BRAIN COMMUNICATIONS

Investigating the cumulative effects of Δ9-tetrahydrocannabinol and repetitive mild traumatic brain injury on adolescent rats

Dhyey Bhatt,[1] Ali Hazari,[1] Glenn R. Yamakawa,[1,2] Sabrina Salberg,[1,2] Marissa Sgro,[2] Sandy R. Shultz[2,3] and Richelle Mychasiuk[1,2]

The prevalence of mild traumatic brain injury is highest amongst the adolescent population and can lead to complications including neuroinflammation and excitotoxicity. Also pervasive in adolescents is recreational cannabis use. Δ9-Tetrahydrocannabinol, the main psychoactive component of cannabis, is known to have anti-inflammatory properties and serves as a neuroprotective agent against excitotoxicity. Thus, we investigated the effects of Δ9-tetrahydrocannabinol on recovery when administered either prior to or following repeated mild brain injuries. Male and female Sprague-Dawley rats were randomly assigned to receive Δ9-tetrahydrocannabinol or vehicle either prior to or following the repeated injuries. Rats were then tested on a behavioural test battery designed to measure post-concussive symptomology. The hippocampus, nucleus accumbens and prefrontal cortex were extracted from all animals to examine mRNA expression changes (*Bdnf*, *Cnr1*, *Comt*, *GR*, *Iba-1* and *Vegf-2R*). We hypothesized that, in both experiments, Δ9-tetrahydrocannabinol administration would provide neuroprotection against mild injury outcomes and confer therapeutic benefit. Δ9-Tetrahydrocannabinol administration following repeated mild traumatic brain injury was beneficial to three of the six behavioural outcomes affected by injury (reducing anxiety and depressive-like behaviours while also mitigating injury-induced deficits in short-term working memory). Δ9-Tetrahydrocannabinol administration following injury also showed beneficial effects on the expression of *Cnr1*, *Comt* and *Vegf-2R* in the hippocampus, nucleus accumbens and prefrontal cortex. There were no notable benefits of Δ9-tetrahydrocannabinol when administered prior to injury, suggesting that Δ9-tetrahydrocannabinol may have potential therapeutic benefit on post-concussive symptomology when administered post-injury, but not pre-injury.

1 Department of Psychology, Alberta Children's Hospital Research Institute, Hotchkiss Brain Institute, The University of Calgary, Calgary, AB T2N 4N1, Canada
2 Department of Neuroscience, Central Clinical School, Monash University, Melbourne, VIC 3004, Australia
3 Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Melbourne, VIC 3050, Australia

Correspondence to: Richelle Mychasiuk, Department of Neuroscience, Central Clinical School,
Monash University, 6th Floor, 99 Commercial Road, Melbourne VIC 3004, Australia
E-mail: richelle.mychasiuk@monash.edu

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Abbreviations: Bdnf = brain derived neurotrophic factor; CB = cannabinoid receptors; Cnr1 = cannabinoid receptor 1; Comt = catechol-O-methyltransferase; EPM = elevated plus maze; ELISA = enzyme-linked immunosorbent assay; GR = glucocorticoid receptor; HPC = hippocampus; Iba-1 = ionized calcium-binding adaptor molecule 1; NAc = nucleus accumbens; mTBI = mild traumatic brain injury; PCS = post-concussive symptomology; PFC = prefrontal cortex; P = postnatal day; PID = post-injury day; RT-qPCR = real-time quantitative polymerase chain reactions; RmTBI = repeated mild traumatic brain injury; TBI = traumatic brain injury; THC = Δ^9-tetrahydrocannabinol; Vegf-2R = vascular endothelial growth factor receptor 2

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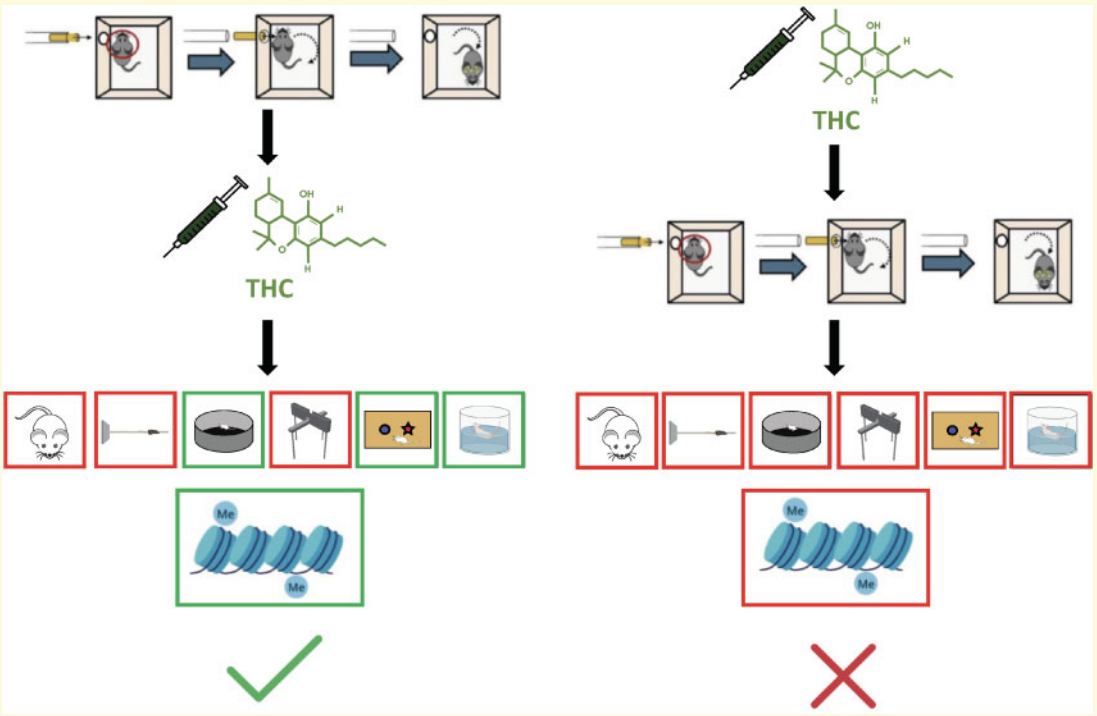
Graphical Abstract



Introduction

Concussions, clinically referred to as mild traumatic brain injuries (mTBIs), are the most common type of traumatic brain injury (TBI), accounting for 80% of all TBI cases (Clark and Guskiewicz, 2016). Children and youth in particular, have the highest rates of mTBI, with more than 23% of hospital-reported mTBIs being diagnosed in adolescents (McKinlay *et al.*, 2008; Barlow *et al.*, 2010; Halstead and Walter, 2010). mTBIs are caused by inertial forces acting on the brain at the moment of impact; these forces result in both linear and rotational acceleration of the brain that lead to brain movement within, and against the skull (Meaney and Smith, 2011). This collision results in compression, straining, and shearing of brain tissue and such brain damage can cause neuronal degeneration and many observable deficits (Meaney and Smith, 2011). The numerous long-term neurophysiological effects associated with mTBI are grouped into what is known as post-concussive symptomology (PCS) (Barlow *et al.*, 2010). Between 10% and 50% of individuals diagnosed with concussion exhibit the persistent symptoms of PCS, which include physical, behavioural, emotional and cognitive symptoms, such as amnesia, insomnia, increased aggression, erratic behaviour and cognitive deficits (Barlow *et al.*, 2010; Solomon *et al.*, 2011). Furthermore, there is growing concern regarding recent evidence demonstrating that repeated mTBIs (RmTBI) can result in cumulative and lasting effects in the adolescent brain (Zhang *et al.*, 2016; Wright *et al.*, 2017; Yamakawa *et al.*, 2017).

Adolescents not only have high rates of mTBIs but also of cannabis use (Barlow *et al.*, 2010; Rotermann and MacDonald, 2018). In Canada, for example, by 18 years of age, more than 31% of youth have reported using cannabis three times or more, making it the most widely used illicit drug by the adolescent population (Arseneault *et al.*, 2002). Δ^9-Tetrahydrocannabinol (THC), the main psychoactive component of cannabis, acts on cannabinoid receptors (CB1) in the central nervous system and (CB2) in the immune system (Bridgeman and Abazia, 2017). Since 1994, increasing rates of recreational THC and cannabis use has been linked to athletes at the international and collegiate levels, as well as in various amateur and leisure sports (Campos *et al.*, 2003). In addition, the high-risk, high-intensity environment generated in sport is associated with a 10–20% increased risk for head collisions and mTBIs (Kelly *et al.*, 2012; Gardner and Yaffe, 2015).

