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Acute and Long-Term Effects of Δ9-tetrahydrocannabinol on Object Recognition and Anxiety-Like Activity are Age- and Strain-Dependent in Mice

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Abstract

Use of exogenous cannabinoids disrupts the fine-tuned endocannabinoid receptor system, possibly leading to alterations in cognition, memory, and emotional processes that endure long after cannabinoid use has stopped. Long-term adolescent use may uniquely disrupt these behaviors when compared to adult use. The current study explored the acute and long-term behavioral effects of six 10 mg/kg Δ9-tetrahydrocannabinol (THC) injections across the adolescent or early adult period in male inbred C57Bl/6J and DBA/2J mice. The acute and prolonged effects of THC on object memory using the novel object recognition task, unconditioned anxiety in the elevated plus maze and open field, and sedative effects in the open field were examined. Acute THC treatment resulted in anxiogenic activity in both strains, but only caused sedation in B6 mice. Repeated THC treatment resulted in a protracted effect on object recognition, but not unconditioned anxiety, assessed 4 weeks later. In both strains, an adolescent history of THC treatment disrupted later object recognition. Interestingly, in B6 mice an adult history of THC exposure appeared to rescue a deficit in object recognition observed in vehicle-treated adults. Repeated THC administration also produced a protracted effected on CB1R protein expression. Animals treated with THC in adolescence maintained increased levels of CB1R protein expression compared to their adult THC-treated counterparts at five weeks following the last injection. These results indicate that THC use may have long-lasting effects with adolescence being a unique period of susceptibility.

Keywords

THC; cannabinoids; adolescence; memory; anxiety; CB1R

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Author Contributions

CRK and SLB were responsible for the study concept, design, and data analysis of behavioral data. CRK, YZ, and SLB were responsible for the design and analysis of Western blot data. CRK was responsible for acquisition of behavioral data, CRK, YZ, and Patricia Muskus were responsible for acquisition of Western blot tissue and data. All authors contributed to writing and reviewing the manuscript.

1. Introduction

Cannabis is the most commonly used illicit drug in the United States across all age groups (National Institute on Drug Abuse, 2014). Exogenous cannabinoids found in cannabis, such as Δ9-tetrahydrocannabinol (THC)[1], as well as endogenous cannabinoids (endocannabinoids), bind to cannabinoid receptors (CBRs). These are inhibitory receptors located on many cell types including neurons, microglia, astrocytes, and endothelial cells in the central and peripheral nervous systems. In an unaltered state, endocannabinoids regulate "fine-tuned" synchronous neuronal outputs which maintain system function and contribute to long term potentiation and depression (Freund & Katona, 2007; Svíženská et al., 2008; Chevaleyre & Piskorowski, 2014). Conversely, administration of cannabinoids, such as following cannabis consumption, broadly disrupts this honed regulation. Repeated exposure to cannabinoids may result in alterations in cognition and memory, focus, mood shifts, and inflammatory and pain responses that persist even after prolonged abstinence. Due to the role the endocannabinoid system plays in neurodevelopment, whether adolescent exposure alters the trajectory of cannabinoid effects should also be studied (Freund & Katona, 2007; Svíženská et al., 2008; Volkow et al., 2016; National Academies of Sciences, 2017).

In a recent review of the literature surrounding both the beneficial and detrimental effects of cannabis use in humans, the National Academies of Sciences (2017) made several recommendations to further development of the cannabis research field. These include focusing on the developmental period of adolescence and the use of preclinical studies that examine both acute and chronic exposure to cannabinoids. The adolescent period in mice is conservatively accepted to range in age from postnatal day (PND) 28–42 (Schneider, 2013) wherein many (NOR), postnatal day (PND), Δ9-tetrahydrocannabinol (THC) behavioral and neurobiological changes occur in rodents that mimic those seen in humans, including the developmental influence of the cannabinoid system (Lee & Gorzalka, 2012). However, there are accepted caveats that some adolescent-like behaviors fall outside of this range, and there are gender-specific differences which may push developmental "milestones" closer to PND60, which is generally considered as adulthood (Spear, 2000; Casey et al., 2008). Nevertheless, adolescent rodent models are increasingly being utilized to explore the neurodevelopmental effects of drug exposure (Casey et al., 2008; Schramm-Sapyta et al., 2009).

The goal of the current study was to characterize whether adolescent and adult mice demonstrate different behavioral consequences following an acute THC injection and a repeated history. THC's effects on memory in a novel object recognition (NOR) task, anxiety-like behavior on the elevated plus maze (EPM), and locomotor activity in the open field were selected as they mimic long-term changes that have been reported in human cannabis users (Freund & Katona, 2007; Svíženská et al., 2008; Volkow et al., 2016). These behaviors exemplify non-spatial memory retrieval, unconditioned anxiety, and sedation behavior, and are independent of motivation to obtain a reinforcer or reward, or to avoid punishment (Cohen & Stackman, 2015; Lee et al., 2015; Mohammad et al., 2016).

[1]C57Bl/6J (B6), cannabinoid receptors (CBR), DBA/2J (D2), elevated plus maze (EPM), novel object recognition

Importantly, as these tasks require little to no training, they are optimal to run during the relatively short period of rodent adolescence.

THC's ability to alter NOR and EPM has been explored under both acute and repeated injection conditions. In the NOR task, acute THC did not alter object discrimination in adolescent or adult rats (Ciccocioppo et al., 2002; Swartzwelder et al., 2012). Conversely, a repeated adolescent history of THC exposure was shown to reduce novel object discrimination in rats (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012; but see O'Tuathaigh et al., 2010). However, of these studies, only Quinn et al. (2008) utilized an adult control and found no effect of adult THC history on later object discrimination. This may indicate that there are important age-related differences in behavior following THC exposure.

The National Academies of Sciences (2017) also recommends recording feelings of anxiety and sedation in all clinical studies, as these are symptoms often associated with cannabinoid use. Preclinical studies have examined the effects of a 30 minute acute THC pretreatment on EPM activity. These studies have produced conflicting findings, with some showing anxiogenic effects (Celerier et al., 2006; Schramm-Sapyta et al., 2007) and others showing anxiolytic effects (Rubino et al., 2007; Braida et al., 2007; Fokos & Panagis, 2010) in both adolescents and adults. In part, this disagreement may be due to differences in strain/genotype sensitivity to THC and/or THC dose, with doses under 1.5 mg/kg generally being anxiolytic. A history of repeated injections in rodents has also produced mixed results. Onaivi et al. (1990) found no effect in mice, but an anxiogenic effect in rats, when THC was administered during adulthood. Conversely, Cadoni et al. (2008) and O'Tuathaigh et al. (2010) demonstrated anxiolytic effects of repeated adolescent administration on later adult behavior.

