Chronic Adolescent Exposure to Δ9-THC Alters Cognition, Metabolite Levels, and Inflammatory Profile in Rats

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Abstract

Cannabis is a prevalent drug that is commonly used both as a therapeutic agent and abused as an illicit drug. Despite the widespread use, little is known about the pharmacokinetics of its main psychoactive component, Δ9-tetrahydrocannabinol (THC), or how it affects the body. In order to understand the long-term consequences of adolescent exposure to THC, adolescent rats were administered with the drug and assessed during adolescence and adulthood for cognitive, developmental, and neurochemical changes. These results suggest lasting cognitive deficits, impairment in the glutamatergic system, and a neuroinflammatory profile, and emphasize the need for further research.

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Introduction

Cannabis is the most commonly used illicit drug worldwide [5, 9, 28, 36-38]. It is obtained from the plant *Cannabis sativa*, which contains over 400 compounds and more than 70 cannabinoids [38, 40]. Cannabis is typically smoked and has been reported to induce behavioral, cognitive, emotional, and physiological changes. These changes include feelings of euphoria, relaxation, altered time perception, lack of concentration, impaired learning, altered memory and mood, rapid changes in heart rate and diastolic blood pressure, dry mouth and throat, increased appetite, vasodilation, and decreased respiratory rate. It has also been shown to elicit anti-inflammatory and analgesic responses [36-38]. Due to the wide range of known effects, cannabis use for therapeutic purposes has increasingly become an area of interest. Presently, it has been approved for medical applications such as treating nausea, vomiting, anorexia, chronic pain, anxiety, epilepsy, glaucoma and asthma, along with a growing list of clinical conditions [5, 31, 36, 38, 43].

In cannabis, geranyl pyrophosphate is synthesized via the deoxyxylulose pathway and serves as a precursor to phytocannabinoids and terpenoids (Figure 1). Geranyl pyrophosphate may couple with olietolic acid to produce pentyl cannabinoids, or divarinic acid to produce propyl cannabinoid acids. Phytocannabinoid acids are typically decarboxylated by heat to form neutral phytocannabinoids, such as the primary psychoactive component of cannabis responsible for its observed effects, Δ9-tetrahydrocannabinol (THC) [35]. THC has a tri-cyclic 21-carbon structure without nitrogen and two chiral centers in *trans* configuration [38]. It has a pKa of 10.6, is highly lipophilic with low aqueous solubility, and has a high volume of distribution of 4-14 L/kg [15, 38].

The pharmacokinetics of THC is dependent on the route of administration. Upon inhalation, THC is rapidly absorbed by the lungs and enters the bloodstream, where it first travels to the heart and is then pumped throughout the body. It is detectable in plasma within seconds, with a peak plasma concentration within 3-10 minutes [38, 40]. As THC circulates the body, it binds to cannabinoid receptors located in the central nervous system, peripheral nerves, spleen, and other immune cells. It also may accumulate in fat tissues due to its lipophilic properties, where it is slowly released back into the bloodstream. A certain portion of the available THC is passed through the liver as the

blood travels throughout the body, where metabolism is catalyzed by enzymes of the P450 (CYP) complex. Upon oxidation, reduction, and/or hydrolysis, THC is first converted to the psychoactive metabolite 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC), which is then oxidized to produce the non-psychoactive metabolite 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH). Following oxidation, it is converted to the glucuronic acid conjugate, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol glucuronide (THC-COOgluc), which increases its water solubility and is excreted in feces and urine [28, 36, 38, 40]. Once THC has passed through the liver, it and its metabolites (11-OH-THC and THC-COOH) continue to circulate until they are eliminated from the body. Metabolism upon oral ingestion is similar; however, THC enters the bloodstream through the walls of the stomach and small intestine at a relatively slower rate, reaching a peak plasma concentration within 1-2 hours. Furthermore, it passes through the liver before reaching the heart and the rest of the body, thus metabolizing some of the THC prior to binding to cannabinoid receptors and lowering its bioavailability [38].

THC exerts its effects through interactions with the endogenous cannabinoid receptors CB1 and CB2. CB1 receptors are most commonly located in the brain on neurons at presynaptic regions, astrocytes, oligodendrocytes, and microglia, and are thought to be involved in neural development as well as elicit responses to regulate inflammation, anxiety, stress, and homeostasis. CB2 receptors are primarily found on immune cells, but they have also been found on neurons at postsynaptic regions, astrocytes, oligodendrocytes and microglia, albeit much less prominent in these regions, and are thought to be involved in the reduction of immune cell function [18, 20, 44]. The exact mechanism of the endocannabinoid system is not completely understood; however, it has been posited that a family of anandamides act as ligands for CB1 and CB2 receptors and regulate neuronal activity through its effects on cAMP dynamics and Ca^{2+} and K^{+} ion transport [38]. THC and its metabolites behave as partial agonists at CB1 and CB2 receptors. Specifically, the psychoactive metabolites THC and 11-OH-THC bind to CB1 receptors and the non-psychoactive metabolite THC-COOH binds to CB2 receptors. The interaction between THC and CB1 receptors are of particular interest, as it reflects its potential as a therapeutic agent through regulatory functions in the brain [18, 31, 43].

Inflammation normally serves as a defense mechanism designed to prevent disease and promote tissue repair; however, an inappropriate inflammatory response can be detrimental to the organism. For example, neuroinflammation has been associated with disorders such as multiple sclerosis, Alzheimer's disease, and ischemia [42]. Inflammation is primarily regulated by the immune system but it appears to be part of an intricate balance including the endocannabinoid system. Interestingly, neuroinflammation is regulated by glial cells of the central nervous system including astrocytes, oligodendrocytes and microglia, cells where CB1 and some CB2 receptors are expressed [18, 42]. Once a site of injury has initiated an immune response, these cells promote an inflammatory response by releasing proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, and/or inhibiting the release of anti-inflammatory cytokines such as TGF-β, IL-4, and IL-10 [42]. Thus, cannabinoids that bind to CB1 and CB2 receptors and induce an anti-inflammatory response through the down-regulation of proinflammatory cytokines or the up-regulation of anti-inflammatory cytokines are attractive methods in combatting neurodegenerative disorders that arise from inappropriate proinflammatory responses.

Although THC has generally become accepted as an anti-inflammatory agent, limited studies have explored the mechanism behind this effect and under what conditions this proves to be true. For instance, Yang et al. (2015) used MG-63 cells, cells used to model osteoblasts, in order to determine the inflammatory effects of THC *in vitro*. The cells had been exposed to lipopolysaccharide (LPS) in order to promote the release of IL-6 and mimic proinflammatory conditions. Upon administration of THC, IL-6 levels dropped, suggesting a short-term anti-inflammatory effect [43]. In contrast, Monnet-Tschudi et al. (2008) observed short-term proinflammatory effects following administration of THC to mixed-cell aggregating brain cell cultures and in cultures enriched in neurons and glia, as indicated by an upregulation of IL-6 expression [20]. Additional studies have also demonstrated contradictory results, including a long-term anti-inflammatory response [11, 18], a long-term proinflammatory response [45, 46], and a short-term anti-inflammatory response switching to a long-term proinflammatory response [21, 22]. In regards to utilizing cannabinoids such as THC as therapeutic agents, it is important that further studies be conducted in order to understand the underlying mechanisms of

these systems and how factors such as age of onset and frequency of use can impact the balance of these systems.