The rotational and linear biomechanical forces that produce mTBI typically result in diffuse damage to white matter throughout the brain as axons are particularly sensitive to stretching induced injury (Bigler, 2001; Buki and Povlishock, 2006; Fidan *et al.*, 2018). A secondary consequence of mTBI in the initiation of neurometabolic cascades whereby cytokines are upregulated, there are changes in the subunits of N-methyl-D-aspartate receptors, broad and non-specific glutamate release, efflux of

potassium ions and influxes of calcium and sodium, which causes the over-activation of ATP-dependent membrane pumps to restore ionic concentrations (Giza and Hovda, 2014; Sun *et al.*, 2019). Although endocannabinoids and cannabinoid drugs have been thoroughly investigated in the context of mTBI [for review see Mechoulam and Shohami (2007)], there has been a void in the literature regarding THC and TBI. This is surprising given that THC, reduces excess glutamate release, offers protection against neuroinflammation and has potent antioxidative properties (Hampson *et al.*, 1998; Hayakawa *et al.*, 2007; Fishbein-Kaminietsky *et al.*, 2014).

The importance of studying THC exposure in the context of mTBI is therefore apparent as there is significant overlap in risk for THC use and mTBI in adolescent populations, and there are notable commonalities between mTBI pathology and the neuroprotective potential of THC. The first study examined the therapeutic potential of THC on RmTBI, providing rats with daily injections of THC while examining PCS. The second study examined the effects of intermittent THC exposure before RmTBI, as this was ethologically relevant and could provide insight into the neuroprotective potential of THC. Using a clinically relevant model of concussion, the lateral impact device (Mychasiuk *et al.*, 2016*a*, *b*), rats were administered RmTBIs or sham injuries, and were then subsequently tested on a behavioural test battery designed to measure post-concussion symptomatology. The hippocampus (HPC), nucleus accumbens (NAc) and prefrontal cortex (PFC) of the rats were also extracted to examine mRNA changes in the brain associated with mTBI and THC exposure. The genes *Bdnf*, *Cnr1*, *Comt*, *GR*, *Iba-1* and *Vegf-2R* were examined to quantify the differential effects of treatment on recovery from mTBI. We hypothesized that THC would offer therapeutic benefits, reducing the severity of PCS in both groups of rats that received THC either before, or following the RmTBIs.

Materials and methods

Animals

Male and female Sprague-Dawley rats were obtained from Charles River Laboratories (QC, Canada) and arrived on postnatal day (P) 21. Rats were housed in groups of four in a temperature- and humidity-controlled husbandry room at 22–24°C with controlled humidity at ~30%. The rats were maintained on a 12:12 light-dark cycle (lights on at 0700). Rats had *ad libitum* access to water and standard rat chow. All experiments were carried out in accordance with the Canadian Council of Animal Care and were approved by the University of Calgary Conjoint Faculties Research Ethics Board.

For Study 1, rats were randomly assigned to a vehicle control group ($n = 12$) or a post-injury treatment group ($n = 20$). Rats were further divided into either an RmTBI group or a sham injury group generating groups of, (i) post-THC + RmTBI ($n = 5$M and 5F), (ii) post-THC + sham ($n = 5$M and 5F), (iii) placebo + RmTBI ($n = 3$M and 3 F) and (iv) placebo + sham ($n = 3$M and 3F). The daily treatment of THC was designed to model a therapeutic paradigm whereby an individual may have been prescribed THC following RmTBI. Twelve daily intraperitoneal (I.P.) injections were administered between P44 and P54, with the first injection occurring the day after the last injury, at a dose of 1.25 mg/kg. All injections were given at least 30 min after the behavioural testing procedure for that particular day was completed. See Fig. 1A for experimental timeline.

For Study 2, rats were randomly assigned to a vehicle control group ($n = 12$) or an intermittent pre-injury treatment group ($n = 20$). Rats were further divided into either an RmTBI group or a sham injury group generating groups of (i) pre-THC + RmTBI ($n = 5$M and 5F), (ii) pre-THC + sham ($n = 5$M and 5F), (iii) placebo + RmTBI ($n = 3$M and 3F) and (iv) placebo + sham ($n = 3$M and 3F). Six I.P. injections of drug or vehicle were given intermittently between P29 and P43, at a dose of 1.25 mg/kg. The intermittent dosage of THC was designed to provide a realistic model of individuals that are casual consumers of THC before RmTBI and then quit when they experience concussion-related symptoms. See Fig. 1B for experimental timeline.

Experimental procedures

Drugs

THC (Sigma-Aldrich, Oakville, ON, Canada) dissolved in dimethyl sulfoxide (DMSO) (10 mg/ml) was mixed to make a 1:1:18, THC/DMSO: Tween80: Saline injection stock for a final 5% drug concentration. The vehicle was prepared similarly for a 1:1:18, DMSO: Tween80: Saline stock solution. Fresh stock was made daily. Rats were I.P. injected with a low-moderate dose of THC (1.25 mg/kg) or an equivalent volume of vehicle stock.

Lateral impact device

This clinically relevant model of concussion emulates the acceleration/deceleration forces associated with sports-related injuries using controlled speeds to deliver precise injuries (Viano *et al.*, 2009). RmTBIs were administered 3 days apart (P36, P40 and P43), at approximately 82 Gs, which is well above the sub-concussion threshold that has been previously defined as ~35Gs (Guskiewicz *et al.*, 2007; Guskiewicz and Mihalik, 2011). The 3-day inter-injury interval was used as we have previously demonstrated this produces greater impairment than a single injury and is more representative of the symptomologies associated with adolescent RmTBI (Wright *et al.*, 2017). The force exerted by the lateral impact propels the rat into a 180-degree rotation, mimicking the lateral acceleration and rotational forces experienced in sports-related

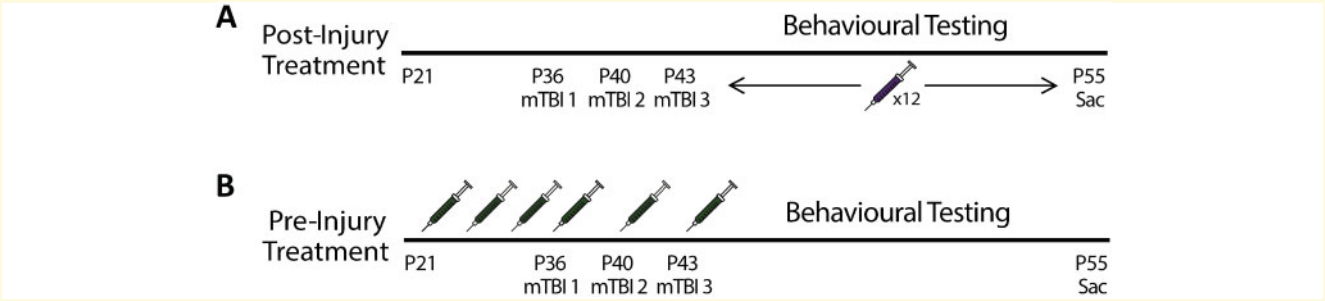


Figure 1 Experimental timelines for (A) Study 1 and (B) Study 2.

injury. Before the administration of the mTBI, rats were anaesthetized with isoflurane gas until the plantar reflex was no longer present. Rats were subsequently placed in a prone position onto a Teflon board, adjacent to the lateral impact device, such that the left side of the head was facing the impactor device. A 50 g cylindrical weight was propelled at an aluminium plate placed against the rat's head at 8.96 ± 0.12 m/s. Xylocaine was applied to the site of impact and rats were allowed to recover in a warm, clean cage before being placed back into their home cage. The time-to-right was also recorded for each rat and was measured from the time of impact to the time the rat was able to right itself to prone position from being laid on its back. The sham animals underwent the same procedure but were removed from the lateral impact device without insult.

Behavioural testing

Following RmTBI, animals were subjected to a battery of behavioural tests designed and temporally sequenced to examine the clinical symptoms associated with post-concussive syndrome (Mychasiuk *et al.*, 2014; Mychasiuk *et al.*, 2015).

Beam walk (P44)

Twenty-four hours following the third injury (post-injury Day 1—PID1), the beam walk task was administered. This task is designed to measure balance and motor co-ordination (Schallert *et al.*, 2002). One-hundred and sixty-five centimetres in length, the beam was tapered such that it narrows towards the home cage (which was used as a reinforcing cue). The beam was raised 1 m above the ground and was equipped with safety ledges, 2 cm in length on either side of the beam to catch the rat's leg if it slipped. Each rat performed five trials, with 1 min between each trial to reinforce the goal of the task. The final four trials were recorded and scored to determine the time to cross the beam, for each trial.

Elevated plus maze (EPM) (P46)

To measure anxiety-like behaviour, the EPM task was administered (Whishaw and Kolb, 2005). The elevated plus maze (EPM) arena consisted of two open arms and two closed arms in the form of a plus, 55 cm above the ground. At the beginning of the trial, rats were placed in the centre of the maze facing a closed arm. Each trial was recorded for 5 min and scored by a blinded observer to measure time spent in the open arms and the closed arms. More time spent in the open arms indicates decreased anxiety-like behaviour.

