breeding: female Long – Evans (LE) rats were paired with male breeders and left undisturbed for 5-10 days. Care was taken to not disturb the dam and her litter of pups for 24 hours post-partum to facilitate maternal bonding. The pups were examined and monitored a day later to ensure they were thriving. In this study, no pups were culled unless pups showed signs of deterioration or could not grow alongside healthy littermates. At PND 21, male pups were removed and paired in cages in pairs or in threes. Females were housed similarly, but not used in this study.

2.1.3. R-mTBI model: Awake Closed Head Injury (ACHI)

This study investigated repeated mild traumatic brain injuries that were induced using the awake closed-head injury model (ACHI) (Christie et al., 2023). This model was adapted from a model that produces mild closed-head injuries in adult mice without the use of anesthesia for

use in juvenile rats and can produce injury deficits that are observable in human concussion cases (Merconi et al., 2018).

To produce injury, rats were restrained in a soft plastic restraint cone that had an opening at the narrow end to allow for rats to breathe adequately (DecapiCones, Fischer Scientific, USA). Once the rat was as far down the cone as possible, with its nose at the opening of the cone, a plastic hair clip was used to secure them from behind. Care was taken to make sure the rats were comfortably positioned with their feet beneath them. A 3D-printed helmet was then placed over the head of the animal and held in place using a rubber elastic band. The raised circular platform of the helmet served as the site of impact and was centred over the left parietal cortex (Figure 6).

The animal was subsequently placed on a soft foam platform that is located below the apparatus used for injury. A modified cortical controlled impact (CCI) device (Impact One, Lecia Biosystems Inc., ON, Canada) was used to induce injury and this device was mounted on a stereotaxic frame. The impactor had been modified by the addition of a 7mm diameter flat rubber tip. This impact tip is lined up square to the raised platform on the 3D-printed helmet. The impact parameters for this experiment included an impact speed of 6 m/s, a 10 mm depth of impact and 0.1 second dwell time (or time for the impact tip to retract). As soon as the animal was still and the helmet and impactor were aligned, the impact was delivered by depressing the lever ‘impact’ on the control box. Animals were quickly removed from the restraint cone following the injury and a neurological assessment was given.

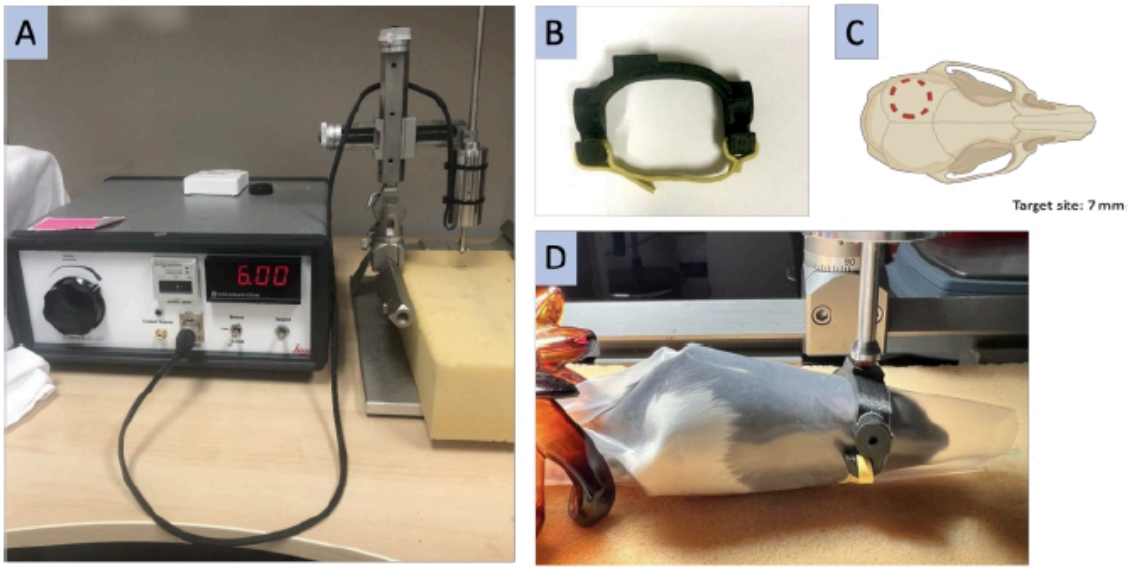


Figure 6. Awake closed head injury (ACHI) model and setup

(A) *A modified cortical controlled impactor mounted on a stereotaxic frame is used to induce injury to animals. This apparatus rapidly displaces the animal's head 10 mm at a velocity of 6.0 m/s.* ***(B)*** *A 3D custom-printed helmet is shown with an elastic band that connects either side of the helmet to secure the helmet to the animal's head* ***(C)*** *A representative image of the animal's skull. The red circle indicates the impact site (where the platform of the helmet is aligned, on the left parietal cortex).* ***(D)*** *Subjects are placed in a plastic restraint cone on a foam platform with the helmet placed over the cone and positioned so the target site is directly under the impactor tip. This figure was taken from our publication:* (Christie et al., 2023)

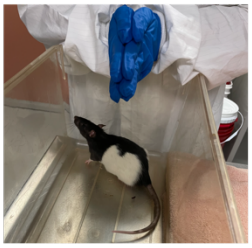
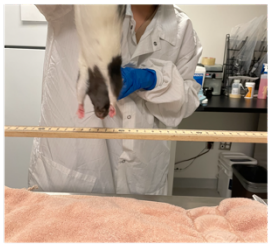
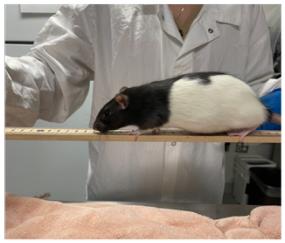
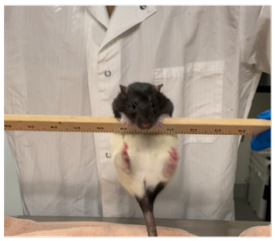
2.1.4. Neurological Assessment Protocol (NAP) Score

The state of the animal was examined as soon as they were removed from the restraint cone. Loss of consciousness, presence of apnea and righting reflex were assessed and the time to recover was recorded if an animal experienced any of the listed ailments. In more detail, righting reflex consists of placing the animal on its back and measuring the time the animal took to right itself. If the animal was exhibiting difficulty breathing, the time it takes for the animal to return to normal breathing is recorded. If loss of consciousness is observed, the researcher performs a toe pinch to assess the animal's reflexes and the time to recover from the loss of consciousness is recorded.

To assess potential neurological changes after each individual ACHI, a behavioural test called the Neurological Assessment Protocol (NAP) was performed. These tests use

standardized scoring criteria to reliably indicate the abilities of the animal's (Christie et al., 2023; Meconi et al., 2018) (**Figure 7**). This assessment consists of four behavioural tests that are scored on a four-point scale. These four tests include startle response, limb extension, balance beam, and rotation beam. Each of these tests is scored as shown in **Figure 7** and these scores are then summed to represent the animal's overall performance. For example, the highest score an animal can receive is 12 which indicates perfect performance whereas a total score of zero shows a complete failure at all tasks. In this study, the NAP score was used to measure neurological function after injury exposure.

The first test performed was the startle reflex test where the animal is placed in the center of an empty standard housing cage. The researcher then claps above the animal's head and the response to the clap is assessed. Then, the limb extension test is performed whereby the researcher held the rat at the base of the tail and raised it 3 – 5 cm above a meter stick (100 cm long x 2 cm wide x 0.75 cm thick) that is balanced between two empty home cages. The researcher notes the limb extension response and scores the animal accordingly. The third test is the balance beam test where the animals are placed on the center of the same meter stick as for the limb extension test. This meter stick is balanced between two empty home cages (~20cm high) and a towel is folded and placed under the beam for cushioning. The researcher watches the animal and their ability to balance and walk on the beam. Lastly, the same beam is again used to assess the ability of the animal to navigate a slowly rotating beam. The animal is placed in the middle of the beam and the researcher raises the beam to chest height (about 40cm above the cushioned workspace). Once ready, the researcher rotates the beam once per second for four rotations.

A  B  C  D 

Test	0	1	2	3
A. Startle Response	No reaction to clapping sound	Only ears react to clapping sound	Slow reaction or slight freezing reaction to clapping sound	Ears react quickly and whole body jumps and freezes
B. Limb Extension	Absence of limb extension; limp body	Intermittent reaction/extension of limbs	Only one limb properly extends	Full extension of both paws, animals grasp beam successfully
C. Beam Walk	Complete absence of movement, with all limbs hanging off beam	Non-locomotive movement ("swimming" or "rowing" motion of limbs without movement across the beam)	Animal walks on beam, but more than 2 limb slips are observed	Animal walks across beam to home cage with 2 or fewer foot slips
D. Rotating Beam	Animals falls during the 1st rotation	Animal falls during 2nd or 3rd rotation	The animal falls during 4th rotation	Animal successfully completes 4 rotations

Figure 7. Neurological Assessment Protocol and Scoring Criteria

Photographs showing the NAP tasks are as followed: ***(A)*** *Startle response,* ***(B)*** *Limb Extension,* ***(C)*** *Beam walk, and* ***(D)*** *Rotating Beam. The scoring criteria for each task are presented in the associated table. All the tests are scored on a scale from 0-3. 0 is the lowest score (shows the greatest amount of impairment) and 3 is the highest score (no impairment). Prior to these batteries of behavioural tests, an assessment of consciousness was made. This assessment is made by observing righting reflexes, apnea, and any loss of consciousness. If any ailments are observed, the time it takes for the animal to return to normal is recorded on the scoring sheet.*

2.1.4.1. Cage side monitoring post-injury

After the ACHI procedure and NAP protocol, animals were monitored closely for any symptoms of pain or discomfort. The animals are placed back into their home cage and checked for any signs of pain or irregular behaviour (arching of the back, vocalizations, pain when touched… etc.) and their appearance (signs of porphyrin, self-injury… etc.). If any signs of ailment are seen, they are recorded on the cage side monitoring sheet (Appendix D). Changes in weight can also be monitored, however, this would only be useful for a protocol spanning several days. If there are signs of serious distress, animal care would be contacted for a professional assessment and for the animal to be given any potential treatment.

2.1.5. Study Design

Animals were randomly assigned to one of three groups: injured, SHAM or control. In this set of experiments, only males were included. Protocol for the experiments was performed on animals when they reached PND 25 and ran until PND 29. Injured animals received eight impacts, two hours apart over the course of a day. SHAM animals were subjected to the same procedure as the injured animals, which includes being placed in the restraint cone, being placed on the injury platform, wearing the 3D printed helmet, and hearing the piston 'impacting', but without receiving any impact. Control animals were not subject to any protocol and were left to live normally in their home cages. Animals were used at post-injury day (PID) 1 and 7 (i.e., 1 or 7 days after the last injury) for electrophysiological experiments (**Figure 8**).

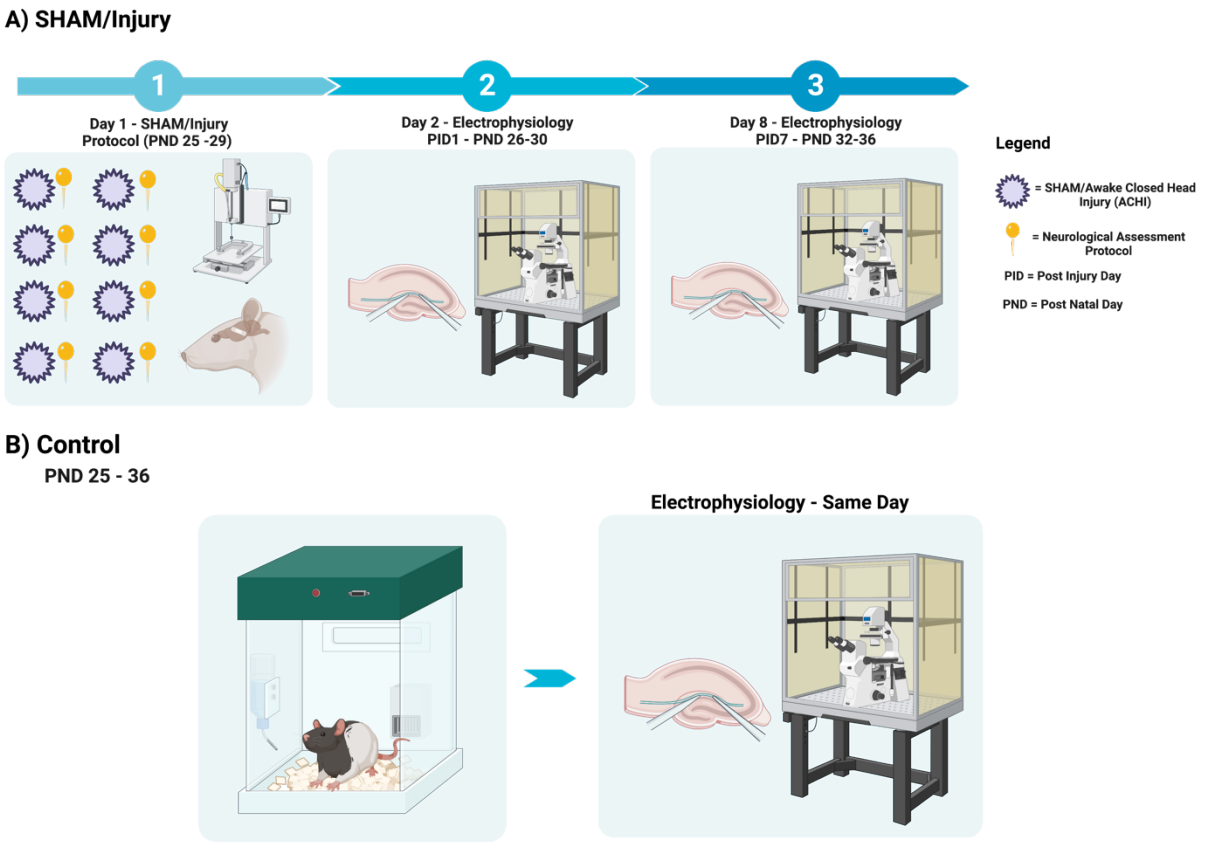


Figure 8. Study Design for r-mTBI and electrophysiology experiments

Male Long Evans rats aged between PND 25-29 were randomly assigned to one of three groups: SHAM, Injury or Control. ***(A)*** *Injury animals received 8 impacts, 2 hours apart over the course of the day, while SHAM animals underwent an identical protocol to those injured, including being placed in the restraint cone, on the injury platform, wearing the 3D-printed helmet and hearing the piston impacting, but without receiving any impact. All animals that underwent a SHAM or Injury protocol also went through a Neurological Assessment Protocol (NAP) immediately following their procedure. Electrophysiological experiments began on day 2 (one-day post-injury) or on day 8 (seven days post-injury).* ***(B)*** *Control animals were not subjected to any protocol and were left to live normally in their home cage. These animals underwent electrophysiological experiments when they were of the correct age (PND: 26 – 36).*

2.1.6. Electrophysiology

Tissue preparation for in vitro field electrophysiological recordings is described below with a visual depiction in **Figure 9**.

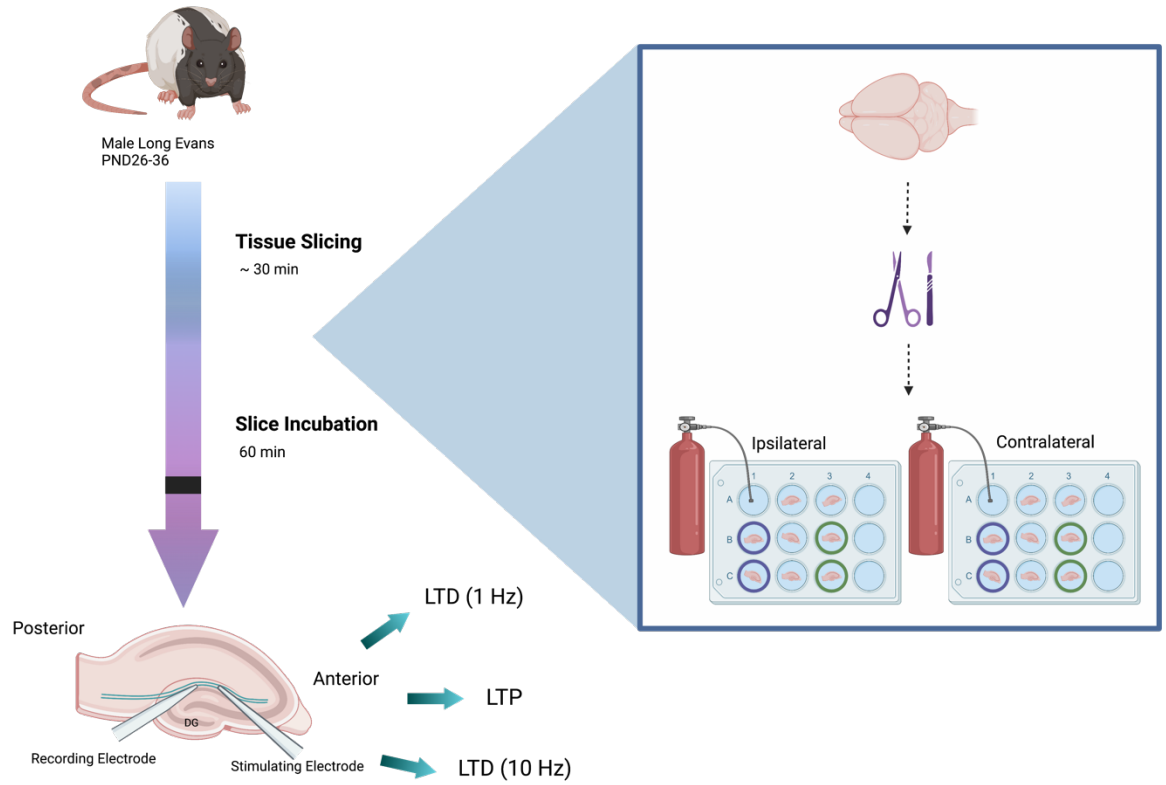


Figure 9. Tissue preparation for in vitro electrophysiology

Following either control, r-MTBI or SHAM protocol, Juvenile male Long Evans rats (PND 26-36) were anesthetized. Their brains are excised, dissected, and fixed to the compresstome piston dorsal side down. Tissue slicing takes about 30 minutes when cutting in 350 µm thick sections with a compresstome. These slices are then transferred into a holding chamber where they are incubated for 60 minutes at 32 ℃. Wells with blue circles indicate most ventral slices, while wells with green circles indicate most dorsal slices (right of the figure). The black line indicates the removal of the holding chambers from the temperature bath to the lab bench where they sit at room temperature (20-21 ℃). Individual slices were moved from the holding bath to electrophysiology rigs and recording (left) and stimulating (right) electrodes were then lowered into the dentate gyrus as shown above to the bottom left of the figure and randomly assigned either long-term potentiation (LTP – 50 pulses,100 Hz x4) or long-term depression (1 Hz x 1 pulse or 10 Hz x 6000 pulses) conditioning stimuli.

2.1.6.1. Slice Preparation

Male Long Evans rats between PND 26-36 from either SHAM, Injury or Control groups were used for in *vitro* electrophysiology for the study of DG synaptic plasticity. The animals were deeply anesthetized through the inhalation of isofluorane USP (Cresenius Kabi Canada Ltd., Toronto, ON, Canada) and then quickly decapitated when they lost their toe pinch reflex. Brains were excised and submerged in ice-cold artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 2.5 mM KCl, 1.25 $NAHPO_4$, 25 mM $NAHCO_3$, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 10 mM dextrose (300 $\pm$ 10 mOSM; pH 7.2 – 7.4)) that is kept oxygenated with carbogen (95% O_2/5%

CO_2). Transverse hippocampal slices (350 µm) (**Figure 10**) were cut in oxygenated ice-cold aCSF using a Compresstome VF – 510 – 0Z (Precisionary Instruments LLC, Natick, MA, USA). Slices were collected from one hemisphere at a time to keep track of ipsilateral (injured) and contralateral (non–injured) hemispheres for the purpose of being able to analyze any potential differences due to injury bias. Slices were placed in recovery wells from most ventral to dorsal. Slices were left undisturbed in the recovery wells for a minimum of an hour in warmed (32°C), oxygenated aCSF. Once this hour had passed, the wells were removed from the temperature bath and left to sit on the lab bench at room temperature (20-21°C).

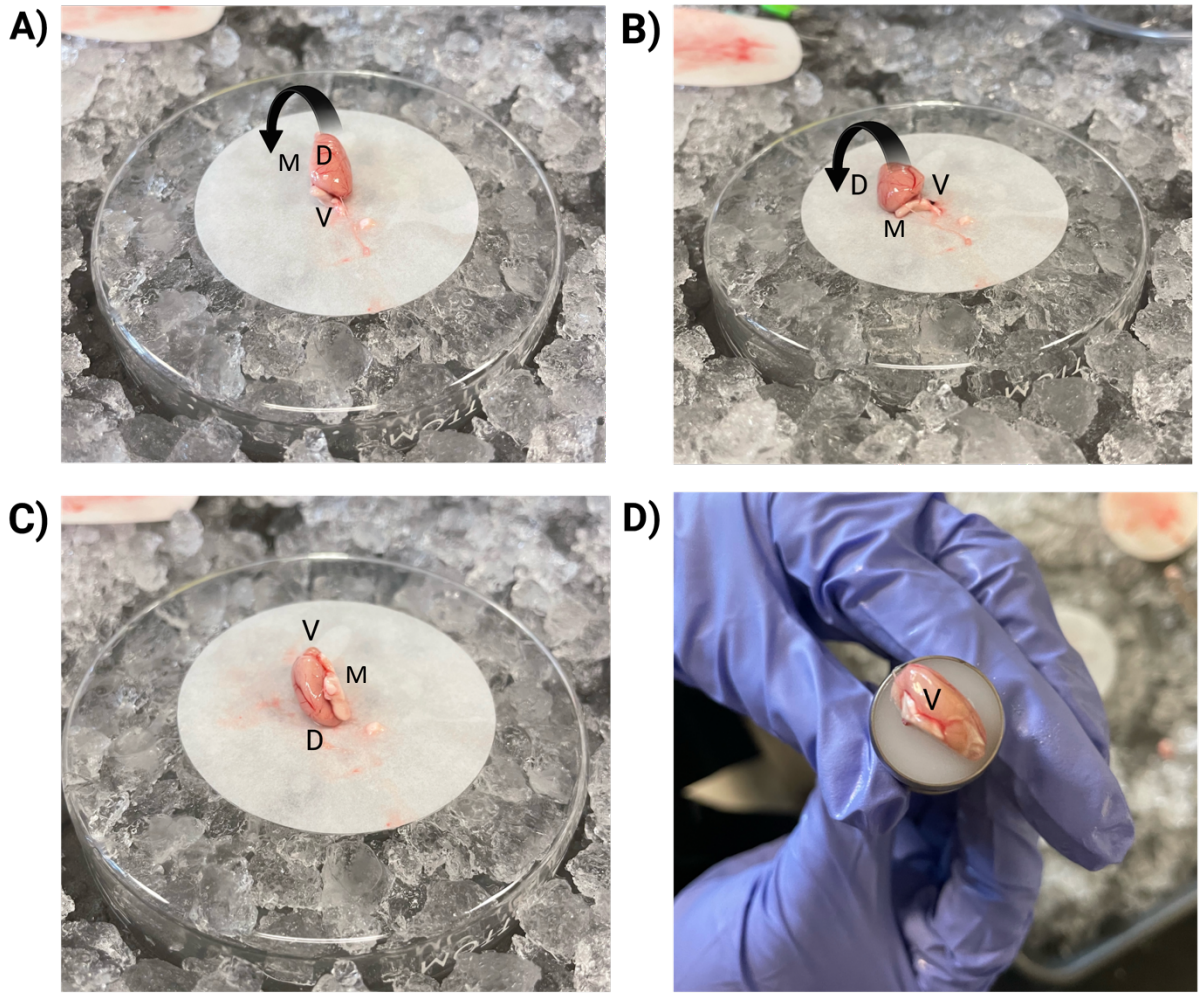


Figure 10. Transverse hippocampal slicing protocol

The rat brain was dissected from the skull and placed on some damp filter paper. The brain was then sectioned down the middle. One hemisphere was removed and placed in a beaker filled with oxygenated aCSF for later use, while the other is prepared for compresstome slicing. ***(A)*** *The chosen hemisphere was placed ventral side down. The cerebellum and a small portion (~3mm) of the prefrontal cortex was removed. The hemisphere was then flipped onto its medial (M) side.* ***(B)*** *Once the hemisphere had*

been flipped so its M side is sitting against the filter paper, a 30º cut is made on the dorsal side. This is to ensure that the brain would sit flat against the piston. After the cut had been made, the brain hemisphere was flipped onto its dorsal (D) side. ***(C)*** *The brain hemisphere was placed so that it is sitting on its dorsal side, with its ventral (V) side on top. This is the correct orientation for transverse slicing.* ***(D)*** *The brain hemisphere was then glued to the compresstome piston with Vetbond (3M, Union City, CA, USA) dorsal side down (ventral side up). When the piston was loaded into the compresstome chamber, the direction of cutting is ventral to dorsal.*

2.1.6.2. Field Electrophysiology

Once the slices had been recovered for an hour, individual slices were transferred to the recording chamber on the electrophysiology rig. Warmed oxygenated aCSF maintained at 30°C is continuously washed over the slice with a flow rate of about 1-2 drips per second or 2mL/min. Slices were visualized using an upright microscope (Olympus, BX50W1, Olympus, Center Valley, PA, USA) which allowed for extracellular field excitatory postsynaptic potentials (fEPSP) to be recorded using a glass pipette filled with aCSF. The glass recording pipette was placed in the dendritic field of DG granule cells. A concentric bipolar stimulating electrode (FHC) was placed near (~ 200nm) the recording pipette in the medial perforant path (MPP) of the DG (**Figure 11**). Electrophysiological data were collected using an Axon Multiclamp 700B amplifier, digitized by an Axon Digidata 1440; recorded using Clampex 11 software (Molecular Devices, San Jose, CA, USA), and displayed using a Dell Latitude PC running Windows 11 (Microsoft, WA). When searching for optimal placement of electrodes and fEPSP, the magnitude of the amplifier (Digital Stimulus Isolation Amplifier, Getting Instruments, Iowa City, Iowa) was set to 0.05 mA. The resulting fEPSP was displayed on a computer screen and the stimulus amplitude was adjusted to determine the maximum amplitude of the response for each slice. Once the maximal amplitude was determined, the intensity of the stimulus was adjusted to elicit a response that was either 50% (for LTP experiments) or 70% (for LTD experiments) of the maximum amplitude for each individual slice. The rationale for this was to ensure that the synaptic plasticity work was not confounded by ceiling (LTP), or floor (LTD) effects related to the maximal size of the fEPSP.

Before the conditioning stimulus is given, a series of pre-recordings are taken by administering a single pulse (0.12 ms; 0.06 Hz or every 15 sec) until the slope of the evoked fEPSP was stable for a minimum of 20 minutes (80 traces). Baseline stability was the first inclusion criterion for slices to be included in the analysis (< ±10% change and a slope of < 0.5).

Following the pre-conditioning recording period, paired pulse (PP) stimuli were administered and then a final input/output (I/O) curve was constructed. These provided the baseline measures needed to evaluate whether our experimental manipulations altered either the pre-synaptic neurotransmitter (NT) release probability (PP ratio) or the stimulus-response relationship (I/O) in our recordings. The paired-pulse stimuli consisted of six pairs of pulses 50 ms apart. The ratio was calculated as the percentage change in the slope of the second EPSP (pulse 2) over the slope of the initial EPSP (pulse 1). I/O experiments were conducted following the administration of the paired-pulse stimuli. Here, I/O curves were elicited by increasing pulse width from 30 to 300 μs at pre-set intervals and analyzing the change in the fEPSP slope for each pulse width.

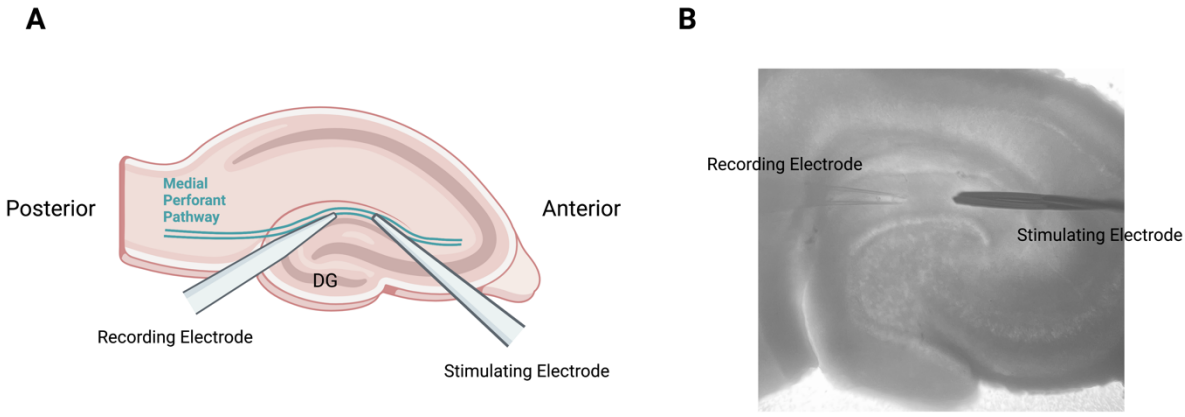


Figure 11. Field electrophysiology electrode placement

(A) *A representative image of the dentate gyrus (DG) of the hippocampus. A glass pipette filled with aCSF is used as a recording electrode (left) while a concentric bipolar stimulating electrode was used for eliciting electrical current to the slice (right). Both electrodes were placed about 200 nm apart in the medial perforant path of the DG.* ***(B)*** *This image shows the same as* ***(A)*** *but shows what we see through the microscope.*

2.1.6.3. Conditioning Stimulus Protocols

Following the collection of the pre-conditioning recordings, each slice from each animal was pseudo-randomly assigned to be administered either the LTP or LTD conditioning protocols as described below. To note, for each hemisphere of the animal, slices were rotated through LTP and LTD protocols (i.e., 10 Hz, 1 Hz, LTP… repeat), to get a sample of each conditioning stimulus per animal.

2.1.6.3.1. Long-Term Depression (LTD)

An innovative aspect of this work was that it used two distinct protocols to induce LTD. The first protocol used low-frequency stimuli (LFS: 900 pulses at 1 Hz) to induce a homosynaptic LTD that has been characterized by us, and others, as being dependent on NMDA receptor activation (Dudek & Bear, 1992; Pinar et al., 2017). The second protocol involved administering 6000 pulses at 10 Hz, a protocol that induces what has been referred to as endocannabinoid LTD (eCB-LTD) by some authors (Fontaine et al., 2020; Peñasco et al., 2019) but which we have recently shown to also require the activation of mGluR receptors (Fontaine et al., 2020). Following the pre-conditioning protocols, a chosen LTD protocol was elicited then followed by a 60-minute post-conditioning recording (1 pulse every 15s).

2.1.6.3.2. Long-Term Potentiation

LTP experiments were run with the $GABA_A$ receptor antagonist picrotoxin (Tocris, 100 μM) during the pre-conditioning recordings (1 pulse every 15 s; 0.067 Hz) until a stable baseline was recorded (20 min). GABA can inhibit the activity of neurons and by blocking these receptors, the neurons are able to remain in a state of excitation which is what we want for inducing LTP (Snyder et al., 2001; Sourdet et al., 2003). A high-frequency stimulus protocol was then delivered (HFS; 50 pulses at 100 Hz, repeated 4 times in 30 s intervals). Following HFS, a 60-minute post-conditioning recording was taken (1 pulse every 15 s) in aCSF.

