CHARACTERIZATION AND TARGETING OF THE ENDOCANNABINOID SYSTEM IN TRAUMATIC BRAIN INJURY

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Abstract

Traumatic Brain Injuries (TBI) are considered one of the leading causes of death and disability worldwide. One emerging area of TBI research is the involvement of the endocannabinoid system (ECS) in response to brain injury. The ECS is modulated by exogenous cannabinoids such as Δ^9-tetrahydrocannabinol (THC) found in *Cannabis sativa.* THC is a partial agonist of both cannabinoid receptors CB1R and CB2R. CB1R activation is associated with neuroprotective effects and contributes to analgesia and anxiolytic effects, whereas CB2R activation reduces inflammation. Therefore, the treatment of rats subjected to TBI with THC post-injury may restore motor function and improve behavioral profiles of injured rats. In order to assess behavioral and physiological changes associated with TBI following a closed head impact injury in a rat model, two experiments were performed. Rats were subjected to a closed-head injury impact equivalent to a mild/moderate TBI, or sham injury, and subsequently treated with THC or vehicle treatment. In the first experiment, 9 rats of both sexes were randomly assigned to: 1) SHAM TBI + Vehicle; 2) SHAM TBI + 1 mg/kg THC; 3) TBI + Vehicle; or 4) TBI + 1 mg/kg THC. Rats were subjected to a number of behavioural measures to assess drug effect, anxiety, working memory, and locomotor function following injury. Rats were assessed prior to TBI to establish a baseline, and on the above measures for 7 days following TBI. In the second experiment, 3 rats of both sexes were randomly assigned to 1) TBI + Vehicle or 2) TBI + 10mg/kg THC and tested for locomotor performance only. According to the data collected, TBI significant decreased male but not female locomotor recovery on the rotarod. Additionally, 1 mg/kg THC administration 1 h post-TBI significantly decreased male Sham-TBI, but not female locomotor activity. In the second experiment, visible differences in locomotor recovery were seen between TBI-VEH vs. TBI+THC 10 mg/kg on day 1-4 following TBI and drug administration, but according to the data collected, 10 mg/kg THC administration 1 h post-TBI did not significantly impact locomotor recovery post injury. THC or TBI did not significantly change other behavioural measures collected. These data demonstrate the importance of exploring the therapeutic potential of cannabinoids such as THC following TBI, which could contribute to reducing the longevity of lasting post-injury symptomology.

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Dedication

This thesis is dedicated to my parents, my partner, my science moms, and all others who supported and inspired me on this excellent adventure.

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List of Abbreviations

AA	Arachidonic Acid
2-AG.	2-Arachidonylglyderol
AEA	Anandamide
AUC	Area Under the Curve
BBB	Blood Brain Barrier
CB1R	Cannabinoid Receptor 1
CB2R	Cannabinoid Receptor 2
CNS	Central Nervous System
CT	Computerized Tomography
ELISA	Immunohistochemistry and Enzyme-Linked Immunosorbent Assay
ENS	Endocannabinoid System
FAAH	Fatty acid amide hydrolase
HPLC/MS	High-Performance Liquid Chromatography with Tandem Mass Spectrometry
I.P	Intraperitoneal
I-CAM-1	Intracellular Adhesion Molecule-1
LASU	Lab Animal Service Unit
MAGL	Monoacylglycerol lipase
NO	Nitric Oxide
OFT	Open Field Test
PTA	Post-traumatic Amnesia
PPCS	Post-Concussion Syndrome
PPAR	Peroxisome Proliferator Activated Receptors
SIS	Second Impact Syndrome
RONS	Reactive oxygen and nitrogen species
RmTBI	Repeated mild traumatic brain injury
SOP	Standard Operating Procedure
TBI	Traumatic Brain Injury
THC	Δ^9-tetrahydrocannabinol
TVP	Transient Receptor Potential Channels
WADA	World Anti-Doping Agency

Chapter 1 Introduction

1.1 Introduction

Traumatic Brain Injury (TBI) is one of the leading causes of death and disability world-wide (1). Brain Injury Canada states that Acquired Brain Injuries, which encompass all-caused brain injuries due to traumatic and non-traumatic etiologies, are the leading cause of death among Canadians aged 40 and under (2, 3). The Center for Disease Control has confirmed that incidents of TBI account for up to 30% of injury-related deaths in the United States (4). According to an epidemiological assessment by Fu et al., 2016, in Ontario alone, confirmed cases of TBI during a 1-year period accounted for up to $1.22 billion in lifetime injury-associated expenses (5). TBIs can range from moderate to severe in nature and occur as a result of external mechanical force causing alterations in acute and chronic cognitive, physical, or psychosocial function (6). TBIs most commonly result from falls, sports-related injuries, car accidents, or physical assaults, and are usually accompanied by loss of consciousness, post-traumatic amnesia (PTA), as well as persistent cognitive and neurological deficits (7). Injuries ranging from mild to severe are often accompanied by persistent memory loss and cognitive deficits lasting for months to years post-injury (8). Men are twice as likely as their female counterparts to suffer a TBI, with the largest number of TBIs occurring in children under the age of 4 or adults over the age of 85 (5). Despite a lack of epidemiological investigation, a review by Langlois in 2006 estimated that globally, sports related TBI were responsible for approximately 1.6 to 3.8 million injuries per year (9). More recently, recognition within Canadian amateur and professional national sport communities has increased, resulting in a surge of TBI incidences being reported, but data is still limited (10). The 5th International Conference on Concussion in Sport (11, 12) released updated clinical assessment tools and return-to-sport guidelines to address lasting gaps in diagnosis and rehabilitation; yet persistent social stigma, the pressure to “return to normal”, and a lack of available pharmacological treatments for TBI leave many at risk (13). Depending on the injury’s severity, cognitive and neurological deficits can persist long-term, causing a lifetime paradigm shift for the persons injured as well as for their loved ones.

One emerging area of TBI research is the involvement of the endocannabinoid system (ECS) following brain injury. The ECS is a system within the human body that is responsible for numerous physiological functions such as temperature, nociception, and locomotion, as well as higher level cognitive and behavioural functions (14, 15). As awareness surrounding post-traumatic complications and return-to-sports activities increases, the need for research examining this intricate relationship is apparent. This is in part because endogenous cannabinoids may possess neuroprotective and inflammatory mediating properties (16). These endocannabinoids, which are produced by neurons and other cells in the body, modulate pain (17), anxiety (18), and general neurotransmitter release through the activation of the type 1 cannabinoid receptor (CB1R) and inflammation (19) through the type 2 cannabinoid receptor (CB2R). CB1R is the most abundant G protein-coupled receptor (GPCR) in the central nervous system and is found in areas of the brain linked with motor learning, memory, reward pathways, and higher cognitive processing (20). Because of these known modulatory roles, the ECS and cannabinoids may play a role in the treatment of TBI. However, to date there has been a lack of research into the effects of TBI on the ECS, pre-clinical or otherwise.

As the ECS is the system on which cannabinoids from *Cannabis sativa* (cannabis) and its derivatives act, the use of cannabis in TBI treatment also deserves investigation. Indeed, Δ^9-tetrahydrocannabinol (THC), the intoxicating constituent of cannabis, exerts its effects by activating both CB1R and CB2R (15). Up until its recent legalization, research into cannabis and its derivitives has been limited. This has left a large gap in our overall clinical understanding of cannabis, cannabinoids, and their potential theraputic effects. The lack of therapeutics available for TBI, in conjunction with recent legalization and the therapeutic potential of cannabis, opens the door to undiscovered drug therapies for this debilitating condition.

Much like available data on sports-related TBI, little is known about the prevalence and patterns of cannabis use in sports (21). Despite THC being banned according to the World Anti-Doping Agency (WADA), cannabis is the 2nd most widely used recreational drug behind alcohol among athletes, and its use is associated with higher-risk sports (22). Numerous anecdotal self-reports exist of contact sport players using cannabis for post-game analgesia, as well reported use to decrease pre-competition anxiety and increase focus, yet available research is exceptionally limited (23). Therefore, research in this area is critical regardless of positive or negative results, as our data will add to the growing body of knowledge on the topic for policy, education, and health care

applications affecting TBI and cannabis use in sport and overall health from a harm reduction perspective.

The goal of the research described here is to explore how the ECS changes in an animal model of TBI, and to measure whether or not the ECS can be targeted with THC to restore motor function and improve behavioral profiles of injured rats.

1.2. TBI Pathophysiology

The term TBI is an umbrella term used to define a complex neurological event, induced by biomechanical forces resulting in mild to serious downstream physiological, behavioural, and cognitive consequences. TBI is a multifaceted injury, characterized by numerous pathophysiological changes within the brain that have been triggered by an original structural injury, known as the primary injury (24). This mechanical primary injury causes immediate contusions to the injured area, diffuse shearing of axons and blood vessels, damage to glial cells, cytoskeletal disruptions, as well as localized hemorrhaging (25-29). TBI severity in its early stages is assessed via the Glasgow Coma Scale (GCS), the current gold standard clinical assessment of TBIs, which grades duration of loss of consciousness and duration of acute memory loss on a scale of 3-15, where high numbers are associated with better patient outcomes (30-31). The primary injury is untreatable but can be prevented by the introduction of safety measures and personal protective equipment.

The secondary injury cascade involves widespread cellular, molecular, and biochemical changes that develop following the primary injury (32-34). These pathophysiological mechanisms are characterized by ion, neurotransmitter, mitochondrial, and cerebrovascular dysregulation leading to eventual cell death and resulting chronic impairments demonstrated in figure 1-1 (27, 35). The secondary injury evolves from minutes to years post-injury due to neuronal hyperexcitability, glial cell dysregulation, lipid degradation, cerebral edema, nitric oxide (NO) synthesis, and eventual widespread neuroinflammation, ultimately resulting in brain cell death, tissue damage, and atrophy and the potential accumulation of protein such as amyloid precursor and tau protein (26, 36-38). The severity of the second injury cascade is difficult to predict due to the heterogeneity of injury morphology and magnitude. This paired with the omnipresent demystification of the intricate neurometabolic cascade is perhaps the reason why modern medicine has failed to provide reliable pharmaceutical intervention.

Scientific investigation has focused on the various known downstream effects of the secondary injury cascade as potential therapeutic targets to decrease functional deficits associated with the primary injury. One specific area of focus has been the widespread CNS excitotoxicity immediately following the onset of adverse biomechanical forces to the cerebrum. Regardless of injury severity,

mechanoporation of axons, caused by shearing forces exerted on axons, leads to adverse molecular perforations of their lipid membrane (32, 39-40). It is suggested that these mechanoporations allow ion leakage, resulting in mass depolarization of voltage- and ligand-gated ion channels, ultimately leading to wholesale glutamate and aspartate release and Ca^{+2} influx in an unregulated and damaging fashion (41-43). The widespread CNS excitation causes largescale influx of Ca^{2+} which is hurriedly sequestered into mitochondria, leading to mitochondrial dysfunction and lasting disruptions of energy production (44). Mitochondrial dysfunction is further perpetuated by injury driven generation of damaging free radicals such as nitrogen and oxygen species (RONS) like NO, synthesized en-masse as part of the second injury cascade (45). When left unchecked, this spread of synaptic depression, cellular Ca^{2+} imbalances, energy deficits, and RONS accumulation contributes to post-injury complications such as seizures, critical dendritic edema and eventual cell death (46). These cellular cascades trigger molecular signals driving downstream immune activation and subsequent cytokine and chemokine release, pushing the initial robust post-traumatic neuroinflammatory response that lasts for weeks to years following injury (47). According to the review of Simon et al. (2017) this inevitable and critical immune cascade resulting in persistent neuroinflammation is increasingly seen as a necessary, but potentially harmful, biological coping mechanism, and an important focal point for pharmaceutical interventions (33).

Another area that has gained interest in brain injury research is the impact of changes incurred by primary injury on cerebrovascular architecture and regulation. The brain is supported by highly sensitive vascular regulatory mechanisms, the most influential of which in TBI is the blood brain barrier (BBB) a functional and metabolic “barrier” between the brain and the body’s circulation characterized by tight junctions between endothelial cells in the surrounding cerebral microvasculature (48, 49). When disrupted during primary injury and further compromised during secondary injury, a lapse in BBB function acts much like the opening of a cerebro-vascular flood gate in its cause and perpetuation of severe edema (49-51). This mass perfusion overwhelms vascular autoregulatory mechanisms and causes dramatic shifts in cerebral blood pressure and oxygenation (52, 53). The dysregulation of these protective mechanisms has been shown to persist for days to weeks post injury (54), contribute to acute risk of reinjury (55) and lasting functional impairments (56).

These compounding dysregulated conditions reflect poorly on neuronal connectivity, and commonly produce lasting behavioural, cognitive and neuromotor symptoms (57). One potential

means of therapeutic intervention for the treatment of the secondary cascade associated with TBI is the use of THC. THC is a compound of interest in TBI research because of its CB1R and CB2R-mediated potential to quell glutamate excitotoxicity, the generation of free radicals and lipid peroxidation, and to mitigate apoptosis, perturbations of cellular calcium homeostasis, vascular dysregulation, and associated cognitive and behavioural deficits, all of which are avenues of focus for the development of pharmacological therapies to treat TBIs (13).

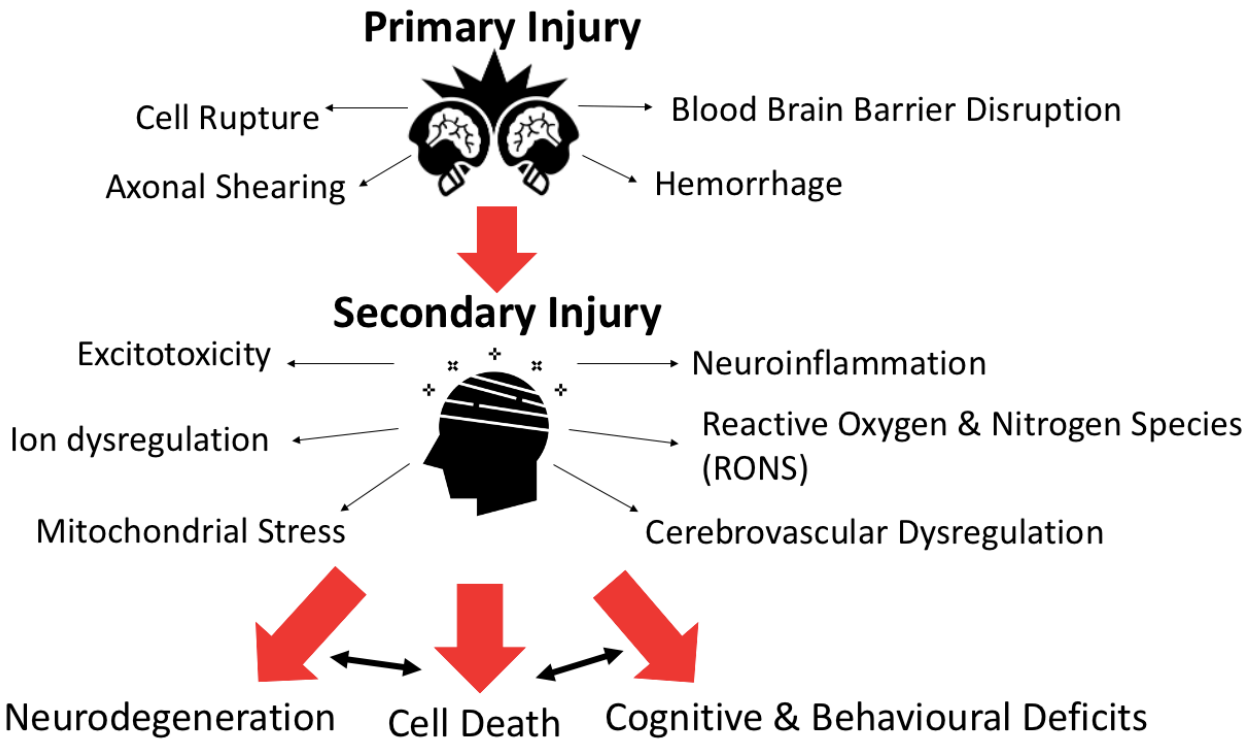

Figure 1-1: Downstream Primary and Secondary Injury Cascades. Summary of secondary injury mechanisms accompanying initial primary injury, responsible for long term impairments. Figure created by author.

1.3 Mild to Moderate Traumatic Brain Injury (mTBI)

According to the most recent consensus statement on mTBI in sport (12), a mild to moderate TBI is defined as a head injury induced by external biomechanical forces incurred by an insult to the head, face, or neck that are frequently accompanied by rapid acceleration and deceleration of the brain within the skull (58). mTBIs are often accompanied by acute impairment of neurological function, neuropathological changes, a GCS measure ≤9, disruptions in normal cognitive function that may include loss of consciousness and PTA, as defined previously. Mild TBIs are characterized by functional disturbances rather than overt structural abnormality, whereas structural abnormalities and non-operative lesions may be visible via neuroimaging in moderate TBI (12, 59).

mTBIs have gained international publicity due to the increased rates of Chronic Traumatic Encephalopathy (CTE), a neurodegenerative disease characterized by chronic behavioural and cognitive changes attributed to cerebral atrophy and neuronal death caused by accumulation of phosphorylated tau and amyloid-β (Aβ) proteins presenting as neurofibrillary and astrocytic tangles and TDP-43 protein pathologies following injury (60-63). CTE has been diagnosed post-mortem in boxers, hockey players, and football players (64-66). mTBI has gained further recognition in Canadian sports communities following the implementation of Rowan's Law in Ontario. Rowan's Law commemorates a young local female rugby player who died suddenly from Second Impact Syndrome (SIS), a syndrome resulting from the increased metabolic vulnerability of a still-symptomatic brain, sensitive to reinjury, which leads to loss of autoregulatory mechanisms and subsequent lethal physiological cascade from a sublethal second impact (55, 67).

mTBI are shown to have acute and lasting chronic impacts. Upwards of 75% of TBIs reported are considered mild to moderate in nature and are diagnosed according to the presence of clinical somatic, cognitive, and emotional symptoms (12, 68). Although mTBIs constitute the most common phenotypes of TBI, mTBIs remain the most underreported and misunderstood type of TBI in both human and animal models (69,70). Despite being illustrated mainly by molecular cascades that rectify within a matter of days, these examples of life-threating chronic and acute pathologies highlight the importance of translational research quantifying systems and targets associated with acute secondary injury cascades following the primary mTBI.