Novel context mismatch (P47–P50)

The novel context mismatch protocol adapted from Spanswick and Sutherland (2010) was used as a measure of short-term working memory. During the training phase (P47–P49) rats were subjected to 5 min in two discrete contexts each containing a pair of unique objects. Context A was a clear rectangular arena containing a pair of novel objects, and Context B was an opaque, blue rectangular arena containing two different novel objects. On the final day (P50), rats were first placed in Context A for 5 min, then Context B for 5 min, and an additional recorded trial in which the rats were placed in the arena from Context B, with an object from the original Context B (familiar) and an object from the original context A (novel) for 5 min after having been given time to rest in their home cage for 5 min. In the probe trial, more time spent exploring the novel object as opposed to the familiar object indicates increased memory recall. The trials were scored to measure time spent interacting with each object by a condition blind researcher.

Open field (P51)

Rats were allowed to explore a circular arena spanning 135 cm in diameter for 10 min. The task was used as a measure of locomotor activity and exploratory behaviour (Whishaw and Kolb, 2005). An overhead camera tracked movement and locomotor activity (distance travelled and velocity). Time spent in the centre of the arena was also used as an additional measure of anxiety-like behaviour.

Forced swim (P55)

The forced swim task was administered on PID12 and was used as a measure of depressive-like behaviour (Yadid *et al.*, 2001). Rats were placed in a 30-cm

diameter by 60-cm high cylindrical container filled with warm water (~25°C) and were recorded for 7 min. The trials were scored to determine time spent immobile as a measure of depressive-like behaviour.

Tissue collection

Animals were placed under light anaesthesia using isoflurane before being euthanized via guillotine decapitation. The HPC, NAc and PFC were extracted from both hemispheres of each rat brain and quickly flash frozen on dry ice and then stored at −80°. An ear notch was also collected from each rat in order to run telomere length analysis. Blood from the trunk was collected for serum enzyme-linked immunosorbent assay (ELISA) analysis. Using serum separator tubes (BD, Franklin Labs, NJ, USA) samples were left to clot for 30 minutes at room temperature, followed by a 15-minute centrifuge at 100 g and 4 degrees. Serum was then stored at -80 degrees.

DNA and RNA extraction for real-time quantitative polymerase chain reactions

RNA was extracted from the brain regions using the Allprep RNA/DNA Mini Kit (Qiagen) according to manufacturer's protocol. Genomic DNA was extracted from ear notch samples using the DNeasy Blood and Tissue kit (Qiagen, Germany). Concentration and purity were determined using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Two micrograms of RNA were reverse transcribed to make cDNA using oligo(dt)20 of the Superscript III First Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA). cDNA was diluted to 10 ng/µl and used to perform real-time quantitative polymerase chain reactions (RT-qPCR) using forward and reverse primers designed and purchased from Integrated DNA Technologies (Coalville, IA). Telomere length analysis was conducted using the protocol described elsewhere (Cawthorn, 2002; Hehar and Mychasiuk, 2016).

Brain regions and gene selection

The HPC was chosen for the role it plays in learning, memory, and emotional regulation. The response of the HPC to THC has been the subject of controversy despite this region being particularly vulnerable to stimuli. Some studies show decreased hippocampal volume following THC use, while others claim there is no effect (Yücel *et al.*, 2016; Koenders *et al.*, 2017). The NAc is the brain's reward system and processes motivation, pleasure, and reinforcement, thus playing an important role in addiction and exposure to THC (Kolb and Whishaw, 2008). The PFC is one of the most vulnerable brain regions to experience-dependent changes during maturation and is important for executive functioning (Mychasiuk and Metz, 2016). Long-term consequences of mTBI experienced during adolescence are associated with deficits of PFC function and include persistent impulsiveness, and subtle deficits in psychomotor speed and visual-spatial skills (Toledo *et al.*, 2012).

This study included six genes of interest. Brain derived neurotrophic factor (*Bdnf*) is a significant regulator of synaptic plasticity, is involved in neurogenesis and cellular differentiation and has shown to be modulated in response to injury (Horch and Katz, 2002; Sagarkar *et al.*, 2017; Ferreira *et al.*, 2018). The cannabinoid receptor 1 gene (*Cnr1*) codes for the CB1 receptor, and is the primary receptor for THC binding (Comings *et al.*, 1997). Catechol-O-methyltransferase (*Comt*) is responsible for the degradation of catecholamine-based neurotransmitters such as dopamine, epinephrine, and norepinephrine. *Comt* is known to modulate the acute effects of THC on neuronal function (Bossong *et al.*, 2019). The glucocorticoid receptor (*GR*) is the primary binding site for corticosterone, which is released in response to stress, and can therefore be used as an indirect marker for functioning of the stress response and the hypothalamus-pituitary-adrenal (HPA) axis (Harris *et al.*, 2013). The ionized calcium-binding adaptor molecule 1 (*Iba1*) is a pro-inflammatory marker, associated with microglial activation, that has been shown to be involved in the neuroinflammatory processes that follow TBI (Sundman *et al.*, 2015). Finally, vascular endothelial growth factor receptor 2 (*Vegf-2R*) mediates the effects of VEGF, promoting angiogenesis and preventing apoptosis (Blázquez *et al.*, 2004), and changes in *Vegf-2R* expression have been associated with TBI outcomes (Xiong *et al.*, 2011).

Enzyme-linked immunosorbent assays

A sandwich ELISA was run in order to measure the stress marker corticosterone and the cytokine IL-1B. The kits (AB108821 Corticosterone ELISA Kit Abcam, Cambridge, USA; BMS630/BMS630TEN- Rat IL-1B, Invitrogen-Affymetrix, Carlsbad, USA) were used according to manufacturer's protocols. Samples were run in duplicate on a 96-well plate and were measured with the BioTek Synergy H.T. plate reader and Gen5 2.00.18 software using a path length correction algorithm. All measured samples were within the standard curve.

Statistical analyses

All statistical analysis was carried out on SPSS 25.0 for Mac. For Study #1, three-way ANOVAs were run with Sex (male; female), Injury (sham; RmTBI) and THC (vehicle; post-THC) as factors. For Study #2, three-way ANOVAs were run with Sex (male; female), Injury (sham; RmTBI) and THC (vehicle; pre-THC) as factors. *Post hoc* pairwise comparisons (LSD) were performed where applicable for interaction effects. Statistical

significance was considered $P < 0.05$ and means ± standard errors are displayed for all figures.

Data availability

All data are available upon request from the corresponding author.

Results study 1

Behavioural outcomes

Administration of THC following RmTBI altered behaviour on 3/6 measures (EPM, novel context mismatch, and forced swim) for both sham and mTBI animals, while RmTBI produced dysfunction in 4/6 tests [time to right, EPM, open field (distance travelled and time in centre) see Fig 2B–D]. A significant TBI × THC interaction was observed in the novel context mismatch task, that was driven by the TBI group, whereby THC exposure improved performance when compared to vehicle treatment, $P < 0.01$, see Fig. 2E. In addition, a significant three-way interaction was identified in the forced swim task that was partially driven by female-THC animals whereby RmTBI animals spent less time immobile than sham animals, $P = 0.01$, and in the female-TBI group where THC animals spent less time immobile that vehicle animals $P = 0.01$, see Fig. 2F. See Table 1 for statistical results and Fig. 2 for graphical representation of the findings.

Serum and telomere results

See Fig. 3 for graphical representation of these findings. The results from the three-way ANOVA for serum *corticosterone* demonstrated a main effect of sex, $F(1,28) = 4.81$, $P = 0.04$, but not of injury, $F(1,28) = 1.79$, $P = 0.19$, or of THC, $F(1,28) = 0.73$, $P = 0.41$, see Fig. 3A. None of the interactions were significant. The results from the three-way ANOVA for serum *Il-1B* failed to identify a main effect of sex, injury or THC, $Ps > 0.05$, but did demonstrate a significant TBI × THC interaction, $F(1, 24) = 4.78$, $P = 0.04$, see Fig. 3B. In addition, a significant TBI × THC interaction was observed in the telomere length analysis, $F(1,30) = 4.33$, $P = 0.05$, whereby TBI reduced telomere length in the vehicle group, but not in the THC group, see Fig. 3C.

Changes in mRNA expression

All statistical results for the mRNA three-way ANOVA results can be found in Table 2 and graphical representation found in Figs 4–6. Although there was not an abundance of main effects for TBI or THC, there were numerous TBI × THC interactions.

