ANALYSIS OF SYNAPTIC PLASTICITY EVENTS CONCERNING THE ENDOCANNABINOID SYSTEM IN THE YOUNG ADOLESCENT (YA) RAT MEDIAL PERFORANT PATHWAY (MPP) FOLLOWING REPETITIVE MILD TRAUMATIC BRAIN INJURY (r-mTBI)

by

Emir Zejnulahovic

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

ANALYSIS OF SYNAPTIC PLASTICITY EVENTS CONCERNING ENDOCANNABINOIDS AND TRPV1 IN THE YOUNG ADOLESCENT MEDIAL PERFORANT PATHWAY (MPP) OF REPETITIVE MILD TRAUMATIC BRAIN INJURY (r-mTBI)

by

Emir Zejnulahovic

Supervisory Committee

Dr. Brian Christie, Division of Medical Science
Supervisor

Dr. Raad Nashmi, Department of Biology
Co-Supervisor

2025

Abstract

Traumatic brain injury (TBI) is a widespread health issue, with mild TBIs (mTBI) making up to 85% of cases, often underdiagnosed due to subtle symptoms. Young adolescents are most at risk for repetitive mTBI (r-mTBI), which compounds mTBI symptoms and outcomes. These injuries can impair synaptic plasticity, including short-term potentiation (STP) and long-term potentiation (LTP), both crucial for cognition. Acute neuronal damage and chronic apoptosis underlie these deficits, especially in the dentate gyrus (DG) and its medial/lateral perforant pathways (MPP/LPP) substructures, whose electrical inputs are essential for adult neurogenesis. Cannabinoid one receptors (CB1Rs) and Transient Receptor Vanilloid Protein 1 (TRPV1), part of the endocannabinoid system (EC), regulate synaptic plasticity in MPP/LPP due to the high concentrations each receptor has in the DG. Altered EC signaling in mTBI models contributes to excitotoxicity. Pharmacological tools for targeting CB1Rs (AM251) and TRPV1 (AMG9810) reveal the receptor's effects indirectly before and after r-mTBI. 53 Sprague Dawley rats were used for recordings, with ninety-nine slices used. Animals were split across six groups (Sham/r-mTBI controls, plus corresponding drug groups). Sham/r-mTBI controls only showed differences in LTP induction, with mechanistic properties of the synapse appearing unchanged via paired pulse and short-term potentiation (STP) recordings. Both AMG9180 and AM251 restored LTP deficits seen between Sham/r-mTBI controls, while TRPV1 also significantly enhanced STP levels, and removed paired-pulse facilitation effects seen in Shams. This suggests that both receptors can be pharmacologically manipulated to aid in synaptic plasticity deficits following r-mTBI, as well as suggesting that both receptors are downregulated following r-mTBI.

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List of Important Abbreviations/Key Terms

2-arachidonoylglycerol (2-AG)

Anandamide (AEA)

AM251 (Cannabinoid 1 Receptor Inverse Agonist)

AMG9810 (Transient Receptor Potential Vanilloid 1 Antagonist)

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)

Bicuculline Methiodide (BMI)

Cannabinoid 1 Receptors (CB1Rs)

Central Nervous System (CNS)

Dentate Gyrus (DG)

Endocannabinoid (eCB)

Diacylglycerol Lipase (DAGL)

Input-Output (IO)

Interpulse Interval (IPI)

Lateral Impact Model (LIM)

Lateral and Medial Perforant Pathway (LPP/MPP)

Loss-of-Consciousness (LOC)

Long Term Potentiation (LTP)

Long Term Depression (LTD)

Mild Traumatic Brain Injury(mTBI)

N-methyl-D-aspartate (NMDAR)

Paired Pulse Ratio (PPR)

Repetitive-mild Traumatic Brain Injury (r-mTBI)

Transient Receptor Potential Vanilloid 1 (TRPV1)

Young Adolescents (YAs)

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Rick Rubin.

Dedications

A man is nothing without his parents; thank you for supporting me, even though you both have no idea what I study in school, you like that I like it, and that is enough for me.

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My friends who are too numerous to list within this document but shall be acknowledged.

Chapter 1: INTRODUCTION

1.1 mTBI Overview

Traumatic brain injuries (TBI) are a chronic health issue worldwide and can negatively affect several aspects of health over time (Dams-O'Connor *et al*., 2023). They account for ~10 million hospitalizations and/or deaths annually worldwide (Schurman *et al*., 2017), with recorded incidents reaching up to 41 million annually (Snowden *et al.*, 2020). Furthermore, due to the heterogeneity of the mechanism of injury, such as work and sports-related injuries, chance events such as falls, (Eyolfson *et al*., 2023), or intimate partner violence (Dow-Fleisner *et al*., 2023), effective treatment of TBI is often relegated to the acute, with little or no regard to the more chronic, long-term effects that present throughout life. The vast majority (75-85%) of TBI are classified as mild TBI (mTBI), with many going either untreated or undiagnosed (Harris *et al*., 2024). An mTBI does not produce skull fractures or brain bleeds, and results in transient behavioural deficits lasting 1–2 weeks, such as increased anxiety, post-concussion headache, decreases in working memory/attention with vast heterogeneity among outcomes (Neale *et al*., 2023; Tseitlin *et al*., 2023; Collins *et al*, 2023). Young adolescents (YAs) are often reported to be at the highest risk of mTBI due to the prevalence of youth sports and risk-taking activities (Wright *et al*., 2017). This is of particular importance, as a history of mTBI has been linked to the development of neurodegenerative disorders later in life, such as Alzheimer's, chronic traumatic encephalopathy, and dementia (Zoppi *et al*., 2011).

There are two mechanisms in which mTBI exert their effects; the primary injury involves acute shearing of axons in the brain due to the physical trauma of the brain impacting the inside of the skull, thus physically damaging neurons; the secondary injuries involve complex biochemical pathways that constitute the apoptosis of neurons (Barkhoudarian *et al*., 2016).

While primary injuries are primarily acute and "simpler" due to their neuronal shearing nature, they serve as the basis for the chronic secondary pathways of injury. These secondary injury cascades are spearheaded by large increases in glutamate released by physically damaged neurons (aka glutamate excitotoxicity), causing postsynaptic efflux of K^+ as a response (Barkhoudarian *et al*., 2016). In turn, this causes increased ion pump activity to restore this gradient to proper form which it needs ATP to do. Thus, more ATP is produced, lactate accumulates, which causes the release of intracellular calcium stores. Finally, mitochondrial function halts ATP production due to Ca^{2+} influx/sequestration, halting oxidative phosphorylation. This leads to hypoperfusion, and then neuronal apoptosis via calpain activation (Barkhoudarian *et al*., 2016). These cascades result from an imbalance between the excitation-based glutamatergic and inhibition-based gamma-aminobutyric acid, or GABAergic signaling (Guerrio *et al*., 2015). Glutamate excitotoxicity is also thought to be the result of increased activation levels of the N-methyl-d-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor (AMPAR). Due to the high permeability for calcium (Ca^{2+}) exhibited by NMDARs, and occasionally AMPARs depending on subunit composition (Gagliardi, 2000), this leads to the initial accumulation of calcium in the neuron due to starting the apoptotic chain of events previously described. These events occur in a similar calcium-related manner in axons, a term deemed axotomy. Although influxes of Ca^{2+} occur differently, mostly due to axolemma disruption, the result of axotomy mirrors cellular fate at synaptic terminals (Barkhoudarian *et al*., 2016).

Following mTBI, NMDA receptors undergo both acute and chronic effects. Immediately following impact, the NMDAR is mechanically activated via stretching of the receptor, altering the binding efficiency of the Mg^{2+} ion within the channel pore (Gabrieli *et al*., 2021). This results

in a hyperacute partial loss of the internal Mg^{2+} "plug" within the channel, leading to enhanced ion conductance, setting the stage of this ionic imbalance (Gabrieli *et al*., 2021). A more chronic glutamate excitotoxicity mechanism occurs due to the immune response following mTBI, which results in altered permeability of neurons and glial cells to glutamate (Mayeux *et al*., 2017), extending this period of increased transmission through the NMDAR. As for AMPAR, increases in expression following mTBI have been recorded via Western blotting of the S845 subunit, which, when phosphorylated, increases the probability of receptor activation in the postsynaptic neuron, indicating hyperexcitability of these receptors is following mTBI (Mayeux *et al*., 2017). Therefore, both NMDA and AMPA receptor activity increases post mTBI acutely, contributing to this initial excitotoxic effect.

1.2 r-mTBI Distinctions, and Experimental Models for mTBI administration

Although many from the pediatric population recover without incident, sustaining a second mTBI while the brain is in a period of cerebral vulnerability can drastically affect outcomes and further exacerbate secondary injury cascades (Prins *et al*., 2013). While mTBI poses significant health risks, repetitive mTBIs (r-mTBI) compound these challenges. This compounding effect has been shown in clinical and preclinical work (White *et al*., 2017). The timing of r-mTBI induction is of importance as the height of neuroinflammatory response in the brain is 3-5 days, due to the influx of neutrophils, and then microglia/astrocytes to the site of injury to perform reparative functions (Mckee and Lukens, 2016). Another injury during this peak immune response renders it less effective, and effectively "resetting" the immune response. A 2005 paper by Longhi *et al* showed that while there were no cognitive impairments in single-concussed rats, there were significant deficits in rats that were concussed a second time 3-5 days

later compared to Sham animals. These r-mTBI rats showed global deficits in all behavioral factors tested (motor skills, balance) as well, when compared to single mTBI (Longhi *et al*., 2005). Furthermore, cytoskeletal/axonal damage were detected in brain regions of interest (ROIs) following single mTBI using Western Blotting, however the amount of damage sustained was significantly increased in r-mTBI cases when compared to a single mTBI (Longhi *et al*., 2005). This supports this compounding theory of mTBI, and how global deficits are associated with such.

There are several pre-clinical models to induce mTBI/r-mTBI all with advantages and disadvantages of their use. Open-head models, such as Controlled Cortical Impact (CCI) and the Lateral Fluid Percussion model (LFP), both make use of craniotomies to create reproducible damage to dura matter in rodents (Alder *et al*., 2011; Romine *et al*., 2014). While more exact in their injury deliverance to a specific brain region, there are several confounding factors, including high anesthesia times, an initial inflammatory response due to the craniotomy, as well as ease of administration.

There are also closed-head injuries that do not require the use of craniotomy and allow free movement of the head. Models such as lateral impact (LIM) and Modified Weight Drop (MWD), where the head is allowed to move freely after impact (Bodnar *et al*., 2019), allow for a more realistic impact following mTBI. The Lateral Impact Model (LIM) applies an equal and opposite 180° rotational/linear biomechanical force to induce an mTBI. This horizontal orientation of impact is akin to a sports injury, which are common in YAs. Other models such as the modified weight drop device (MWD), impact the head in a more-so vertical/sagittal orientation of impact (Mychasiuk *et al*., 2016), which would be more akin to a vehicle crash than a sport injury. Behavioural/cognitive differences have been noted between different models, as

MWD groups have greater impairment in working memory in novel context mismatch tasks, while LIM groups have increased anxiety in elevated maze tasks (Mychasiuk *et al*., 2016). In short, different models have different impacts, have specific purposes, and show different outcomes in both biomarker expression and behavioural tasks (Mychasiuk *et al*., 2016).

The LIM is a novel way to induce mTBI non-invasively and is widely used experimentally in both mTBI and r-mTBI studies (Eyolfson *et al.*, 2024a; Eyolfson *et al*., 2021; Mychasiuk *et al*., 2009). Closed-head injuries were chosen for this study due to the representativeness of real-world conditions where mTBIs would occur, as humans do not have to undergo a craniotomy for mTBI induction, and this needs to be recapitulated in rodent models.

