EFFECTS OF ACUTE AND REPEATED CANNABINOID INJECTIONS ON IMMEDIATE AND DELAYED OBJECT MEMORY AND UNCONDITIONED ANXIETY – A DEVELOPMENTAL TRAJECTORY

by

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This work is dedicated to my family.

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ABBREVIATIONS

B6 C57Bl/6J
CBR Cannabinoid Receptor
CBD Cannabidiol
EPM Elevated Plus Maze
GPCR G-Protein Coupled Receptor
ITI Inter-trial Interval
LPS Lipopolysaccharide
NOR Novel Object Discrimination
OF Open Field
PND Postnatal Day
THC Δ9-tetrahydrocannabinol

ABSTRACT

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Cannabinoid receptors (CBRs) are inhibitory G-protein coupled receptors (GPCRs) that bind endogenous and exogenous cannabinoids. In an unaltered state, endogenous cannabinoids regulate system function and synchrony. Administration of cannabinoids such as Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD), which are found in the cannabis plant, can lead to disruptions in well-maintained inhibitory signaling. Although marijuana usage rates have been relatively stable since 2002, the number of young adolescents and adults that report perceiving marijuana as a "no risk" drug has doubled to more than 17% in each age group (Azofeifa et al., 2016). However, no drug is fully without risks. Preclinical studies have indicated that a history of THC during adolescence, but not adulthood, results in object memory impairments following a period of no-drug administration. In tests of unconditioned anxiety, acute THC evokes anxiety-like activity at higher doses. Conversely, CBD blocks object memory impairment in models that produce inflammation and also produces anxiolytic activity. Although THC and CBD are often used together for recreational and medical purposes, no study has observed the acute and long-lasting effects of THC+CBD in a battery of tests.

The current work sought to fulfill three specific aims of research to identify both age and sex differences in response to cannabinoids. In Aim 1, a dose-response to acute THC or CBD was assessed in male and female adolescent and adult mice on the elevated plus maze (EPM) and open field (OF) activity. In Aim 2, acute vehicle, 10 mg/kg THC, 20 mg/kg CBD, and THC+CBD were assessed for their effects on memory consolidation, EPM, and OF activity in male and female mice during adolescence or adulthood. Mice from Aim 2 received a total of 8 injections over a 3 week period, then were given 3 weeks of rest. In Aim 3, all mice were tested again for object memory, EPM, and OF

activity under no-drug conditions to assess the effects of an adolescent or adult history of cannabinoids in male and female mice.

Results of Aim 1 indicated that adult mice, regardless of sex, were more sensitive to the acute effects of THC on unconditioned anxiety and locomotor activity. A rapid tolerance to THC may develop, as mice tested on the EPM in Aim 2 following their second injection of THC did not demonstrate anxiety-like activity that was present in Aim 1. However, anxiety-like activity persisted in the open field, and acute administration of THC+CBD resulted in synergistic effects on anxiety in adult females over THC alone. Interestingly, acute THC in adolescent males rescued a deficit in object memory in the vehicle group, whereas only adult males receiving vehicle showed significant object discrimination. Females were relatively resistant to effects of acute cannabinoids on object memory, with adolescents being completely insensitive. Results of Aim 3 indicated minimal effects of a history of cannabinoids on behavior. In contrast to previous experiments, an adolescent history of THC did not impair object memory. Interestingly, females administered THC+CBD during adulthood demonstrated impaired object memory following a no-drug period. Although CBD is often considered to lack a psychoactive profile, it is hypothesized that this impairment may be due to actions of CBD on 5HT1a receptors and require a fully-developed stress and gonadal system. The current results indicate that acute cannabinoid administration results in anxiety-like behavior when administered during adulthood, and that an adult history of THC+CBD in females results in impaired cognitive behavior. As the effects of cannabinoids were primarily present in adults, this may suggest that the fully-developed brain is more susceptible to interruption by acute and repeated exogenous cannabinoid administration and that adolescents may have a higher threshold for impairment of behavior.

1 BACKGROUND

1.1 Cannabinoid Use and Mechanism of Action

Use of cannabinoids, such as marijuana, may lead to alterations in cognition and memory, focus, mood shifts, inflammatory and pain responses, and modification in awareness of body senses and time that may persist even after prolonged abstinence (Freund & Katona, 2007; Svizenska et al, 2008). Marijuana, which contains both THC and CBD, is the most commonly used illicit drug in all age groups. Azofeifa et al. (2016) reported past month usage as 7.2% in 12-17 year olds, 19.6% in 18-25 year olds, and 12.6% in 26-34 year olds. Although usage rates have remained relatively stable since 2002, the number of individuals who view marijuana as a "no risk" drug has doubled to more than 17% in young adolescents and adults, and has hit 36.6% in the 18-25 age group (Azofeifa et al., 2016). The United States is actively embracing marijuana for both medical and recreational use. Currently, 28 states and the District of Columbia have laws permitting medical marijuana use, with some of those states also moving to permit recreational use and/or decriminalize the possession of small amounts of marijuana (Bestrashniy & Winters, 2015; National Academies of Sciences, 2017). Although it is unclear whether medical marijuana laws contribute to the views and patterns of cannabis use in adolescents (Cerdá et al., 2017; Johnson et al., 2017), the susceptibility of adolescents and adults to long-term consequences of cannabis use is an important consideration.

A recent review by the National Academies of Sciences (2017) evaluated the literature surrounding both the beneficial and detrimental effects of cannabis use to guide both research and policy. Their assessment indicates that adolescent exposure may be particularly detrimental to cognitive development. Human research has demonstrated impairments in learning in memory even after cannabis use has ceased, and adolescent use is linked to lower levels of educational and employment achievement. However, these conclusions are relatively weak, as the limited number of studies that examine the relationship suffer from methodological differences and limitations, as well as the inability to link cannabis use to later impairment in anything but a correlational manner.

There is no strong evidence indicating that repeated cannabis use is linked to development of non-social anxiety disorders. However, positive or negative changes in anxiety levels as well as sedation are often self-reported as an outcome of cannabinoid use. The National Academies of Sciences makes several recommendations for development of the cannabis research field. These include evaluating feelings of anxiety and sedation in all studies, focusing on the developmental period of adolescence, and including the use of preclinical studies examining both acute and chronic exposure to guide clinical research.

Deficits induced by cannabinoid use may be due to mechanism of action. Both endogenous (endocannabinoids) and exogenous cannabinoids, like those found in the cannabis plant, bind to cannabinoid receptors (CBRs) (Svizenska et al., 2008). CBRs are inhibitory G-protein coupled receptors (GPCRs) and fall under two major subtypes – cannabinoid receptor 1 (CB1R) and cannabinoid receptor 2 (CB2R) – although it is accepted that more CBRs exist than have yet to be identified. CB1R is found in more elevated levels in the central nervous system than CB2R. Although each receptor type is found on most cell types, CB1Rs are typically located on neurons whereas CB2Rs are concentrated on microglia, astrocytes, and endothelial cells (Pertwee, 2008; Fernandez-Ruiz et al., 2008; Hu & Mackie, 2015). CBRs located on immune cells modulate cytokine release in response to immune challenges (Svizenska et al., 2008). In an unaltered state, endocannabinoids operate to maintain system function and synchrony typically via retrograde signaling that maintains adequate inhibitory signaling. Conversely, administration of endogenous and exogenous cannabinoids such as Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD) can result in disruption of the inhibitory cycle by blocking inhibitory interneuron function. Further, repeated use may lead to long-term adaptations in the CBR system. Acute cannabinoid administration or neuroadaptation that persists following abstinence may contribute to negative effects of cannabinoid use (**Fig. 1**). (Freund & Katona, 2007; Svizenska et al, 2008).

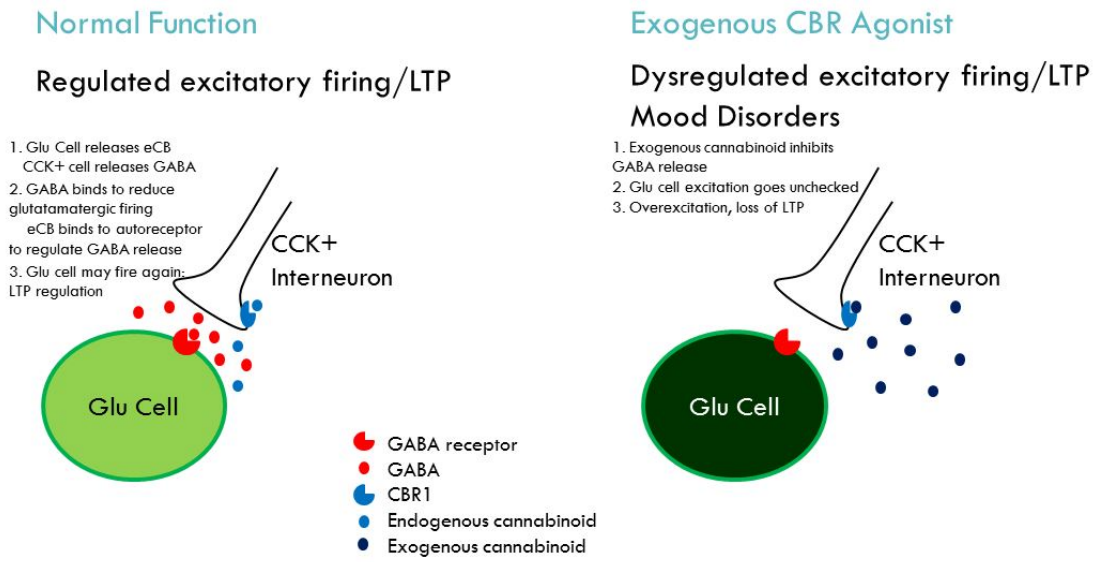

Figure 1 depicts the normal regulatory function of endocannabinoids versus the disruptive function of exogenous cannabinoids. Example is based on hippocampal CCK+ interneuron firing (Freund & Katona, 2007; Chevaleyre & Piskorowski, 2014).

1.2 THC and CBD

Pharmacologically manipulating the cannabinoid system is of major interest to develop treatments for a range of disorders, such as memory, anxiety, pain, neuroinflammatory, and spasticity disorders (Svizenska et al., 2008). It is also important to consider the long-term effects of medical and recreational use as cannabis, which contains both THC and CBD, is the most commonly used recreational drug (Azofeifa et al., 2016). CBR agonists are separated into classical, non-classical, aminoakylindole, and eicosanoid classes based on their method of derivation, selectivity, affinity, and chemical makeup. THC and CBD are both classical cannabinoids, whereas endocannabinoids are part of the eicosanoid group, which has significant structural differences (Svizenska et al., 2007; Pertwee, 2005; 2008). These differences can promote functional selectivity of each class of drug, leading to differential receptor binding as well as downstream and behavioral effects (Svizenska et al., 2007; Pertwee, 2005; 2008) thereby making comparison of different CBR agonists in the same batteries of tests difficult.

Although THC and CBD are both classical cannabinoids with similar CB1R/CB2R affinity, they may still have different behavioral effects (Pertwee et al., 2005; 2008). The most notable is the psychoactive effects of the cannabis plant, which

are primarily attributed to THC (Pertwee et al., 2008). While much less is known about CB2Rs due to difficulty of identifying the receptors (Hu & Mackie, 2015), much is known about THC and CB1Rs. THC is highly efficacious as a partial agonist at CB1Rs. It may work as an antagonist at both CB2Rs and CB1Rs, particularly in areas with lower CB1R density, such as the ventral tegmental area. Further, repeated cannabinoid exposure reduces CB1R density and G-protein coupling, leading to rapid sensitization to some behavioral effects of THC (Pertwee et al., 2005; 2008). Conversely, CBD works as a CBR antagonist and may potentiate certain physiological effects of THC while also reducing its rate of metabolism, with females being particularly sensitive to changes in metabolic rate (Klein et al., 2011; Britch et al., 2017). As both THC and CBD are metabolized by cytochrome P-450 enzymes (Stout & Cinimo, 2014), it is conceivable that THC administration could also work to reduce the rate of CBD metabolism.

Both drugs also show actions at other receptor systems including the orphan receptor GPR55, mu and delta opioid receptors, the monoamine system, and GABAergic systems (Pertwee, 2005; 2008; Kathman et al., 2006; Shore & Reggio, 2015). However, CBD appears to be more effective in moderating other systems than THC. CBD is more efficacious than THC at opioid receptors (Shore & Reggio, 2015). Further, it enhances adenosine signaling by reducing re-uptake and some of its effects may be due fully or in part by working as an inverse agonist at the serotonin receptor 5HT1a (Russo et al., 2005; Mechoulam et al., 2007; Campos & Ruimaraes, 2008; Resstel et al., 2009; Gomes et al., 2011; Campos et al., 2012; Fogaca et al., 2014; Marinho et al., 2015). To consider drugs for pharmacological treatment it is important to understand the profile of THC and CBD separately, as well as how a combination of the two drugs may alter behaviors and neurochemical biomarkers of interest when administered at different points in development.