In the PFC, the sex by THC interaction for *Bdnf* was driven by females whereby animals in the THC group exhibited lower levels of *Bdnf* than vehicle rats $P < 0.01$. The sex by TBI by THC interaction for *Bdnf* was driven by the sham rats whereby males differed from females in the vehicle and in the THC groups, $P = 0.02$, see Fig. 4A. For *Cnr1* the TBI by THC interaction was moderated by the vehicle group where RmTBI animals exhibited significant reductions in *Cnr1* compared to sham animals, $P = 0.03$. The sex by TBI by THC interaction for *Cnr1* was driven by females whereby vehicle sham animals displayed higher levels of *Cnr1* expression compared to THC sham animal $P = 0.04$, and the THC group where females with RmTBI also exhibited higher levels of *Cnr1* than THC sham animals $P = 0.01$, see Fig. 4B. For the *GR*, the three-way interaction was driven largely by females, where sham-vehicle animals had higher levels of *GR* than sham THC rats $P < 0.01$, and in the female-THC group where RmTBI animals exhibited higher levels of *GR* than sham animals, $P = 0.01$. With respect to *Iba-1*, the three-way interaction was driven by the injury groups whereby male and female rats in the sham-vehicle group differed significantly $P = 0.02$, and also in the TBI–THC groups, $P < 0.01$, see Fig. 4D. In addition, the sham and RmTBI animals in the female-THC group exhibited significantly different levels of *Iba-1* expression, $P < 0.01$, see Fig. 4E. Finally, for *Vegf-2R* the TBI by THC interaction was driven by animals in the vehicle group, where RmTBI rats exhibited significant reductions in *Vegf-2R* compared to sham animals $P < 0.01$, see Fig. 4F.

In the HPC, the significant sex by TBI interaction for *Bdnf* was driven by the sham animals where the males displayed higher levels of *Bdnf* than the females, $P = 0.02$, see Fig. 5A. For *Cnr1*, the TBI by THC interaction was identified in the vehicle groups where RmTBI animals have a reduction in *Cnr1* compared to sham animals, $P < 0.01$, and in the TBI animals whereby THC increased *Crn1* compared to vehicle, $P < 0.01$, see Fig. 5B. Finally, for *GR*, the TBI by THC interaction was moderated by the sham group, where animals exposed to THC had reductions in *GR* expression compared to animals in the vehicle group, $P = 0.01$, see Fig. 5D.

With respect to the NAc, the three-way interaction for *Bdnf* was largely driven by the RmTBI-vehicle animals where females exhibited significant elevations in *Bdnf* compared to males, $P < 0.01$, and in the female-RmTBI animals where THC significantly reduced *Bdnf*, $P < 0.01$, see Fig. 5A. The TBI by THC interaction for *Comt* was driven by the sham animals, whereby rats in the vehicle group exhibited significant increases in *Comt* levels compared to animals that received THC, $P = 0.01$, see Fig. 5C. Lastly, the TBI by THC interaction for *Vegf-2R* was driven by the sham animals whereby THC significantly decreased *Vegf-2R* levels as compared to vehicle treated animals, $P = 0.03$, see Fig. 5F.

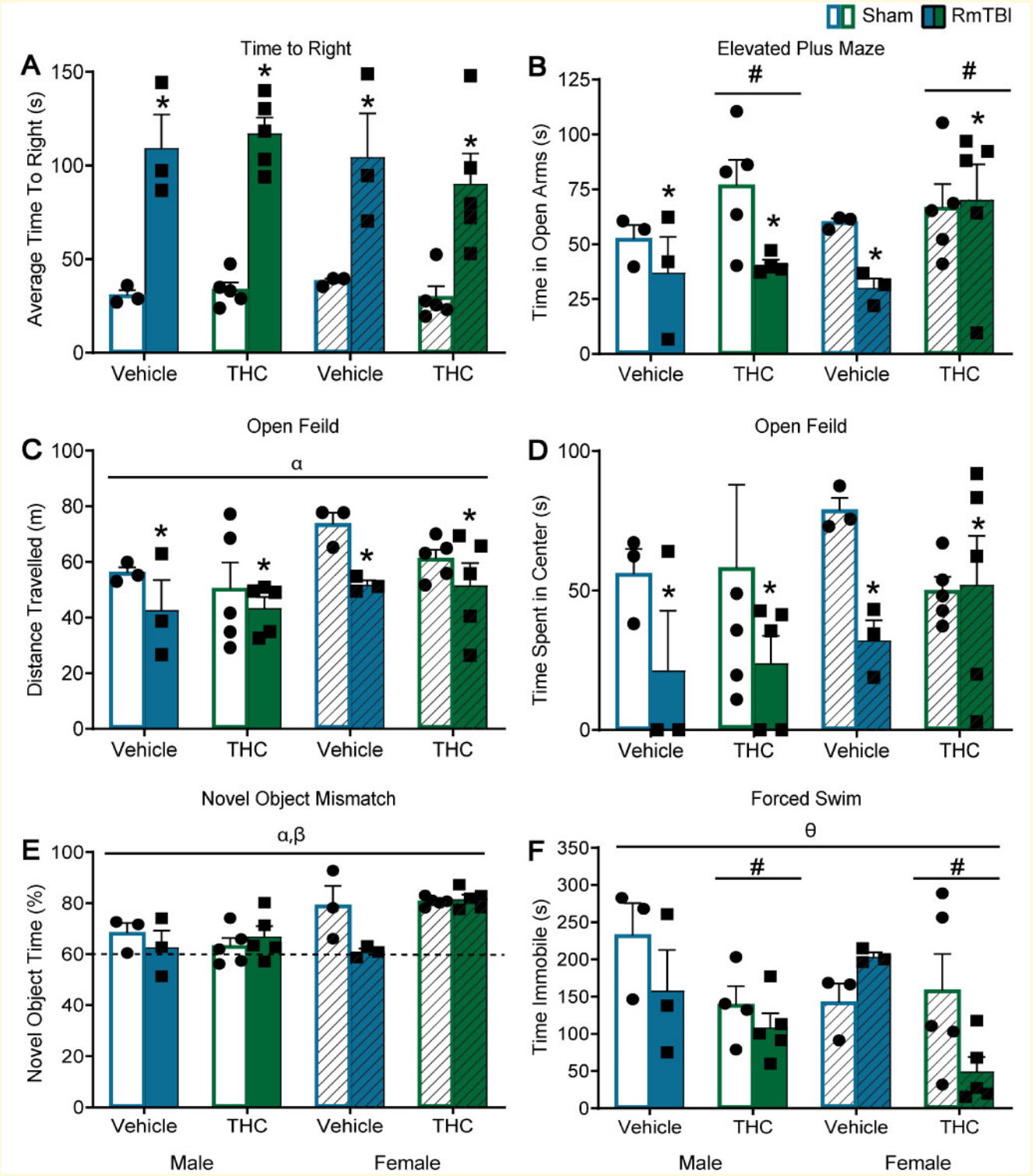


Figure 2 Graphical depictions of the behavioural testing results displayed as mean ± SEM for the post-injury THC administration groups. White and white hashed bars display sham groups (males and females, respectively), while blue and green bars display RmTBI groups. Statistical significance symbols are displayed above the bars (α) indicates a main effect for sex, (*) indicates a main effect for RmTBI, (#) indicates a main effect for THC, (β) indicates a RmTBI by THC interaction and (θ) indicates a sex by RmTBI by THC three-way interaction. (**A**) The mean time to right following injury or sham procedures. (**B**) The mean time spent in the open arm of the EPM. (**C** and **D**) The mean distance travelled and time spent in the centre of the open field respectively. (**E**) The percentage of time spent investigating the novel object in the novel context mismatch with the dotted line representing the minimum expected level of recognition (60%). (**F**) The mean time spent immobile during the forced swim test.

Table 1 Statistical results for the three-way ANOVAs for the behaviour tasks in Study #1

Behavioural task	Effect of sex F (*P*)	Effect of TBI F (*P*)	Effect of THC F (*P*)	Significant interactions F (*P*)
Time to right	0.42 (0.53)	68.32 (0.00)	0.23 (0.64)	N/A
Beamwalk	1.12 (0.30)	2.13 (0.16)	0.59 (0.45)	N/A
EPM	0.29 (0.59)	4.64 (0.04)	4.28 (0.05)	N/A
Novel context mismatch	12.77 (0.00)	2.67 (0.11)	3.42 (0.07)	TBI × THC—6.08 (0.02)
Open field (distance travelled)	4.72 (0.04)	5.84 (0.02)	0.73 (0.40)	N/A
Open field (time in centre)	0.91 (0.35)	4.32 (0.05)	0.01 (0.96)	N/A
Forced swim	0.64 (0.43)	2.41 (0.16)	7.78 (0.01)	Sex × TBI × THC—4.53 (0.04)

Results study 2

Behavioural outcomes

See Table 3 for statistical results and Fig. 7 for graphical representation of the findings. Intermittent administration of THC prior to RmTBI did not alter behaviour with the exception of time to right, while mTBI produced dysfunction in 4/6 tests (time to right, beamwalk, EPM, forced swim, Fig. 7A, B and F, respectively) and there was a trend towards an effect in the novel context mismatch task (Fig. 7E). There was also a significant Sex × THC interaction in the time spent in the centre of the open field whereby females exposed to THC spent more time in the centre of the open field than males in the THC groups, $P < 0.01$, and where males treated with vehicle spent more time in the centre than males treated with THC, $P = 0.01$, see Fig. 7D.