Aside from its role in the immune system, the endocannabinoid system is also associated with opioids, GABAergic, dopaminergic, noradrenergic, serotonergic, cholinergic, glucocorticoid and prostaglandin systems; this intricate network accounts for the many observed effects of cannabis use and emphasizes the complexity of understanding the entire mechanism [31, 38, 44]. As previously stated, these effects include behavioral, cognitive, emotional, and physiological changes. This is especially critical when considering neural development, during which synaptic modifications are made. As the brain develops, it undergoes changes that improve efficiency through structural and neurochemical refinement. As a result, neuronal maturation produces synaptic rearrangements, myelination of nerve fibers, and changes in dendritic spine density, neurotransmitter concentrations and their receptor levels. Synaptic rearrangements, or pruning, reduce cortical grey matter by getting rid of unnecessary neural connections. Myelination forms myelin sheaths around white matter fiber tracts, thus improving neuronal plasticity and efficiency of neural conductivity [16, 31, 33, 34]. Neuronal maturation predominately occurs in the prefrontal and temporal cortices, and in subcortical structures. This includes the prefrontal cortex, hippocampus, nucleus accumbens, striatum, and thalamus, [4, 16, 33, 44, 46]. Interestingly, the prefrontal cortex and hippocampus are especially rich in CB1 receptors [4, 16, 31, 33, 41]. The prefrontal cortex is strongly associated with the GABAergic system and perception of fear, anxiety, and pain while the hippocampus is associated with cognition and memory consolidation. Both the prefrontal cortex and hippocampus also play a role in the emotional circuit [4, 31, 41]. Given the high expression of CB1 receptors in these regions, the cannabinoid system not only affects fear, cognition, etc., but it is also involved in neuronal development [4, 18, 31, 34, 44, 46].

Pruning and synaptic refinement during neuronal development have been shown to primarily affect glutamatergic neurotransmission [14, 32, 33]. Glutamate is the major excitatory neurotransmitter in the mammalian brain, while γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter. Tight regulation of both neurotransmitters is critical in cognitive and sensory processing; disturbances in this regulatory system has been linked to deleterious effects such as decreased neuronal regeneration, cell death, and

neurological dysfunction [23, 32]. Furthermore, the glutamatergic/GABAergic system plays a big role in other systems that maintain homeostasis. Despite its importance, its underlying molecular mechanisms or pathway remain equivocal. It has been suggested that glutamate is a precursor for GABA formation, which is recycled and later converted by the tricarboxylic acid cycle back into glutamate through crosstalk between neurons and astrocytes [8, 14, 23, 24]. Aside from glutamate and GABA levels, the corresponding receptors and transporters also play a role in the regulation of this system [8, 30, 32]. Since neurotransmitter and receptor levels are modified during neuronal development, and the endocannabinoid system has been shown to play a role in this development, negative consequences from cannabinoids such as THC may have lasting implications on glutamate/GABA regulation and thus on neuronal integrity.

Adolescence is an important developmental stage characterized by physical, psychological, interpersonal, and sociocultural changes [10]. The brain continues to grow and develop, a process that does not reach complete maturation until the third decade of life [6, 44]. Thus, it has been postulated that adolescence is a vulnerable period during which alterations in the developmental process may produce lasting effects extending into adulthood. This is of particular concern considering the prevalence of cannabis abuse among adolescents. Cannabis is the most commonly used illicit drug among adolescents, who comprise about two-thirds of new users [16, 34, 45, 46]. Age of onset typically ranges between 12-18 years of age; however, onset as early as 9-10 years of age has been reported. Furthermore, in the United States more than 20% of twelfth graders had reported using cannabis within the past 30 days and about half of twelfth graders had reported using cannabis at some point in their lives [6, 21, 22, 31]. Given its prevalence, it is important to understand the immediate and long-term ramifications of cannabis use. With respect to adolescents, cannabis use and abuse have been associated with increased risk of dependence, use of other illicit drugs, mental health disorders, psychosis, poor academic performance, future unemployment, violence, and crime; however, as with neuroinflammation, factors such as age of onset and frequency of use must be considered [6, 16, 33, 38, 40]. For example, in contrast to the lasting impairments of adolescent cannabis use previously posited, Fried et al. (2005) observed deficits in cognition and memory in heavy adolescent users as compared to controls but found no differences after at least

three months of abstinence [12]. Furthermore, Pope et al. (2001) observed no differences in neurocognition between heavy adult users and controls after 28 days of abstinence; however, Bolla et al. (2002) did find differences in cognition and manual dexterity between heavy and light adult users after 28 days of abstinence [3, 25]. As evidenced by these contradictory results, longitudinal studies must be conducted in order to understand the long-term implications of cannabis on behavior, cognition, and emotion while taking into consideration factors such as age of onset and frequency of use.

In order to further understand the effects of cannabis *in vivo*, we seek to assess cognition, memory, and brain development in relation to metabolite expression following chronic adolescent exposure to THC. Although it is important to investigate its effects as it pertains to humans, it is difficult to perform longitudinal studies in the human model, as it requires years of study examining the effects during adolescence extending into adulthood. It is also difficult to analyze cytokine production in brain tissue without postmortem samples, as well as account for factors such as concomitant drug use; consistency; and bioavailability due to body type, past history, and depth of inhalation (if smoked). Therefore, we will use a rat model in order to perform behavioral tests, noninvasive brain imaging, and biochemical assays over the life span of the developing rat.

Materials and Methods

Drugs

Delta-9-tetrahydrocanninol (THC) was dissolved in ethanol and saline (1:1:18). (Sigma-Aldrich)

Animals

Male Sprague-Dawley rats (Harlan) aged postnatal day (PND) 21 at the time of arrival were obtained and were housed in clear plastic cages in groups of two on a 12h light-dark cycle (lights on 08:00 A.M.) and in a temperature and humidity controlled environment. All animals had free access to food and water. All efforts were made to minimize the number of animals used and their suffering.

Treatments

Rats were injected subcutaneously (s.c.) with increasing doses of THC, or vehicle, twice a day from PND 37 to PND 47 (2.5 mg/kg, PND 37-39; 5 mg/kg, PND 40-43; 10 mg/kg, PND 44-47), according to the protocol used in Rubino et al. (2009), as shown in Figure 2 [34]. This protocol has been used in previous studies in order to resemble heavy use of marijuana in humans as well as to minimize the possibility of developed tolerance.