1.3 Cannabinoid System and Development

Much of the work that has looked at the cannabinoid system during development focuses on the prenatal developing brain and how prenatal exposure to cannabinoids via maternal consumption alters normal developmental trajectory. Based on such studies, it is clear that the endocannabinoid system plays a role in metabolic support, cell proliferation

and migration, and axonal elongation in the developing brain as well as regulation of other neurotransmitter systems. Prenatal exposure to cannabinoids alters the normal role of endocannabinoids in the brain and later development and may result in miscarriage, verbal, cognitive, and visual delays, and mood disorders such as anxiety and depression (see Fernandez-Ruiz et al., 1999; Ramos et al., 2005; Anavi-Goffer & Mulder, 2009; Gaffuri et al., 2012 for review).

Less is known about how CBRs are involved in development from birth through adulthood. Although CB1Rs are expressed highly both in the neonatal and adult brain, neonatal CB1R expression is primarily limited to the hippocampus and amygdala. As reviewed by Lee & Gorzalka (2012), it appears that CB1R expression and functionality peaks during postnatal days (PND) 25-29, considered early adolescence, and then declines over time to adult levels. Conversely, levels of CB2R mRNA in the hippocampus are stable across development and adulthood in C57Bl/6J (B6) mice (Li & Kim, 2015). The distribution of total functional CBRs between gray and white matter also changes over time. Romero et al. (1997) report high levels of cannabinoid binding in white matter areas at PND5 and PND21. Around PND30 this trend begins to shift, with cannabinoid binding being primarily in gray matter areas by the time of adulthood. The authors suggest that this may reflect the necessity of CBRs in elongating axon terminals from white matter areas to their final gray matter destinations, as well as their overall role in neural development. Data from Verdurand et al. (2011) describing CB1R-specific binding indicate that rats at PND70 show higher CB1R binding than those at PND30 specifically in gray matter areas, although overall functionality may be higher in adolescents (Lee & Gorzalka, 2012). As Romero (1997) showed similar overall levels of CBR binding at these ages, it may indicate a shift in CB1R:CB2R ratios with adolescents displaying higher levels of CB2Rs. Sex differences in non-selective CB1R binding have also been observed. Rodriguez de Fonseca et al. (1993) reported subtle sex differences in both the striatal and mesencephalic regions, with females showing lower levels of CBR binding at PND10 in both areas and at PNDs 15, 40, and 70 in the mesencephalic regions. However, females showed higher levels of binding at PND20 in the striatal region. This may suggest that females might display altered sensitivity to adolescent cannabinoid exposure.

1.4 THC, CBD, and Behavior

1.4.1 Novel Object Recognition

A report recently released by the National Academies of Sciences (2017) indicates that there is moderate evidence of cannabis use impairing learning and memory, but that evidence for this impairment lasting beyond sustained abstinence is limited. The novel object recognition (NOR) task is based on the observation that "normal" rodents tend to significantly prefer investigating a novel object over a familiar one. Novel preference reflects that the previously exposed "familiar" object was properly encoded, consolidated, and retrieved during the training and test sessions. NOR represents an optimal task to assess non-spatial memory, as it is free of stress, independent of external motivation, reward, or punishment, and requires minimal training (Cohen & Stackman, 2015). Further, the NOR task is a preclinical analogue of the human visual paired comparisons task. In humans, this task is used to examine perceptual-cognitive skills during development and is related to indicators of cognitive strengths, such as language development and IQ level (Burbacher & Grant, 2012).

The task consists of habituation, training, and test sessions as well the inter-trial interval (ITI) between these sessions (Cohen & Stackman, 2015). Drug administration may take place acutely before training to interfere with memory formation, after training to interfere with memory consolidation, before testing to interfere with memory recollection, or repeatedly before the task to observe an effect of drug history.

Acute THC administration prior to training with a short ITI, as well as acute administration following training with a long ITI, have not been shown to alter discrimination index (Ciccocioppo et al., 2002; Swartwelder et al., 2012). However, these were relatively low doses of 1, 2, or 5 mg/kg. Our lab, using a 10 mg/kg dose administered after training with a long 24 hour ITI also demonstrated no effect of acute THC in adolescent or adult C57Bl/6J males (Kasten et al., under review). Conversely, repeated THC administration has been shown to alter later NOR performance in group-housed rats, specifically when THC is administered during adolescence and behavior is observed in adulthood (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012). Quinn et al. (2008) was the only study to use an appropriate adult control and found no

effect of adult THC history on later NOR discrimination index. Our lab has demonstrated differential effects of repeated 10 mg/kg THC treatment in adolescence or adulthood using singly-housed C57Bl/6J males. Mice administered vehicle during adolescence showed significant object discrimination, which was not present in THC-treated mice. Conversely, mice treated with vehicle during adulthood did not significantly discriminate objects, whereas THC-treated mice did (Kasten et al., 2017 under review). In opposition to these findings are O'Tuathaigh et al. (2010), who found no effect of repeated adolescent or adult THC in group-housed male and female COMT knockout wild type mice. However, their highest dose was 8 mg/kg and their training session was limited to 5 minutes, which may not have offered enough time for mice to familiarize to the object (Cohen & Stackman, 2015).

CBD's ability to acutely alter NOR has not been reported, and one study by Cadoni et al. (2013) suggests that repeated CBD treatment is unable to alter NOR on its own. This finding is supported by other studies that have shown no independent effect of CBD, but an ability of CBD to rescue NOR deficits and proinflammatory responses in other models that induce an inflammatory response such as injection of iron or a malaria-like infection (Fagherazzi et al., 2012; Campos et al., 2015; Gomes et al., 2015). These results indicate that co-administration of CBD with THC may inhibit the impairment in NOR seen in adolescents treated with THC by mediating inflammatory processes.

1.4.2 Elevated Plus Maze

The EPM measures unconditioned anxiety by comparing an animal's drive to remain in a "safe" enclosed space versus the drive to explore open areas. More time on the two open arms of the maze indicates less anxiety. Measures of unconditioned anxiety in the EPM may reflect changes in anxiety levels following acute marijuana use, which the National Academies of Sciences (2017) indicates should be recorded in humans and investigated using preclinical studies. Preclinical studies indicate that cannabinoids mediate unconditioned anxiety, often showing an inverted U-shape curve with low to moderate doses reducing anxiety and higher doses increasing anxiety (Lee et al., 2015). Many studies have investigated how THC and CBD alter anxiety responses in the EPM using various dose ranges and regimens in a variety of strains, ages, and different sexes.

A 30-minute pretreatment of THC has been shown to be both anxiogenic (Celerier et al., 2006; Schramm-Sapyta et al., 2007) and anxiolytic (Rubino et al., 2007; Braida et al., 2007; Foko & Pangis, 2010) in both adolescents and adults. However, strain/genotype appear to play a role in THC sensitivity, with doses under 1.5 mg/kg generally being anxiolytic. Our own lab has demonstrated an age and genotype sensitivity to 10 mg/kg THC, with an acute 30-minute pretreatment being anxiogenic in male adult B6 mice and adolescent DBA/2J mice (Kasten et al., 2017 under review). A strong anxiolytic profile of acute CBD pretreatment has been demonstrated, with CBD increasing time in the open arms of the EPM on its own (Guimaraes et al., 1990; Onaivi et al., 1990; Schiavon et al., 2016) and in response to stress (Resstel et al., 2009; Campos et al., 2013; 2015). Site-specific studies have demonstrated anxiolytic effects of CBD administered into brain regions including the infralimbic PFC, bed nucleus of the stria terminalis, and dorsolateral periacquiductal gray region (Campos & Guimaraes, 2008; Gomes et al., 2011; Marinho et al., 2015), but anxiogenic when administered into the prelimbic cortex (Fogaca et al., 2014) and that these effects are dependent upon actions at the serotonin 5HT1a receptor. Two studies have looked at whether acute administration of THC+CBD alters EPM activity and observed no effects (Onaivi et al., 1990; Stern et al. 2015). However, these studies used low drug doses and administered the dose 24 hours before testing, which may have been too long of a pretreatment window.

A history of repeated cannabinoid injections has also produced mixed results. Onaivi et al. (1990) found no effect of repeated THC in mice, but an anxiogenic effect in rats when THC was administered during adulthood. Cadoni et al. (2008) and O'Tuathaigh et al. (2010) found repeated THC administration during adolescence in rats and mice was anxiolytic when the EPM was tested in adulthood. Our lab has not seen an effect of repeated adolescent or adult THC treatment on later EPM behavior in either adolescent or adult mice (Kasten et al., 2017 under review). Reports of effects of repeated CBD alone on EPM activity are sparse. Campos et al. (2015) reported an anxiolytic effect of repeated CBD in vehicle and malaria-like infected mice and Mayer et al. (2014) reported that repeated CBD treatments were able to rescue anxiogenic activity produced by predator stress. However, Gomes et al. (2015) found no effects of CBD in vehicle or MK801-

treated mice. No studies have investigated the effects of repeated THC+CBD treatment on anxiety-like behavior in the EPM.

1.5 Conclusion

As cannabinoids become legalized and continue to be popular drugs of choice, it is important to understand the profile of each drug of interest as well as how combinations of these drugs affect behavior. The current experiments focus on the role of THC, CBD, and THC+CBD on basic memory, anxiety, and sedation phenotypes: NOR, EPM, and OF. These tasks are preclinical representations of behaviors indicated to be of importance in directing the future of cannabinoid research and policy (National Academies of Sciences, 2017). Although both THC and CBD have been investigated in these paradigms in adolescents and adults, most studies have only looked at one sex or have failed to use appropriate age controls. Further, no study has completed systematic dose responses of THC and CBD in adolescent and adult, male and female B6 mice. Most importantly, no study has looked at the combined effects of acute or previous experience with THC+CBD in adolescence and adulthood even though recreational cannabinoid use in humans often involves consumption of both compounds. The current studies report how acute THC and CBD affect non-spatial memory and anxiety in adolescence and adulthood when administered separately or together. Additionally, the studies report how repeated administration in adolescence and adulthood affects later cognition and anxiety when drug is not on-board.

1.6 Specific Aims

1. *Assess a dose-response to acute THC and CBD in adolescent and adult B6 mice on the elevated plus maze and open field activity.* Across ages and sexes, it was hypothesized that both acute THC and CBD would dose-dependently alter anxiety in the EPM with THC being anxiogenic and CBD being anxiolytic. It was also hypothesized that THC would reduce locomotor activity in the open field (OF), but that CBD would have no effect in both ages and sexes.
2. *Assess how acute THC alone, CBD alone, or THC+CBD injections affect memory consolidation in the novel object recognition task in adolescent and adult B6*

mice. It was hypothesized that all mice would develop novel object discrimination when given an acute vehicle injection. It was also hypothesized that acute THC, CBD, or THC+CBD following training would not significantly alter object discrimination in any group compared to vehicle. It was expected that THC would result in anxiogenic and sedative activity in the EPM and OF for all groups, and that THC+CBD would attenuate these behaviors.

3. *Assess how an adolescent or adult history of repeated THC alone, CBD alone, or THC+CBD injections affect later performance in novel object recognition, elevated plus maze, and OF activity.* It was hypothesized that aged mice with an adolescent history of THC exposure would not be able to successfully discriminate in the novel object task, and that administration of CBD or THC+CBD would rescue this deficit. There were no expected effects of previous drug injections on EPM or OF activity.

2 METHOD

2.1 General Design

For Aim 1, THC and CBD dose responses were conducted in adolescent male, adolescent female, adult male, and adult female B6 mice. Each animal received one dose of THC (1, 5, or 10 mg/kg), CBD (5, 10, or 20 mg/kg), or vehicle 30 minutes before being placed on the EPM, immediately followed by OF. These dose ranges were chosen based on previous studies that have demonstrated effects of THC (Onaivi et al., 1990) or CBD (Guimares et al., 1990; Onaivi et al., 1990) on EPM activity. Further, no studies observing the history of THC on NOR surpassed a 10 mg/kg dose (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012).

The general design with a representative timeline for Aims 2 and 3 is shown in Table 1. The acute effects of vehicle, THC, CBD, or THC+CBD on NOR, EPM, and the OF activity tasks were evaluated in adolescents starting on PND28 or adults starting on PND70. Mice sharing a cage received the same drug. Based on the results of Aim 1, 10 mg/kg of THC was chosen for its ability to produce anxiogenic effects in adults and sedative effects across both ages and sexes. Although CBD produced minimal effects in Aim 1, 20 mg/kg of CBD was chosen because it falls within the range of doses used in previous studies which observed a rescue effect of CBD on object memory impairment (Fagherazzi et al., 2012; Campos et al., 2015; Gomes et al., 2015). For the combination, a higher level of CBD to THC may reduce THC binding levels and also slow metabolism of THC to active metabolites, thereby blocking more of the psychoactive effects of THC while promoting the rescuing effects of CBD (Pertwee et al., 2008; Klein et al., 2011). Following the initial two doses to assess acute behavior, mice received six maintenance injections for a total of eight injections. Eight injections were chosen so that adolescent exposure concluded on PND45, similar to previous studies demonstrating an adolescent exposure effect (Realini et al., 2011; Zamberletti et al., 2012; Kasten et al., under review) Mice then remained undisturbed until aging to PND69 or PND111. Aim 3, which determined how an adolescent or adult history of vehicle, THC, CBD, or THC+CBD

affected NOR, EPM, and OF activity without drug on-board, began at that point. In all studies, THC and CBD shared the same control group to minimize animal usage.