1.3 Dentate Gyrus as a Region of Interest, and Hippocampal Circuitry Overview

As previously mentioned, cognitive deficits are among the most common persistent post-concussive symptomologies, even presented in rodent models (Mychasiuk *et al*., 2016). Uncovering the mechanism responsible for these deficits would be beneficial in the development of potential therapeutics. A primary ROI for these symptomologies is the hippocampus and in particular the dentate gyrus (DG) subregion. Whilst the hippocampus itself is classically associated with spatial navigation, memory formation, and general learning mechanisms (Peñasco *et al*., 2019), the dentate gyrus is associated with a phenomenon known as adult neurogenesis, the creation of new neurons throughout adult life via generated neural stem cells (White *et al*., 2017). These specialized stem cells require electrical input from the perforant pathways (bisected into medial and lateral pathways [MPP/LPP]) to mature properly, with MPP inputs being more effective in delivering this input due to shorter latencies of input and higher amplitudes (Ferbinteanu *et al*., 1999). Acting as a “highway,” these pathways are connected to

layers II/III of the entorhinal cortex, which itself serves as the interface between the prefrontal cortex and the hippocampus. Synapses from the perforant pathways connect to the DG, then to the cornu ammonis 3 (CA3) pyramidal cells, to the CA1, and then out to the rest of the brain. These connectivity features are often described as a "unidirectional loop" (Pinar *et al*., 2020), and due to connectivity within the DG involving both feed-forward and feed-back inhibition, it is impossible to stimulate LTP without the use of a GABAα antagonist, such as bicuculline methiodide, or BMI (Eyolfson *et al*., 2024). Feed-forward inhibition is blocked with use of GABAα antagonism during these recordings. Ideal concentrations of specifically BMI were determined to be 10 μM in previous studies conducted in-lab (Eyolfson *et al*., 2024).

1.4 Synaptic Plasticity following mTBI.

This initial input via the perforant pathways is known to be dampened in mTBI cases, due to deficits in long-term potentiation (LTP), a classical neurological basis of learning and measure of increased synaptic plasticity (Eyolfson *et al*., 2024b; Peñasco *et al*., 2019; Bliss and Lømo, 1970) and was discovered based off groundwork laid by Dr. Cajal in 1894. Coincidentally, the MPP-DG granule cell synapse was the site of the discovery of LTP in a mammalian model (Walling *et al*., 2023; Lomo, 1966). The hippocampus displays elevated levels of NMDA-dependent LTP, which, in short, constitutes increases in intracellular Ca^{2+} upregulating AMPA receptors onto the surface of the postsynaptic neuron, increasing response sensitivity to presynaptic stimulation (Peñasco *et al*., 2019; Bliss and Lomo, 1970). The link between TBI and LTP deficits in the hippocampus is well documented, with studies showing deficits following TBI in the CA1/3 regions, which is further exacerbated by the suppression of the immune response (Zhang *et al*., 2011). However, DG remains underrepresented in pre-clinical research.

A recent study by White and colleagues found that the DG and CA1 subregions respond differently to juvenile mTBI, where the DG was more susceptible to damage than CA1 (White *et al*., 2017). Furthermore, recent studies in-lab show reductions in LTP in the DG following r-mTBI as well (Eyolfson *et al*., 2024). Due to the connectivity between these different areas in the hippocampal formation, assessing neurotransmitter mechanisms in the perforant pathways is imperative to further understand mTBI pathology within the DG.

1.5 Endocannabinoid System, and Implications in mTBI

Of further interest in the DG is the endocannabinoid (eCB) system, a retrograde regulatory neurotransmitter system in the brain due to elevated levels of expression of both TRPV1 and CB1Rs (White *et al*., 2017). Within this system, neurotransmitters are released from the postsynaptic neuron and travel "backward" to inhibit the presynaptic vesicle release (Eyolfson *et al*., 2024). This synthesis of this neurotransmitter system is dependent on incoming presynaptic input, specifically the high influxes of Ca^{2+} following depolarization (White *et al*., 2017, see Figure 1). This neurotransmitter system has been highly implicated within the DG previously, with studies suggesting endocannabinoids (eCBs) are needed for LTP and LTD induction in the DG specifically in the LPP, with inhibition of the main enzyme for eCB synthesis finding comparable results (Wang *et al*., 2016). Furthermore, previous results published in the Christie lab have shown that endocannabinoids have a role in several forms of long-term depression in the MPP (Fontaine *et al*., 2020), as well as other research groups providing a link to LTP (Gambino *et al*., 2020). These results form the basis of interest for this regulatory system present in this study.

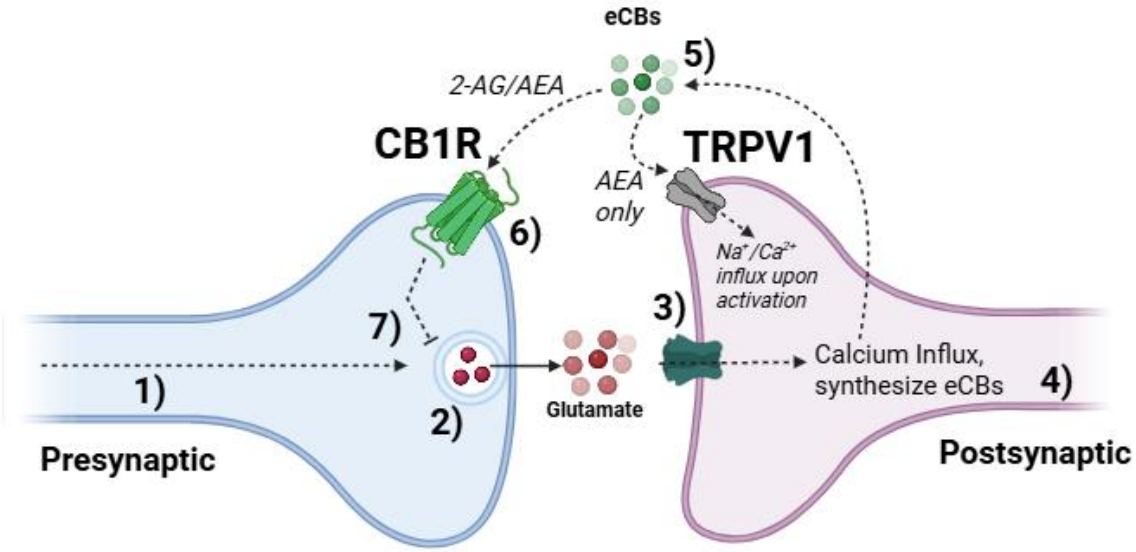

Figure 1. Schematic describing the method of synthesis and action of endocannabinoids within the DG-MPP synapses. **1)** Presynaptic stimulation travels down the neuron, reaching the synaptic terminal. **2)** Glutamatergic vesicles are created, as glutamate is the major excitatory neurotransmitter in DG. They are released into the synaptic cleft. **3)** Stimulation of the NMDA causes large influxes of Ca^{2+} and more minorly Na^{+} into the cell following postsynaptic stimulation. **4)** Endocannabinoid (eCB) synthesis ensues, due to high postsynaptic Ca^{2+} levels being required for the initiation of their synthesis. **5)** Endocannabinoids (eCBs) diffuse across the membrane due to their lipid-based nature and diffuse out of the neuron. This transport is facilitated with carrier proteins. Interactions with the postsynaptic TRPV1 occur due to their partial affinity of binding to only AEA (not 2-AG), opening the channel, initiating further cationic influx. **6)** Both eCBs can interact with CB1Rs where they exert their effects through the adenylyl cyclase (AC) pathways. **7)** Prolonged activation of the CBR1 is associated with decreased NT release.

The eCB system is composed of two major ligands; N-arachidonoylethanolamide (anandamide/AEA) as a partial agonist with a high affinity, and 2-Arachidonoylglycerol (2-AG) as a full agonist with a lower affinity (Schurman *et al*., 2017). Studies have shown that 2-AG is the primary ligand involved in this retrograde regulatory mechanism, despite its lower affinity nature (Justinová *et al*., 2011). Post-synaptic release eCB ligands are considered Ca^{2+}-dependent, through a variety of channels, and are dependent on pre-to-postsynaptic depolarizations for their

synthesis (Wilson and Nicoll, 2001). Interacting through the Cannabinoid Receptor 1 (CB1R, neuronal GPCR) and Cannabinoid Receptor 2 (CB2R, immune cell-based GPCR) receptors primarily (but not exclusively), the neurotransmitters interact with the presynaptic CB1 receptor to modulate vesicular output. CB1Rs were identified within the brain by Devane *et al*. in 1988, particularly in regions responsible for memory, emotion, and motor control (Bietar *et al*., 2023). CB1R is encoded by the *CNR1* gene and is the most abundantly expressed among 100 G-protein-coupled receptors in the brain (Bietar *et al*., 2023). Due to both the CB1 and CB2 receptors primarily coupling with $G_{i/o}$ proteins, they are associated with reducing the levels of adenylate cyclase (AC) within the neuron, promoting mitogen-activated protein kinase (MAPK) levels, and stimulating phosphoinositide 3-kinase (PI3K) pathways (Bietar *et al*., 2023). These biochemical events have the end goal of either 1) downregulation of certain voltage-gated calcium channels (P/Q and N types primarily, allowing Ca^{2+} into the cell), or 2) upregulation of certain potassium channels (KA and/or GIRK channels, both of which flux K^{+} out of the cell) (Balezina *et al*., 2021). Both channel regulation mechanisms act to decrease vesicular release and hyperpolarize the neuron more quickly, respectively.

In addition to the two eponymous receptors of the system, the Polymodal Transient Receptor Protein Vanilloid 1 (TRPV1) has been implicated in the endocannabinoid system within the DG as a postsynaptic receptor, due to its interactions with AEA (Schurman *et al*., 2017). TRPV1 has been suggested to be a "primary postsynaptic ionotropic receptor counterpart" to the GPCR-based CB1/2 receptors and has high co-expression within the MPP/LPP (Gambino *et al*., 2020). Due to its interactions with endocannabinoid ligands (specifically AEA), TRPV1 should be considered when investigating this area of the DG (Gambino *et al*., 2020). Egaña-Huguet and colleagues found that a low-frequency stimulation typically associated with long-

term depression (LTD) is shifted to LTP within MPP-DG synapses in a TRPV1 knockout mouse model, implying that LTD in the MPP is partially dependent on TRPV1. This raises the question of what its role in LTP within these synapses is, and how this role may change in following mTBI. While there is little in the field of mTBI, there are correlates for TRPV1 function in the more moderate-to-severe TBI, such as the blockage of TRPV1 in humans has been associated with alleviation from post-traumatic headache, a common TBI symptom (Fiorina *et al*., 2020). It is also referenced in general nociception, due to TRPV1 being most commonly implicated within these pain systems (Fiorina *et al*., 2020; Jurik *et al*., 2014).

1.6 Endocannabinoids in relation to TBI

Following TBI, studies show that endocannabinoid signalling recruits microglia via immune-oriented CB2 receptor interactions to the site of injury as a response to glutamate excitotoxicity, as well as both 2-AG/AEA expressions being enhanced acutely and chronically (Eyolfson *et al*., 2024; Xu *et al*., 2019). Specifically, the full agonist 2-AG has been implicated in a neuroprotective role following TBI after inhibition of monoacylglycerol lipase (MAGL), its major degradation enzyme (Mayeux *et al*., 2017). Furthermore, glial cells also can synthesize eCBs following injury (Xu *et al*., 2019). Behavioural studies are also present in the literature as well, with MAGL inhibition being found to ameliorate deficits in beam-walk, Y-maze and Morris water maze tests as a measure of fine motor movement/control (Selvaraj *et al*., 2021) However, most studies focus on TBI, not mTBI/r-mTBI, thus more research is needed to determine potential differences between the TBI and mTBI pathologies in terms of endocannabinoid signalling.