Although previous findings have been inconclusive on how THC affects behavior, an adolescent history of THC has reliably led to protracted deficits in object discrimination in the NOR task and anxiolytic behavior in the EPM. However, these studies have not consistently included the assessment of adult groups, which is necessary to conclude whether adolescents are differentially susceptible to the effects of THC. To observe how acute and repeated treatment with THC may differentially affect behavior when administered during adolescence or adulthood we used inbred C57Bl/6J (B6) and DBA/2J (D2) mice from Jackson Laboratories. These strains have been previously demonstrated to exhibit strain- and age-specific differences in NOR, EPM, and open field behaviors at the time of acute adolescent exposure used in the current study (Moore et al., 2011; Balsevich et al., 2014). Identification of strain-specific differences may help to identify genetic markers of THC-related susceptibility. Based on previous research, we hypothesized that acute treatment would be ineffective in altering object recognition in both ages and strains, but would be anxiogenic in the EPM and would elicit a sedative response in the open field. Following acute assessment, mice received repeated injections of THC or vehicle and were tested again following a period of no drug exposure to assess whether an adolescent history of THC resulted in different behavioral consequences than exposure occurring during adulthood. We hypothesized that a repeated history of adolescent injections would impair later adult object discrimination but be anxiolytic in the EPM compared to vehicle groups,

whereas an adult history would have no effect on later behavior. Finally, CB1R binding increases across the brain during the transition from adolescence to adulthood (Verdurand et al., 2011), but repeated treatment may result in receptor downregulation (Breivogel et al., 1999). Therefore we hypothesized that adolescent treatment with THC would cause long-term changes in CB1R receptor expression compared to adult treated mice.

2 Method

2.1 Animals

Eighty B6 and D2 mice were purchased from Jackson Laboratories and arrived at age 3 weeks (20 per genotype) or 8 weeks (20 per genotype). Mice were singly housed upon arrival and maintained on a 12:12 light cycle in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Single-housing was chosen to avoid detrimental effects of subordinate/dominant hierarchies which could affect outcomes of the behavioral tasks in an uncontrolled manner (Blanchard et al., 2001; Singewald et al., 2009). Food and water was available at all times apart from during behavioral tests. Testing began at PND27 and PND68 for adolescent and adult mice, respectively. All procedures were approved by the IUPUI School of Science Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals (The National Academic Press, 2003).

2.2 Drugs

THC was obtained from the National Institutes of Health/National Institute on Drug Abuse (Bethesda, MD) at a concentration of 1 mg per 50 microliters of 200 proof ethanol. For the work describe herein, the THC was then diluted to a concentration of 10 mg in vehicle comprised of 0.9% saline, Tween 80 (Sigma Aldrich, St. Louis, MO), and 200 proof ethanol (Pharmco, Inc., Brookefield, CT). Vehicle was similarly composed of 90% saline, 5% Tween 80, and 5% ethanol. Animals received 6 injections of THC or vehicle throughout the course of the study. THC or vehicle was delivered via intraperitoneal injection in a volume of 0.1 ml per 10 g of body weight. Although previous studies finding object memory deficits employed a twice/day injection paradigm (Realini et al., 2011; Zamberletti et al., 2010), even the heaviest adolescent users do not use cannabinoids on a daily basis (Scalco & Colder, 2016). Therefore, we chose to administer injections every 72 hrs. A 10 mg/kg injection was chosen for its ability to produce anxiogenic activity in the EPM following acute administration (Onaivi et al., 1990). Weights were recorded for every injection day and on the day of brain extraction. Table 1 outlines the general experimental procedure and mouse age (PND) at each test. All behavioral assays were run under red light conditions (approximately 8 lux) from PND27-29 or PND68-70.

2.3 Acute Exposure Tests

2.3.1 Novel Object Recognition—The NOR task consists of a training session, wherein animals are given two identical objects, and a test session, wherein one familiar object is replaced with a novel object. The tendency to explore the novel object during the test session reflects non-spatial, hippocampal-based memory (Cohen & Stackman, 2015). NOR sessions were conducted in a 40 x 40 cm wooden box painted ochre brown. Our procedure followed

that of Fritz et al. (2014) with the exception that mice were individually transported into the behavioral testing room and given approximately 10 minutes to acclimate before each session and removed from the testing room immediately following each session. Following the training session mice received their first injection of THC or vehicle upon being placed back in the vivarium. This injection time point was chosen so that the sedative properties of THC would not interfere with object exploration. Only data from the first 5 min of the novel object session are reported due to the rapid familiarization with the novel object that has been previously reported (Antunes & Biala, 2012) and observed in the current study. The order of familiar and novel object familiarization, as well as novel object zone, was counterbalanced across strain, age, and treatment group. All behavior was recorded using ANY-maze Software (Stoelting, Wood Dale, IL). Object interaction was recorded as time spent sniffing or directly contacting each object.

2.3.2 Elevated Plus Maze—The EPM test was run approximately 72 hours following the NOR test on PND32 or PND73. The test was run under dim red light; lux was approximately 5 in the closed arm, 10 in the center, and 15 on the open arms. Although EPM is commonly run under normal light conditions, red light gave us the ability to observe either anxiolytic or anxiogenic drug effects compared to control group behavior. As described in Moore et al. (2011), a smaller scaled version of the maze was used for adolescents and a full-sized maze was used for adults. The black Plexiglas mazes had an arm length of 57 cm and 76 cm for adolescents and adults, respectively, and both were elevated 74.5 cm from the ground. THC or vehicle was administered 30 minutes before behavioral testing. Mice were injected in the animal vivarium and then walked to the EPM room immediately prior to placement on the maze to avoid interruption of the previous ongoing EPM test. Behavior was recorded for 5 minutes by a camcorder mounted to the ceiling above the apparatus. Videos were scored by a blind rater who recorded time spent in the open arms and number of open arm entries.

2.3.3 Open Field Activity—Immediately following the EPM task (72 hours following the NOR test on PND32 or PND73), mice were walked into another room and placed in Versamax activity monitors (Accusan Instruments, Columbus, OH) for 10 minutes. These chambers measure 40 x 40 cm with photocell beams located 2 cm above the Plexiglas floor which record locomotor activity. Output is sent to a Dell computer. They are contained in sound attenuating chambers with a house light. The house light remained off for the current studies. Total distance traveled and total time spent moving were used as indicators of sedation. The ratio of percent time spent moving in the center versus total time spent moving was used as a secondary quantification of anxiety-like behavior.

2.4 Maintenance Injections

Following the two injections given during behavioral testing (PND28 and 32 for adolescents, PND69 and 73 for adults), mice received four more THC or vehicle injections for a total of 6 injections. Whereas the subsequent adolescent exposure injections occurred on PND35, 38, 41, and 44, the subsequent adult exposure injections occurred on PND76, 79, 82, and 85. All injections took place in the vivarium. Mice were then left undisturbed for approximately four weeks before initiation of the second pass of behavioral testing (see below).

2.5 Effects of Adolescent or Adult History on Later Behavior

To observe how a previous history of THC alters behavior in abstinence, mice were run through the same battery of behavioral tasks in the same manner as described in sections 2.3.1–2.3.3 beginning on PND71 (adolescent exposure group) and PND117 (adult exposure group) (see Table 1). During this second set of tests there were no injections administered.