Behavioral Tests

Classic and Spatial Versions of the Novel Object Recognition (NOR) Test

The apparatus used for the object recognition test was an open-field arena (90 x 75 x 60 cm) made of white poster board. The experiments took place in a dimly illuminated room. Animals performed each test individually. Each test was carried out in two trials, according to the protocol used in Realini et al. (2011) [29]. During the first trial (familiarization), each animal was placed in the arena with two identical objects placed according to behavioral schemes developed to counterbalance and eliminate bias for 5 minutes. During the second trial (testing), one of the familiar objects was replaced by a novel object and the animal was returned to the arena and allowed to explore for 5 minutes. In the spatial variation of the test, two objects were used during the familiarization trial then one of the familiar objects was placed in a different position during the testing trial. Each trial of the spatial variation was run for 5 minutes.

Both the classic and spatial versions of the NOR test were performed during the pre-treatment phase, post-treatment phase, and adult phase as shown in Figure 2. Each

trial was videotaped and recorded. The time spent exploring the familiar object (or familiar location) and the novel object (or novel location) during the test trials was determined and the novel object preference ratio was calculated for each animal as follows: [(novel time) / (novel time + familiar time)]. The arena was wiped clean with 70% ethanol and dried after each trial.

Social Interaction Test

The apparatus used for the object recognition test was an open-field arena (90 x 75 x 60 cm) made of white poster board. The experiments took place in a dimly illuminated room. Animals performed each test individually. Each test was carried out in two trials. During the first trial, each animal was placed in the arena with a novel object and caged congener placed according to behavioral schemes developed to counterbalance and eliminate bias for 5 minutes. During the second phase, the congener was replaced by a novel congener and the animal was returned to the arena and allowed to explore for 5 minutes. Similarly, the social interaction test was performed during the pre-treatment phase, post-treatment phase, and adult phase. Each trial was videotaped and the total duration of time spent engaged in social investigation was recorded. The social memory and social preference was calculated for each animal as follows: social memory [(novel congener time) / (novel congener time + familiar congener time)]; social preference [(total congener time) / (total object time)]. The arena was cleaned with 70% ethanol and dried after each trial.

Magnetic Resonance Spectroscopy

Each cohort was anesthetized by inhalation of isoflurane (<1%) and constantly monitored during the scans for vital signs. The standard anesthetic agent isoflurane was chosen for its properties of rapid metabolism and potentially higher reproducibility, lending it high suitability for longitudinal brain investigations of brain function [13].

All ^{1}H MR scans were performed using a 4.7T/40 cm horizontal MRI magnet (Oxford, UK) interfaced with a Biospec Bruker console (Bruker, Germany) and equipped with a 20G/cm magnetic field gradient. A custom-built ^{1}H radiofrequency (RF) volume and head coil was used for transmission and reception. High-resolution T_2-weighted TurboRARE images were used for MRS voxel positioning using the following parameters: repetition time (TR) = 36 ms, echo time (TE) = 36 ms, slice number = 24,

slice thickness = 0.8 mm, average number = 8, RARE factor = 8, field of view (FOV) = 32 mm x 32 mm, matrix = 256 x 256. A point-resolved spectroscopy sequence (PRESS) was used to acquire ^{1}H MRS data along the dorsal hippocampus with the following parameters: TR = 2,500 ms, TE = 6 ms, average number = 512, voxel size = 8 x 2 x 3 mm. ^{1}H MRS data was similarly acquired along the prefrontal cortex, specifically the anterior cingulate cortex, with the following conditions: TR = 2,500 ms, TE = 6 ms, average number = 640, voxel size – 4 x 2 x 5 mm.

Proton spectra were fit using Linear Combination Model (LCModel) software (Version 6.2-2B), which analyzed *in vivo* proton spectrum as a linear combination of model *in vitro* spectra from individual metabolite solutions98 and generated data as absolute fits (in institutional units) and SD%. Spectroscopic analysis measured metabolite levels of glutamate (Glu), glutamine (Gln), N-acetyl aspartyl glutamate (NAAG), and γ-amino butyric acid (GABA), with inclusion criteria of SD<25%. Mixed analysis of variance (ANOVA) was used for comparing group mean metabolite levels (age as within-group factor and drug condition as between-group factor), with a significance threshold of $p<0.05$.

Biochemical Studies

Tissue Preparation

Rats were immediately decapitated following MRS while still anaesthetized by isoflurane and brains quickly removed. The extracted brains were immediately frozen in liquid isopentane on dry ice and stored at -80°C until regional dissection.

The prefrontal cortex and hippocampus were obtained using a cryostat and stored at -80°C until RNA extraction.

RNA Extraction and Real-Time PCR

Total RNA was extracted from prefrontal cortex and hippocampus samples using the Qiagen RNeasy Lipid Tissue Mini Kit. TRIzol reagent (600 μL) and stainless steel beads were added to each tube and homogenized using the TissueLyser for 2 minutes at 20 Hz. The homogenates were then transferred to new 1.5 mL microcentrifuge tubes and incubated at room temperature for 5 minutes. Chloroform (200 μL) was added to each tube, vortexed, and incubated for 2 minutes at room temperature. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, each upper,

aqueous phase was transferred to a new tube and 70% ethanol (400 μL) was added to each tube and mixed thoroughly by vortexing. Then, the samples were transferred to RNeasy Mini spin columns placed in 2 mL collection tubes and centrifuged at 8,000 x g for 15 seconds at room temperature. The flow-through was discarded and the collection tubes were washed with Buffer RW1 (350 μL). The samples were centrifuged under the previous conditions, and the flow-through discarded. The samples were then washed with DNase I stock solution (10 μL) and Buffer RDD (70 μL) and incubated for 15 minutes at room temperature. Buffer RW1 (350 μL) was added to each tube and centrifuged under the previous conditions. The flow-through was discarded, and Buffer RPE (500 μL) was added to each tube before centrifugation under the previous conditions. The flow-through was discarded, and Buffer RPE (500 μL) was added once more to each tube and centrifuged at 8,000 x g for 2 minutes at room temperature. The RNeasy spin columns were transferred to new 2 mL collection tubes and centrifuged at full speed for 1 minute at room temperature. The spin columns were then transferred to new 1.5 mL tubes and the RNA was eluted by adding RNase-free water and centrifuged at 8,000 x g for 1 minute at room temperature.

The mRNA was quantified using a NanoDrop, and an equal amount of mRNA (500 ng) underwent reverse transcription using the Bio-Rad iScript cDNA Synthesis Kit under the following conditions: priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C, and RT inactivation for 1 minute at 95°C. The cDNA was then diluted with RNase-free water (1:1) and used as a template for real-time PCR. The PCR mix (12.5 μL) was comprised of 1X SYBR Green PCR master mix, primers, and H_2O. All measurements were performed in duplicate and quantified using the ΔΔCt method. The Ct values were normalized to the Ct value of the internal control (18s) and the comparative Ct model ($2^{-\Delta\Delta Ct}$) was used to obtain the fold change of the THC-treated rats relative to the vehicle-treated rats.