2.2.Statistics and Analysis

2.2.1. Statistical tests

All statistical analysis was conducted using JASP version 0.16.3 (JASP Team, University of Amsterdam, The Netherlands, 2022). Graphs were made using python, an open-source software (Python Software Foundation, https://www.python.org/). Assumption tests for each statistical test were run where appropriate (Levene's test for homogeneity of variance, Shapiro Wilk for normality). The *average NAP score* data per injury and input-output analysis were

tested using a two-way repeated measures ANOVA with the Geiser-Greenhouse Correction for sphericity with group and r-mTBI as factors. Between-group factors for the two-way repeated measures ANOVA included trial (ACHI) and pulse width (for I/O analysis) and between-subject factors were group (Injury or SHAM). All *synaptic plasticity overtime* data, *dorsal vs. ventral* data and *ipsilateral vs. contralateral data* were tested using two-way ANOVAs. Fixed factors included either (1) group and day post-injury, (2) hemisphere and group or (3) location (dorsal or ventral) and group, while the dependent variable for all these analyses was the % Change EPSP. PPR analysis was done by one-way ANOVA, where fixed factors included the group, and the dependent variable was the PPR itself. Note that group refers to an injured or SHAM animal.

2.2.2. Electrophysiological Analysis

Paired-Pulse Measurements

Paired pulse ratios were determined by dividing the average slope of the second pulse over that of the first.

Input/Output (I/O) curve Analysis

I/O curves were created through the comparison of the initial fEPSPs slopes to the stimulation pulse width (from 30-300 μs). All data is represented as the mean ± the standard error of the mean (SEM).

Synaptic Plasticity Analysis

For all synaptic plasticity experiments, the fEPSP slopes from the pre-conditioning recordings (the last 20 minutes of the recording) were established and used to calculate an average pre-conditioning slope as a reference for all slope measurements. For post-conditioning recordings, they were expressed as a percentage change from the average pre-conditioning slope. The assessment of short-term and long-term plasticity was done by

averaging the percentage change in the post-conditioning recordings from 0-1 minute and 55-60 minutes respectively.

Inclusion Criteria:

To make accurate synaptic plasticity comparisons, starting with a steady baseline recording is important. The slices were determined to be stable when the fEPSP slope didn't deviate more than ±10% of the average fEPSP slope, and when there was no slope drift (the line of best-fit slope didn't exceed ±0.5). In addition, to confirm any changes in synaptic plasticity magnitudes, the fEPSP slopes during the pos-conditioning recording (minutes 55-60) had to be stable. If the recordings are unstable, it can lead to inaccurate average values that do not truly reflect the stability or magnitude of synaptic plasticity, and this could suggest an experimental setup that is not stable. Therefore, only slices that maintained a stable fEPSP slope (line of best-fit slope didn't exceed ±1.5) from minutes 55-60 were included in the analysis. The data is displayed as the mean ± SEM.

3. Chapter 3 – Results

3.1. Consciousness Assessment and Neurological Assessment Protocol

Each animal underwent a loss of consciousness (LOC) assessment immediately after each ACHI or SHAM procedure and this assessment consisted of the observations of apnea and righting reflexes. For all animals tested, none exhibited any indication of LOC given the observation of these two indicators and therefore a graphical representation of this data is not presented in this thesis.

Following each ACHI or SHAM protocol, animals were subjected to 4 sensorimotor and reflex tasks which include: startle response, limb extension, beam walk and rotating beam. Each of these individual tasks was scored on a scale of 0 to 3, with 0 being a complete failure at the task and 3 being a perfect score. A total NAP score was calculated out of 12 by summing the scores of the 4 individual tasks. A Geisser-Greenhouse corrected repeated measures ANOVA was performed on non-spherical data which revealed significant effects of treatment (SHAM or injury) ($F(1,49) = 100.37$, $p < 0.001$, $\eta^2 = 0.672$). These findings suggest that behaviour performance declines in injury animals compared to SHAM animals (**Figure 12**).

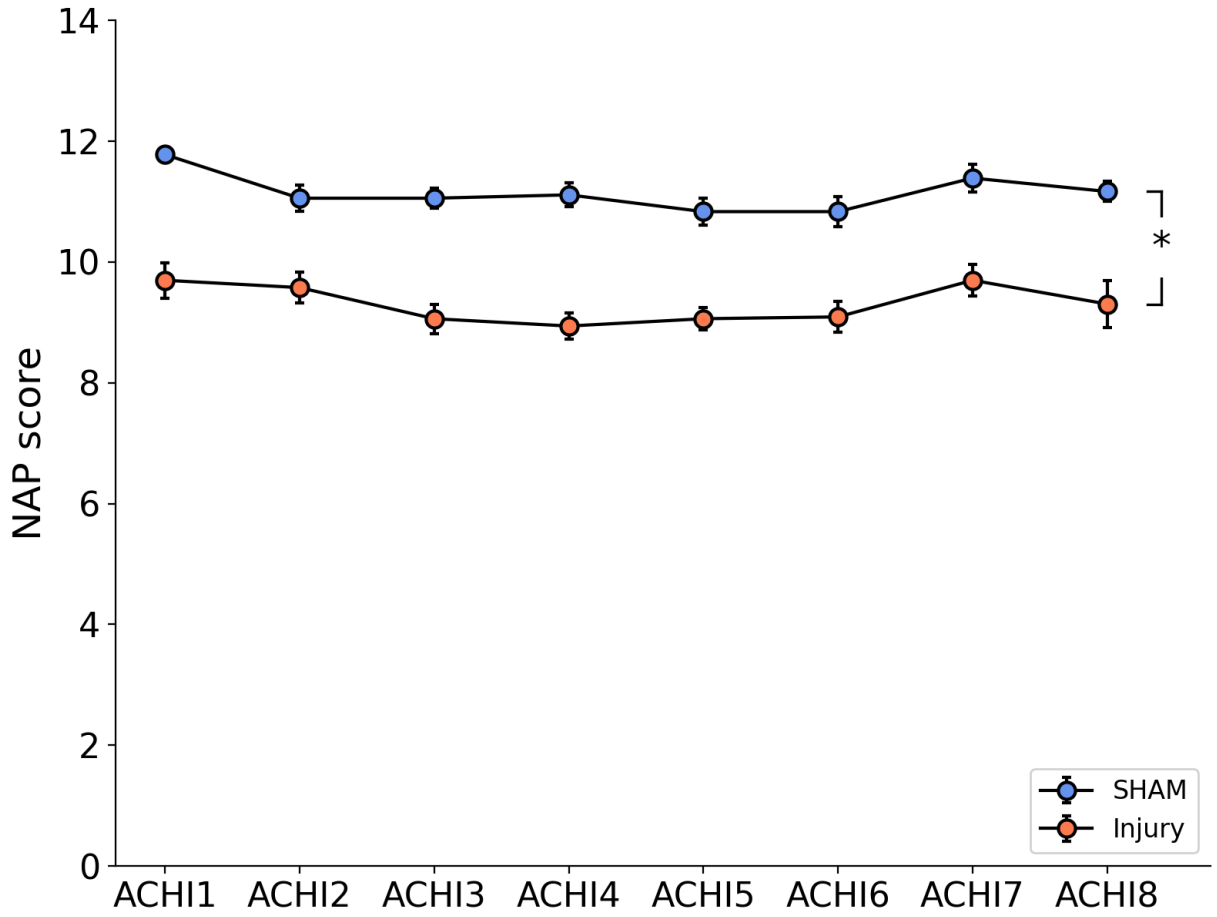


Figure 12. Neurological Assessment Protocol shows acute neurological impairment after r-mTBI.

*Animals that underwent ACHI show a significantly lower NAP score across all ACHIs as compared to their SHAM counterparts. Statistical test: Two-way repeated measures ANOVA with Geisser-Greenhouse correction for sphericity). Bars represent the standard error of the mean (SEM); * Represents that injured animals (ACHI scores) are significantly different from SHAM.*

3.2. Paired Pulse Plasticity

The purpose of examining paired-pulse plasticity was to investigate whether r-mTBI had any effects on neurotransmitter release. Paired pulse stimulation was elicited through six pairs of pulses 50 ms apart and the ratio was calculated as the percentage change in the slope of the second EPSP (pulse 2) over the slope of the initial EPSP (pulse 1). **Figure 13** shows that our results indicate that 8 r-mTBI impacts do not significantly affect the paired-pulse plasticity.

PPR data were analyzed using a one-way ANOVA. R-mTBI animals did not show significant differences between groups on PID1 (**Figure 13A.** F (2,153) = 2.08, p = 0.129, η_p^2 = 0.026) or on PID7 (**Figure 13B.** F (2,121) = 0.40, p = 0.68, η_p^2 = 0.006).

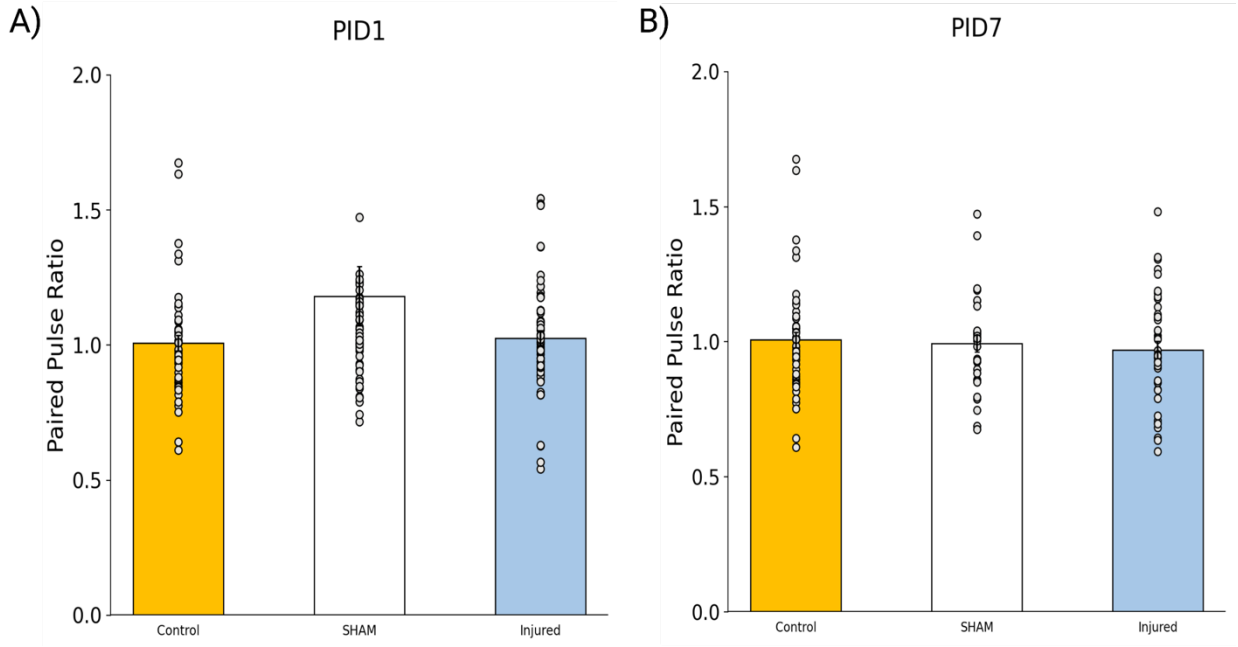


Figure 13. Paired pulse plasticity is not significantly affected by r-mTBI

(A, B) *The ratios of the slopes of the second pulse relative to the slopes of the first pulse were found for Control, SHAM and injured animals and compared through one-way ANOVA analysis. Results show that there are no significant differences between paired-pulse plasticity at post-injury day 1 (A) or post-injury day 7 (B)*

3.3. Input – Output (I/O) Functions

Before measuring long-term plasticity, I/O curves were investigated to analyze whether 8 r-mTBI impacts influenced the responsiveness of postsynaptic neurons. This study employed a consistent stimulation intensity while gradually increasing the pulse width (ranging from 30 to 300 µs at 15-second intervals) to generate the I/O curves. These curves were used to evaluate the response of the slice to increasing stimulation, which in turn allows the interpretation of slice health. The results of the I/O curves can be found in **Figure 14**.

R-mTBI did not significantly affect the ability of slices to react to increasing pulse width one-day post-injury (**Figure 14A.** F (2,149) = 1.92, p = 0.150, η_p^2 = 0.025) or seven days post-injury (**Figure14B.** F (2,118) = 2.78, p = 0.066, η_p^2 = 0.045).

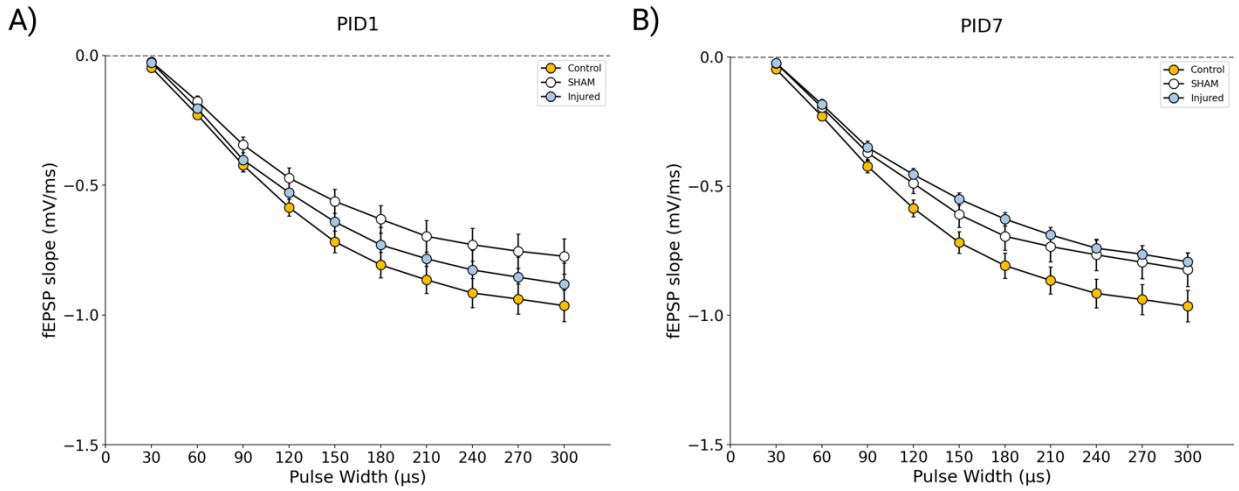


Figure 14. r-mTBI does not affect post-synaptic responsiveness to increasing stimulation

(A, B) *Increasing the pulse width does not significantly alter post-synaptic responsiveness in animals after r-mTBI on PID1 (A) or on PID7. A repeated measures ANOVA was used to compare I/O curves and the individual points represent the average fEPSP slopes by group and error bars are represented as the standard error of the mean (SEM).*

3.4. Lateralization of injury – Is there an Ipsilateral or Contralateral difference in the amount of synaptic plasticity recorded?

The nature of the ACHI model is that injury to the animal is sustained on the left hemisphere of the skull. This lateralization of injury may induce a bias in the amount of synaptic plasticity recorded (i.e., secondary injury cascades being more prominent on the left) from the ipsilateral hemisphere (hemisphere that was injured) compared to the contralateral hemisphere (uninjured hemisphere). The analysis of ipsilateral and contralateral hemispheres was thus conducted to elucidate any potential lateralization bias.

3.4.1. Long-Term Potentiation

To investigate the impact of r-mTBI on LTP in the DG, both injured and uninjured animals were tested for their ability to exhibit PTP and LTP at 1 day and 7 days post-injury. Slices were separated into two different holding chambers to keep track of which slices came from which

hemisphere. LTP was induced by stimulating the MPP with an HFS protocol consisting of 4 trains of 50 pulses at 100 Hz, with a 30-second interval. The degree of LTP was assessed by calculating the average fEPSP responses during the last 5 minutes, while PTP was measured during the first minute after the conditioning stimulus. As shown in **Figure 15** and **Figure 16**, the results implicate that there are no significant differences in the amount of LTP recorded in the ipsilateral or contralateral hemispheres on PID1 or PID7.

A 2-way ANOVA analysis indicated that there are no significant differences between hemispheres on PID1 (**Figure 15B.** $F(1,36) = 0.28$, $p = 0.60$, $\eta_p^2 = 0.008$) or on PID7 (**Figure 16B.** $F(1,20) = 0.06$, $p = 0.81$, $\eta_p^2 = 0.003$). Moreover, no significant differences were observed between the SHAM and injury groups in relation to the hemisphere on PID1 (**Figure 15B.** $F(1,36) = 0.23$, $p = 0.63$, $\eta_p^2 = 0.006$) or on PID7 (**Figure 16B.** $F(1,20) = 1.95$, $p = 0.17$, $\eta_p^2 = 0.089$). The magnitude of PTP in relation to hemisphere and protocol (SHAM or injury) for both PID1 (**Figure 15A.** $F(1,36) = 0.022$, $p = 0.88$, $\eta_p^2 = 5.98 \times 10^{-4}$) and PID7 (**Figure 16A.** $F(1,20) = 0.067$, $p = 0.80$, $\eta_p^2 = 0.003$) is not significantly affected.

Table 1. Long-Term Potentiation by hemisphere PID1: average STD, LTD and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM Ipsilateral	151.76 ± 17.10	106.79 ± 14.43	N = 5, n = 8
SHAM Contralateral	139.95 ± 14.88	94.24 ± 10.89	N = 6, n = 9
Injury Ipsilateral	131.49 ± 18.34	79.60 ± 10.72	N = 5, n = 12
Injury Contralateral	114.32 ± 19.10	77.9 ± 12.86	N = 8, n = 11

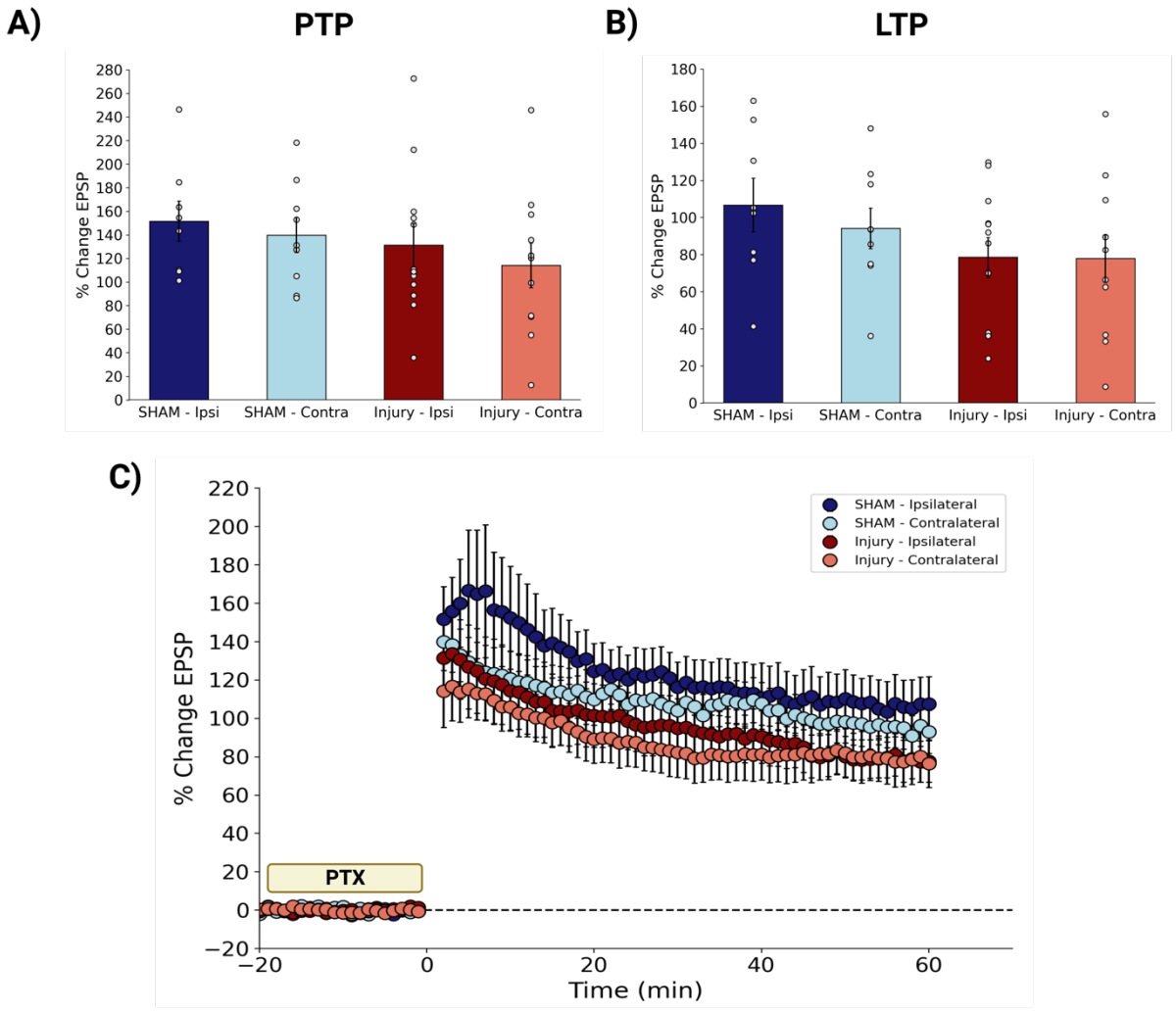


Figure 15. Long-Term Potentiation (LTP) shows no significant differences between hemispheres on post-injury day 1 (PID1)

(A) *Post-Tetanic Potentiation (PTP) is measured as the percent change in the fEPSP slope after the first minute following the high-frequency stimulus (HFS; 4 trains-50 pulses at 100Hz; 30s intervals). R-mTBI does not significantly affect PTP.* ***(B)*** *Long-Term potentiation (LTP) was measured as the average percent change in the fEPSP slope relative to its baseline in the last 5 minutes following HFS (55-60 minutes). It was found that there are no significant lateralization effects in LTP following r-mTBI induced through the ACHI model on post-injury day 1.* ***(C)*** *The average traces of LTP recordings from the beginning of the baseline to the end of post-conditioning recording for post injury day 1 recordings for injury contralateral and ipsilateral hemispheres and their SHAM counterparts (SHAM – ipsilateral and SHAM – contralateral). All error bars represent the standard error of the mean (SEM). All comparisons were analyzed using a two-way ANOVA and individual points for PTP (A) and LTD (B) represent the average PTP or LTP for each individual slice in this dataset. PTX indicates that the $GABA_A$ antagonist PTX was washed over the recorded slices throughout the duration of the baseline recording.*

Table 2. Long-Term Potentiation by hemisphere PID7: average STD, LTD and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM Ipsilateral	120.69 ± 7.25	103.40 ± 14.63	N = 3, n = 6
SHAM Contralateral	140.55 ± 8.44	76.72 ± 24.06	N = 3, n = 4
Injury Ipsilateral	123.56 ± 20.52	56.39 ± 12.06	N = 6, n = 6
Injury Contralateral	133.72 ± 20.86	75.12 ± 14.63	N = 4, n = 8

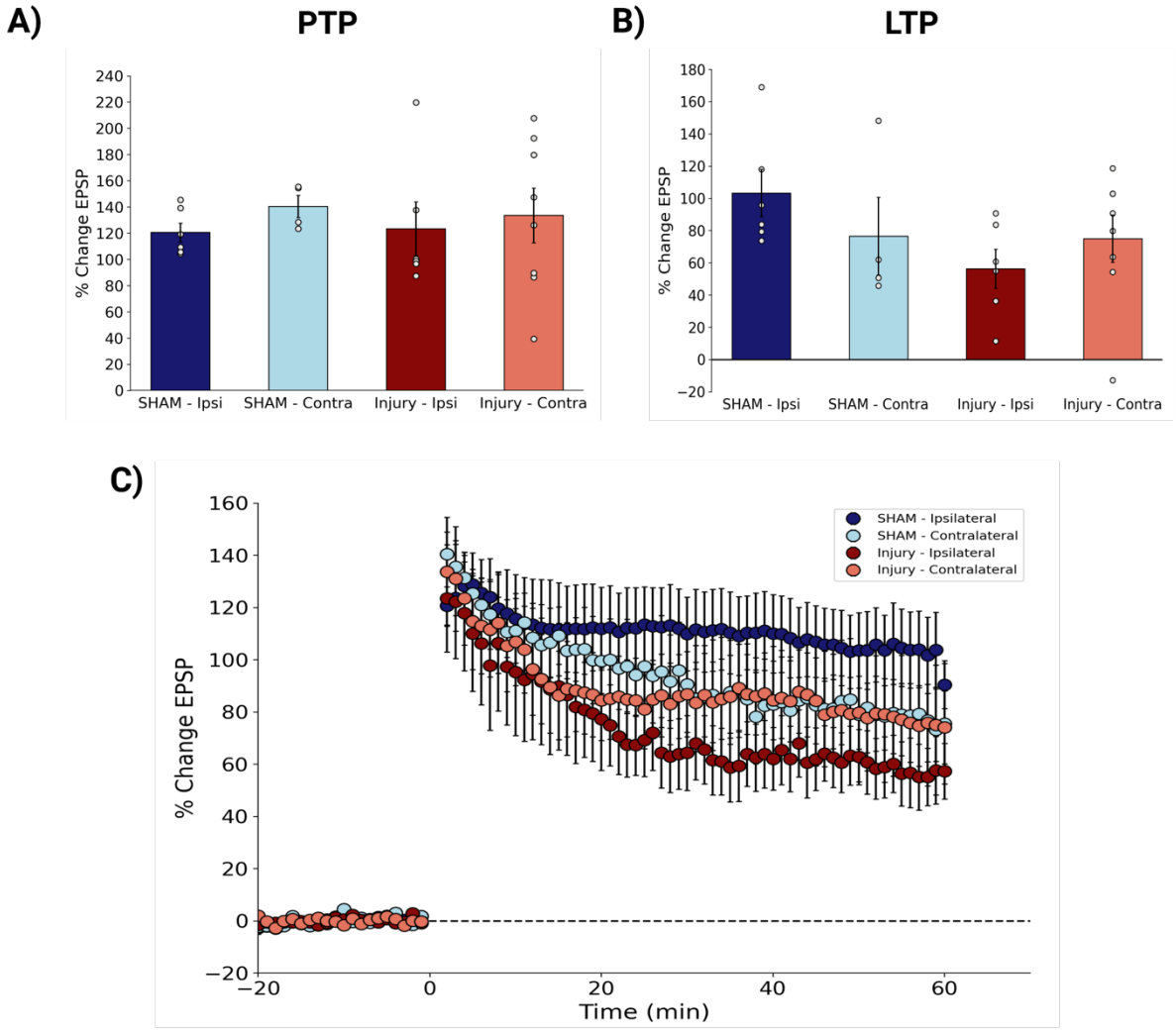


Figure 16. Long-Term Potentiation (LTP) shows no significant differences between hemispheres on post-injury day 7 (PID7)

(A) *Post-Tetanic Potentiation (PTP) is measured as the percent change in the fEPSP slope after the first minute following the high-frequency stimulus (HFS; 4 trains-50 pulses at 100Hz; 30s intervals). R-mTBI does not significantly affect PTP.* ***(B)*** *Long-Term*

potentiation (LTP) was measured as the average percent change in the fEPSP slope relative to its baseline in the last 5 minutes following HFS (55-60 minutes). It was found that there are no significant lateralization effects in LTP following r-mTBI induced by the ACHI model on post-injury day 7. ***(C)*** *The average traces of LTP recordings from the beginning of the baseline to the end of post-conditioning recording for post-injury day 7 recordings for injury contralateral and ipsilateral hemispheres and their SHAM counterparts (SHAM – ipsilateral and SHAM – contralateral). All error bars represent the standard error of the mean (SEM). All comparisons were analyzed using a two-way ANOVA and individual points for PTP (A) and LTD (B) represent the average PTP or LTP for each individual slice in this dataset. PTX indicates that the $GABA_A$ antagonist PTX was washed over the recorded slices throughout the duration of the baseline recording.*

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3.4.2. Long-Term Depression (1Hz - LTD and 10 Hz - LTD)

To assess the impact of r-mTBI on both 1 Hz - LTD and 10 Hz - LTD, animals with and without injury were compared based on their ability to exhibit STD and LTD at both PID1 and PID7. Slices were separated by hemisphere and LTD was elicited in the MPP of the DG using either the 900 x 1 Hz protocol (1 Hz - LTD) or the 6000 x 10 Hz protocol (10 Hz - LTD). The degree of LTD was evaluated by computing the average fEPSP responses during the last 5 minutes of the post-conditioning recording, while STD was measured in the first minute following the conditioning stimulus. **Figure 17**, **Figure 18**, **Figure 19** and **Figure 20** present that there are no significant differences in the amount of either type of LTD elicited in the ipsilateral or contralateral hemisphere on PID1 or PID7.

In the case of 1 Hz - LTD, the results of a 2-way ANOVA indicated that there are no significant differences between hemispheres on PID1 (**Figure17B.** $F(1,26) = 3.93$, $p = 0.06$, $\eta_p^2 = 0.128$). The capacity to elicit STD after SHAM or injury in both hemispheres was also not significantly affected on PID1 (**Figure 17A.** $F(1,21) = 0.68$, $p = 0.42$, $\eta_p^2 = 0.032$). Due to the lack of observations in the SHAM–ipsilateral group for PID7, an ANOVA was not run for LTD or STD data. More data is required for the group ipsilateral SHAM-PID7 to elucidate any significant differences between groups (Figure 18).

Results from 10 Hz - LTD data indicated that the amount of LTD recorded per hemisphere was not significantly different on PID1 (**Figure19B.** $F(1,38) = 0.082$, $p = 0.78$, $\eta_p^2 = 0.002$) or PID7 (**Figure20B.** $F(1,34) = 1.01$, $p = 0.32$, $\eta_p^2 = 0.029$). 1-day post-injury (**Figure 19B.** $F(1,38) = 27.0$, $p < 0.001$, $\eta_p^2 = 0.41$), there was a significant difference in LTD recorded in SHAM and injury groups, however, this difference was not seen in PID7 (**Figure20B.** $F(1,34) = 0.45$, $p =$

0.51, $\eta_p^2 = 0.013$). STD was not significantly affected by hemisphere in SHAM or injury groups on PID1 (**Figure19A.** F (1,37) = 0.68, p = 0.41, $\eta_p^2 = 0.018$) or PID7 (**Figure19B.** F (1,30) = 0.0.016, p = 0.90, $\eta_p^2 = 5.37 \times 10^{-4}$).

Note, dorsal and ventral differences were also analyzed for all conditioning stimuli elicited. No significant differences were elucidated, except for 10 Hz - LTD on PID7 and the data can be found in Appendix A. While there was a difference in the dorsal and ventral region in 10 Hz - LTD on PID7, the sample size was small and further investigation would be needed to extract definite differences in dorsal and ventral regions in this group which is outside of the scope of this thesis.