2.6 Brain Extraction and Western Blot

Brains were harvested on PND77 or PND123, approximately 24 hours following conclusion of behavioral testing. Mice were euthanized by cervical dislocation and the brain was rapidly removed and frozen in liquid nitrogen. Western Blot was used to assess levels of hippocampal CB1R expression. The hippocampus was bilaterally removed and homogenized in 300ul of RIPA buffer with protease inhibitor (1ml of RIPA buffer containing 100ul of 10X PI and 10ul of 0.1M PMSF) (Thermo Fisher) for about 30 seconds until the tissue thoroughly homogenized using a Pellet pestles cordless motor. Protein concentration was determined using a Bio-Rad Protein Assay kit. Samples were denatured using 200ug of sample protein with 5ul of 4x Loading Dye, and adding 5% of 1M DTT. The final volume was adjusted to 20ul with RIPA buffer and denature at 95°C for 5 minutes. Two hundred micro grams of total protein in 20 ul volume was loaded into each well of a 12% gel (mini-pro TEAN TGX Gels, Bio-Rad), and the protein was separated by electrophoresis at 120V in a 1X Tris/Glycine/SDS buffer (Bio-Rad). The gel was transferred using a mini format 0.2 uM nitrocellulose single application Trans-Blot turbo (Bio-Rad) for 7 minutes at 2.5A/25V. Primary antibody (Anti-Cannabinoid Receptor 1, Rabbit polyclonal to Cannabinoid Receptor 1, Abcam) was added to the PBS buffer (5% nonfat milk in 1x PBS with 0.1% Tween 20) at 1:1000 dilution and incubated at 4°C overnight on a rotator. After incubation the membrane was washed with PBS (PBS+ 0.1% Tween 20) 3 times, 10 min each. Secondary antibody was then added at a 1:5000 dilution (IRDye 800 CW Goat anti-Rabbit IgG (H+L), LI-COR). The membrane was again washed with PBS 3 times for 10 mins each. The image was then scanned from membrane with a CLx Odyssey scanner. B-actin was used as the reference (primary antibody β-actin mouse monoclonal antibody, secondary antibody IRDye 680RD Donkey anti-Mouse IgG (H+L), LI-COR). CB1 protein expression for each mouse was calculated as the signal strength of CB1 expression normalized to the signal strength of β-actin expression.

2.7 Statistical Analysis

Statistical analyses were run using SPSS 24 (IBM) or GraphPad Prism. Significance was set at $p < .05$ and corrected for post-hoc analyses. To conserve power, data for B6 and D2 mice were analyzed separately due to differences in baseline behavior seen previously. Independent samples t-tests were also used to determine drug effects in each age group due to previously documented age effects in these tasks (Moore et al., 2010; 2011; Balsevich et al., 2014). Alpha level for t-tests were corrected to .0125. Cohen's d (d) is reported as a measure of effect size for significant independent samples t-test. Weight over the course of the study was analyzed using a repeated measures Day*Treatment*Age at Treatment ANOVA. Training effects on object discrimination were analyzed by running a Pearson's bivariate correlation on training investigation time and novel object discrimination, as well

as by determining whether training zone preference affected discrimination. Training zone preference in the NOR task was calculated as a ratio of time spent in zone A compared to zone B. A ratio of 0.75–1.25 = no zone preference, < 0.75 = zone A preference, and > 1.25 = zone B preference. Whether the novel object was placed in the preferred training zone was coded as 0 = no preference, 1 = yes, and 2 = no. A one-way ANOVA was then run to analyze whether the novel object being placed in the preferred training zone significantly influenced novel object discrimination.

Two-way age at treatment*treatment type ANOVAs were used to analyze discrimination index in the NOR task, time spent in the open arms and number of open arm entries in the EPM, and total distance moved, total time spent moving, and percent of time spent moving in the center of the open field. Discrimination index for the NOR task was calculated as [(novel investigation time-familiar investigation time)/(total investigation time)]. The discrimination index value ranges from −1 to +1 with positive numbers indicating novel object discrimination and 0 indicating no preference. A preference value that was significantly greater than 0 as analyzed by a one-sample t-test indicated significant discrimination for a group. This method of analysis is preferred as it factors in the total time spent exploring both objects (Cohen & Stackman, 2015). Percent of time spent moving in the center of the open field was calculated as (Time spent in center/Total time spent moving) as reported in the Accuscan output.

Genotype differences were not present on CB1R expression in vehicle-treated mice. Therefore, effects of THC on CB1R expression were analyzed as a ratio of vehicle expression and compared using a genotype*age at treatment ANOVA. This ratio was calculated by finding the average CB1R expression for each vehicle group (B6/adolescent treated, B6/adult treated, D2/adolescent treated, and D2/adult treated) then dividing the expression of each THC treated mouse by the respective vehicle group average.

3 Results

3.1 Weight

A repeated measures ANOVA with day as the within subjects variable and age at treatment and treatment type as between subjects variables was run to assess the weights of B6 and D2 mice independently across the course of the study. For B6 mice there was a significant effect of day with a linear and cubic trend; $F(6,156) = 40.50$, $p < .001$. There was also a significant effect of age at treatment, with adolescents having lower weights; $F(1,26) = 51.81$, $p < .001$. Day and age at treatment also showed a significant interaction with a linear trend; $F(6,156) = 2.638$, $p < .05$. Weights were significantly different between the adolescent and adult treated mice at all time points (p's < .001). However, an independent samples t-test comparing the final weight of adolescent mice at PND77 to the weight of adult treated mice at PND76 was not significantly different, indicating that adolescent-treated mice did not display long-term weight changes ($p > .05$). There were no significant main effect of or interactions with treatment type (p's > .05) (Fig. 1A).

D2 mice also displayed a significant effect of day with a linear, quadratic, and cubic trend; $F(6, 150) = 150.59$, $p < .001$. There was also a significant effect of age at treatment, with

adolescent treated mice having lower weights; $F(1,25) = 208.69$, $p < .001$. Day and age at treatment also showed a significant interaction with a linear, quadratic, and cubic trend; $F(6,150) = 45.46$, $p < .001$. Weights were significantly different between the adolescent and adult treated mice at all time points (p's < .001). An independent samples t-test comparing the final weight of adolescent mice at PND77 to the weight of adult treated mice at PND76 demonstrated that adolescent treated mice showed a long-term deficit in weight gain; $t(27) = 4.234$, $p < .001$. There were no significant main effect of or interactions with treatment type (p's > .05) (Fig. 1B).

3.2 Effects of Acute Administration

3.2.1 Novel Object Recognition: B6 Mice—The non-significant correlation of time spent investigating the objects during the training session and discrimination index during the test session indicated that training investigation did not predict later object discrimination in B6 mice; $r(40) = -0.238$, $p > .05$ (data not shown). Whether the novel object was placed in the zone that was preferred during the training session also did not influence discrimination index; $F(2,39) < 1$, $p > .05$ (data not shown).

Effects of age (adolescent or adult) and treatment (THC or vehicle) on discrimination index were analyzed using a two-way ANOVA. There were no significant effects of age, acute injection following training, or interaction of age*treatment on discrimination index during the initial NOR task. Alpha-corrected independent t-tests indicated no significant effect of drug treatment at either age group (p's > .05). One-sample t-test assessment of novel object discrimination determined that only the adult mice that received THC displayed significant object recognition in the acute task ($p < .05$) (Fig. 2A).

3.2.2 Novel Object Recognition: D2 Mice—Similar to that for B6 mice, the non-significant correlation of time spent investigating the objects during the training session and discrimination index during the test session indicated that training investigation did not predict later object discrimination in D2 mice; $r(40) = -0.036$, $p > .05$ (data not shown). Whether the novel object was placed in the zone that was preferred during the training session also did not influence discrimination index; $F(2,39) = 1.70$, $p > .05$ (data not shown).