1.4 Cognitive and Behavioural Implications of TBI

TBI is a complex pathology due to the overlapping nature of persistent symptoms. The complex onset of neurocognitive, behavioural, and motor changes resulting from the intricate neurobiological second injury cascade have a profound long-term impact on the relationships, mental health, and independence of severely injured individuals (71). Acutely, in the first hours to days following the injury, all but the mildest TBIs are associated with short term memory gaps immediately following injury, referred to as PTA. PTA manifests as temporary periods of confusion and amnesia accompanied by agitation; disturbances in sleep/wake patterns, arousal, and general disorientation (72). Seventy percent of patients suffering from mTBI are expected to make a full recovery within 3-6 months post-injury (12). However, in a study of 375 emergency room confirmed mTBI cases, over 82% of those assessed were plagued by persistent neurophysiological symptoms 1 year post-injury (73). These persistent symptoms (months to years) are known as Post-Concussion Symptoms (PCS), and are characterized by headaches, confusion, sleep difficulties, nausea, decreased cognitive capacity and memory, dizziness, and gait changes (8, 29). Persistent symptoms are omnipresent in moderate to severe TBI, where basic functional recovery stretches for 3-12 months. For this reason, data in more severe cases of TBI typically focus on lifelong impact of injury, where upwards of 65% of patients report persistent cognitive, psychiatric, and behavioral dysfunction for years following TBI (74).

Symptoms of both short-term behavioural and cognitive impairments, as well as TBI-induced neurodegeneration, largely result from alterations in executive function due to direct injury to the frontal lobes, to changes in system connectivity resulting from diffuse axonal damage, or to a combination of the 2 (75). According to an analysis of lateral impact versus dorsal impact methods for mTBI by Mychasiuk et al. 2016, despite a similar injury severity and acute neurological symptoms, the location and rotation of impact resulted in significantly different emotional and cognitive function between groups (76). These results demonstrate that injuries implicating different white matter tracks may contribute to variation in lasting cognitive deficits following injury. It is of note that chronic neuromotor dysfunction is a generally a function of injury severity and is therefore more prominent and long-lasting in severe TBI, which is not the current focus of this research. Typical behavioural changes associated with mild to severe TBI are characterized by decreases in attention and concentration, decreased processing speed, increased impulsivity, irritability and aggression, variable judgment, and changes in memory acquisition and mood (77,

78). Both acute and chronic symptoms are aggravated in the presence of co-morbidities, such as pre-existing diagnosed or undiagnosed psychiatric conditions, migraines, history of previous TBI, as well as concomitant injuries sustained in conjunction with the TBI (8). Age and sex can also impact the development of acute and chronic symptoms (8). Research indicates that various aspects of acute injury pathophysiology play a role in the manifestation of cognitive and behavioural changes. More and more, acute (less than 3 months) and persistent (over 6 months) cognitive, behavioural, and neuromotor complications have been associated with an inflammation-induced neurodegenerative sequela (33,79). Of note, Shultz et al. demonstrated in a 2012 study that as much as 1 sub-concussive injury increases markers of neuro-inflammation without remarkable behavioural dysregulation (80). This suggests that a less-than-clinical injury can easily contribute to neurodegenerative damage. In another rodent model of mTBI, cognitive and behavioural discrepancies were ameliorated 7-days post injury by interrupting synthesis of inflammatory cytokines such as TNFα (81), further demonstrating the interplay between inflammation and behaviour changes. The diagnosis of TBI-associated neurodegenerative pathologies, such as Alzheimer's disease and CTE, are frequently diagnosed post-mortem in individuals with a history of mild repetitive neurotrauma such as uncontrolled epilepsy, physical abuse, and repeat mTBI in sport (61, 82), as well as after a single moderate to severe TBI (83). Importantly, a review by McInnes et al. (2017) examining short and long-term cognitive function in individuals with a single mTBI determined that 55% of study participants assessed experienced cognitive impairments lasting upwards of 1-year post-injury, which further highlights the indiscriminatory impact of a single mTBI on overall health (84).

Due to the unpredictability of injury severity and underlying comorbidities, the extent of cognitive and behavioral deficits present in the early stages of recovery are difficult to predict and encompass a poorly researched area of traumatic brain injury. Due to the associated changes in behavior, cognitive function, and mood seen in TBI, animal models serve as a functional way to assess biomechanical impact on behavioral outcomes, such as anxiety- and depression-like behaviour, sociability, motor function, and memory (85). Using targeted behavioural analysis paired with appropriate animal models replicating human injury mechanisms is an important way to further elucidate complex lasting impacts of the spectrum of TBIs.

1.5 Animal Models of TBI

Animal models serve as a bridge to examine TBI severity and complexity by creating various models of injury mechanisms to explore resulting pathophysiology and behavioural changes. Despite our best efforts, not all animal models of TBI remain equal. Notably, there remains a disconnect between injury severity in humans and animals because the common means of quantifying injury severity in humans, such as injury-associated somatic, cognitive, and emotional symptoms are unavailable in rodent models. Furthermore, use of anesthetics and analgesics post-injury can alter symptom manifestation, leaving judgment of severity largely to physiology, histopathology, and to measures of rodent behaviour, which are themselves imperfect. According to a review by Bodnar et al. (2019), the most commonly used models of TBI are the focal, diffuse, and mixed model injuries; and choice of model largely depends on what is being examined (86). Focal injuries, named from the latin word, *focus,* indicate an injury resulting from direct impact to a specific region of the skull causing potential fracture and significant contusions and bleeding (87). A focal injury is frequently performed on an open head, meaning the animal undergoes surgery to expose the skull or dura to which a weight is dropped or a pressurized impact, also known as a Controlled Cortical Impact (CCI) is then administered to the focalized site of injury. A focalized injury may be administered on a fixed head or unfixed and is most commonly used to explore the injury pathology to further understand the secondary mechanism of injury (88).

Diffuse injuries on the other hand can occur without any direct contact to the head, and are used to emulate the shearing forces due to acceleration and deceleration of the brain commonly responsible for behavioural phenotypes of mTBI (89). The injury incurred by this type of mechanism is highly variable, and therefore mimics some of the variability seen in human motor vehicle accidents, sports injuries, and blast injuries (90). In pre-clinical research, due to a lack of homogeneity between diffuse injury mechanisms, diffuse injuries are best emulated with non-penetrating blast wave models, such as fluid percussion, emulating injuries emerging from military engagement, and therefore require specialized equipment (89).

Most commonly, mTBI etiology results from a combination of both focal and diffuse injury mechanisms, defined in pre-clinical research as a mixed-model injury. The mixed model injury is administered via variations of the weight drop model as well as CCI where animals are placed on foam or allowed to rotate or free fall following the weight drop or CCI (89, 76). Helmet-like devices

are occasionally used to increase or decrease the injury severity and modify the ratio of focal to diffuse injury type (90).

Regarding injury severity, a significant amount of the research available on rodent models of TBI has been centered around the extremes of the injury spectrum (severe and mild), leaving to question the investigation of moderate TBI. I highlight the variation because according to a 2019 systematic review of closed head mild TBI by Bodnar et al. (86), the largest proportion of rodent models of mild TBI used was the weight drop, with the majority of these mimicking the classic Marmarou et al. (1994) model which uses a 450-500 g weight dropped from a 1-2 m height (91). The Marmarou method cites intubation as part of the procedure or else the death toll in rats subjected to the injury ≅ 50% (91), symptoms rarely seen in clinical mTBI. Upon closer examination of other adapted mTBI weight drop models featured in this review, weight drops as low as 10 g and as high as 1,600 g in rats with or without the use of protective head gear were considered "mild" (86). This disparity in weight drop models only highlights the need for widespread consensus on models of sub-concussive, mild to severe, and repeat injury between weight drop and other more complex mixed method injury models. Although I have highlighted gaps in research pertaining to symmetry between injury methods, this is once again not the goal of this project; yet still more critical when actively contributing to the growing body of knowledge on this topic.

For this reason, consensus and universal use of functional and uniform mixed models of brain injury that are easily replicable and encompass the diffuse axonal injuries present in rotational and acceleration injuries akin to sports and motor vehicle collisions, in addition to focal injury characteristics of direct head trauma, are important when examining behavioural sequelae following mild-moderate injury.

1.6 The Endocannabinoid System

Research into the ECS entered mainstream biomedical research in 1964 with the discovery of THC, and has since gained popularity within the research community at an exponential rate despite its longstanding controversial nature. As previously noted, the ECS is a system within the human body that regulates numerous critical physiological and behavioural functions. More specifically, the ECS actively participates in potentiating synaptic plasticity and neurogenesis and when dysregulated, it has been associated with numerous neurodegenerative and psychiatric conditions such as Huntington's disease, Parkinson's disease, and mood disorders such as schizophrenia (92, 103). The biological importance of the ECS is further amplified by its presence and modulating capacity in all vertebrates (93). Much like other commonly known systems, the ECS consists of endogenous ligands, and anabolic and catabolic ligand-specific enzymes and receptors.

The two main receptors within the ECS are CB1R and the CB2R (94). These 2 receptors are GPCRs, and, like other GPCRs, are composed of 7 transmembrane α-helices and play a critical role in initiating downstream signalling of hormones and neurotransmitters (95). They are considered the main receptors of the ECS and are both activated by endogenous and exogenous cannabinoids.

CB1R is one of the most common GPCRs within the CNS (96) and is largely expressed in the CNS, on neurons, with its highest concentrations in the cerebral cortex, striatum, hippocampus, and cerebellum (15, 97). These are all areas that are highly implicated in TBI-related mood and motor deficits. The activity of CB1R depends on the cell type (excitatory or inhibitory), as well as the location on the cell. Importantly, CB1R participates in the activation and deactivation of numerous critical cell signalling pathways associated with cellular communication, proliferation, and apoptosis such as MAP kinase phosphorylation, cAMP inhibition, Ca^{2+} channel inhibition, and inflammatory pathways via NO and arachidonic acid (AA) (15, 98, 99). Its activation is considered neuroprotective because it dynamically depresses glutamatergic excitotoxicity (100), which in turn slows the production of RONS and associated cell death (71).

CB2Rs, on the other hand, are found primarily in the periphery located on virtually all types of immune cells, in both the innate and adaptive immune system (macrophages, monocytes, and T and B cells), as well as on microglia in the CNS, and to a lesser degree on neurons (101, 102).

Importantly, CB2R activity is upregulated in the presence of inflammation and chronic neurological diseases (15, 101, 13). According to a review of CB2R pharmacology, conducted by Turcotte et al. (2016), agonists of CB2R consistently produce anti-inflammatory downstream signalling, mainly through the modulation of leukocytes (98). In a TBI-specific example of CB2R mediated anti-inflammatory effects, Amenta et al. (2014) (104) demonstrated that both CB2R knockout mice and those treated with CB2R antagonist exhibited significantly higher levels of TNFα, inducible NO synthase (iNOS), and intracellular adhesion molecules-1 (ICAM-1). iNOS is an enzyme present only during inflammatory episodes associated with free radical accumulation during TBI, and ICAM-1 is a protein responsible for enhanced endothelial permeability enabling leucocyte migration across the BBB resulting in subsequent allocation of edema into the brain and signaled by TNF-α (105). Similarly, when fetal astrocytes were treated with WIN55,212-2, a synthetic CB1R and CB2R agonist, iNOS and associated NO production was interrupted in addition to the inhibition of TNF-α and a number of inflammatory chemokines (106). The above research indicates that both CB1R and CB2R possess unique and intimate modulatory roles in the maintenance of homeostatic control in the face of inflammatory insult.

Of the 15 known endogenous compounds synthesized on demand to activate the ECS, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the most abundant and well-studied (107). Both AEA and 2-AG, in addition to a few lesser known endocannbionids, (14) are known to work on CB1R and CB2R, in addition to other non-cannabinoid-based receptors such as non-cannabinoid GPCRs, transient receptor potential channels (TRP), ionotropic receptors, and peroxisome proliferator activated receptors (PPAR) (14). The primary pharmacological effects of the cannabinoids are thought to be on the GPCRs CB1R and CB2R.

At homeostasis in the CNS, 2-AG is found in higher concentrations than AEA, and acts as a full agonist at both CB1R and CB2R (108). AEA, on the other hand, acts as a partial agonist at CB1R and to a lesser extent at CB2R (109). AEA anabolism is complex but it is largely synthesized by N-arachidonoyl phosphatidyl ethanolamine-specific phospholipase D (NAPE-PLD) (110), and catabolized by fatty acid amide hydrolase (FAAH) (111). 2-AG is synthesized from arachidonoyl-containing phosphatidyl inositol bis-phosphate (PIP2) by diacylglycerol lipase (DAGL), and is catabolized by enzymes monoacylglycerol lipase (MAGL) and ABHD6/12 (112, 113). Notably, 2-AG acts as a precursor to arachidonic acid (AA), which is important for prostaglandin synthesis

and thus critical for inflammatory pathways (COX-1 and COX-2) (114). CB1R resides on the pre-synaptic membrane of neurons, and its endogenous ligands travel in a retrograde direction from post-synaptic membrane to pre-synaptic membrane to suppress pre-synaptic neurotransmitter release in a stimulus-response-dependent fashion (Figure 1-2)(71). Both of these endocannabinoids are manufactured on demand according to intracellular levels of Ca^{2+} (115), which is indicative of their ability to modulate neuronal activity.

Another important part of the ECS and its function are the exogenous cannabinoids that interact with the system. Over 120 unique phytocannabinoids have been identified from the *Cannabis sativa* plant (116), with the main 2 constituents being THC and cannabidiol (CBD). THC acts as the main psychoactive component of *Cannabis*, and much like AEA, acts as partial agonist at the CB1R and CB2R receptors. It therefore possesses both potential neuroprotective activity at CB1R, and the ability to subsequently mediate excitatory and inhibitory neurotransmissions (117) and anti-inflammatory activity at CB2R by regulating inflammatory cytokine release and movement of immune cells in the CNS (103, 118). CBD, on the other hand, is less well understood in its action and does not possess psychoactive properties. CBD has been shown to act as a possible antagonist or negative allosteric modulator at CB1R, and as a partial agonist at CB2R (119-121). Evidence also suggests that the bulk of CBD's activity is seen at non-cannabinoid receptors (98), further contributing to the complexity of the ECS. The ubiquity and importance that the ECS, its endogenous ligands, exogenous cannabinoids, and non-cannabinoid-based receptors makes this system a promising target for complex disorders such as TBI that impact numerous physiological systems.

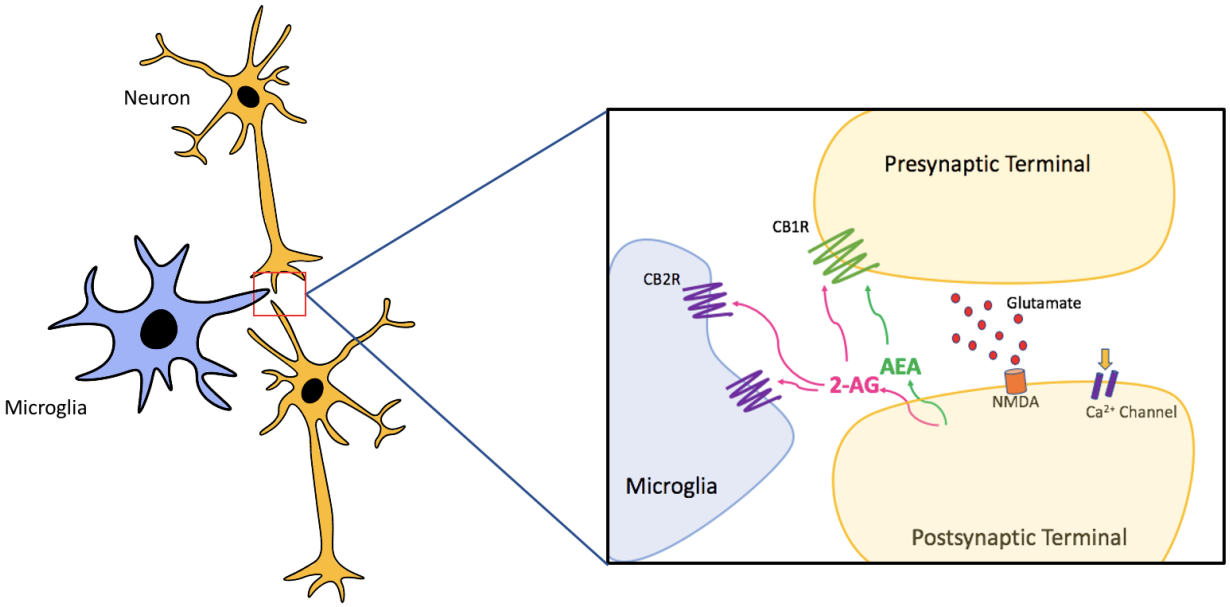

Figure 1-2. Snapshot of Retrograde Signaling of the CB1R and CB2R. A visualization of endocannabinoid action at CB1R and CB2R in microglia and neurons. Figure created by author, using information from (207).

1.7 The Endocannabinoid System In TBI

The complex pathophysiology of TBI and the overlap of the ECS present an interesting intersection in the exploration of pharmacological interventions, but until this point, all investigation into the ECS and TBI has remained pre-clinical in nature. It is established that in a healthy state, 2-AG is present in the CNS in 2 to 3 times higher concentrations than AEA (122) and in a TBI state of glutamatergic excitotoxicity, CNS concentrations of both AEA (123) and 2-AG (124-126) increase over the following hours to days post-injury. This suggests that the ECS acts as a self-regulatory mechanism to reduce Ca^{2+} flux and decrease accumulation of RONS (127, 128). Research into the inhibition of FAAH, MAGL, and ABHD6 to increase endogenous AEA and 2-AG has been linked to decreased inflammatory cytokine production, microglial activation, BBB breakdown, and improvements in behavioural measures (Figure 1-3) (123, 128-130). Administration of other lesser known endocannabinoids such as, *N*-arachidonoyl-L-serine (131), palmitoylethanolamide (132) promote neurogenesis and vascular recovery, respectively, post TBI. Early research examining the neuroprotective properties of the exogenous cannabinoid THC at 1 mg/kg *i.p*. and AEA transport inhibitor AM404 at 2 mg/kg *i.p*. administered 5 min after a carotid occlusion ischemic injury protected gerbils from any damaging effects of the injury, according to behavioural and EEG measures (133). A study by Belardo et al. (2019), 1 of the few publications available exploring the therapeutic action of phytocannabininoids in TBI, found that CBD (30 µL with 10% CBD), administered orally, partially recovered behavioural, neurological and biochemical deficit post mTBI in mice, but failed to indicate glutamate levels (134). Another study by Amenta et al. (2012) demonstrated that the treatment of murine models of TBI with the CB2R agonist O-1966 decreased BBB disruption and neuronal damage, and improved behaviour when compared to vehicle control (135). A 2020 publication by Bhatt et al. indicated that the repeat administration of 1.25 mg/kg *i.p.* THC after repeated mild TBI via a 50 g lateral impact device led to partially recovered anxiety- and depression-like measures and deficits in working memory, but did not when THC was administered pre-injury (136). It is clear that more research is necessary despite the pre-clinical research available exploring the therapeutic possibilities of endocannabinoids, synthetic cannabinoids, and inhibition of catabolic enzymes, as well as the limited research available to indicate the efficacy of exogenous plant cannabinoids – such as the CB1R and CB2R partial agonist THC – as a therapeutic intervention (71).