The goals of this study were to further characterize the pathology of r-mTBI as it relates to the DG, as well as to elucidate the changes in the roles of the endocannabinoid system regarding LTP. This will be done with pharmacological manipulation (AM251/AMG9810) and the electrophysiological induction of LTP, with all effects measured indirectly. This study will advance knowledge in the field by determining if there are significant changes in endocannabinoid signalling within the MPP in a YA r-mTBI model. The novelty of the study lies within the trifecta of endocannabinoid signalling, YAs, and r-mTBI; a combination of which has not been analyzed before within the literature. We hypothesize that both AM251 and AMG9810 drugs will ameliorate these deficits in LTP and will have a negligible effect on other STP-based protocols administered in the study.

Chapter 2: MATERIALS AND METHODOLOGY

2.1 Animal Subjects

2.1.1 Animal Care

All experiments were done using fifty-three male/female Sprague Dawley rats bred in-house at the University of Victoria, Canada. Subjects were given at least one week to acclimate to handling/pristine environment prior to experimental use. Animals were used for experimental protocols starting at Postnatal Day (PND) thirty. All animals were housed in polyethylene cages, with a 12-hour light/dark cycle at constant temperature (21 ± 1 °C) and humidity (50% ± 7%), with *ad libitum* access to standard rat chow and water. Animal procedures were conducted in accordance with the University of Victoria and Canadian Council on Animal Care (CCAC) principles of laboratory animal care.

2.1.2 r-mTBI induction/protocol

The goal of rmTBI administration in the present study was to ensure that mTBIs were delivered in a valid mammalian model, in an analogous manner to the forces experienced in sports-related injuries. To ensure homogeneous impacts, the Lateral Impact Model (LIM) was used to deliver these brain injuries (Mychasiuk *et al*., 2009). All rats were anesthetized for ~60 seconds prior to use in the LIM with isoflurane. mTBIs were delivered with a pneumatically powered 50-gram weight, propelled at 9.01 ± 0.12 m/s towards the rat's head. Rats were then rotated 180 ° by impact, experiencing horizontal acceleration/deceleration. Rats were then placed in a supine position and their time-to-right was compared (**Figure 2)**, as a proxy measurement for loss-of-consciousness (LOC, Bhatt *et al*., 2020), with an unpaired t-test to ensure statistical difference.

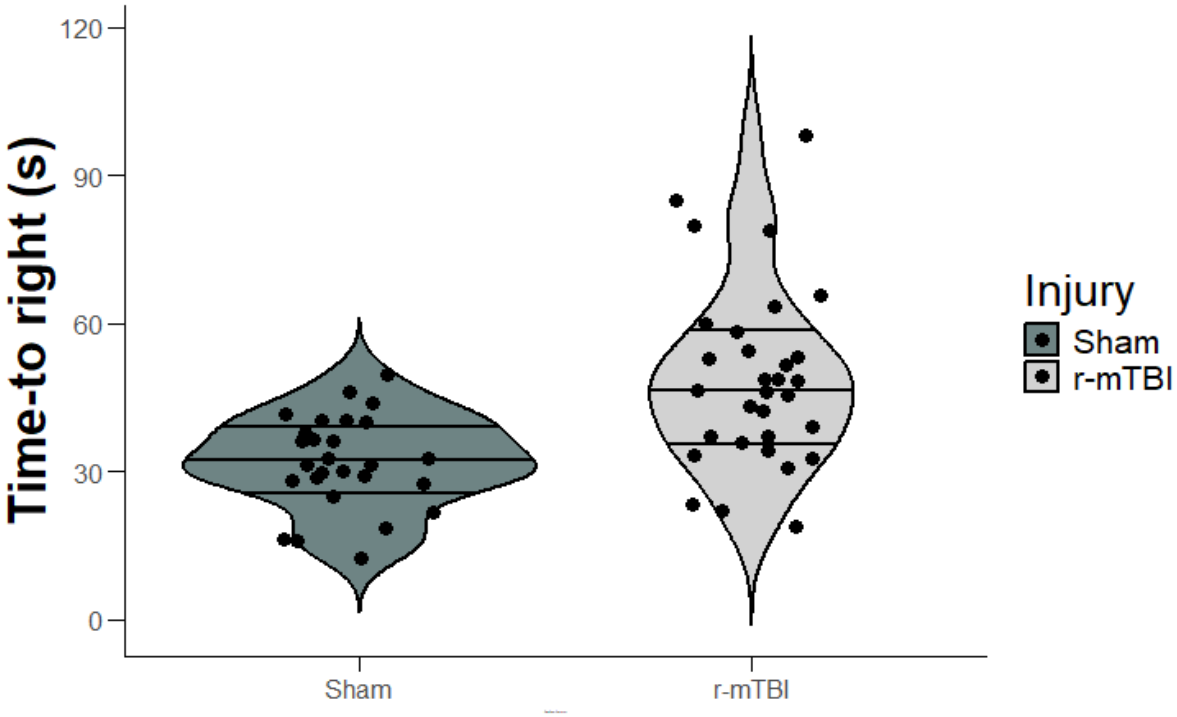

Figure 2. Violin plot of TIR, a method used for confirmation of concussion deliverance. Rats were placed in a supine position initially, with time measurements ending when rats were stable on all-fours. Averages for groups were as follows: Sham (31.9 ± 9.4, n = 27) and r-mTBI (50.7 ± 21.1, n = 32). $t_{(57)} = 4.29$, p = <0.0001. 53 total animals were used: 31 Sham, 28 r-mTBI.

2.2 Brain isolation/Slice preparation

2.2.1 Artificial Cerebrospinal Fluid (aCSF) specifications

Using ddH_2O, aCSF was created with the following ionic composition (in millimoles): 125 NaCl, 3.0 KCl, 1.25 NaH2PO4, 25, NaHCO3, 2 CaCl2, 1.3 MgCl2, and 10.4 dextrose. The solution then had its pH adjusted to ~7.3-7.4 and used throughout the study.

2.2.2 Surgical Procedure

All rats were anesthetized with gaseous isoflurane in a sealed chamber. Once animals were no longer reactive to a toe-pinch, subjects were quickly decapitated by guillotine. The head was then moved to a stable surface for brain removal, which was done via a longitudinal cut along the midline of the skull up to the accessory olfactory bulb, to expose the brain. Another longitudinal cut was then done along the sides of the skull to remove the flaps created by the previous cut. The brain was chilled in ice-cold carbogenated (95% O2– 5% CO2) aCSF until dissection.

2.2.3 Transverse hippocampal sectioning

Brains were left to rest in ice-cold aCSF for one minute before being halved along the midline fissure. One hemisphere was sectioned at a time, with the second hemisphere being placed into ice-cold aCSF until sectioning for preservation (left and right hemispheres were counterbalanced for first dissection). To create transverse slices, the brain was flipped onto its medial surface, and a small 30° cut was made to the dorsal cortex. The brain was mounted with cyanoacrylate glue on the dorsal surface and immediately covered in 2% agarose in phosphate-buffered saline (PBS), and chilled using a specially fitting clamp. Sectioning with a Precisionary

model VF-510-0Z compresstome took place in ice cold aCSF constantly bubbled with carbogen. 400 µm slices were recovered from slicing well into a custom built 9-well holding chamber at 32 °C with bubbling aCSF. All slices were incubated for 1 hour at 32 °C and then allowed to recover at room temperature until recordings (1-6 hours). Ninety-nine total slices were obtained.

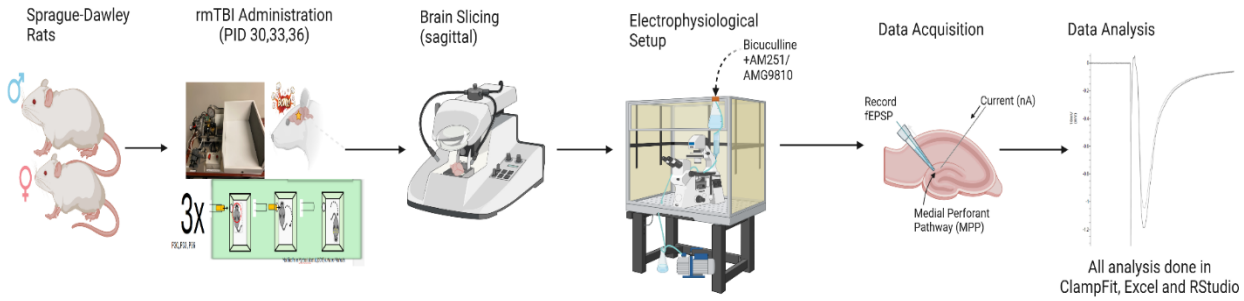

Figure 3. Flowchart overview of basic methodology for brain preparation. All data accumulation was done within the young-adolescent developmental time frame in rats (Bethlehem *et al*., 2022). Injuries were delivered on postnatal days 30, 33, and 36. Brains were collected 7 days later for recordings. All data were collected using Clampfit10 software and filtered with a 10kHz low-pass filter.

2.3 Electrophysiological Field Recordings

2.3.1 General placement/Current magnitude adjustments

Following one hour incubation at 32oC slices were transferred into a perfusion chamber with carbogenated aCSF (1-2 mL/min) and maintained at 30°C (Temperature Controller, Scientific Systems Design, Montclair, NJ). Transverse slices were secured in place with handcrafted weights. Using a 4x objective on Olympus BX51WI, an optimal view of the medial perforant pathway was obtained, allowing visually guided placement of stimulating and recording electrodes. A concentric bipolar electrode and recording electrode (pulled to 1-2 MΩ of resistance and filled with ACSF) were positioned in the medial perforant pathway

approximately 200 µm apart (Figure 4). Initial assessment of field excitatory postsynaptic potentials (fEPSPs) was simulated every 15s (0.12 µs stim width) and visualized using Clampfit 10.1 software (Axon Instruments, Molecular Devices). Electrode positions were adjusted accordingly to obtain optimal responses from the tissue, with a minimum response of 0.7mV, before being set to 50-60% of the maximum fEPSP (found via 1.0-1.5 nA current adjustments).

2.3.2 Electrophysiological protocols

A preliminary 5-minute baseline was obtained to determine a stable signal before moving on to experimental recordings for data. Following this we sought to determine if r-mTBI could induce changes in presynaptic neurotransmitter release and basal synaptic transmission with the Pulse (PP) and Input-Output (IO) curve protocols, respectively. For PP, six paired pulses were administered with a 50-millisecond interpulse interval (IPI), and IO recordings were obtained with a stepwise current increase of 0.03 mA (0.03 to 0.30 mA).