Serum and telomere results

The results from the three-way ANOVA for serum *corticosterone* failed to demonstrate any main effects, but did identify a significant TBI × THC interaction, $F(1,23) = 5.38$, $P = 0.04$. The results from the three-way ANOVA for serum *Il-1B* failed to identify any main effects or significant interactions, $Ps > 0.05$. The three-way ANVOA for *telomere length* did, however, identify a main effect of sex, $F(1,31) = 5.38$, $P = 0.03$, and a main effect of Injury, $F(1,31) = 12.71$, $P < 0.01$, but not of THC, $F(1,31) = 0.17$, $P = 0.69$; none of the interactions were significant. Telomere length was significantly reduced in response to RmTBI and in males compared to females.

Given that there were no significant effects of intermittent pre-injury THC exposure on behavioural outcomes, nor any significant TBI × THC interactions, despite significant main effects of RmTBI, we did not examine changes in mRNA expression for Study #2.

Discussion

The objective of this study was to observe the effects of THC on the adolescent brain in the context of RmTBI. Specifically, the study aimed to examine the efficacy of THC as a therapeutic for remediation of PCS, and also investigate whether or not intermittent THC exposure prior to RmTBI would affect recovery. We hypothesized that THC would have both therapeutic and neuroprotective potency against the negative consequences of mTBI. The results of this study suggest that THC exposure post-injury has some beneficial potential and warrants further investigation.

Study 1—therapeutic potential of THC for RmTBI

Although the risks associated with THC use in adolescence have been a topic of significant debate, substantial evidence has suggested that THC is a potent antioxidant with neuroprotective properties (Hampson *et al.*, 1998, 2000). In this study, RmTBI affected 4/6 of the behaviours examined, with therapeutic administration of THC following RmTBI producing beneficial outcomes in three of these measures. In vehicle treated animals, RmTBI increased loss of consciousness, increased anxiety-like behaviour in both the EPM and the open field, reduced exploratory behaviour and produced sex-dependent effects on depressive-like behaviours (reducing depression in males and increasing depression in females). Interestingly, daily administration of THC reduced anxiety in the EPM and reduced depressive-like behaviour in the forced swim task. These findings are in line with previous literature demonstrating that THC (at lower doses) reduces anxiety and emotional responses to psychosocial stress (Crippa *et al.*, 2009; Childs *et al.*, 2017), and suggests that THC may offer therapeutic potential for the treatment of RmTBI-induced anxiety. In addition, daily administration of THC mitigated RmTBI-induced impairments in short-term working memory. This is consistent with prior studies demonstrating that THC administration improves cognitive performance (Suliman *et al.*, 2018). In summary, results from the behavioural test battery suggest that daily administration of THC following RmTBI has potential therapeutic efficacy for the treatment of PCS.

Telomeres are evolutionarily conserved sequences of repetitive DNA at the ends of chromosomes that maintain the integrity of the genome and protect the DNA from oxidative stress (Zhu *et al.*, 2011). While telomere shortening has been recognized as a marker of biological aging and neurodegeneration (Klapper *et al.*, 2001), research in our laboratory has demonstrated that telomere

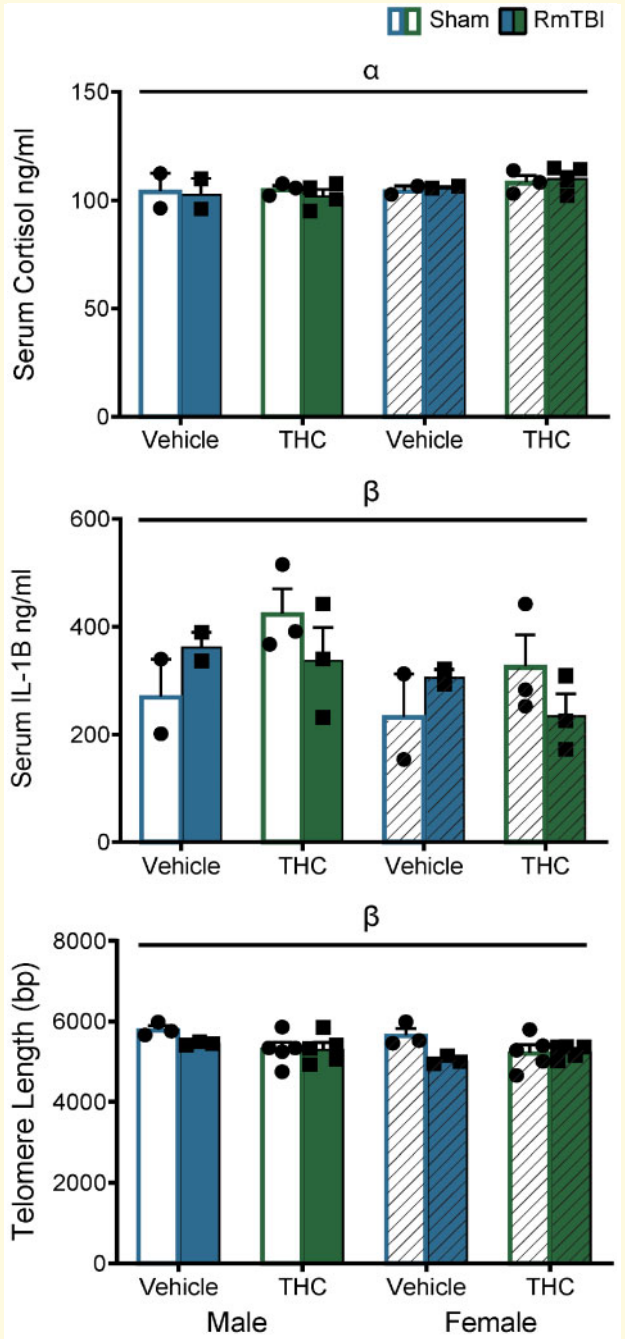


Figure 3 Graphical depictions of the ELISA serum cortisol and IL1B in addition to the telomere lengths displayed as means ± SEM for the post-injury THC administration groups. White and white hashed bars display sham groups (males and females, respectively), while blue and green bars display RmTBI groups. Statistical significance symbols are displayed above the bars (α) indicates a main effect for sex, (β) indicates a RmTBI by THC interaction.

shortening is a characteristic of mTBI and can be used as a prognostic tool in rodent models (Hehar and Mychasiuk, 2016; Wright *et al.*, 2018). Consistent with our previous studies, RmTBI reduced telomere length but interestingly, there was no RmTBI-induced reduction in telomere length for animals who were administered THC providing further support for the therapeutic benefits of THC.

The analyses of mRNA expression changes across the brain regions revealed numerous important neurological alterations associated with THC and RmTBI at the molecular level. First, we did not see any THC-induced changes in hippocampal *Bdnf* expression. This is important as evidence suggests that exogenous THC can affect long-term synaptic plasticity and remodel neurocircuitry (Gerdeman and Lovinger, 2003), and *Bdnf* has recently been implicated as a critical moderator of cannabinoid-mediated neurogenesis (Ferreira *et al.*, 2018). We did, however, see downregulation of *Bdnf* in the PFC and NAc of THC treated rats. This is contradictory to previous studies that have found an upregulation of *Bdnf* in the NAc and ventral tegmental area of adult rats who were chronically administered THC (Butovsky *et al.*, 2005). As studies have found that serum *Bdnf* levels are associated with whether or not individuals have a history of cannabis use (D'Souza *et al.*, 2009), the timing of sample collection, age of the rats and history of exposure likely contribute to the discrepant changes in *Bdnf* expression identified in this study.

The numerous interactions identified in mRNA expression changes of *Cnr1* are of particular relevance given that chronic THC exposure is known to alter the expression of *Cnr1* (Zhuang *et al.*, 1998) and cannabinoid receptor activation is critical for attenuation of brain damage following injury (Mechoulam and Shohami, 2007; Fishbein-Kaminietsky *et al.*, 2014). Exactly how the endocannabinoid system assists in neuroprotection and acute neuronal injury is not fully understood. However, the activation of CB1 receptors by THC causes an alteration in GABA/glutamergic neurotransmission, inhibition of acetylcholine release and modulates immune responses and the release of inflammatory mediators (Pacher *et al.*, 2006; Boggs *et al.*, 2018). Moreover, CB1 receptors located on post-synaptic neurons control N-methyl-D-aspartate ion channel activity and therefore, provide protection against acute excitotoxicity (Paloczi *et al.*, 2018). Given these functions of CB1 receptors, THC appeared to provide therapeutic benefit with respect to expression of *Cnr1* in the HPC of both males and females; while *Cnr1* mRNA levels were reduced in RmTBI animals treated with vehicle, they were increased in RmTBI animals treated with THC. This therapeutic benefit was also identified in the PFC, but only for female rats treated with THC.