Another issue for consideration is the ever-growing presence of anecdotal evidence touted in the media for the therapeutic effects of phytocannabinoids. Advertisers are appealing to professional and recreational athletes alike for the use of cannabis and cannabinioids for anything from sleep enhancement, to post-training recovery, to supposed performance enhancement, despite an astounding lack of data. One of the few limited sources of information on potential clinical implications of cannabinoids is indicated in a 3-year retrospective study at the UCLA medical center by Nguyen et al. (2014), which explores the correlation between injury mortality and THC concentrations in urine. They found that in TBI patients, those who screened positively on urine toxicology for THC had a decreased mortality in comparison to patients who screened negatively (137). Although there is a lack of research on the topic, this is an area of science that requires investigation due to the potential utility and harms associated with cannabinoid use post-TBI.

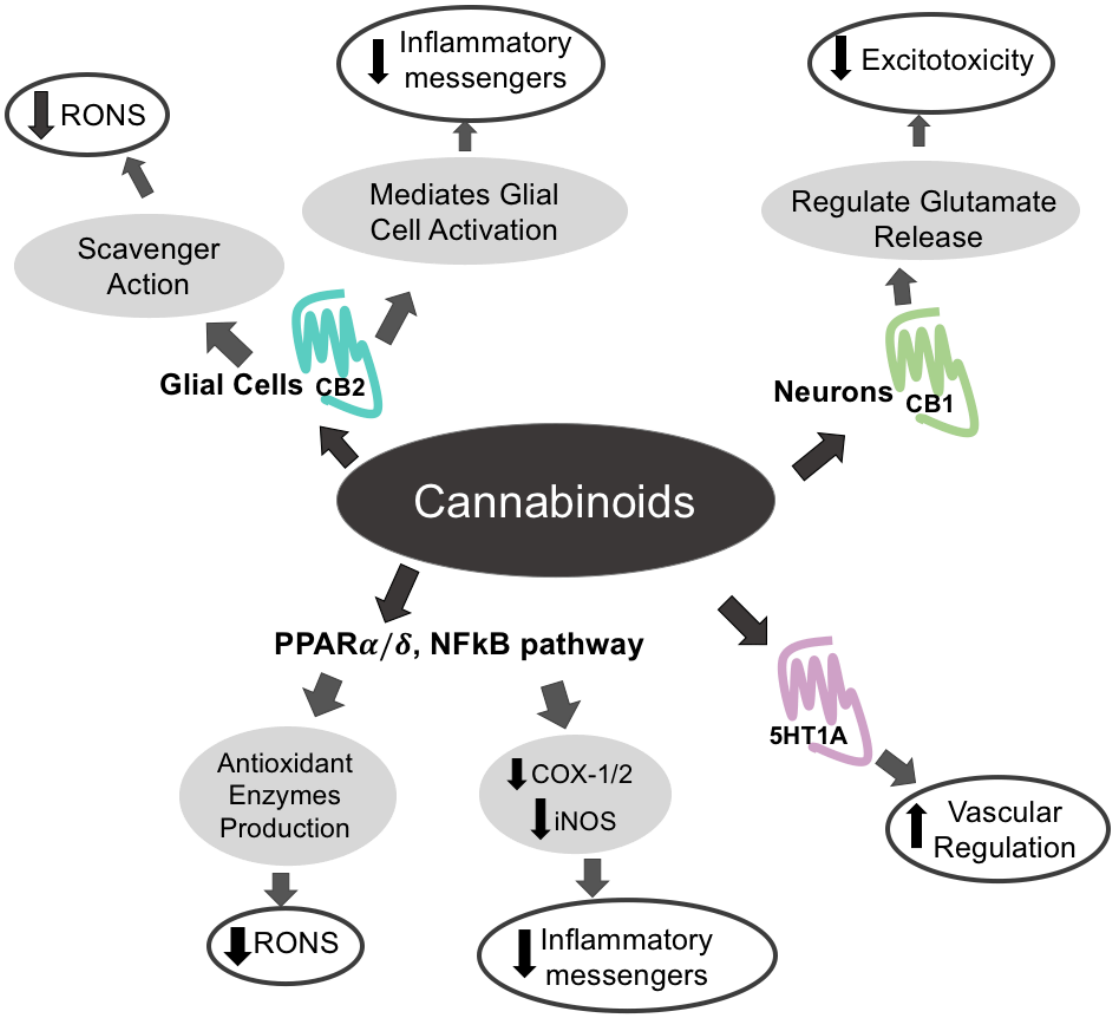

Figure 1-3. Cannabinoids in TBI. Summary of cellular and molecular pathways by which cannabinoids exhibit pharmacological therapeutic potential in TBI induced secondary injury cascade. Figure created by author, using information from (192).

1.8 Hypothesis

According to previous research, TBI treatments have been split into neuroprotective strategies, which are aimed at decreasing the secondary injury and the associated neuronal cell death; or neurorestorative strategies, which focus on neurovascular remodeling, as well as neurogenesis, oligodendrogenesis, and dendrite and axon extension (13). Recent preclinical research indicates that the ECS and exogenous cannabinoids possess numerous promising modulatory pathways to intervening in the secondary injury cascade of TBI (138, 71).

Past research has shown that following TBI: (i) the endocannabinoids AEA and 2-AG are significantly upregulated (139, 124); (ii) levels of the excitatory neurotransmitter glutamate increase whereas levels of the inhibitory neurotransmitter GABA decrease (140); (iii) CB1R and CB2R are upregulated (141, 142); and (iv) inflammatory cytokine production increases (27). Prior research in rodent models of TBI has shown a significant decrease in exercise capacity, motor coordination, stamina, grip strength, balance, working memory, and spatial learning (143); as well as increased anxiety compared to healthy controls (144). THC is a partial agonist of CB1R and CB2R. Activation of these receptors via THC reduces glutamate release, cytokine production, spasticity, and anxiety (16). Based on our pre-existing understanding of the ECS, TBI, and THC, **the hypothesis of this study is that treatment of rats subjected to TBI with THC post-injury will improve higher level locomotor function and improve behavioral profiles of injured rats.**

Chapter 2 Materials & Methods

2.1. Animal Care

Sixty-three Sprague-Dawley rats of both sexes, approximately 8-12 weeks old at the time of experiment, were obtained from Charles Rivers Laboratories Inc. (Senneville, QC, Canada). Rats were chosen as an adequate translational model because the human and rat CB1R and CB2R respectively hold 97% and 93% amino acid congruency (15). Rats were housed 2 per cage, in same-sex pairs with appropriate environmental enrichment, bedding materials, and *ad libitum* access to food and water. Animals were maintained on a 12 h light and dark cycle established by the Lab Animal Service Unit (LASU) at the University of Saskatchewan. Following the completion of *in vivo* data collection, all rats were euthanized by overdose of isofluorane (5%) followed by decapitation. Whole brains were collected and stored at -80 C.

All procedures and protocols described below were performed with approval from the of University Animal Care Committee (UACC) and Scientific Merit Review Committee for Animal-Based Research (SMRCABR) and are in keeping with the guidelines of the Canadian Council on Animal Care (CCAC) and the ARRIVE guidelines.

2.1.1 Terminal endpoints

During housing and interventions described above, any animal was removed from the experiment if they qualified for any 3 of the possible terminal endpoints. These terminal endpoints include animals who appeared pre-moribund; experienced weight loss exceeding 15%; showed a decrease in body temperature greater than 6°C; or had any ulceration, bleeding, self-mutilation, skull fractures, or severe and prolonged lethargy or stress in addition to any severe reactions such as unanticipated seizure activity, depression, or pre-comatose state. Over the course of this project, 2 animals were removed from the experiment due to the meeting terminal criterion.

2.2 TBI Protocol

Based on extensive analysis of previous research, in accordance with results from Bodnar et al. 2019, as well as previous research by Mychasiuk et al. 2014, a modified weight drop model with additional PPE was chosen for this experiment due to its ability to produce, at low cost, a reliable and reproducible injury, producing similar behavioural deficits characterized by other more complex pre-clinical models (76, 89, 90).

Prior to TBI, rats were habituated to the procedure room for 15 min. Rats were anesthetized (3% Isofluorane) and subjected to a modified closed head impact to simulate motor vehicle and sports induced TBI. Isofluorane 3% was the approved anesthetic for use by the animal care committee and is frequently used in close head TBI models (145). This TBI model has been adapted from Mychasiuk et al. (2014), and Qin et al. (2018), whose injury models marry focal and diffuse injury patterns associated with TBI (90, 146, 147), thereby replicating a functional model where contact forces, inertia forces and rotational acceleration are combined (24) (Figure 2-1).

Set up of the TBI apparatus required a metal guide tube (15 mm inner diameter x 1.25 m height), a ring stand, a clamp, a weight with metal ring at the top, parachute cord (2 mm), a metal key ring, and a metal pin. A weight, 300 g total (comprised of 1 detachable 100 g weight and 4 detachable 50 g segments with a total length at 248.5 mm long) was designed and fabricated by the Engineering Shops, University of Saskatchewan. A hole was drilled into the metal guide tube using a drill press, 248.5 mm from the top of the guide tube, where a metal holding pin was inserted to suspend the 300 g weight. One and one-half metre of parachute cord was attached to the metal loop of the weight and the metal key ring was attached to the end of the cord. In order to produce the accelerative and rotational forces that often accompany sports- and vehicular-related TBI, rats were suspended on a sheet of aluminum foil that had been taped to the top of a clear plastic box (38 x 25 x 35 cm^3) positioned below the TBI apparatus and scored horizontally four times with a razor. Placed within the box was a foam collection sponge (38 x 25 x 10 cm^3). According to previous models, the use of aluminum foil to suspend the rat allows for it to rotate 180 degrees following the impact of the weight being dropped, producing a more functional mechanism of injury.

In order to elicit a TBI, 1 rat at a time was placed in a sealed transparent box and anesthetized with 1.5 L/min oxygen and isoflurane (3%) until their pedal reflex was no longer responsive. Following

anesthesia, the rat was briefly transferred to a head cone and heating pad where the dorsal part of their cranium was shaved, and a stainless-steel disc ("helmet") (10 mm x 2 mm) was fixed with dental cement on the sagittal mid-line between the inter- aural line and bregma to indicate the impact site and protect the skull demonstrated in Figure 2-2. Once the helmet was securely fastened, the rat was quickly transferred to the aluminum foil, and placed in the prone position with their helmet positioned below the guide tube. At this point, the animals ceased to receive anesthetic. In total, anesthetic duration was < 15 min for all animals except for one (< 30 min) due to a fire alarm. The weight was then dropped to produce a TBI. Following impact, the rat was transported to a recovery cage placed on top of a heating pad with a pulse oximeter, in order to monitor heart rate and blood oxygen content until the rat regained consciousness. Heart rate and blood oxygen were monitored directly before and after injury (Appendices 1-3) and the day before the procedure rats were weighed (Appendix 4).

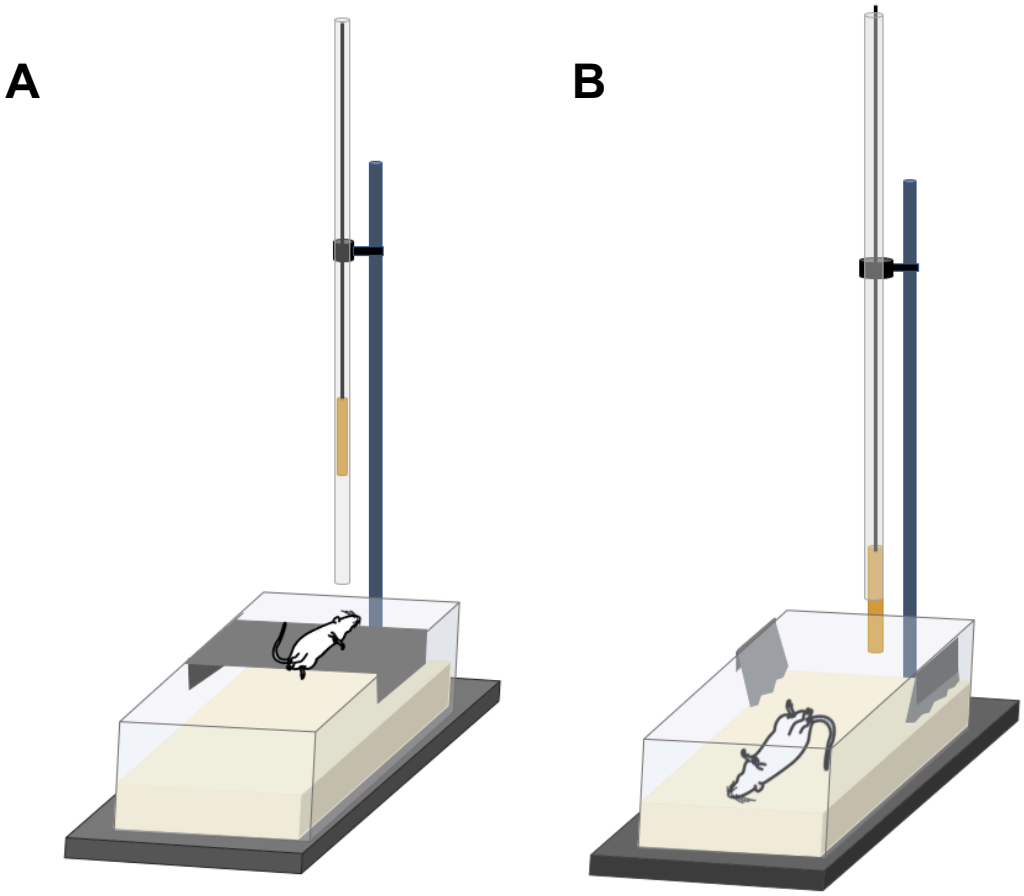

Figure 2-1. Modified Weight Drop Model. Graphical representation of mixed injury model used to emulate a combination of diffuse and focal injuries characteristics commonly seen in sports-related and motor vehicle accidents. Figure created by author in reference to model adapted from Mychasiuk et al. 2016 (147).

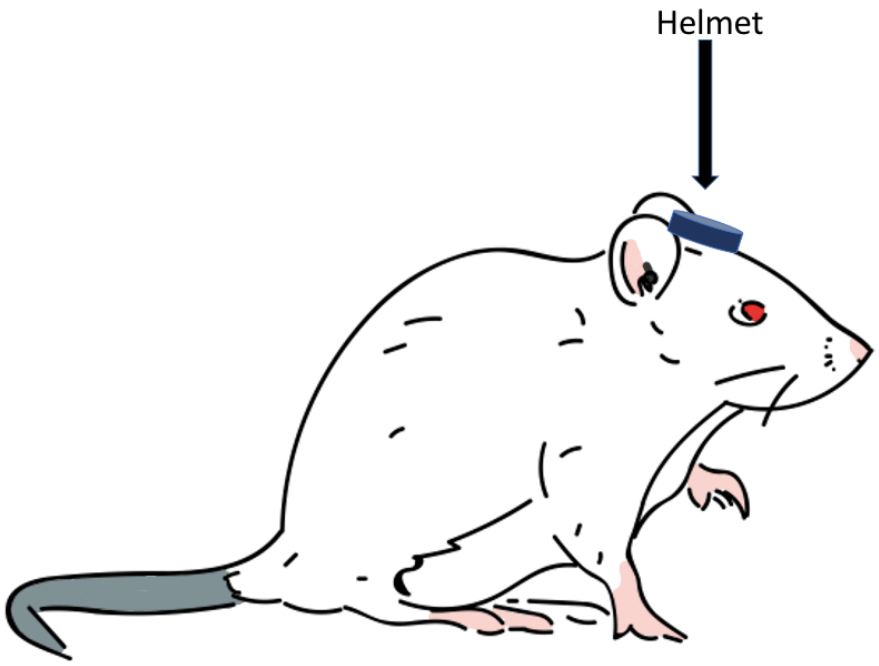

Figure 2-2. Protective Stainless-Steel Disc. A helmet-like structure used to diffuse the 300 g impact to create an injury model more closely resembling a moderate TBI. Figure created by author.

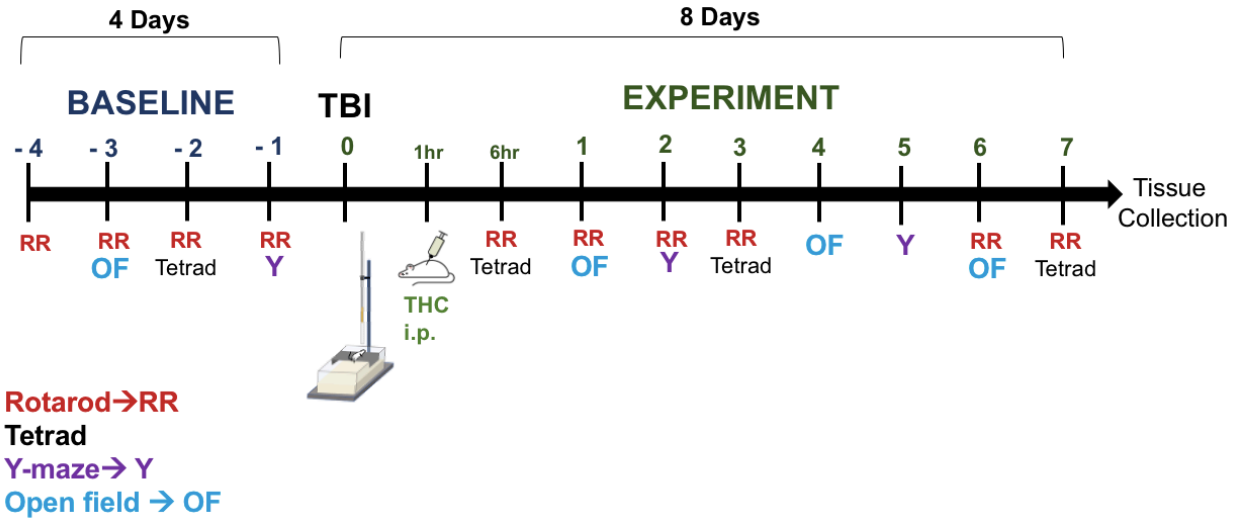

Figure 2-3. Experimental Timeline. This timeline illustrates both baseline measure collections (day -4 to -1), Injury and THC Injection date (Day 0), as well as the following 7 days of post-injury data collection (day 1-7).