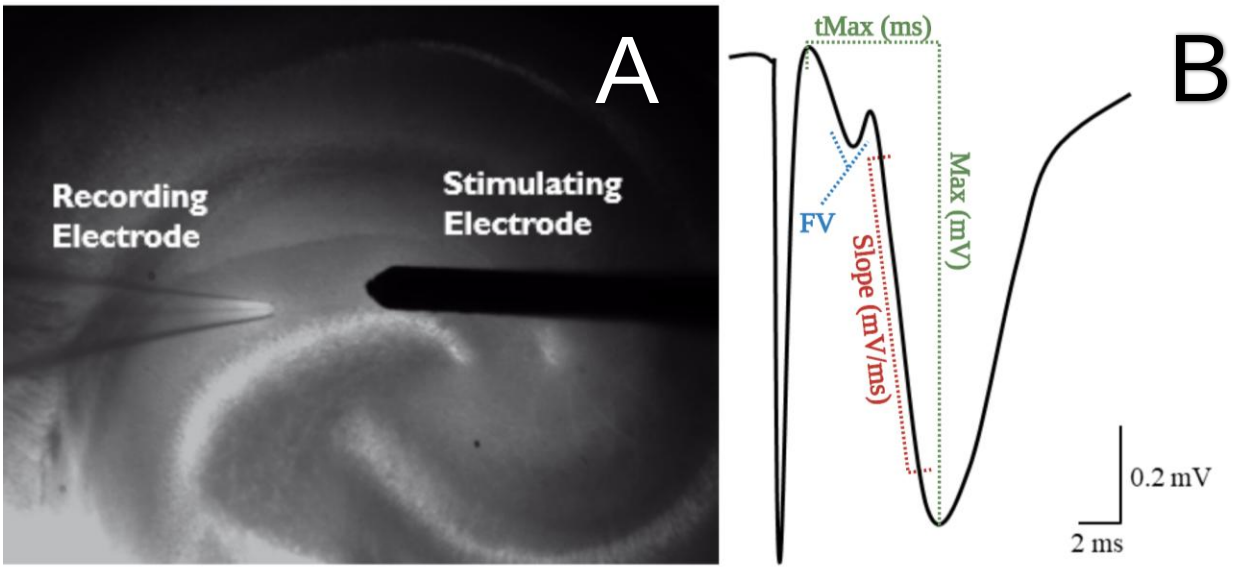

Figure 4. Anatomy and measurement of fEPSP recordings. **A)** A photo at 4x magnification depicting electrode placement for electrophysiological recordings. Stimulating electrodes were typically placed first in the tissue, followed by the placement and micro adjustment of the recording electrode. Recording electrodes were filled with aCSF and contained a silver-coated wire. **B)** Anatomy of measurements used on resulting recordings for data analysis, The slope of the fEPSP following the fiber volley (FV) was of primary concern, and changes of which were used to quantify LTP. Data was filtered at 10 kHz using a Multiclamp 700B (Axon Instruments) and digitized at 100 kHz using a Digidata 1440A data acquisition system.

All pre-conditioning recordings were obtained in the presence of bicuculline methiodide (BMI; 10 μM) to block feed-forward inhibition in the DG (Eyolfson *et al*., 2024). All drug administration was blinded to the researcher. For AM251 or AMG9810 trials, a second PP was obtained directly after the baseline recording to determine if drug administration altered presynaptic neurotransmitter release probabilities.

Following pre-conditioning recordings, a high-frequency stimulation (HFS) protocol was then delivered (4 x 100 Hz, with a 0.24 us pulse duration). Post-conditioning recordings occurred for 60 minutes in regular aCSF (1 pulse/15 seconds, 0.12 μs).

All electrophysiological signals were filtered at 10 kHz using a Multiclamp 700B (Axon Instruments) and digitized at 100 kHz using a Digidata 1440A data acquisition system. All statistical analysis was done in RStudio, PRISM, and Excel.

2.3.3 Drugs and Chemicals

Baseline and HFS recordings were recorded in the presence of BMI, as LTP induction is blunted due to feed-forward inhibition in the DG (Eyolfson, 2024). For trials assessing the contribution of the eCB AM251 (4 μM; Cat. No. 1117), and AMG9810 (3 μM; Cat. No. 2316) were dissolved in a 10 μM BMI and aCSF solution in preparation for perfusion. Drugs were purchased from Tocris Bioscience (Bristol, United Kingdom), and perfused over slices during baseline and HFS.

2.3.4 Data Analysis

All data measurements were recorded in ClampFit, with slope measurements (millivolts/millisecond) taken to discuss the rate of change seen within the various protocols employed (Figure 4). Slope was used as the measurement of choice, due to it being a more efficacious measure of synaptic transmission, and is typically less affected by population spikes, a common interference in field recordings (Fagni *et al*., 1987). For PP protocols, a ratio between the first and second pulse was obtained for comparison, with ratios created via PP1/PP2. For recordings that employed a second paired pulse directly following the baseline in the protocol, measurements were taken in the same method and used to compare the changes in PP ratio before and after drug application (expressed as Delta/Δ PPR). I/O recordings were built over increasing pulse widths (0.03 → 0.30 mA), and the maximum amplitude was taken from each

step. Differences in current response between Sham and r-mTBI groups were analyzed, to compare levels of basal synaptic transmission between groups. All values are given as mean ± SD with p-values and sample size.

Small “n” was used to denote the sample size of a group, with the viable slice recordings (1-4 slices per animal) being averaged together for final data values per animal. 6 groups were obtained for comparison: Sham Controls (n = 11) , r-mTBI Controls (n = 13), AM251/AMG9810 Controls (n = 7 and 9 respectively), and AM251/AM9810 r-mTBI groups (n = 7 and 11, respectively). For all recordings, post-conditioning responses were compared as percentage change from baseline responses. STP was calculated as the average of the first minute following HFS and LTP was calculated as the average of the final five minutes of post-conditioning recordings (55-60 min post-HFS).

Not every recording was deemed appropriate for further analysis. Out of 123 slices, only ninety-nine slices were permitted. Stringent inclusion criteria were determined if the slope of the baseline response was less than 0.5 and had less than 10% variance; the slope of the last 10 minutes of post-conditioning response was less than 1.5, and general irregularities in recordings, such as large gains in slope over decay time baseline. All data are presented in-text as mean ± standard deviation (SD) of the data. Following Shapiro-Wilk tests to confirm normality and Levene's test to confirm appropriate variance differences and parametric assumptions were met, unpaired Student's t-tests were conducted between groups of interest ($p<0.05$ for significance initially). Test statistics (t), p-values (p), and degrees of freedom ($t(_{XX})$) were reported. Bonferroni's corrections were applied to analysis where applicable, as multiple comparisons between the same data group were conducted.

Chapter 3: RESULTS

3.1 Sham versus r-mTBI protocols

3.1.1 r-mTBI induces changes in LTP, but not post-HFS STP

Before proceeding with drug trials, it was imperative to establish that r-mTBI resulted in deficits in LTP in the MPP (**Figure 5),** with visualization of the data shown in **Figure 5A.** Following HFS, r-mTBI (97.81 ± 46.26 SD) resulted in a non-significant reduction in STP compared to Sham animals (120.26 ± 22.93) (unpaired t-test; $t_{(20)} = 1.39$, $p = 0.18$) (**Figure 5B).** Therefore, within the first minute following HFS there were no differences between Sham and r-mTBI groups in the capacity to induce STP.

There was a significant difference in the capacity to induce LTP (**Figure 5C)** between Sham/r-mTBI groups**.** Sham animals displayed a greater capacity to induce LTP (81.6 ± 29.34) compared to r-mTBI animals (52.4 ± 30.3) (unpaired t-test; $t_{(20)} = 2.28$, $p = 0.03$).

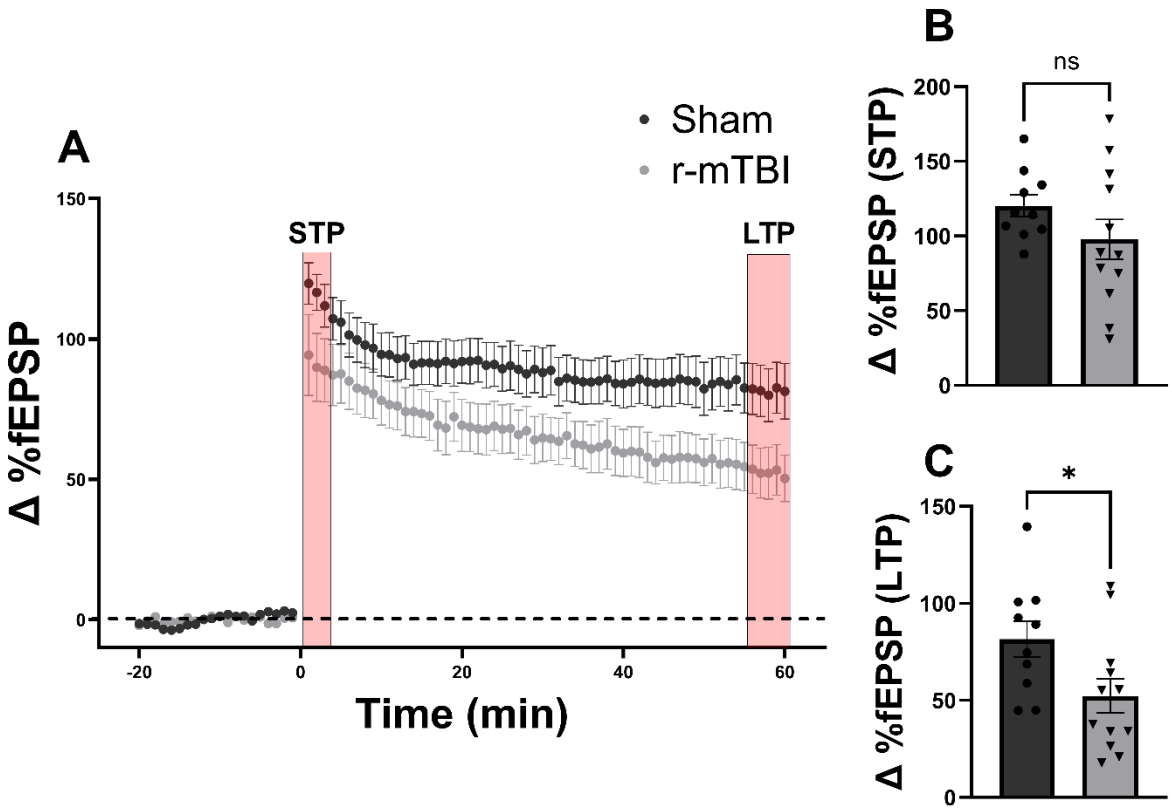

Figure 5. LTP recording data from Sham/r-mTBI groups. **A)** Graphical depiction of 1-hour long LTP decay recordings. Black arrow indicates HFS delivery (4x 100Hz). All points are averages of four stimulations, one every 15 seconds (i.e., 1 minute of decay per point). STP and LTP were defined as the first 3 minutes/points, and the final 5 minutes/points, respectively. Control groups contained 10 Sham rats, and 12 r-mTBI. Vertical bars through points represent standard error measurements (SEM). **B)** Histogram comparing the differences in STP within control groups. No significant differences were found in STP **C)** Histogram comparing LTP differences between groups. Groups were compared using unpaired t-tests. Significant p-values were defined at $p < 0.05$. Figures were created in GraphPad PRISM 10. Sham n = 10, r-mTBI groups n = 12 All data are expressed as mean ± SEM.

3.1.2 Sham vs. r-mTBI PP Analysis

As another measure of STP, a series of paired pulses were administered to assess levels of presynaptic neurotransmitter responses (**Figure 6)**. Pulses were administered 50ms apart and a ratio was taken from the slope of the second pulse divided by the slope of the first pulse (**Figure 6A)**. Means for Sham and r-mTBI ratios were as follows; 0.96 ± 0.11, and 0.99 ± 0.12).

Comparing the two groups' ratios shows no significant differences in PP ratios ($t_{(20)} = 0.60$, $p = 0.56$), as seen in **Figure 6B.** This supports the literature-based hypothesis of no mechanistic differences in individual synaptic activity pre- and post-r-mTBI.

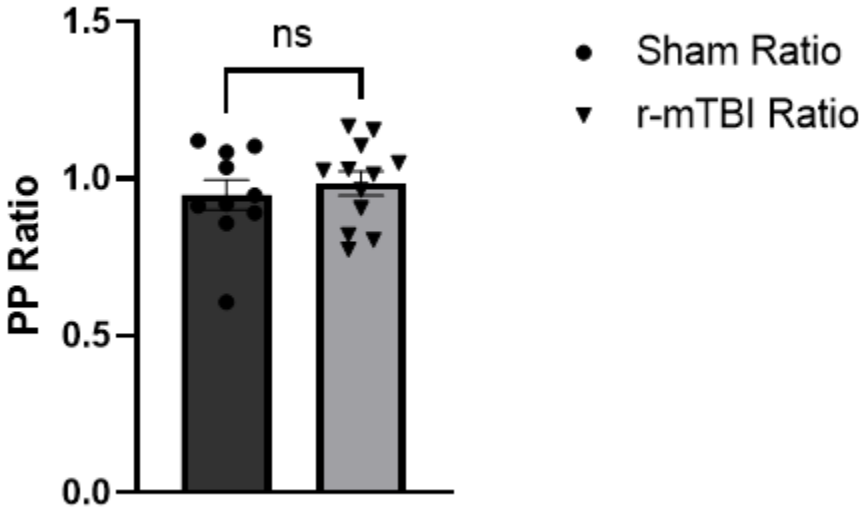

Figure 6. Analysis of paired-pulse experiments conducted in control Sham/r-mTBI groups. Pulses were administered with 50 millisecond intervals, with 6 total pairs stimulated and averaged. No significant differences were found, consistent with literature. Sham n = 10, r-mTBI groups n = 12. All data are expressed as mean ± SEM.