Table 1 details a representative timeline of Aims 2 and 3. Aim 2 experiments take place during Week 1 and Aim 3 experiments take place during Week 7.

	Monday	**Tuesday**	**Wednesday**	**Thursday**	**Friday**
Week 1		NOR habituation	NOR Training Injection 1	NOR Test	Injection 2 EPM & OF
Week 2	Injection 3		Injection 4		Injection 5
Week 3	Injection 6		Injection 7		Injection 8
Weeks 4-6	REST				
Week 7	NOR habituation	NOR Training	NOR Test	EPM & OF	Brain Extraction

2.2 Animals

A total of 440 male and female B6 mice were purchased from Jackson Laboratories and arrived at PND21 or PND56. Mice were singly-housed for Aim 1 and pair-housed throughout the durations of Aims 2 and 3. Single-housing was chosen for Aim 1 to remove the need to individually house animals following injection before behavioral assays. Pair-housing was chosen for Aims 2 and 3 due to the long-term nature of the study. Animals were housed on a 12:12 light/dark cycle (lights off 8 am) with *ad libitum* access to food and water at all times. All procedures adhered to the protocol approved by Indiana University-Purdue University Indianapolis School of Science Institutional Animal Care and Use Committee and conform to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (The National Academies Press, 2003). All injections and behavioral tasks were conducted in red light during the dark phase of the light cycle. Conducting behavior under red light conditions reduces potential anxiety-like conditions, potentially resulting in more exploration time in the NOR task and higher levels of open arm time in the EPM which allow for sensitivity to anxiogenic drug effects.

2.3 Drugs

Both THC and CBD were supplied by the National Institutes of Health (Bethesda, MD). THC (1, 5, and 10 mg/kg), CBD (5, 10, and 20 mg/kg), or the combination (10 mg/kg THC + 20 mg/kg CBD) were dissolved in a vehicle solution of 5% Tween80, 5% 100 proof ethanol, and 90% saline. All solutions were delivered via intraperitoneal injections in a volume of 0.1 mL per 10 g of body weight. To reduce the stress of multiple injections during Aims 2 and 3, THC and CBD were combined in one solution.

2.4 Elevated Plus Maze

For Aims 1 and 2, mice received a vehicle, THC, CBD, or THC+CBD injection 30 minutes prior to the EPM task and were placed in an individual cage. Drug was administered in a pseudorandomized order, with each drug being equally represented in every cohort. The injection took place in the animal vivarium. For Aim 3, mice received no injection prior to EPM. Mice were individually transported to the testing room immediately prior to the EPM task. Mice were placed in the EPM facing an open arm and given 5 minutes to explore. Two separate black Plexiglas plus mazes (Med Associates, Inc., St. Albans, VT) that are adjusted for size were used. Adult mice were tested on a standard maze with two open arms and two closed arms elevated at 74.5 cm from the floor with distance from end to end of opposing arms being 76 cm. The walls encasing the closed arms are 20.5 cm. Mice tested in adolescence were placed on a maze that is approximately 25% smaller. Although still 74.5 cm above the ground and walls of 20.5 cm encasing the closed arms, the distance from end to end of the opposing arms on the adolescent maze is 57 cm. The apparatus was cleaned between each mouse with 20% ethanol. Each session was video recorded and scored. Time in the open arms was recorded when all four of the animal's paws crossed the center zone into the open arm. Each occurrence of four paws crossing into an open arm was counted as one open arm entry.

2.5 Open Field

Immediately following the EPM in Aims 1, 2, and 3, mice were individually transferred to the OF testing room. Each mouse was placed in a Versamax Animal Activity Monitor (Accuscan Instruments, Columbus, OH) for 10 minutes. Activity was recorded by eight pairs of intersecting photocell beams (2 cm above the chamber floor) evenly spaced along the walls of the 40×40 cm test chamber. Sound-attenuating box chambers (inside dimensions, 53 cm across × 58 cm deep × 43 cm high) equipped with a house light and fan for ventilation and background noise encased the test chamber. For this study, the house light was not turned on. The chambers were attached to a Dell computer which recorded activity counts every minute. Following the end of the session, the chambers were cleaned with 20% ethanol and animals were immediately returned to their home cage in the vivarium.

2.6 Novel Object Recognition

The NOR apparatus consists of a 40x40x40 cm wooden chamber painted light brown and sealed to block any spatial cues and allow for cleaning. The NOR task took place over three days, with each session being spaced 24 hours apart. Sessions were recorded by a video camera and object investigation was hand-scored. On each day, the mice were individually walked into the testing room immediately prior to their session and returned to the vivarium immediately following their session. On the habituation day, animals were placed in the arena for 10 minutes without any objects present. On the training day, animals were placed into the arena with two identical objects and given 10 minutes to explore. The objects were placed approximately 10 cm out from diagonal corners. For Aim 2 only, mice received an injection of vehicle, 10 mg/kg THC, 20 mg/kg CBD, or THC+CBD 10 minutes following return to the vivarium post-training session (see Table 1). On the test day, one familiar object was replaced with a novel object and mice were given 5 minutes to explore. Exploration time is time the animal spent oriented towards the object sniffing within 2 centimeters or in physical contact with the object. The apparatus was cleaned between each mouse with 20% ethanol.

In an attempt to control for potential dominant/subordinate effects, mice in the same cage received the same drug and the same order of familiar-novel objects. Zone placement of the novel object was counterbalanced within the cage whereas object order was counterbalanced between cages. Drug order was pseudorandomized across cages, with each drug being administered to 2-3 cages (4-6 mice) per cohort. Objects used in the NOR task were optimized in pair-housed naïve adolescent and adult male and female mice. An object pair was considered optimal when significant discrimination was reached during the probe and discrimination indices were similar regardless of which object was novel. These two conditions indicated that mice could significantly discriminate the novel object under naïve conditions and that preference for one object was not driving investigation during the test session. See Table 2 for objects used for each sex and age-point.

Table 2 indicates the objects used for the NOR task for each treatment and sex group at each time-point.

Treatment/Sex	**PND28-30**	**PND70-72**	**PND111-113**
Adolescent Males	Small "5 Hour Energy" & opaque drug vial	Small Erlenmeyer Pyrex & mini brown ceramic mug	
Adult Males		Small Erlenmeyer Pyrex & mini brown ceramic mug	Small "5 Hour Energy" & opaque drug vial
Adolescent Females	Small Erlenmeyer Pyrex & mini brown ceramic mug	Small conical tube & white plastic slide case	
Adult Females		Small conical tube & white plastic slide case	Small Erlenmeyer Pyrex & mini brown ceramic mug

2.7 Maintenance Injections

For Aim 2, animals received one injection following NOR training and a second injection prior to the EPM and OF tasks. Six more injections were given on Monday, Wednesday, and Friday of the following two weeks for a total of eight injections (see **Fig. 4**). All injections took place in the animal vivarium.

2.8 Statistical Analyses

All analyses were run separately in males and females to conserve statistical power to assess the primary question of these studies: does adolescent administration of cannabinoids differentially affect behavior compared to adult administration? Therefore, omnibus tests were Dose*Age at administration for each sex independently. For all statistical analysis, the omnibus significance was set at $p < .05$ and corrected for follow-up tests. For Aim 1, time in open arms, open arm entries, and activity in the OF was analyzed using a Dose*Age factorial ANOVA for THC and CBD. There was an *a priori* hypothesis that each age*sex group may have different sensitivities to THC and CBD, so a one-way ANOVA analyzing dose response to each drug were run for all groups to determine dosage for Aims 2 and 3. Dunnet's post-hoc tests were used to compare all drug doses to the vehicle group. For Aims 2 and 3, a Drug*Age factorial ANOVA was run to assess acute or prior history effects of vehicle, THC, CBD, or THC+CBD on novel object behaviors, time in open arms, open arm entries, and activity in the OF for each sex independently. One-way ANOVAs were run to assess the effect of dose or drug on each age group. Dunnet's tests were used to analyze whether drug groups were significantly different than vehicle for discrimination index, time in open arms, open arm entries, and OF activities. An independent-samples t-test was also used to compare THC to THC+CBD. The significance level for this test was adjusted to .0125. All NOR groups were also analyzed using a one-sample t-test comparing each group to 0 to determine if significant novel object discrimination occurred. Tukey's post-hoc analyses were used to examine differences in all other metrics of the novel object task. These metrics were total training and test investigation time, ratio of training investigation time in zone A:B, number of investigative bouts with the familiar and novel objects, average investigative

bout length for each object, total time spent with each object during the test session, total investigative bouts during the test session, percent of novel bouts during the test session, and average novel-average familiar bout length. Pearson correlations within each age*sex*drug were used to determine whether investigation during the training session influenced discrimination index, as it has been previously suggested that more investigation during the training session may increase object memory (Cohen & Stackman, 2015).

The discrimination index was calculated as (time spent with novel object – time spent with familiar object)/total object investigation time. It ranges from -1 to +1, with more positive numbers indicating more time spent with the novel object and 0 indicating no preference. Percent of novel investigative bouts were calculated as [(novel bouts/total bouts)*100]. Percent of total distance and time spent in the center of the OF, an alternative measure of anxiety, were calculated as [(center activity/total activity)*100].

3 RESULTS

3.1 Aim 1: THC and CBD Dose Responses

3.1.1 Time in the Open Arms

In males, a Dose*Age ANOVA revealed a significant interaction of THC on time spent in the open arms on the EPM; $F(3,61) = 3.214$, $p < .05$. There was no significant main effect of age ($p >. 05$), but there was a significant main effect of dose; $F(3,61) = 7.71$, $p < .001$. One-way ANOVAs for each age group indicated that the 10 mg/kg dose of THC reduced time spent in the open arms only in adult mice ($p < .001$) (**Fig. 2A**). A Dose*Age ANOVA revealed no significant interaction or main effect of dose of CBD on time spent in the open arms on the EPM (p's > .05). However, there was a main effect of age; $F(1,56) = 17.13$, $p < .001$, with adults spending more time in the open arms (**Fig. 2B**). One-way ANOVAs to assess the effect of CBD dose at each age were not significant (p's > .05).

In females, Dose*Age ANOVA revealed no significant interaction or main effect of age of THC on time spent in the open arms (p's > .05). There was a main effect of dose; $F(3,60) = 8.076$, $p < .001$. One-way ANOVAs for each age revealed that the effect of dose was limited to adult mice, with both the 5 and 10 mg/kg doses significantly reducing time in the open arms (p's < .05) (**Fig. 2C**). A Dose*Age ANOVA revealed no significant interaction or main effect of dose of CBD on time spent in the open arms on the EPM (p's > .05). However, there was a main effect of age; $F(1,59) = 27.75$, $p < .001$, with adults spending more time in the open arms (**Fig. 2D**). One-way ANOVAs to assess the effect of CBD dose at each age were not significant (p's > .05).

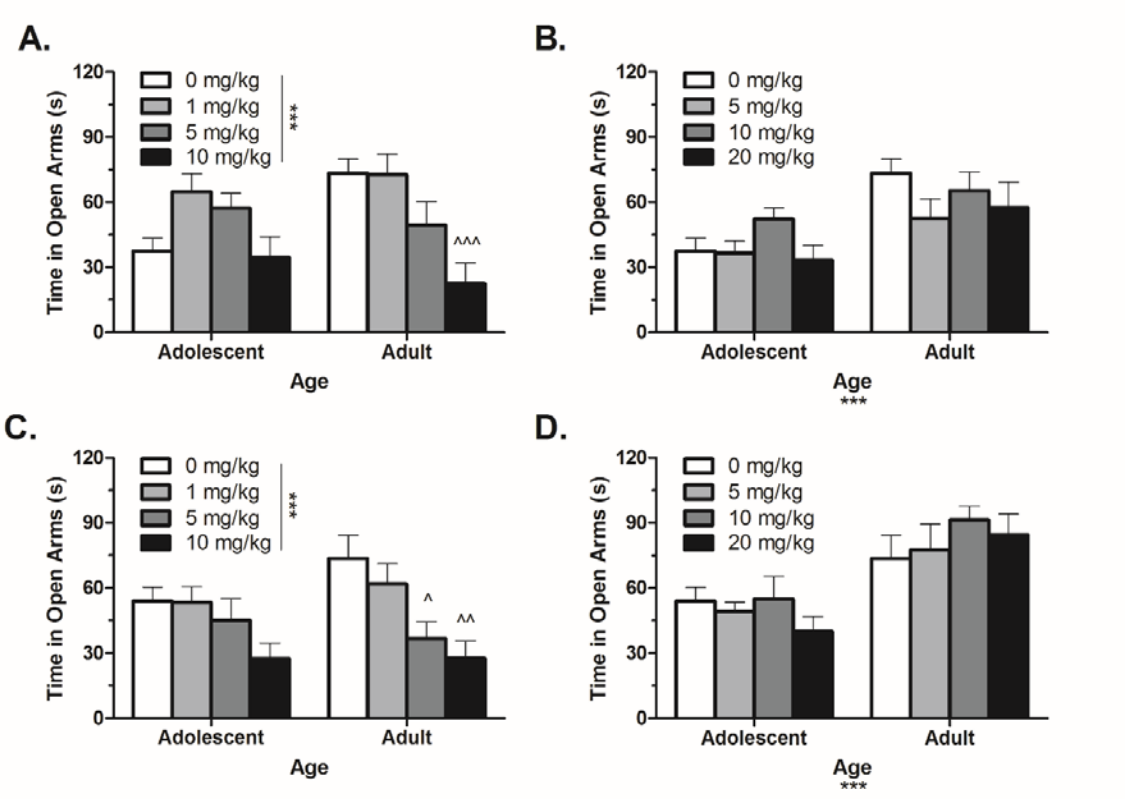

Figure 2 depicts time spent in the open arms of the EPM for males injected with THC (A), males injected with CBD (B), females injected with THC (C), and females injected with CBD (D). Three asterisks (***) indicates a main effect with significance of $p < .001$. Carrots indicate significant compared to that group's control at $p < .05$ (^), $p < .01$ (^^), and $p < .001$ (^^^). n's = 7-9 per group.