2.3 The Rotarod

The rotarod is a test used to quantify vestibulo-motor function in rats following TBI by testing grip strength, coordination, and balance of the animal. The Rotarod (ROTO-ROD, Series 8 IITC Life Science) is a motorized cylindrical rod that rotates at varying speeds, as demonstrated in Figure 2-4. Rats were pre-trained on the rotarod for four days prior to TBI (Day -4 to -1) and tested on days 0, 1, 2, 3, 6 and 7 following the TBI on day 0 (see Figure 2-3). Animals were habituated to the treatment room and instrument for 3 days prior to data collection. Rats were allowed to habituate in a neighbouring room for a minimum of 10 min, and to habituate in the rotarod room for 5 min pre-rotarod. Rotation was turned on with a smooth acceleration (2 rpm/5 sec) beginning from 4 rpm to a maximum of 36 rpm (achieved in 1 min 20 sec and maintained for the duration of the trial). The latency to fall was measured in sec to a maximum trial time of 5 min. Each animal was repeated 3 times with a 5 min rest between trials. The mean of all 3 trials was used for each rat. The rotarod used was thoroughly cleaned before and after use with the general virucide disinfectant PerCept$^{TM/MC}$RTU. Females were tested first, followed by males. Between sexes, the room was swept, cleaned, and allowed a 10 min airing-out period before male rats were brought in to habituate.

Figure 2-4. The Rotarod. Graphical demonstration of the rotarod enclosure, comprised of an elevated rotating beam, used to measure strength, balance, endurance and coordination following injury.

2.4 Tetrad

The tetrad is a collection of 4 different tasks used to determine cannabinoid-based drug effect in the CNS as cannabinoids are known to effect voluntary movement, body temperature, nociception and fear-based behaviour in rodents (148). Male and female rats were assigned to volume matched *i.p*. injections of 1 or 10 mg/kg THC or Vehicle as 1:1:18 (ethanol: emulphor: saline) according to previous randomization. One hour post TBI, rats were injected and tetrad tests – with the exception of the open field described below – were performed following a previously described protocol (149), at 6 h, 3 days and 7 days post-injection, prior to rotarod, to determine whether there was any indication of lasting cannabinoid receptor-mediated effects Animals were habituated to the treatment room and instruments for 3 days prior to data collection. Prior to data collection, rats were habituated in a neighboring room for minimum of 10 min prior to a 5 min procedure room habituation. It should be noted that CB1R activation commonly produces a hypolocomotive response (150). Therefore, the tetrad was also selected in order to verify that no significant drug effects were confounding the locomotor performance of the rats (151).

2.4.1 Catalepsy

To test catalepsy, rats were placed such that their forepaws rested on a 0.7 cm diameter bar 4.5 cm above a clean table. The amount of time spent holding the bar was recorded up to a 60 sec threshold. Time was stopped when the rat moved off of the bar or when the rat moved their head to the left or the right (Figure 2-5). Three trials were performed on each measurement day, and the mean was collected.

2.4.2 Body Temperature

Body temperature was measured with a rectal thermometer (Thermalert TH-5, Physitemp^{TM}) 10 min after catalepsy was tested. Vaseline^{TM} was used to facilitate insertion of probe to the appropriate depth. Once a stable temperature recording was collected, the probe was removed and cleansed using 70% ethanol

2.4.3 Tail Flick

Anti-nociception was measured 15 min after catalepsy using the Tail Flick Analgesia Meter (Series 8, IITC Life Science). Rats were wrapped in a towel and positioned such that the heat light shone at 5 cm from the base of the tail. Time to tail removal was recorded up to a 20 s threshold (Figure 2-6).

2.4.4. Open Field Test (OFT):

The OFT is a measure of locomotion activity and used to interpret levels of anxiety by making use of rodents' aversion to open environments (90). This test is generally performed directly after the tail flick, but due to equipment scheduling as well as the OFT being a go-to measure of fear-based behaviour in TBI, OFT measures were collected separately from the other tetrad measures, but analyzed similarly. OFT baseline was collected on day -1 and experimental data was collected on days 1, 4, and 6 following TBI, as indicated in Figure 2-7. Animals were habituated to the treatment room for 2 days prior to data collection. Prior to data collection, all rats were habituated in a neighboring room for a minimum of 10 min before being transferred into the OFT room for 5 min of habituation before OFT. The OF chamber (diameter 1.5 m, inner area diameter 1 m) was swept and thoroughly cleaned with 40% ethanol by sponge and allowed to air-dry before and after each animal. Four floor lamps were turned on, and overhead lights were dimmed. Rats were placed in the open field for 10 min, and total movement was recorded using a video camera suspended from the roof. Locomotor movement was tracked using Ethovision XT (Noldus). Female rats were tested first, followed by male rats. Between sexes, the room was swept, cleaned and allowed a 10 min airing-out period before male rats were brought into habituate.

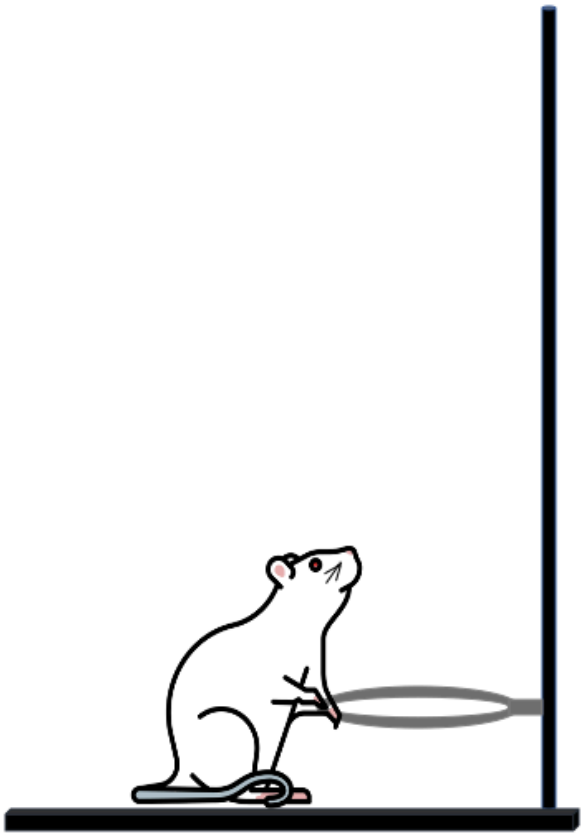

Figure 2-5. Catalepsy. The bar hold test is used to functionally assess cannabinoid-mediated effect in the CNS.

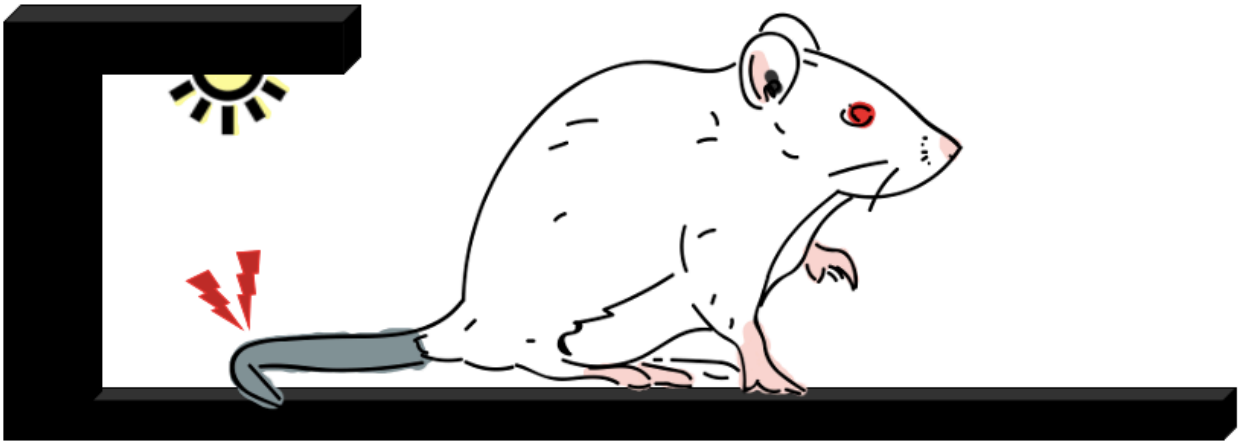

Figure 2-6. Tail Flick. This test is used to measure the impact of cannabinoids on spinal-mediated analgesia. Figure created by author.

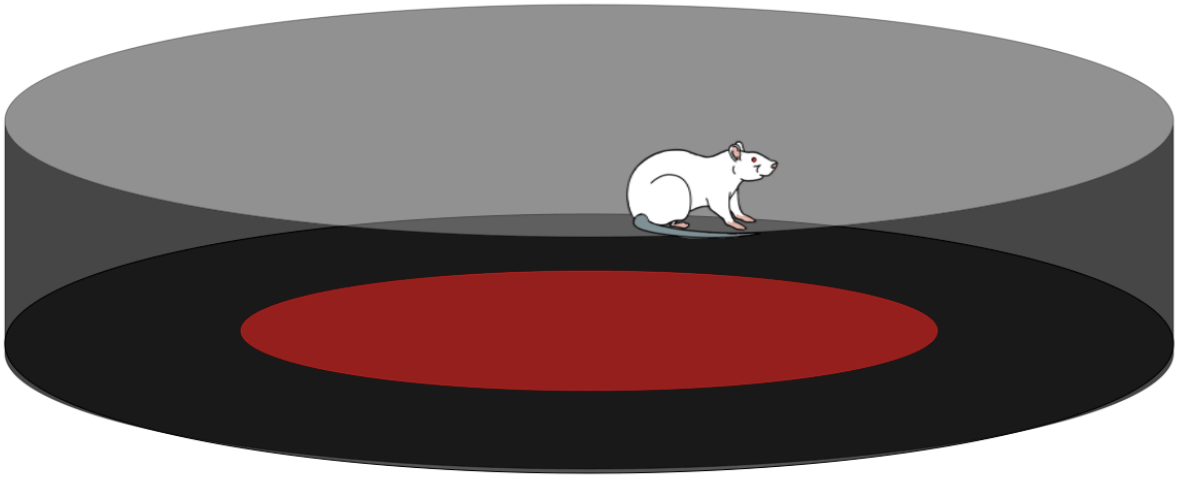

Figure 2-7. Open Field. A test used to quantify locomotive and associated fear-based behavioural phenotypes in rodents. Rodents have a natural tendency to explore novel spaces and will preferentially stick to the parameter of an enclosure to stay hidden. Time spent in the center and total locomotion can give indication of anxiety-like behaviour of a rodent. Increased time spent in the center and lower locomotor activity indicate a decrease in anxiety-like behavior. Figure created by author.

2.5 Y-Maze

Y-maze is a preliminary tool frequently used to detect overt changes in working and spatial memory (152) (Figure 2-8). This test is based on assumptions made about exploratory behavior of rodents exposed to a novel habitat. When placed in the Y-maze, a healthy rodent will normally explore the least explored area, resulting in an increased alternation ratio between arms of the Y-maze (153). The Y-maze consists of 3 arms equally separated at 120° from one another. Animals were habituated to the treatment room for 1 day prior to data collection. All equipment was cleaned with 70% ethanol before and after each animal and allowed to air dry to mitigate odors between sessions. Female rats were trained and tested before male rats. Equipment was cleaned and room was allowed to air out for 10 min between sexes. A baseline measure was established on day -1 pre-TBI. Rats are put into a different arm for each recording session as per the established timeline (Figure 2-3) and recorded for 8 min. Videos were recorded by a video camera suspended from the roof. Videos were hand scored and the percentage of spontaneous alternations as well as the number of entries into each arm were calculated. The percentage of alternation is equal to the number of correct alternations demonstrated in figure 11A, divided by the total number of entries multiplied by 100. Videos were also analyzed to obtain total locomotion using Ethovision XT (Noldus). The entry arm was not counted in the total number of entries (153).

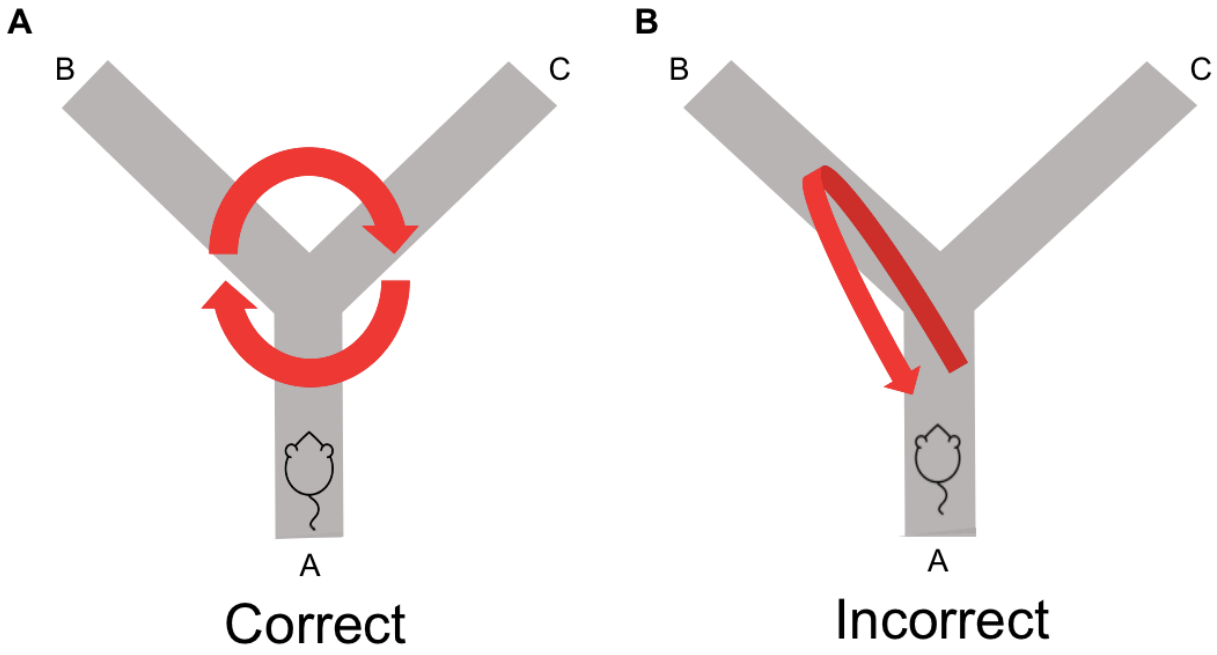

Figure 2-8. Y-maze Spontaneous Alternations. Rodents have a natural inclination to explore novel environments. The Y-maze is used to detect overt changes in rodent working memory.

2.6 Experimental Design

2.6.1 TBI Method Validation: Pilot Experiment and CT Scans

A pilot study (n=6) was conducted first for experimental calibration using the protocol listed above with conditions of 300 g dropped from 1 m. Animals were euthanized with isoflurane (5%) and assessed for cranial microfracture by computerized tomography (CT) at the cyclotron facility (Fedoruk Center, University of Saskatchewan). CT images were acquired using the VECTor^{4}CT (MILabs), with a source of voltage of 55 kV and a current of 0.27 mA with a scan angle set to full and scan mode accurate. Reconstruction of each scan was performed using PMOD V3.703, and 3D images were rendered using AMIRA (2019.1). According to the images collected, there were no signs of overt microfractures occurring at 300 g x 1 m, indicating the magnitude of injury sustained by the animals was not greater than a moderate TBI, as intended for our rodent model (Figure 2-9).

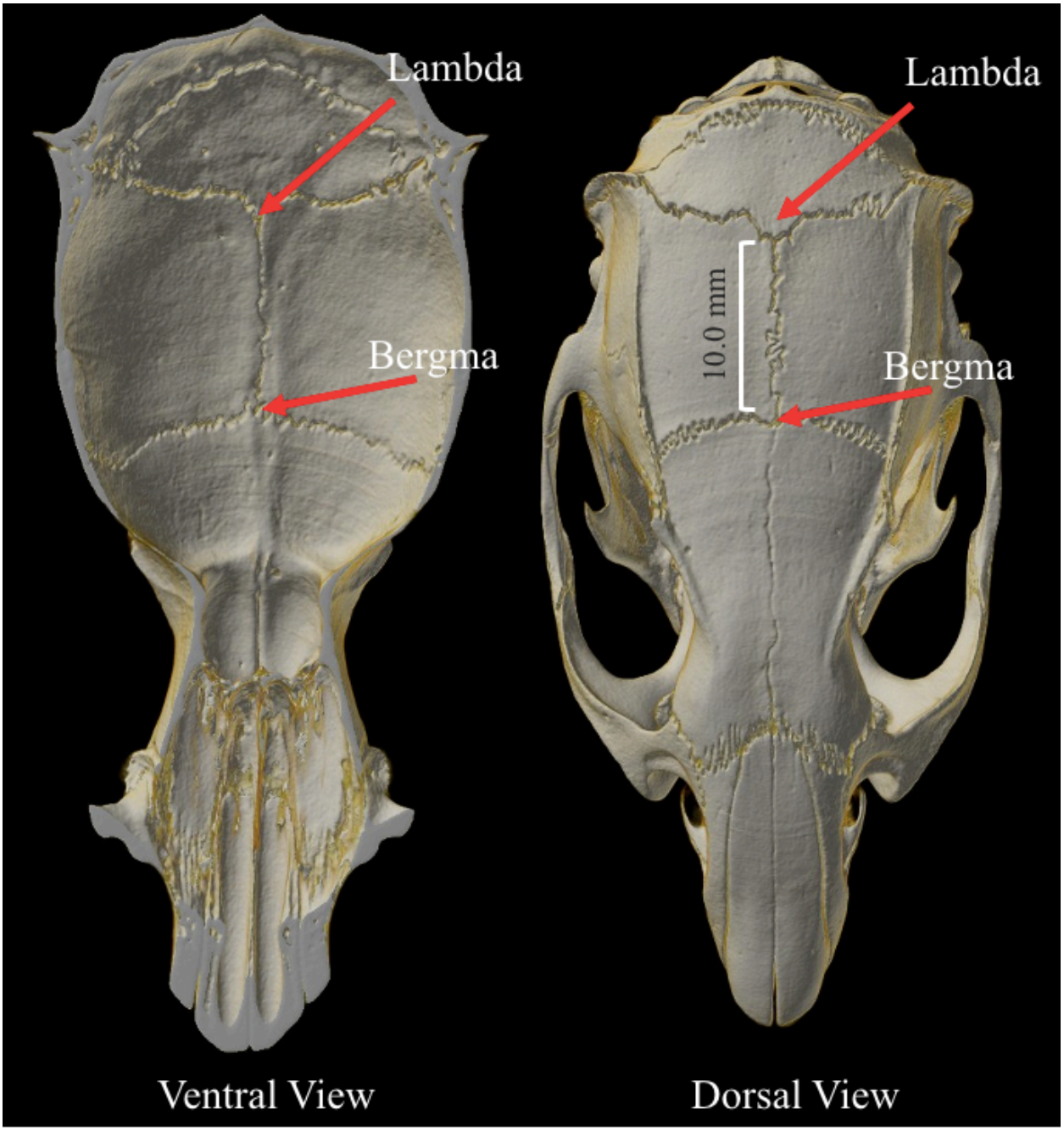

Figure 2-9. CT Images of Rat Skull Post- TBI. Representative images for method validation of 6 pilot rats used to determine the occurrence of microfractures from the modified weight drop model (300 g dropped from 1 m). Image reconstructed using AMIRA™.