There were no significant effects of age, acute injection following training, or interaction of age*treatment on discrimination index during the initial NOR task in D2 mice, and alpha-corrected independent t-tests indicated no significant effect of drug treatment at either age group (p's > .05). Finally, one-sample t-test assessment of novel object discrimination determined that no D2 groups displayed significant novel object recognition (p's > .05) (Fig. 2B).

3.2.3 Elevated Plus Maze Activity: B6 Mice—Although there were no main effects of treatment age or type on time spent in the open arms during the EPM for B6 mice, there was a significant interaction of age at treatment*treatment type; $F(1,36) = 5.76$, $p < .05$. This interaction was driven by THC reducing time spent in the open arms in the adult mice; $t(18) = 2.50$, $p < .05$, $d = 0.92$ (Fig. 3A). There were no significant effects of age at treatment, treatment type, or age*treatment on number of open arm entries (data not shown).

3.1.4 Elevated Plus Maze Activity: D2 Mice—D2 mice displayed no main effect of age at treatment or interaction of age*treatment type (p's > .05). There was a significant effect of treatment type, with THC reducing time in the open arms; F(1,36) = 5.809, $p < .05$. This effect was driven by THC reducing time spent in the open arms in the adolescent mice; t(18) = 2.57, $p < .05$, $d = 1.15$ (Fig. 3B). There were no significant effects of age at treatment or age*treatment on number of open arm entries. There was a main effect of treatment type, with THC significantly reducing number of open arm entries; F(1,36) = 5.54, $p < .05$. Alpha-corrected independent samples t-tests did not reveal a significant effect of THC for adolescents or adults (p's > .05) (data not shown).

3.2.5 Open Field Activity: B6 Mice—There was a significant main effect of treatment type on total distance in the open field in B6 mice, with THC reducing activity; F(1,36) = 15.16, $p < .001$. Alpha-corrected independent samples t-tests revealed that the sedative effect was seen in both age groups, with a greater effect in adults (p's < .05, adolescent $d = 0.96$, adult $d = 1.61$). Although the main effect of age did not reach significance there was a trend towards adolescents being more active ($p = .074$). There was not a significant interaction of age at treatment*treatment type ($p > .05$) (Fig. 3C).

There was a significant main effect of treatment type on total movement time in the open field, with THC reducing activity; F(1,36) = 13.543, $p < .001$. Alpha-corrected independent samples t-tests revealed that the reduction in time spent moving only reached significance in adult mice; t(18) = 3.60, $p < .01$, $d = 1.70$. Although the main effect of age did not reach significance there was a trend towards adolescents spending more time moving ($p = .054$). There was not a significant interaction of age at treatment*treatment type ($p > .05$) (data not shown).

There were no significant main effects or interaction effects on percent of time spent in the center of the open field (p's > .05). Alpha-corrected independent samples t-tests indicated that there was a significant reduction in percent of time spent in the center for adolescent mice only, with THC reducing this metric; t(18) = 4.23, $p = .001$, $d = 1.89$ (Fig. 3E).

3.2.6 Open Field Activity: D2 Mice—There was a significant main effect of age on total distance in the open field in D2 mice, with adults showing reduced activity; F(1,36) = 5.84, $p < .05$. There was no significant interaction of age at treatment*treatment type or overall treatment effect ($p > .05$). However, THC significantly reduced locomotion in adult mice; t(18) = 2.57, $p < .05$, $d = 1.15$ (Fig. 3D).

There was a strong trend towards a significant main effect of age on total time spent moving in the open field for D2 mice ($p = .05$). Alpha-corrected independent samples t-tests revealed that this trend was driven by a reduction in adults only; t(18) = 2.27, $p < .05$, $d = 1.07$. There was not a significant effect of treatment type or interaction of age at treatment*treatment type ($p > .05$) (data not shown).

There was a significant main effect of treatment type on percent of time spent moving in the center of the open field, with THC reducing this metric in D2 mice; F(1,36) = 13.543, $p < .001$. However, alpha-corrected independent samples t-tests revealed that the reduction did

not reach significance in either age group (p's > .05). There were no significant effects of age at treatment or age*treatment type (p's > .05) (Fig. 3F).

3.3 Effects of Repeated Administration History

3.3.1 Novel Object Recognition: B6 Mice—The non-significant correlation of time spent investigating the objects during the training session and discrimination index during the test session indicated that training investigation did not predict later object discrimination in B6 mice; r(40) = 0.104, p > .05 (data not shown). Whether the novel object was placed in the zone that was preferred during the training session also did not influence discrimination index (p > .05) (data not shown).

Effects of age (adolescent or adult) and treatment (THC or vehicle) on discrimination index were analyzed using a two-way ANOVA. Although there was a trend towards a significant interaction of age at treatment*treatment history; F(1,36) = 3.52, p = .069, there were no significant effects of age at treatment, treatment history, or their interaction on discrimination index during the NOR task following a history of repeated injections in B6 mice. Alpha-corrected independent t-tests indicated no significant effect of drug history for either age group (p's > .05). One-sample t-test assessment of novel object discrimination determined that only the mice with an adolescent history of vehicle or an adult history of THC displayed significant object recognition (p < .05) (Fig. 4A).

3.3.2 Novel Object Recognition: D2 Mice—One D2 adult treatment mouse was dropped from the history analyses due to development of seizures over the course of THC treatment. The non-significant correlation of time spent investigating the objects during the training session and discrimination index during the test session indicated that training investigation did not predict later object discrimination in D2 mice; r(39) = −0.174, p > .05 (data not shown). Whether the novel object was placed in the zone that was preferred during the training session also did not influence discrimination index; F(2,38) < 1, p > .05 (data not shown).

A two-way ANOVA revealed no significant effects of age at treatment, treatment history, or interaction effect on discrimination index during the NOR task in D2 mice (p's > .05). Alpha-corrected independent t-tests indicated no significant effect of treatment history at either age group (p's > .05). One-sample t-test assessment of novel object discrimination determined that only D2 mice with an adolescent vehicle history displayed significant novel object recognition (p < .05) (Fig. 4B).

3.3.3 Elevated Plus Maze Activity: B6 Mice—A two-way ANOVA revealed no main effect of treatment history or interaction of age at treatment*treatment history on time spent in the open arms during the EPM for B6 mice (p's > .05). Alpha-corrected independent t-tests confirmed no significant effects of drug history in either age group (p's > .05). However, there was a significant main effect of age at treatment; F(1,36) = 9.36, p < .01 (Fig. 5A). The same pattern was demonstrated for open arm entries, with only age at treatment displaying a significant main effect; F(1,36) = 6.27, p < .05 (data not shown). B6 mice with an adult treatment history, aged to PND122, spent significantly less time in the

open arms and made fewer open arm entries than mice with an adolescent history that were aged to PND76 (p's < .05).

3.3.4 Elevated Plus Maze Activity: D2 Mice—A two-way ANOVA of D2 data revealed no significant effects of age at treatment, treatment history, or interaction effect on time spent in the open arms during the EPM task (p's > .05). Alpha-corrected independent t-tests indicated no significant effect of treatment history at either age group (p's > .05) (Fig. 5B). The same patterns were demonstrated for number of open arm entries in D2 mice, with no significant main effects of age at treatment, treatment history, interactive effects, or treatment history for each age group independently (p's > .05) (data not shown).