3.1.2 Number of Open Arm Entries

For males, a Dose*Age ANOVA revealed a significant interaction of THC on number of open arm entries on the EPM; $F(3,61) = 3.101$, $p < .05$. There was no main effect of age ($p > .05$), but there was a significant main effect of dose; $F(3,61) = 6.763$, $p < .001$. Although one-way ANOVAs for each age group indicated a significant effect of THC dose for adolescents ($p < .05$), no dose was significantly different from the control group (p's > .05). 10 mg/kg of THC significantly reduced open arm entries in the adults ($p < .01$) (**Fig. 3A**). A Dose*Age ANOVA revealed no significant interaction or main effect of dose of CBD on number of open arm entries on the EPM (p's > .05). However, there was a main effect of age; $F(1,56) = 16.31$, $p < .001$, with adults making more

entries (**Fig. 3B**). One-way ANOVAs to assess the effect of CBD dose at each age were not significant (*p*'s > .05).

In females, Dose*Age ANOVA revealed no significant interaction or main effect of age of THC on number of open arm entries (*p*'s > .05). There was a main effect of dose; F(3,60) = 3.345, *p* < .05. One-way ANOVAs for each age revealed that the effect of dose was limited to adult mice, with the 10 mg/kg doses significantly reducing open arm entries (*p*'s < .05) (**Fig. 3C**). A Dose*Age ANOVA revealed no significant interaction or main effect of dose of CBD on number of open arm entries on the EPM (*p*'s > .05). However, there was a main effect of age; F(1,59) = 27.62, *p* < .001, with adults making more entries (**Fig. 3D**). One-way ANOVAs to assess the effect of CBD dose at each age were not significant (*p*'s > .05).

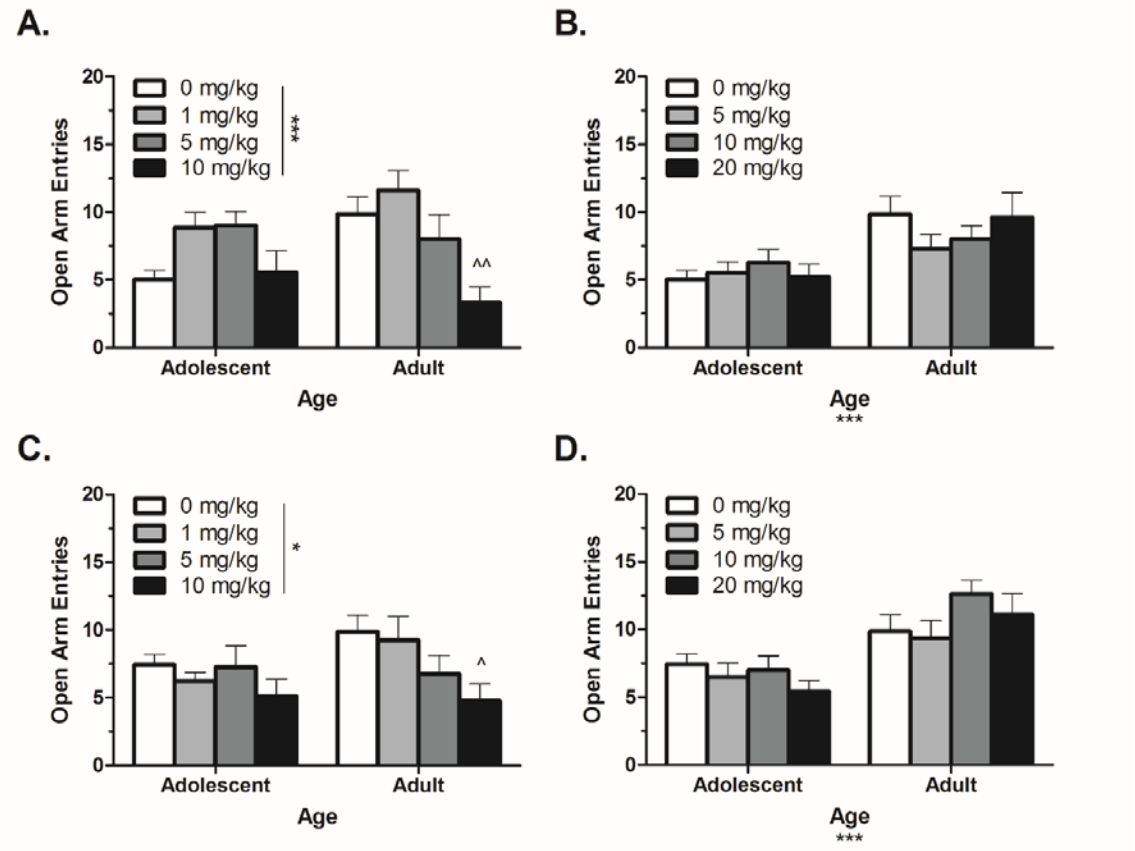

Figure 3 depicts the number of open arm entries on the EPM for males injected with THC (A), males injected with CBD (B), females injected with THC (C), and females injected with CBD (D). Asterisks indicate a main effect with significance at p < .05 (*) and p < .001 (***). Carrots indicate significant compared to that group's control at p < .05 (^) and p < .01 (^^). n's = 7-9 per group.

3.1.3 Open Field Activity

For males, a Dose*Age ANOVA revealed no significant interaction or main effect of age on THC-induced locomotor activity in the open field (*p*'s > .05). There was a significant main effect of dose; $F(3,62) = 15.5$, $p < .001$. One-way ANOVAs for each age group revealed a significant effect of dose in adolescents ($p < .01$), although no dose was significantly different from the control group (*p*'s > .05). In adults, both the 5 and 10 mg/kg dose of THC reduced activity (*p*'s < .05) (**Fig. 4A**). For CBD, Dose*Age ANOVA revealed a significant interaction; $F(3,61) = 3.418$, $p < .05$. There was no main effect of dose, but there was a significant main effect of age with adults moving more; $F(1,61) = 9.195$, $p < .01$. One-way ANOVAs assessing dose for each age group revealed no effects of CBD in adolescents ($p > .05$), but a significant effect in adults with the 5 mg/kg dose reducing activity ($p < .05$) (**Fig. 4B**).

For females, a Dose*Age ANOVA revealed no significant interaction on THC-induced locomotor activity in the open field ($p > .05$). There was a significant main of age; $F(1,61) = 6.95$, $p < .05$. There was also a significant effect of dose; $F(3,61) = 26.83$, $p < .001$. One-way ANOVAs for each age group indicated that the 10 mg/kg dose of THC significantly reduced locomotor activity at both ages (*p*'s < .01) (**Fig. 4C**). A Dose*Age ANOVA revealed no significant interaction or main effect of dose on CBD-induced locomotor activity in the open field (*p*'s > .05). One-way ANOVAs for each age group also revealed no significant effects of dose (*p*'s > .05). There was a significant main effect of age, with adults moving more; $F(1,59) = 59.23$, $p < .001$ (**Fig. 4D**).

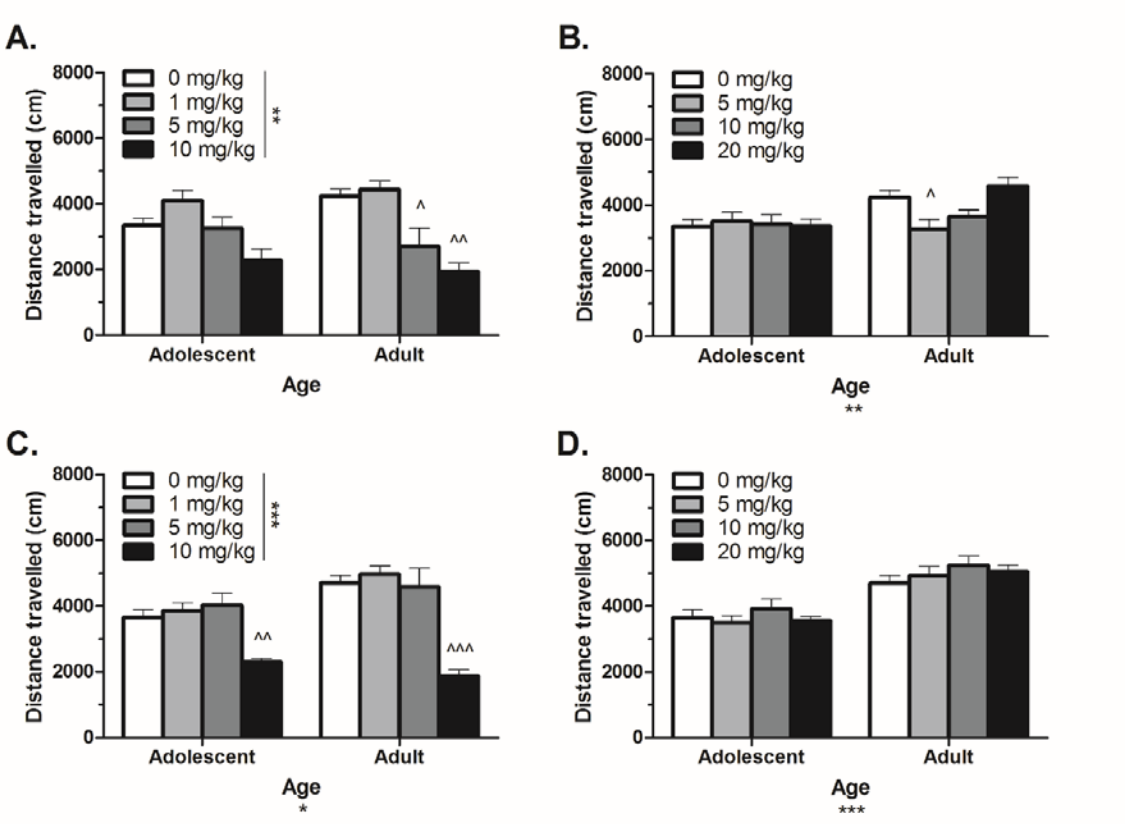

Figure 4 depicts the total distance traveled in the OF for males injected with THC (A), males injected with CBD (B), females injected with THC (C), and females injected with CBD (D). Asterisks indicates a main effect with significance of $p < .05$ (*), $p < .01$ (**), or $p < .001$ (***). Carrots indicate significant compared to that group's control at $p < .05$ (^) and $p < .01$ (^^). n's = 7-9 per group.

3.2 Aim 2: Acute Cannabinoid Administration

3.2.1 Object Optimization

An Age*Sex ANOVA revealed no significant interaction or main effects on object discrimination index in naïve mice using the optimized objects. Independent samples t-tests indicated that all groups were able to significantly discriminate the novel object under a no-injection condition (**Fig. 5**).

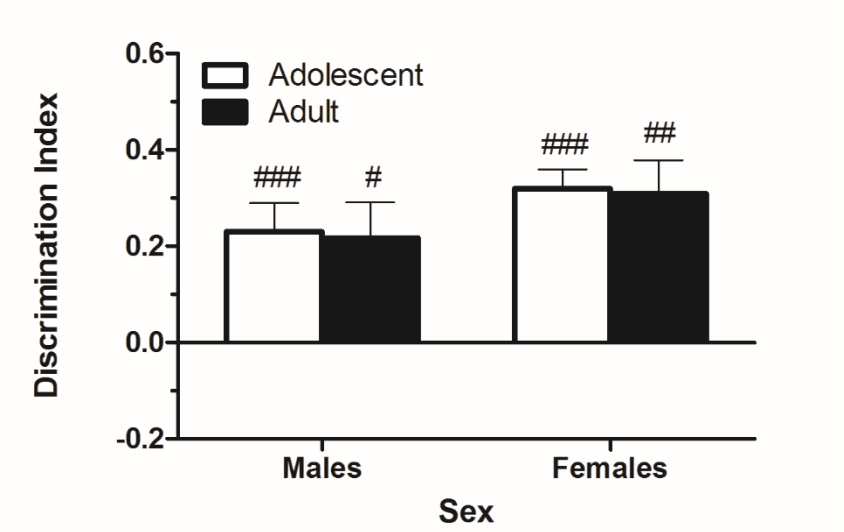

Figure 5 depicts discrimination indices for naïve animals using the optimized objects chosen for Aims 2 and 3. Pound signs indicate significantly different than 0 at $p < .05$ (#), $p < .01$ (##), and $p < .001$ (###). n's = 8-10.