Results

Behavioral Tests

Adolescent exposure to THC may suggest differences in brain development. The classic and spatial versions of the NOR test are commonly used to assess learning and memory utilizing the rodent's natural propensity to explore novel objects. Due to this natural tendency, it is more likely that a rodent will spend more time interacting with a novel object over a familiar object, and an object in a novel location over an object in a familiar location [7, 17, 29]. Moreover, since the hippocampus is associated with memory and spatial learning, the NOR tests are also thought to be an indication of hippocampal development [4, 31, 41, 45]. The results of the NOR tests are presented in Figure 3. The novel object preference ratio of each animal was calculated according to the following: [(novel time) / (novel time + familiar time)]. The average preference ratio of the THC group was compared to the average preference ratio of the vehicle group both during adolescence immediately following administration as well as during adulthood, accounting for baseline results (pre-exposure) by using it as a covariant, in order to determine short-term versus long-term effects. The classic version of the NOR test did not produce statistically significant results; however, the was an observable increase in the time spent with the novel object between adolescence and adulthood in the vehicle group in contrast to a decrease in the time spent with the novel object between adolescence and adulthood in the THC group. This could potentially indicate a difference in brain development between the two groups, although it should be emphasized that this speculation be taken with caution, as the results were not considered significant. The spatial version of the NOR test did exhibit significant results, indicating a preference for the object in the novel location during adolescence in the vehicle group as opposed to a preference for the object in the familiar location in the THC group, in accordance to what was expected. Surprisingly, the results were reversed in adulthood. Once again, this could exhibit differences in brain development due to exposure to THC; however, these results are inconsistent with previous studies.

The effect of adolescent exposure to THC in the social interaction test is illustrated in Figure 3. The social interaction test has become an accepted measure of anxiety-like behavior in rodents [29, 31, 39, 46]. Anxiety is tied into the emotional circuit that

has been proposed to correspond to the prefrontal cortex and GABAergic system [31]. The social memory analysis also utilizes the rodent's natural tendency to explore novel objects, in this case a novel congener. Furthermore, anxiety-like behavior due to THC is typically assumed from less time engaging in social activity. The mean time spent engaged in social behavior was calculated according to the following: social memory [(novel congener time) / (novel congener time + familiar congener time)]; social preference [(total congener time) / (total object time)]. As with the NOR tests, the results of the THC group were compared to the results of the vehicle group both during adolescence immediately following administration as well as during adulthood, accounting for baseline results (pre-exposure) by using it as a covariant, in order to determine short-term versus long-term effects. Social memory analysis produced significant results similar to that observed in the spatial NOR test. As expected, the vehicle group preferred the novel congener to the familiar congener during adolescence while the THC group preferred the familiar congener to the novel congener during adolescence. However, in adulthood both groups preferred the novel congener. The results for social preference did not yield significant results. As can be seen, there were longitudinal differences between the two groups; the vehicle group spent less time overall engaging in social activity as compared to the THC group during adolescence, but spent more time overall engaging in social activity in adulthood. Based solely on the results of the behavioral tests, the effects of THC on behavior cannot be elucidated and further testing is required to determine conclusive results.

Brain Metabolite Levels

Changes in metabolite levels throughout development. Neuronal development is characterized by changes in brain plasticity as well as in neurotransmitter levels and their receptors [16, 32]. Furthermore, these changes are heavily associated with the glutamatergic system [14, 32]. Therefore, it is important to understand how changes in the glutamatergic system affect synaptic pruning and neuronal refinement. The metabolites of interest in the glutamatergic system were assessed using MRS, which has become an increasingly promising approach in identifying the relationship between brain metabolism and function through a noninvasive method [2, 13, 24]. Figure 4 shows the mean metabolite levels for Glu, Gln, NAAG, and GABA in the hippocampus as well as the anterior cingulate

cortex. The ratios between Gln/Glu and GABA/Glu levels are also presented, serving as indications of glia and neuron coupling and thus excitatory/inhibitory balance. Glu is the major excitatory neurotransmitter and GABA is the major inhibitory neurotransmitter [24, 30]. NAAG is a dipeptide that helps regulate the glutamatergic system by acting as an agonist of the metabotropic type-3 Glu receptor (mGluR3) and as a weak antagonist of the N-Methyl-D-aspartate receptor (NMDAR) [23, 30]. Furthermore, NAAG and Gln both serve as intermediates in the Glu/GABA cycle and play a role in the regulation of neurotransmission. Abnormalities in the balance of these metabolites have been associated with a number of disorders. For example, elevated extracellular glutamate and reduced glutamine levels have been found in epileptic and schizophrenic patients [8, 30]. The effects of exogenous drugs such as THC on the glutamatergic system remains ambiguous, as increases or decreases in metabolite levels could be attributed to decreased production of precursors, deficiencies in transporters or receptors, etc. Thus, it is useful to compare altered metabolite levels to what is considered healthy in order to determine the effects of drugs and further understand the pathways by which they are metabolized. As shown in Figure 4, there were significant differences in hippocampal NAAG, GABA, and GABA:Glu levels during adolescence as well as in Gln:Glu levels in the anterior cingulate cortex during adulthood.

Biochemical Studies

Adolescent THC treatment alters the glutamatergic system and produces signs of neuroinflammation in adult rats. Similar to the MRS studies, the hippocampus and PFC were the primary regions of interest due to their known association with cognition and memory. Furthermore, the microglia in these regions have also been shown to play a role in neuroinflammation. It is for these reasons that the hippocampus and PFC were extracted from each cohort immediately following MRS imaging and later analyzed for the expression of the following: GFAP, GLT-1, GRIN2B, IL-1β, IL-6, and TNF-α. The results are reported in Figure 5. Glial fibrillary acidic protein, or GFAP, is the main intermediate filament of astrocytes and is used as an astrocyte marker, which is an indication of neurotoxicity [18, 19, 45]. GLT-1 is a glutamate transporter in astrocytes. It is a member of the excitatory amino acid transporters (EAATs), which prevent neuronal excitotoxicity through regulation of glutamate uptake [1, 4]. GRIN2B (glutamate receptor,

ionotropic, N-methyl-D-aspartate 2B) is a subunit of the major glutamate receptor, NMDAR, and is also highly involved in modulating the glutamatergic system [2, 47]. Interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) are proinflammatory cytokines that are used to determine levels of inflammation [20, 22, 27]. Although the results were not considered significant, there were trends consistent with previous studies worth noting. In the hippocampus, there were trends toward increases in TNF-α ($p=0.18$) and in GFAP ($p=0.15$) levels. In the PFC, there was a trend toward a decrease in GLT-1 ($p=0.13$). IL-1β and IL-6 were not detected in either brain region. These results are suggestive of a proinflammatory profile and impairment in neuroglutamatergic transmission, respectively [4, 45].

Discussion

In this study, we attempted to link the cognitive and behavioral phenotypes associated with adolescent exposure to THC with changes in genotype, and how these changes progress into adulthood. Despite its high prevalence, little is know about the mechanisms underlying its observed effects, as well as its long-term implications [8, 16, 18, 25].