2.6.2 Timeline

Four cohorts of 4-5 males and 4-5 female Sprague-Dawley rats were utilized for this study (total animals = 34). Upon arrival at the Lab Animal Services Unit (LASU), animals were acclimatized for a 7-day period without handling. The rats were housed in pairs in green-transparent plastic temperature-controlled cages and maintained on a standard 12:12 light dark cycle throughout the length of the experiment. Following initial acclimatization, animals were handled for 7 days prior to commencement of experimentation. Prior to formal training on specific tasks, rats were extensively held and handled for 2 days in the rat suite, where TBI and injections would take place. On day 3 of acclimatization, rats were transported through the vivarium by cart to the hallway of the behaviour suits via the elevator and left to acclimatize for 45 min in the behaviour hallway, adjoining behaviour suits. Day 4, rats were once again brought to the behavior hallway, left for 15 to 30 min, and then transported into the allocated testing room for 10 min with all equipment running. Day 5, rats were left for 10 min in the behaviour hallways, transported into the testing room and left for 10 min. Rats were then individually placed in bottom of the Rotarod for 2 min. Day 6 and 7 followed the same 10 min and 10 min protocol from Day 5, but on Day 6 rats were placed for the first time on a moving rotarod. On Day 7, rats were placed on a moving rotarod three times, following the established rotarod protocol, but no measures were collected. Day 7 was the last day of formal acclimatization. For all following days of training, testing and treatment, rats were left in the behaviour hallway to acclimatize for 10-15 min and then transported into the testing room for 10 min. Following acclimatization, rats were pre-trained for 4 days on the rotarod and a pre-injury baseline measure was collected one day before TBI. Baseline measures were also collected for Y-maze one day prior to TBI, for tetrad 2 days prior, and for OFT 3 days prior to TBI (Figure 2-3).

Rats from 3 of the 4 cohorts were randomly assigned into 4 experimental groups of 9 rats (4-5 male and 4-5 female): (1) Sham TBI treatment (anesthetized with isoflurane) + *i.p.* vehicle injection [ethanol: emulphor: saline (1:1:18)]; (2) Sham TBI treatment + THC [*i.p.* injection 1 mg/kg]; (3) TBI (under isoflurane anesthesia) + *i.p.* vehicle injection; (4) TBI + 1 mg/kg THC group: TBI + *i.p.* THC. These 3 cohorts were subjected to the battery of behavioural measures including Y-maze, Tetrad, and Rotarod. For the 4th cohort, 10 rats (2-3 male and 2-3 female) were randomly assigned to 2 experimental groups: (1) TBI treatment (anesthetized with isoflurane) + intraperitoneal (*i.p.*) vehicle injection [ethanol: emulphor: saline (1:1:18)] or (2) TBI treatment

(anesthetized with isoflurane) + *i.p.* THC (10mg/kg). Rotarod was selected as the only behavioural measure to be administered for the 4th cohort.

The observer assessing the rats post-treatment was blinded to the treatment type. Purified THC (98%) was sourced from Aurora Cannabis, Saskatoon, Saskatchewan. In past experiments with rats, 1 mg/kg THC *i.p.,* as per the first experiment, represents an approximate ED_{50} dose (i.e. 50% maximal effect) (154), and acted as a therapeutic dose for neuroprotection without adverse side effects classically associated with higher doses of THC (155).

A 10 mg/kg dose, as per the second experiment, produces cannabinoid receptor-dependent effects (hypolocomotion, reduced anxiety, reduced body temperature) but effects remain non-toxic, unlike doses observed exceeding 10 mg/kg *i.p.* in rodents (156).

2.7 Statistical Analysis

In order to determine the effect of 1 mg/kg of THC and TBI on all measures of catalepsy, body temperature and anti-nociception, data (n=4-5/group) were separated for sex due to known locomotor, temperature, and nociceptive differences between males and females, and analyzed according to 2 two-way repeated measures (RM) analysis of variance (ANOVA) test, 2x2 completely randomized design. Data for OFT (total movement and time in center) were normalized as a measure of fold change from the baseline measure for each subject and analyzed according to a three-way RM ANOVA, 2x2x2 completely randomised design. Y-maze percentage correct alternations were analyzed according to a three-way repeated measure ANOVA, 2x2x2 randomized design. Rotarod for the 1 mg/kg THC group Rotarod was normalized as a measure of fold change from mean baseline (days -4 to -1), and then analyzed according to a three-way ANOVA, 2x2x2 completely randomized design. Normalization was performed to account for biological variability in performance between animals and account for scale differences, so all animals appear to have the same starting point. When necessary, data was broken down for sex or injury and follow-up analyses were conducted using two-way ANOVA, 2x2 completely randomized design, when time was not considered a significant factor. The 10 mg/kg THC experiment (n=4-5) was analyzed according to a one-way RM ANOVA. Correction for multiple comparisons was done using Tukey's *post-hoc* analysis. Data is presented as mean ± standard error of the mean (SEM) from each group for 1 mg/kg THC experiment and for 10 mg/kg THC experiment, $*p \leq 0.05$ is considered statistically significant. All data was analyzed using GraphPad Prism 8.0 and IBM SPSS (27).

Chapter 3 Results

3.1 Tetrad

The tetrad test consists of bar holding, body temperature, tail flick, and open field assays and is used to assess the most-common physiological and behavioural responses to CB1R agonists: catalepsy, hypothermia, anti-nociception, and hypolocomotion and anxiety (both in the open field), respectively. To determine whether there was a significant effect of TBI or 1 mg/kg *i.p.* THC administered on day 0 on animal physiology and behaviour, the tetrad tests were administered, and data was separately analyzed for sex.

According to the two-way RM ANOVA of male catalepsy, sphericity was not assumed (Mauchley's: $p<0.001$), time was not significant (Greenhouse-Geisser: $p=0.58$) and there was no significant interaction or main effects of injury or drug on measures of catalepsy (two-way RM ANOVA: $F_1 = 0.063$, $p=0.81$) (Figure 3-1A). For females, sphericity was assumed (Mauchley's: $p=0.077$), time was not significant ($p=0.85$), and there was no significant interaction or main effects of injury or drug on measure of catalepsy (two-way RM ANOVA: $F_1=0.38$, $p= 0.55$) (Figure 3-1B).This analysis indicates that neither THC or TBI had a significant effect on catalepsy in male and female rats.

For both male and female measures of body temperature, sphericity was assumed (Mauchley's Male: $p=0.26$; Female: $p=0.22$) and time was significant ($p<0.024$) but neither males of females exhibited any interaction or main effects of injury or drug treatment on body temperature (two-way RM ANOVA Male: $F_1=0.93$, $=0.35$; Female: $F_1= 0.64$, $p= 0.44$) (Figure 3-1C, D). This analysis indicates that neither THC or TBI had a significant effect on body temperature in male and female rats.

In the tail flick assessment anti-nociception, sphericity was assumed for both sexes (Mauchley's Male: $p=0.96$; Female: $p=0.68$), time was not significant ($p>0.39$) and there was no significant interaction or main effects of injury or drug on latency to tail flick (two-way RM ANOVA Male: $F_1=0.21$, $p=0.65$; Female: $F_1=0.34$, $p=0.57$) (Figure 3-1E, F). These results indicate that there was

no significant effect of THC or TBI on nociception as assessed by the tail flick assay.

Locomotion was assessed by total movement in the open field. Anxiety-modelling effects were measured by time in the central quadrant of the open field. The data are described elsewhere because open field tests were conducted on different days from the bar-holding, body temperature and tail flick assays (Figure 2-3).

Overall, catalepsy, body temperature, and tail flick assessments indicate that 1 mg/kg THC administered *i.p.* did not evoke cannabinoid-dependent effects in rats whether they received TBI or not and TBI did not alter THC-dependent effects in these tests. Although THC administered acutely at this dose has been shown previously to produce tetrad effects (148), we did not detect such effects here. This may be because tetrad assessments took place hours and days after drug administration when overt signs of cannabinoid receptor-dependent intoxication had dissipated.

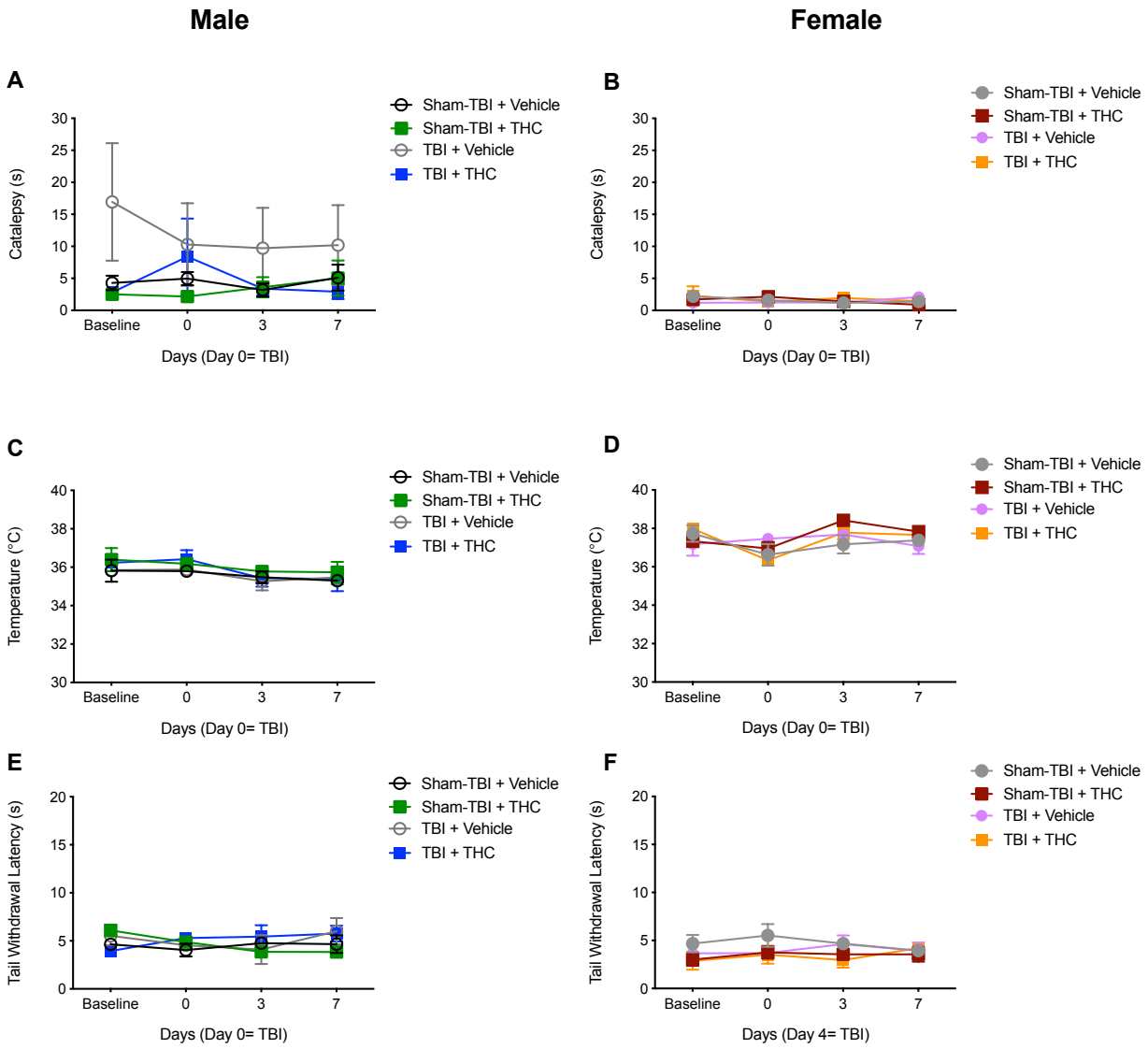

Figure 3-1. Assessment of catalepsy, body temperature, and nociception in rats following TBI and 1 mg/kg THC treatment. Seventeen Sprague-Dawley rats of both sexes were administered a Sham-TBI or TBI and injected with 1 mg/kg THC *i.p.* or Vehicle. 1 mg/kg THC *i.p.* nor TBI significantly induced catalepsy in males **(A)** or females **(B)**. 1 mg/kg THC *i.p.* nor TBI significantly lowered body temperature in males **(C).** or females **(D)**. 1 mg/kg THC *i.p.* nor TBI significantly effect tail flick responsiveness in males **(E)** or females **(F)**. Data presented as mean ± S.E.M. n=4-5 per group (males and females). Statistical analyses were two-way RM ANOVA.

3.2 Open Field

In order to determine whether 1 mg/kg THC and TBI effected total locomotion or fear-behaviour in rats, the OFT was conducted to measure total movement and time spent in the center of the open field as a model for anxious or fearful behaviour in rats.

3.2.1 Measure of Total Locomotion

Total movement in the OFT (measured in m) was normalized to the baseline measurement taken prior to TBI. Normalized data were analyzed according using three-way RM ANOVA. Assumptions of sphericity were not met (Mauchley's: $p=0.003$). Time was significant (Greenhouse-Geisser: $p=0.022$); however, no main effects of sex, injury, or drug treatment, or interactions between these variables were detected (three-way RM ANOVA: $F_{1,28}=0.056$, $p=0.81$) (Figure 3-2). These results indicate no significant effect of TBI or THC on total locomotor behaviour in male and female rats.

3.2.2 Time in Center Quadrant

Time spent in the center quadrant of the OFT was normalized to the baseline measurement taken prior to TBI and analyzed according to a three-way RM ANOVA. Assumptions of sphericity were not met (Mauchley's: $p<0.001$). Time was considered significant (Greenhouse-Geisser: $p=0.034$); however no main effects of sex, injury, or drug treatment, or interactions between these variables were detected (three-way RM ANOVA: $F_{1,28}=1.38$, $p=0.25$) (Figure 3-3). These results indicate no significant effect of TBI or THC on time spent in the center quadrant between male and female rats.

As with catalepsy, body temperature, and nociception, data from the OFT demonstrates that 1 mg/kg THC administered *i.p.* did not produce cannabinoid-dependent reductions in locomotion or anxiety-like behaviour. Although acute treatment with 1 mg/kg THC has been shown previously to reduce movement and anxiety (148), these were not detected here. Despite this, conducting these tetrad analyses was crucial to determine whether cannabinoid-mediated intoxication had occurred in these animals.

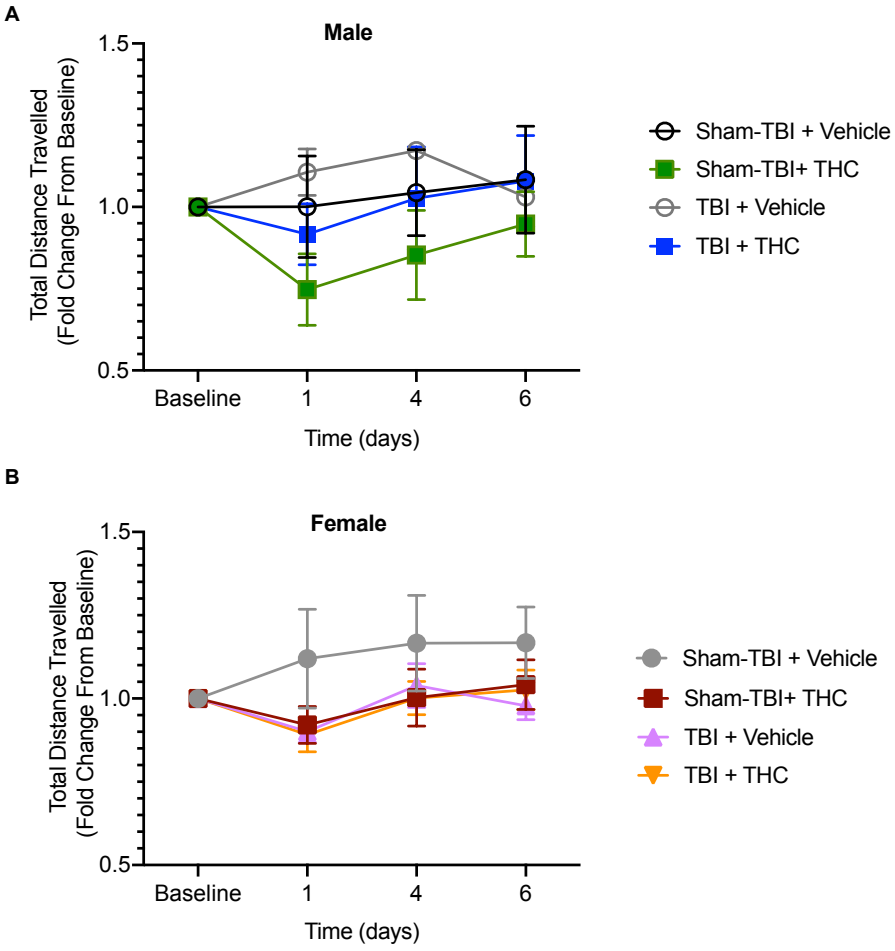

Figure 3-2. Assessment of total locomotion in the open field test following TBI and 1 mg/kg THC treatment. Seventeen Sprague-Dawley rats of both sexes were administered a Sham-TBI or TBI and injected with 1 mg/kg THC *i.p.* or Vehicle. 1 mg/kg THC *i.p.* nor TBI significantly impacted total locomotion of male of female rats. Data is normalized as a fold change from baseline and displayed by sex, **(A)** Male **(B)** Female. Data presented as mean ± S.E.M. n=4-5 per group (males and females). Statistical analyses were three-way RM ANOVA.