TBI is believed to causes disruption to the catecholaminergic system, specifically because these neurons in the PFC are vulnerable to axonal injury, metabolic stress and mechanical injury, which often results in cognitive impairment (Jenkins *et al.*, 2016). In addition, and of importance for this study, *Comt* modulates the acute neuronal effects of THC and is involved in the release of striatal

Table 2 Statistical results for the three-way ANOVAs for mRNA expression from Study #1

Brain region	Gene	Effect of sex, *F* (*P*)	Effect of TBI, *F* (*P*)	Effect of THC, *F* (*P*)	Significant interactions, *F* (*P*)
PFC	*Bdnf*	0.01 (0.96)	0.37 (0.55)	2.69 (0.12)	Sex × THC—5.95 (0.03) Sex × TBI × THC—6.96 (0.02)
	Cnr1	0.01 (0.91)	0.29 (0.60)	0.18 (0.68)	TBI × THC—7.50 (0.02) Sex × TBI × THC—5.88 (0.03)
	Comt	3.65 (0.07)	4.46 (0.05)	11.06 (<0.01)	N/A
	GR	0.64 (0.44)	0.11 (0.75)	2.46 (0.14)	Sex × TBI × THC—7.17 (0.01)
	Iba-1	8.25 (0.01)	0.01 (0.94)	2.64 (0.12)	Sex × TBI × THC—10.26 (<0.01)
	Vegf-2R	0.13 (0.72)	2.60 (0.13)	16.31 (<0.01)	TBI × THC—5.77 (0.03)
HPC	*Bdnf*	1.79 (0.20)	0.00 (0.99)	2.05 (0.17)	Sex × TBI—4.95 (0.04)
	Cnr1	0.39 (0.54)	0.92 (0.35)	1.72 (0.21)	TBI × THC—12.05 (<0.01)
	Comt	0.44 (0.52)	0.13 (0.72)	0.01 (0.94)	N/A
	GR	1.42 (0.25)	0.04 (0.85)	1.35 (0.26)	TBI × THC—6.48 (0.02)
	Iba-1	5.72 (0.02)	0.13 (0.73)	0.00 (0.98)	N/A
	Vegf-2R	6.39 (0.02)	0.49 (0.49)	0.02 (0.89)	N/A
NAc	*Bdnf*	8.57 (0.01)	0.87 (0.37)	14.14 (<0.01)	Sex × TBI × THC—6.22 (0.02)
	Cnr1	14.32 (<0.01)	4.12 (0.06)	0.23 (0.64)	N/A
	Comt	2.51 (0.13)	0.10 (0.75)	1.12 (0.31)	TBI × THC—7.31 (0.01)
	GR	0.01 (0.95)	1.38 (0.26)	6.93 (0.02)	N/A
	Iba-1	0.69 (0.42)	0.01 (0.92)	0.24 (0.63)	N/A
	Vegf-2R	0.08 (0.78)	0.75 (0.40)	0.27 (0.61)	TBI × THC—6.15 (0.03)

dopamine (Bossong *et al.*, 2019). The changes in *Comt* expression in the PFC were consistent with both of these concepts; RmTBI reduced *Comt* expression in vehicle animals but not THC animals, suggesting that THC administration provided therapeutic restoration of TBI-induced reductions in *Comt* expression. THC also altered expression of *Comt* in the NAc, corroborating evidence that *Comt* modulates THC-induced dopamine release in the striatum.

We examined *GR* expression because it can be used as a measurable indicator of the stress response. The endocannabinoid system is intricately involved in regulating the stress response and numerous studies have demonstrated that THC can directly modulate the HPA axis and glucocorticoid release (Manzanares *et al.*, 1999). While current literature indicates that endocannabinoid signalling suppresses HPA activity (Hill and Tasker, 2012), stressful stimuli such as a traumatic brain injuries do the opposite and activate this system (Grundy *et al.*, 2001). In this study, we found that while sham animals exposed to THC had reductions in *GR* mRNA expression in the HPC, RmTBI animals exhibited *GR* expression levels equivalent to vehicle-sham animals, suggesting that THC may have provided neuroprotection. These alterations in *GR* expression may have also contributed to the reduced anxiety-like behaviour on the EPM and open field tasks. Interestingly, *GR* expression was upregulated in the NAc in response to THC in both sham and RmTBI. Given the NAc and HPC work synergistically in feedback loops involved in drug administration and stress (Piazza and Le Moal, 1998), the reductions of *GR* in the HPC may be associated with the increased expression in NAc. To our knowledge, this is the first study to examine changes in *GR* expression in the NAc following THC exposure and this finding warrants further investigation.

In addition, neuroinflammation, such as microglial activation, is hypothesized to play a significant role in the pathophysiology of mTBI (Patterson and Holahan, 2012). Microglial activation has been found to persist in areas remote from the area of focal damage after TBI and has been related to various cognitive and behavioural impairments (Ramlackhansingh *et al.*, 2011). We examined *Iba1* mRNA expression as an indicator of neuroinflammation and microglial activation and found that THC exposure actually increased *Iba-1* mRNA expression in the PFC and had no effect in the HPC or NAc. While the expression of *Iba-1* is typically upregulated in microglia following TBI (Ito *et al.*, 1998), in this study, THC administration did not confer therapeutic benefit.

Finally, RmTBI is believed to pose a threat to *Vegf-2R* expression, angiogenesis and tissue repair, with inhibition of *Vegf-2R* being associated with worse TBI outcomes, including greater lesion size and decreased neurogenesis (Xiong *et al.*, 2011). Given that the cerebral vasculature is crucial for the delivery of oxygen and other nutrients to the brain, functional impairment and dysregulation of this system following TBI may inhibit recovery (Wendel *et al.*, 2019). In both the PFC and NAc, RmTBI significantly reduced *Vegf-2R* mRNA expression levels in vehicle-treated animals, but this RmTBI-induced reduction was absent in THC-treated animals. Similar to the results identified for *Comt* and *Crn1*, post-injury THC administration appears to have provided some therapeutic benefit.

Neuroprotective potential of THC prior to RmTBI

Given that cannabis use is gaining popularity in adolescents, and is now more common than cigarette use (Chadwick *et al.*, 2013), we sought to examine the effects of intermittent THC exposure prior to RmTBI on PCS symptom resolution. This intermittent dosage of THC was designed to provide a realistic model of individuals

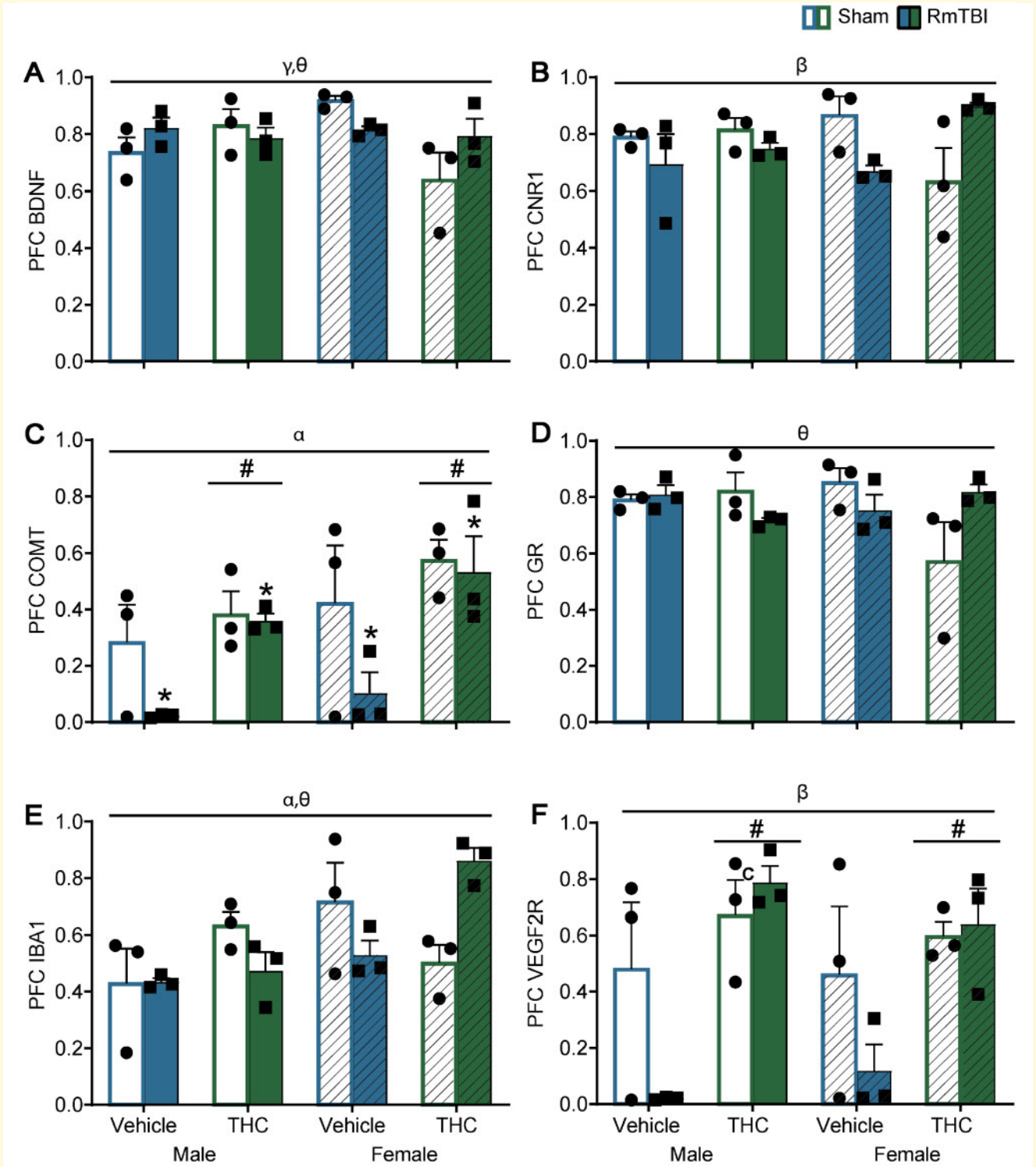


Figure 4 Graphical depictions of the RT-qPCR results in the PFC as mean ± SEM for the post-injury THC administration groups. White and white hashed bars display sham groups (males and females, respectively), while blue and green bars display RmTBI groups. Statistical significance symbols are displayed above the bars (α) indicates a main effect for sex, (*) indicates a main effect for RmTBI, (#) indicates a main effect for THC, (β) indicates a RmTBI by THC interaction, (γ) indicates a sex by THC interaction and (θ) indicates a sex by RmTBI by THC three-way interaction.