3.1.3 Sham vs. r-mTBI IO curve analysis

To observe the basal synaptic rate of transmission between control and r-mTBI cases, IO analysis was done **(Figure 7A)**. Fiber volley (FV) amplitudes and total fEPSP amplitudes were obtained to create a ratio between FV and fEPSP values, describing the efficacy of neural communication based on the fiber volley value (**Figure 7B**). 0.03mA stepwise increases (0.03-3.0mA) show no significant differences between the two groups ($t_{(19)} = 1.52$, $p = 0.15$)

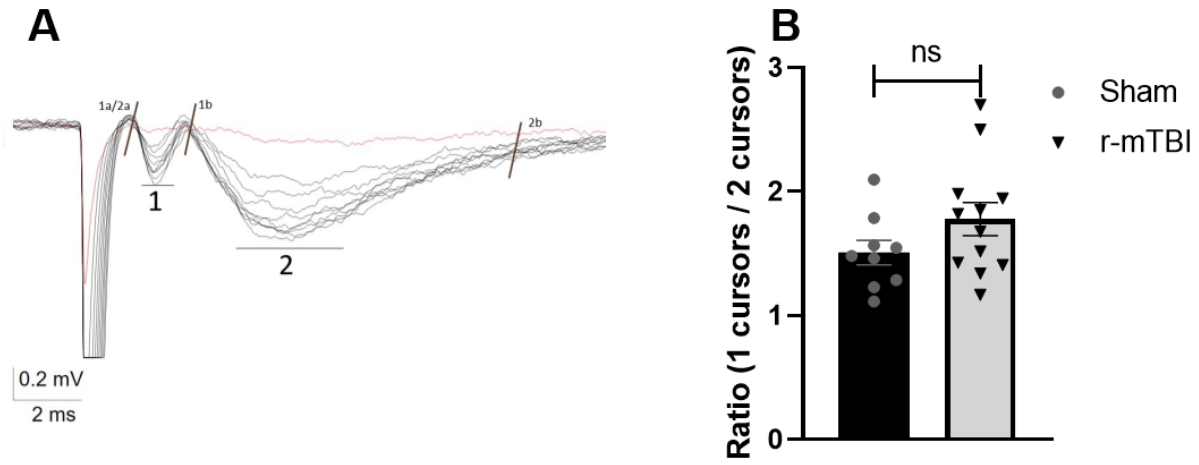

Figure 7. Graphs depicting input-output results between Sham and r-mTBI groups. **A)** Example IO recording trace, with cursor placements shown. One indicates the fiber volley in recording, and 2 indicates the amplitudes measured. 1a/b cursors are how fiber volleys/input currents are obtained. 2a/b are how the amplitude/output responses are obtained. 10 current steps of increasing pulse width were used (0.03 → 0.30 mA). **B)** Ratios between fiber volley and fEPSP amplitudes were plotted. This measures synaptic efficacy, of which there is no difference between Sham/r-mTBI. Sham = 1.51 ± 0.29 SD, r-mTBI = 1.77 ± 0.46. Sham n = 10, r-mTBI groups n = 12 All data are expressed as mean ± SEM.

3.2 AM251 protocols

3.2.1 STP/LTP recordings

Based on initial deficits in LTP following r-mTBI we wanted to examine the influence of the eCBs with the administration of a CB1 inverse agonist (AM251). As we saw in the control conditions there was no difference in the induction of STP between Sham (80.4 ± 33.9 SD)**,** and r-mTBI animals (103.3 ± 36.3) ($t_{(10)}$ = 1.13, p = 0.29). This trend did not change with the application of AM251, with non-significant effects on STP when comparing AM251 Sham/r-mTBI groups ($t_{(10)}$ = 1.13, p = 0.29), as shown in **Figure 8B**. Sham animals (120.3 ± 22.9) displayed higher levels of STP compared to AM251 Sham animals (80.4 ± 33.9) ($t_{(14)}$ = 2.82, p = 0.01). This suggests that CB1 receptors may have a role in STP mechanisms post-HFS within MPP-DG synapses. r-mTBI was seen to induce significant decreases in the capacity to induce

LTP. Following AM251 perfusion, there was no difference between Sham (59.4 ± 33.9) and r-mTBI (73.9 ± 36.3) groups ($t_{(10)}$ = 1.27, p = 0.23)**.** Relative to control r-mTBI cases, perfusion of AM251 in r-mTBI individuals resulted in a 29.18% increase in LTP, rendering LTP levels between AM251 Sham and r-mTBI groups to non-significance ($t_{(16)}$ = 1.53, p = 0.15). What this shows is that AM251 perfusion restores the initial deficit seen between control groups to non-significant levels (**Figure 8C),** supporting that inverse agonism of the CB1 receptor can aid in restoring LTP deficits seen in r-mTBI victims.

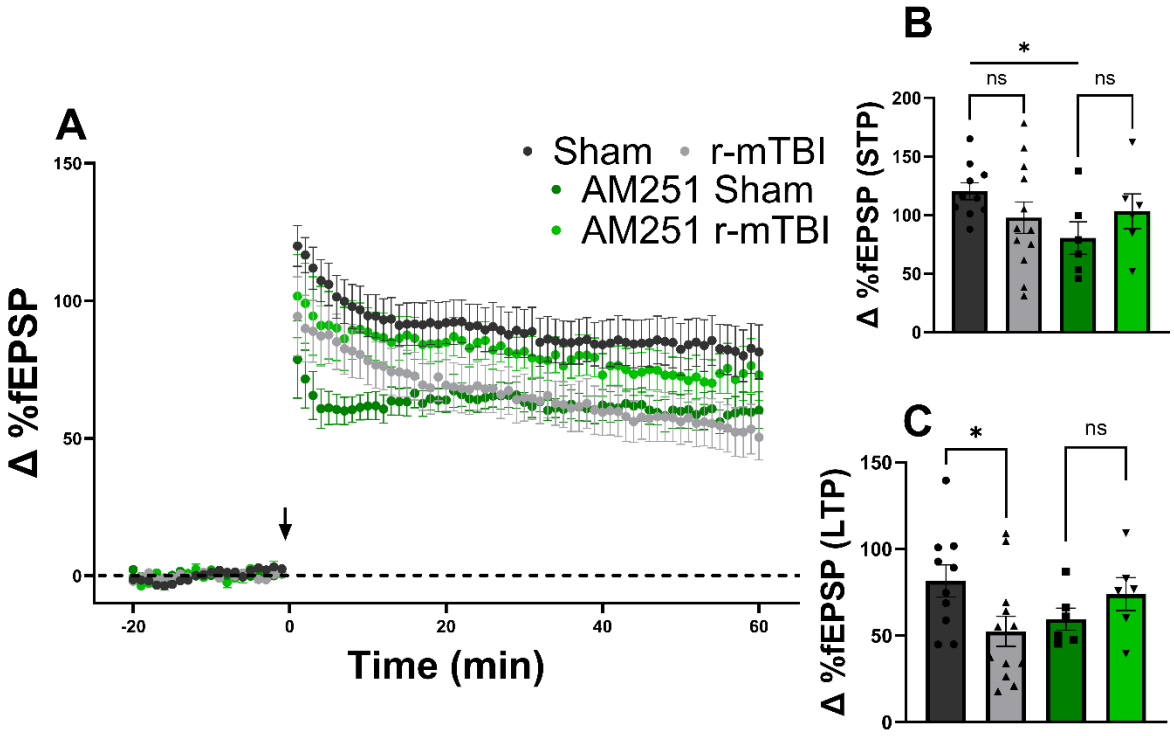

Figure 8. Graphical depiction of AM251 trial LTP data. **A)** LTP decay of AM251 (CB1 inverse agonist) trials (12 total recordings, 6 control/ 6 rm-TBI) as compared to control conditions. All procedures were identical to control protocols. **B)** Histogram comparing STP levels between conditions (defined as the first 3 minutes of decay). No significant differences were found between the groups. **C)** Histogram comparing the differences in LTP between groups conditions (defined as the last 5 minutes of decay). Note LTP alleviation seen in Sham AM251 trials versus r-mTBI AM251. AM251 treatments in r-mTBI cases saw recoveries in LTP back to Control Sham levels. Significant p-values were set to p<0.05. Sham n = 10, r-mTBI groups n = 12, AM251 Sham/r-mTBI groups n = 6 for both groups. All data are expressed as mean ± SEM on graph.

3.2.2 AM251 PP Analysis

Paired pulse experiments were also conducted for AM251 trials, once again regarding ratio differences before and after drug application (**Figure 9A)**. All procedures were conducted the same, apart from pre- and post-drug applications. Therefore, drug effects were analyzed from the changes in ratio before/after drug application, called the delta ratio (**Figure 9B).** There was no significant difference found between AM251 Sham/r-mTBI changes in ratios ($t_{(9)}$ = 0.81, p = 0.44), with means of -0.07 ± 0.1, and -0.11 ± 0.05), respectively. Therefore, while there appears to be a slight PP depression ensuing from AM251 application, these depressive events are not statistically significant.

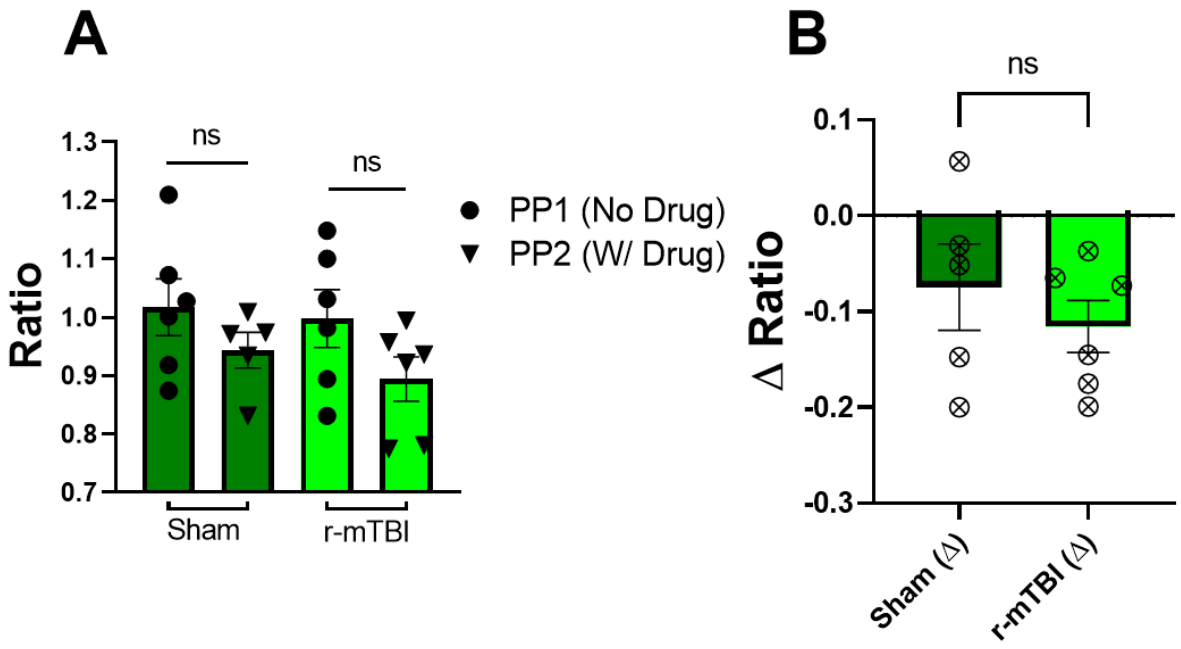

Figure 9. PP analysis of AM251 trials. No significant differences were recorded between groups. Lighter colours signify r-mTBI groups, and darker colours for Sham groups. **A)** Pre-AM251 signifies that a record that conducted a PP before and after drug administration, colours coded to Sham (black) and r-mTBI animals (grey). **B)** Depiction of that amount of change that occurred before and after drug administration, where no effect was seen. AM251 Sham/r-mTBI groups n = 6 for both groups. All data are expressed as mean ± SEM.