3.3.5 Open Field Activity: B6 Mice—A two-way ANOVA revealed no significant effects of age at treatment, treatment history, or an interaction effect on open field activity in B6 mice (p's > .05). Alpha-corrected independent t-tests confirmed a lack of significant effect of drug history on locomotor activity in both age groups (p's > .05) (Fig. 5C).

There were also no significant effects of age at treatment, treatment history, or an interaction effect on time spent moving in the open field in B6 mice (p's > .05). Alpha-corrected independent t-tests confirmed a lack of significant effect of drug history on time spent moving in both age groups (p's > .05) (data not shown).

Finally, there were no significant effects of age at treatment, treatment history, or an interaction effect on percent of time moving in the center of the open field in B6 mice (p's > .05). However, alpha-corrected independent t-tests indicated that mice with an adult history of THC spent more of their time moving in the center of the open field compared to vehicle history; $t(18) = -2.16$, $p < .05$, $d = -0.95$ (Fig. 5E).

3.3.6 Open Field Activity: D2 Mice—A two-way ANOVA revealed no significant effects of age at treatment, treatment history, or an interaction effect on open field activity in D2 mice (p's > .05). Alpha-corrected independent t-tests confirmed a lack of significant effect of drug history on locomotor activity in both age groups (p's > .05) (Fig. 5D).

Significant effects were also not observed for age at treatment, treatment history, or their interaction on time spent moving in the open field in D2 mice (p's > .05). Alpha-corrected independent t-tests confirmed a lack of significant effect of drug history on time spent moving in both age groups (p's > .05) (data not shown).

Finally, there was no observable age at treatment or interaction effects on percent of time moving in the center of the open field in D2 mice (p's > .05). However, there was a trend towards an effect of injection history, with a history of THC reducing this metric; $F(1,35) = 3.961$, $p = .054$. Alpha-corrected independent t-tests indicated that this trend was driven by a significant effect of adolescent history of THC reducing percent of movement time spent in the center; $t(18) = 2.73$, $p < .05$, $d = 1.22$ (Fig. 5F).

3.4 CB1R Expression

The two-way ANOVA did not reveal a significant effect of genotype, age at treatment, or an interaction of these factors on normalized CB1R expression in animals receiving vehicle (p's > .05) (Fig. 6A). However, in the animals that received THC, there was a significant main effect of age at treatment; $F(1,35) = 4.35$, $p < .05$. Mice with an adolescent history of THC exposure exhibited elevated expression of CB1R. However, the effect of genotype and interaction of genotype*age at treatment were not significant (p's > .05). The effects of adolescent THC exposure were also observed when normalized CB1R expression levels were examined as a ratio to their respective vehicle control group ($p < .05$) (Fig. 6B). No age*genotype THC treated group was significantly different from their respective vehicle control group (p's > .05).

4 Discussion

The outcomes of the current research supported some, but not all of the hypotheses based on previous findings. Acute THC resulted in anxiogenic and sedative effects that were entirely age- and genotype-specific. An adolescent history of THC was shown to significantly reduce subsequent adult weight in D2 mice, and to impair significant novel object discrimination in both strains. Interestingly, an adult history of THC appears to rescue an effect of aging on object discrimination demonstrated in the adult history vehicle mice. Further, THC history did not alter later unconditioned anxiety-like activity in the EPM in either age or strain. However, percent of time in the center of the open field, another measure of unconditioned anxiety-like activity, was altered in an age- and strain-specific manner. Finally, an adolescent THC history produced an upregulation of hippocampal CB1R protein expression compared to an adult history across strains that was present 5 and a half weeks following the last injection.

An adolescent history of THC has been well-documented to cause impairments in later novel object discrimination (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012; but see O'Tuathaigh et al., 2010). The current study observed similar disruption of novel object discrimination in both strains of adolescent treated mice, wherein the control groups showed significant discrimination and the THC groups did not. It should be noted that THC did not significantly reduce discrimination levels compared to the control group, but rather disrupted the expression of significant object discrimination. Previous studies using rats have shown robust, significant disruption of novel object recognition following an adolescent history of THC. However, these studies employed a 3 minute (Realini et al., 2011; Zamberletti et al., 2012) or 1 hour (Quinn et al., 2008) intertrial interval (ITI) between the training and test sessions of the NOR task. This relatively short ITI may have enhanced the ability to detect THC-induced impairments compared to the 24 hour ITI used in the current study.

In the current study, mice with an adult history of vehicle did not show significant discrimination, but the adult mice with a history of THC did (Fig. 4). In part, this may be due to the significant reduction of hippocampal CB1R protein seen in the adult THC treated mice compared to the adolescent treated mice (Fig. 6). It has been previously documented that repeated THC administration reduces CB1R density rapidly in areas such as the

hippocampus, and that THC may work to antagonize CB1Rs in areas with low CB1R density (Breivogel et al., 1999; Pertwee, 2008). Conversely, THC agonizes CB1Rs in high-density areas (Pertwee, 2008), and leads to alterations in neuroinflammatory processes when administered during adolescence, resulting in a pro-inflammatory shift in the hippocampus during adulthood following repeated adolescent injection (Zamberletti et al., 2015; Moretti et al., 2015). Moderate increases in inflammatory and stress responses are also seen following an acute saline administration, whereas stress response, neuroinflammation, and prolonged single-housing have been shown to reduce performance in the NOR task (Võikar et al., 2005; Carey et al., 2009; Fishbein-Kaminietsky et al., 2014; Freiman et al., 2016). The ability of THC to protect against disruption of novel object discrimination in adult treated mice may have been due to reduced neuroinflammatory responses across treatment that were present in the adult control- and adolescent THC-treated mice, in part due to stress response and CB1R receptor levels, respectively. The impairments seen in adolescent-treated animals may also be a result of the role CBRs play in development of the hypothalamopituitary adrenal axis and stress responsivity (Lee & Gorzalka, 2012), thereby exacerbating the effects of immediate single-housing following shipment stress, repeated injection stress, and THC exposure during adolescence. Although single-housing was chosen to eliminate uncontrollable subordinate/dominant relationship stress (Blanchard et al., 2001; Singewald et al., 2009), pair-housing in the current study may have mediated some of the negative effects of repeated THC administration and resulted in a different pattern of behavior.

It is important to note that in the current study significant discrimination was not reached by the control groups in the acute task (Fig. 2) and there were no statistically significant differences between control and THC groups in the NOR task at either exposure time point, likely due to the high levels of variability seen in our NOR paradigm specifically during the acute task. Variability could arise from multiple aspects of the current study including a stressful injection occurring immediately post-training, the use of a relatively long inter-trial interval to assure that the test session did not occur under the influence of THC, and mice generally not performing as well in the NOR task as rats which were used in the previous studies (Cohen & Stackman, 2015). As both B6 and D2 control mice tested at PND73 during abstinence reached significant novel object discrimination (Fig. 4), it appears that the timing of the injection following training during the acute task may be of primary concern. Future studies might consider delaying drug injection so that it does not immediately follow the training session, reducing the inter-trial interval so that it falls outside of the time-course of THC's pharmacological effects but is shorter than 24 hours, and/or increasing the training interval.