Table 3. 1 Hz Long-Term Depression by hemisphere PID1: average STD, LTD and slice & animal count

Group	STD	LTD	Animal (N) and slice (n) values
SHAM Ipsilateral	-36.53 ± 3.62	-5.84 ± 3.86	N = 5, n = 7
SHAM Contralateral	-35.00 ± 6.57	-16.23 ± 6.25	N = 5, n = 5
Injury Ipsilateral	-24.20 ± 4.06	-10.34 ± 10.25	N = 2, n = 4
Injury Contralateral	-31.65 ± 5.13	-16.67 ± 7.13	N = 6, n = 9

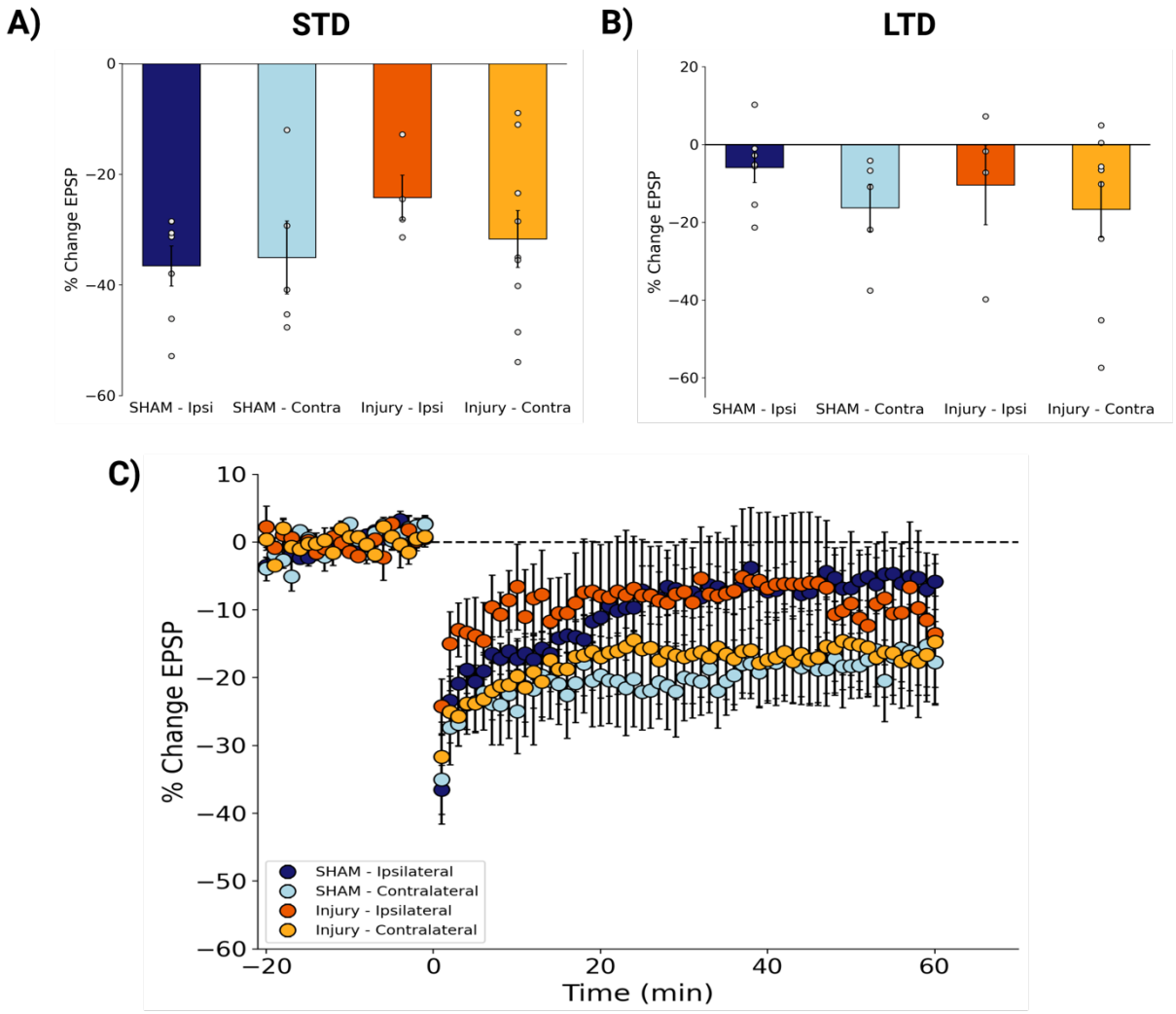

Figure 17. 1 Hz - Long-Term Depression (LTD) shows no significant differences between hemispheres on post-injury day 1 (PID1)

(A) *Short-Term Depression (STD) is measured as the percentage change in fEPSP slope in the first minute following the low-frequency stimulus (LFS; 900 x 1 Hz). Results showed no significant differences in STD across groups.* ***(B)*** *LTD was measured as the average percentage change in the fEPSP slope relative to its baseline during the last five minutes following LFS (55-60 minutes). Statistical analysis showed that there are no significant lateralization effects in 1 Hz - LTD on post-injury day 1.* ***(C)*** *The average traces of LTD recordings from the beginning of the baseline to the end of the post-conditioning recording for the injury condition in both ipsilateral and contralateral hemispheres are plotted along with their SHAM counterparts (SHAM – ipsilateral and SHAM – contralateral). All error bars represent the standard error of the mean (SEM), and all comparisons were analyzed using a two-way ANOVA. Individual points for STD (A) and LTD (B) represent the average STD or LTD for each individual slice in this dataset.*

Table 4. 1 Hz - Long-Term Depression by hemisphere PID7: average STD, LTD and slice & animal count

Group	STD	LTD	Animal (N) and slice (n) values
SHAM Ipsilateral	-33.69 ± 2.42	8.19 ± 3.58	N = 2, n = 2
SHAM Contralateral	-33.17 ± 5.45	-19.92 ± 4.12	N = 3, n = 4
Injury Ipsilateral	-31.17 ± 7.39	-0.54 ± 7.98	N = 3, n = 4
Injury Contralateral	-33.32 ± 4.81	-13.61 ± 6.10	N = 4, n = 6

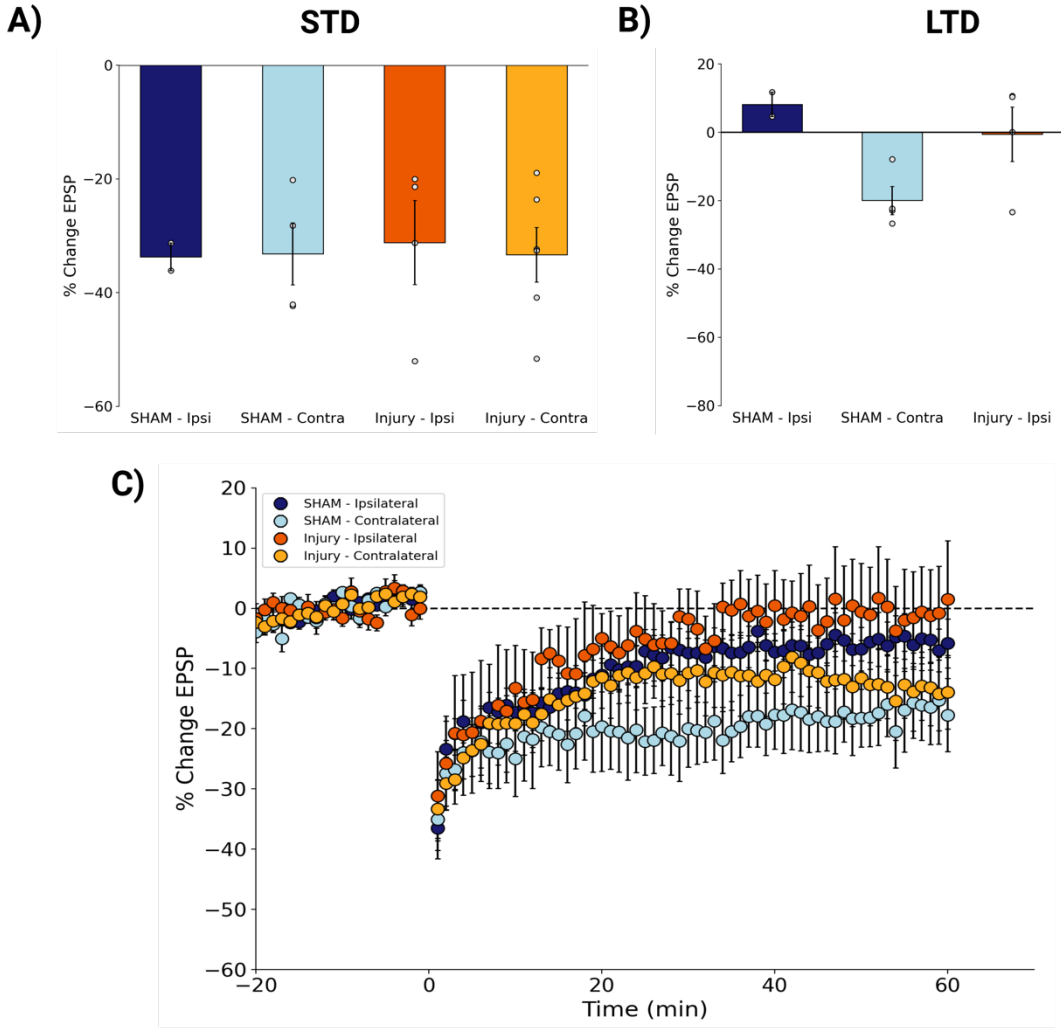

Figure 18. 1 Hz - LTD is potentially unaffected between hemispheres on post-injury day 7 (PID7)

(A) *Short-Term Depression (STD) is measured as the percentage change in fEPSP slope in the first minute following the low-frequency stimulus (LFS; 900 x 1 Hz).* ***(B)*** *LTD was measured as the average percentage change in the fEPSP slope relative to its baseline during the last five minutes following LFS (55-60 minutes).* ***(C)*** *The average traces of LTD recordings from the beginning of the baseline to the end of the post-conditioning recording for the injury condition in both ipsilateral and contralateral hemispheres are plotted along with their SHAM counterparts (SHAM – ipsilateral and SHAM – contralateral). All error bars represent the standard error of the mean (SEM). Individual points for STD (A) and LTD (B) represent the average STD or LTD for each individual slice in this dataset. Note: Unfortunately, lack of data in the group SHAM – ipsilateral prevented statistical analysis, in that the effect size may affect statistical power.*

Table 5. 10 Hz - Long-Term Depression by hemisphere PID1: average STD, LTD and slice & animal count

Group	STD	LTD	Animal (N) and slice (n) values
SHAM Ipsilateral	-89.69 ± 4.73	-54.67 ± 8.62	N = 6, n = 13
SHAM Contralateral	-87.96 ± 3.81	-31.83 ± 8.86	N = 5, n = 8
Injury Ipsilateral	-87.41 ± 4.76	7.71 ± 5.98	N = 7, n = 11
Injury Contralateral	-93.50 ± 4.41	-6.42 ± 5.03	N = 5, n = 9

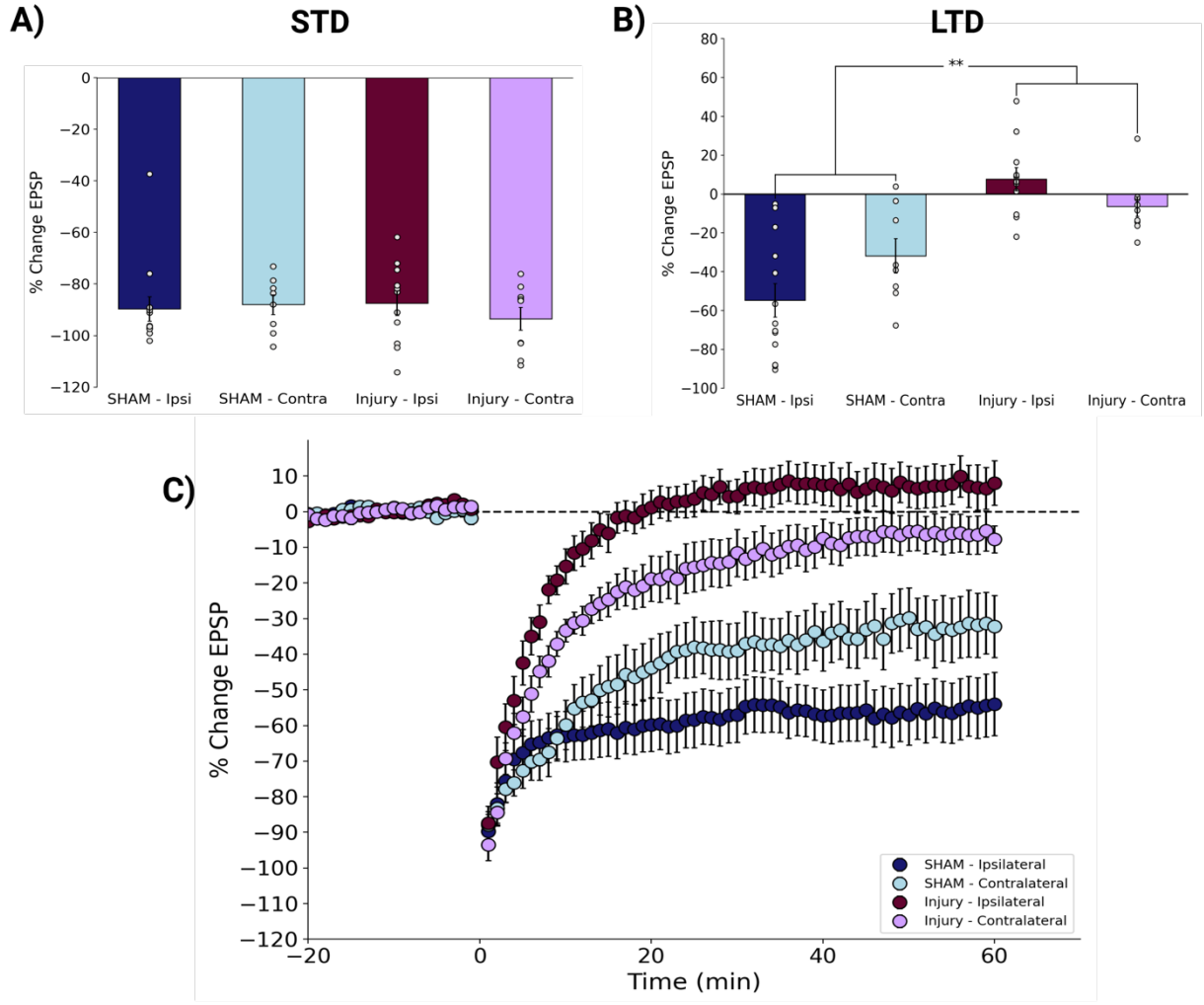

Figure 19. 10 Hz - LTD shows no significant differences between hemispheres on post-injury day 1 (PID1)

(A) *The percentage change in fEPSP slope during the first minute after the low-frequency stimulus (6000 x 10 Hz) was used to measure short-term depression (STD), and there were no significant differences observed among the groups.* ***(B)*** *To measure long-term depression (LTD), the average percentage change in fEPSP slope relative to the baseline during the last 5 minutes of the LFS (55-60 minutes) was calculated, and while there were no significant differences in lateralization of injury on post-injury day 1, there was a significant difference between injury and SHAM groups.* ***(C)*** *The average LTD traces were recorded from the beginning of the baseline to the end of the post-conditioning recording for ipsilateral and contralateral hemispheres in injury animals and for their respective non-injured counterparts (SHAM – ipsilateral and SHAM – contralateral). The standard error of the mean (SEM) is represented by black bars. A two-way ANOVA was used to analyze all comparisons, and individual points for STD (A) and LTD (B) show the average STD or LTD for each slice in this dataset. ** indicates a $p < 0.001$.*

Table 6. 10 Hz - Long-Term Depression by hemisphere PID7: average STD, LTD and slice & animal count

Group	STD	LTD	Animal (N) and slice (n) values
SHAM Ipsilateral	-93.07 ± 1.69	-24.87 ± 11.58	N = 4, n = 8
SHAM Contralateral	-98.21 ± 2.74	-51.20 ± 13.35	N = 4, n = 8
Injury Ipsilateral	-91.19 ± 19	-32.16 ± 11.74	N = 5, n = 10
Injury Contralateral	-95.72 ± 2.69	-28.71 ± 8.73	N = 6, n = 8

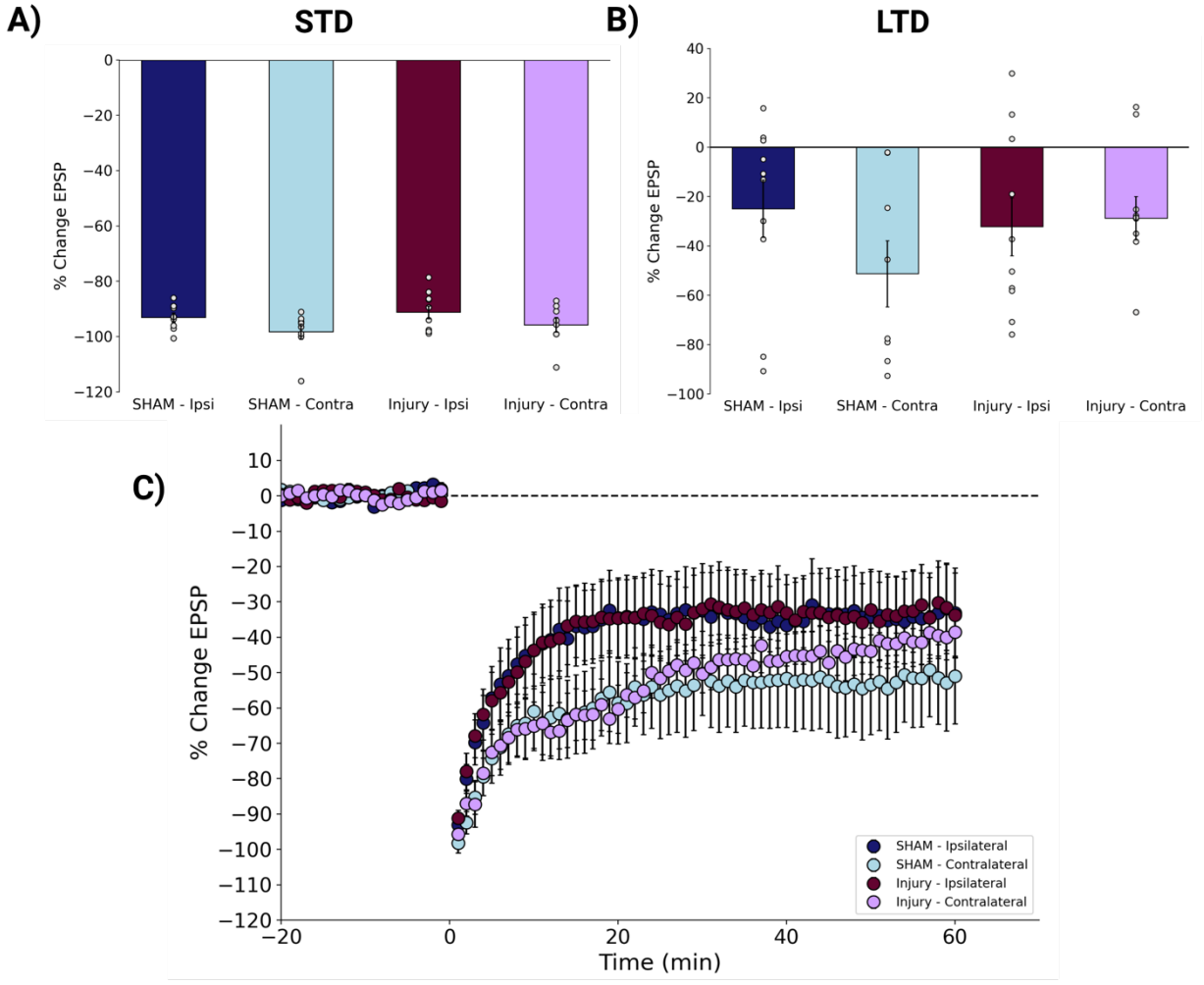

Figure 20. 10 Hz - LTD shows no significant differences between hemispheres on post-injury day 7 (PID7)

(A) *The percentage change in fEPSP slope during the first minute after the low-frequency stimulus (6000 x 10 Hz) was used to measure short-term depression (STD), and there were no significant differences observed among the groups.* ***(B)*** *To measure long-term depression (LTD), the average percentage change in fEPSP slope relative to the baseline during the last 5 minutes of the LFS (55-60 minutes) was calculated, and no significant differences were observed between hemispheres on post-injury day 7.* ***(C)*** *The average LTD traces were recorded from the beginning of the baseline to the end of the post-conditioning recording for ipsilateral and contralateral hemispheres in injury animals and for their respective non-injured counterparts (SHAM – ipsilateral and SHAM – contralateral). The standard error of the mean (SEM) is represented by black bars. A two-way ANOVA was used to analyze all comparisons, and individual points for STD (A) and LTD (B) show the average STD or LTD for each slice in this dataset.*

3.5. Synaptic plasticity after injury – Does synaptic plasticity after r-mTBI ameliorate over time? (PID1 → PID7)

As there was no significance between the ipsilateral and contralateral hemispheres in rats subjected to 8 r-mTBI impacts, the data from both hemispheres were combined based on the conditioning stimulus and then divided into time intervals (PID1 or PID7) and injury or uninjured groups.

3.5.1. Long-Term Potentiation

To assess whether LTP in the DG was affected after 8 r-mTBI impacts, the ability to display PTP and LTP was evaluated in animals with injuries and without at both 1 day and 7 days after the injury. The MPP of the DG was stimulated to induce LTP via an HFS protocol comprising 4 trains of 50 pulses at 100 Hz, with a 30-second interval. LTP was measured by averaging fEPSP responses for the last 5 minutes (LTP) and PTP was measured in the first minute after the conditioning stimulus. As depicted in **Figure 21**, the results suggest that 8 r-mTBI impacts significantly affect the overall magnitude of LTP recorded between SHAM and injured animals.

A two-way ANOVA was used to examine the impact of both time (PID1 and PID7) and protocol assigned (SHAM or Injury) on LTP. The main effect of protocol (whether the animal was injured or not) had a statistically significant effect on the amount of LTP measured (last 5 minutes) (**Figure21B.** $F(1,60) = 5.15$, $p = 0.03$, $\eta_p^2 = 0.08$), however, LTP did not ameliorate or change significantly over time (**Figure21B.** $F(1,60) = 0.007$, $p = 0.94$, $\eta_p^2 = 1.13 \times 10^{-4}$). The magnitude of PTP was also unaffected by the 8 r-mTBI impacts (or 'impacts') over time (**Figure21A.** $F(1,60) = 0.72$, $p = 0.40$, $\eta_p^2 = 0.01$).

Table 7. Long-Term potentiation over time: average PTP, LTP and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM PID1	145.51 ± 11.00	100.16 ± 8.76	N = 9, n = 17
SHAM PID7	128.63 ± 6.13	92.73 ± 12.94	N = 5, n = 10
Injury PID1	123.28 ± 12.05	78.30 ± 8.12	N = 11, n = 23
Injury PID7	129.37 ± 14.33	69.25 ± 10.00	N = 7, n = 14

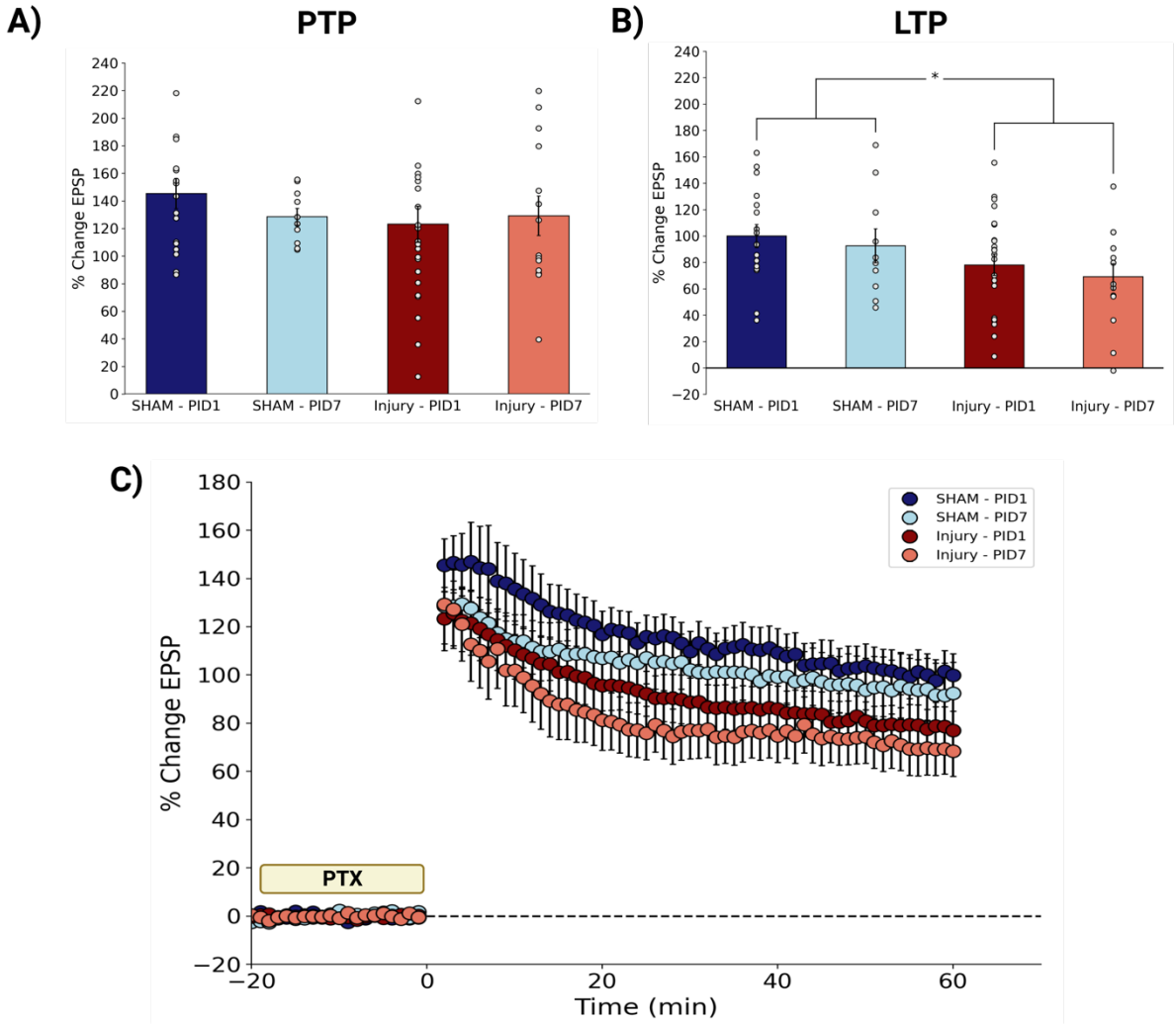

Figure 21. Long-Term Potentiation (LTP) shows significant differences between SHAM and Injury groups

(A) *Post-Tetanic Potentiation (PTP) is measured as the percentage of change in the fEPSP slope in the first minute immediately following the high-frequency conditioning stimulus (HFS; 4 trains-50 pulses at 100Hz; 30s intervals). PTP showed no significant*

*differences over time. (**B)** LTP was measured as the average percentage change in the fEPSP slope relative to its baseline during the last 5 minutes following HFS (55-60 minutes). While there was no significant difference in LTP after r-mTBI over time, LTP in SHAM groups was significantly different from injury groups. (**C)** The average traces of LTP recordings from the beginning of the baseline to the end of the post-conditioning recording at both post-injury days 1 and 7 (PID1 and PID7) and their respective non-injured counterparts (SHAM – PID1 and SHAM – PID7). PTX indicates that the drug was washed over the slice throughout the duration of the baseline (20min). All error bars represent the standard error of the mean (SEM). All comparisons were analyzed using a two-way ANOVA and individual points for PTP (A) and LTP (B) represent the average STD and LTD for each individual slice in this dataset Significance is denoted as * = $p < 0.05$*

3.5.2. Long-Term Depression (1 Hz - LTD and 10 Hz - LTD)

The examination of whether LTD was affected after r-mTBI was performed by analyzing the capacity to exhibit STD and LTD and then comparing injured and uninjured animals at PID1 and PID7. LTD was induced in the MPP of the DG and was done so through two different conditioning stimuli. 1 Hz - LTD was elicited through an LFS protocol of 900 x 1 Hz while 10 Hz - LTD was elicited through the LFS protocol 6000 x 10 Hz. LTD was measured by averaging fEPSP responses over the last 5 minutes of the post-conditioning recording and STD was measured in the first minute after the conditioning stimulus. The data presented in **Figure 22** indicates that there is no significant difference in 1 Hz - LTD due to the effect of 8 r-mTBI impacts over time, while the data presented in **Figure 23** shows that there is a significant reduction in eCB – dependent LTD that ameliorates over time after r-mTBI.

An examination using a 2-way ANOVA revealed that SHAM compared injured animals exhibited no significant change in 1 Hz - LTD (**Figure 22B.** $F(1,47) = 0.08$, $p = 0.78$, $\eta_p^2 = 0.002$). Additionally, the amount of this type of LTD remained significantly unaltered over time (**Figure 22B.** $F(1,47) = 2.02$, $p = 0.16$, $\eta_p^2 = 0.041$). The capacity of slices to elicit STD was also unaffected by r-mTBI over time (**Figure 22A.** $F(1,37) = 0.54$, $p = 0.48$, $\eta_p^2 = 0.014$).

Results from the 10 Hz - LTD data show that there is a significant difference in the amount of LTD measured (last 5 minutes) dependent on which protocol animals underwent (injured or uninjured SHAM) (**Figure 23B.** $F(1,76) = 13.79$, $p < 0.001$, $\eta_p^2 = 0.15$). The amount of LTD after r-mTBI is significantly different depending on the length of time post-injury (**Figure 23B.** $F(1,76) = 7.97$, $p = 0.006$, $\eta_p^2 = 0.095$). Post-Hoc analysis indicates injured animals one-day post-injury are significantly affected ($p < 0.01$) and that this deficit ameliorates seven days post-injury ($p =$

0.009). The capacity of slices to elicit STD was unaffected by r-mTBI over time (**Figure 23A.** $F(1,71) = 0.41$, $p = 0.52$, $\eta_p^2 = 0.006$).