3.3 AMG9810 protocols

3.3.1 STP/LTP recordings

Based on previous reports of endocannabinoids interacting with the TRPV1 receptor, we also ran a set of experiments with a TRPV1 antagonist AMG9810. STP in the presence of AMG9810 showed significant difference between drug groups ($t_{(16)} = 2.33$, $p = 0.034$), with shams versus r-mTBI drug groups having 87.38 ± 18.8 and 114 ± 28.2 (Δ 26.6%), respectively. Furthermore, non-significant results between Control Shams and AMG r-mTBI groups were found ($t_{(18)} = 0.488$, $p = 0.63$), showing a rescue effect back to control Sham levels (120.25 ± 22.93, and 114.64 ± 28.23, respectively). This would indicate the TRPV1 antagonism influences STP post-HFS

In LTP analysis, much like AM251 trials, comparison between AMG9810 Sham/r-mTBI groups showed no statistically significant differences ($t_{(16)} = 0.77$, $p = 0.46$), with averages of sham (68.304 ± 18.89 SD) and r-mTBI (78.91 ± 28.23) drug groups only showing a 10.6% difference. This indicates a rescue of LTP levels in r-mTBI cases following drug application, implying TRPV1 has a role in LTP induction in r-mTBI cases. Furthermore, Sham trials show no differences between ($t_{(16)} = 1.05$, $p = 0.31$), with AMG Shams showing 68.30 ± 18.88 LTP levels, compared to the 81.57 ± 22.93 of Sham controls.

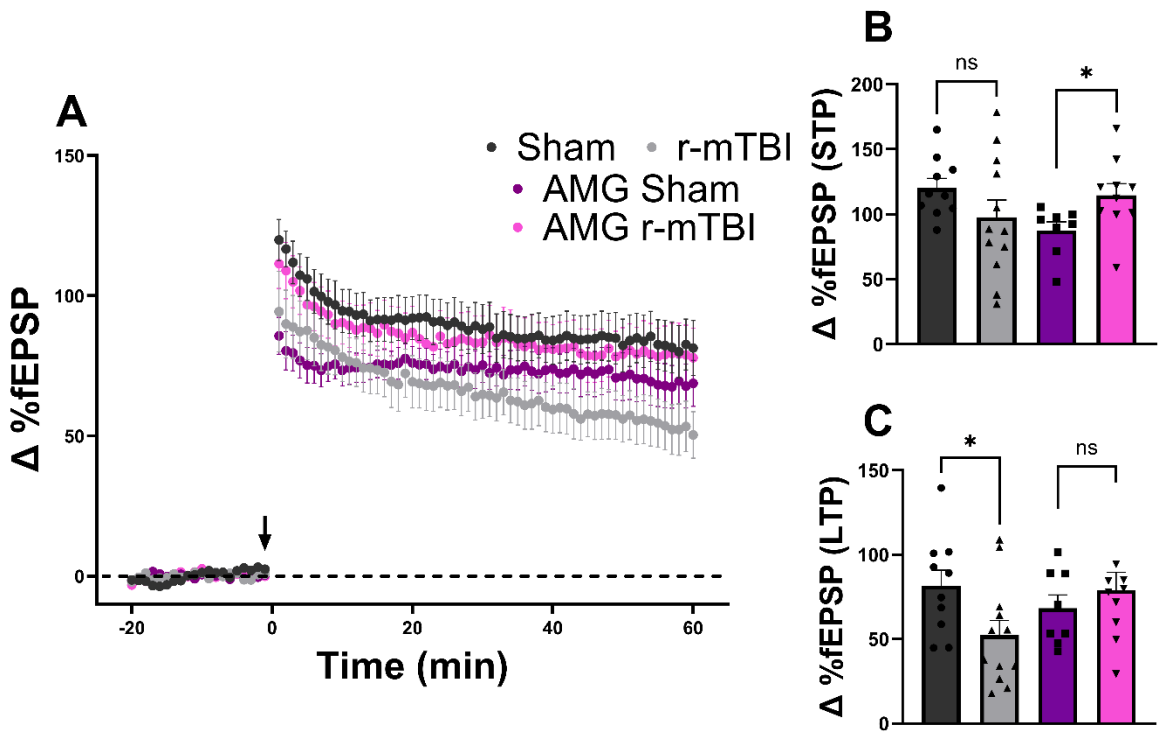

Figure 10. Graphical depiction of AMG9810-based LTP trials **A)** LTP decay of AMG9810 (TRPV1 antagonist) trials (18 total recordings, 8 control/10 rm-TBI). All procedures were done the same as controls **B)** Histogram comparing the differences in faciliatory STP between groups. No significant difference was found between groups. **C)** Histogram comparing differences in LTP levels between conditions. r-mTBI groups treated with AM251 showed recovery to Control LTP levels. Groups were compared using separate unpaired t-tests. Figures were created in GraphPad PRISM 10. Significant p-values were set to $p<0.05$.Sham n = 10, r-mTBI groups n = 12, AMG9810 Sham/r-mTBI groups n = 8 and 10, respectively. All data are expressed as mean ± SEM.

3.3.2 AMG9810 PP Analysis

Much like AM251 trials, AM9810 was investigated within a PP protocol (same as AM251 trials). Significant differences between Sham groups before/after AMG9810 application were recorded ($t_{(14)} = 2.82$, $p = 0.01$) following Bonferroni corrections (**Figure 11)**. This effect disappears in r-mTBI AMG9810 trials ($t_{(15)} = 0.24$, $p = 0.81$), indicating a change in the circuitry following r-mTBI, or that the TRPV1 receptor has decreased involvement in r-mTBI cases. Means were as follows; Sham PP1 (1.15 ± 0.20), Sham PP2 (0.98 ± 0.063), r-mTBI PP1 (0.96 ± 0.12), and r-mTBI PP2 (0.98 ± 0.11). Despite the differences found between ratios. no significant

differences between the ΔRatios were found (-0.08 ± 0.10) and r-mTBI groups (-0.004 ± 0.13) via unpaired t-test ($t_{(15)}$ = 1.29, p = 0.22). Curiously, a large level of PPF is seen within the AMG9810 Sham PP1R values, and upon further analysis, were found to be significantly different than the dedicated Sham PP1R values ($t_{(15)}$ = 2.48, p = 0.025), although this is not pictured on **Figure 11.**

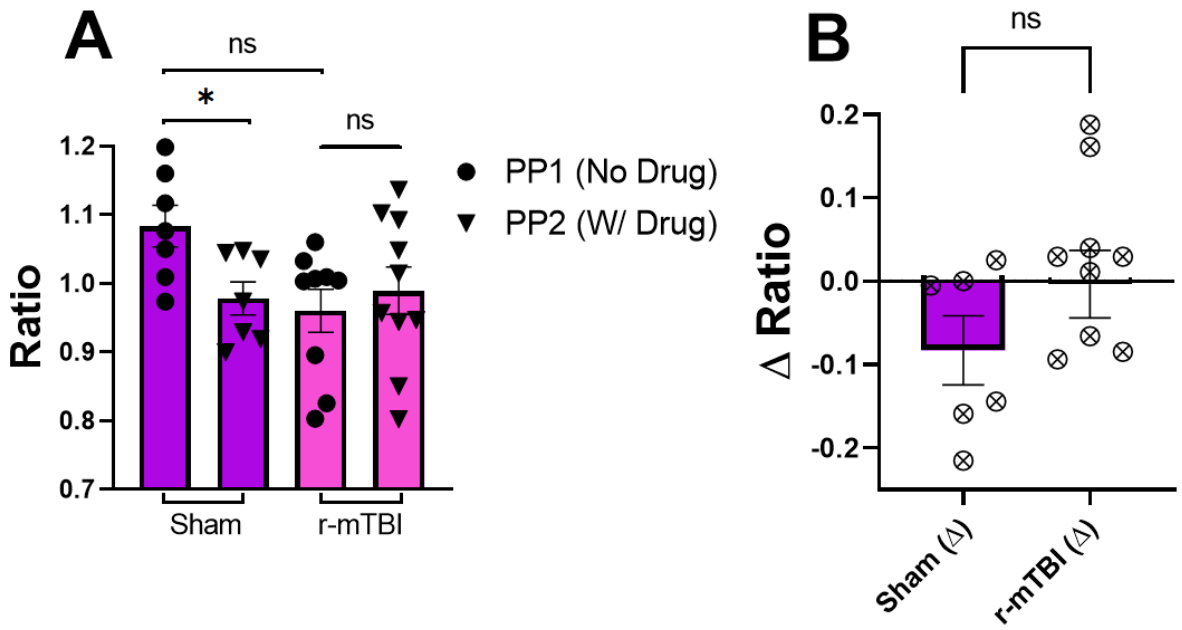

Figure 11. AMG9810 PP data analysis. Comparison between the AMG9810 Sham and r-mTBI groups were made for PP ratios. **A)** AMG9810 had a PP depressive effect on Sham groups, but this difference did not maintain in r-mTBI groups (n = 10/12 for Sham/r-mTBI groups, respectively). PP1R ratios were found to be NS, congruent with control values. 1 data point not pictured on graph, excluded for visual reasons. **B)** No effects were seen when comparing the difference between ratios of AMG9810 drug groups, congruent with control data. One data point for Sham Δ points was excluded from the graph for visual purposes. AMG9810 Sham/r-mTBI groups n = 8 and 10. All data are expressed as mean ± SEM.

Chapter 4: DISCUSSION

In the current study we examined if pharmacological manipulation of the EC system within the DG has potential for amelioration of LTP deficits that are common in mTBI. Sham/r-mTBI groups were analyzed to establish differences in LTP/STP/IO, where significant differences were only seen in LTP protocols. Once established, two drug trials were administered to cover for interactions of endogenous eCBs with both CB1 (inverse agonized with AM251) and TRPV1 (antagonized with AMG9810). Numerous studies suggest a role for endocannabinoids following TBI (Mayeux *et al*, 2017; Frazier, 2007; Lee *et al*., 2015), however very few have analyzed their effects following r-mTBI, with none intersecting YAs. YAs were of interest in this study due adolescence being a critical period of neural development (Kelly, 2012; Gotlieb *et al*., 2024), with mTBI being linked to long-term cognitive deficits and increased risk of other neurodegenerative diseases, such as Parkinsons and Alzheimer's (Zoppi *et al*., 2011). It was found that AM251 had negative effects on STP slope when comparing shams, but not when comparing r-mTBI groups. A rescue in LTP deficits between Sham and r-mTBI groups was seen, restoring the difference to non-significance following AM251 application in r-mTBI groups. AMG9180 based-protocols were also seen to cause the disappearance of PPF in Shams, with r-mTBI removing this effect between PP1/PP2 ratios, leaving no significant difference before/after drug application in r-mTBI trials. A rescue of initial LTP deficits was seen as well, like that of AM251 trials.