One of the observed phenotypes of THC is impairment in cognition and memory [18, 33, 41, 44]. Furthermore, performance in memory has been correlated with hippocampal development [7, 17]. The novel object recognition (NOR) test has become the standard assessment for memory consolidation and recollection in animal studies, which utilizes a rodent's natural tendency to explore novel objects [7, 11, 17]. Although designs may vary, most studies conduct the tests in a square or rectangular box with high walls that eliminate any spatial or contextual clues. Memory is characterized by encoding, consolidation, and recollection; the classic NOR test assesses for any impairments in these stages of learning and development through an initial familiarization phase, a testing phase, and an intermittent phase in between the two [7]. This was the behavioral design that was used in this study to assess the effects of adolescent exposure to THC on cognition and memory. There were no significant differences in the classic NOR test; the novel object was preferred over the familiar object regardless of drug condition or the time at which the tests were conducted. This is in stark contrast to what would be expected given past results of similar studies and the wide association of THC exposure with cognitive impairment; however, there are several important points that could explain these observations. As stated, there are standard stages used to test memory acquisition, consolidation, and recollection, although there is high variability among published studies in terms of the time spent in each phase, which can have significant implications on results. For example, a low novel object preference score could be indicative of impaired memory, but it could also be a direct reflection of inadequate time spent exploring the samples during familiarization [7]. It is reasonable to postulate that if memory acquisition is insufficient, then the recollected memory will also be insufficient, resulting in a failure to distinguish novel from familiar. Furthermore, if a cohort is not given sufficient time in between the familiarization and test phase, it may not have the chance to forget the

objects adequately enough for recollection. This can be seen in a study by Kenney et al. (2011) [17], who observed no indications of memory impairment when a 24-hour delay between familiarization and testing was applied, but observed differences with a 48-hour delay. It should be noted, however, that this was a study on the effects of nicotine; nevertheless, the time spent in each phase of testing appears to have an impact and must be taken into consideration when interpreting results.

A spatial variation of the NOR test was also applied in order to assess spatial memory, which the classic NOR test fails to do. Similarly, a rodent will tend to explore an object in a location it recognizes as novel over one in a familiar location. Our findings demonstrated that there was an effect of both age and drug condition. Interestingly, during adolescence only the vehicle group preferred the object in the novel location to the object in the familiar location, suggesting impairment in spatial memory due to exposure to THC. In adulthood, however, only the THC group preferred the object in the novel location to the object in the familiar location, suggesting that impaired spatial memory from the THC group may have recovered, at least to some degree, after a washout period. The results seen during adulthood seem to conflict with results published from other studies testing for behavior in adult rats treated with THC during adolescence; however, it is important to note that many of these results are seen in female rodents as opposed to male rodents [18, 29, 33, 44]. Interestingly, Zamberletti et al. tested adolescent exposure to THC in both female and male rats in two separate studies and found that adult female rats exhibited cognitive impairment through the classic and spatial NOR tests, while adult male rats only exhibited impairment in the spatial NOR test [44, 45]. Although these results oppose what has been observed in our study, it indicates that gender may play a role in the effects of THC on cognition and behavior.

In addition to cognitive and behavioral effects, THC has also been reported to affect emotion and social functioning [29, 33, 44]. Disruptions in psychosocial development may have lasting implications, such as anxiety and depression [6, 10, 16, 29]. Adolescence is a critical time window for development, thus we sought to investigate how adolescent exposure to THC might affect emotional and social functioning. According to previous studies, THC has been reported to induce anxiety-like symptoms as seen by a reduction in social interaction [6, 10, 16, 29]. Our study showed no significant results in terms of

preferring a social target to an inanimate object (social preference), but yielded significant results in social memory. The vehicle group was seen to spend more time with the novel congener than the familiar congener in both adolescence and adulthood, while the THC group only spent more time with the novel congener during adulthood. These results suggest that there were no significant impairments in social behavior or signs of anxiety; however, the difference in time spent with the novel congener over the familiar congener between the groups during adolescence may signify a role in memory, similar to that of the spatial NOR test. Nevertheless, it is difficult to make a generalization based on the results of the social interaction test. Using a similar test for assessing anxiety- and depressive-like symptoms, Realini et al. (2011) acknowledged that the results observed from this test may not accurately represent anxious behavior, at least in comparison to other behavioral tests such as the elevated plus maze and open field test [29]. Furthermore, there appear to be varying results in social behavior dependent on gender, similar to that observed in the NOR tests. Female rodents appear to be more prone to social withdrawal and anxiety-like behaviors than their male counterparts, which may explain the inconclusive responses observed in this study [29, 39, 44-46].

Adolescence is also characterized by neurological changes and development. These changes include synaptic rearrangements, myelination of nerve fibers, and changes in dendritic spine density, neurotransmitter concentrations and their receptor levels [16, 31-33]. Many endogenous systems are involved in brain maturation, including the cannabinoid system. Thus, exogenous cannabinoids such as THC can significantly alter neuronal development [38, 44]. Determining abnormal changes in neuronal development can help identify how THC operates and what systems are affected as a result. ^{1}H MRS studies have become an increasingly important method of noninvasive imaging to track drug and metabolite levels [2, 24, 26]. Neuroimaging studies have shed light on how changes in the glutamatergic system can have an effect on developing mental health disorders [2, 16, 26]. For example, Rowland et al. (2013) found that younger schizophrenic patients have increased NAAG levels in the centrum semiovale and lowered glutamate, glutamine, and GABA levels regardless of brain region, while older schizophrenic patients have lower NAAG levels in the centrum semiovale and a similar effect in glutamate, glutamine, and GABA levels [30]. Due to the predictive power of MRS, we sought to determine the effects

of THC on the glutamatergic system through its metabolite levels in relation to controls. Our study produced no effect of time in the THC group, with the exception of a decrease in hippocampal NAAG levels during adolescence. In contrast, the only effect of time in the vehicle group was in hippocampal GABA levels, and consequently the GABA:Glu ratio. There were significant effects of drug condition in hippocampal NAAG levels during adolescence, hippocampal GABA levels during adolescence, and Gln:Glu in the anterior cingulate cortex during adulthood. Due to limited data, particularly in respect to *in vivo* adolescent exposure to THC in animal studies, it is difficult to connect these changes in the glutamatergic system to any specific effects of THC. It can be seen that THC has some effect on the glutamatergic system, implying an effect on neuronal development as well; however, further studies are required to map its effects in its entirety.

Other neurological diseases that have been attributed to THC use are multiple sclerosis and Alzheimer's disease, consequences of neuroinflammation [42]. In order to assess the inflammatory profile, as well as further indications of glutamatergic changes, we extracted the hippocampus and prefrontal cortex of each cohort and determined expression of GFAP, GLT-1, GRIN2B, IL-1β, IL-6, and TNF-α. Our findings suggested a trend towards a proinflammatory state, as indicated by an increase in TNF-α and GFAP levels [45]. Furthermore, there appeared to be signs of impaired glutamatergic neurotransmission, as seen by the decrease in GLT-1 [4]. As previously stated, these results were not significant; however, they were worth noting due to the trend towards correlation and consistency with previous studies.