3.2.2 Acute NOR: Males

In adolescent and adult males, time spent investigating the objects during the training session was not significantly correlated with discrimination index in any drug group (*p*'s > .05) (**Fig. 6A, B**). A Drug*Age ANOVA and follow-up one-way ANOVAs analyzing dose revealed no differences in total training investigation or the ratio of investigative time spent with the object in Zone A:Zone B during the training session (*p*'s > .05). These results indicate that there were no differences in baseline investigation or zone preferences.

Some differences in behavior during the novel object test session were present in males. Omnibus Drug*Age ANOVAs indicated no significant interactions of the variables on test session behaviors (*p*'s > .05). However, there were significant main effects of both drug and age on number of investigative bouts with the familiar object; $F(3,71) = 4.32$ and $F(1,71) = 4.88$, *p*'s < .05, respectively. Overall, mice treated with CBD had more interactions with the familiar object than the THC and THC+CBD groups (*p*'s = .06 and .046, respectively), and adults had significantly more interactions with the familiar object ($p < .05$).

One-way ANOVAs (**Table 2**) examining the effect of drug within each age group for males revealed no significant effects on the test-day behaviors in adolescent male mice (p's > .05). In adult male mice, drug administration significantly affected number of familiar and total investigative bouts (p's < .01) and trended towards influencing number of novel bouts (p = .069). Post-hoc analyses revealed that CBD significantly increased both familiar and total bouts compared to THC and THC+CBD (p's < .05), and no effects on number of novel bouts were revealed (p's > .05).

Table 3 displays group means ± standard error for investigative behaviors of male mice in the novel object task following acute cannabinoid administration. Displayed p values are for one-way ANOVAs assessing drug effects in each age group.

	Adolescents					Adults				
	Veh	**THC**	**CBD**	**THC+ CBD**	***p* value**	**Veh**	**THC**	**CBD**	**THC+ CBD**	***p* value**
Training Investigation (s)	66.4 ±5.80	67.6 ±7.76	67.4 ±7.32	66.6 ±8.71	> *.05*	68.2 ±7.01	54.3 ±7.41	61.0 ±6.19	75.2 ±8.17	> *.05*
Zone A:Zone B Training Time	0.85 ±0.10	0.84 ±0.07	1.00 ±0.12	0.95 ±0.01	> *.05*	0.97 ±0.07	1.02 ±0.08	1.01 ±0.08	0.84 ±0.11	> *.05*
Familiar Bouts	11.0 ±0.92	8.9 ±0.89	10.1 ±1.10	9.5 ±0.76	> *.05*	12.2 ±1.01	10.0 ±1.03	13.8 ±0.92	9.3 ±0.70	< ***.01***
Novel Bouts	10.7 ±1.47	11.4 ±1.92	12.6 ±1.19	11.2 ±1.49	> *.05*	13.5 ±1.57	9.8 ±0.68	13.1 ±1.13	10.5 ±1.06	= ***.069***
Total Bouts	21.7 ±2.21	20.3 ±2.23	22.7 ±2.0	20.7 ±1.67	> *.05*	25.7 ±2.31	19.8 ±1.41	26.9 ±1.29	19.8 ±1.47	< ***.01***
% Novel Bouts	47.5 ±2.73	54.4 ±4.45	55.7 ±2.62	52.9 ±3.85	> *.05*	51.8 ±2.40	49..9 ±2.47	48.4 ±2.99	52.6 ±2.55	> *.05*
Avg. Familiar Bout (s)	1.12 ±0.13	0.90 ±0.12	0.84 ±0.10	0.96 ±0.12	> *.05*	0.99 ±0.17	1.25 ±0.36	0.75 ±0.09	1.44 ±0.27	> *.05*
Avg. Novel Bout (s)	1.26 ±0.12	1.45 ±0.20	1.19 ±0.17	1.12 ±0.23	> *.05*	1.66 ±0.23	1.51 ±0.31	1.22 ±0.22	1.57 ±0.27	> *.05*
Avg. Nov – Avg. Fam (s)	0.14 ±0.12	0.55 ±0.25	0.35 ±0.20	0.16 ±0.14	> *.05*	0.67 ±0.11	0.26 ±0.34	0.47 ±0.22	0.12 ±0.21	> *.05*
Familiar Investigation (s)	12.8 ±2.14	8.2 ±1.3	9.2 ±2.14	9.14 ±1.34	> *.05*	11.7 ±1.93	12.7 ±4.11	10.3 ±1.35	13.0 ±2.09	> *.05*
Novel Investigation (s)	13.6 ±2.41	16.0 ±3.11	14.7 ±1.55	14.0 ±4.57	> *.05*	21.9 ±3.39	14.3 ±2.81	15.3 ±2.77	16.3 ±2.86	> *.05*
Total Test Investigation (s)	26.4 ±4.23	24.1 ±3.53	24.0 ±3.15	23.1 ±5.65	> *.05*	33.6 ±4.91	27.1 ±5.23	25.6 ±3.01	29.3 ±4.30	> *.05*

Finally, discrimination index was evaluated. Significant novel object discrimination occurred in THC- and CBD-treated adolescent males and vehicle-treated adult males (*p*'s < .05). There were no main effects of drug or age (*p*'s > .05), but there was a trend towards an overall interaction of Drug*Age; F(3,71) = 2.47, *p* = .069. One-way ANOVAs examining drug effects within each age group indicated a trend towards a significant effect in adolescents; F(3,38) = 2.51, *p* = .074, with THC trending towards increasing object discrimination compared to vehicle (*p* = .071). Although not all adult groups significantly discriminated, there were no significant effects of drug administration (*p*'s > .05) (**Fig. 6C**). Importantly, the lack of differences in total test investigation time (*p*'s > .05) indicate that these differences in discrimination index are not due to motivational differences in investigation.

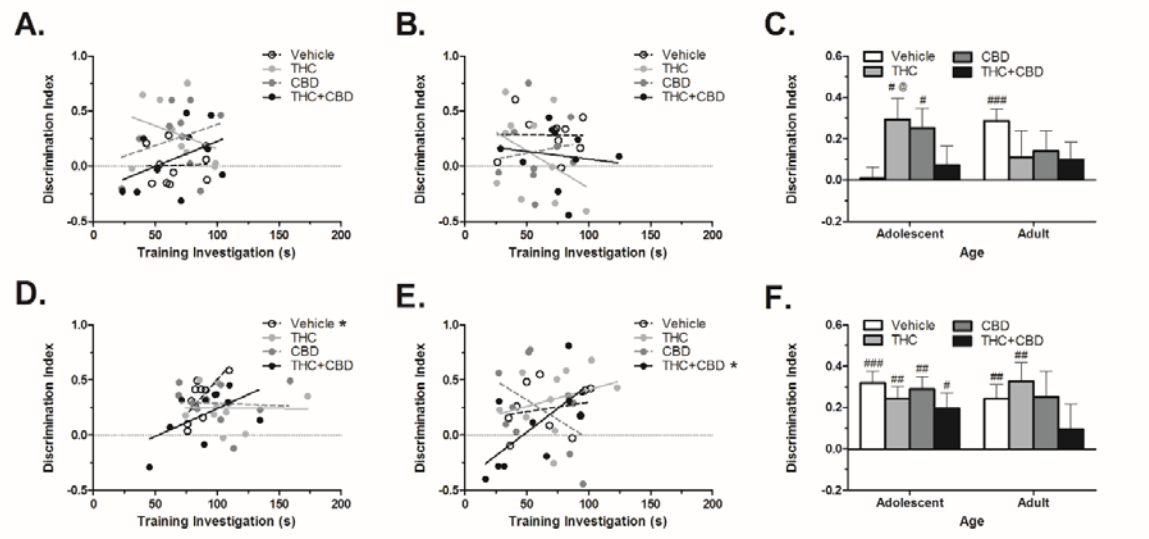

Figure 6 depicts novel object recognition behavior when a post-training injection was administered. Adolescent (A) and adult (B) male training investigation for each drug group was not correlated with discrimination index during the test session (C). Adolescent vehicle (D) and adult THC+CBD (E) female training investigation was correlated with discrimination index during the test session (F), with the exception of adolescent females treated with THC. Asterisk (*) indicates a significant correlation at p < .05. "At" sign (@) indicates a trend towards different than respective vehicle at p < .075. Pound signs indicate significantly different than 0 at p < .05 (#), p < .01 (##), and p < .001 (###). n's = 9-10.

3.2.3. Acute NOR: Females

In females, adolescent mice treated with vehicle and adult mice treated with THC+CBD showed strong and significant correlations between training investigation time and object discrimination; r(10) = .701 and r(10) = .683, *p*'s < .05, respectively (**Fig. 6D, E**). Omnibus Drug*Age ANOVAs on training investigation and ratio of investigative time in Zone A:Zone B indicated significant main effects of age for each metric, wherein adolescents spent more time investigating and did not show a zone preference, whereas adults had a slight preference for Zone B (*p*'s < .05). However, there was no main effect of drug or interaction, and one-way ANOVAs indicated no drug effects on these metrics within each age group (*p*'s > .05). This indicates that, within each age group, there were no baseline differences in investigation or zone preference.

There were many differences in behavior during the test session in females, primarily as a result in differences between adolescent and adult activity. Drug*Age ANOVAs revealed a trend towards differences in number of novel and total investigative bouts (*p*'s = .068 and .061, respectively). There was a significant main effect of age on familiar, novel, and total test investigation; number of familiar, novel, and total bouts; and average familiar and novel bout length (*p*'s < .01). Adolescents had significantly higher averages across all metrics (*p*'s < .001), thereby indicating significantly more overall investigative activity across all parameters of the test session. However, a lack of difference in percent of total bouts that were novel and average novel-average familiar bout length (*p*'s > .05) indicates that this increase in investigative activity did not translate to increased novel preference in adolescents. There was a significant main effect of drug on novel and total test investigation and familiar, novel, and total investigative bouts (*p*'s < .05). Post-hoc tests revealed that CBD increased novel and total investigative time compared to THC+CBD (*p*'s < .01). Generally, vehicle and CBD also increased number of bouts compared to THC and THC+CBD (*p*'s < .05).

When drug effects were examined for each age independently (**Table 3**), there was a significant effect on novel object and total test investigation and number of familiar, novel, and total bouts in adolescent females (*p*'s < .05). Drug effects in adult females were relegated to number of novel and total bouts (*p*'s < .01). In adolescents, CBD significantly increased total investigation time compared to all other groups and

novel investigation time compared to the THC and THC+CBD groups (p's < .05). CBD also increased number of familiar bouts compared to THC+CBD and number of novel and total investigative bouts compared to all other groups (p's < .05). Vehicle treated adolescent mice also made more total investigative bouts than THC+CBD mice (p < .05). In adults, vehicle- and CBD-treated females displayed significantly more novel and total investigative bouts than the THC+CBD-treated mice (p's < .05). Interestingly, these results indicate that acute CBD treatment in females appears to result in greater motivational drive to explore the novel object, particularly in adolescent mice.

Table 4 displays group means ± standard error for investigative behaviors of female mice in the novel object task following acute cannabinoid administration. Displayed p values are for one-way ANOVAs assessing drug effects in each age group.

	Adolescents					Adults				
	Veh	**THC**	**CBD**	**THC+ CBD**	***p* value**	**Veh**	**THC**	**CBD**	**THC+ CBD**	***p* value**
Training Investigation (s)	85.6 ±3.10	107.7 ±8.40	100.7 ±9.12	91.0 ±8.17	*> .05*	67.2 ±8.23	72.0 ±8.65	59.7 ±8.09	58.0 ±4.26	*> .05*
Zone A:Zone B Training Time	1.11 ±0.13	1.14 ±0.14	0.99 ±0.11	0.97 ±0.07	*> .05*	0.87 ±0.08	0.94 ±0.08	0.86 ±0.09	0.78 ±0.05	*> .05*
Familiar Bouts	11.2 ±0.73	9.6 ±0.98	12.4 ±0.82	9.3 ±0.68	***< .05***	10.2 ±1.06	7.5 ±0.72	8.8 ±0.70	7.6 ±1.01	*> .05*
Novel Bouts	13.9 ±0.69	13.2 ±0.59	18.4 ±0.58	11.5 ±0.83	***< .001***	12.4 ±0.65	9.5 ±1.13	12.3 ±1.19	7.2 ±0.92	***< .01***
Total Bouts	25.1 ±1.07	22.8 ±0.94	30.8 ±1.03	20.8 ±1.11	***< .001***	22.6 ±1.22	17.0 ±1.51	21.1 ±1.57	14.8 ±1.70	***< .01***
% Novel Bouts	55.5 ±1.86	58.5 ±2.90	60.0 ±1.57	55.1 ±2.65	*> .05*	55.5 ±2.87	55.2 ±3.51	57.9 ±2.92	49.0 ±3.19	*> .05*
Avg. Familiar Bout (s)	1.02 ±0.08	1.39 ±0.18	1.32 ±0.14	1.33 ±0.16	*> .05*	0.75 ±0.12	0.74 ±0.06	0.84 ±0.18	0.71 ±0.09	*> .05*
Avg. Novel Bout (s)	1.60 ±0.10	1.56 ±0.11	1.58 ±0.13	1.59 ±0.18	*> .05*	1.00 ±0.16	1.46 ±0.33	1.01 ±0.12	1.04 ±0.19	*> .05*
Avg. Nov – Avg. Fam (s)	0.58 ±0.12	0.17 ±0.19	0.26 ±0.12	0.26 ±0.15	*> .05*	0.25 ±0.16	0.72 ±0.33	0.17 ±0.24	0.33 ±0.22	*> .05*
Familiar Investigation (s)	11.4 ±1.12	12.8 ±1.69	16.1 ±1.77	12.0 ±1.49	*> .05*	7.36 ±1.16	5.65 ±0.73	7.49 ±1.86	5.51 ±1.04	*> .05*
Novel Investigation (s)	21.8 ±1.05	20.6 ±1.66	29.2 ±2.76	18.3 ±2.42	***< .01***	12.4 ±1.80	13.2 ±2.51	12.7 ±2.07	8.72 ±2.55	*> .05*
Total Test Investigation (s)	85.6 ±3.10	107.7 ±8.40	100.7 ±9.12	91.0 ±8.17	*> .05*	67.2 ±8.23	72.0 ±8.65	59.7 ±8.09	58.0 ±4.26	*> .05*

Finally, discrimination index was evaluated. All adolescent and adult female mice treated with vehicle or THC demonstrated object discrimination to varying levels of significance (p's < .05). There were no significant main effects of age or drug, nor interaction of the two variables, on discrimination index (p's > .05). Further, there were no significant drug effects within each age group (p's > .05) (**Fig. 6F**).