A 30 minute pretreatment of THC resulted in age- and strain-specific alterations in anxiety-like and sedative behaviors (Fig. 3), whereas prior THC treatment had minimal effects on anxiety-like behavior five weeks following the last injection (Fig. 5). The basis for these age- and strain-specific differences in anxiety-like behavior is unclear. The amygdala, which has been implicated in anxiogenic effects of THC and evaluation of uncertainty (Rosen & Donley, 2006; Rubino et al., 2008), differs from the hippocampus in that adolescent and adult Wistar rats display similar levels of available CBRs (Verdurand et al., 2011). All ages and strains treated with acute THC demonstrated anxiety-like behavior in at least one paradigm, indicating a nebulous anxiogenic effect of THC. The EPM and open field

measures of anxiety-like activity are both considered to fall under the same umbrella of "approach-avoidance conflict tests." Thus, it is not immediately clear why the two different assessments of THC-induced anxiogenesis did not produce similar outcomes for adolescent and adult B6 and D2 mice. However, a recent meta-analysis determined that studies assessing anxiety-related interventions using the EPM and open field do not reliably reproduce each other (Mohammad et al., 2016). Furthermore, in the current study the open field task was run in the absence of white or red light. Therefore, discrepancies between these paradigms may be expected due to a lack of difference in lux in the center versus edges of the field, unlike the open versus closed arms of the EPM.

It is important to interpret the anxiogenic effects of THC in light of the sedative properties of drug that are also produced. Adolescent and adult mice exhibited a significant reduction in distance moved and total time spent moving following acute THC. However, movement was not completely abolished, indicating that although a sedative effect was present mice were still able to perform in the EPM. This is further reinforced by the lack of effect on number of entries into the open arms made by B6 mice treated with THC. Conversely, THC treatment resulted in a reduction in open arm entries across ages in D2 mice, but no sedative effect in the open field, also indicating that D2 mice were able to perform in the EPM task without interfering sedative effects.

These genotype-specific effects are both novel and important. Genetic differences in basic behaviors such as sedation or anxiety-like activity, which are analogous to anecdotal reports of short-term negative consequences of human use (National Academies of Sciences, 2017), may serve as the basis for identification of genetic markers of THC-related susceptibility. Strategies such as assessing behavior in a panel of recombinant inbred strains, such as the B6 by D2 cross (BXD RI), allow for genetic mapping of quantitative trait loci (QTLs) or chromosomal regions that may contain genes that influence susceptibility or resistance to THC-related behaviors (Plomin et al., 1991; Williams & Williams, 2017). Newer techniques in RNA and chromatin sequencing also make it possible to identify epigenetic alterations following THC treatment (Scott-Boyer & Deschepper, 2013; Goldowitz et al., 2014; Yeo et al., 2016). These preclinical advances are critical in identifying translational biomarkers of risk and consequences of drug use in human populations (Crabbe, 2016).

The current study has several limitations. The NOR paradigm was not fully optimized, as control animals in the acute task failed to show significant novel object discrimination. Individual housing may have led to heightened stress levels and exacerbation of novel object recognition impairment. Stress may cause deficits in NOR discrimination (Võikar et al., 2005) and adolescent behavior is particularly sensitive to the influence of stress (Lee & Gorzalka, 2015). Due to rapid development, adolescent animals in the current study began testing one week following arrival at IUPUI, whereas adult animals had two weeks to acclimate to the vivarium. Thus, it is possible that mice never exposed to shipping stress may display different behavioral responses to THC than that seen in the current study. The use of only male mice in the current study is also a major drawback, as females may show sex-specific differences in these tasks as well as susceptibility to THC treatment in adulthood and across development (Rodriguez de Fonsesca et al., 1993; Podhorna & Brown, 2002; Moore et al., 2007; Llorent-Berzal et al., 2013; Balsevich et al., 2014). Future studies should

include female mice and may consider pair housing animals to reduce the harm of long-term isolated housing. It should also be cautioned that 4 weeks of abstinence may not be representative of the true “long-term” effects of THC. A recent review by Ganzer et al. (2016) concludes that, although adolescent cannabis use in humans is related to many cognitive and structural functions in abstinence, the term “long-term abstinence” is ill-defined and these deficits may improve given more time. Cause-and-effect of cannabinoid use on these constructs cannot be determined in humans, thereby necessitating the use of preclinical models to observe the parameters of these deficits and inform clinical studies. Although marijuana is the most commonly used illicit drug, use is relatively infrequent when compared to legal drugs (Azofeifa et al., 2016), with the heaviest young adolescent users report use that averages about once per week (Scalco & Colder, 2016). Bioavailability of THC in humans is widely inconsistent, even when the dose and parameters of administration are tightly controlled (Huestis, 2005). However, it is difficult to extrapolate how preclinical parameters, such as the 10 mg/kg dose given every 72 hrs in the current study, mimic the nature of adolescent and adult cannabinoid usage. Finally, THC is only one of many cannabinoids that are currently being researched. As a classical cannabinoid, it may show different binding potential, selectivity, and result in different behavioral effects than other cannabinoids, such as Win55212, a prominently researched aminoalkylindole cannabinoid (Pertwee, 2005).

In conclusion, the current study comprehensively demonstrated the acute and prolonged effects of adolescent and adult THC treatment in two strains of inbred mice. Our findings replicated previous studies that have demonstrated that an adolescent history of THC interferes with later novel object recognition after prolonged abstinence. In adults this may be due to CB1R downregulation and neuroinflammatory responses, whereas adolescent effects may be due to THC’s developmental interactions with the HPA-axis. These proposed mechanisms may be investigated by concurrently antagonizing pro-inflammatory and stress-activated pathways. Further, previous findings of acute anxiogenic effects of high doses of THC have been replicated in an age- and strain-specific manner. No repeated administration effects of THC were seen in B6 or D2 mice, replicating Onaivi et al.’s (1990) findings that long-term anxiolytic effects in the EPM may be specific to rats and of a much shorter duration in mice. Given the current trends in cannabinoid research, combining THC with cannabidiol may work to reduce these deficits. Cannabidiol is recognized for its lack of psychoactive effects, stimulation of hippocampal cell proliferation and neurogenesis, and has been demonstrated to reduce novel object memory impairment in other models that produce neuroinflammation (Pertwee, 2008; Fagherazzi et al., 2012; Fishbein-Kaminietsky et al., 2014; Campos et al., 2015; Schiavon et al., 2016). With the use of medical cannabis on the rise, it is important to understand how the addition of cannabidiol to THC may mediate negative side effects.

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Highlights

- Acute THC is anxiogenic in adult C57Bl/6J and adolescent DBA/2J mice.
- Acute THC is sedative in adolescent and adult C57Bl/6J mice.
- An adolescent history of THC causes object recognition impairment in both strains.
- An adolescent history of THC increases CB1R protein expression.