In conclusion, this study presented mixed results, both consistent and inconsistent with studies similar in nature. Behavioral findings suggested short-term impairment of spatial memory, which recovered after a washout period. THC did not seem to have a significant effect on object recognition or social behavior. Metabolite levels of the glutamatergic system appeared altered overall between the vehicle group and the THC group throughout development, implying some effect of THC on the glutamatergic system and neuronal development. Finally, biochemical assays proposed a long-term proinflammatory profile as well as lasting effects on glutamatergic impairment. Due to non-significant findings, further studies must be conducted in order to understand the full

implications THC has in all regards. Moving forward, several factors should be taken into consideration based on this study. Gender may play a role in differences in observed behavior and cognition, possibly due to differences in brain development or CB1 expression. A criterion on sample size acquisition during NOR testing might be beneficial to ensure that each cohort is exposed and familiarized to the objects equally, and further consolidate them in an appropriate time according to literature published for the species used. Furthermore, an elevated plus maze or open field test could be considered if testing for anxiety, or included in addition to social interaction if social behavior remains a target of interest. With respect to MRS acquisition, it has been known that the method for anesthesia affects the observed results; therefore, different anesthetics should be considered depending on the study at hand [13]. In addition, the concentrations of desired markers or their respective primers may not have been sufficient for proper quantification, thus it may be necessary to use alternative methods in order to determine gene expression. Finally, an increase in sample size could provide stronger results. It is important that the cannabinoid system be further studied and understood. Mapping its path in the body will justify whether or not cannabis should be used as a therapeutic agent, but perhaps more importantly, reveal areas in the endocannabinoid system or other related systems that can be targeted by other drugs to alleviate disorders and diseases.

Figures

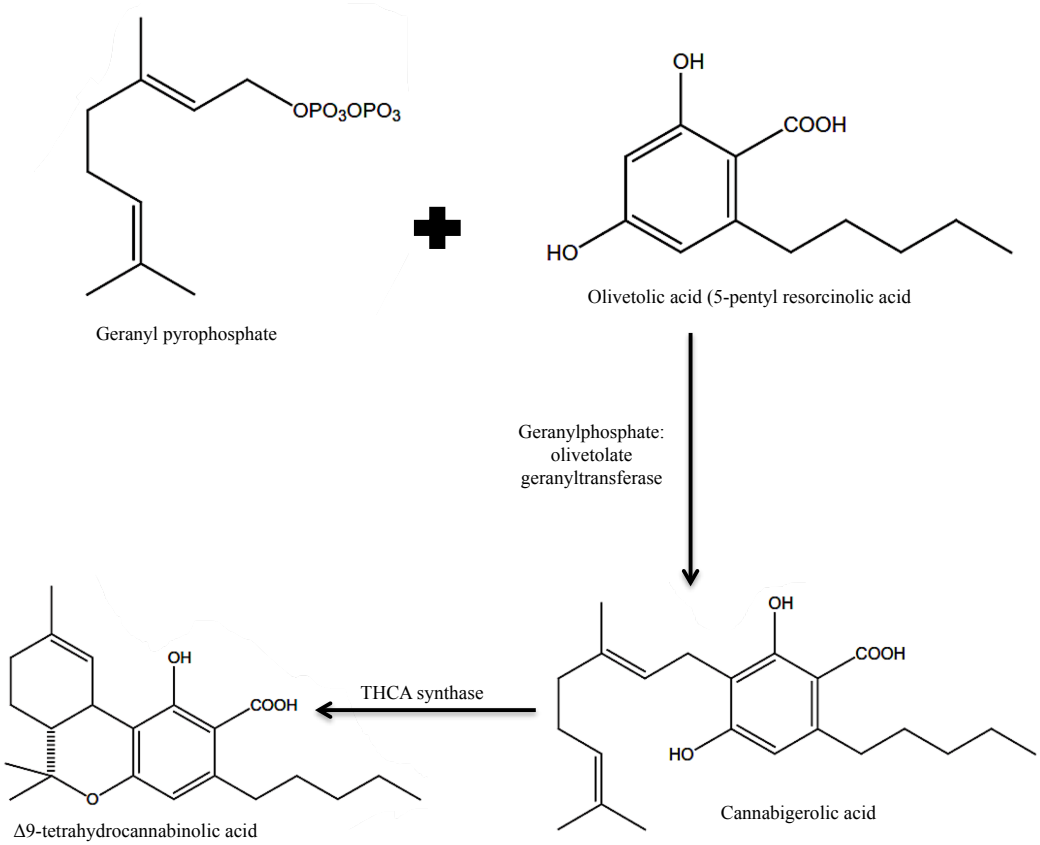


Figure 1. Synthesis of Δ9-tetrahydrocannabinol (THC) from geranyl pyrophosphate.

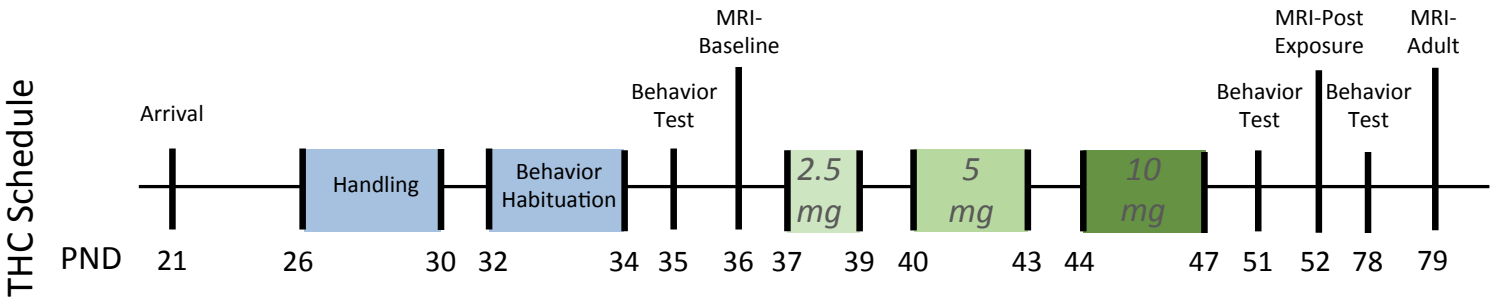
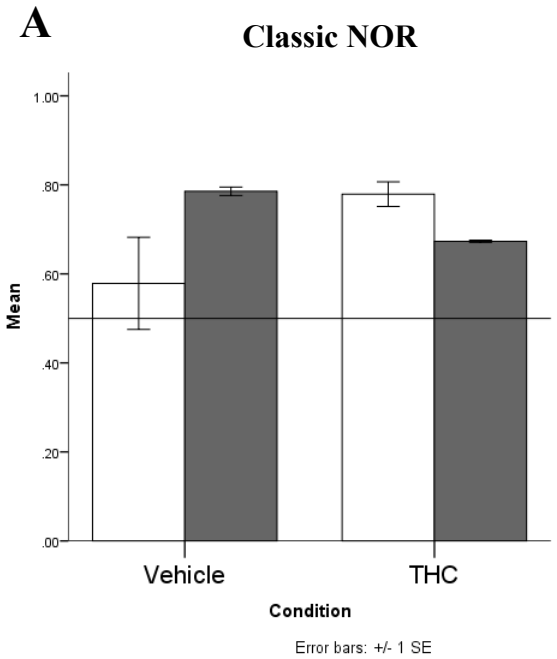


Figure 2. Experimental schedule detailing behavioral testing, magnetic resonance imaging (MRI), and THC administration.