3.2.4 Acute EPM

In males, a Drug*Age ANOVA revealed a strong trend towards an interaction on time spent in the open arms of the EPM; $F(3,67) = 2.733$, $p = .0505$. There was no significant main effect of age ($p > .05$), but there was a main effect of drug; $F(3,67) = 2.97$, $p < .05$. One-way ANOVAs for each age group revealed that this effect was driven by a significant reduction of time in the open arms in adults administered THC+CBD ($p < .05$) (**Fig. 7A**). In males, there was a significant interaction of Drug*Age on number of open arm entries in the EPM; $F(3,67) = 2.857$, $p < .05$. There was no significant main effect of age ($p > .05$), but there was a significant main effect of drug; $F(3,67) = 2.905$, $p < .05$. A one-way ANOVA assessing drug effects revealed a trend towards a significant effect in adolescents ($p = .066$), with THC significantly increasing number of open arm entries compared to vehicle ($p < .05$). In adults, there was a significant effect of drug on number of open arm entries ($p < .05$), but no group was significantly different (p's > .05) (**Fig. 7B**). One-way ANOVAs assessing drug effects on time spent in the open arm per each open arm entry were also run for each age group to assess the relationship between total time in the open arms and number of open arm entries. The effect of drug did not reach significance in adolescents or adults (p's > .05) (data not shown).

In females, a Drug*Age ANOVA revealed no significant interaction or main effects on time spent in the open arms of the EPM (p's > .05). One-way ANOVAs assessing drug effects for each age group also revealed no significant effects in adolescents or adults (p's > .05) (**Fig. 7C**). In females, a Drug*Age ANOVA revealed no significant interaction or main effects on number of open arm entries on the EPM (p's > .05). Although a one-way ANOVA revealed a trend towards a significant effect in adolescents ($p = .066$), no drug significantly changed number of open arm entries (p's > .05). There was no significant effect of drug on open arm entries in adults ($p > .05$) (**Fig.**

7C). A one-way ANOVA assessing drug effects on time spent in the open arm per each open arm entry revealed a significant effect in adolescents ($p < .01$), with the THC+CBD significantly reducing this metric compared to vehicle ($p < .05$). There was not a significant drug effects in adults ($p > .05$) (data not shown).

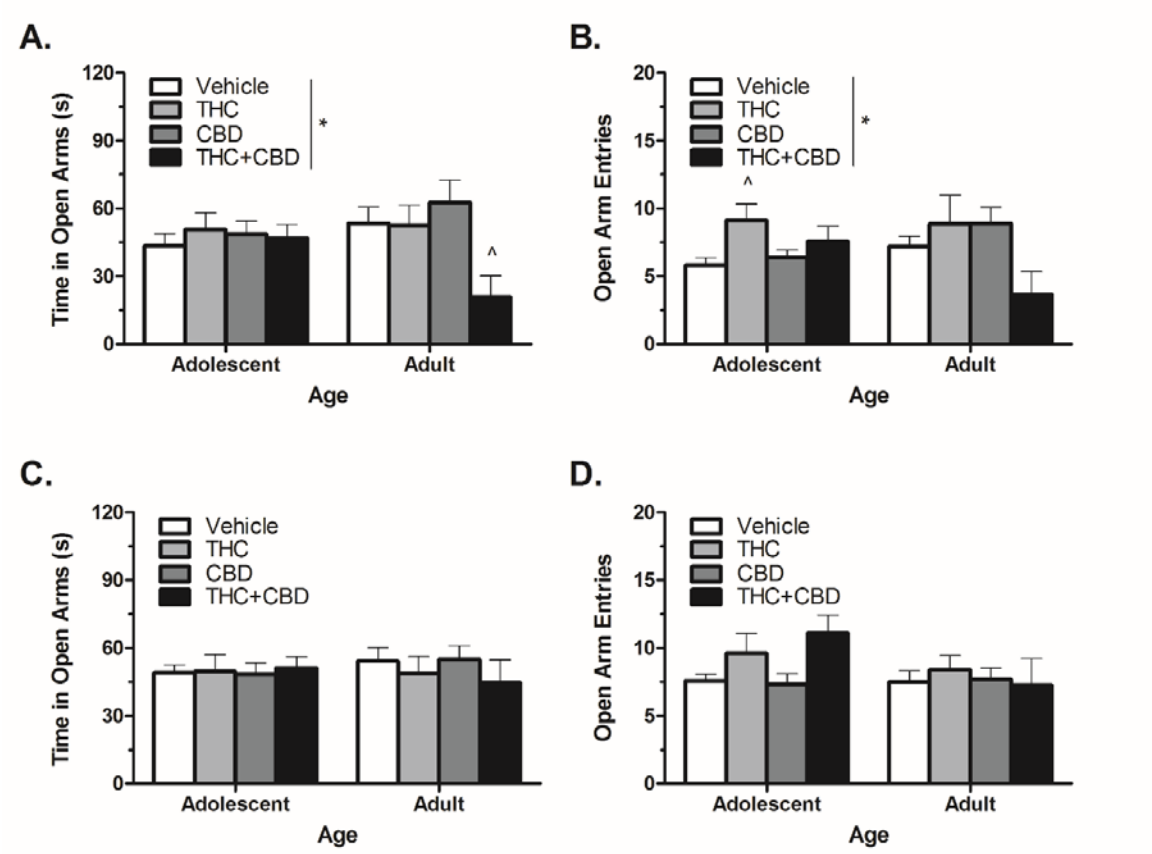

Figure 7 depicts time in the open arms and number of open arm entries for males (A, B) and females (C, D) during the acute EPM behavioral task. Asterisk (*) indicates main effect at p < .05. Carrot (^) indicates significantly different than respective vehicle group at p < .05. n's = 7-10.

3.2.5 Acute OF: Total Distance and Time

In males, a Drug*Age ANOVA revealed no significant interaction on total distance traveled in the open field ($p > .05$). There was a significant effect of age; $F(1,72) = 5.912$, $p < .05$. There was also a significant effect of drug $F(3,72) = 17.46$, $p < .001$. One-way ANOVAs indicated a significant effect of drug in both ages, with both THC and THC+CBD reducing total distance traveled (p's < .05) (**Fig. 8A**). In males, a Drug*Age ANOVA revealed no significant interaction on total time spent moving in the

open field ($p > .05$). There was a significant effect of age $F(1,72) = 13.57$, $p < .001$. There was also a significant effect of drug; $F(3,72) = 12.34$, $p < .001$. One-way ANOVAs indicated a significant effect of drug in both ages, with THC reducing total time moving in adolescents and THC+CBD reducing total time moving in adults compared to vehicle (p's $< .001$). THC trended towards reducing total time moving compared to vehicle in adults ($p = .07$) (**Fig. 8B**).

In females, a Drug*Age ANOVA revealed no significant interaction or main effect of age on total distance traveled in the open field (p's $> .05$). There was a significant effect of drug; $F(3,71) = 10.43$, $p < .001$. One-way ANOVAs indicated a significant effect of drug for each age group, with THC reducing locomotion in both age groups but THC+CBD only reducing total locomotion in adults compared to vehicle (p's $< .05$) (**Fig. 8C**). In females, a Drug*Age ANOVA also revealed no significant interaction or main effect of age on total time spent moving in the open field (p's $> .05$). There was a significant effect of drug; $F(3,71) = 6.394$, $p < .001$. One-way ANOVAs indicated a trend towards an effect of drug in adolescents ($p = .064$) and a significant effect in adults, with THC+CBD reducing total time spent moving compared to vehicle (p's $< .01$) (**Fig. 8D**).

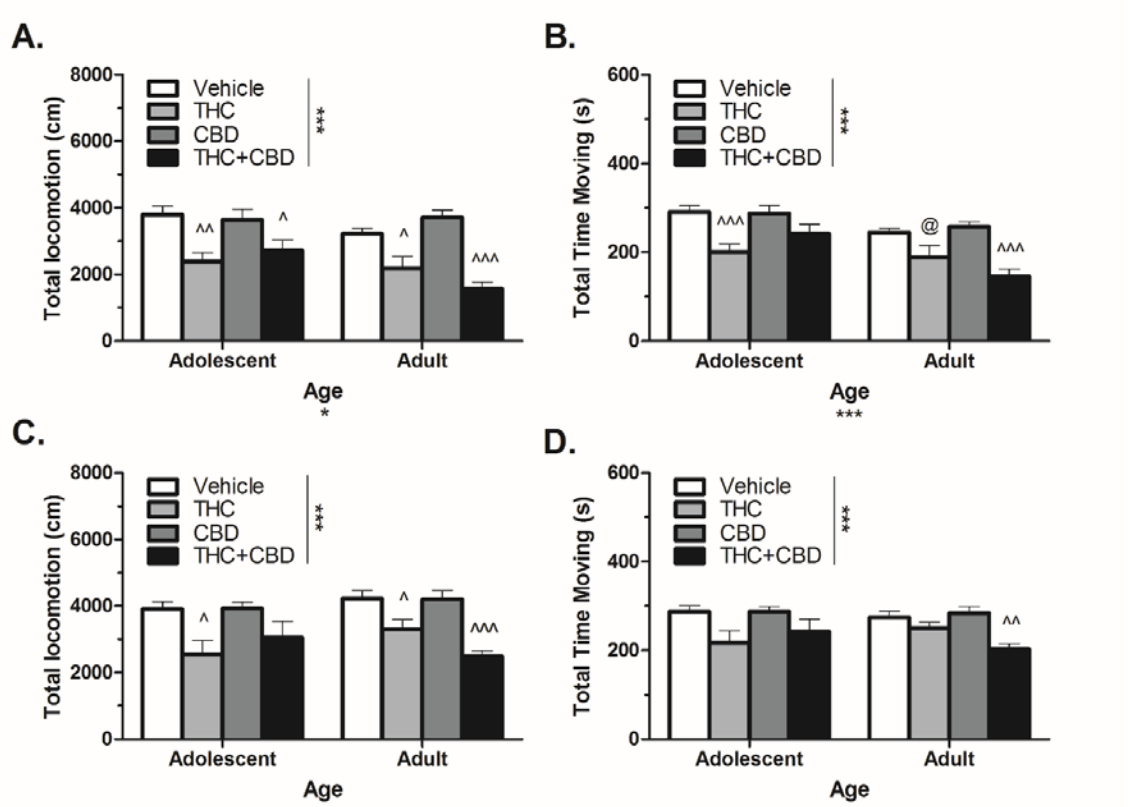

Figure 8 depicts the total distance and total time spent moving in the open field for males (A, B) and females (C, D). Asterisks indicate a main effect with significance of p < .05 (*) and p < .001 (***). Carrots indicate significance compared to that group's control at p < .05 (^), p < .01 (^^), and p < .01 (^^). "At" symbol (@) indicates a trend of p = .051 - .075. n's = 9-10 per group.

3.2.6 Acute OF: Center Distance and Time

In males, a Drug*Age ANOVA revealed no significant interaction on distance traveled in the center of the open field ($p > .05$). There was a significant effect of age; $F(1,72) = 5.105$, $p < .05$. There was also a significant effect of drug $F(3,72) = 15.16$, $p < .001$. One-way ANOVAs indicated a significant effect of drug in adolescents and adults, with THC reducing center time in both age groups and THC+CBD reducing center time only in adults compared to vehicle (p's < .05) (**Fig. 9A**). In males, there was a significant interaction of Drug*Age on the amount of time spent moving in the center of the open field; $F(3,27) = 3.188$, $p < .05$. There was a trend towards a significant effect of age ($p = .074$), and there was a significant main effect of drug; $F(3,72) = 14.45$, $p < .001$. One-way ANOVAs indicated a trend towards a significant effect in adolescents ($p = .058$) and a

significant effect of drug in adults ($p < .001$). In adolescents, there was a trend towards reduction of time spent moving in the center following THC administration compared to vehicle ($p = .067$). In adults, THC and THC+CBD significantly reduced time spent moving the center compared to vehicle (p's < .001) (**Fig. 9B**).