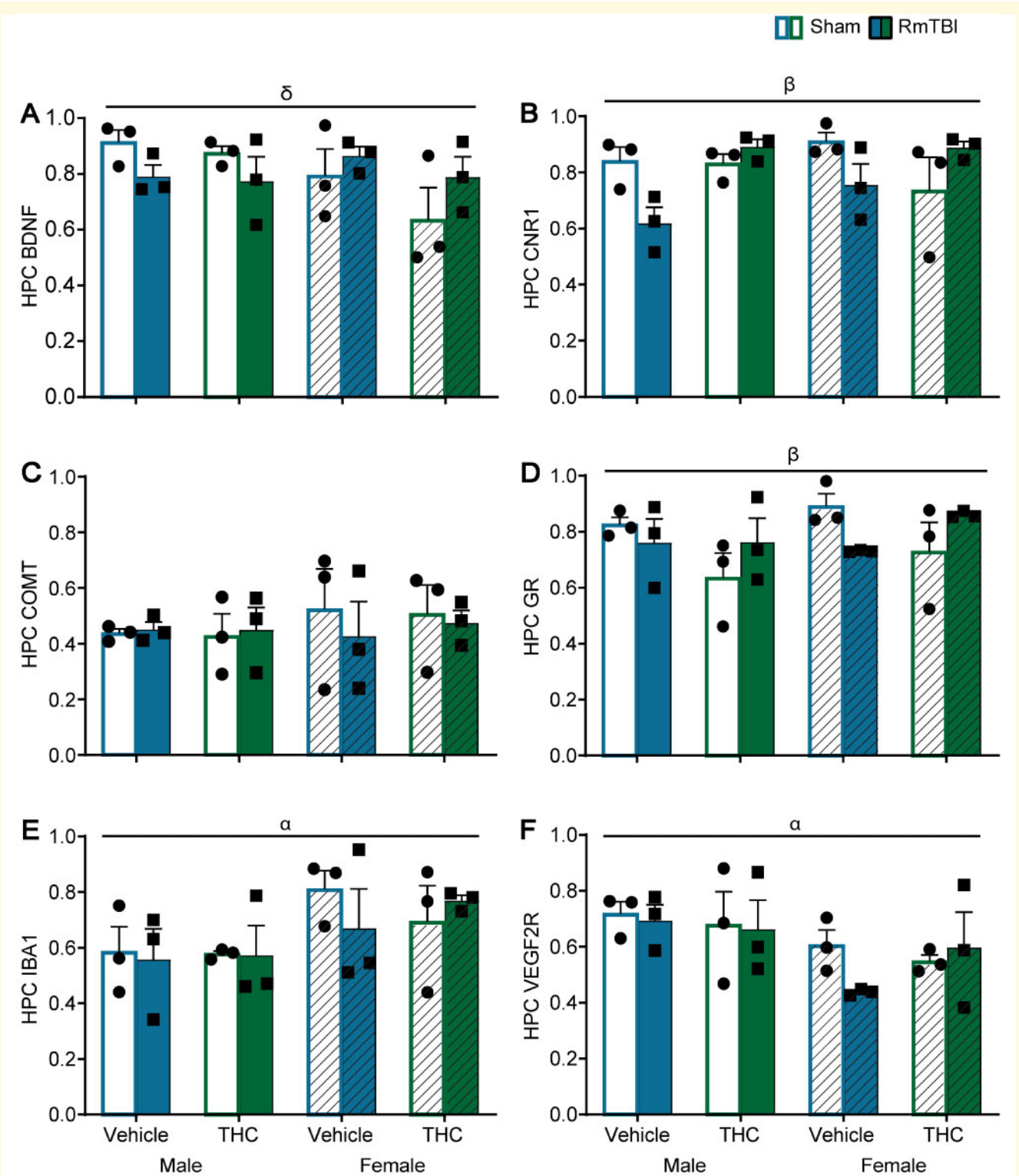


Figure 5 Graphical depictions of the RT-qPCR results in the hippocampus displayed as mean ± SEM for the post-injury THC administration groups. White and white hashed bars display sham groups (males and females, respectively), while blue and green bars display RmTBI groups. Statistical significance symbols are displayed above the bars (α) indicates a main effect for sex, (β) indicates a RmTBI by THC interaction and (δ) indicates a sex by RmTBI interaction.

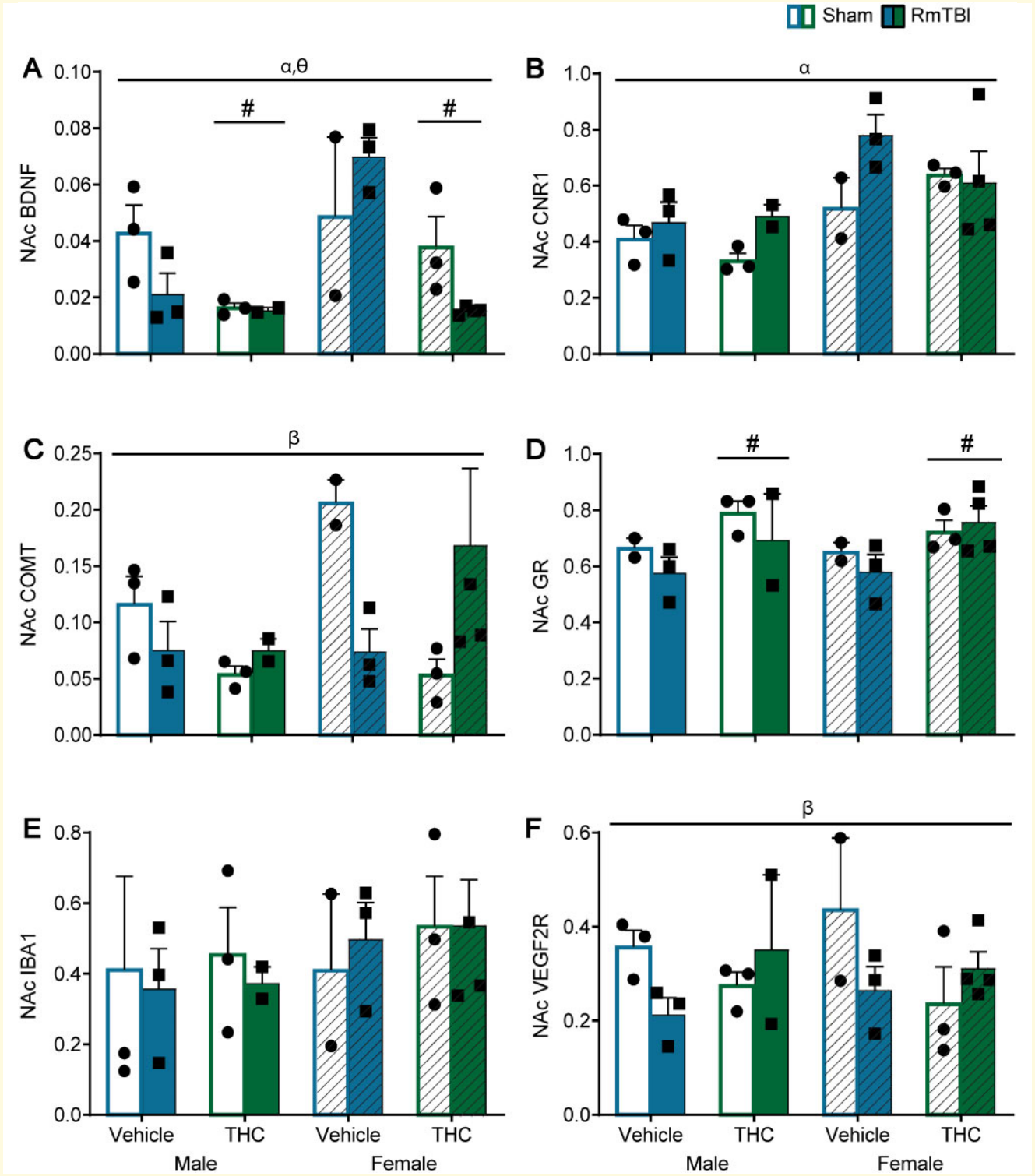


Figure 6 Graphical depictions of the RT-qPCR results in the nucleus accumbens displayed as mean ± SEM for the post-injury THC administration groups. White and white hashed bars display sham groups (males and females, respectively), while blue and green bars display RmTBI groups. Statistical significance symbols are displayed above the bars (α) indicates a main effect for sex, (#) indicates a main effect for THC, (β) indicates a RmTBI by THC interaction and (θ) indicates a sex by RmTBI by THC three-way interaction.