Table 8. 1 Hz - Long-Term depression over time: average STD, LTD and slice & animal count

Group	STD	LTD	Animal (N) and slice (n) values
SHAM PID1	-35.89 ± 3.28	-10.17 ± 3.58	N = 8, n = 12
SHAM PID7	-33.34 ± 3.51	-10.55 ± 6.54	N = 3, n = 6
Injury PID1	-29.36 ± 3.8	-14.73 ± 5.68	N = 7, n = 13
Injury PID7	-32.46 ± 3.89	-8.38 ± 5.05	N = 6, n = 10

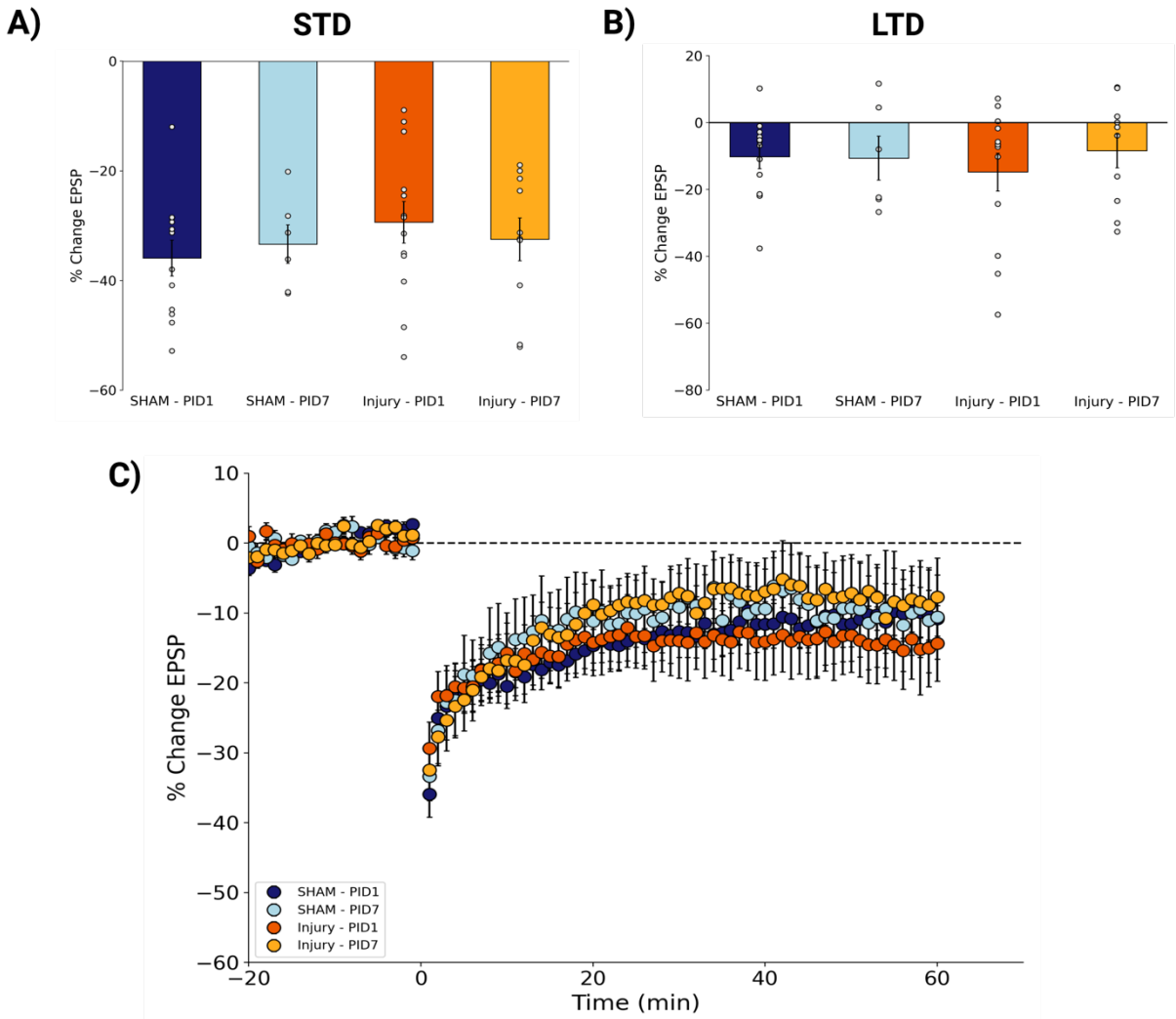

Figure 22. 1 Hz - LTD shows no significant differences over time

(A) *Short-Term Depression (STD) is measured as the percentage of change in the fEPSP slope in the first minute immediately following the low-frequency stimulus (LFS; 900 x 1 Hz). STD showed no differences across groups.* ***(B)*** *LTD was measured as the average percentage change in the fEPSP slope relative to its baseline during the last 5 minutes following LFS (55-60 minutes). There were no significant differences elucidated in 1 H - LTD over time.* ***(C)*** *The average traces of LTD recordings from the beginning of the baseline to the end of the post-conditioning recording at both post-injury days 1 and 7 (PID1 and PID7) and their respective non-injured counterparts (SHAM – PID1 and SHAM – PID7). All error bars represent the standard error of the mean (SEM). All comparisons were analyzed using a two-way ANOVA and individual points for STD (A) and LTD (B) represent the average STD and LTD for each individual slice in this dataset.*

Table 9. 10 Hz - Long-Term depression over time: average STD, LTD and slice & animal count

Group	STD	LTD	Animal (N) and slice (n) values
SHAM PID1	-89.03 ± 3.20	-43.77 ± 6.71	N = 9, n = 22
SHAM PID7	-95.64 ± 1.69	-36.59 ± 9.07	N = 5, n = 18
Injury PID1	-90.15 ± 3.27	1.34 ± 4.21	N = 8, n = 20
Injury PID7	-93.21 ± 1.78	-30.44 ± 7.13	N = 8, n = 20

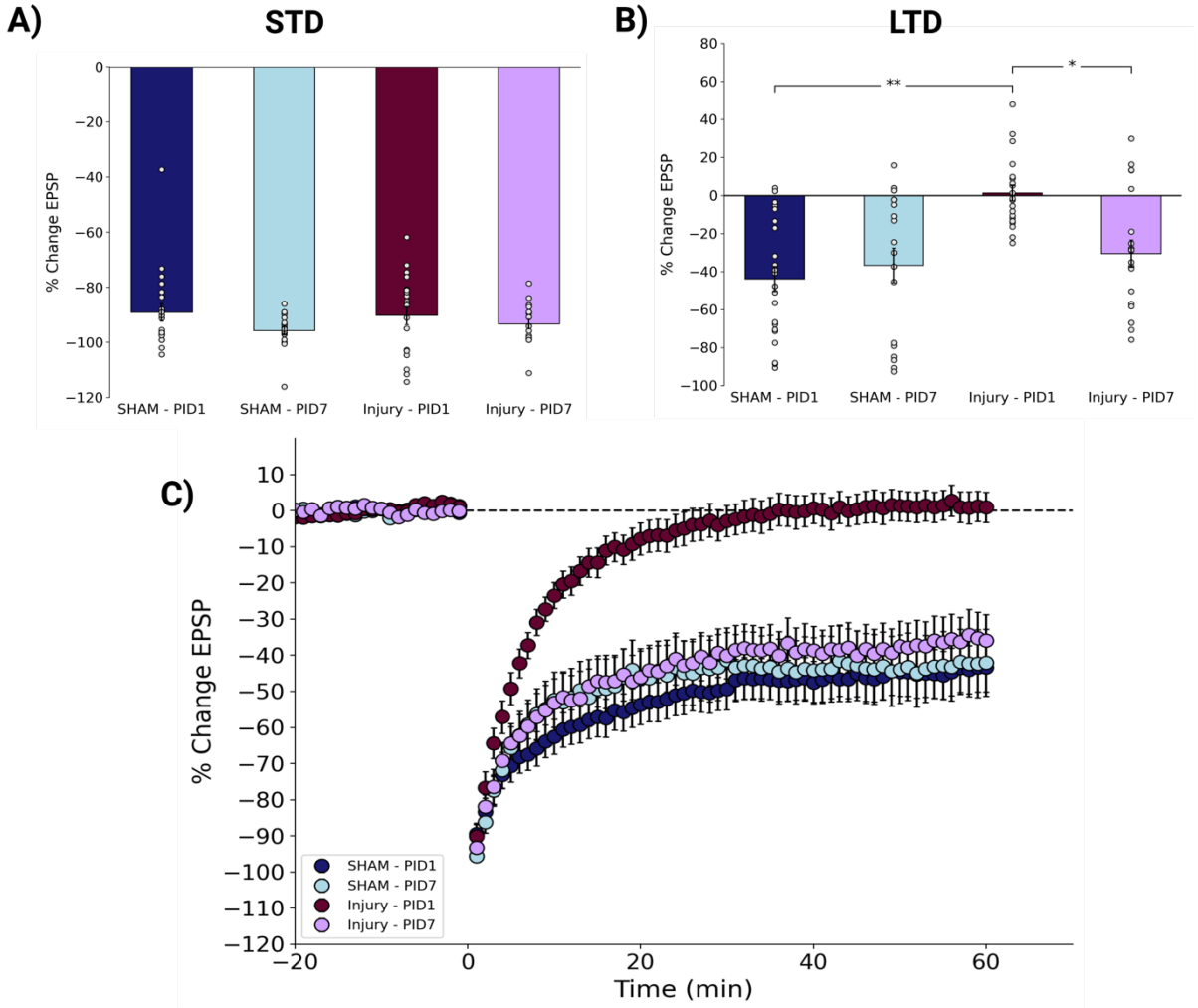

Figure 23. 10 Hz - LTD is significantly affected on post-injury day 1 (PID1) and ameliorates by post-injury day 7 (PID7)

(A) *Short-Term Depression (STD) is measured as the percentage of change in the fEPSP slope in the first minute immediately following the high-frequency conditioning stimulus (LFS; 6000 x 10 Hz). STD across groups showed no significant differences.* ***(B)*** *LTD was measured as the average percentage change in the fEPSP slope relative to its baseline during the last 5 minutes*

*following LFS (55-60 minutes). LTD was significantly affected one-day post-injury compared to its SHAM counterpart and ameliorated a week later (PID7) (**C)** The average traces of LTD recordings from the beginning of the baseline to the end of the post-conditioning recording at both post-injury days 1 and 7 (PID1 and PID7) and their respective non-injured counterparts (SHAM – PID1 and SHAM – PID7). This data shows the same as (B), whereby eCB-LTD is significantly affected on PID1 and ameliorates on PID7. All error bars represent the standard error of the mean (SEM). All comparisons were analyzed using a two-way ANOVA and individual points for STD (A) and LTD (B) represent the average STD and LTD for each individual slice in this dataset Significance is denoted as * = p < 0.05 and ** < 0.001*

3.5.3. Control and SHAM analysis

Aside from analyzing animals that underwent the same procedure as the injured animals but did not sustain any injury (referred to as SHAM animals), an analysis of control animals was also conducted. The purpose of comparing SHAM animals to control animals was to uncover any possible distinctions between animals that underwent a procedure and those that lived in standard conditions.

LTP and both LTD protocols analysis was conducted for both SHAM and Control animals. As detailed previously, LTP protocol was elicited LTP via an HFS protocol comprising 4 trains of 50 pulses at 100 Hz, with a 30-second interval and LTD was elicited by either an LFS of 900 x 1 Hz or 6000 x 10 Hz protocol. LTP or LTD was measured by averaging fEPSP responses over the last 5 minutes of the post-conditioning recording and PTP or STD was measured in the first minute after the conditioning stimulus. The data in **Figure 24** show that SHAM LTP and SHAM 1 Hz LTD differ significantly from their control counterparts while 10 Hz - LTD SHAM showed no difference compared to its control.

The analysis for SHAM compared to controls was conducted through two-tailed T–tests and is shown graphically in **Figure 24**. SHAM LTP (97.41 ± 7.19; n =27[14]) is significantly different from their control counterparts (46.18 ± 5.05; n = 15[7]; $p = 8.32 \times 10^{-7}$) (**Figure 24A**), and this held true as well in the comparison of 1 Hz - LTD SHAM animals (-10.30 ± 3.12; n = 16[9]) compared to controls (-22.17 ± 4.38; n = 18[9]; p = 0.03) (**Figure 24B**). 10 Hz - LTD SHAMs (-44.39 ± 5.41; n = 37[13]) are not significantly different from their control equivalents (-29.67 ± 6.49; n = 18[10]; p = 0.08) (**Figure24C**). The magnitude of PTP is significantly affected between groups (SHAM: 139.26 ± 7.36, Control: 83.35 ± 8.77; $p = 2.78 \times 10^{-5}$, **Figure24A (right)**) and STD

is not significantly affected for 1 Hz - LTD (SHAM: -35.04 ± 2.43, Control: -43.39 ± 6.13; p = 0.21, **Figure24B (left)**) and 10 Hz - LTD (SHAM: -91.88 ± 2.01, Control: -89.83 ± 2.24; p = 0.50, **Figure24C (right)**).

Note: data is expressed as: Mean % EPSP ± SEM, n = (number of slices [number of animals])

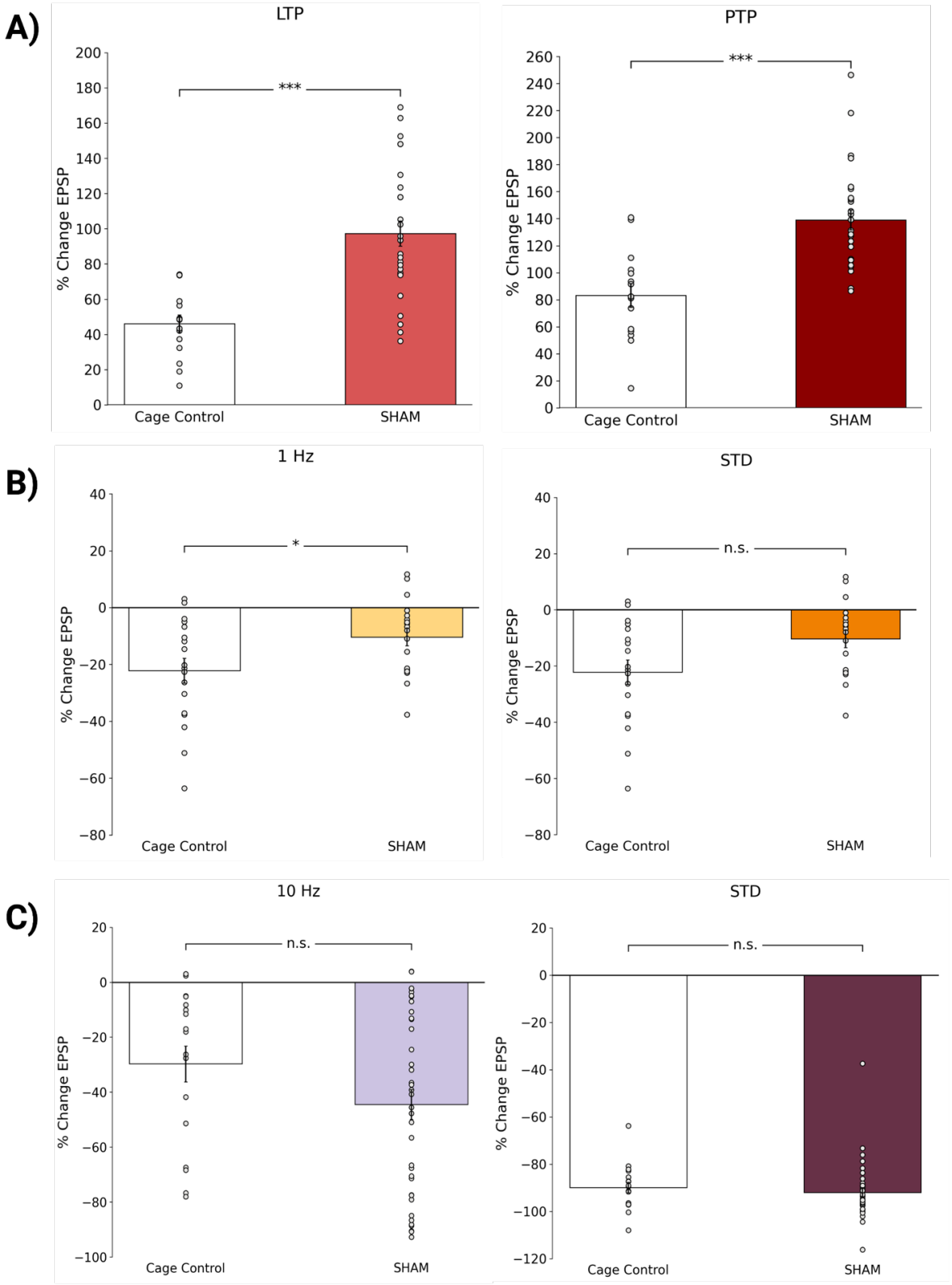

Figure 24. Cage Control compared to SHAM groups showed significant differences between slices that underwent 1 Hz and LTP stimulating conditions, but not 10 Hz

(A) *Long - Term Potentiation (LTP - left) was measured as the average percent change in the fEPSP slope relative to its baseline during the last five minutes (55-60 minutes) following a high-frequency stimulation (HFS; 4 trains-50 pulses at 100Hz; 30s intervals). Post-tetanic potentiation (PTP - right) was measured as the percentage of change in the fEPSP slope in the first minute immediately following the HFS. LTP and PTP were both significantly affected when comparing between groups.* ***(B)*** *1 Hz Long-Term Depression (LTD – left) was measured as the average percent change in the fEPSP slope relative to its baseline during the last five minutes (55-60 minutes) following a low-frequency stimulation (LFS; 900 x 1 Hz). Short-Term depression (STD - right) was measured as the percentage of change in the fEPSP slope in the first minute immediately following the LFS. 1 Hz - LTD was significantly different in the SHAM group compared to the control, while STD was not significantly affected.* ***(C)*** *10 Hz - Long-Term Depression (LTD - left) was measured identically to what was described in (B) for both LTD and PTP. LTD and PTP for 10 Hz - dependent LTD was not significantly affected between SHAM and control groups. All comparisons were analyzed using two-tailed t-tests and individual points for PTP, STD, LTP and LTD represent the average PTP, STD, LTP or LTD for each individual slice in the dataset. *** = p < 0.0001, * = p < 0.05, n.s = not significant.*

3.6. Do acute neurological deficits correlate to the amount of synaptic plasticity recorded?

The correlation between the average NAP score and the amount of LTP or LTD recorded was determined using a linear regression line. This analysis was performed to elucidate any significant relationships between the deficit animals experience after r-mTBI and the amount of synaptic plasticity recorded post-injury on day 1 or day 7.

The NAP consists of 4 tasks: startle response, limb extension, beam walk and rotating beam and each of these tests was scored on a scale of 0 to 3. A score of 3 indicates that the animal performed the task with no difficulty or impairment and 0 indicates a complete failure at the task. These tasks are then summed to give a score out of 12. For this analysis, a total NAP score was determined by summing the individual ACHI NAP scores. This means that now, the highest score for an animal is 96 (i.e., 12 (highest score) x 8 (8 impacts) = 96). The scores were then turned into a percentage, to allow for a more intuitive understanding of the data. The data were plotted on a scatter by pairing the % of NAP scores to their corresponding % Change EPSP data for either LTP or LTD. A linear regression line was plotted using python and the R^2 value was calculated to determine the correlation between the NAP score and the amount of LTP/LTD observed. An R^2 value closer to 1 indicates that there is a correlation between the dependent and independent variables, whereas 0 indicates there is no association between the variables.

The data presented in **Figure 25**, **Figure 26**, Figure **27** correspond to the correlation analysis for LTP, 1 Hz - LTD and 10 Hz - LTD respectively. All data showed that there is no correlation

between % NAP score and the amount of LTP/LTD observed; in other words, there is no correlation between deficits after r-mTBI and synaptic plasticity observed (**LTP** – **Figure 25** - PID1: SHAM R^2: 0.24, Injury R^2: 0.01, PID7: SHAM R^2: 0.01, Injury R^2: 0.00, **1 Hz LTD** – **Figure 26** - SHAM R^2: 0.05, Injury R^2: 0.10, PID7: SHAM R^2: 0.00, Injury R^2: 0.05, **10 Hz – LTD** – **Figure 27** - PID1: SHAM R^2: 0.01, Injury R^2: 0.10, PID7: SHAM R^2: 0.16, Injury R^2: 0.00). These graphs do however depict visually that on average, injured animals score lower than their SHAM counterparts.

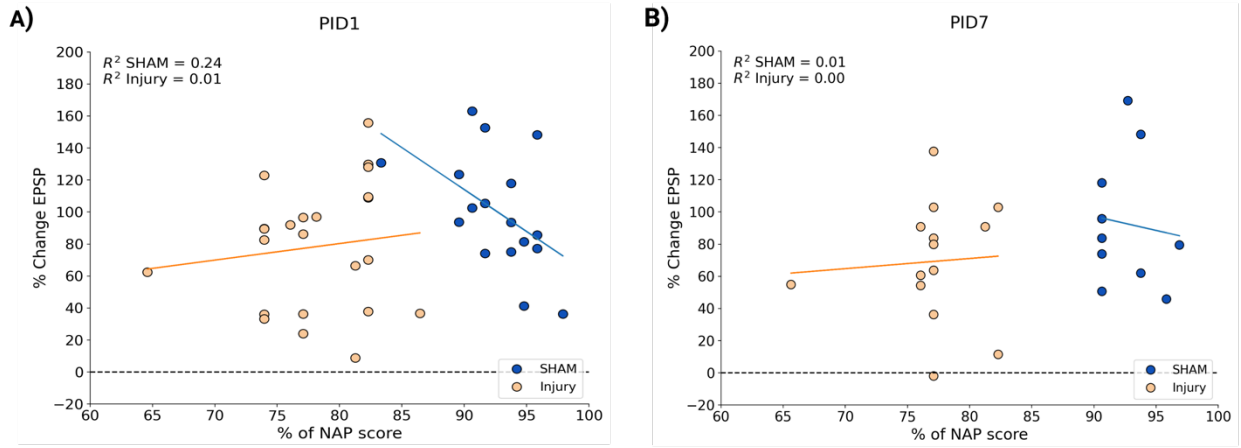

Figure 25. The correlation between the amount of long-term potentiation (LTP) and the % score from the neurological assessment protocol shows no significant relationship

(A) *The correlation between the amount of LTP and % score from the NAP was plotted with a regression line for animals that were examined on post-injury day 1 (PID1). R^2 analysis (values shown on graph) shows that there is no significant correlation between the NAP score and the amount of LTP recorded.* ***(B)*** *This plot shows the same as (A), but for post-injury day 7 (PID7). At PID7, there are no significant relationships between the NAP score and the amount of LTP recorded which is shown by the R^2 values. The coefficient of determination (R^2) was used to determine the relationship between the % of NAP score and % Change EPSP for LTP. Each data point represents an individual slice.*

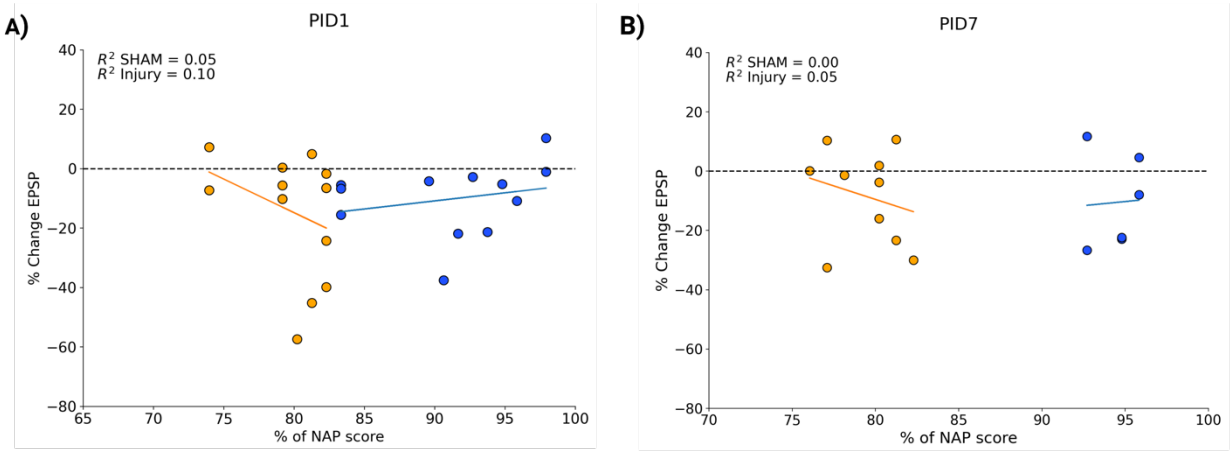

Figure 26. The correlation between the amount of 1 Hz - long-term depression (LTD) and the % score from the neurological assessment protocol shows no significant relationship

(A) *The correlation between the amount of 1 Hz - LTD and % score from the NAP was plotted with a regression line for animals that were examined on post-injury day 1 (PID1). R^2 analysis (values shown on graph) shows that there is no significant correlation between the NAP score and the amount of LTD recorded.* ***(B)*** *This plot shows the same as (A), but for post-injury day 7 (PID7). At PID7, there are no significant relationships between the NAP score and the amount of LTD recorded which is shown by the R^2 values. The coefficient of determination (R^2) was used to determine the relationship between the % of NAP score and % Change EPSP for LTP. Each data point represents an individual slice.*

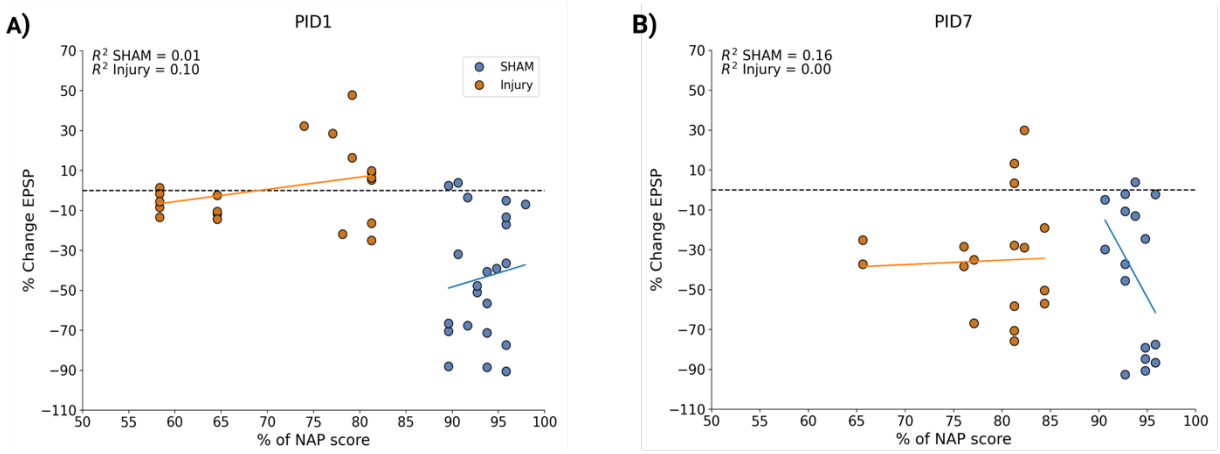

Figure 27. The correlation between the amount of 10 Hz - long-term depression (LTD) and the % score from the neurological assessment protocol shows no significant relationship

(A) *The correlation between the amount of 10 Hz - LTD and % score from the NAP was plotted with a regression line for animals that were examined on post-injury day 1 (PID1). R^2 analysis (values shown on graph) shows that there is no significant correlation between the NAP score and the amount of LTD recorded.* ***(B)*** *This plot shows the same as (A), but for post-injury day 7 (PID7). At PID7, there are no significant relationships between the NAP score and the amount of LTD recorded which is shown by the R^2 values. The coefficient of determination (R^2) was used to determine the relationship between the % of NAP score and % Change EPSP for LTP. Each data point represents an individual slice.*

4. Chapter Four – Discussion

4.1. Summary of Major Findings

The objective of this research was to examine how r-mTBI impacts synaptic plasticity, which is believed to play a crucial role in the capacity for learning and memory. The investigation focused on bidirectional synaptic plasticity in the dentate gyrus of juvenile Long Evans rats. This study is unique because to my knowledge, it is the first to identify endocannabinoid-dependent impairments in synaptic plasticity through *in vitro* electrophysiology following r-mTBI using the ACHI model.

In the absence of lateralization of injury, slices from the ipsilateral and contralateral hemispheres were combined to observe the overall effect of bidirectional synaptic plasticity. LTP after injury was found to be significantly different from its SHAM counterparts, while 1 Hz - LTD was unaffected by r-mTBI over time. 10 Hz - LTD showed a significant decrease on PID1 which then ameliorated by PID7. Control and SHAM animals were also compared to elucidate any possible distinctions between animals that underwent a procedure and those that lived in standard conditions. LTP and 1 Hz - LTD indicated significant differences between control and SHAM groups, while 10 Hz - LTD was unaffected. The comparison of % of total NAP score and the amount of synaptic plasticity recorded revealed that there was no significant relationship between the behavioural deficit's animals experience after r-mTBI and the amount of synaptic plasticity recorded on PID1 or on PID7. This study observed that synaptic plasticity is affected after r-mTBI, and the results suggest certain molecular mechanisms may be affected more than others. Specifically, deficits were noted in LTP and 10 Hz - LTD, while 1 Hz - LTD remained unaffected.

4.2. The ACHI model does not cause lateralization of injury

Due to the nature of the ACHI model, there is a more direct injury to the hemisphere in which the injury occurs. While the 3-D helmet is designed to dissipate the load of this force throughout the skull, there is still the possibility that injury severity may be skewed towards the injured side as compared to the uninjured side. For this reason, the analysis of ipsilateral (injured) to contralateral (uninjured) hemispheres was performed to explore the potential differences in electrophysiological recordings due to injury lateralization resulting from the ACHI model.

This study did not find evidence of lateralization of injury in the amount of synaptic plasticity recorded in the DG with the use of the ACHI model. This finding suggests that the force delivered by the impactor was effectively dissipated by the 3D-custom-printed helmet (Meconi et al., 2018). This is important, as mTBIs are a primary example of diffuse brain injuries, which are the most common type of head injury seen in humans (Bales et al., 2018). While open head injuries such as those induced by FPI and CCI systems are easily reproducible, the extensive tissue destruction and absence of skull displacement (due to stereotaxic head restraint) in these models reduce their resemblance to most actual injuries experienced by humans (Namjoshi et al., 2014). Closed head injuries (CHI) in contrast, are believed to allow a greater movement of the skull upon impact, and the utilization of flexible platforms can result in a shift of the injury from a localized pattern to a more diffuse pattern (Namjoshi et al., 2013). For this reason, models of CHI are generally preferred as they are thought to stimulate TBI cases that are seen in humans.

While this study supports the assertation that CHI causes diffuse injury in the brain, there is still controversy in the field of TBI. White *et al*. (2017) utilized a weight drop model to examine the effects of mTBI on LTP, and their findings indicated that there were notable disruptions in LTP that were localized to the hemisphere on the same side as the impact site (White et al., 2017). Other research that employed the FPI model found that the hippocampus on the side of the injury had a substantial neuronal loss that persisted for 30 days, whereas the hippocampus

on the opposite side was only impacted for 7 days (Tran et al., 2006). While there were deficits due to injury in both hemispheres (indicating a diffuse injury), there was still a lateralization injury bias, shown by the more rapid amelioration in the uninjured hemisphere. Future studies should comprise the analysis of lateralization to elucidate any potential site of injury differences. This is important, as using the most clinically relevant model in the lab allows us as researchers to bridge the gap between preclinical studies and patient care.

4.3. R-mTBI significantly reduces long-term potentiation

Although this study did not observe a significant change in the amount of LTP recorded following r-mTBI over time, there was a cumulative PID1-PID7 difference between SHAM and injury groups. TBI can result in damage to the hippocampus, which can lead to cognitive impairments - a common occurrence in those with an mTBI (de Freitas Cardoso et al., 2019). Other studies have similarly found deficits in LTP in the DG (and CA1) regions of the hippocampus after mTBIs through both open head injury using the FPI model (Sick et al., 1998) and closed head injury using the weight drop model (White et al., 2017), however, these deficits were more pronounced than what was found in this study. This may be due to differences in injury model paradigms. FPI models include craniotomy procedures which may exacerbate secondary injury cascades that may not be representative of what occurs after mTBI. Weight drop models, while preferable for mTBI injury as it is performed on closed heads has been known to have a high variability in injury severity (Xiong et al., 2013a). Despite differences in models, several implications for the deficits behind a decrease in LTP following TBI have been elucidated.

Traumatic brain injuries can result in significant dysregulation, including neuroinflammation, neurodegeneration, and alterations in the transmission of excitatory signals in the hippocampus (Jamjoom et al., 2021). Disrupted synaptic transmission is believed to involve important receptors, such as AMPARs and NMDARs, with consistent findings indicating that NMDARs are particularly impacted after r-mTBI (Almeida-Suhett et al., 2015; Grossman et al., 2003; Schwarzbach et al., 2006). Research conducted by Aungst et al (2014) in

the stratum radiatum in the CA1 region, demonstrated through pharmacological experiments that NMDA receptors play a crucial role in the induction of LTP. Using DNQX, an AMPAR antagonist, the researchers found that animals exposed to a single mTBI, and SHAM 'injury' showed NMDAR mediated LTP responses. However, this response was absent in animals that received r-mTBIs. Additionally, by using D-AP5, an NMDAR antagonist, the researchers showed that AMPAR responses were unaffected in all groups tested (Aungst et al., 2014). Although the decreased NMDA activity could explain the absence of LTP in the CA1, it is possible that this impairment also occurs in the DG following r-mTBI. Differences in injury induction between Aungst and colleagues and the current study (FPI vs ACHI) may have resulted in smaller decreases in LTP. The FPI requires a craniotomy which may exacerbate secondary injury cascades and induces a full concussive injury compared to the ACHI model which induces sub-concussive injuries. However, despite differences in injury inductions it is likely that both models induce similar mechanisms of disruptions for NMDARs and subsequent changes in LTP.

After TBI, inflammation can also cause further complications. Tumour necrosis factor-alpha (TNFα), a proinflammatory cytokine, can enter injury sites and decrease the expression of AMPA GluR2 subunits while also increasing the expression of AMPA receptors lacking this subunit (Beattie et al., 2002). TNFα has also been shown to impede late LTP through p38 MAP kinase (Pickering et al., 2005). Alterations in AMPA receptors can result in an increase in intracellular glutamate, leading to the exacerbation of calcium intake into the postsynaptic synapse. This phenomenon is referred to as glutamate excitotoxicity. The presence of decreased AMPA-receptor desensitization and excessive synaptic glutamate can trigger hyperexcitability, cellular damage and death (Jamjoom et al., 2021; Park et al., 2008), all of which may contribute to deficiencies in LTP. While this study did not investigate neuroinflammation after r-mTBI, it is still relevant to consider that this type of phenomenon may be partially responsible for deficiencies in LTP after injury.

4.4. R-mTBI does not significantly affect 1 Hz - long-term depression

The induction of 1 Hz - LTD was not found to be affected in this study after r-mTBI. While LTP in the literature has shown consistent attenuation in LTP responses after TBI, LTD has received less attention and findings regarding its effects after TBI have been inconsistent (Albensi & Janigro, 2009).