Consistent with other in-lab studies concerning MPP electrophysiology (Petersen *et al*., 2013; Patten *et al*., 2013), fEPSP slopes were taken for measurement and analysis. The initial findings of r-mTBI negatively affecting LTP found in this experiment are well supported in the literature, with no differences being found in faciliatory, STP and IO curve trials also being

supported (Eyolfson *et al*., 2024; Christie *et al*., 2023). Both pharmacological manipulations were found to recover r-mTBI-induced deficits in LTP, whereas the Sham/r-mTBI groups showed differences. This indicates that the eCB system participates in LTP mechanisms within the DG, with their expressions changing following injury.

4.1 Control Experiments Establish LTP Deficits in r-mTBI models, but not in STP/IO

Congruent with previous research, mTBI induces deficits in LTP events, and none of significance in STP/PP/IO at 7 days post-final injury in the DG (Eyolfson *et al*., 2024; Guerriero *et al.,* 2015; Harris *et al*., 2024). This result appears to extend to r-mTBI cases and is thought to be more consistent than single mTBI results, which are more sensitive to methodological differences such as age, method of mTBI administration, and species of rat (Guerriero *et al.,* 2015; Mychasiuk *et al*., 2016). These deficits in LTP following a single mTBI have been seen to last up to 28 days in an r-mTBI model (White *et al*., 2017). These results are quite limited in literature however, and future research is needed to bolster this relationship.

STP mechanisms within the MPP were found to not be affected by r-mTBI, consistent with previous research (White *et al*., 2017). The slight PPD seen at these synapses is present in literature at the specific pulse used in this experiment (Petersen *et al*., 2013), although reports are varied and are subject to different experimental protocols. Furthermore, increases in pulse width in IO experiments (0.03 → 0.30 mA, 0.03 steps) showed no significant differences as well, also consistent with previous literature (White *et al*., 2017). These results support the hypothesis that r-mTBI primarily affects LTP-based plasticity but does not change the basic presynaptic NT release properties of neurons in the MPP-DG synapses (White *et al*., 2017).

4.2 AM251 recovers LTP levels to NS levels in rmTBI cases.

Endocannabinoids primarily interact with the CB1R which is located presynaptically (Eyolfson *et al*., 2024a; Monory *et al*., 2015). This proposed effect of eCBs on the excitatory presynaptic terminals is referred to as "depolarization-induced suppression of excitation (DSE)" via these retrograde mechanisms inhibiting vesicular release (Mackie, 2006). Here we show that the perfusion of AM251 can ameliorate deficits in the induction of LTP following r-mTBI. Previous studies targeting CB1R and LTP have shown that CB1R agonism results in decreased fEPSP slope in the DG (Hoffman *et al*., 2017), as well as links to impairment of hippocampal-dependent learning tasks (Eyolfson *et al*., 2024a: Bonilla Del Río *et al.,* 2021). Hoffman and colleagues suggest that reductions in fEPSP slope are due to inhibition of glutamate release, particularly due to CB1Rs role in calcium modulation at excitatory synapses (Hoffman *et al*., 2017). We should expect to see one would then expect a similar effect would be observed in the DG, but to a lesser extent due to the nature of inverse agonism (i.e., a decrease in slope but to the effect of a true agonist, as per Nazari *et al*., 2023).

In the current study we applied inverse agonism on CB1Rs, due to the higher affinity/specificity for the receptor than natural eCBs, this results in holding CB1R in an inactive position during HFS induction and outcompeting the endogenous ligands that would activate the receptor (Frazier *et al*., 2007). It also allows for a more exact interpretation of the effects of these receptors, as using antagonism at these receptors does not account for the baseline activity of the receptor, whereas inverse agonism removes it (Nie and Lewis, 2001).

Previous in-lab and collaborative research using immunogold electron microscopy report a decrease of CB1Rs in the DG on both excitatory and inhibitory terminals following r-mTBI (Eyolfson *et al*., 2024) an area with high levels of CB1Rs (some ~26% localization on excitatory

synaptic terminals in the middle ⅓ of the dentate molecular layer, as per Peñasco *et al*., 2019). This suggests less modulation via the CB1Rs in r-mTBI cases, as CB1Rs function in presynaptic inhibition in the hippocampus/DG (Eyolfson *et al*., 2024a; Borilla-Del Rio *et al*., 2021). Therefore, we hypothesized that an increase in slope would be observed in r-mTBI cases when AM251 is applied, relative to Sham AM251 groups, due to decreased inhibition of vesicular release via CB1Rs.

Although there is no significant difference between Sham/r-mTBI groups in LTP, AM251 resolves the deficit seen between Sham/r-mTBI groups, restoring LTP to non-significantly various levels post-application. If we consider the previous in-lab result of the downregulation of CB1Rs in the DG following r-mTBI, it is plausible that a further reduction of CB1R activity allows for this recovery effect to ensue. For this, it is important to understand the distinction between antagonism, and inverse agonism, and their different receptor interactions. While the former simply blocks the receptor, inverse agonism acts via reducing receptor activity to below baseline levels by "locking" it in the inactive position (Pertwee, 2004) but notably does not stop all activity. If coupled with a pathological reduction of CB1Rs in r-mTBI cases, further reduction in receptor concentrations would explain an increase in LTP levels in r-mTBI cases following AM251 application, due to decreased interactions with CB1Rs therefore preventing downregulation of key Ca^{2+}/K^{+} receptors on the presynaptic. This would allow for more Ca^{2+} influx during HFS due to the heightened number of receptors, thus more glutamatergic release onto the postsynaptic NMDARs, and so on. To further support this theory, there are studies that find that mice with CB1 -/- in glutamatergic hippocampal cells have enhanced LTP (Monory *et al*., 2015). While the experiment did not specifically involve DG, CB1Rs are presynaptically

localized in the CA1/3 neurons as well (Monory *et al*., 2015). Thus, this study further supports the theory of enhanced LTP effects in r-mTBI models due to reductions in CB1 activity.

These effects are also assumed to not be restricted to the MPP, as electrophysiological studies also using α-GABAergic inhibition (picrotoxin) and AM251 in the lateral perforant pathway synapses also showed AM251 had no significant effect on LTP (Wang *et al*., 2016), albeit with no r-mTBI focus. This suggests that this endocannabinoid form of LTP modulation is broader than the MPP and provides a future direction of research in the LPP of r-mTBI models for comparison.

It is also worth noting due to the pharmacological nature of AM251, it is possible that TRPV1 interactions with endocannabinoids may be increased. Due to its synthetic nature, AM251 binds to CB1Rs with a higher affinity than the endogenously produced eCBs, 2-AG/AEA (Dao and François, 2021). This competition could potentially increase postsynaptic response due to primarily postsynaptic TRPV1 localization within DG. A comparison between "AM251 + BMI" and "AM251 + BMI+ FAAH" (FAAH = fatty acid amide hydrolase, involved in the breakdown of eCBs in synaptic cleft) groups would determine the extent of these postsynaptic interactions, and provide a future avenue for research. An antagonist for the CB1R, such as rimonabant, should also be considered (Gorelick *et al*., 2011). Furthermore, TRPV1 is most common, but not restricted to a postsynaptic orientation in the DG. AEA/TRPV1 interactions on the presynaptic neuron could be influencing Ca^{2+} levels in the presynaptic, thus increasing synaptic release, and so on. TRPV1 antagonism therefore needed to be attempted in this study, to further isolate the effects of CBR1s, and to control for these pre/postsynaptic contributions.

4.3 AM251 shows no effects on STP post HFS.

While there was a difference between Sham groups of the two, Sham/r-mTBI and its AM251 equivalents showed no significant differences between injuries for STP measurements. An explanation for the differences in Shams could be attributed to the neuron having an influx of Ca^{2+} depleting its vesicular stores during this HFS. Without the proper activity of the CB1R due to the AM251, there would be no regulation of vesicular release via Ca^{2+}/K^{+} channel downregulation, due to CB1R activity. However, despite this initial difference between Shams, there is no difference between r-mTBI groups. This could indicate that during HFS, there is a reduced effect of CB1Rs, rendering the r-mTBI trials to NS, or that STP becomes less dependent on CB1R following r-mTBI.

This loss of regulation could also be indicative of a loss of neuroprotective effects via eCB signaling. eCBs have been implicated within this role in other excitotoxic diseases such as cerebral ischemia, although their expression has typically been recorded as upregulated to regulate glutamate excitotoxicity (Gagliardi, 2000) in CA1. Our results would contradict this, as we see less CB1R activation following r-mTBI via increasing levels of STP, therefore a loss of regulation of vesicular release could be inferred. Given Sham groups are significantly different in STP levels following HFS, a loss of this acute regulation of glutamate transmission via CB1Rs in the MPP could be indicated in r-mTBI, as this effect disappears following injuries. Therefore, the "rescue" of STP levels following AM251 application in r-mTBI cases indicates a loss of CB1Rs on the presynaptic terminal, further supporting the findings in LTP trials.

4.4 AM251 Shows no Difference in PPRs between Sham/r-mTBI groups.

PP stimulation results were also recorded for each trial, with drug trials receiving one PP protocol pre- and post-drug application. Thus, the ratios between the two rounds were compared, with this difference (delta) also being compared to the appropriate r-mTBI group. Between Sham/r-mTBI groups, AM251 caused no differences within AM251 protocols, with no differences recorded in the PP ratio before/after drug application. This suggests that CB1R are insignificantly/not involved in PP mechanisms with 50ms IPIs, as no differences were recorded between control groups. CB1Rs having no effect on glutamatergic-mediated PPRs have been recorded in literature before (Nazari *et al*., 2023; Monory *et al*., 2015), however these results were obtained *in vivo* for the former, and did not include r-mTBI in the latter.

Despite what the results suggest, CB1Rs are active in PP mechanisms in certain recording protocols. In a 2001 study by Kreitzer and Regehr, a model of DSE was suggested, with AM251 being shown to prevent this regulation in parallel and climbing fiber inputs into Purkinje cells. This model specifically precludes the CB1R via AM251 and is found to reduce presynaptic Ca^{2+} influx which causes this DSE. This is congruent with our results in the STP section, but not in our paired pulse section, as no differences were found in PPRs during either Sham/r-mTBI cases. However, the Kreitzer and Regehr paper was done via whole-cell voltage clamps, quite different from fEPSP due to their intracellular nature. Therefore, due to this action present in the whole-cell recordings, we cannot say CB1Rs are not involved in PP mechanisms at all, but we can conclude their effects are not present in fEPSP measurements taken during this study.

In consideration with our data, the suggestion that CB1Rs are not acutely active during extracellularly recorded PP protocols is supported. An analysis of differences in

facilitation/depression following increased IPIs in PP protocols could offer insight into the pharmacokinetics of CB1Rs within PP protocols.

4.5 AMG9810 has a positive impact on LTP induction in r-mTBI.

Research has shown that TRPV1 receptors are expressed at elevated levels in the DG on both inhibitory (minorly) and excitatory (majorly) synapses receiving perforant pathway input in the DG (Puente *et al*., 2015; Chávez *et al*., 2014) and interact with endocannabinoids (Chávez *et al*., 2010). Furthermore, previous studies have shown that TRPV1 is implicated in EC-dependent LTD within the MPP, with genetic knockouts of TRPV1 shifting this LTD to LTP (Egaña-Huguet *et al*., 2021). Separate studies also suggest that LTP in MPP-DG synapses is postsynaptically dependent and mediated through postsynaptic neurotransmitter receptors, which can include TRPV1 (Nazari *et al*., 2023). Therefore, the postsynaptic TRPV1 interactions are necessary to account for when investigating the endocannabinoid system and are unexplored within an r-mTBI context.

Since TRPV1 is multimodally activated via a myriad of stimuli (heat, pH, etc.), and cation non-specific, it is harder to elucidate the effects it has on a neuron in most circumstances (Chávez *et al*., 2010). However, with the use of in vitro electrophysiology, with AMG9810 and BMI as a TRPV1/GABAα antagonist respectively in an in vitro model, we can attempt to isolate the effects of endocannabinoids interacting with CB1Rs within the DG when inducing LTP. Majorly, there is a trend toward significance between r-mTBI controls and AMG9810 r-mTBI groups, with a p-value of 0.065 calculated in an unpaired t-test. This is, however, not a significant result, as the p-value is set to 0.05, therefore this trend is irrelevant.