that are casual consumers of THC before RmTBI and then quit when they experience concussion-related symptoms. Despite numerous injury related impairments (loss of consciousness, beamwalk, increased anxiety- and depressive-like behaviour), intermittent THC use only affected loss of consciousness and time spent in the centre

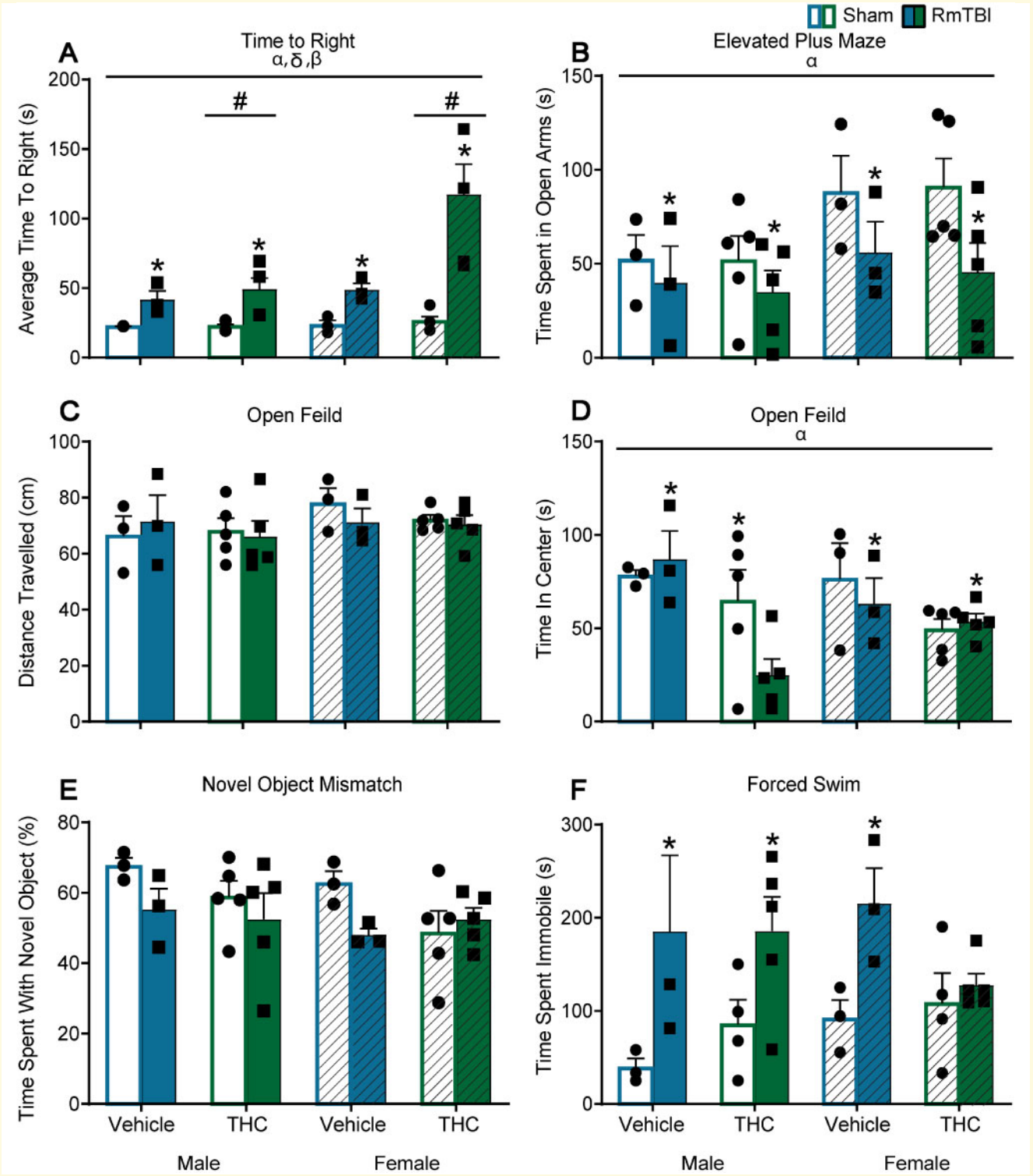


Figure 7 Graphical depictions of the behavioural testing results displayed as mean ± SEM for the pre-injury THC administration groups. White and white hashed bars display sham groups (males and females, respectively), while blue and green bars display RmTBI groups. Statistical significance symbols are displayed above the bars (α) indicates a main effect for sex, (*) indicates a main effect for RmTBI, (#) indicates a main effect for THC, (β) indicates a RmTBI by THC interaction and (δ) indicates a sex by RmTBI interaction. (**A**) The mean time to right following injury or sham procedures. (B) The mean time spent in the open arm of the EPM. (**C** and **D**) The mean distance travelled and time spent in the centre of the open field, respectively. (**E**) The percentage of time spent investigating the novel object in the novel context mismatch. (**F**) The mean time spent immobile during the forced swim test.

Table 3 Statistical results for the three-way ANOVAs for the behaviour tasks in Study #2

Behavioural task	Effect of sex, *F* (*P*)	Effect of TBI, *F* (*P*)	Effect of THC, *F* (*P*)	Significant interactions, *F* (*P*)
Time to right	6.33 (0.02)	25.99 (0.00)	6.18 (0.02)	Sex × TBI—4.92 (0.04) TBI × THC—5.29 (0.03)
Beamwalk	8.71 (0.01)	5.01 (0.03)	0.03 (0.86)	N/A
EPM	5.18 (0.03)	5.65 (0.02)	0.08 (0.77)	N/A
Novel context mismatch	2.01 (0.17)	3.52 (0.07)	1.77 (0.19)	N/A
Open Field (distance travelled)	2.54 (0.12)	0.23 (0.68)	0.09 (0.77)	N/A
Open field (time in center)	4.43 (0.04)	1.67 (0.21)	2.05 (0.17)	Sex × THC—5.50 (0.03)
Forced swim	0.22 (0.65)	14.62 (<0.01)	0.06 (0.82)	N/A

of the open field for female rats, failing to influence any of the other behavioural measures examined. While there is currently conflicting literature regarding early life THC exposure and neurodevelopmental outcomes [for review see Jacobus and Tapert (2014); Lorenzetti *et al.* (2016)], our study found very little evidence for behavioural impairments in adolescence associated with intermittent THC use at this developmental time period. It is possible that the six injections over 20 days was not frequent enough, or at a high enough dosage, to produce lasting changes, as many of the human studies do report greater behavioural and neurological impairment in heavy cannabis users (Lorenzetti *et al.*, 2016; Koenders *et al.*, 2017). The sex-dependent THC effects in time to right and open field, do however suggest that females may be more sensitive to the effects of THC at this time period. This finding is consistent with accumulating evidence demonstrating that sex is an important modulator of cannabinoid sensitivity resulting in a sexually dimorphic endocannabinoid system (Struik *et al.*, 2018). Overall however, we failed to support our hypothesis that THC prior to RmTBI conferred neuroprotection. Moreover, as this regimen of THC exposure failed to produce significant changes in behaviour or produce significant interactions with our RmTBI model, we did not investigate changes in mRNA expression in this cohort.

Conclusions and limitations

These studies demonstrated that THC before and after RmTBI differentially affected outcomes. While the results indicate that there are some therapeutic benefits of THC when administered after RmTBI, further investigation regarding this association is warranted. To gain a better understanding of the identified effects, it would be important to allow these adolescent rats to mature and then assess various cognitive and emotional measures in adulthood to obtain a comprehensive picture of the long-term effects of adolescent THC exposure. Future studies would also benefit from testing THC at multiple doses and via different administration routes, such as vaporized cannabis exposure, in order to replicate human experiences in an ecologically valid manner (McLaughlin, 2018). In summary, we did not find evidence to support our hypothesis that THC prior to RmTBI provided neuroprotection. However, we did find that therapeutic administration of THC following RmTBI was beneficial for short-term working memory, and anxiety- and depressive-like behaviours. In addition, chronic administration of THC following the injuries appeared to have beneficial effects on many of the genes examined in the PFC, HPC and NAc. Overall, this study suggests that THC has potential therapeutic efficacy for the treatment of RmTBI-induced symptomology but requires additional examination.

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Competing Interests

The authors report no competing interests.

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