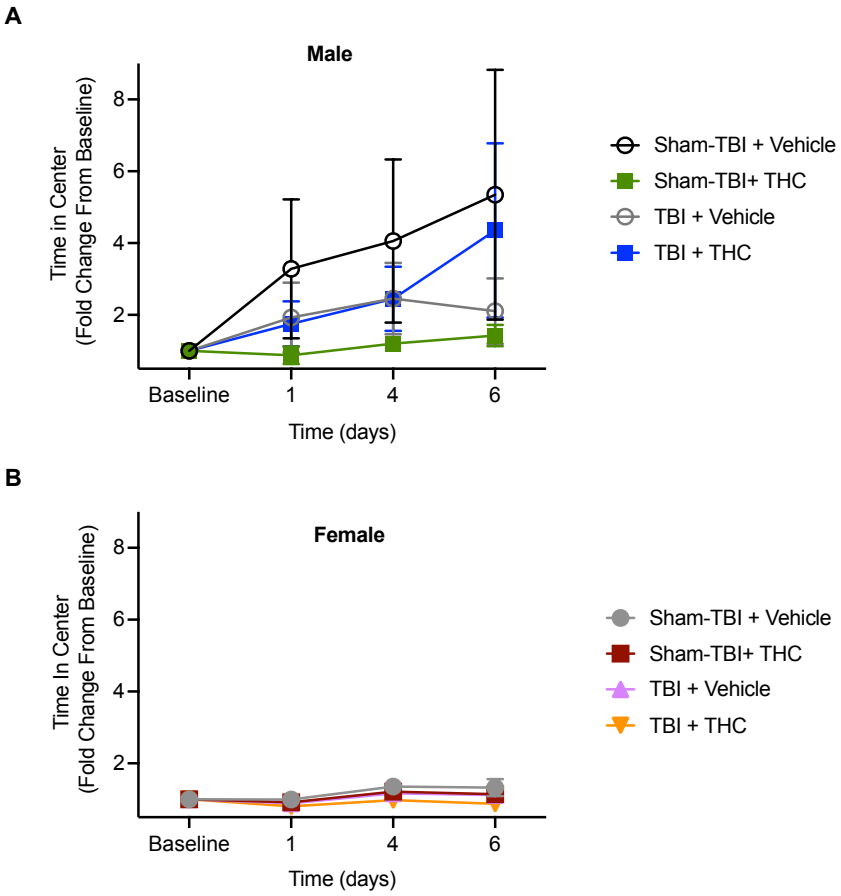

Figure 3-3. Assessment of time spent in the center quadrant of the open field following TBI and 1 mg/kg THC treatment. Seventeen Sprague-Dawley rats of both sexes were administered a Sham-TBI or TBI and injected with 1 mg/kg THC *i.p.* or Vehicle. 1 mg/kg THC *i.p.* nor TBI significantly impacted time spent in the center quadrant of male of female rats. Data is normalized as a fold change from baseline and displayed by sex, **(A)** Male **(B)** Female. Data presented as mean ± S.E.M. n=4-5 per group (males and females). Statistical analyses were three-way RM ANOVA.

3.3 Y-Maze Percentage Spontaneous Alternations

Y-maze is a measure of overt changes in spatial working memory. In order to determine the effect of 1 mg/kg THC and TBI effect on working memory, the percentage of correct alternations between all 3 arms of the Y-maze were analyzed. Data met assumptions of sphericity (Mauchly's: p=0.93) and the results from the three-way RM ANOVA on percentage correct alternations of male and female data indicated time was significant (p= 0.048) but no significant main effects of sex, injury, or treatment, or interactions between these variables were detected (three-way RM ANOVA: $F_{1,27}$=0.022, p=0.88) (Figure 3-4).

For analysis of total arm entries in the Y-maze, data met assumptions of sphericity (Mauchly's: p=0.29), and the results from the three-way RM ANOVA indicate that time was significant (p= 0.001), and sex was considered a main effect (three-way RM ANOVA: $F_{1,27}$=6.453, p=0.017). No other main effects or interactions between sex, injury or treatment were considered significant (three-way RM ANOVA: $F_{1,27}$=0.036, p=0.85) (Figure 3-5). Therefore, neither TBI or THC treatment had an overt effect on locomotion, which is in line with our data in the OFT (Figure 14), but females consistently entered more arms than males did. One female Sham TBI + VEH was removed from the analysis due to a breach of test parameters, when the rat jumped out of the maze. This analysis indicates that Y-maze total number of total arm entries significantly differed between sexes, but TBI or THC played no part in this effect. It can therefore be concluded that, neither 1 mg/kg THC, TBI, or the combination of these significantly altered working memory in the Y-maze task.

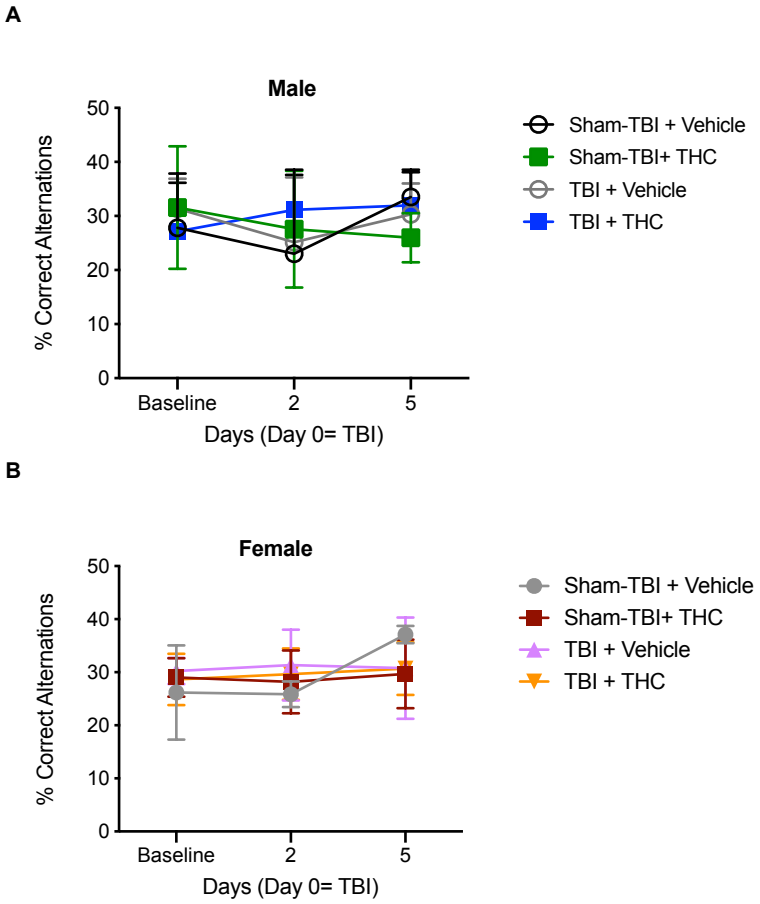

Figure 3-4. Assessment of working memory through measurement of spontaneous alternations in the Y-maze task following administration of TBI or treatment with 1 mg/kg THC. Seventeen Sprague-Dawley rats of both sexes were treated with a Sham-TBI or TBI and injected with 1 mg/kg THC *i.p.* or Vehicle. Sex, 1 mg/kg THC *i.p.* nor TBI significantly impacted performance on Y-maze. Data is displayed by sex, **(A)** Male **(B)** Female. Data presented as mean ± S.E.M. n=4-5 per group (males and females). Statistical analyses were three-way RM ANOVA.

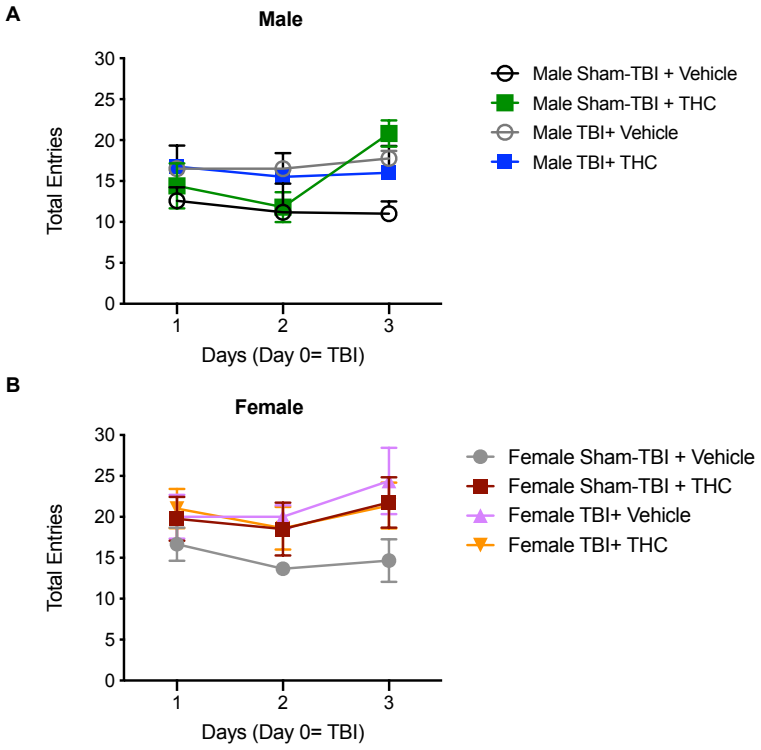

Figure 3-5. Total number of male and female Y-maze arm entries. This figure illustrates the mean number of arm entries by each treatment group across the experimental timeline. 1 mg/kg THC *i.p.* nor TBI significantly impacted total number of arm entries for Y-maze, but there was a significant difference between males and females as determined via three-way RM ANOVA: $F_{1,27}$=6.453, p=0.017. Data are presented as mean ± S.E.M. n=4-5 per group (males and females),.

3.4 Rotarod

3.4.1 Treatment with 1 mg/kg THC

Rotarod is a commonly used behavioural used to asses higher-level motor function in rodents following TBI. In order to determine the effect of 1mg/kg THC and TBI on rotarod, data was normalized relative to the mean of baseline measurements taken prior to TBI, expressed as fold change relative to this mean, and analyzed via a three-way repeated measures ANOVA. One male Sham-TBI + VEH was removed from the assessment after an outlier's analysis was performed (observation was greater than 2 SD from the group mean). Data did not meet assumptions of sphericity (Mauchly's: $p<0.001$). Time was not considered significant (Greenhouse-Geisser: $p=0.29$) (Figure 3-6). According to the analysis, there was no significant three-way interaction between sex, injury, and treatment: (three-way RM ANOVA: $F_{1,27}=3.65$, p=0. 67), but the analysis indicated a significant interaction between sex and injury (three-way RM ANOVA: $F_{1,27}=0.01$) (Figure 3-7A). These results indicate that males and females reacted significantly differently to injury. THC and vehicle treatment data were aggregated and compared between sexes and injury using a two-way ANOVA (Figure 3-7B). The two-way ANOVA indicated a significant interaction between sex and injury (two-way ANOVA=F1,32=10.03, p= 0.0034), but the Tukey *post-hoc* indicated no significant differences between groups (Figure 3-7B). Based on this analysis, data was segregated by sex and 2, two-way ANOVAs were conducted for male and female data separately.

The two-way ANOVA of male data indicated a main effect of injury (two-way ANOVA: $F_{1,14}=11.27$, $p=0.0047$) and no significant interaction between injury and drug (Figure 3-7C). The Tukey *post hoc* however indicated significant differences between Sham-TBI + VEH vs. TBI + VEH (p= 0.01), Sham-TBI + VEH vs. TBI + THC (p=0.01), and Sham-TBI+ VEH vs. Sham-TBI+THC(p=0.049), Figure 3-7C. Therefore, these results indicate that TBI robustly reduced rotarod performance in both VEH and THC treated groups, and THC treatment of non-injured rats reduced latency to fall in comparison to controls.

The two-way ANOVA of female data indicated no significant main effects of injury or drug and no interactions between these variable (two-way ANOVA: $F_{1,13}=3.14=0.10$) (Figure 3-7D). Interestingly, this indicates that female performance on the rotarod was not significantly changed by TBI or THC.

Based on these results, TBI significantly reduced motor coordination in male rats but had no effect in female rats, and THC measurably decreased motor coordination in non-injured male rats compared to vehicle controls, an effect that was not present in their injured counterparts or in female rats. Moreover, the magnitude of the injury did not significantly change over the course of the study because time was not significant in our analyses. Similarly, THC treatment did not alter rotarod performance in rats with or without TBI. These results differ from our initial hypothesis in that we expected both TBI and THC to have a significant effect on recovery in both male and female rats. importantly, the data clearly indicate the TBI altered behaviour and motor performance in a sex-dependent manner and support the use of this model for TBI in rats (90). Given the absence of clear effect brought about by THC treatment in these assessments, we next chose to test a higher dose of THC – 10 mg/kg – in this rodent model of TBI.

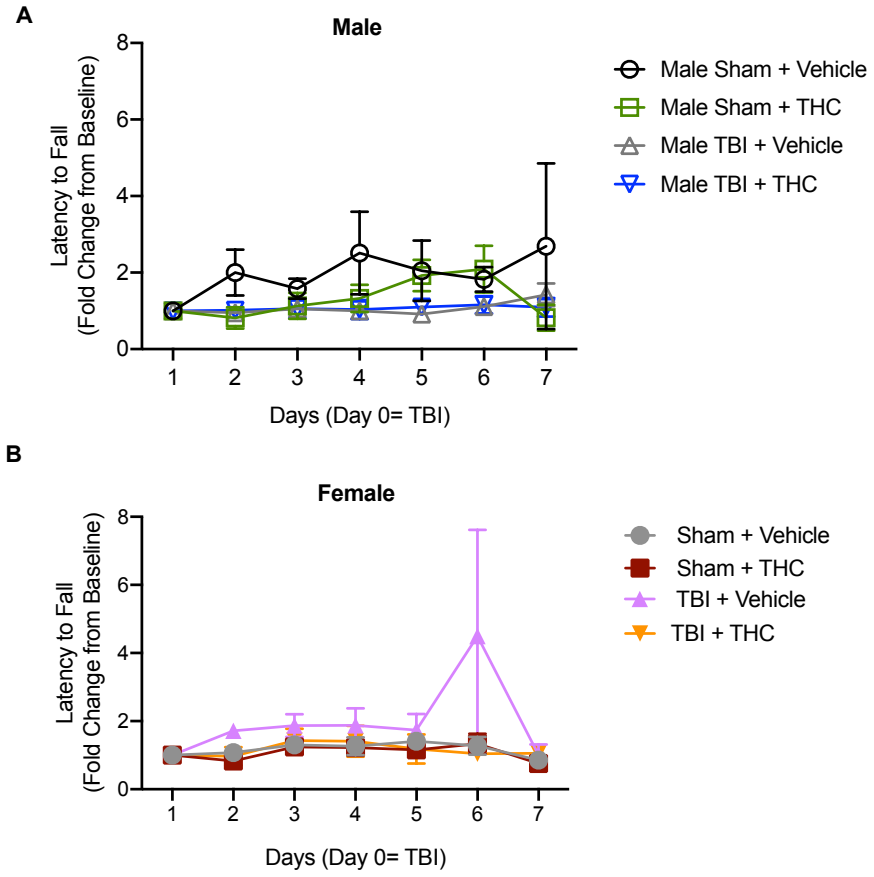

Figure 3-6. Visualization of rotarod data by day following administration of TBI or treatment with 1 mg/kg THC. Seventeen Sprague-Dawley rats of both sexes were treated with a Sham-TBI or TBI and injected with 1 mg/kg THC *i.p.* or Vehicle. Data is displayed by sex, **(A)** Male **(B)** Female. Because time was not found to have a significant effect, statistical analyses were conducted between group means and are displayed in figure 19. Data are presented as mean ± S.E.M. n=4-5 per group (males and females).

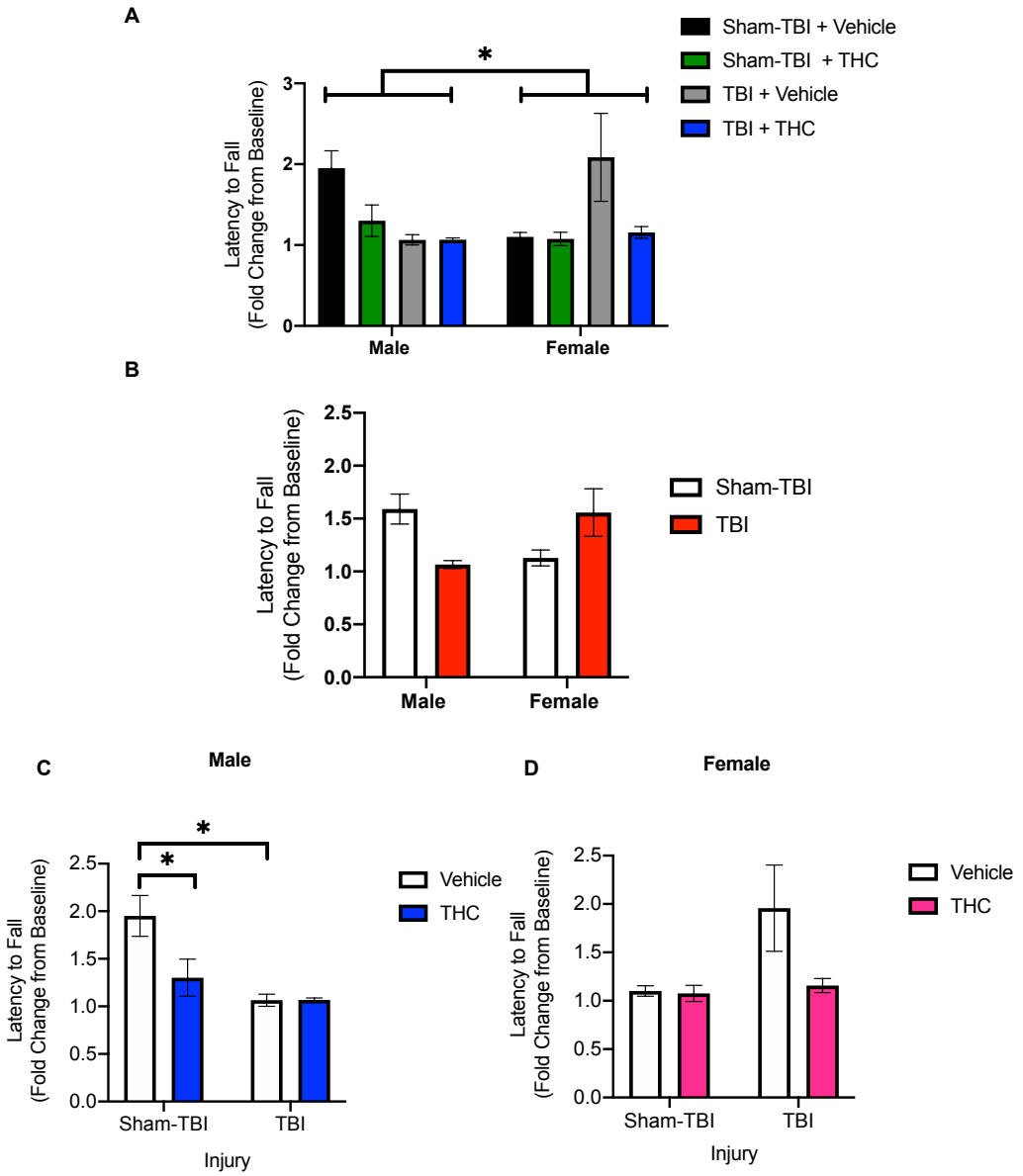

Figure 3-7.**The effect of 1 mg/kg THC and TBI on male vs. female rotarod latency to fall.** Seventeen Sprague-Dawley rats of both sexes were treated with a Sham-TBI or TBI and injected with 1 mg/kg THC *i.p.* or Vehicle. **(A)** Data are displayed by treatment group where (*) indicates a significant interaction between sex and injury ($p<0.05$), as determined via three-way RM ANOVA**.** Data are presented as mean ± S.E.M. n=4-5 per group (males and females). **(B)** Data are displayed with vehicle and THC treatment aggregated for a comparison of sex and injury. Data are presented as mean ± S.E.M. n=8-10 per group (males and females), Analysis was done by two-way ANOVA. Data are displayed by sex for males **(C)** and **(D)** females. **(C)** TBI decreased rotarod performance in male TBI rats compared to Sham-TBI controls, and THC decreased rotarod performance in Sham-TBI rats compared to Sham-TBI rats treated with vehicle**. (D)** Neither TBI or THC altered rotarod performance in female rats. Data are presented as mean ± S.E.M. n=4-5 per group (males and females). Analyses were done by two-way ANOVA followed by Tukey's *post-hoc* test, where (*) indicates a significant difference ($p<0.05$).