A study using the FPI model found that LTP was significantly reduced after moderate TBI while LTD was unaffected. The hypothesis behind this finding was that the inability to induce LTP may be due to a potentiation that is not dependent on a specific synaptic drive. This interpretation is supported by the fact that LTD was consistently induced and sustained in slices from injured animals. As both forms of synaptic plasticity rely on the activation of NMDARs, it is highly improbable that the FPI model caused a selective loss of these receptors. These researchers also suggested that, following mechanical injury, the relief of the Mg^{+2} ion blocking NMDARs could cause LTP saturation, thereby preventing further induction of LTP while leaving LTD unaffected (D'Ambrosio et al., 1998; L. Zhang et al., 1996). The difference in the degree of LTP reduction between the current study and the research by D'Ambrosio and colleagues may be due to differences in injury severity. However, both studies employed similar protocols to induce LTD, utilized similarly aged animals, and both yielded consistent results showing that TBI had no effect on LTD. This implies that the mechanisms underlying LTD remain intact and unaltered after TBI despite differences in severity of injury. Impairment in LTP while 1-Hz LTD remains unaffected in individuals with TBI may contribute to deficits in memory. Synaptic plasticity requires a delicate balance between strengthening and weakening of synapses for proper memory formation (Zhong & Gerges, 2010), and in this case where LTD is unaffected, it is now "increased" relative to LTP, therefore disrupting the normal balance required. While this may be a plausible reasoning for unaltered LTD after TBI, there is debate in the literature.

Albensi *et al* (2000) found that after cortical contusion, LTP was impaired while LTD was enhanced. Their hypothesis aligns with a theoretical model known as the "sliding modification threshold" which states that synapses with higher levels of previous activity may favour the

induction of LTD and inhibit LTP, causing a shift in the LTP/LTD threshold (Albensi et al., 2000). The precise mechanisms underlying LTD following TBI are not completely understood, and although the differences in LTD observed in the previously mentioned studies may be attributable to the specific injury model employed (more invasive techniques) compared to this study, further research is necessary to comprehend the dynamics of LTD after TBI.

4.5. R-mTBI significantly affects 10 Hz - long-term depression one-day post-injury, which then ameliorates by post-injury day 7

The results of this study showed that 10 Hz - LTD (which is thought to involve the ECS) in the DG of juvenile LE rats was significantly reduced one-day post-injury. However, it was observed that this impairment in LTD had improved by the seventh day after injury. Impairment in 10 Hz – LTD is quite novel and there is a lack of specific literature on this subject, however, it is feasible to deduce inferences about these deficits by examining the eCB system as a whole.

As previously mentioned, 2-AG is an endogenous cannabinoid that is a strong agonist for CB1 receptors. Following a TBI, levels of this eCB were observed to be significantly elevated in the hippocampus. Specifically, 2-AG was found to be 6.7 times higher than baseline one day after injury and gradually decreased over time, returning to normal levels after seven days (Xu et al., 2019). While 2-AG has been shown to be a potential neuroprotectant against TBI (Panikashvili et al., 2001; Shohami et al., 2011) its degradation can have detrimental effects. 2-AG is a very unstable fatty acid that is rapidly metabolized by enzymes upon its release (J. Zhang et al., 2015). The enzyme monoacylglycerol lipase (MAGL) is responsible for breaking down 2-AG and converting it into arachidonic acid (AA) (Schurman & Lichtman, 2017). AA is a precursor to several pro-inflammatory mediators such as prostaglandins (I.e., prostaglandin D2 (PGD2)) and other pro-inflammatory cytokines and chemokines (i.e., IL-1β and TNFα) (B. Wang et al., 2021). 2-AG can also be directly oxidized by cyclooxygenase-2 (COX-2) to form prostaglandins (Yang et al., 2008). The process of eCB oxidation can cause cannabinoid receptors to become inactive, while also generating bioactive lipids that participate in inflammatory responses during the initial phases of injury (Schurman & Lichtman, 2017). Investigation of the eCB system

has indicated that TBI-induced lesions result in a decrease in CB1 receptor levels, which has been linked to significant neurological impairments (Lopez-Rodriguez et al., 2015).

One possible hypothesis that can be derived from the given information is that the deficiencies observed in 10 Hz - LTD could be a result of an increase in the concentration of 2-AG in the hippocampus, which ultimately leads to an increase in pro-inflammatory mediators. This increase could be due to the breakdown and oxidation of 2-AG by MAGL and COX-2 enzymes. On the other hand, the observed improvement in 10 Hz - LTD after 7 days could be linked to the restoration of 2-AG levels back to their baseline values. While the reason for the deficiency in 10 Hz - LTD is not certain, it could be caused by the inactivation or reduction of CB1 receptors, or by an increase in neuroinflammation. However, further investigation is needed to fully understand the underlying mechanisms involved.

4.6. SHAM animals compared to controls exhibit significant differences in the amount of LTP and 1 Hz - LTD recorded

The analysis of control animals to SHAM was conducted to determine if there was an effect of synaptic plasticity between animals that underwent a procedure versus those that were left to live normally in their home cages. The expected result of this comparison was that there would be a similar amount of synaptic plasticity recorded (LTP or LTD) between the two groups. However, this was not the case. The results of this comparison showed a significant increase in LTP between the control and SHAM groups, while there was a smaller but significant decrease in 1 Hz - LTD. A potential explanation for the difference in synaptic plasticity in SHAM animals compared to controls could be linked to the acute stress animals experience when placed into the restraint cones.

When organisms encounter a stressor, whether originating from the external environment or within the body, signals conveying information about the stressful event are transmitted to various regions of the brain, such as those located in the limbic system and those responsible for sensory processing. Activation of hormonal systems due to stress causes the body to

increase the levels of adrenaline and corticosterone (cortisol in humans) and these two hormones not only affect peripheral organs but also the brain. Two receptor types in the brain are thought to mediate stress responses: mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) (De Kloet et al., 2005). In the DG, when activated under normal conditions, the receptors will alternate between mainly activating MRs or activating both MRs and GRs simultaneously. When corticosteroids were found to bind to receptors in the brain, many decided to study the effect of stress on memory performance and more specifically the effect of stress on LTP (McEwen et al., 2016). The reduction of LTP in the CA1 after stressful events was first seen when administering an inescapable shock to rats. Subsequent studies concurred with this first study, and showed decreases in LTP (and subsequent increase in LTD) after the administration of high doses of corticosterone in both *in vivo* and *in vitro* studies, which indicated that corticosterone may be the leading hormone in the effects observed after stress (see (Joëls & Krugers, 2007) for review). While many studies have observed a decrease in LTP after stress, this is not always the case.

Interestingly, a study by Kavushansky *et al* (2016) found that rats exposed to an uncontrollable stressor (no means of escaping the Morris water maze) demonstrated an enhancement in LTP compared to those exposed to a controlled stress (where subjects could escape the water by locating a hidden platform). The amygdala was found to exhibit a heightened baseline activity after uncontrollable stress (implying stress-induced plasticity), but LTP in this region was equally inhibited by all stressors. The increase in baseline activity found by these researchers in the amygdala has been proposed to be linked to why there is an increase in LTP in the DG. The basolateral amygdala has a role in modulating memory processes in other brain regions and it has been found there is an interconnection between the amygdala, hippocampus, and EC (Brothers & Finch, 1985). The activation of the basal amygdala has also been found to enhance LTP in the perforant path inputs to the DG (Akirav & Richter-Levin, 1999). Kavushansky et al (2016) have shown that when the amygdala is activated 30 seconds before or after LTP induction, LTP was enhanced in the DG but decreased in the CA1. Therefore, it is hypothesized that uncontrollable stress enhances amygdala activity which in turn

differentially influences plasticity in the DG and CA1 (Kavushansky et al., 2006). The ventral hippocampus makes direct connections to the amygdala (Kim & Cho, 2017) and if amygdalar-induced stress was responsible for the rise in LTP, it could be hypothesized that ventral slices would show increased LTP levels. In this study, this difference was not seen, however, the dorsal hippocampus is still in contact with the amygdala indirectly through the EC (Kim & Cho, 2017) and therefore amygdala-induced stress may not only be localized to the ventral hippocampus. Further investigation would be needed to elucidate the effects of the potential amygdalar stress on the hippocampus in the DG.

Our animals experienced uncontrollable stress from being confined in restraint cones for a brief duration of approximately 2-5 minutes, but this exposure occurred eight times throughout the day which would total up to 40 minutes. This exposure to stress is similar to Kavushansky and colleagues' study, where their animals were subjected to the Morris water maze for 45 minutes (12 trials). This repeated stress may have a cumulative impact, and the animals may also begin to associate the researcher with the 'stressful' event (i.e., being placed in the restraint cone). Large increases in corticosterone levels have been associated with increased LTP and it is possible that when being handled and prepared for electrophysiology, corticosterone could be released in large amounts which would account for the increase in LTP that was recorded (Joëls & Krugers, 2007). While this hypothesis may be conceivable for animals on PID1, animals should not be as stressed on PID7. Additional research is required to examine the possible cumulative impact of stress on synaptic plasticity after r-mTBI using the ACHI model, and future investigations utilizing this model should consider measuring stress hormones in blood samples.

4.7. There are no relationships between the deficit animals experience after r-mTBI and the amount of synaptic plasticity recorded post-injury on day 1 or day 7

We utilized the NAP to evaluate potential impairments in animals after mTBI, which is similar to the SCAT5 clinical tool employed in sports to diagnose concussions. This study revealed deficits in sensorimotor function and synaptic plasticity in injured animals compared

to SHAM groups, which suggests a potential correlation between these two factors. Specifically, animals with higher NAP scores may exhibit greater synaptic plasticity, while those with lower scores (due to injury) may display less.

Our study however did not find any correlation between the deficits observed in animals after r-mTBI and the amount of synaptic plasticity recorded on post-injury day 1 or 7. Animals with higher scores on the NAP indicated that their sensorimotor function and reflexes were unaffected by the injury, while lower scores indicated deficits in these factors and signaled the presence of a head injury. The animals displayed the greatest impairment following the first or second impact during the ACHI protocol, but deficits did not worsen with each subsequent hit. The plateau in NAP scores may partially explain their lack of correlation with synaptic plasticity. In this study, the lowest post-injury score was 75%, with little variation being observed in subsequent scoring. A more sensitive test or increased injury severity may be necessary to distinguish differences in sensorimotor deficits and the amount of synaptic plasticity observed. One interesting observation in this analysis is that uninjured (SHAM) animals demonstrate a markedly higher NAP score compared to injured animals, thus confirming that injured animals demonstrate motor and reflex impairments after ACHI.

4.8. Limitations

4.8.1. Awake Closed Head Injury Model

While the ACHI model can avoid potential confounding factors such as anesthesia, surgery, or head immobilization, there are a few constraints that need to be addressed. Stress due to restraint may cause increased glial cell activation, glutamate release and changes in neuronal plasticity (Orellana et al., 2015). Blood was not collected in this study and therefore, corticosterone levels could not be measured. In the future, it would be worth collecting blood in order study the effect of stress post-ACHI procedures. While animals in this injury paradigm are only restrained for a short period of time (~2-5 minutes), further investigation of the impact of acute stress may be warranted.

The paradigm used for this study was 8 injuries, spaced two hours apart over the course of the day. This paradigm may miss out on many injury cascades that occur over a longer period of time. Neuroinflammatory compounds for example is time dependent: cytokines and chemokines reach their peak at one-day post injury and decline readily thereafter, while microglia and macrophages are most active seven days post injury (Simon et al., 2017). Deficits seen after injury may therefore be different if the injury paradigm were spaced out.

4.8.2. Data collection

Due to strict inclusion and exclusion criteria used in this study, a few groups were underpowered and the need for increased data in those groups to elucidate any differences may be needed. The addition of data in these groups may be performed by myself or by future members of the Christie laboratory.

4.9. Looking ahead – Future Directions

4.9.1. Potential treatment options for 10 Hz - LTD

As discussed in a previous section, 10 Hz - LTD may be affected by the increased levels of the eCB 2-AG. When this eCB is metabolized by MAGL, it is converted into AA which plays a major role in inflammatory pathways. Inhibition of MAGL through JZL184 (an irreversible inhibitor for MAGL) was found to boost 2-AG levels, decrease AA and prostaglandins, and reduce CTE-like neuropathologic changes found in mice after r-mTBI. Decreased neuroinflammation, neurodegeneration and deficits in LTP were also shown through the inhibition of MAGL (J. Zhang et al., 2015). These findings have also been supported by other research that also found that inhibition of MAGL (and thus increased 2-AG levels) showed protective effects against deficits in LTP and was able to decrease the induction of microglia. This is due to the fact that lower levels of AA can reduce the production of pro-inflammatory mediators (Schurman & Lichtman, 2017). While these studies focused on LTP, it would be valuable to explore whether the inhibition of the breakdown of 2-AG could ameliorate the deficits in 10 Hz - LTD following r-mTBI, given that the mechanism underlying this type of synaptic plasticity heavily involves 2-AG.

Due to its status as an agonist for CB1 receptors, which are abundantly found in the hippocampus, Δ9-THC may have the potential as a therapeutic agent for targeting synaptic plasticity. However, previous studies have demonstrated that exposure to Δ9-THC or synthetic cannabinoids may disrupt synaptic plasticity (Hoffman et al., 2020). Despite this knowledge, as cannabis has been legalized in Canada, it has become an increasingly popular treatment option for TBI survivors and others. While some pre-clinical TBI research suggests that exogenous cannabinoids have neuroprotective and psychotherapeutic properties, other studies have revealed detrimental effects (refer to Hergert et al., 2021 for a review of cannabis use in TBI treatment). With inconsistent findings regarding the benefits or drawbacks of cannabis use following TBI, further research is necessary.

4.9.2. Sex differences

Because of time limitations, the scope of this research was restricted to exploring synaptic plasticity exclusively after mTBI in male LE rats. However, there is a compelling rationale for investigating synaptic plasticity in females after mTBI. TBI shows variability in many factors between sexes (see (Gupte et al., 2019) for a review) and synaptic plasticity can also be influenced by sex hormones (see (Pinar et al., 2017) for a review).

The male population is most frequently affected by TBI, with men having 2.2 times greater odds of experiencing TBI compared to women. This difference can be explained by the fact that men are more likely to be involved in physical altercations, military service and contact sports. While this is true, sports related injuries in young women have been found to be equal to or higher than incidences in men. Women are also more likely to experience post concussive symptoms, have poorer outcomes and experience a slower recovery time than men (Biegon, 2021). These differences between outcomes post TBI in men compared to women or vice versa, may be due to sex hormones that can influence inflammation, edema, oxidative stress, excitotoxicity, and mitochondrial functioning. With these differences in mind, studying sex

differences in animal models is important for improving and developing treatments for those suffering from TBI.

Although sex hormones are commonly associated with the reproductive organs, it is worth noting that the brain can produce these hormones locally. Compared to systemic hormone release, locally synthesized hormones have a more rapid and specific impact on the brain. Throughout the estrous cycle, the concentration of estradiol fluctuates naturally, with its highest concentration occurring proestrus and its lowest during diestrus. It has been well established that estrogens can induce changes in synaptic plasticity, with evidence suggesting that they can enhance LTP in the hippocampus. However, the effect of estrogens on LTD is a subject of debate. Some studies have shown that female rats lacking ovaries exhibit impaired LTD, which can be restored through the administration of exogenous 17β – estradiol (E2) and male rats treated with E2 exhibited enhanced LTD. However, other studies have failed to observe the effect of E2 on LTD in both female and male rats. It is also possible that progesterone may have a role in LTP, however, its impact on LTD has not yet been explored. Future studies should aim to report or control hormone levels in both sexes, especially when working with TBI as this type of injury may alter puberty (Rose & Auble, 2012).

4.9.3. Experimental paradigm

The ACHI model offers a unique advantage for studying repeated injuries, as researchers can employ various injury induction timelines. Our experimental design is clinically relevant, as football players, for example, may experience an average of 6.3 impacts per practice and 14.3 impacts per game (Crisco et al., 2010), which exceeds the number of impacts utilized in this study. Other timelines are also worth studying to model other types of sports-related injuries, like soccer players who experience an average of 4.8 impacts per hour of play time (Huber et al., 2021). Additionally, secondary injury cascades differ based on the amount of time elapsed following TBI, and the release of neuroinflammatory compounds is also time dependent. For example, cytokines and chemokines reach their peak one-day post-injury and decline rapidly thereafter, while microglia and macrophages are the most active seven days post-injury (Simon

et al., 2017). These differences in neuroinflammation may have varying effects on synaptic plasticity and thus warrant further investigation.

Our lab has begun investigating two different induction timelines for the ACHI model: one which involves animals being injured twice per day for four consecutive days with the injuries being spaced two hours apart and the other being this study's timeline (8 injuries, 2 hours apart over the course of the day). A preliminary comparison of synaptic plasticity recorded between these two different paradigms can be found in Appendix C. Future research in the lab aims to incorporate the 10 Hz - LTD protocol into current injury paradigms and to explore synaptic plasticity in animals with r-mTBI using a more prolonged injury paradigm involving a single injury over 8 days.

4.10. Overall Conclusions

The objective of this thesis was to explore how synaptic plasticity is affected after r-mTBI. The ACHI model was found to cause a diffuse injury which closely imitates injuries most seen in humans. This result allowed for the combination of data between hemispheres, and it was found that subjecting test subjects to 8 r-mTBI hits within a single day had a significant impact on synaptic plasticity in both acute and sub-acute timepoints. While there was no impact on 1 Hz -LTD in the MPP-DG synapse, there was a discrepancy in the amount of LTP recorded between SHAM and injury groups (PID1 and PID7 time points combined). To my knowledge, this is the first study investigating the effects of 10 Hz – LTD, a type of LTD thought to be induced by the ECS, where a significant decrease in LTD on PID1 was observed, which then recovered by PID7. These deficits in synaptic plasticity did not correlate to the animals' respective NAP scores and this may be due to a lack of sensitivity in the evaluation paradigm.

Overall, these results indicate that r-mTBI causes modifications in synaptic plasticity that exist in the DG area of the hippocampal formation across three different pathways, one of which is a novel area of investigation. Future research is needed to elucidate specific impairments in mechanisms that occur during synaptic plasticity, particularly in the context of

10 Hz - LTD and 1 Hz - LTD. Together, these results may be relevant to the cognitive impairments related to learning and memory that are frequently observed in young people who have suffered from a concussion.

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6. Appendix A – Dorsal vs. Ventral: criteria for slices

6.1. Histology

A histological stain (cresyl violet) was performed to acquire representative images of transverse hippocampal slices. The assignment of 2 slices per hemisphere was made for dorsal and ventral analysis post-electrophysiological experiments.

6.1.1. Intracardiac Perfusion

In preparation for cresyl violet staining, intracardiac perfusion was performed on a Long Evans rat (~PND 130). The solutions used for this perfusion were 0.1M heparinized phosphate-buffered saline (PBS, stock: (10xPBS, PH 7.4) 1.37 mM NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4. Then diluted to a 1x solution (2L): 200mL 10x PBS and 1800mL dH_2O. Heparinized PBS: from a 1000 IU/µL heparin stock, 250 µL heparin per 250 mL heparinized PBS) and 4% paraformaldehyde (PFA, 10 g PFA and 200 mL 1xPBS dissolved at 65°C, 5N NaOH, 6N HCl (pH 7.4), 250mL 1xPBS). A Masterflex pump (VWR International LLC, Mississauga, ON, Canada) was calibrated to deliver 11-12 ml/min and once complete, one side of the influent tube was placed into a beaker containing 250 mL heparinized PBS. The other side (the effluent portion of the tube) is attached to an 18g needle which is used to puncture the heart. The pump is allowed to run to allow air bubbles to exit the tube. The pump is stopped, and the animal is prepared to be perfused. Anesthetizing the animal was done with isoflurane in a bell jar. Once completely anesthetized (lack of toe pinch response and blink response), the animal is moved to the perfusion tray. A falcon tube filled with isoflurane-soaked cotton was placed over the rat's nose to keep them anesthetized during surgery. Scissors were used to cut through the skin and muscle layers of the abdomen and chest to expose the heart. The needle attached to the effluent tube was inserted through the apex of the heart and into the right ventricle. The right atrium was then cut, and the pump was turned on to allow the heparinized PBS to flow through. Once the liver looks clear, the influent tube was moved to a beaker containing 250 mL

of 4% PFA and this was allowed to be pumped into the animal until the animal was stiff to the touch or until all the solution had been run through. Once the perfusion was complete, the head was decapitated, and the brain was extracted. The brain was placed in a falcon tube filled with 4% PFA (to post-fix) and left overnight at 4°C. The following day, the brain was then moved to a sucrose solution (30% sucrose) and stored at 4°C until sectioning occurred (the following day).

6.1.2. Brain sectioning and Cresyl Violet Staining

50 µm sections were cut with a vibratome (Leica VT100S, Concord, ON, Canada) and slices were then mounted onto Superfrost Plus slides and dried at room temperature for at least 30 minutes prior to staining. Slices were first rinsed in double distilled water (ddH_2O) for a minute, then rinsed in ethanol (EtOH) of decreasing concentrations for a total of 16 minutes (70% EtOH 1 min, 95% EtOH 5 min, 100% EtOH 10 min). Citrisolv was then washed over the slices for 20 minutes. Another wash of decreasing ethanol concentration was performed for a total of 7 minutes (100% EtOH 5 min, 95% EtOH 1 min, 70% EtOH 1 min). Slices were then stained with 0.5% cresyl violet for 10-20 minutes or until the slices were very dark purple in appearance. Once stained, slices were rinsed in ddH_2O and destained in 70% EtOH with 1% acetic acid before one last dehydration step with 100% EtOH. Sections were cleared in Citrisolv, then cover slipped with Permount and left to dry overnight prior to imaging.

Representative images were taken using an Olympus brightfield BX51F microscope (MBF Bioscience, Williston, VT, USA) and Stereo investigator software version 11.03 (MBF Bioscience, Williston, VT, USA) using the 10X objective lens (**Figure 28**).

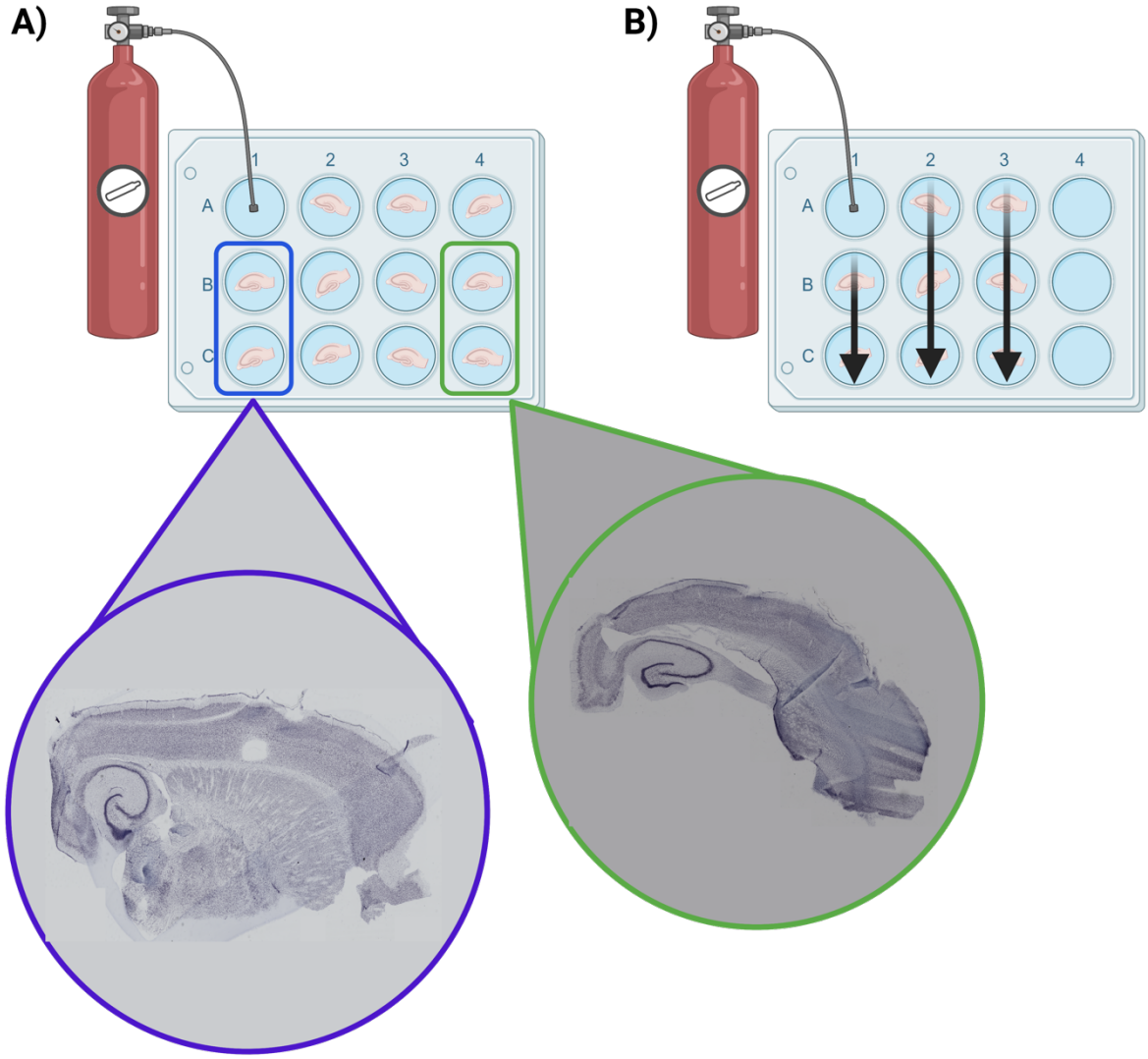

Figure 28. Dorsal and Ventral criteria and well placement

(A) *Representative image of holding wells for electrophysiological slices. Blue boxed wells represent slices that were considered ventral and green boxed wells represent slices that were considered dorsal. Both regions have spotlights that show cresyl violet stained slices that show representative dorsal and ventral hippocampal slices.* ***(B)*** *Black arrows through the representative image of the holding well shows the orientation in which slices are placed in the well after cutting. The first slice is placed into B1 which would be the most ventral slice and then the rest of the slices are placed in a vertical there onward.*

7. Appendix B – Dorsal vs. Ventral Analysis

7.1. Is there a Dorsal or Ventral difference in injury?

The analysis of dorsal and ventral differences was performed to elucidate any potential differences in synaptic plasticity from top to bottom. This analysis would show whether there is a coup-contra-coup-like effect in the ACHI model (coup-contra-coup like referring to a top-to-bottom injury instead of a forward-to-back).

7.1.1. Long-Term Potentiation

To study how 8 r-mTBI impacts affect LTP in the DG, both injured and uninjured animals were evaluated for their ability to show post-tetanic potentiation (PTP) and ant LTP at 1 day and 7 days after the injury. The determination of dorsal or ventral slices were made by taking the 2 most dorsal slices and the 2 most ventral slices. Due to the nature of the cutting protocol, slices are cut from ventral to dorsal, meaning the first 2 slices in the wells were ventral and the last 2 were dorsal. LTP was induced by applying a high-frequency stimulation (HFS) protocol of 4 trains of 50 pulses at 100 Hz with a 30-second interval to stimulate the MPP. The degree of LTP was measured by calculating the average fEPSP responses during the last 5 minutes of the post-conditioning recording, while PTP was measured during the first minute after the conditioning stimulus. The results, illustrated in **Figure 29** and **Figure 30** suggest that there are no significant differences in the amount of LTP recorded in the dorsal or ventral regions on PID1 or PID7.

A 2-way ANOVA analysis revealed there were no significant differences between dorsal and ventral regions on PID1 (**Figure 29B.** $F(1,19) = 0.28$, $p = 0.60$, $\eta_p^2 = 0.014$) or on PID7 (**Figure30B.** $F(1,16) = 0.052$, $p = 0.82$, $\eta_p^2 = 0.003$). Moreover, no significant differences were observed between the SHAM and injury groups in relation to dorsal or ventral regions on PID1 (**Figure 29B.** $F(1,19) = 0.12$, $p = 0.73$, $\eta_p^2 = 0.006$) or on PID7 (**Figure 30B.** $F(1,16) = 1.23$, $p = 0.29$, $\eta_p^2 = 0.071$). The magnitude of PTP in relation to dorsal or ventral region and protocol (SHAM or injury) for both PID1 (**Figure 29A.** $F(1,19) = 0.26$, $p = 0.61$, $\eta_p^2 = 0.014$) and PID7 (**Figure 30A.** $F(1,16) = 1.74$, $p = 0.21$, $\eta_p^2 = 0.098$) is not significantly affected.

Table 10. Long-Term Potentiation, dorsal and ventral regions PID1: average STD, LTD and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM Dorsal	137.57 ± 10.96	100.38 ± 16.00	N = 6, n = 7
SHAM Ventral	107.43 ± 28.70	83.84 ± 23.15	N = 3, n = 4
Injury Dorsal	131.66 ± 27.92	74.58 ± 15.80	N = 5, n = 7
Injury Ventral	125.52 ± 21.21	71.26 ± 21.10	N = 5, n = 5

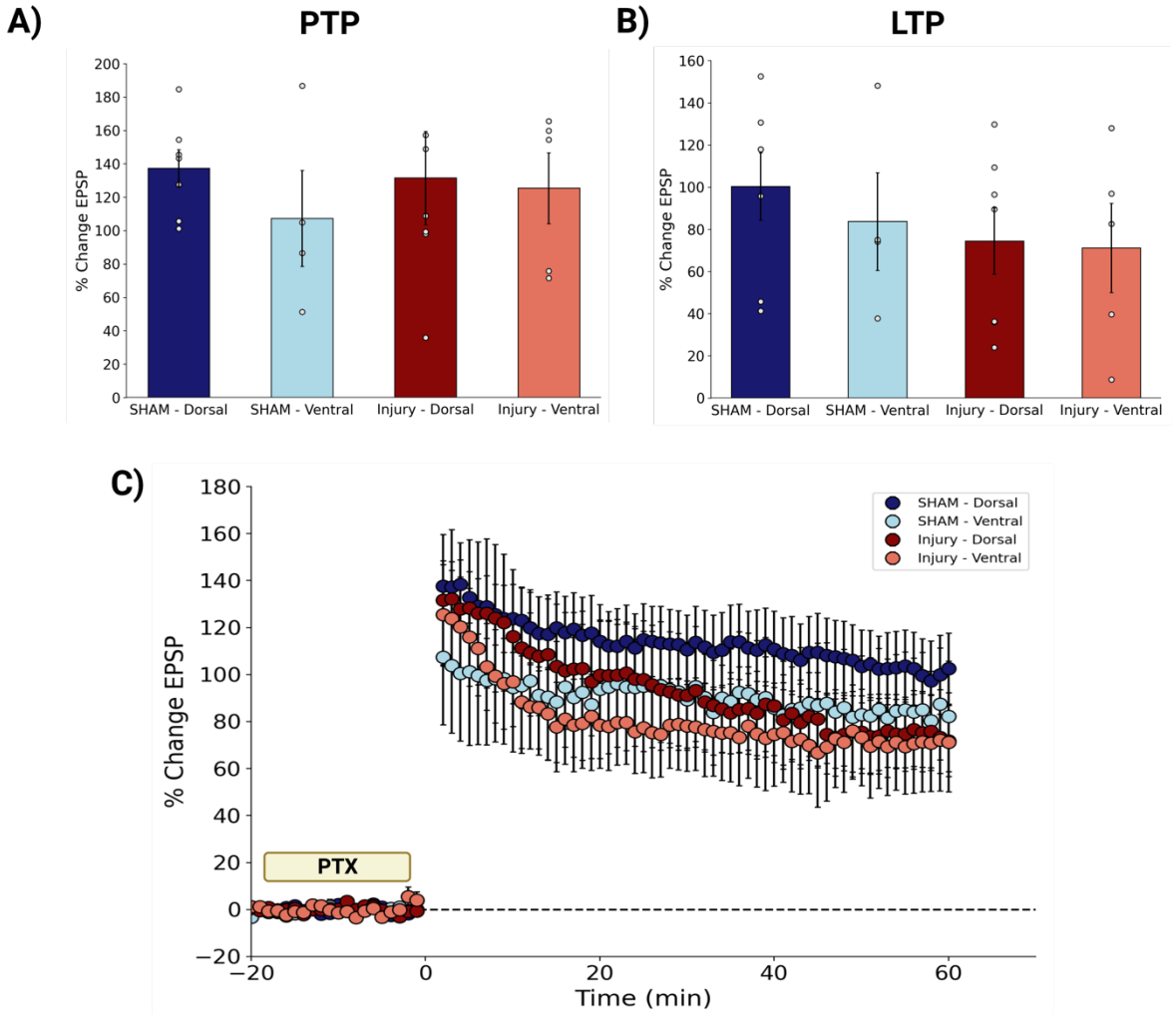

Figure 29. Long-Term Potentiation (LTP) shows no significant differences between dorsal and ventral regions on post-injury day 1 (PID1).