Nevertheless, this is indicative of a rescue effect observed for LTP between AMG9810 groups, with the drug Shams and r-mTBI groups non-significantly differing, as opposed to the significant difference seen between control Shams and r-mTBI groups. Importantly, r-mTBI pathology itself has been associated with decreased TRPV1 expression in the hippocampus (Yousuf *et al*., 2020; Hsieh *et al*., 2017), suggesting an endogenous compensatory response aimed at restoring plasticity by reducing TRPV1-mediated suppression of excitatory signaling. Therefore, pharmacological TRPV1 antagonism via AMG9810 may further recapitulate or enhance this compensatory mechanism.

It is also important to reiterate that AMG9810 allows for further isolation of CB1 interactions following r-mTBI, as TRPV1 is the only other relevant major EC-receptor interaction when measuring synaptic plasticity in vitro. While AM251 trials aimed to elucidate the effects of CB1R-EC through direct interactions, AMG9810 works to isolate its effects by removing an additional interaction from the endocannabinoid system to observe the effects of the endogenous eCBs on CB1Rs. Therefore, based on known reductions in CB1Rs following r-mTBI (Eyolfson *et al*., 2024), we can further determine that CB1 is involved in r-mTBI pathologies, as AMG9810 works to allow eCBs (specifically AEA) to interact with the CB1R more often. This is reflected in the literature as well, as the binding pocket of TRPV1 (residues Y512, S513, T551, and E571 specifically) has been shown to interact with AEA, thus reducing the compound's overall probability of interaction with CB1 within a synapse (Benítez-Angeles *et al*., 2020).

With TRPV1 being primarily postsynaptically located, antagonizing the receptor during LTP induction gives insight into the net activity of the receptor during and post-HFS. If we compare the effects of a TRPV1 -/- in the DG, we see that reduced TRPV1 activity shifts LTD to

LTP within the same low-frequency stimulation paradigm in the MPP-DG synapses, further indicating the two receptor classes have an interactive role during LTP/LTD (Egaña-Huguet *et al*., 2021). This LTP switch was abolished after the application of DAGL, the major 2-AG degradation enzyme; however, no effect was seen with AEA, the TRPV1-interacting eCB (Egaña-Huguet *et al*., 2021). What this implies is that AEA is not necessary during LTP induction when TRPV1 interactions are limited, due to evidence its production is not affected within this TRPV1 -/- KO model but does have a role within these synapses. This is via indirect actions such as disinhibition (less eCB tone, less CB1 inhibition).

Therefore, by using AMG9810 to antagonize postsynaptic TRPV1 receptors during LTP induction, we can better isolate and assess the contribution of CB1R-mediated signaling in the DG following r-mTBI. This pharmacological isolation is particularly valuable given the known downregulation of CB1Rs post-injury (Eyolfson *et al*., 2024), as it removes a competing site of endocannabinoid action, namely TRPV1, allowing for more direct interaction of endogenous AEA with CB1Rs. These findings suggest that alterations in TRPV1 activity (whether through injury-induced changes or pharmacological manipulation) can modulate synaptic plasticity outcomes in the DG. This further suggests interplay between the two receptors when mediating synaptic plasticity. Thus, the use of AMG9810 in this model provides critical insight into how endocannabinoid signaling dynamics are reshaped following r-mTBI and supports the notion that CB1R signaling remains a key modulator of synaptic function in the injured brain, particularly in the context of disrupted TRPV1-mediated signaling. Further research is needed to confirm specific changes in TRPV1 signaling post-rmTBI directly, but it is clear within these results that its activity is changed following injuries.

4.6 AMG9810 has a positive effect on STP following HFS in r-mTBI models.

AMG9810 increasing STP values post-HFS in r-mTBI models when compared to appropriate controls suggests that this initial STP value is correlated with LTP response levels. This can be attributed to decreased levels of inhibition via the CB1Rs. Firstly, TRPV1 fluxes Ca^{2+} into the cell when agonized via AEA. This ionic influx is a necessary step for EC synthesis (Grandes *et al*., 2021). This would theoretically be in a "loop," where a decrease of Ca^{2+} influx causes less EC synthesis, therefore less inhibition via the CB1Rs postsynaptically. However, TRPV1 is not the only receptor present on the postsynaptic, and not the only one that displays Ca^{2+} permeability, such as NMDARs (Egaña-Huguet *et al*., 2021). Furthermore, within an r-mTBI context, where CB1Rs are already downregulated (Eyolfson *et al*., 2024), the removal of TRPV1 via AMG9810 is unlikely to critically impair EC synthesis to a meaningful degree. Instead, antagonizing TRPV1 would allow for greater AEA availability at CB1Rs, as TRPV1 has been shown to sequester AEA via direct binding (Benítez-Angeles *et al*., 2020). This would therefore result in a net reduction of inhibitory action on the presynaptic neuron, due to increased CB1R interaction with AEA, thus allowing for increased vesicular release following HFS. This is consistent with the observed increase in STP values post-HFS in AMG9810 r-mTBI groups.

4.7 AMG9810 Has Significant Effect on Sham, but not r-mTBI PP Ratios

AMG9810 was found to eliminate paired-pulse facilitation (PPF) in medial perforant path-dentate gyrus (MPP-DG) synapses in Shams. PPF, indicative of lower release probability from the initial pulse, facilitates subsequent pulses by allowing residual Ca^{2+} to remain inside the neuron, which enhances vesicular/quantal release via Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein microdomains (Tahmasebi *et al*., 2016; Shahrezaei

and Delaney, 2004). Due to fEPSP being a postsynaptic response measure, and PP being a protocol used to determine presynaptic mechanisms, a decrease in PPR following AMG9810 application would be indicative of the receptor being involved in the postsynaptic response, as it is primarily postsynaptically located in DG (Puente *et al*., 2015)

It is important to note that PPF has varied results in MPP. *In vitro* studies suggest a range of slight PP depression to PPF effects when measuring the fEPSP slope in the MPP (Petersen *et al*., 2013). Within this study this variability was seen as well, as AMG9810 Sham levels of PPF before drug application were found to be significantly more than the dedicated Sham groups. As there was no difference in data filtration, treatment of slices, and recording conditions, it can only be assumed that this variability is random. It does, however, conveniently offer an indirect analysis of TRPV1 activity changes post-rmTBI.

These results suggest TRPV1 receptor downregulation following repetitive mild traumatic brain injury (r-mTBI), like changes observed in CB1 receptors (CB1Rs) (Eyolfson *et al*., 2024). TRPV1 receptors, primarily postsynaptic in the dentate gyrus, contribute to excitatory postsynaptic potentials (fEPSP) via nonspecific cation influx (Schurman *et al*., 2017; Chávez *et al*., 2010; Egaña-Huguet *et al*., 2021; Tahmasebi *et al*., 2016). Although PPF primarily reflects presynaptic mechanisms, the absence of presynaptic effects in AM251 trials supports the hypothesis that TRPV1 down-regulation alters postsynaptic responses, and therefore fEPSP responses.

Furthermore, TRPV1 downregulation in r-mTBI cases may increase calcium channel expression on presynaptic neurons. CB1R activation typically reduces Ca^{2+} permeability via internalization of P/Q and N-type calcium channels, or upregulation of potassium channels (KA and GIRK) through the adenylyl cyclase pathway (Balezina *et al*., 2021). Lower CB1R activity

via TRPV1 downregulation post-rmTBI could therefore increase vesicular release, diminishing the inhibitory actions of CB1R. Further investigation is required to confirm this mechanism, where the use of a calcium chelating agent could be considered, as this would limit excessive Ca^{2+} influx causing excitotoxicity that may be occurring post-mTBI due to lower CB1R regulation.

Endocannabinoid (EC) signaling regulation depends on distinct synthesis pathways: AEA synthesis involves phospholipase D hydrolysis of lipid precursors, while 2-AG synthesis relies on phospholipase C cleavage (Frazier, 2007). AEA, which interacts with postsynaptic TRPV1 receptors, requires large postsynaptic Ca^{2+} influxes triggered by depolarization or excitotoxicity events to be synthesized (Frazier, 2007). AMG9810 disrupts these AEA-TRPV1 interactions by blocking access to the channel, potentially explaining the disappearance of PPF in Shams. However, in r-mTBI cases, TRPV1 downregulation prevents these PPF effects indirectly. While fEPSP measurements primarily capture postsynaptic responses, further studies should examine TRPV1 receptor localization pre- and post-r-mTBI to better understand their role.

Chapter 5: Concluding Statements

Limitations of Current Study

It would be remiss to not consider confounding variables within this study. Firstly, considering the precision needed for a swift brain removal following decapitation, variable removal times could contribute to some level of inconsistency within the data. Physical damage caused in the dissection could also alter levels of synaptic plasticity in recording, due to the shearing of neurons during surgery. Furthermore, transitions from aCSF to drug protocols were often far from seamless, with perfusion systems occasionally stopping flow of solution into

perfusion chambers entirely, or the drip rate changing out of nowhere. While the researchers were diligent in preventing these events from occurring, a non-zero number of recordings that were passable otherwise were lost. This negatively affected the n of this experiment and thus lowered the power of analysis. Additionally, slices were often thrown out if a signal was not easily reached within the first five attempts of electrode placement. While this convenience of recording easily is a blessing, it is possible that slices were thrown out even if they had the potential for a stable recording.

In studies, rats are typically used as a mammalian model, due to a variety of factors (generation/maturation time, ease of care relative to other animals, and so on). However, they are not perfect analog for humans. Particularly for mTBI studies, rats have less developed skull structure, offering less protection from impacts as humans would. This could exacerbate the effects of mTBI induction on synaptic plasticity. Furthermore, the rat hippocampus/DG is more dorsal than that of the human, which could cause more shearing forces on the neurons within ROIs upon impact. Using humans for a study like this, however, is clearly unethical, and rats should be used.

Conclusions/Future Research

It was concluded that endocannabinoids have a modulatory role in both PP and LTP forms of YA synaptic plasticity in an r-mTBI model. Control Sham/r-mTBI groups were found to have no differences in basic synaptic mechanisms such as PP and IO ratios, but differed in levels of LTP, consistent with previous research. AM251 was found to have no effects on STP/PPRs, however, it did perform a rescue of LTP levels to no significance between r-mTBI groups, indicating that the presynaptically-based CB1Rs have an effect primarily in LTP-based

synaptic plasticity mechanisms. Furthermore, AM9810 was found to affect both STP/PPR as a slight PPF effect was abolished following drug application. This PPF effect also disappeared in r-mTBI models, with no significant differences found between before/after ratios, suggesting that TRPV1 is downregulated following r-mTBI due to its primarily excitatory influx of cations. As CB1Rs have been noted to downregulate themselves following r-mTBI, TRPV1 can be considered a post-synaptic mirror to the CB1R pathology. LTP was also rescued in AMG9810 trials to non-significance between r-mTBI trials, suggesting TRPV1 has a role in these known r-mTBI LTP deficits, although this was theorized to be due to decreased endocannabinoid synthesis.

Future studies should interrogate the effects of agonists/antagonists such as THC on the CB1Rs within a r-mTBI model, as it would provide a counterpart to the inverse agonism seen within this system. In this same vein, TRPV1 agonists such as capsaicin should be considered to view potential differences in pre- and post-rmTBI induction. Both approaches could supply more information via indirect measurement on the effects of these receptors before and after r-mTBI induction and could be contrasted with different age ranges to see how YA electrophysiology differs following r-mTBI. For non-electrophysiology analysis, Western Blotting/immunohistochemistry could be used to directly measure the differences in receptor compositions. This, in addition to immunogold imagery, would confirm the indirect measures/proposed receptor downregulation in this experiment.

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