3.4.2. Treatment with 10mg/kg THC

In order to assess the effect of THC and TBI on latency to fall further, a higher dose of THC was used. Male and female rats were treated with 10 mg/kg THC + TBI or TBI + VEH. Data were not separated according to sex because too few animals of either sex were utilized. A one-way RM ANOVA was performed. According to the analysis, assumptions of sphericity were not met (Mauchley's: $p< 0.001$). Time was considered significant, (Greenhouse-Geisser: $p=0.025$), but according to the one-way RM ANOVA THC treatment was not significant (one-way RM ANOVA: $F_{1,8}= 0.449$, $p=0.52$) (Figure 3-8). Therefore, a greater dose of THC that approximates an ED_{80} (150) failed to produce a significant effect on rotarod performance in Sham and TBI rats. Given the small sample size of these data and inability to segregate data on the basis of sex, future studies with larger group sizes are warranted to fully understand how higher THC doses may alter rotarod performance in male and female TBI rats.

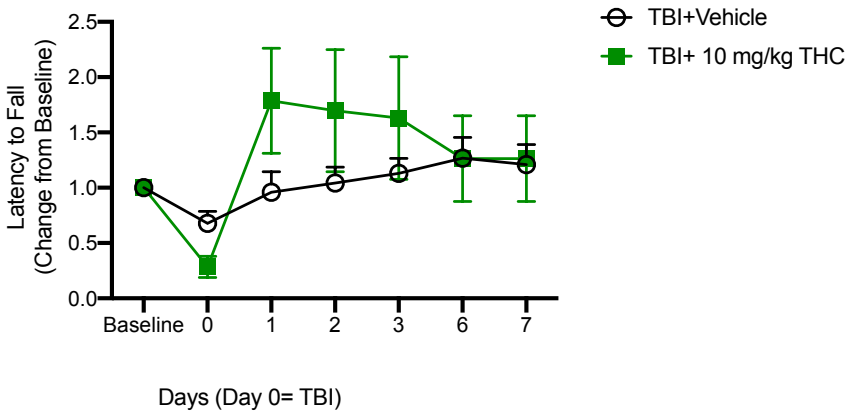

Figure 3-8. Assessment of 10 mg/kg of THC and TBI on combined male and female rotarod latency to fall. Five Sprague-Dawley rats (male and female, data not segregated by sex) were treated with a TBI and injected with 10 mg/kg THC *i.p.* or Vehicle. Analysis of normalized data indicated no significant difference between treatment groups across experimental timeline. Data presented as mean ± S.E.M. Statistical analyses were one-way RM ANOVA. n=5 per group.

Chapter 4 Discussion

4.1 Hypothesis of this Research

The data described here provide novel information regarding the effects of THC on the modulation of the ECS following mTBI in male and female rats. We postulated that the treatment of rats subjected to TBI and THC 1 h post-injury would restore motor function and improve behavioural profiles of injured rats across the experimental timeline.

Our results show that 1 mg/kg of THC and TBI did not alter catalepsy, body temperature, or anti-nociception and failed to impact open field total locomotion or time spent in the center quadrant. In contrast, both THC and TBI demonstrated sex specific differences in rotarod performance as measured by latency to fall. In males, THC and TBI independently reduced performance on the rotarod in males, but THC did not enhance or diminish rotarod performance in injured male rats. In females, neither THC or TBI altered rotarod performance. **Therefore, our initial results do not agree with our hypothesis because THC treatment did not restore motor function or improve the rat's behavioural profiles. On the contrary, THC itself was deleterious to male rotarod performance and neither beneficial or detrimental in injured male and female rats.** Moreover, we anticipated a symmetrical effect of TBI on rotarod in both males and females and potential sex-dependent differences in response to 1 mg/kg THC; however, male and female rats responded differently to our TBI model and THC, demonstrating potential sex-dependent differences in both that must be accounted for in future research.

Importantly, we observed that our TBI model produced a sustained reduction in higher level locomotor function in the rotarod test in male rats – demonstrating the validity of this model (143) and observed that 1 mg/kg THC *i.p* 1 h post-injury did not change this motor function reduction.

4.2 Tetrad

The tetrad battery of tests was selected in order to identify any lasting cannabinoid-based impact on animal physiology. The tests were strategically implemented to identify any lasting cannabinoid activity in areas of the CNS where CB1R are most abundant that could interfere with other behavioural measures. Non-significant catalepsy, body temperature and anti-nociceptive results held true to our expectations, as these tests measure acute effect of cannabinoids which generally peak at 30 mins following an *i.p.* injection in rodents (156). Importantly, these tests also served as confirmation that TBI had no measurable effect on catalepsy, body temperature and anti-nociception of the rodents 1 h out of administration that could have confounded other behavioural measures.

OFT is a measure frequently used in pre-clinical research to assess locomotion and anxiety-like behaviours in rodents via the quantification of total locomotion and time spent in the center quadrant that can be present due to both cannabinoid-based drug effect as well as TBI. Anxiety like-behaviour manifests acutely following TBI in rodents and humans as well as acting as a common symptom of chronic post-concussive symptoms (157). For this reason, according to our hypothesis, we expected to see measurable deficits in OFT of total distance traveled following TBI, but there were no measurable or visual changes in data during the experimental timeline. This disconnect between observed and expected results may have to do with the injury magnitude. Quite simply, the injury incurred may have been too subtle to produce persistent large-scale changes of fear-based behaviour in rodents detectable in 10 min measures of OFT. Given these results, there are a few modifications to consider for future experimentation. To detect more subtle injury and drug induced behaviours, it is imperative to modify test parameters, for example, implementing longer recording time (>10 min), increasing or decreasing the light or environmental stimulus or plainly substituting more complex behaviour measures could serve as beneficial modification to detect more subtle injury and drug induced behaviours.

4.3 Y-maze

Y-maze percentage correct alternations is a behavioural modality frequently used to assess the acute and chronic changes in spatial memory for both TBI and pharmacological investigation in rodents (158). Much like the OFT, we expected to see a measurable deficit in Y-maze performance following TBI, but there was no significant difference between Sham-TBI and TBI across the experimental timeline for percentage correct alternations. This most likely speaks to the lack of specificity of the Y-maze, which seems most likely to detect overt changes in behaviour only when animals are very ill or heavily affected by pharmacological agents. Importantly, females consistently scored higher total number of arm entries in comparison to males, indiscriminate of treatment or injury. Although we expected to detect this difference in OFT as well, this difference in total Y-maze entries serves to highlighting sex differences in exploratory behaviour. Although this test has been applied in evaluating the full spectrum of TBI (86, 166), it may be more suited to injury models that are on the severe end of the spectrum.

4.4 1 mg/kg Rotarod

The rotarod is a device that resembles an elevated treadmill for rodents suspended above a sensor plate to detect the animal when it falls from the treadmill. In pre-clinical TBI research, the rotarod is a commonly used methodology to measure higher level neuromotor capacity such as coordination, musculoskeletal strength, endurance, and balance post TBI (159). Interestingly, according to the results, sex, injury and drug effect played a significant factor in the performance of animals on the rotarod.

More specifically, TBI significantly decreased male latency to fall, but had no comparable effect in females. From a drug effect, the Tukey's *post-hoc* analysis revealed that 1 mg/kg THC effected Male Sham-TBI groups, as the Sham-TBI + THC groups performed significantly worse on the rotarod in comparison to control. This reaction to THC in male Sham-TBI is interesting as it reinforces circulating knowledge about sex-dependant modulatory differences in the ECS (160, 161). THC normalizes glutamate release via CB1R and reduces inflammation via CB2R, which are beneficial actions in the event of an injury, but could be intoxicating and detrimental under "normal conditions, as seen in results between Sham-TBI + TBI and Sham-TBI+ VEH.

In contrast, female drug treatment approached significance as main effect ($p = 0.051$). Additionally, upon visual inspection of female rotarod data (Figure 19D), the TBI + VEH group shows a non-significant increase in latency to fall, between TBI + VEH and Sham-TBI groups as well as its female TBI+ THC counterpart. Likely, the almost significant p-value from drug effect and the difference in performance between female sham-TBI + VEH and Sham groups and TBI+ THC may speak to the need to increase group size and tighten the SD to clearly indicate outliers to concretely show a statistical difference.

4.5 Rotarod 10 mg/kg

With regard to our supplementary analysis with 10 mg/kg of THC, we hypothesized that 10 mg/kg of THC would restore motor function in comparison to a TBI control group. Our results showed that the administration of 10 mg/kg of THC did not significantly improve performance of THC treated rats on the rotarod in comparison to a vehicle control group. Visual inspection of the data according to day (Figure 20) indicates that THC may have offered some therapeutic benefit, as the rats treated with THC exhibited a "rebound effect" in their performance on day 1 to 3, performing on average 0.5 fold better than the vehicle treated TBI group. Importantly, this data represents pooled male and female data, which according to previous analyses of the 1 mg/kg cohort, demonstrated significant interactions between sex and injury. Therefore, an appropriate follow-up experiment to this data would be to separate for sex and increase the number of male and female subject to fully quantify sex specific reactions to 10 mg/kg THC.

4.6 Sex differences in TBI

In pre-clinical research, females have been known to survive and functionally recover better in brain injury than males (162, 163). Administration of exogenous estrogen and progesterone to males and ovariectomized females has improved molecular, and histological outcomes (164), but behavioural outcomes seem to vary largely according to mechanism of injury. Previous research demonstrated that in a model of diffuse TBI, under 3 different anesthetics, female rats consistently scored better than males on rotarod, and isoflurane exhibited enhanced neuroprotective properties in females more so than males in comparison to pentobarbital and halothane according to results on Barnes Maze and OFT (165). In comparison, in a study of CCI, a focal injury administered in male and female mice, no differences in comparable behaviour measures were found (166). Females may recover function better than males because of antioxidant and anti-inflammatory effects of estrogen and progesterone on the preservation of BBB integrity and regulation of edema.

By comparison, clinical observational data indicates that females are known to be at higher risk, have poorer outcomes in TBI, and are more likely to report persistent post-concussive symptoms (167). This may be due to differences in size as well as sex hormones which may impact recovery and influence injury severity, but also may be influenced by the increased willingness of females to report symptoms , as well as perceived social pressures for females to forgo treatment and return

to their role as caregivers (168, 169, 170). A large-scale observation study by Davis et al. (2006), indicated that when aged-matched, there was no significant difference in recovery between pre-menopausal females and males, but post-menopausal females recovered better than their male counterparts (171). Differences between pre-clinical sex differences in models of TBI and observational data in humans on the bases of sex and gender underscore the limitations of animal models in studying the complete disease etiology of TBI. Future pre-clinical work that probes more subtle behavioural and cognitive changes following injury that were not assessed here would provide valuable data for model validation the translational potential of this work.

Most importantly, the robust reaction of males to TBI according to rotarod performance confirm the injury model, but specifically highlight the difference in female to male reaction to injury and the complexities associated with comparing behavioural data between sexes in preclinical research. The discrepancies highlighted between pre-clinical and clinical research indicate the importance of continued inclusion of female subjects in future research.

4.7 Sex differences in the Endocannabinoid System

As previously stated, the endocannabinoid system is a complex system within the human body that modulates numerous physiological functions such as locomotion, pain, body temperature as well as cognitive and behavioural functions (14, 15). With CB1R being the most abundant receptor within the CNS, it can be assumed that its relationship with other systems in the human body is complex and multifaceted. Current pre-clinical research has reliably shown persistent sex differences in cannabinoid-based drug; where females reliably response physiologically and behaviourally stronger and faster to lower doses, and exhibit higher plasma concentrations of active metabolites (172, 173). As proof of concept, Craft and Leitl (2008), demonstrated that the anti-nociceptive effects of THC in females fluctuated according to estrus cycle, and were more sensitive and different from males between late proestrus and estrus (174). Similarly, increasing estradiol was shown to mitigate decreases in body temperature, performance in operant tasks, and hyperphagia, associated with cannabinoid exposure (160, 174, 175). From an addictions perspective, pre-clinical research has shown that females are at high risk of acquiring and reinforcing behaviours associated with cannabinoid abuse (177, 178). Furthermore, sex differences have been shown at a cellular level, where female CB1R and male CB1R display significantly different binding affinities most notably in the prefrontal cortex, amygdala, hippocampus and

cerebellum (179). According to Cooper and Craft (2018), this increase bioavailability and associated potency in females is attributed to fundamental sexual differences occurring early in development in addition to acute effects of gonadal hormones acting on the system causing lasting changes in hormones pharmacokinetics and pharmacodynamics (see 180 for in-depth review). Notably, these sex differences are present, but less clear in adolescent animals, which supports the relevance of estradiol concentrations to pharmacokinetic and pharmacodynamic differences in females (180).

Known behavioural differences between females and males exhibited in this study highlight the need for future molecular examination of sex-dependant differences of the ECS and recovery in TBI. Mechanisms for these differences are yet unknown, but a study by Xing et al. (2014), who used a stress mechanism to modulate the ECS of male and female rats, highlights specific differences in CB1R mRNA expression level and protein levels in the prefrontal cortex of stressed female rats compared to their male counterparts (182). Their work represents early postulation about how these sex- and stress-dependant modulatory changes could participate in resiliency following brain injury (182). For this reason, on-going multifaceted investigations of inflammatory cytokine profiles, cortical CB1R receptor concentration as well and cortical endocannabinoid levels on these animals following injury and administration of 1 mg/kg and 10 mg/kg THC, respectively, will provide validity to current behaviour results and elucidate pending modulatory sex dependent effects on recovery.

The sex-dependant effects of cannabinoids are consistent in human studies but are reflected in different ways. However, data are limited. Clinically, females and males exhibit different use patterns for cannabis (180). Women are less likely to gain analgesic benefits from THC, but maintain similar abuse criterion (183). As in animal models, human females have high levels and greater exposure to THC's main metabolite 11-OH-THC after oral dose (184). These persistent sex differences that translate across both the animal and human development highlight the importance and clinical relevance of research with both sexes for therapeutic and addiction purposes. Research has illustrated the complex relationship between sex and TBI, and the endocannabinoid system is not absolved from this biological phenomenon therefore emphasising the importance of continued investigation of the interplay between these complex systems.

4.8 Locomotor Activity

An important distinction to be made in TBI research is the difference between motor function and locomotor activity. Motor function speaks to basic neurological function eliciting low level motor function following injury. By comparison, locomotor activity speaks to high level locomotor capacity associated with health subjects and outcomes where changes in direction, rate changes and balance are involved (185). Within pre-clinical data, the lines between these 2 entities often blur. Throughout my literature review, due to the blur of distinction across levels of injury severity, the language used to describe varying degrees of motor impairment following injury also blurred. In order to encourage translation within brain injury research, it is imperative that researchers distinguish between high-order locomotor activity and learning required to participate in behavioural activities such as the rotarod, versus low level motor function associated with more consequential injury phenotypes.

4.9 Comparisons to Current Data

Only 1 paper was identified in the literature to date using THC as a pharmacological intervention in the treatment of TBI in both male and female rats. This paper, published by Bhatt (2020) (136), repeatedly administered a lateral impact method to illicit three diffuse repeated mTBIs (RmTBI) using 50 g. In their first experiment, animals were treated with 6 *i.p* injections of 1.25mg/kg THC, and during the last 3 administrations of THC animals received 3 RmTBIs (136). Following RmTBIs, behavioural data were collected. Results from the first experiment indicated that THC administration increased righting time in rats following TBI as well as persistent sex differences, but no other significant differences in THC administered animals (136). For the second experiment, 3 RmTBIs were administered followed by repeated THC administration for 12 consecutive days via *i.p* injection at a dose of 1.25 mg/kg (136). Animals were then tested to assess neurological function, anxiety-like behaviour, short term working memory and depressive-like behaviour (136). According to their results, repeated administration of 1.25 mg/kg of THC improves working memory in the Novel Object Recognition Test, anxiety-like symptoms in Elevated Plus Maze but not OFT, and depressive-like symptoms in Force Swim Test in RmTBI groups (136). Additionally, sex and RmTBI interacted for depressive-like symptoms and sex was a main effect for working memory (136). Importantly, although this experiment used a completely different battery of

behaviour tests from the ones used in this study, the sex differences identified with injury are consistent with our experiment, and the more precise models of anxiety and working memory serve as a good reference for following experimentation.

The study of Bhatt et al. (2020) demonstrated that post-injury THC was able to rescue the injured phenotype symmetrically for males and females in in working memory, and anxiety like behaviour (136). Is important to note however that the rats in this study were adolescents when testing occurred (post-natal day: 21-55) which is problematic considering the previously highlighted roles that estrogen and progesterone can play on TBI resilience, as well as on the formation and modulation of the ECS (161). The comparison between our results and the results from Bhatt et al. (2020) indicate that THC can provide therapeutic effect independent of sex when administered before sexual maturity, and therefore provides indication that a similar repeated THC administration in adult animals is worth performing.

4.10 Limitations

According to our results, the existing experimental design did not provide as forthright a behavioural response as anticipated, indicating that further research is required to determine potential avenues of change in the ECS following TBI. More specifically, the inclusion of more precise behavioural and neurological assessment tools is critical to increase the precision of mTBI appraisal, increasing the n for both groups, as well as the continued inclusion and further investigation of TBI- and ECS-based sex differences. Above all, it is important to note the limitations the low number of test subjects in the 10 mg/kg experiment presented. Each group had an n=5 combined males and females, and based off of the sex differences brought to light from the rotarod, it is imperative to analyze them separately. Sex desegregated data in mTBI research is sorely lacking (86, 186) and according to a review by Fattore and Fratta (2010), cannabinoid research is not absolved from this problem (187). The differences seen between sexes in the 1 mg/kg experiment amongst rotarod measures indicate that pooled data does not accurately paint the whole data picture.

Firstly, the substitution of more sensitive assessment tools to accurately gauge subtle forms of physiological and cognitive change associated with mTBI are necessary as follow-up analysis to

this study. Despite the frequent use of the Y-maze for cognitive assessment following brain injury, the injury model in this study may not have been severe enough to elicit overt cognitive changes required for detection in the Y-maze test of percentage correct alterations. Inclusion of more focused assessment tools providing a multi-faceted investigation of memory and cognition such as novel object recognition, Barnes maze, sociability, and valuable gold standard tools such as trial unique non-matching to location task (TUNL) and 5-Choice Serial Reaction Time Task (5-CSRTT) (188, 188) would serve to enhance the understanding and translation of more subtle acute and chronic behaviour changes associated with mTBI.