(A*) Post-Tetanic Potentiation (PTP) is measured as the percent change in the fEPSP slope after the first minute following the high-frequency stimulus (HFS; 4 trains-50 pulses at 100Hz; 30s intervals). R-mTBI does not significantly affect PTP.* ***(B)*** *Long-Term potentiation (LTP) was measured as the average percent change in the fEPSP slope relative to its baseline in the last 5 minutes following HFS (55-60 minutes). It was found that there are no significant differences in dorsal or ventral regions in LTP following r-mTBI induced through the ACHI model on post-injury day 1.* ***(C)*** *The average traces of LTP recordings from the beginning of the baseline to the end of post-conditioning recording for post-injury day 1 recording for injury dorsal and ventral regions and their SHAM counterparts (SHAM – dorsal and SHAM – ventral). All error bars represent the standard error of the mean (SEM). All comparisons were analyzed using a two-way ANOVA and individual points for PTP (A) and LTD (B) represent the average PTP or LTP for each individual slice in this dataset. PTX indicates that the $GABA_A$ antagonist PTX was washed over the recorded slices throughout the duration of the baseline recording.*

Table 11. Long-Term Potentiation, dorsal and ventral regions PID7: average STD, LTD and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM Dorsal	137.57 ± 10.96	100.37 ± 16.00	N = 6, n = 7
SHAM Ventral	107.43 ± 28.70	83.84 ± 23.15	N = 3, n = 4
Injury Dorsal	103.70 ± 30.85	37.27 ± 17.41	N = 4, n = 5
Injury Ventral	132.81 ± 19.0	62.45 ± 17.91	N = 3, n = 4

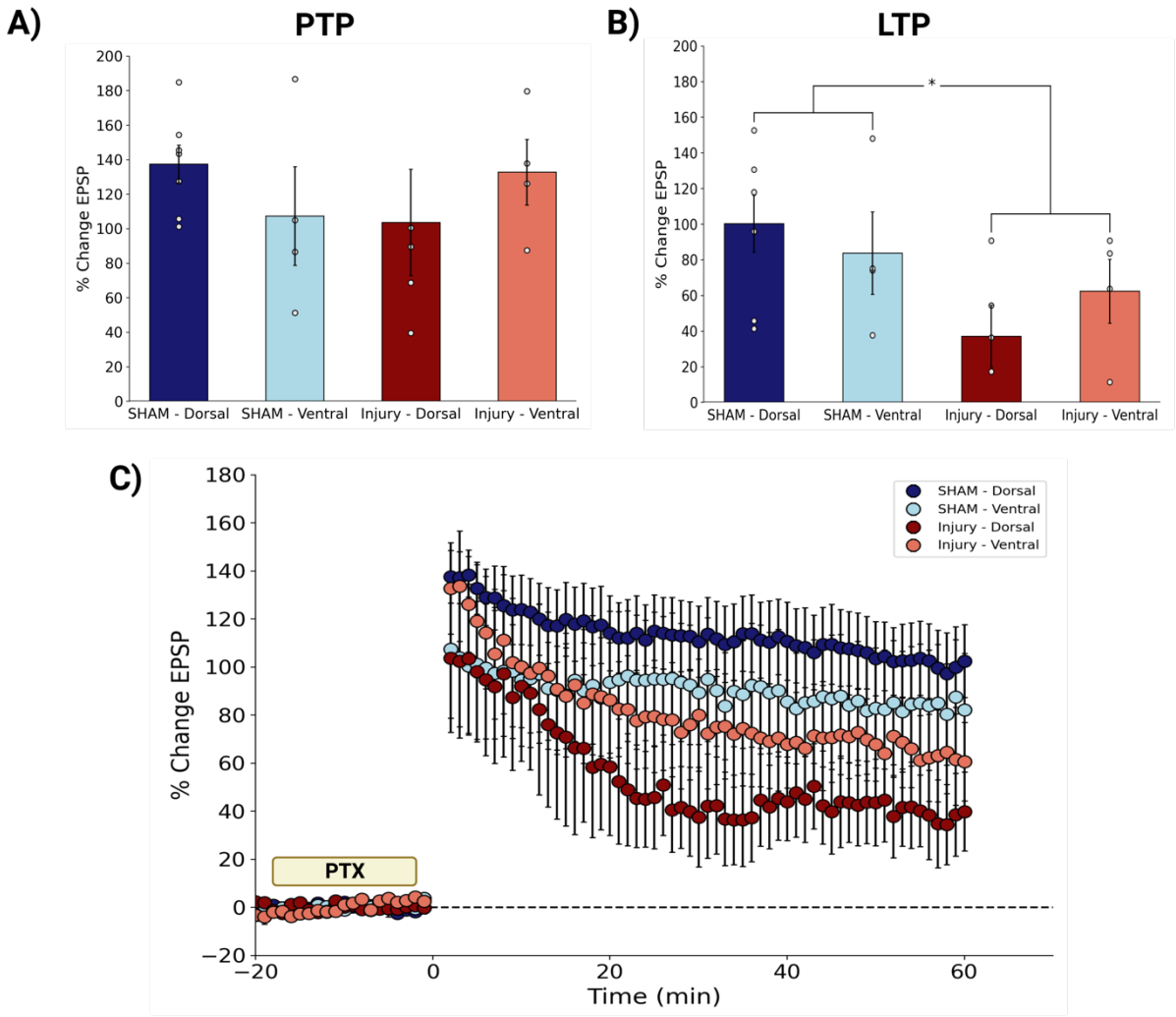

Figure 30. Long-Term Potentiation (LTP) shows no significant differences between dorsal and ventral regions on post-injury day 7 (PID7).

(A*) Post-Tetanic Potentiation (PTP) is measured as the percent change in the fEPSP slope after the first minute following the high-frequency stimulus (HFS; 4 trains-50 pulses at 100Hz; 30s intervals). R-mTBI does not significantly affect PTP.* ***(B)*** *Long-Term potentiation (LTP) was measured as the average percent change in the fEPSP slope relative to its baseline in the last 5 minutes following HFS (55-60 minutes). It was found that there are no significant differences in dorsal or ventral regions in LTP following r-mTBI induced through the ACHI model on post-injury day 7, however, there were significant differences between groups (SHAM and Injury)* ***(C)*** *The average traces of LTP recordings from the beginning of the baseline to the end of post-conditioning recording for post-injury day 1 recording for injury dorsal and ventral regions and their SHAM counterparts (SHAM – dorsal and SHAM – ventral). All error bars represent the standard error of the mean (SEM). All comparisons were analyzed using a two-way ANOVA and individual points for PTP (A) and LTD (B) represent the average PTP or LTP for each individual slice in this dataset. PTX indicates that the $GABA_A$ antagonist PTX was washed over the recorded slices throughout the duration of the baseline recording. * = $p < 0.05$.*

7.1.2. Long-Term Depression (1 Hz - LTD and 10 Hz - LTD)

To assess the impact of r-mTBI on both 1Hz - LTD and 10 Hz - LTD, animals with and without injury were compared based on their ability to exhibit STD and LTD at both PID1 and PID7. To distinguish between dorsal and ventral slices, the two most dorsal and two most ventral slices were selected. The cutting protocol followed a ventral to dorsal direction, hence the first two slices in the holding chamber were ventral while the last two were dorsal. Slices were then elicited in the MPP of the DG using either the 900 x 1 Hz protocol or the 6000 x 10 Hz protocol for. The degree of LTD was evaluated by computing the average fEPSP responses during the last 5 minutes of the post-conditioning recording, while STD was measured in the first minute following the conditioning stimulus. **Figure 31**, **Figure 32**, **Figure 33**, and **Figure 34** shows that there are no significant differences in the amount of either type of LTD elicited in the dorsal or ventral regions on PID1 or PID7.

In the case of 1 Hz - LTD, the results of a 2-way ANOVA indicated that there are no significant differences between dorsal and ventral regions on PID1 (**Figure 31B.** $F(1,23) = 0.002$, $p = 0.96$, $\eta_p^2 = 9.52 \times 10^{-5}$) or PID7 (**Figure 32B.** $F(1,15) = 1.56$, $p = 0.23$, $\eta_p^2 = 0.094$). Additionally, no significant differences were observed between the SHAM and injury groups in relation to dorsal or ventral regions on PID1 (**Figure31B.** $F(1,23) = 0.089$, $p = 0.77$, $\eta_p^2 = 0.004$) or on PID7 (**Figure 32B.** $F(1,15) = 1.69$, $p = 0.21$, $\eta_p^2 = 0.10$). The capacity to elicit STD after SHAM or injury in both dorsal or ventral regions was also not significantly affected on PID1 (**Figure 31A.** $F(1,23) = 2.48$, $p = 0.13$, $\eta_p^2 = 0.097$) or PID7 (**Figure 32A.** $F(1,15) = 0.002$, $p = 0.97$, $\eta_p^2 = 1.0 \times 10^{-4}$).

Results from 10 Hz - LTD data indicated that the amount of LTD recorded in dorsal and ventral regions was not significantly different on PID1 (**Figure 33B.** $F(1,23) = 2.70$, $p = 0.11$, $\eta_p^2 = 0.105$). Injury and SHAM groups, however, were found to be significantly different on PID1 (**Figure33B.** $F(1,23) = 11.14$, $p = 0.003$, $\eta_p^2 = 0.021$). PID7 interestingly showed the opposite of PID1. PID7 resulted in a significant difference in dorsal and ventral regions (**Figure 34B.** $F(1,20) = 10.06$, $p = 0.005$, $\eta_p^2 = 0.34$), but not a significant difference between injury and SHAM groups

(**Figure 34B.** F (1,20) = 0.078, p = 0.78, η_p^2 = 0.004). STD was not significantly affected by dorsal or ventral regions in SHAM or injury groups on PID1 (**Figure 33A.** F (1,23) = 0.90, p = 0.35, η_p^2 = 0.038) or PID7 (**Figure 34A.** F (1,20) = 0.29, p = 0.59, η_p^2 = 0.014).

Note: while PID7 10 Hz – LTD showed significant differences between dorsal and ventral regions, the number of data is quite low, and more data collection may be required to elucidate true effects which was out of scope for the purpose of this study.

Table 12. 1 Hz - Long-Term Depression, dorsal and ventral regions PID1: average STD, LTD and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM Dorsal	-36.48 ± 6.47	-16.78 ± 10.29	N = 5, n = 6
SHAM Ventral	-28.40 ± 3.80	-16.29 ± 6.50	N = 6, n = 7
Injury Dorsal	-22.25 ± 4.94	-6.00 ± 12.42	N = 5, n = 8
Injury Ventral	-31.18 ± 6.40	-8.60 ± 7.98	N = 6, n = 6

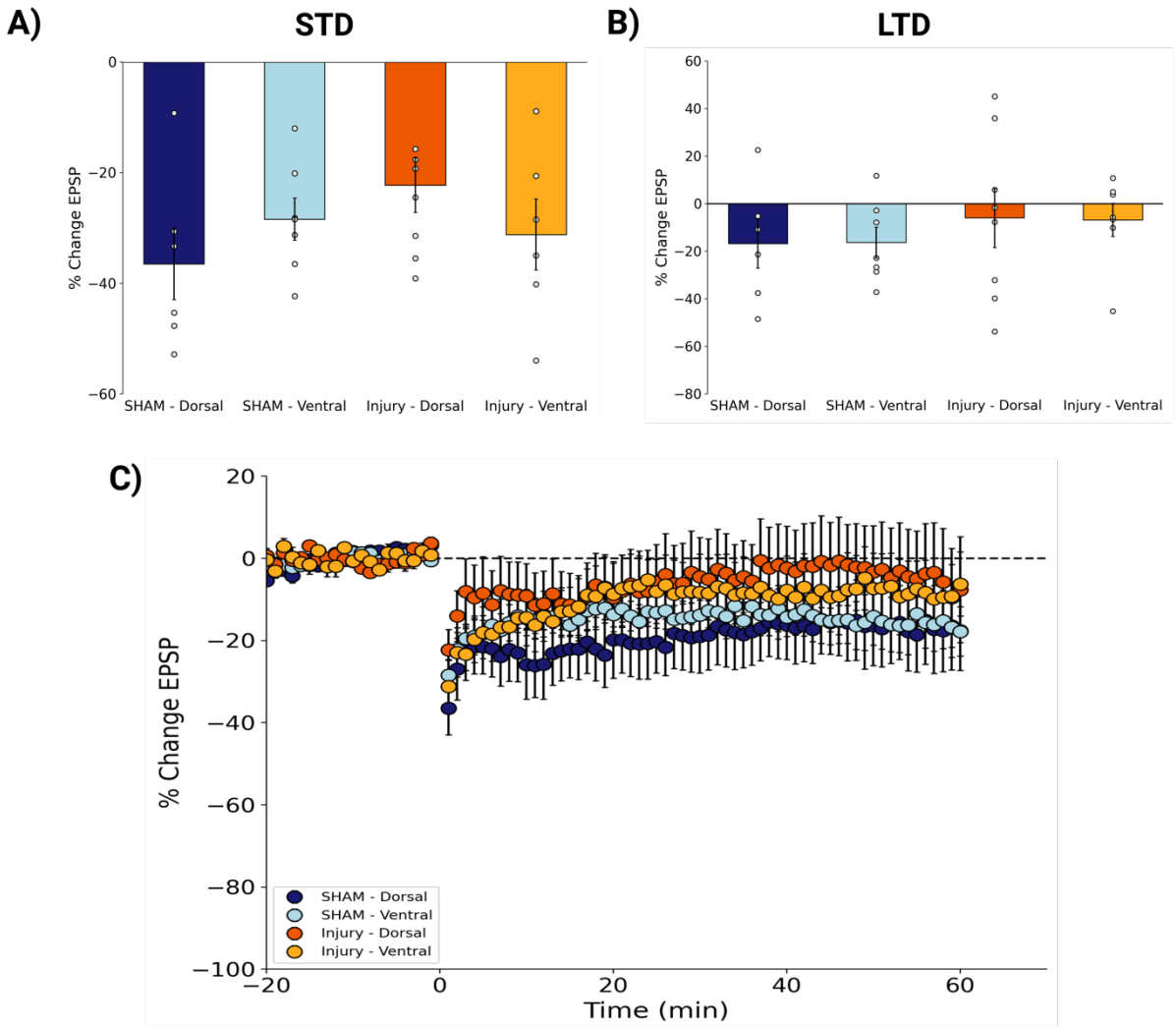

Figure 31. 1 Hz - Long-Term Depression (LTD) shows no significant differences between dorsal and ventral regions on post-injury day 1 (PID1).

(A) *Short-Term Depression (STD) is measured as the percentage change in fEPSP slope in the first minute following the low-frequency stimulus (LFS; 900 x 1 Hz).* ***(B)*** *LTD was measured as the average percentage change in the fEPSP slope relative to its baseline during the last five minutes following LFS (55-60 minutes). Dorsal and ventral regions showed no significant differences in the amount of LTD recorded on post-injury day 1.* ***(C)*** *The average traces of LTD recordings from the beginning of the baseline to the end of the post-conditioning recording for the injury condition in both dorsal and ventral regions are plotted along with their SHAM counterparts (SHAM–dorsal and SHAM–ventral). All analysis was done by way of a 2-way ANOVA. All error bars represent the standard error of the mean (SEM). Individual points for STD (A) and LTD (B) represent the average STD or LTD for each individual slice in this dataset.*

Table 13. 1 Hz - Long -Term Depression, dorsal and ventral regions PID7: average STD, LTD and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM Dorsal	-36.48 ± 6.47	-16.78 ± 10.29	N = 5, n = 6
SHAM Ventral	-28.40 ± 3.80	-16.29 ± 6.50	N = 6, n = 7
Injury Dorsal	-29.35 ± 4.89	19.99 ± 8.93	N = 3, n = 3
Injury Ventral	-20.83 ± 1.42	-5.89 ± 12.28	N = 3, n = 3

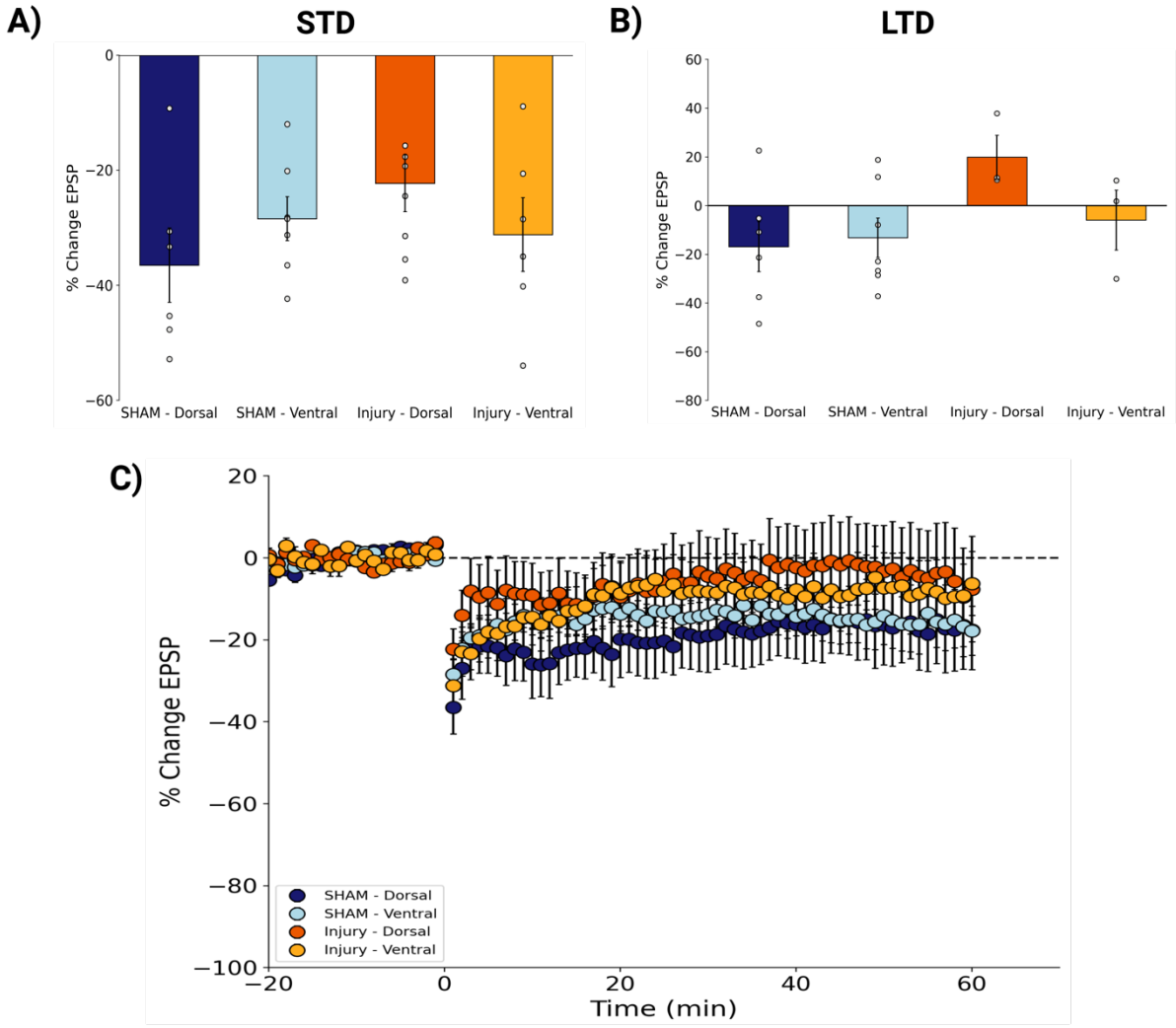

Figure 32. 1 Hz - Long-Term Depression (LTD) shows no significant differences between dorsal and ventral regions on post-injury day 7 (PID7).

(A) *Short-Term Depression (STD) is measured as the percentage change in fEPSP slope in the first minute following the low-frequency stimulus (LFS; 900 x 1 Hz).* ***(B)*** *LTD was measured as the average percentage change in the fEPSP slope relative to its baseline during the last five minutes following LFS (55-60 minutes). Dorsal and ventral regions showed no significant differences in the amount of LTD recorded on post-injury day 7.* ***(C)*** *The average traces of LTD recordings from the beginning of the baseline to the end of the post-conditioning recording for the injury condition in both dorsal and ventral regions are plotted along with their SHAM counterparts (SHAM–dorsal and SHAM–ventral). All analysis was done by way of a 2-way ANOVA. All error bars represent the standard error of the mean (SEM). Individual points for STD (A) and LTD (B) represent the average STD or LTD for each individual slice in this dataset.*

Table 14. 10 Hz - Long-Term Depression, dorsal and ventral regions PID1: average STD, LTD and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM Dorsal	-96.83 ± 3.19	-34.83 ± 10.32	N = 8, n = 9
SHAM Ventral	-94.73 ± 3.34	-12.87 ± 7.21	N = 4, n = 5
Injury Dorsal	-90.28 ± 5.27	2.96 ± 5.99	N = 5, n = 7
Injury Ventral	-96.63 ± 5.19	11.65 ± 9.48	N = 6, n = 6

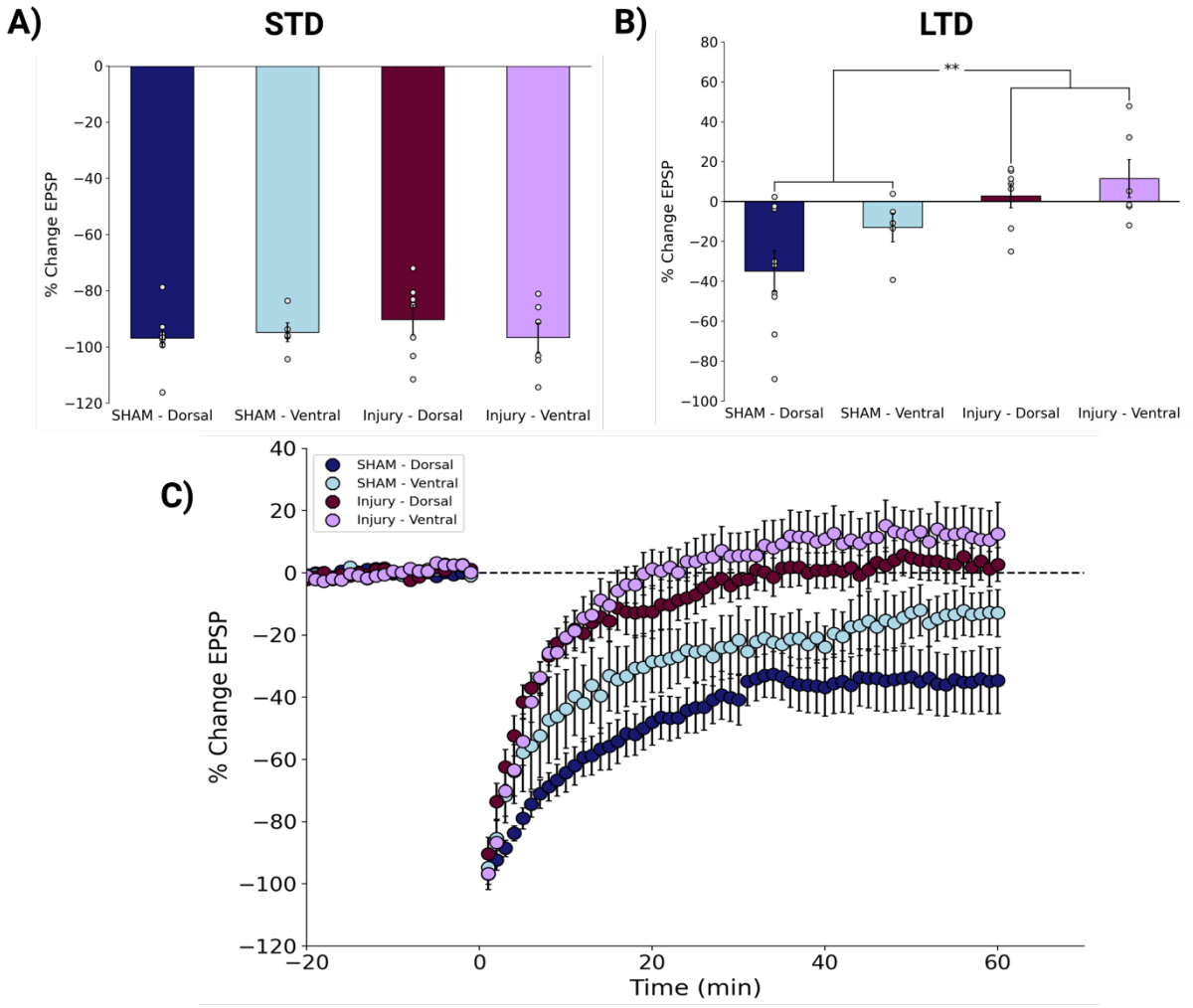

Figure 33. 10 Hz - Long-Term depression shows no significant differences in dorsal or ventral regions on post-injury day 1 (PID1).

(A) *The percentage change in fEPSP slope during the first minute after the low-frequency stimulus (6000 x 10 Hz) was used to measure short-term depression (STD), and there were no significant differences observed among the groups.* ***(B)*** *To measure long-term depression (LTD), the average percentage change in fEPSP slope relative to the baseline during the last 5 minutes of the LFS (55-60 minutes) was calculated, and while there were no significant differences in dorsal or ventral regions after injury on post-injury day 1, there was a significant difference between injury and SHAM groups.* ***(C)*** *The average LTD traces were recorded from the beginning of the baseline to the end of the post-conditioning recording for dorsal and ventral regions in injury animals and for their respective non-injured counterparts (SHAM – dorsal and SHAM – ventral). The standard error of the mean (SEM) is represented by black bars. A two-way ANOVA was used to analyze all comparisons, and individual points for STD (A) and LTD (B) show the average STD or LTD for each slice in this dataset. ** indicates a p < 0.001.*

Table 15. 10 Hz - Long-Term Depression, dorsal and ventral regions PID7: average STD, LTD and slice & animal count

Group	PTP	LTP	Animal (N) and slice (n) values
SHAM Dorsal	-96.83 ± 83	-34.83 ± 10.32	N = 8, n = 9
SHAM Ventral	-94.73 ± 3.35	-12.87 ± 7.21	N = 4, n = 5
Injury Dorsal	-94.10 ± 1.71	-48.09 ± 8.13	N = 6, n = 6
Injury Ventral	-88.59 ± 2.13	7.17 ± 21.60	N = 4, n = 4

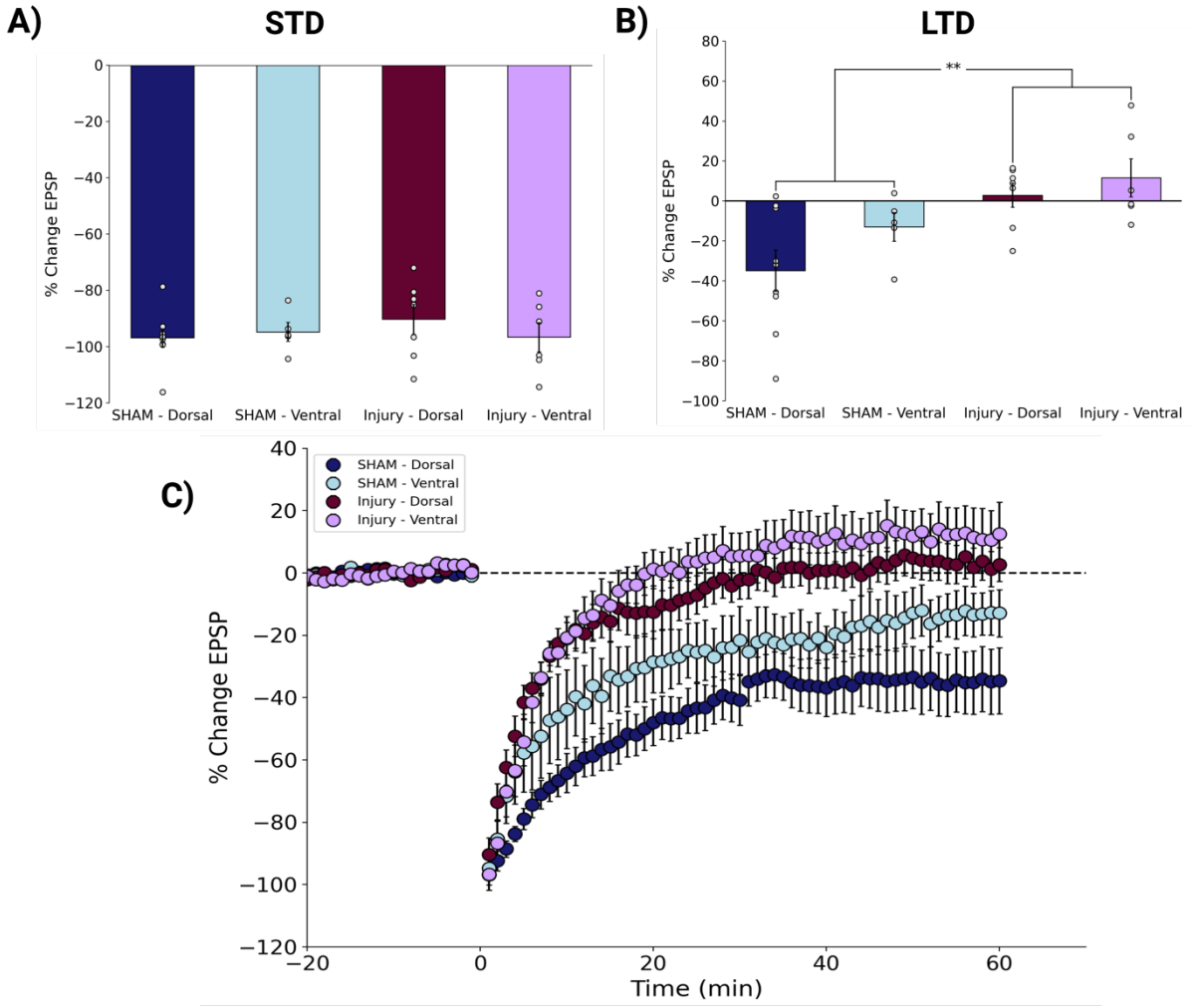

Figure 34. 10 Hz - Long-Term depression shows significant differences in dorsal or ventral regions on post-injury day 7 (PID7).

(A) *The percentage change in fEPSP slope during the first minute after the low-frequency stimulus (6000 x 10 Hz) was used to measure short-term depression (STD), and there were no significant differences observed among the groups.* ***(B)*** *To measure long-term depression (LTD), the average percentage change in fEPSP slope relative to the baseline during the last 5 minutes of*

the LFS (55-60 minutes) was calculated, and while there were no significant differences between injury and SHAM groups, there was a significant difference between in the amount of LTD recorded in dorsal and ventral regions in the DG post- r-mTBI ***(C)*** *The average LTD traces were recorded from the beginning of the baseline to the end of the post-conditioning recording for ipsilateral and contralateral hemispheres in injury animals and for their respective non-injured counterparts (SHAM – ipsilateral and SHAM – contralateral). The standard error of the mean (SEM) is represented by black bars. A two-way ANOVA was used to analyze all comparisons, and individual points for STD (A) and LTD (B) show the average STD or LTD for each slice in this dataset. * Indicates a $p < 0.05$.*

8. Appendix C: Preliminary results – Difference between ACHI paradigms

Our lab has investigated synaptic plasticity (LTP and 1 Hz - LTD) after two different ACHI paradigms: the first involved 8 injuries spaced two hours apart through the course of the day (**data collection acquired by me, 1 x 8**), while the second involved two injuries per day for four consecutive days, each also spaced two hours apart (**data collection performed by Dr. Christina Pinar and Dr. Luis Bettio, 4 x 2**). I conducted a comparison between the synaptic plasticity recorded and the different injury paradigms (through 2-way ANOVA) and found that 1 Hz -LTD was unaffected between the different injury paradigms (**Figure 35B.** $F(1,58) = 3.01$, $p = 0.09$, $\eta_p^2 = 0.005$) (Figure 35). It was found however that LTP recorded after the 1x8 injury paradigm was significantly larger than LTP recorded after the 4x2 injury paradigm (**Figure 35B** $F(1,56) = 12.28$, $p < 0.001$, $\eta_p^2 = 0.180$) (Figure 36). This data suggests that injury paradigms do induce different effects on synaptic plasticity.