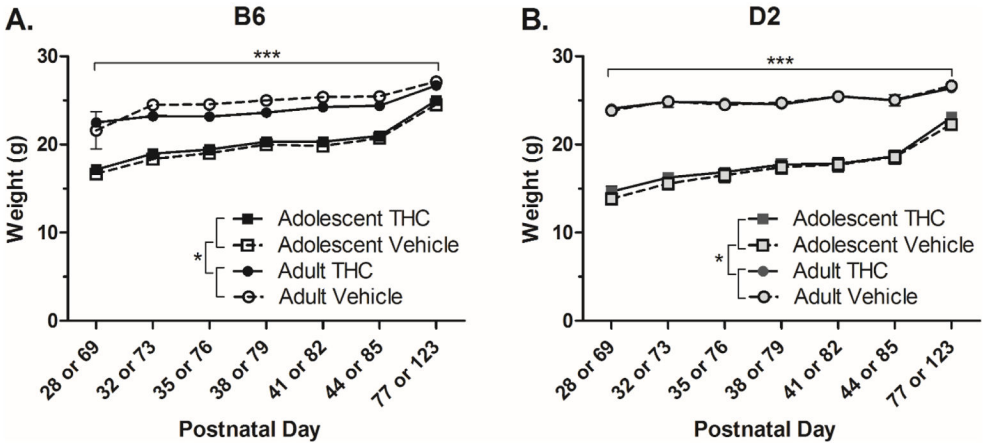

Figure 1.

depicts the weights of B6 (**A**) and D2 (**B**) mice across the study. Asterisk indicates a main effect at $p < .05$ (*) and $p < .001$ (***). n's = 9–10.

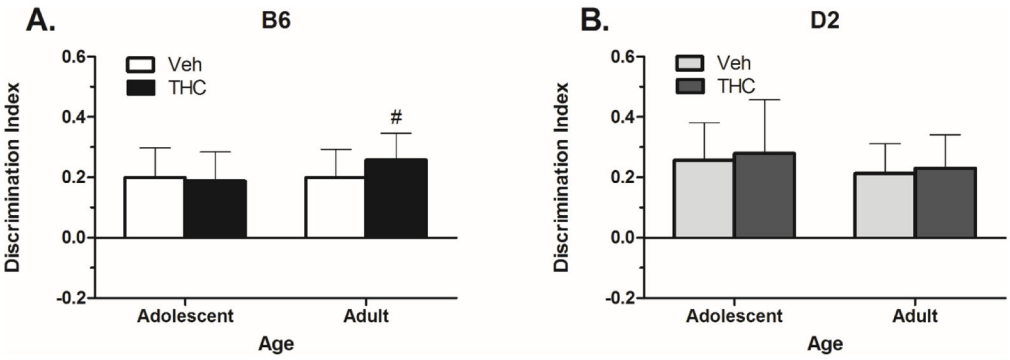

Figure 2.

depicts novel object discrimination in B6 (**A**) and D2 (**B**) mice. Hashtag (#) indicates significantly different than 0 at the $p < .05$ level. n's = 10.

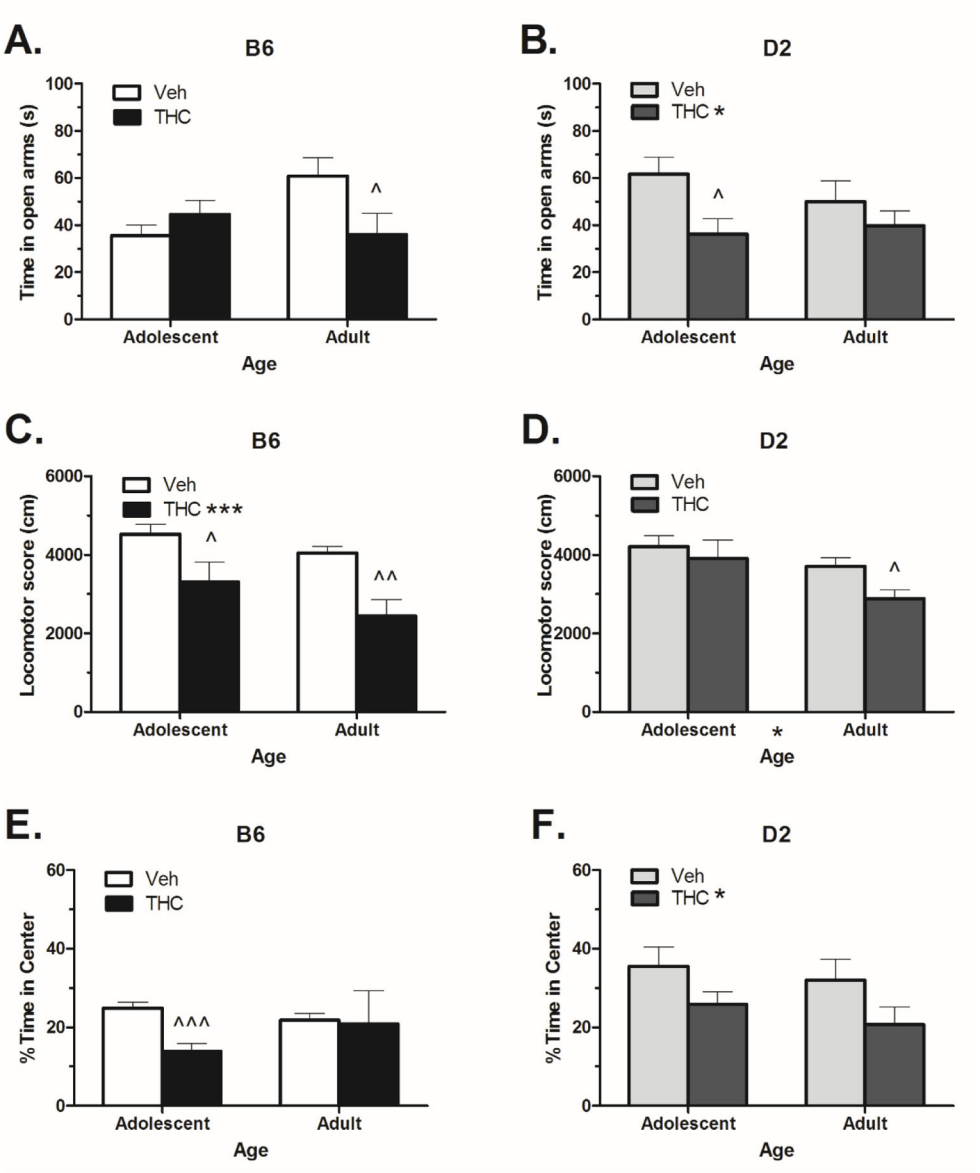

Figure 3.

depicts time spent in the open arms on the EPM (**A, B**), distance moved in the open field (**C, D**), and percent of time moving in the center of the open field (**E, F**) in B6 and D2 mice. Asterisk indicates a main effect at $p < .05$ (*) and $p < .001$ (***). Carrot indicates significantly different from respective vehicle group at $p < .05$ (^), $p < .01$ (^^), and $p < .001$ (^^^). n's = 10.

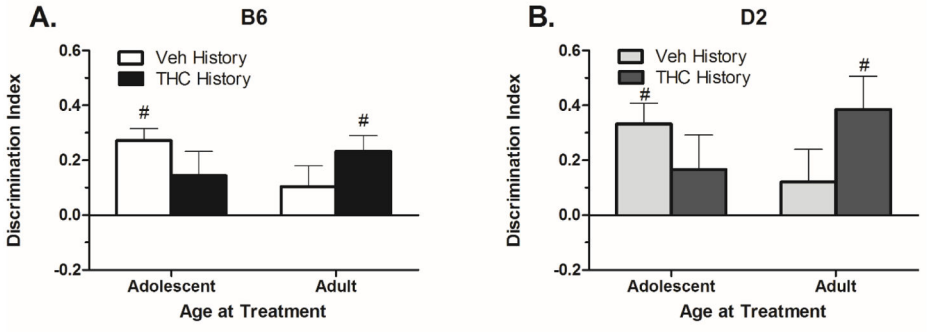

Figure 4.

depicts the effects of repeated THC or vehicle on later novel object recognition in B6 (**A**) and D2 (**B**) mice. Hashtag (#) indicates that a group is significantly different from 0 at the $p < .05$ level. n's = 9–10.