In females, a Drug*Age ANOVA revealed a significant interaction on distance traveled in the center of the open field; $F(3,71) = 3.31$, $p < .05$. There was no main effect of age ($p > .05$), but there was a significant main effect of drug; $F(3,71) = 17.17$, $p < .001$. One-way ANOVAS indicated a significant effect of drug in both adolescents and adults (p's < .05). In adolescents, no drug was significantly different from vehicle (p's > .05). In adults, THC and THC+CBD reduced distance traveled in the center of the open field compared to vehicle and mice that received THC+CBD traveled significantly less distance in the center than mice receiving only THC (p's < .01) (**Fig. 9C**). In females, a Drug*Age ANOVA revealed a significant interaction on time spent moving in the center of the open field; $F(3,71) = 3.15$, $p < .05$. There were significant main effects of both age and drug (p's < .001). There was a trend towards a significant effect of drug in adolescents ($p = .068$), but no group was significantly different from vehicle (p's > .05). There was a significant effect of drug in adults, with all treatment groups significantly different than vehicle and THC+CBD being significantly less than THC alone (p's < .05) (**Fig. 9D**).

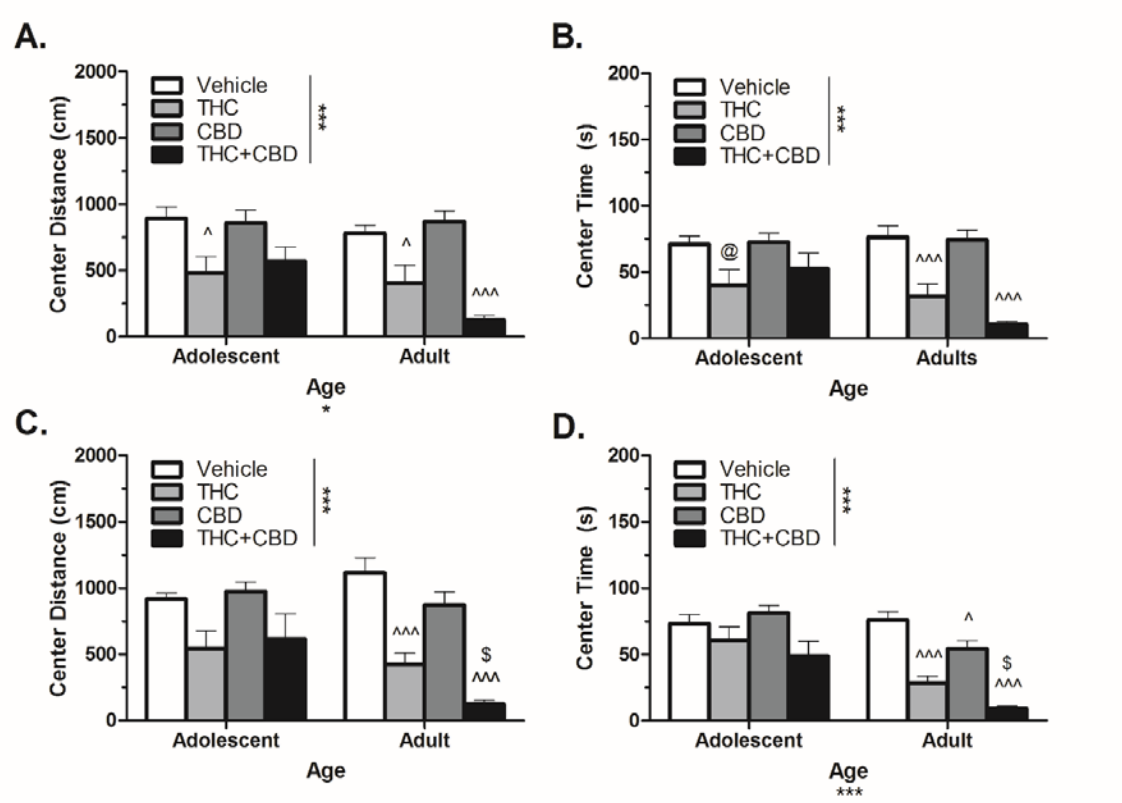

Figure 9 depicts the distance and time spent moving in the center of the open field for males (A, B) and females (C, D). Asterisks indicate a main effect with significance of $p < .05$ (*) and $p < .001$ (***). Carrots indicate significant compared to that group's control at $p < .05$ (^), $p < .01$ (^^), and $p < .01$ (^^). Dollar sign ($) indicates significantly different than THC at $p < .01$. "At" symbol (@) indicates a trend of $p = .051$ - $.075$ n's = 9-10 per group.

3.2.7 Acute OF: Percent of Distance and Time in the Center

To observe the extent that measures of activity in the center of the open field reflected similar patterns to total distance and time traveled, the percent of total distance and total time spent moving in the center [(center activity/total activity)*100] was analyzed. In males, a Drug*Age ANOVA indicated a significant interaction on percent of distance travelled in the center; $F(3,72) = 3.534$, $p < .05$. There were also significant main effects of age and drug (p's < .05). One-way ANOVAs indicated no significant effect of drug in adolescents ($p > .05$). There was a significant effect of drug in adults, with THC and THC+CBD significantly reducing the percent of total distance spent in the center compared to vehicle ($p < .05$) (**Fig. 10A**). In males, A Drug*Age ANOVA revealed no

significant interaction on percent of total time spent in the center ($p > .05$). There was a significant main effect of age; $F(1,72) = 8.524$, $p < .01$. There was also a significant effect of drug; $F(3,72) = 6.855$, $p < .001$. One-way ANOVAs indicated no significant effect of drug in adolescents ($p > .05$), and the visually suggested difference between vehicle and THC did not reach significance ($p = .09$). There was a significant effect of drug in adults, with THC and THC+CBD significantly reducing percent of time spent in the center of the OF compared to vehicle (p's $< .001$) (**Fig. 10B**).

In females, a Drug*Age ANOVA indicated a significant interaction on percent of distance travelled in the center; $F(3,71) = 6.584$, $p < .001$. There were also significant main effects of age and drug (p's $< .001$). One-way ANOVAs indicated significant drug effects in both age groups, with THC+CBD reducing percent of distance traveled in the center for both ages, but THC reducing percent of distance only in adults compared to vehicle (p's $< .05$). In adults, THC+CBD induced a significantly greater reduction than THC administered alone ($p < .01$) (**Fig. 10C**). A Drug*Age ANOVA also indicated a significant interaction on percent of total time spent in the center of the open field; $F(1,71) = 4.568$, $p < .01$. There were also significant main effects of age and drug (p's $< .001$). One-way ANOVAs indicated no significant drug effect in adolescents ($p > .05$). In adults, there was a significant drug effect with all treatments reducing percent of time spent in the center of the OF compared to vehicle and THC+CBD inducing a significantly greater reduction than THC alone (p's $< .01$) (**Fig. 10D**).

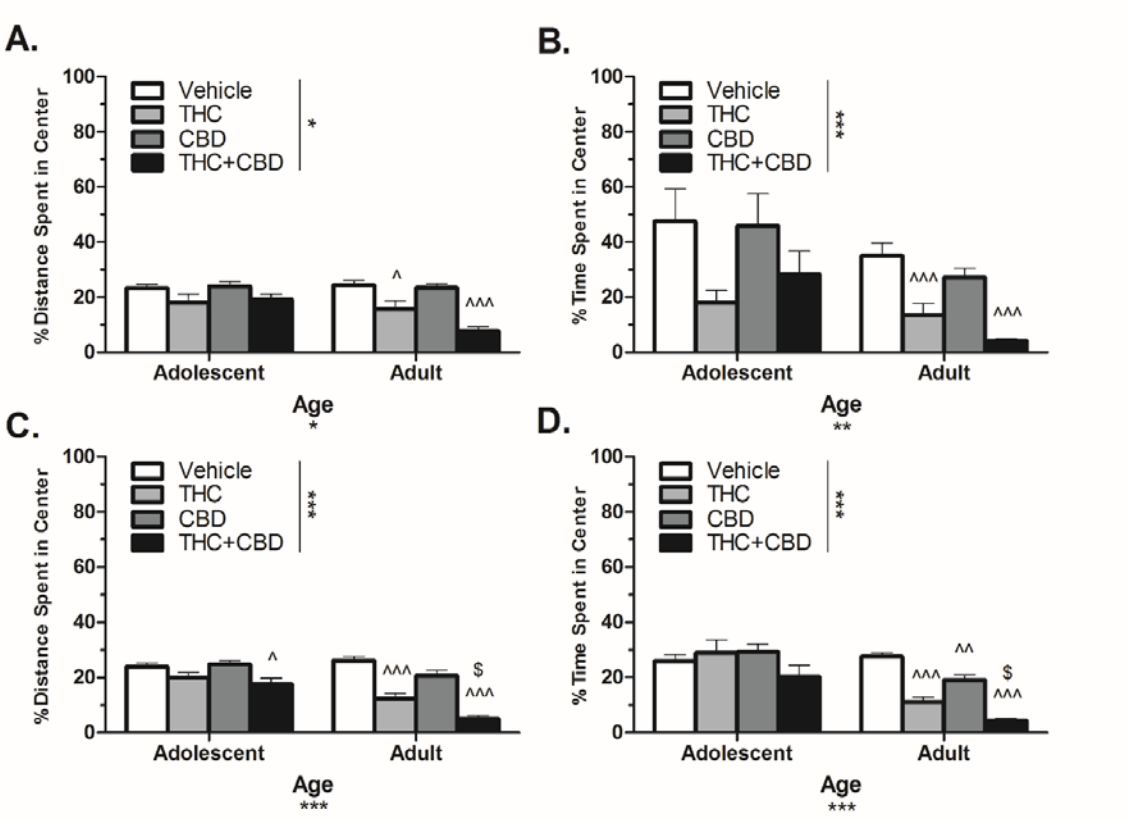

Figure 10 depicts the percent of distance and time spent moving in the center of the open field for males (A, B) and females (C, D). Asterisks indicate a main effect with significance of $p < .05$ (*), $p < .01$ (**) and $p < .001$ (***). Carrots indicate significant compared to that group's control at $p < .05$ (^), $p < .01$ (^^), and $p < .01$ (^^). Dollar sign ($) indicates significantly different than THC at $p < .01$. n's = 9-10 per group.

3.3 Aim 3: Repeated Cannabinoid History

3.3.1 Weights

To determine whether repeated drug exposure affected developmental weight gain, weight across testing was analyzed using repeated measures Drug*Day ANOVA for each age and sex group independently. Greenhouse-Geisser statistics are reported when warranted. In adolescent-treated males there was a significant interaction; $F(6.633,79.596) = 2.317$, $p < .05$. There was not a significant main effect of drug ($p > .05$), but there was a main effect of day, which displayed a significant linear, quadratic, and cubic trend (p's $< .001$). One-way ANOVAs assessing drug effects at each day

indicated a significant effect only on PND43, with the THC+CBD group weighing significantly less than the vehicle-treated group ($p < .05$) (**Fig. 11A**).

In adult-treated males the interaction of drug*day did not reach significance under Greenhouse Geisser parameters; $F(5.258,63.101) = 1.626$, $p > .05$. There was a strong trend towards a main effect of drug ($p = .056$), but no group was significantly different in overall weight than the vehicle group (p's > .05). There was a significant effect of day with both linear and cubic trends (p's < .05) (**Fig. 11B**).

In adolescent-treated females there was no significant interaction of drug*day nor a significant main effect of drug (p's > .05). There was a significant main effect of day; $F(2.382,85.754) = 209.995$, $p < .001$. The main effect of day displayed significant linear, quadratic, and cubic trends (p's < .001) (**Fig. 11C**).

In adult-treated females there was a significant interaction of drug*day; $F(14.946,174.374) = 2.185$, $p < .01$. There was no significant main effect of drug ($p > .05$), but there was a main effect of day which displayed a significant linear, quadratic, and cubic trend (p's > .001). One-way ANOVAs assessing drug effects at each day indicated a significant effect on PND78 ($p < .05$), with the THC-treated animals trending towards having a lower weight than the vehicle-treated group ($p = .054$) (**Fig. 11D**).

To assess whether adolescent drug treatment affected long-term weight gain, the weight of adolescent-treated mice at PND73 (four weeks after the last injection) was compared to the weight of adult-treated mice at PND73 (their weight prior to their fifth injection). In males, a Drug*Age at Treatment ANOVA revealed no significant interaction on weight ($p > .05$). There was a trend towards a main effect of drug ($p = .056$), and a significant main effect of age at treatment ($p < .001$). Independent-samples t-tests revealed that adolescent mice that received CBD and THC+CBD had lower weights on PND73 than adult mice receiving the same drug treatment ($p < .05$) (**Fig. 11E**). In females, there were no significant interaction or main effects of drug or age at treatment (p's > .05). Independent-samples t-tests also revealed no significant differences between adolescent- and adult-treated mice that received the same treatment at PND73 (**Fig. 11F**).