***Table 16.** 1 Hz - Long-Term depression between injury paradigms: average STD, LTD and slice & animal count*

Group	STD	LTD	Animal (N) and slice (n) values
Control	-42.0 ± 3.78	-21.7 ± 2.82	N = 17, n = 34
PID1 – 4 x 2	-24.8 ± 3.57	-17.5 ± 3.68	N = 8, n = 16
PID7 – 4 x 2	-34.02 ± 3.55	-12.12 ± 4.30	N = 9, n = 16
PID1 – 1 x 8	-29.36 ± 3.8	-14.73 ± 5.68	N = 7, n = 13
PID7 – 1 x 8	-32.46 ± 3.89	-8.38 ± 5.05	N = 6, n = 10

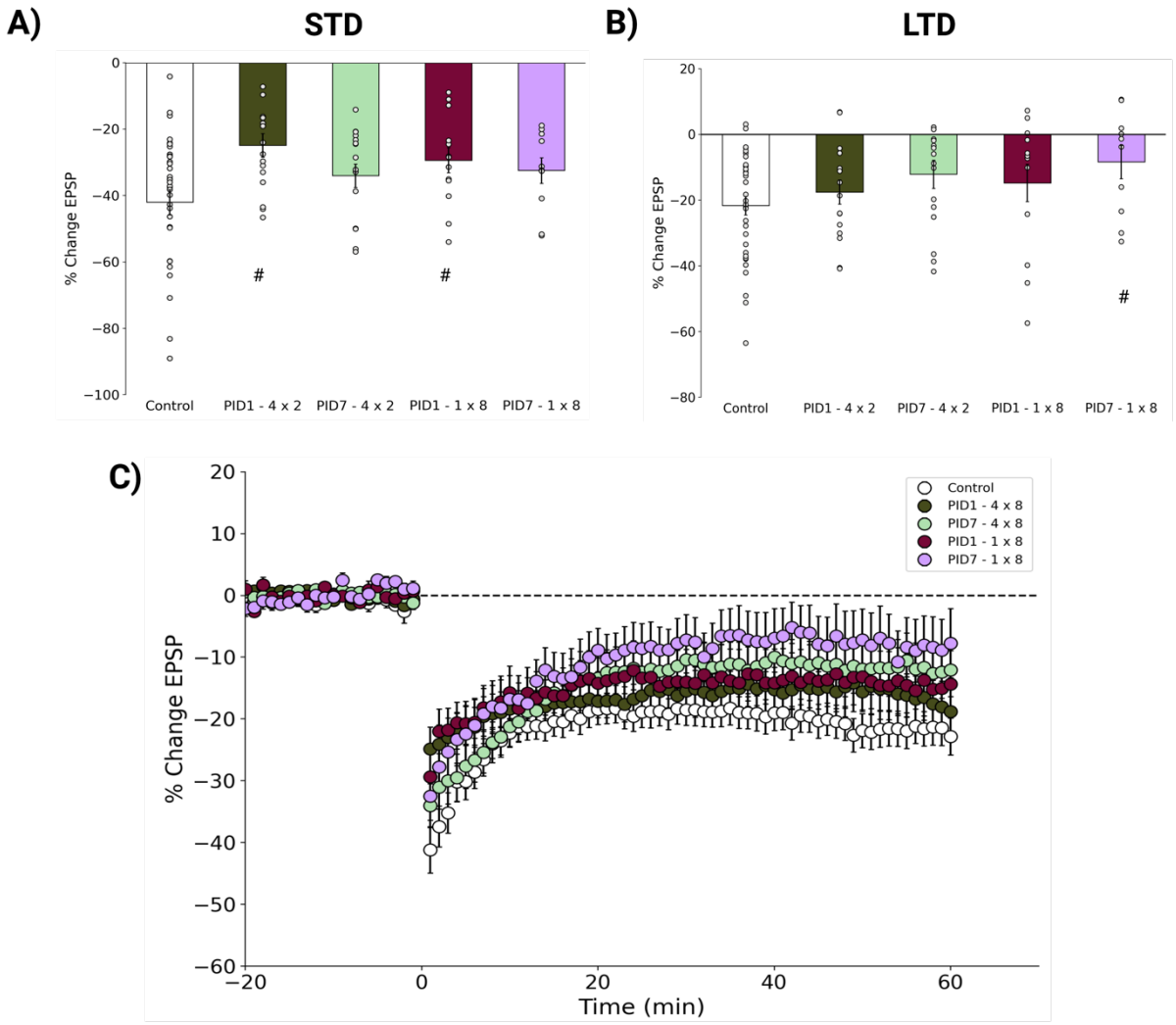

Figure 35. 1 Hz - LTD does not show significant differences between different injury paradigms

(A) *Short-Term Depression (STD) is measured as the percentage change in fEPSP slope in the first minute following the low-frequency stimulus (LFS; 900 x 1 Hz). There was a significant difference between the PID1- 4x2 and PID1 - 1x8 groups compared to the control (indicated by #)* ***(B)*** *LTD was measured as the average percentage change in the fEPSP slope relative to its baseline during the last five minutes following LFS (55-60 minutes). No statistical significance was found between injury paradigms, however, injury elicited by the 1x8 protocol on PID7 showed a significant reduction in LTD compared to control (indicated by #)* ***(C)*** *The average traces of LTD recordings from the beginning of the baseline to the end of the post-conditioning recording for different injury paradigms (1x8 or 4x2) are plotted for both post-injury day 1 (PID1) and 7 (PID7) along with a control group. All error bars represent the standard error of the mean (SEM). Individual points for STD (A) and LTD (B) represent the average STD or LTD for each individual slice in this dataset. # indicates $p < 0.05$ against control. All 1x8 data was collected by me, while the 4x8 data was collected by Dr. Christina Pinar and Dr. Luis Bettio.*

Table 17. Long-Term potentiation between injury paradigms: average STD, LTD and slice & animal count

Group	STD	LTD	Animal (N) and slice (n) values
Control	93.9 ± 6.72	51.8 ± 4.70	N =~ 14, n = 28
PID1 – 4 x 2	104.8 ± 17.1	42.3 ± 10.0	N =? n = 10
PID7 – 4 x 2	103.4 ± 7.08	41.7 ± 5.09	N =? n = 11
PID1 – 1 x 8	123.28 ± 12.05	78.30 ± 8.12	N = 11, n = 23
PID7 – 1 x 8	129.37 ± 14.33	69.25 ± 10.00	N = 7, n = 14

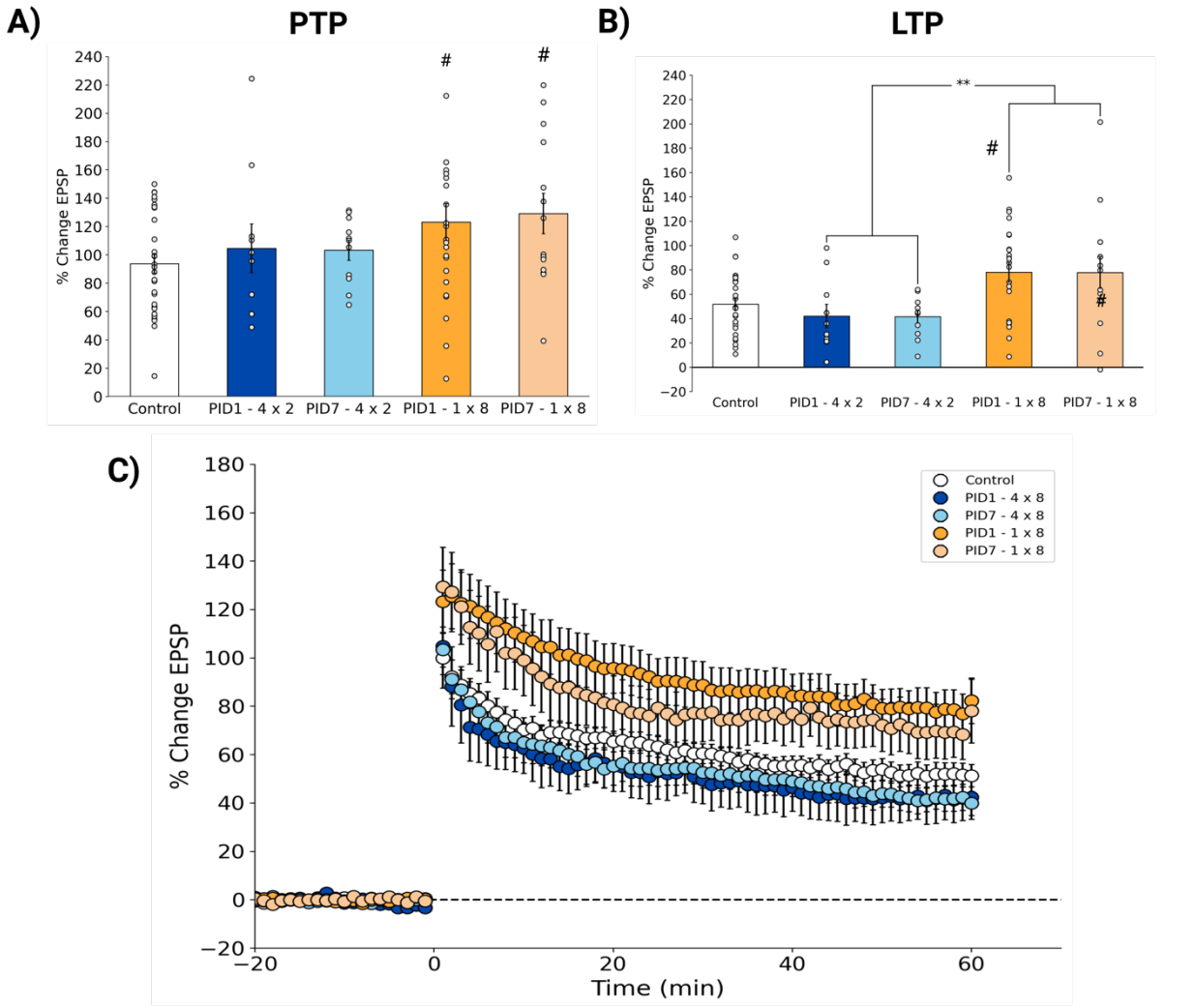

Figure 36. Long - Term potentiation shows significant differences between injury paradigms

(A) *Post-Tetanic Potentiation (PTP) is measured as the percent change in the fEPSP slope after the first minute following the high-frequency stimulus (HFS; 4 trains-50 pulses at 100Hz; 30s intervals). R-mTBI significantly affected the 1x8 protocol on PID7 compared to the control (indicated by #).* ***(B)*** *Long-Term potentiation (LTP) was measured as the average percent change in the fEPSP slope relative to its baseline in the last 5 minutes following HFS (55-60 minutes). It was found that there was a significant difference between injury paradigms (1x8 and 4x2) and the amount of LTP recorded. LTP on post-injury day 1 (PID1) for the 1x8 protocol was also found to be significantly elevated compared to control (indicated by #) (**C**) The average traces of LTP recordings from the beginning of the baseline to the end of post-conditioning recording for different injury paradigms (1x8 or 4x2) are plotted for both post-injury day 1 (PID1) and 7 (PID7) along with a control group. All error bars represent the standard error of the mean (SEM). All comparisons were analyzed using a two-way ANOVA and individual points for PTP (A) and LTD (B) represent the average PTP or LTP for each individual slice in this dataset. PTX indicates that the $GABA_A$ antagonist PTX was washed over the recorded slices throughout the duration of the baseline recording. # indicates $p < 0.05$ against control. All 1x8 data was collected by me, while the 4x8 data was collected by Dr. Christina Pinar and Dr. Luis Bettio.*

9. Appendix D – Cage Side Monitoring Sheet

Observer:	AUP#		
Species:	PI:		
Animal ID#			
Date			
Time			
Appearance			
Physical Signs			
Behaviour			
Weight (%)			

10. Appendix E – AUP

University of Victoria

Office of Research Services
Animal Care Committee
Administrative Services Building, Room B202

Research & teaching with ~~animals~~ website (ACC)

Animal housing & care website (ACS)

Protocol Number:	Start Date:	Expiry Date:
2019-002 (4)	14-Jul-22	31-Mar-23
Amendment Dates:		

For Administrative Use Only (AUP4)
last revised November 20-2015

Application to Use Animals for Research

Click on blue text to display "help boxes" while completing this form.
The Application HELP SHEET is available on the ACC website to assist you.

1. Project

Title

Repeated mild traumatic brain injury (mTBI) in rats

STATUS ☐ New Application ☐ Pilot Project ☐ Amendment of Protocol #: continuation w/o amendment

2. Contact Information

PRINCIPAL INVESTIGATOR

Surname	First Name	Initial
Christie	**Brian**	**R.**

Rank / Position (please indicate)	Department
Professor	**Division of Medical Sciences**

Business & Laboratory Telephone	Residence Telephone	Emergency / Cell Telephone
250-472-4244/ 250-721-8798	**250-477-4973**	**250-508-8780**

Laboratory Address	E-mail Address
Room 250, Medical Sciences Building, 3800 Finnerty Road, UVic, Vi	**brain64@uvic.ca**

NAME OF DESIGNATED ALTERNATE FOR EMERGENCIES

A. (mandatory)

Surname	First Name	Emergency Telephone
Reid	**Hannah**	**(604) 785-1063**

B. (optional)

Surname	First Name	Emergency Telephone
Snowden	**Taylor**	**(250) 713-0522**

3. Declaration

The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of the University of Victoria. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. **I understand that this approval is valid for one year and must be approved on an annual basis.**

Principal Investigator	Signature	Date
Dr. Brian R. Christie		

UVic Department Chair	Signature	Date
Dr. Bruce Wright		

(When the Department Chair is the Principal Investigator, the signature of the Dean is required)

Page 1

4. Approvals

University Veterinarian Signature

Chairman, University Animal Care Committee Signature

5. Amendments made to Protocol

☒ **N/A (New protocol; go directly to section 6)**

5a) Please indicate the section/subsection numbers where the amendments have been made.

☐ 6	☐ 7	☐ 8	☐ 9a	☐ 9b	☐ 10a	☐ 10b	☐ 10c
☐ 11	☐ 12a	☐ 12b	☐ 12c	☐ 12d	☐ 13	☒ 14a	☐ 14b
☐ 14c	☐ 14d	☐ 14e	☐ 15a	☐ 15b	☐ 15c	☐ 16a	☐ 16b
☐ 17a	☐ 17b	☐ 17c	☐ 17d	☐ 17e			

5b) Justification for amendment(s):

6. Primary Funding Information

Agency: **CIHR (renewal)** ☒ External ☐ Internal ☐ Other

Status: ☒ Awarded ☐ Pending

Peer Reviewed: ☐ No ☒ Yes

Funding Start Date: **1-April-2021** Funding End Date: **30-March-2026**

Funds Administered by: ☒ UVic ☐ Other – Provide Details:

Billing Account Number (required for invoicing of project costs): **39090-59420**

7. Research Timelines

Proposed Start Date of Research: **1-Jul-20**

Proposed End Date of Research: **30-April-2026**

8. Category of Invasiveness

☐ A ☐ B ☐ C ☒ D ☐ E

9. Animal Use Data for CCAC

9a) Purpose of Animal Use

If the application includes breeding, select 0. If the application also includes research, select one additional option of 1 - 5.

☒ 0. Breeding colony / stock
☐ 1. Studies of a fundamental nature / basic research
☒ 2. Studies for medical purposes relating to human / animal diseases / disorders
☐ 3. Studies for regulatory testing of products, for the protection of humans, animals or the environment
☐ 4. Studies for the development of products or appliances for human or veterinary medicine
☐ 5. Education and training of individuals in post-secondary institutions or facilities

9b) Study Details

i. Will field studies be conducted? ☐ Yes ☒ No

ii. Will research involve surgery? ☒ Yes ☐ No ☒ recovery surgery or ☒ terminal

Please review the Policy on Survival Surgery of Rodents, Recovery Surgery checklist and Surgical record template.

iii. If breeding transgenics, knockouts or mutant animals, please complete the Transgenic Information Sheet

10. Summary

10a) Lay description (in language that will be understood by members of the general public): (2-3 sentences please)

Mild traumatic brain injury (mTBI) can lead to many impairments in learning and memory, and depression. These impairments tend to be more severe and prolonged after multiple mTBI, and may even progress to lifelong disability and neurodegenerative disorders. We aim to assess the learning and memory impairment, depressive behaviour, and related pathological processes that occur following single or multiple traumatic brain injury. Importantly, previous research on this subject has been completed in anesthetized animals, which is not representative of typical human mTBI.

10b) Specific scientific objectives of the study (2 – 3 sentences):

We aim to induce single and repeat mTBI in our awake rat model, and assess deficits in behavioural outcomes as they relate to cellular and molecular pathological and inflammatory processes in the brain. Previous research in this area has primarily been completed in anesthetized animals. This is not representative of typical human mTBI, and can confound results because common anesthetics are neuroprotective and would affect the interpretation of data following mTBI. To provide a comparison, we will continue also using our weight drop model that creates an mTBI in anaesthetized animals.

10c) Keywords (click here for a list of recommended keywords):

Research, breeding, stereotaxic surgery, survival surgery, tissue/organ collection, minor surgery, behavioural observation, short duration physical restraint, blood sampling (small volume), special diet, injection (subcutaneous, intraperitoneal, intravenous), rat.

11. Alternatives

State why animals must be used in the study. If alternatives to animals are available, indicate why they are not being used in this study.

Alternatives to animals are not available for this type of study as this work addresses how a mild injury affects subsequent behaviour, and how this reflects neuropathological processes at a cellular and molecular level. Additionally, rats provide an ideal model for the study of TBI as they mimic the physiological conditions seen in humans, which may be reliably observed and measured. Such relationships cannot be assessed using cell culture, or computerized models.

12. Animal Data

12a) Describe the rationale for using this specific species or strain.

Sprague-Dawley and Long Evans rats are commonly used in this type of research and will provide a model that is easily compared to existing literature. The types of deficits they show following mTBI are similar to those seen in humans, and they perform reliably in behavioural tests that are commonly used to assess these deficits. SD rats are preferred for research using aged animals, while Long Evans animals perform better on many behavioural tasks. We are finishing a number of experiments with the Long Evans animals that were started on the prior version of this protocol, and forsee shifting more to using Sprague Dawley animals for aging work over the course of this protocol.

12b) Are there special housing requirements? (Normal housing parameters can be found at the ACS website **here**)

☐ No ☒ Yes

Please review the ACC Procedure on Alternative Housing of Experimental Animals.

If yes, please list them (including the species or strain):

Animals will be housed with a red hut (red huts are red transparent huts used for enrichment provided by animal care services) once weaned and post surgery (if required i.e. weight drop model). Animals will be singly housed to a maximum of 72 hours, following any surgery to allow them to recover without interference

from other animals." If surgery is required, animals will be monitored post-surgery as described in CL010: Weight drop model of traumatic brain injury. Post-surgery, food will be placed on the floor of the cage directly to facilitate access.

12c) Indicate the number of animals required.

☒ Lab animals (mammalian) required?

Delete Strain Record

MAMMALIAN - Species			Common Name			Supplier / Source		Strain		
Rattus norvegicus			Rat			Charles River		Long-Evans		
Housing Location	**Experimental Location**	**# Acquired / Purchased**	**# females breeding**	**# males breeding**	**# females culled**	**# males culled**	**# females for experiment**	**# males for experiment**	**# Needed at One Time**	**Total # Per Year**
MSB	MSB	38	18	18	96	20	270	420	30	846

Delete Strain Record

MAMMALIAN - Species			Common Name			Supplier / Source		Strain		
Rattus novergicus (Drinking Cohort)			rat			Charles River (for breeders); colony will be bred internally		Long-Evans		
Housing Location	**Experimental Location**	**# Acquired / Purchased**	**# females breeding**	**# males breeding**	**# females culled**	**# males culled**	**# females for experiment**	**# males for experiment**	**# Needed at One Time**	**Total # Per Year**
MSB	MSB	6	4	2	4	4	16	16	8	46

Delete Strain Record

MAMMALIAN - Species			Common Name			Supplier / Source		Strain		
Rattus norvegicus (Physiology Experiments)			rat			Charles River		Long-evans		
Housing Location	**Experimental Location**	**# Acquired / Purchased**	**# females breeding**	**# males breeding**	**# females culled**	**# males culled**	**# females for experiment**	**# males for experiment**	**# Needed at One Time**	**Total # Per Year**
MSB	MSB	26	20	6	67	67	402	402	30	964

Delete Strain Record

MAMMALIAN - Species			Common Name			Supplier / Source		Strain		
Rattus norvegicus (aging)			Rat			Charles River & in-house colony breeding		Sprague-Dawley		
Housing Location	**Experimental Location**	**# Acquired / Purchased**	**# females breeding**	**# males breeding**	**# females culled**	**# males culled**	**# females for experiment**	**# males for experiment**	**# Needed at One Time**	**Total # Per Year**
MSB	MSB	80					40	40	20	80

Add Strain

☐ Field animals required?

☐ Lab animals (aquatic species) required?

12d) Indicate how you arrive at your total number of animals (click here for an example calculation):

Awake Closed Head Injury (ACHI) Experimental Work
Group N: Animal numbers for our experiments are based on a maximum n=10 animals for any experiment. This is common in this type of work and provides the N necessary for adequate power for the histology and electrophysiology experiments required in most experiments.
Time Points: We have found that we require up to 5 main time points for our experiments: 1, 3, 7, or 28 days post-injury; 1 yr post injury.
Experimental Groups: We will use both male and female animals for the following four groups. (1) Control animals (no intervention); (2) SHAM (undergo procedures but no injury); (3) mTBI (animals administered awake closed head injury); (4) repeat mTBI (animals are administered ACHI repeatedly. In some cases we will perform these procedures with the animals anaesthetized to evaluate the neuroprotective capacity of anaesthesia.
Animal Calculation for each experiment: Behaviour: 2 time points x 1 experiment x 9 group types x 10 per group) + (Awake immuno and molecular: 4 time points x 2 experiments x 6 group types x 10 per group) + (Anaesthetised immuno and molecular: 2 time points x 2 experiments x 3 group types x 10 per group) = 690
12 animals per litter will be used, and an average of 2 animals per litter will be culled. 690 animals / 12 per litter = ~58 litters.

Weight Drop TBI Protocol: We are retaining this protocol as it provides a means to administer a greater impact force than the ACHI model.
5 female breeders + 5 male breeders, each dam bred ~3 times = 15 total litters x 12 pups used experimentally
15 x 12 = 180 pups used experimentally (total) + 2 pups (average) culled/litter = 180 + 30 = 210 total offspring
210 total offspring + 10 breeders = 220

TBI Drinking Cohort:
Groups: repeat mTBI (male, female), sham (male,female)
Calculation:
4 group types x 8 per group = 32
4 litters x approx. 2 pups culled = 8
58 litters x 2 pups culled per litter = 116 animals culled. 150 more males are needed than females, so males will only be culled when required to allow weaning into groups of 3. The small discrepancy between females culled and extra males needed will be accommodated by actual variation in litter size and ratio of males and females born.

Physiology Projects
Time Points: 1 day, 3 days, 7 days, 28 days, or up to 1 yr post-injury.
Experiments: In vitro electrophysiology, in vivo electrophysiology, stress assessment
Groups: repeat mTBI (male, female), sham (male, female), cage control (male, female)

Animal Calculation: For the electrophysiology work we typically require additional animals to achieve adequate experimental power.
In vitro electrophysiology: 5 time points x 1 experiments x 6 group types x 12 per group = 360
In vivo electrophysiology: 5 time points x 1 experiments x 6 group types x 12 per group = 360
Stress Assessment: 1 time point x 1 experiment x 14 group types x 6 per group = 84
Total experimental animals: 804

Aging cohort: For the aging cohort we will also work with Sprague Dawly rats, using retired breeders for experiments rather than just culling them at the end of their effective breeding period.

Groups: repeat mTBI (male, female), sham (male, female)
Calculation:
4 group types x 20 per group = 80 animals total.
Animals will usually not be bred, and will be ordered at the correct age-point we wish to study.

13.

Standard Operating Procedures (SOP's) (click here to access all SOP folders on the ACS website)

Please click on the subject and choose the SOP's that apply to your research or teaching project:

- ☐ **Antibodies**
- ☐ **Aquatics - General Procedures**
- ☐ **Aquatics - Health**
- ☒ **Facility Procedures (mammalian)**
  - ☐ AC1021 BWC - Entry/Exit Procedures
  - ☐ AC1015 Disposal of Soiled Animal Bedding Containing Infectious Waste
  - ☒ AC1016 MSB - Entry/Exit Procedures
  - ☐ AC1045 AIMS Tattoo Machine Care and Maintenance
  - ☐ AC1031 BWC - Intra-facility Movement of Personnel
  - ☐ AC1033 Procedure Room Cleaning: BWC
  - ☐ AC1034 Procedure Room Cleaning: MSB
  - ☐ AC1038 BWC Sanitation
  - ☐ AC1039 MSB Sanitation
  - ☐ AC1040 BWC - Transportation of Laboratory Animals
  - ☐ AC2059 Fecal Flotation Test
  - ☐ AC2066 Perineal and Fur Testing for Endo/Ectoparasites
  - ☐ AC2070 Procedures for Escaped Rodents
  - ☐ AC3001 Surgical Equipment Sterilization Techniques
  - ☐ AC3009 BWC Dumpstation Use and Maintenance
  - ☐ AC3011 MSB Dumpstation Use and Maintenance
  - ☐ AC3014 Use of Freight Elevator - BWC
  - ☐ AC3017 Processing of Cytotoxic Cages
  - ☐ AC3021 Use of Class 3B Laser
- ☐ **Field studies**
- ☒ **General Animal Procedures**
  - ☒ AC2009 Rodent Analgesia - Adult
  - ☒ AC2013 Rodent Euthanasia
  - ☐ AC2015 Chicken- Blood Collection
  - ☐ AC2027 Gavage Procedure in Adult Rats
  - ☒ AC2030 Vaginal Lavage in Rats
  - ☐ AC2032 Tail Tip Biopsy - Mice
  - ☒ AC2038 Monitoring of Animals with Abnormalities
  - ☐ AC2061 Necropsy - Rodents & Lagomorphs

☐ AC2069 Housing Immunocompromised Mice in a Ventilated Rack
☐ AC2072 Rodent ear notching for genotyping: weanling and adult
☐ AC2073 Mouse Gavage Procedure
☐ AC2074 Rodent Tattoo Identification
☐ AC2075 Use of a Heating Lamp of Coils - Rodents
☒ AC2076 Guillotine and Sharp Scissor Use

☒ Husbandry

☐ AC1013 BWC - Mouse Cage Changing
☒ AC1014 MSB - Rat Cage Changing
☐ AC1023 BWC - Biosafety Level II Containment: Cage Changing Procedures for Mice Housed in a Negative Air Rack
☒ AC1030 BWC - Rat Cage Changing
☐ AC1037 MSB - Mouse Cage Changing
☐ AC1042 Temporary Laboratory Housing of Experimental Animals
☐ AC1043 Mouse Husbandry in the C.R. Semi-Rigid Isolator
☐ AC1047 Receiving Rodents and Rabbits from a Commercial Source
☐ AC2048 Rodent Husbandry - Breeding Mice (all animal units)
☐ AC2069 BWC - Housing Immunocompromised Animals in a Ventilated Rack
☐ AC3017 Processing of Cytotoxic Cages

Rabbits

☒ Rodents - Anaesthesia & Surgery

☒ AC2003 Anaesthesia - Adult
☐ AC2005 Castration
☐ AC2008 Ovariectomy
☒ AC2013 Rodent - Euthanasia
☐ AC2040 Implantation of LinBit Insulin Release Implants
☐ AC2041 Irradiation of Tumours - Mice

☒ Rodents - Blood Collections & Injections

☒ AC2007 Handling & Injection Techniques
☒ AC2018 Cardiac Blood Collection in Rodents
☐ AC2019 Jugular Blood Collection in Rodents
☒ AC2020 Lateral Saphenous Vein Blood Collection in Rodents
☐ AC2029 Tail Blood Sampling - Rodents
☐ AC2049 Medial Saphenous Vein Blood Collection in the Mouse
☐ AC2064 Intranasal Inoculation - Adult Mice

Will Animal Care Services Staff be assisting with the use of SOP's?

☐ Yes ☐ No

Research Lab specific or other SOPs? Please list:

CL001 Behaviour Object Context Rodents
CL003 Open Field Test rats

CL004 Elevated Plus Maze rats
CL005 Forced Swim Test Rodents
CL010 Weight Drop Model
CL011 Social Behaviour
CL015 Awake mTBI Impact Model
CL016 Morris Watermaze Rats
CL017 Sucrose Preference Test
CL019 Rat Neurological Severity Scale
CL020 Rotarod Test Rodents
CL021 Beam Walk Rodents
CL022 Barnes Maze Rodents
CL023 Play Fighting
CL024 Spontaneous Alternation
CL026 Chronic Unpredictable Stress
CL027 Intermittent Access to Ethanol in a 2-Bottle Choice Procedure
AC1027 MSB Daily Rounds
AC2078 Rodent Euthanasia by open drop Isoflurane Delivery

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14. Description of Procedures

14a) Describe all procedures and techniques (reference SOPs where possible):

Mating and birth: Animals will begin breeding after postnatal day 60 (P60), and when they have grown to 250 g. Breeding pairs will remain housed together. Males and females may be used for multiple litters (Max 4) but we will stop breeding animals at 12-18 months of age, or if they are unable to produce at least ten healthy pups in two consecutive litters.
Litters: Dam and pups will not be disturbed for 24 hours postpartum to facilitate bonding. Pups will be counted and monitored by ACS staff to ensure they are thriving. Litters will be culled to 12 pups where possible on P2-3 by Christie lab personnel.
Weaning: Pups will be weaned at P22 by ACS staff . Males and females will be separated, and housed in groups of 2-3. Groups will be limited to 3 in order to allow animals to remain housed in triplets after the impact procedure. Animals will be marked for identification with a tail band by the researcher at the time of any procedure. One band will indicate sham, two bands will indicate mTBI, no bands will indicate rmTBI.
Aging Animals: Animals that remain in the colony for any aging studies will be housed in groups of 2-3 and monitored by ACU staff following AC1027 (MSB Daily Rounds) to ensure they are in good health. Animals exhibiting any abnormalities will be reported to the investigators and monitored using SOP AC2038 (Monitoring rodents and rabbits with abnormalities).

Awake mTBI Cohort:
Male and female animals will undergo the impact procedure as is described in SOP#CL015 and undergo a Neurological Severity Assessment (CL019) after each procedure (Experimental or SHAM). Animals will be divided into three experimental groups: repeat mTBI (rmTBI) , single mTBI (mTBI), and sham. rmTBI animals will undergo up to 2-8 procedures per day for up to 2-7 days. Most animals will receive 8 impacts to continue our rmTBI studies, but the studies require the flexibility to deliver up to 42 impacts in one cohort of animals as in the Petraglia study (2014). While this may appear excessive, these authors report "*There were no post-traumatic apneic episodes or seizure activity observed, and no mortalities secondary to any of the impacts.*" We have a very low mortality rate for our work (<1%) as this is in reality a very mild procedure. We anticipate requiring up to 10 animals for this cohort initially. Single mTBI animals will undergo sham procedures followed by a single impact. This will follow the same time line as the rmTBI animals. Sham animals will undergo only sham procedures following the same time line as the rmTBI animals. An impact record will be kept for each animal (See CL015). Animals will be monitored following each impact, and will be removed from the experiment and euthanized if they reach a clinical endpoint

(Refer to section 14c Clinical Endpoints and section 14d Monitoring). Animals will remain housed in triplets following the procedure, and will be differentiated by marking tails.
For the aging cohort, as animals grow larger, we may switch to double housing, allowing more space for the animals. Aging animals will be monitored for age related ailments (e.g. tumor growth) and will be removed from the experiment and euthanized if they reach a clinical endpoint (refer to section 14c) during the aging process. Because aging animals may naturally exhibit several clinical signs that would indicate significant morbidity in younger animals, and may also experience certain benign ailments at an increasing incidence, clinical endpoints will be determined in consultation with ACU staff.