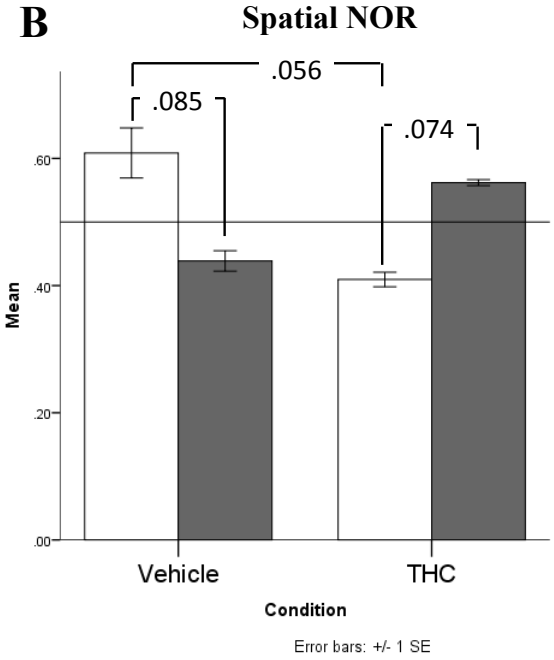
A



Adolescence
Adult

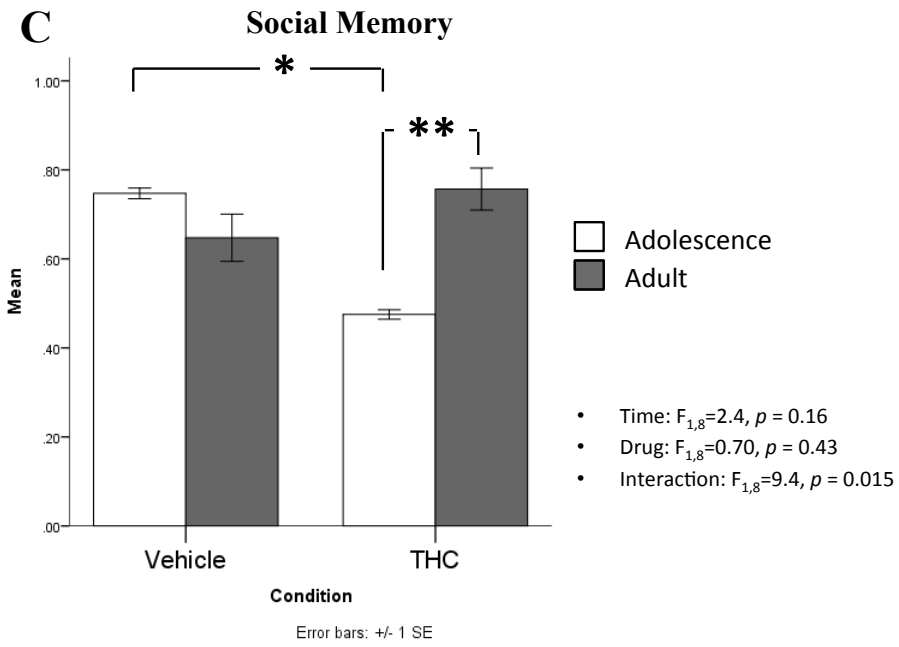
- Time: $F_{1,9}$=3.3, p = 0.10
- Drug: $F_{1,9}$=1.4.0, p = 0.26
- Interaction: $F_{1,9}$=0.02, p = 0.88

B



Adolescence
Adult

- Time: $F_{1,9}$=1.5., p = 0.25
- Drug: $F_{1,9}$=0.38, p = 0.55
- Interaction: $F_{1,9}$=7.6., p = 0.022



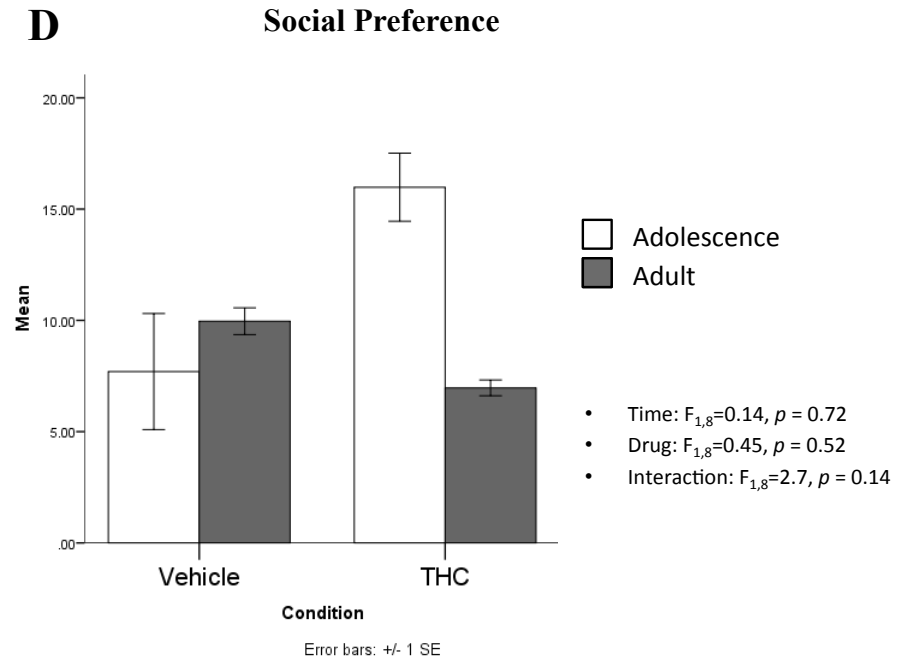


Figure 3. Effect of adolescent THC exposure on object recognition, spatial memory, social memory, and social preference during adolescence and adulthood. (A) Average preference ratios of the classic novel object recognition test; (B) Average preference ratios of the spatial object recognition test; (C) Social memory in the social interaction test; (D) Social preference in the social interaction test.