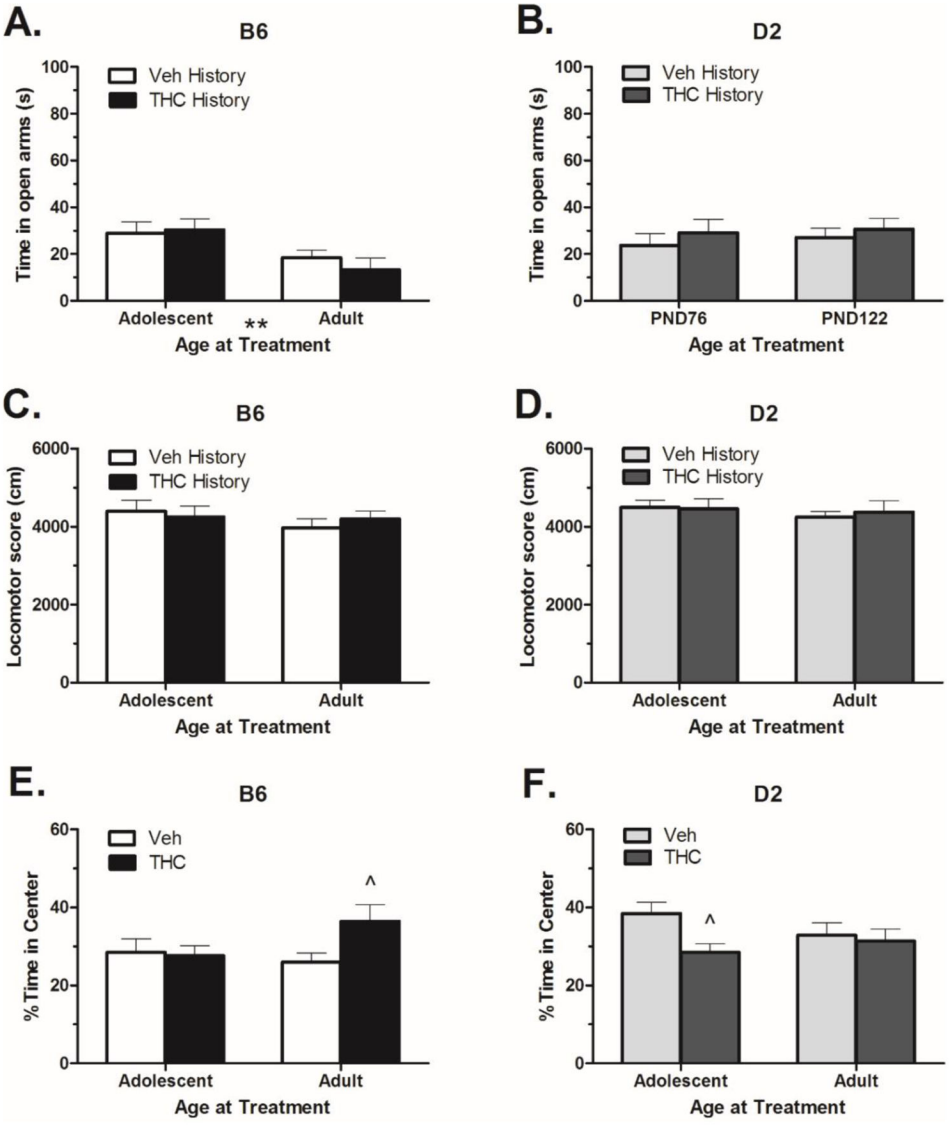

Figure 5.

depicts the effects of repeated THC or vehicle on later time in the open arms of the EPM (**A, B**), distance moved in the open field (**C, D**), and time spent moving in the center of the open field (**E, F**). Carrot (^) indicates significant difference from respective vehicle group at $p < .05$ level. n's = 9–10.

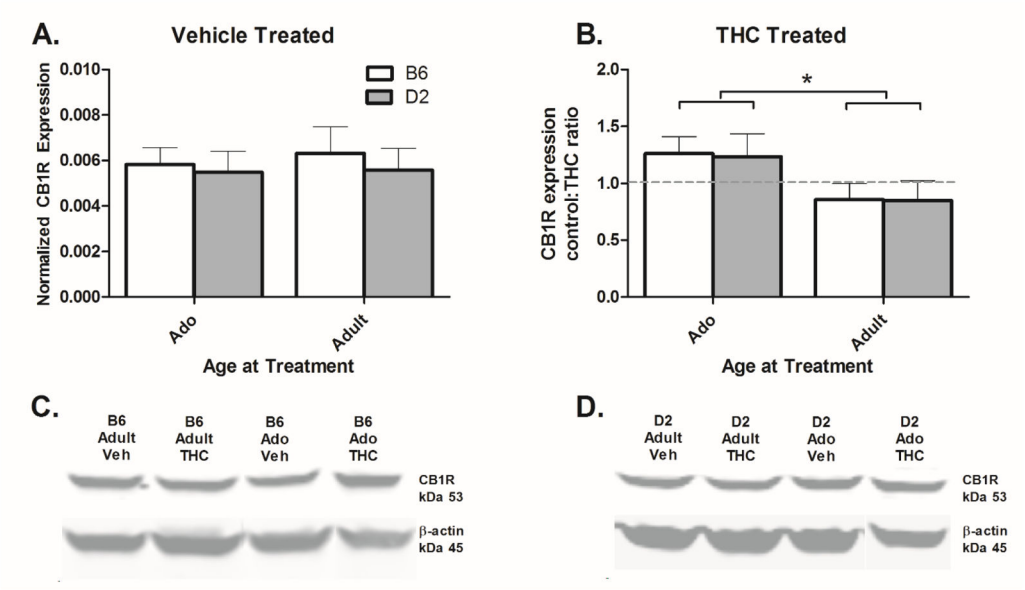

Figure 6.

depicts CB1R expression levels in mice with a history of repeated vehicle (**A**) or THC (**B**) injections. Representative CB1R and β-actin Western Blot expression for B6 (**C**) and D2 (**D**) mice are also shown. Samples for B6 mice were cut from one gel while D2 samples were cut from another. Asterisk (*) indicates a main effect at the $p < .05$ level. n's = 9–10.

Table 1

indicates mouse age (PND) at each behavioral test, as well as test order throughout the course of the study. Ado = adolescent.

	NOR1 Habituation	NOR1 Training Injection 1	NOR1 Test	Injection 2 EPM1 OF1	Injections 3–6 (every 72 hours)
Acute Ado	27	28	29	32	35–44
Acute Adult	68	69	70	73	76–85

	NOR2 Habituation	NOR2 Training	NOR2 Test	EPM2 OF2	Brain Extraction
Ado History	71	72	73	76	77
Adult History	117	118	119	122	123