Anesthetized mTBI Cohort:
In order to demonstrate the necessity for an awake model of mTBI, a subgroup of male rats will undergo single, repeat, and sham procedures while under anaesthesia. Anaesthesia will be applied following SOP#AC2003: Rodent-Anaesthesia. The animals will undergo the impact procedure as is described in SOP#CL015 with the following additions: Isoflurane anaesthesia will be induced (2-3% iso; 1 L/min O_2), and the rat will be transferred to the restraint bag when no longer able to right itself in the induction chamber. Anaesthesia will be maintained (1-3% iso; 0.6 L/min O_2) by placing the face mask around the protruding nostrils/ breathing hole of the restraint bag, and the rat will be secured in the bag when toe-pinch reflex is absent. The rat will be moved to the impact platform, and the anesthetic hose will be taped in place. Immediately after impact, anaesthesia will be removed and the rat will be removed from the restraint bag. When consciousness is regained (indicated by the ability to stand and some mobility) the rat will be moved to a dark recovery cage lined with a towel, warmed with a water circulating heat pad. When the animal is behaving normally (e.g. grooming, exploring) it will be returned to its home cage and monitored in the same way as anaesthetised rats.

Weight Drop TBI:
The TBI weight drop model procedure will be carried out exactly as outlined in SOP CL010: Weight Drop Model of Traumatic Brain Injury. Sham controls will undergo the same procedure, minus the weight-drop event.

For animals receiving "multiple TBI" a second identical impact procedure will be performed 24hrs after the initial procedure. Animals will receive the same pre-surgery analgesics as mentioned in SOP# CL010: Weight Drop Model of Traumatic Brain Injury. If animals are experiencing clinical problems associated with the first injury, they will be removed from the study and euthanized, as animals that are treated differently from the rest will affect the purpose of the study. An animal that has undergone a single TBI will not go through with a second injury unless it has fully recovered with no abnormalities (refer to clinical endpoints in section 14c).

Behavioural Assessment:
In order to determine if the deficits observed with long term potentiation (LTP) are correlated to changes in hippocampal dependent behaviours that are described in individual SOPs. Typically we will assess animals for anxiety related behaviours: CL003 (Open Field); CL004 (Elevated Plus Maze); and/or CL005 (Forced Swim task) and/or CL017 (Sucrose Preference); or for motor related behaviours such as CL020 (Rotarod test); CL021 Beam Walk Test; or for learning and memory related behaviours: Typically one of CL001 (Behaviour Object Context Rodents); CL016 (Watermaze); CL022 (Barnes Maze); CL023 Play Fighting); CL024 (Spontaneous alternation). In some cases we expose cohorts of animals to unpredictable stress (CL026) to determine if rmTBI reduces the capacity to deal with stressful situations. These animals would then be assessed using CL003 (Open Field); CL004 (Elevated Plus Maze); and/or CL005 (Forced Swim task) and/or CL017 (Sucrose Preference).

Drinking Cohort:
These experiments will look at whether TBI impacts addictive behaviours. This is outlined in SOP# CL027.

Mating, birth and weaning will be conducted as described above except that in the present set of experiments rats will be singly housed in order to accurately measure alcohol consumption. Awake rmTBI will occur as described in CL015.
Experimental groups will consist of males and females randomly assigned to either the sham or rmTBI (described above) condition. TBI or sham procedures will be conducted on P 26 and 27. The drinking protocol described herein is a modified procedure used for adolescent Sprague-Dawley rats (Vetter et al., 2007 Alcoholism: Clinical and Experimental Research). On P28, drinking water will be removed for 2 hours (afternoon; 1-3pm) and animals will be weighed. At the end of this delay animals will be subjected to a standard 2-bottle voluntary choice experimental design where one bottle contains regular tap water and the other bottle contains 10% Ethanol diluted in tap water. The positions of these bottles on the cage top will be randomized between animals and the positions will be alternated daily in order to eliminate bottle position preferences. Drinking liquids will be removed daily for 2 hours (1-3pm) and experimenters will measure the volume of each liquid consumed. Animals will also be weighed every 2 days during this time. This procedure will continue from P28 or P60 until P50 or P82 (22 days of drinking for either young adult or adult animals) where at the beginning of the deprivation time (1pm) ~1mL of blood will be collected from the lateral saphenous vein as per animal care SOP 2020 in order to assess blood alcohol concentrations of animals. Blood will be collected only once, approximately midway (~P39) through the drinking period. The animals will then be euthanized via inhaled isofluorane anesthesia followed by rapid decapitation.

Molecular experiments: Animals will be anesthetized with isoflurane (bell jar, >5% inhalant) and immediately decapitated at the appropriate experimental endpoint.

Immunohistochemistry experiments: Animals may be anesthetized with isoflurane (vaporizer, 2-3% inhalant) and then tracers such as Evan's Blue dye (1-4%, 0.5mL) or FITC-dextran (0.5mL of 300mg/mL) solution in 0.9% saline will be injected and allowed to circulate for 30-45 minutes. Animals will be deeply anesthetized (bell jar, >5% inhalant) and transcardial perfusion with 0.9% saline and 4% paraformaldehyde.
Injection of Bromodeoxyuridine (BrdU): BrdU is a thymidine analogue that incorporates into the DNA during the S-phase of the cell cycle and is commonly used in research as an exogenous marker of cell proliferation and neurogenesis. BrdU will be given as an intra-peritoneal (i.p.) injection up to 2x/day 4 hours (minimum) apart over a period of up to 7 days (maximum 200 mg/kg/injection, prepared in 0.1 M TBS; Sigma). Animals will be sacrificed by transcardial perfusion 2 hours (for cell proliferation experiments), 2 weeks or 4 weeks (for cell survival experiments) after the last BrdU injection. All cages housing animals that received BrdU injections will be visibly labeled with cage tags with the word “BRDU” written on them.

Note: BrdU will be administered following the injection protocols described in SOP# AC2007 Rodent-Handling and Injection Techniques. All drug solutions will be prepared in a certified fume hood in MSB#250 using sterile techniques and at physiological pH (pH = 7.4). All personnel preparing and handling the listed compounds will use appropriate protective equipment.

Physiology Experiments:
In vitro electrophysiology: Animals will be deeply anesthesized with inhaled isofluorane and will be rapidly decapitated following loss of response to the toe and tail pinch reflexes. Brains will be rapidly extracted and sliced for experiments in cold artificial cerebrospinal fluid.

In vivo electrophysiology: Animals in these experiments will undergo in vivo electrophysiology as per previous work in the Christie laboratory (Sickmann et al., 2014 Hippocampus; Patten et al., 2013 Neuroscience Letters). Following rmTBI at the 5 time points indicated above, animals will be anesthetized with urethane (1.5 mg/kg, I.P) and brought to the lab (MSB250) for acute in vivo electrophysiological experiments and placed in a stereotaxic instrument where the skull will be exposed by making an 3-4 cm long incision. Subsequently, bregma coordinates will be determined for calculation and marking of drilling

spot. Four holes are made in the skull (each 2 mm wide) for insertion of the grounds, stimulating and recording electrodes, respectively. The recording and stimulating electrodes are gently lowered into the hippocampal area of interest. The studies will proceed for 3 hours during which the level of anaesthetic will be monitored using toe pinch reflex. A top up of 0.15 - 0.25mg/kg of urethane will be given if the animal shows signs of waking up. All manipulations will stop until the animal is deep enough. Animals will be euthanized at the end of the experiment by administering an anaesthetic overdose of urethane (1.5mg/kg I.C) followed with decapitation.

Blood Withdrawal for Stress Assessment:
In order to assess whether the mTBI and sham procedures are causing stress that could confound the plasticity experiments above trained individuals will collect blood from the lateral saphenous veins as per AC SOP 2020 immediately following the completion of the Neurological Assessment Protocol (NAP) following the final rmTBI or sham procedures. Immediately following blood collection these animals will be deeply anesthesized by inhaled isoflurane and rapidly decapitated upon loss of the toe pinch and tail pinch reflexes.

Neurological Assessment Protocol:
In order to rapidly and objectively assess cognitive and motor function after mTBI or sham, each rat will be assessed using the neurological assessment protocol (NAP). (See Christie Lab SOP entitled Rat Neurological Severity Score). Briefly, each rat will undergo a rapid and simple assessment of consciousness (apnea; toe pinch reflex; righting reflex) and neuromotor function (startle response; limb extension; flat beam walk; rotating beam walk). Each rat will be tested at baseline 1 hour before initial impact/sham, and then retested after each impact/sham, and also retested one hour before final endpoint. Every subject within an individual experiment will undergo this assessment so that performance may be correlated with subsequent physiological observations. The neuromotor assessment will also aid in the acute stage of post-impact monitoring described in 14d) as the total neuromotor score will aid in assessing each subject's recovery. That is, a perfect score indicates a rat is not experiencing significant symptoms of their injury, or has recovered, and a low score indicates that a rat may not have recovered and requires further observation until returned to home cage.

14b) Experimental endpoint:

Experimental endpoints for most procedures will be 1, 3, 7, or 28 days post-procedure, however a cohort will also be allowed to age up to 24 months after receiving the procedure as an adolescent. Please note that all animals will have cage-mates unless in the immediate phase of recovering from anesthesia.

Experimental endpoints for weight drop TBI are at 1 hr, 1 day, 7 days or 28 days after 1x, 2x or sham TBI surgery unless they need to be removed from the experiment because they have reached a clinical endpoint.

BrdU endpoints: BrdU will be given as an intra-peritoneal (i.p.) injection up to 2x/day 4 hours (minimum) apart over a period of up to 7 days (maximum 200 mg/kg/injection, prepared in 0.1 M TBS; Sigma). Animals will be sacrificed by transcardial perfusion 2 hours (for cell proliferation experiments), 2 weeks or 4 weeks (for cell survival experiments) after the last BrdU injection.

For stress assessment experiments experimental endpoints are immediately following blood collection that occurs immediately following the completion of the NAP following the final impact.

For the drinking cohort, the experimental endpoint is following 22 days of drinking after the final impact.

If an animal reaches a clinical endpoint (Outlined in 14c) before the assigned experimental endpoint they will

be euthanized and removed from the experiment.

Males and females may be used for multiple litters (Max 4) but we will stop breeding animals at 12-18 months of age, or if they are unable to produce at least ten healthy pups in two consecutive litters.

14c) Clinical endpoint:

Clinical end-points refer to Guidelines for Endpoints Setting, Monitoring and Humane Euthanasia for Animals Used for Research. Because animals are bred in house, any problems with breeding (i.e. dystocia, cannibalism, poor mothering/neglect) will result in animals being removed the experiment and euthanized as appropriate, and in consultation with ACU staff. Commonly used signs of moribundity for aged rats include, but are not limited to: a) lack of responsiveness to manual stimulation; b) immobility; and/or c) an inability to eat or drink. For breeding animals we will also work with ACU staff to keep alert for clinical endpoints that include dysotcia, cannibalism, poor mothering/maternal neglect, etc. In these studies, animals exhibiting these signs will be evaluated in consultation with ACU staff to determine if animals should be euthanized. Similarly Animals that receive a TBI will be removed from the experiment and euthanized if they show the following signs of distress or illness (refer to Appendix 3 for pain scores):

- Complete limb paralysis resulting in self-trauma
-Weight loss (>15% body weight relative to age and sex-matched litter mate) regardless of supportive care
-Urinating on self resulting in perineal urine scalding
-Chewing at impaired limbs
-Inability to independently consume food and water
-Pain score of 3 in any pain chart category
-Combined pain score of 6 or greater
-Age greater than 24 months

Because there are no known issues associated with consumption of 10% ethanol diluted in tap water, animals in the drinking cohort will be removed from the experiment following the same signs of distress of illness.

Supportive action to address symptoms will be taken for any animals receiving a pain score of 2 in at least one category, with a combined pain score of 3-5 (See pain scale in Appendix 3). Actions include increased monitoring, application of heat, subcutaneous fluids, softened food, and increased access to food and water. Animals requiring supportive care will not receive multiple impacts. Animals will also be removed from the study if pain score does not recover within 12 hours of supportive care. Animals with symptoms that cannot be addressed with supportive care will be removed from the study because pain relief such as NSAIDs, opioids, and steroids may affect experimental results. We are interested in looking at inflammation in the hippocampus, and how this affects learning and memory at a molecular, cellular, functional, and behavioural level. NSAID's reduce inflammation by inhibiting cyclooxygenase family of enzymes and preventing the production of signaling molecules such as prostaglandins, which are normally elevated after traumatic brain injury in the rat (DeWitt et al., 1988). In addition to modulating blood flow through vasoconstriction and vasodilation, prostaglandins interact with a family of receptors that are abundant in the brain and hippocampus, and can regulate cell signaling and survival through as Ca++ mobilization, and cAMP production (Reviewed in Hein & O'Banion, 2009). Administration of NSAIDs has been shown to alter plasticity (Chen & Bazan, 2003) and neurogenesis (Monje, Talda, & Palmer, 2003); (Reviewed in Ajmone-cat, Cacci, & Minghetti, 2008) in the hippocampus. Corticosteroids like dexamethasone interact with glucocorticoid and mineralocorticoid receptors, which are abundant in the hippocampus (Herman, Pate,, & Watson, 1989). In addition to reducing inflammation and pain, these are involved in stress signaling and can affect neurogenesis (Kim et al., 2004) and plasticity (Reviewed in Chaouloff & Groc, 2010) in the hippocampus. Opioid administration alters cellular signaling by changing the composition of signaling molecules and receptors at the synapse, and has been shown to affect both neurogenesis (Eisch et al., 2000), and synaptic plasticity (Pu et al., 2002) in this structure.

Injection of BrdU: Any given animal will be considered to have reached clinical endpoint if they present any of the features listed above. No supportive care will be provided and the animal will be euthanized.

A pilot experiment showed that restraint stress was not a significant issue for this procedure. Animals

showed minimal resistance to entering restraint, and were not distressed while secured in the bag. When receiving the last of four impacts over two days, rats were less willing to enter the bag, but they were not distressed and the procedure could be completed easily. However, while unlikely it is still possible that an animal could react poorly to restraint. In order to better monitor this a restraint score has been included in the impact record (Appendix 1: Impact Record and Restrain Score). The degree of restraint resistance will be scored for each impact according to Table 2 in Appendix 1, and in the event that an animal resists restraint such that the impact cannot be completed properly appropriate action will be taken according to Table 2. In the event that an animal resists restraint excessively, food reward may be used to encourage them into the restraint bag. If an animal is persistently and severely resisting restraint (i.e. continuous forceful struggling and loud vocalization) they will be removed from the bag and returned to the home cage. If an animal displays these behaviors intermittently or mildly, but to the extent that the impact cannot be initiated within 5 minutes after the animal is closed in the bag, they will be removed from the bag and returned to their home cage. They will remain in the home cage for at least 30 minutes before trying again. This will be repeated up to 3 times, and if the impact cannot be completed on the third trial the animal will be removed from the experiment.

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14d) Monitoring:

Single Impact:

Following impact animals will be returned to their home cage and monitored by the researcher. A cage-side assessment will be performed one hour after impact and recorded in Appendix 2: Cage-side Monitoring Checklist. If the rats are behaving normally, and displaying no physical signs of abnormality they will be returned to the colony room. Cage-side monitoring by Christie lab personnel will occur once every 24 hours

for 3 days after impact, and then every 7 days until the experimental endpoint is reached, and recorded in Appendix 2. If there is any indication of abnormality (i.e. any score that is not N) during cage-side monitoring a full pain score will be taken for the animal and an advanced monitoring checklist will be initiated according to Appendix 3: Pain Score and Advanced Monitoring Checklist. Action will be taken according to the total pain score, as is outlined in Appendix 3. If an animal has a score of 0-1 in a single category (s) or 0-2 combined (c), monitoring will be increased. If an animal has a pain score of 2(s) or 3-5(c) supportive action will be taken for up to 12 hours. If an animal has a pain score of 3(s), or ≥6 (c), or if their pain score remains above 2(s), or 3-5(c) for 12 hours, it will be removed and euthanized.

Multiple Impact:
Animals receiving multiple impacts will undergo cage-side assessments prior to impact 2 and 4 (Appendix 2), and total pain scoring prior to impact 3 (Appendix 3). If cage-side assessments indicate any abnormalities, a total pain score will be determined. Animals with a pain score of ≥2 (s) or ≥3 (c) that have only sustained one impact will begin supportive care and be used as a single impact animal when possible. Animals with a pain score of ≥2 (s) or ≥3 (c) that have sustained 2-3 impacts will not proceed with subsequent impacts and will be removed and euthanized. Monitoring after the final impact will proceed exactly as monitoring for single impact animals. Animals in the drinking cohort will also be monitored prior to impacts 2 and 4 impact and after the final impact.

Aging Animals: Animals that remain in the colony for any aging studies will be housed in groups of 2-3 and monitored by ACU staff following AC1027 (MSB Daily Rounds) to ensure they are in good health. Animals exhibiting any abnormalities will be reported to the investigators and will continue to be monitored using SOP AC2038 (Monitoring rodents and rabbits with abnormalities).

Weight Drop Surgeries:
For recovery from the TBI or sham surgery, the rat will first be placed in a recovery cage and kept warm on a heating pad designed for use with rodents (Harvard Apparatus). The post-operative condition of the rat will be carefully monitored by the researcher every 15 minutes for 1 hour. Once the rat has fully recovered from the anesthetic (isoflurane) – as indicated by its ability to right itself, walk around, and eat/drink – the rat will be returned to the animal colony room (MSB ACU), where monitoring will continue. These rats will then be checked on by Christie lab personnel twice daily for the first 3 days and every 24 hours for the following 2 weeks to ensure there are no post-operative complications in the days to weeks following a surgery. Pain or discomfort will be rated according to a pain scale (Appendix 3: Pain Scale for Rodents after Cranial Surgery, and AC2009 Rodent - Analgesia (Adult)) which will dictate the appropriate action. Use of the pain scale will be implemented 2 hours following recovery from anesthesia. If an animal experiences any significant indicators of endpoints (as outlined in Appendix 2: Endpoint Monitoring Checklist or the pain scale in Appendix 3), a monitoring record will be initiated for that specific animal. Pain control (in addition to intraoperative anaesthesia) is not normally required following mild TBI, as the brain does not contain pain receptors; animals requiring pain control (pain score of 2 in any category, or a combined pain score of 4 or greater) will be euthanized as treatment with analgesics will affect the purpose of the study. Additionally, the incision site will be checked daily and the following interventions applied if required:
- incision open <1 cm = apply neosporin, increase monitoring to twice daily until incision edges heal
- incision open >1 cm = re-suture and apply EMLA, maintain monitoring once daily
- incision infected = euthanize

The incision will be re-sutured a maximum of one time. If the incision opens >1 cm following re-suturing, the animal will be euthanized. For the cohort of animals used for MWM behavioural testing, incisions will be closed with tissue glue. If the tissue glue dehisces, the animal will be anesthetized, the old glue removed, the tissue edges debrided, and the incision re-adhered with tissue glue once again. The incision will be re-sutured a maximum of one time.

If intervention is required (pain score of 2 in one or two categories, for a combined pain score of 4), supportive action will be taken immediately. If non-responsive to supportive actions after 72 hours, animals will be removed from the study and euthanized.

BrdU Injections:
We do not expect to observe changes in behavior or side effects associated with the administration of BrdU. The doses chosen have been repeatedly used by our laboratory and other groups in various studies.

Nevertheless, all animals receiving BrdU will be monitored for one hour immediately after the injection and subsequently once a day for the next three days by the researchers/ACU staff for any signs of distress or illness (such as drowsiness and lethargy).

Weight Monitoring
Weight will be measured by Christie Lab personnel and recorded in the cage-side monitoring checklist (Appendix 2) on the day of (each) impact, 72 hours after (final) impact, 1 week after (final) impact, and then twice per week (i.e. the 3rd and 7th day of the week) until the experimental endpoint is reached. Weight will also be measured and recorded in Appendix 3 whenever a full pain score is taken. Weight loss will be calculated as percentage body weight relative to the average of age and sex-matched litter mates. Animals in the drinking cohort will also be weighed every 2 days during the 22 days they are given access to ethanol.

Note that it is important that the housing and handling of all experimental animals is exactly the same in order to limit potential confounders. In order to help ensure all animals are treated the same, pain scores of lower than 2 will not require the initiation of a detailed monitoring record and additional monitoring. This is because mild behavioural abnormalities associated with mTBI in rats are very similar to common pain indicators, but they do not necessarily indicate the animal is in pain. For example reduced locomotion, changes in food intake, increased aggression, or reduced sociability are all symptoms commonly associated with mTBI. Additionally, some criteria in category 1 of Appendix 3 such as vocalization are occasionally displayed by uninjured rats during normal handling or activity. This means is possible for an animal that is not in pain to receive a score of 1 in one or more categories, which could cause an animal to receive increased monitoring or supportive care or be removed from the study unnecessarily. Although "unnecessary monitoring or supportive care" does not seem like an issue, continually disturbing cages and handling animals to weigh and palpate injury sites can be stressful,and providing extra care, especially to an animal that is not in pain, is a potential confounder that might alter their recovery and make it difficult to identify more subtle effects of the injury.

14e) Morbidity rate:

ACHI Model: So far in our work, no animals have reached a clinical endpoint outlined in 14c, and no animals received a total pain score greater than 1 in the 7 days examined following impact. This model aims to produce a mild injury that can be compared to a typical human concussion. This means the injury should not lead to severe clinical symptoms.
Weight Drop Model: Previous experiments conducted by this laboratory using the weight drop model to produce mTBI have shown that the probability of mTBI resulting in an animal reaching a clinical endpoint (14c) is negligible, and that post-procedural pain relief was not required. mTBI is more likely to produce symptoms related to altered cognition, and not increased pain. Morbidity arising from potential alterations in cognition such as reduced food and water intake, self mutilation, and abnormal behavior will be monitored as is outlined in section 14d and recorded in Appendix 2, and supportive care will be provided if necessary.

It is possible that movement or excessive restraint resistance during the impact procedure could lead to an impact in the wrong position. The restraint scoring guideline in Appendix 1 details the appropriate response to this circumstance. If an impact appears to be inaccurate the researcher should examine the animal for signs of more severe trauma or fracture. If there are signs of more severe trauma or fracture, the animal will be removed from the study and euthanized. If the animal appears normal, and the impact is within 2mm of the target site, the animal will remain in the study. If the impact is displaced more than 2mm the animal will be removed and euthanized. If the impactor misses entirely the animal will be kept as a sham.

Skull fracture is common concern with some closed head injury models, however several features of our model negate the risk of skull fracture and no skull fractures have occurred in any of our work. The impactor has a rubberized tip, and the animal wears a protective helmet during impact. The rubber tip reduces the magnitude of shock during impact by spreading the change in the head's acceleration over time. The helmet increases the surface area of the impact force by dispersing it across the skull. Together, these model a mild concussive head injury by using a blunted force to create a rapid change in acceleration in the head, causing the brain to move within the skull without directly damaging the skull. Petraglia et al. (2014) noted no incidence of skull fracture using this model in mice. Prins et al. (2011) developed a similar model for anesthetized rats, but they did not use a helmet. They also report that an impact of similar magnitude did not produce skull fracture. In order to verify that our impact parameters do not pose a risk of skull fracture the

impact procedure was tested on three euthanized rats using the exact same apparatus that will be used for experimental animals. These tests confirmed that this impact model does not produce skull fractures.

Weight Drop:
The most vulnerable periods during the trauma procedure are avoiding skull fracture (during weight-drop), and the recovery from anesthesia following surgery (<10%). We are aware that rounding the tip of the Telflon-tipped cone should reduce the incidence of skull fracture. Non-experimental factors of morbidity include opening of incision site by the rat itself. During our TBI pilot study we experienced 5% incision dehiscence due to self-trauma. Eight animals required re-suturing after opening their incision and exposing their skull following surgery.

BrdU Injections: I.p. injections of BrdU will be performed by experienced researchers that were previously certified in these techniques by the ACU staff or the DMS Animal Technician. The techniques employed will follow the SOP AC2007 Rodent — Handling and Injection Techniques. Furthermore, at the doses used and based on previous studies, these drugs should not be toxic to the animals. Thus, we expect to observe a morbidity rate of <1% in animals that will undergo these procedures.

The drinking cohort is subject to the same morbidity rate as the awake mTBI cohort; we do not expect to observe morbidity as a result of consumption of 10% Ethanol diluted in tap water.

References:

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Prins, M., Hales, A., Reger, M., Giza, C. & Hovda, D. (2011) Repeat traumatic brain injury in the juvenile rat is associated with increased axonal injury and cognitive impairments. Developmental neuroscience 32, 510-518.

15. Drugs / Chemicals / Biologicals / Anaesthetic

15a) Pre-Anesthetic / Anaesthetic / Analgesic Agents

Add Item

	Species	Agent	Dosage	Total volume (ml) per administration	Route	Frequency / Duration	Purpose
X	Rattus norvegicus	Isoflurane	>5%	n/a	inhalation bell jar	once	anesthesia for anesthesized TBI or prior to euthanasia
X	Rattus norvegicus	Isoflurane	Induction: 2-3%; Maintenance: 1-3%	n/a	inhalation; vaporizer	continuous/ ~5 minutes (< 2 minutes maintenance after < 3 minutes induction)	anesthesia
X	Rattus norvegicus	Urethane	1.5 ml/kg	max 1.5 ml	I.P.	1 injection	termination of experiment by transcardial perfusion
X	Rattus norvegicus	Urethane	1.5 mg/kg	max. 5ml	i.p	1 injection	acute in vivo electrophysiology (terminal experiment)

X	Rattus norvegicus	Urethane	0.15-0.25 mg/kg	max. 1ml	i.p	1-2 i.p. injections	top up of aneasthetic during in vivo electrophysiology
X	Rattus norvegicus	Lidocaine	0.125mg/kg	0.02ml	topically to open scalp incision (weight drop)	once, allowed to sit for 60 seconds	analgesic

15b) Clinical drugs including antibiotic

Add Item

	Species	Agent	Dosage	Total volume (ml) per administration	Route	Frequency / Duration	Purpose
X	Rattus norvegicus	Evan's Blue Dye	1-4%	max 0.5ml	I.V.	1 injection	to trace extravasation from the bloodstream into the concussed brain region
X	Rattus norvegicus	FITC-dextran	300mg/ml	0.5ml	I.V.	1 injection	to trace extravasation from the bloodstream into the concussed brain region
X	Rattus norvegicus	tear-gel	n/a	n/a	topical	once/as necessary	eye lubrication during surgery
X	Rattus norvegicus	neosporin	n/a	n/a	topical	every 12 hours until incision healed	to reduce postoperative incision site irritation or inflammation
X	Rattus norvegicus	EMLA	n/a	1cm strip to surgery site (scalp)	topical	once	reduce incision related to pain of the scalp

15c) All other substances administered to animals

Add Item

	Species	Agent	Dosage	Total volume (ml) per administration	Route	Frequency / Duration	Purpose
X	Rattus norvegicus	Ethanol	10% v/v	Max 150mL/day	ingestion	Voluntary self-administration for 22 hours day for 22 days	Examine drinking behavior following TBI
X	Rattus norvegicus	0.9% saline	n/a	3ml (1.5ml per side)	s.c.	once	hydration
X	Rattus norvegicus	Tissue glue (VetBond)	n/a	Cover incision	topical	once	adhere incision for MWM experiments
X	Rattus norvegicus	Bromoxyuridine (BrdU)	200mg/kg	Max 10ml/kg	I.P	Twice per day for up to 7 consecutive days	Labelling of DNA synthesis

16. Euthanasia

16a) Method of euthanasia

Add Item

	Species	Method	List Agent / Dose / Route (if applicable)
X	Rattus norvegicus	>5% isofluorane (bell jar) followed by rapid decapitation	Isoflurane (>5% inhalant)
X	Rattus norvegicus	Deep anesthesia followed by perfusion	Isoflurane (>5% inhalant) followed by urethane (1.5ml/kg, i.p.), followed by transcardial perfusion with 0.9% saline and 4% paraformaldehyde
X	Rattus norvegicus	anaesthetic overdose followed by rapid decapitation	urethane, 0.15mg/kg, i.c.

16b) Final disposition of animals if not euthanized:

Retired breeders are occasionally transferred to the ACU for training purposes.

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17. Hazardous Agents

It is the responsibility of the investigator to obtain the necessary UVic Biosafety Committee and/or UVic Radiation Safety Committee permits before this protocol is submitted for review.

☐ No hazardous materials will be used in this study.

17a) Indicate which of the following will be used in animals:

☐ Infectious/Biological agents (includes vectors) ☒ Hazardous chemicals ☒ Carcinogens

☐ Transplantable tumours and/or tissues ☐ Radioisotopes

UVic Radiation Certificate Number: _____ UVic Biosafety Certificate Number: _____

17b) After administration the animals will be housed in:

☐ Bob Wright Animal Care Unit ☒ Medical Sciences Animal Care Unit

☐ Outdoor Aquatic Unit Investigator/Teaching Laboratory

☒

☐ Other (please specify building and room number) _____

Please note that cages must be appropriately labeled at all times.

17c) Describe potential health risk(s) to humans or animals:

Potential health risks associated with urethane exposure as per Appendix 7: Urethane MSDS:
Acute Effects: Very hazardous in case of contact with eye (irritant). Hazardous in case of skin contact (irritant, permeator), ingestion, or inhalation. Eye inflammation characterized by redness, watering, and itching.
Chronic Effects: Potential carcinogen. Toxic to kidneys, nervous system, liver, and digestive tract. Repeated or prolonged exposure can damage target organs.

Potential health risks to humans of isoflurane:
Note: Inhalation at a concentration of 0.5%-3.0% can induce general anesthesia in 7 to 10 minutes with analgesia, muscle relaxation and loss of consciousness.
Acute toxicity: Anaesthesia, respiratory depression, coughing
Chronic: No present evidence that isoflurane is a mutagen, teratogen or carcinogen.

Target Organs: Respiratory, cardiovascular and central nervous system.

Potential health risks associated with Evans Blue exposure as per attached MSDS: hazardous in case of inhalation (respiratory tract irritation), skin contact (permeant and irritant), eye contact (irritant). Toxic if swallowed. Potential carcinogen.

Potential health risks to humans of BrdU (see material safety data sheet, MSDS):
- Acute Effects: May be harmful if swallowed, inhaled or absorbed through skin. May cause irritation.
- Chronic Effects: May cause reproductive disorders. May alter genetic material.
Potential health risks to rats of BrdU:
- At the dose used (200 mg/kg), no major risks to the health of animals have been reported.

17d) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:
Urethane will be administered to rats in a fume hood in MSB250, by trained research personnel only. ACU staff will not be exposed. The researcher will use proper personal protective equipment: gloves, lab coat, eye protection, and respirator when preparing the solution and administering it to the animals. All areas where urethane has been handled will be properly cleaned. All liquid and solid waste will be immediately disposed of into the appropriate biohazard waste containers as per UVic OHSE policy.

Isoflurane: Isoflurane will be stored in a well-ventilated area and will be used under a certified fume hood. The researcher will use proper personal protective equipment: gloves, lab coat, eye protection when handling this substance. All areas where isoflurane have been handled will be properly cleaned.

Evans Blue will be prepared in a fumehood in MSB 250, and administered in MSB 250 of in MSB ACU immediately prior to transport to MSB 250. The researcher will use proper personal protective equipment: gloves, lab coat, and eye protection, when preparing the solutions and administering them to the animals. ACU staff will not be exposed.

BrdU: Solutions will be prepared under a certified chemical fume hood. The researcher will use proper personal protective equipment: gloves, lab coat, protective eyewear, closed toed shoes, and a N95 respirator when preparing the solutions and administering them to the animals. All areas where BrdU, have been handled will be properly cleaned. All liquid and solid waste will be immediately disposed of into the appropriate waste containers as per UVic OHSE policy. All cages with animals that BrdU will be clearly labeled. As per consultation with OSHE, there are no special disposal requirements. Disposal of bedding $

17e) If using cell lines, have they been tested?

☐ Yes If yes, what human and/or animal pathogens have been tested:

☐ No If no, please justify:

Upon completion of the form, save and then email to acsc@uvic.ca. Print pages one & two, obtain the necessary signatures, and forward to the Animal Ethics Liaison, Office of Research Services (ASB room B202).