In addition to quantifying cognitive changes, neurological symptoms were assessed using the rotarod. Recent evidence indicates that exercise activates the ECS and contributes to physiological and psychological adaptations consistent with regular physical activity (190). Over the last several decades, exercise has become recognized as a therapeutic agent. As a general lifestyle intervention, exercise is known to increase mitochondrial function and endorphin levels, increase cerebral blood flow and cognitive performance, regulate neurotransmitter levels, and decrease inflammation (191, 192). Exercise facilitates recovery when implemented pre-TBI (193), immediately post-TBI (194), as well as weeks after the initial injury (195) in both animal models and human studies. Several studies indicate that endogenous cannabinoids are upregulated post-exercise and contribute to exercise induced analgesia, also known as “runners high” (196, 197). Since exercise is therapeutic in TBI models on its own, the combination of exercise and cannabinoid-based therapeutics may provide additive or synergistic benefit. Therefore, the administration of rotarod as a behavioural measure in this study may have blunted the detection of more sensitive behavioural symptoms by providing enhanced recovery rates, such as OFT or Y-maze. For this reason, in follow up investigation, non-confounding assessments of neurological function, such as beam walk or reflex testing (76, 198), could serve as effective means of detecting subtle and acute changes in neurological function that may resolve quickly following injury, especially when paired with exercise.

Another consideration is the role of the ECS in circadian rhythm disruption frequently associated with mTBI. Symptoms of sleep/wake disturbances following injury range in severity and can play a significant role in patient outcomes and quality of life (199-200). In terms of mTBI, a review of cannabis and cannabinoids on sleep by Babson et al. (2017) indicated that acute THC use can improve latency to sleep and frequency of wake after sleep onset (201). That same review

highlighted research indicating that low dose CBD supports wakefulness and high dose CBD improves latency to sleep. Therefore, the inclusion of sleep wave analysis via EEG to monitor the more subtle impacts of cannabinoid administration on sleep quality, wakefulness, and recovery of injured animals merits investigation.

4.11 Future Directions

In order to build on the findings described here, various avenues of investigation should be addressed. From a brain injury perspective, the injury mechanism, frequency, and severity all supply possible opportunities for exploration as well as the investigation of other potentially therapeutic cannabinoids, different dosing regimens, unexamined therapeutic avenues of cannabinoids, and most importantly, the continued prioritization of sex desegregated data.

Injury magnitude and frequency of injury deserve additional investigation given the inconsistency of injury severity classification within the mTBI research field. As mentioned previously, to date, pre-clinical TBI researchers using variations of the weight drop model have classified injuries as mild when dropping a weight ranging between 10 g and 1600 g (86). Moreover, CCI, another injury model highlighted earlier has its own methods for classifying injury severity, and it classifies an impact as mild when a craniotomy is performed, and an impact administered by a 3.0 mm steel tip, driven into the brain at 5.0 m/s, to a depth of 1 mm, resulting in significant visual and behavioural abnormality upon examination (166). In a clinical setting, mild TBIs are characterized by functional abnormalities without the presence of structural abnormalities. When structural abnormalities are present, the injury is then classified as a moderate TBI (12, 59). This limited comparison within and between injury methods indicates that mild to moderate brain injury classification vary significantly across pre-clinical investigations, and these differences dilute behavioural data due to misalignment of translation and ultimately misrepresents applicable knowledge to the clinical field.

Modification of the injury mechanism and the use of anesthetic are worth examination. Anesthetic, more specifically isoflurane, is neuroprotective with administration pre-injury (202, 145), and is simply not part of normal sport or motor vehicle mechanism of injury. Within models of severe TBI, isoflurane's neuroprotective properties may not be considered significant given the depths of the injury, but within a mild to moderate TBI model, this may account for the lack of significance

within the OFT measures and Y-maze % alternations. Consequently, a novel model of conscious mTBI where no anesthetic is used, such as the one developed by Pham et al. (2019) (203), warrants consideration.

In terms of ECS modulation, THC was selected as the treatment because it is the most abundant cannabinoid found in *Cannabis sativa plant*, and it possesses potential neuroprotective and anti-inflammatory potential thanks to its unique pharmacology. Yet it's psychoactive component makes potentially less suitable intervention in a clinical setting. Due to the overarching lack of research examining the modulatory effect of various phytocannabinoids in TBI, and THC's promising CB1R and CB2R pharmacology, THC served as a perfect candidate to begin these studies. One and 10 mg/kg were selected as appropriate doses thanks to aforementioned dose response curves from *i.p* injection resulting in ED_{50} and ED_{80} respectively, and therefore established a starting point (154, 155). Regarding the experimental timeline, THC was administered in a single dose following injury. As immune activation is present for weeks to months following injury (83) exploration of diverse dosing regimens post injury targeting chronic secondary injury mechanisms is necessary. Quite simply, 1 dose of THC represented a necessary starting point for this research but may not have been enough to elicit a lasting therapeutic response. Additionally, *i.p* injection is not the main mode of administration for cannabinoids when used in a recreational setting, thus emphasizing the importance of investigating smoked, vaped and oral administration of cannabis flower and oil in an injury model. Future research in this area could therefore utilize chronic daily dosing regimens through inhalation or oral routes of exposure.

Previous research highlighted in review by Singh and Neary (2020) shows promise for other cannabinoids such as CBD for endocannabinoid and non-cannabinoid-based modulation in the treatment of post-traumatic inflammation, cerebrovascular dysregulation, ROS accumulation and epithelial disruption via TRPV-1, 5-hydroxytryptamine receptors (5-HT1A), PPARγ, and adenosine receptors (204). Preliminary data shows that when administered together, CBD and THC elicit significantly different behavioural and metabolic effects than when administered alone (205), potential due in part to dual modulation of CB1R and CB2R in addition to other remote and lesser explored receptor targets. CBD alone also deserved future exploration due to its activity at CB2R, who's activity has already been shown to produce robust CNS anti-inflammatory activity (103, 206, 207). A point of interest emerging from the results of CARE-E trials of pediatric treatment-resistant epilepsy by Huntsman et al. (2019) have found that a 1:20 ratio of THC to CBD using

whole plant extract is more effective than its isolated counterpart, suggesting potential entourage effects of lesser-known cannabinoids that could translate to TBI-induced excitotoxicity and seizures (208). As this available information suggests a plethora of pharmacotherapeutic avenues of cannabinoids deserving of investigation.

4.12 Conclusion

The TBI model used in this study decreased rotarod performance for male and not female rats, but did not impact performance in the OFT or Y-maze. THC treatment decreased rotarod performance in Sham-TBI male rats compared to Sham-TBI male rats treated with vehicle but had no other lasting behavioural or physiological effects in males or females. Thus, a single dose of 1 mg/kg THC was deleterious with respect to motor function in otherwise healthy male rats; but this single 1 mg/kg THC dose was otherwise benign in otherwise healthy female rats and male and female TBI rats. Therefore, we can conclude that (1) consequences of both TBI and THC treatment are sex-dependent in rats; and (2) in an animal model of mTBI, THC did not overtly change mTBI recovery according to the behaviour measures used in this study. These mixed effects validate the importance of studying sex as a biological variable of pathophysiological and pharmacological responses.

Ongoing investigations of CNS cytokine, endocannabinoid, and CB1R and CB2R concentration are required to further elucidate modulatory effects and sex differences. The lack of pharmacological treatment and impaired quality of life associated with TBIs makes the continued investigations of the endocannabinoid system and the role it plays in the modulation of secondary injury mechanisms in TBI key to the discovery and understanding of novel treatment strategies.

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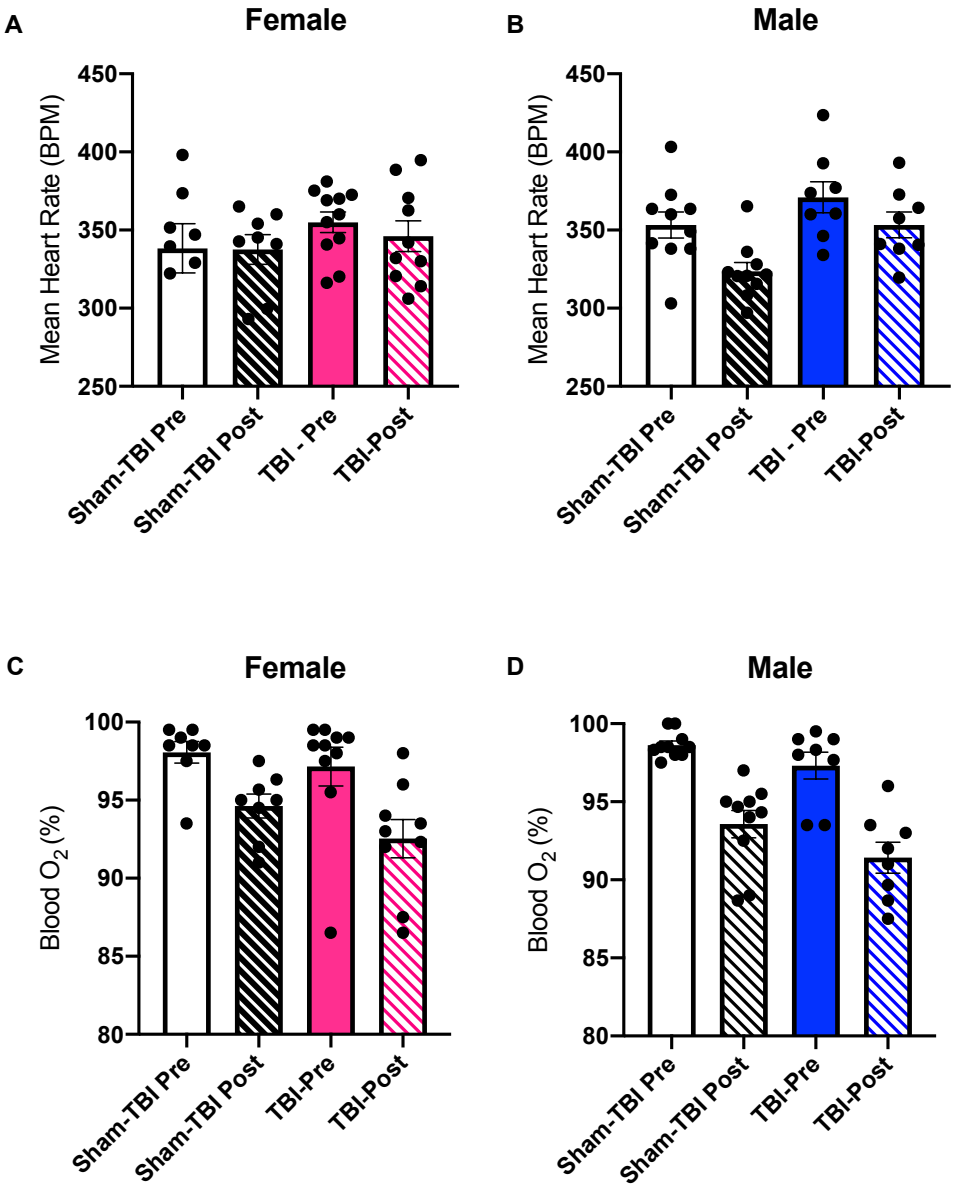
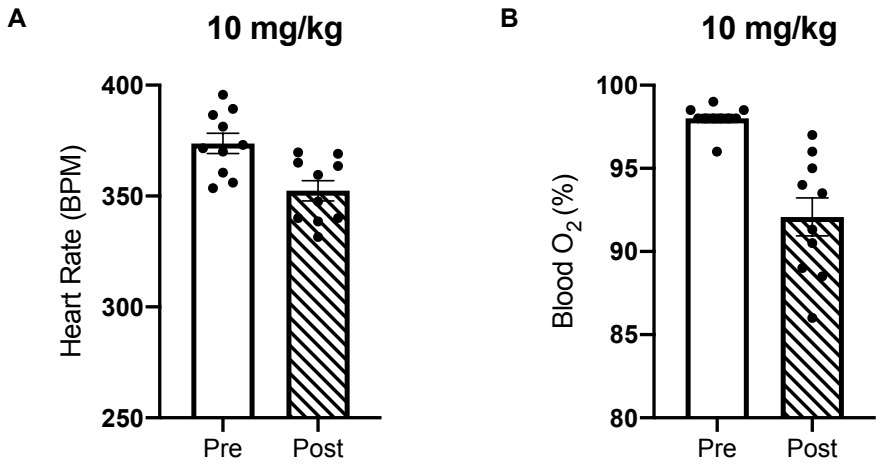
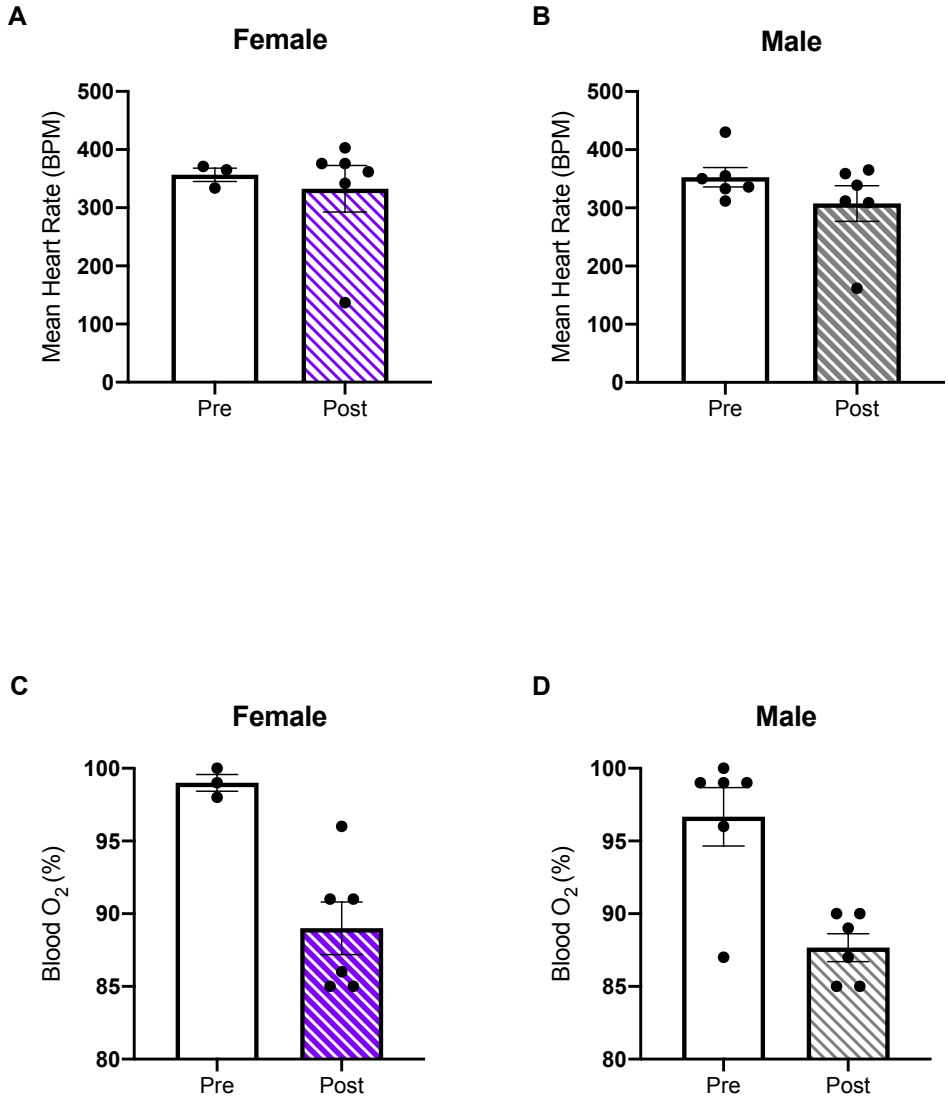
Appendices



A.1. Heart rate and blood oxygen levels of 1 mg/kg THC experiment Heart rate (**A, B**) and blood oxygen levels (**C,D**) for male and female rats treated with 1 mg/kg THC *i.p*. Data are grouped as pre- and post-TBI or Sham-TBI**.** Data are presented as mean ± S.E.M. with symbols for individual observations n=8-10 per group (males and females).



A.2. Heart rate and blood oxygen levels of 1 mg/kg THC experiment. Heart rate (**A***)* and blood oxygen levels (**B**) for male and female rats (combined) treated with 10 mg/kg THC *i.p.* Data are grouped as pre- and post-TBI**.** Data are presented as mean ± S.E.M. with symbols for individual observations n=10 per group (males and females, combined).



A.3. Heart rate and blood oxygen levels of pilot experiment. Heart rate (**A, B**) and blood oxygen levels (**C, D**) for male and female rats in the pilot group experiment. Data are grouped as pre- and post-TBI**.** Data are presented as mean ± S.E.M. with symbols for individual observations n=6 per group (males and females).

A

Male 1 mg/kg	Treatment:	Body weight (g)	Female 1 mg/kg	Treatment:	Body weight (g)
C1M4	SHAM TBI +THC	468	C1F1	SHAM TBI +THC	241
C1M6	SHAM TBI +THC	449	C3F1	SHAM TBI +THC	265
C3M5	SHAM TBI +THC	651	C3F3	SHAM TBI +THC	299
C2M2	SHAM TBI +THC	528	C2F6	SHAM TBI +THC	299
C2M3	SHAM TBI +THC	500	C1F3	SHAM TBI +VEH	250
C1M3	SHAM TBI +VEH	450	C3F2	SHAM TBI +VEH	276
C1M5	SHAM TBI +VEH	442	C3F5	SHAM TBI +VEH	284
C3M6	SHAM TBI +VEH	525	C2F3	SHAM TBI +VEH	303
C2M4	SHAM TBI +VEH	527	C3F6	TBI+THC	230
C2M5	SHAM TBI +VEH	509	C1F2	TBI+THC	230
C3M1	TBI+THC	416	C1F6	TBI+THC	284
C3M4	TBI+THC	536	C2F4	TBI+THC	246
C1M2	TBI+THC	446	C2F5	TBI+THC	301
C2M1	TBI+THC	499	C3F4	TBI+VEH	210
C3M2	TBI+VEH	472	C1F4	TBI+VEH	233
C3M3	TBI+VEH	504	C1F5	TBI+VEH	249
C1M1	TBI+VEH	516	C2F1	TBI+VEH	261.1
C2M6	TBI+VEH	521	C2F2	TBI+VEH	287

B

Cohort: 10 mg/kg	Treatment:	Body weight (g)
C4F2	THC	453
C4F4	THC	387
C4M2	THC	725
C4M4	THC	769
C4M6	THC	777
C4F1	Vehicle	452
C4F3	Vehicle	403
C4M1	Vehicle	760
C4M3	Vehicle	785
C4M5	Vehicle	697

A.4. Recorded weights of male and female rats at the time of injury. (**A**) Represents weight in grams of male and female rats from the 1 mg/kg experiment. (**B**) Represents weight in grams of combined male and female rats from the 10 mg/kg experiment.