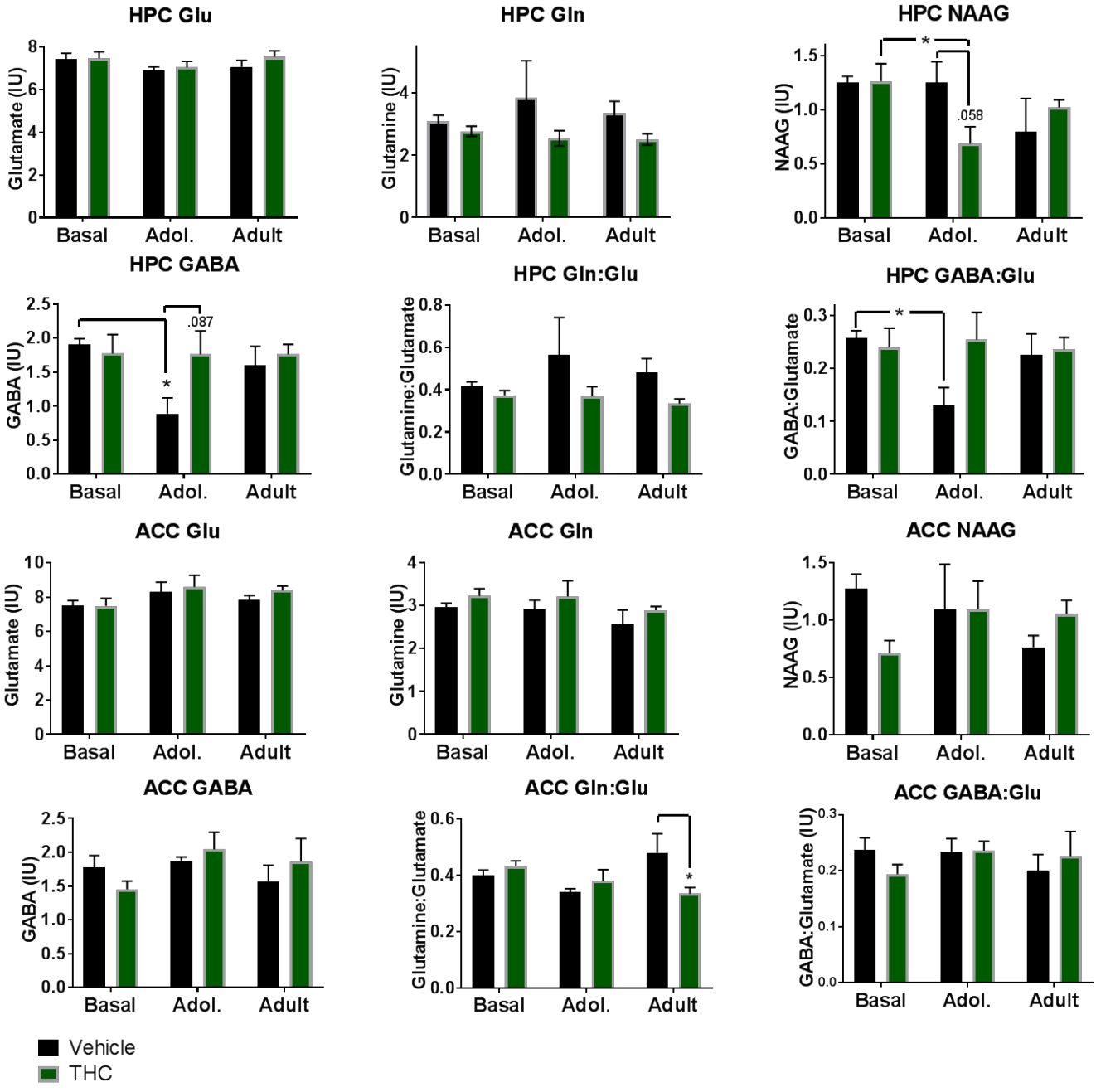


Figure 4. Effect of adolescent THC exposure on metabolite levels in the hippocampus (HPC) and anterior cingulate cortex (ACC).

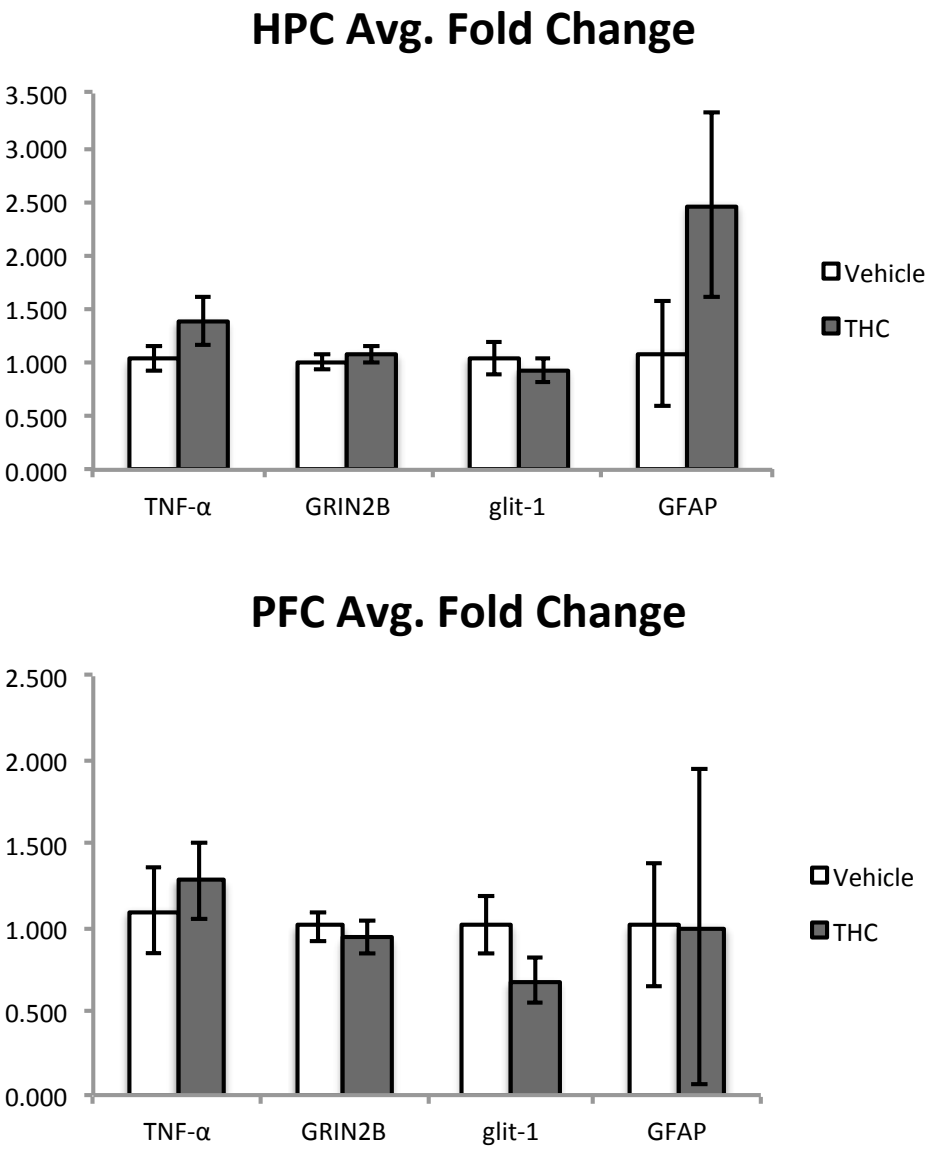


Figure 5. Effect of adolescent THC exposure on expression of inflammatory markers (TNF-α), glutamate receptors (GRIN2B), glutamate receptors (GLT-1 or glit-1), and astrocyte markers (GFAP).

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