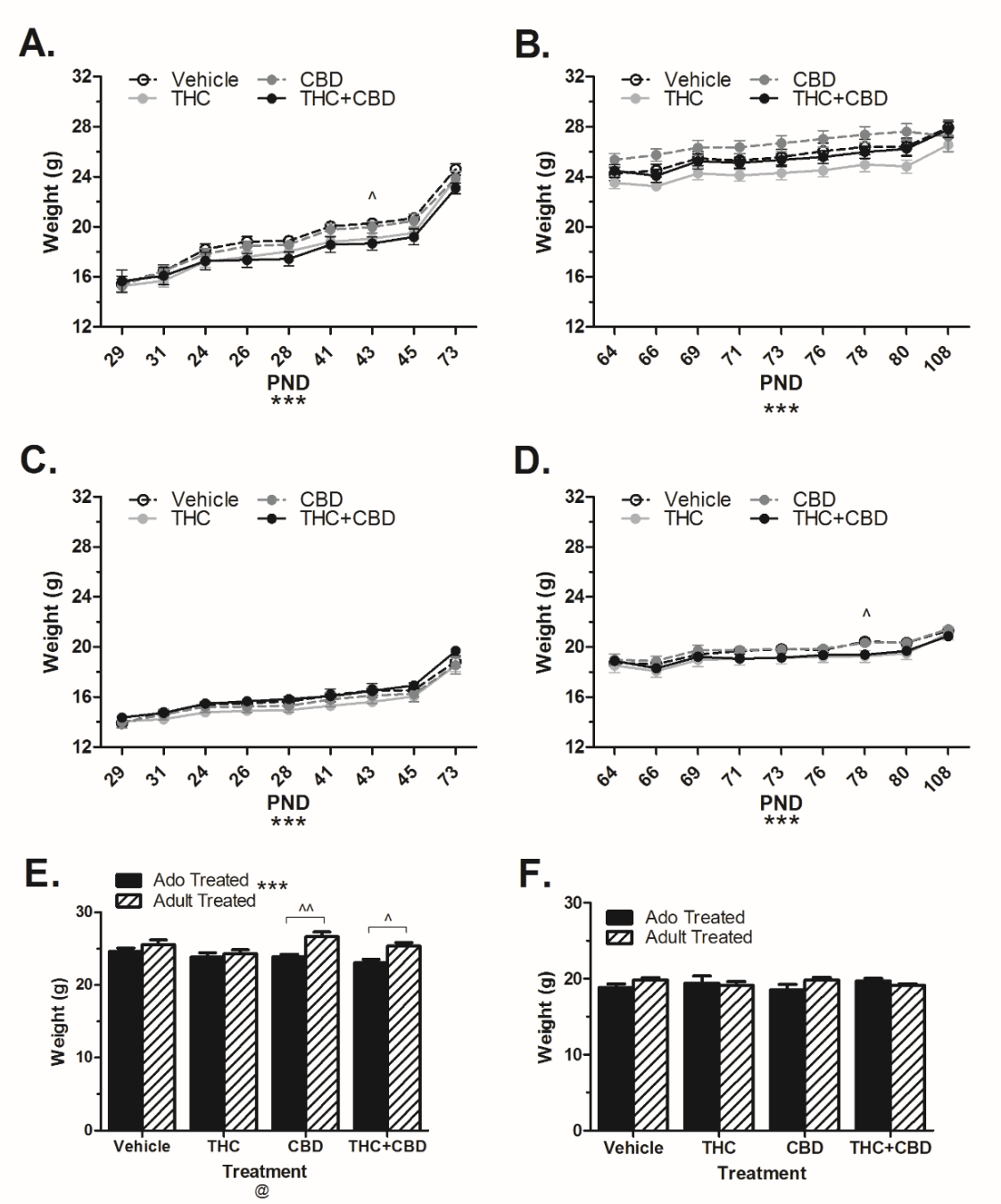

Figure 11 depicts the weights across treatment in adolescent-treated males (A), adult-treated males (B), adolescent-treated females (C), and adult treated females (D). Weight for adolescent- and adult-treated males (E) and females (F) at PND73 is also shown. Three asterisks (***) indicate a main effect with significance of $p < .001$. Carrots indicate significant compared to that group's control at $p < .05$ (^), and $p < .01$ (^^). "At" symbol (@) indicates a trend of $p = .051$ - $.075$ n's = 9-10 per group.

3.3.2 Aged NOR: Males

In males treated during adolescence or adulthood, time spent investigating the objects during the training session was not significantly correlated with discrimination index in any drug group (*p*'s > .05) (**Fig. 12A, B**). A Drug*Age at Treatment ANOVA revealed no interaction or main effect of drug on total training investigation or ratio of investigative time spend with the object in Zone A:Zone B during the training session (*p*'s > .05). There was a significant main effect of age for total training investigation, with mice with an adolescent history spending more time interacting with the objects ($p < .001$). One-way ANOVAs examining drug effects on training activity also confirmed no effect of previous drug exposure on baseline investigative activity in either age group (*p*'s > .05).

There were differences in behavior during the novel object test session in males, with differences due primarily to main effects of age. Drug*Age at Treatment ANOVAs revealed no significant interaction effects on test session behaviors (*p*'s > .05). A main effect of age was revealed for investigative time of the familiar and novel objects; total investigative time; number of familiar, novel, and total investigative bouts; and average familiar bout length (*p*'s < .05). Differences in all of these metrics were due to higher investigative activity levels in mice treated during adolescence (*p*'s < .05). Interestingly, there was only a main effect of drug history on average novel bout length – average familiar bout length; mice treated with THC had a larger difference in novel to familiar bout lengths than those treated with CBD or THC+CBD (*p*'s < .05).

One-way ANOVAs within each age group further confirmed no significant effect of drug history on any metric in males (*p*'s > .05) (**Table 4**). The overall effect of drug history on average novel – average familiar bout length was limited to a trend in adolescent treated mice ($p = .061$), wherein no history group was significantly different from another (*p*'s > .05).

Table 5 displays group means ± standard error for investigative behaviors of male mice in the novel object task following a history of cannabinoid exposure. Displayed p values are for one-way ANOVAs assessing drug effects in each age group.

	Adolescents					Adults				
	Veh	**THC**	**CBD**	**THC+ CBD**	***p* value**	**Veh**	**THC**	**CBD**	**THC+ CBD**	***p* value**
Training Investigation (s)	55.9 ±5.51	65.5 ±5.86	67.8 ±7.16	62.0 ±6.80	*> .05*	49.6 ±4.38	49.9 ±3.86	51.9 ±5.73	48.2 ±3.96	*> .05*
Zone A:Zone B Training Time	0.93 ±0.07	1.16 ±0.13	1.05 ±0.08	1.04 ±0.11	*> .05*	0.94 ±0.12	0.90 ±0.07	1.01 ±0.10	1.13 ±0.06	*> .05*
Familiar Bouts	11.4 ±0.92	11.3 ±1.05	12.7 ±0.96	12.7 ±0.50	*> .05*	9.6 ±0.78	9.9 ±1.14	10.4 ±0.92	8.8 ±1.02	*> .05*
Novel Bouts	14.5 ±0.64	12.4 ±0.84	15.4 ±0.60	14.3 ±1.15	*> .05*	11.9 ±0.86	11.1 ±1.14	12.3 ±0.90	10.2 ±1.20	*> .05*
Total Bouts	25.9 ±1.27	23.8 ±1.74	28.1 ±1.45	27.0 ±1.39	*> .05*	21.5 ±1.41	21.0 ±1.84	22.7 ±1.37	19.0 ±1.67	*> .05*
% Novel Bouts	56.4 ±1.89	52.8 ±1.68	55.3 ±1.33	52.3 ±2.06	*> .05*	55.1 ±2.26	52.9 ±3.02	54.3 ±2.60	53.5 ±4.06	*> .05*
Avg. Familiar Bout (s)	0.83 ±0.08	0.99 ±0.11	1.08 ±0.11	0.98 ±0.06	*> .05*	0.85 ±0.08	0.69 ±0.08	0.83 ±0.06	0.89 ±0.13	*> .05*
Avg. Novel Bout (s)	1.25 ±0.17	1.60 ±0.23	1.13 ±0.11	1.02 ±0.13	*> .05*	1.17 ±0.16	1.19 ±0.20	1.00 ±0.08	1.03 ±0.12	*> .05*
Avg. Nov – Avg. Fam (s)	0.41 ±0.20	0.61 ±0.22	0.05 ±0.08	0.04 ±0.14	= ***.061***	0.33 ±0.17	0.50 ±0.19	0.17 ±0.09	0.14 ±0.11	*> .05*
Familiar Investigation (s)	9.28 ±0.97	11.0 ±1.47	13.5 ±1.72	12.2 ±1.62	*> .05*	8.02 ±0.91	6.81 ±1.17	8.52 ±0.86	7.92 ±1.46	*> .05*
Novel Investigation (s)	18.3 ±2.75	20.0 ±3.25	17.5 ±1.90	15.2 ±2.61	*> .05*	13.7 ±2.10	13.9 ±3.81	12.3 ±1.25	10.7 ±1.96	*> .05*
Total Test Investigation (s)	27.6 ±2.39	31.0 ±4.52	31.0 ±3.42	27.4 ±3.71	*> .05*	21.8 ±2.30	20.7 ±4.11	20.8 ±1.39	18.6 ±3.23	*> .05*

Finally, discrimination index was evaluated. Significant novel object discrimination occurred in male mice with an adolescent and adult history of vehicle or THC as well as mice with an adolescent history of CBD (*p*'s < .05). A Drug*Age at Treatment ANOVA revealed no significant main effects or interaction of the variables on discrimination index (*p*'s > .05). Further, there were no effects of drug history within either age group (*p*'s > .05) (**Fig. 12C**).

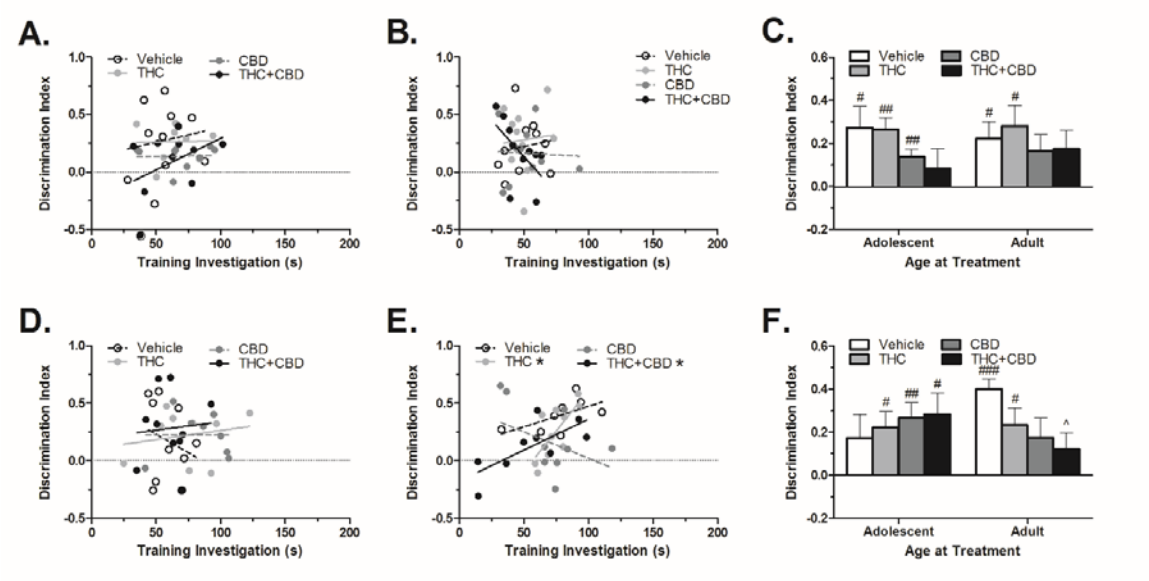

Figure 12 depicts novel object recognition behavior following a history of repeated cannabinoid injections. Correlations of training investigation and discrimination index are shown for adolescent-treated males (A), adult-treated males (B), adolescent-treated females (D), and adult-treated females (E). Discrimination index on test day is shown for males (C) and females (F). Asterisk indicates a significant correlation at p < .05 (*) or p < .01 (**). Pound signs indicate significantly different than 0 at p < .05 (#), p < .01 (##), and p < .001 (###). Carrot (^) indicates significantly different than vehicle at p < .05. n's = 9-10.

3.3.3. Aged NOR: Females

In females, mice with an adult history of treatment with THC and THC+CBD showed strong and significant correlations between training investigation time and object discrimination; r(10) = .740 and r(9) = .726, *p*'s < .05, respectively (**Fig. 12D, E**). Omnibus Drug*Age at Treatment ANOVAs on training investigation and ratio of investigative time in Zone A:Zone B revealed no significant main effects or an interaction (*p*'s > .05). One-way ANOVAs indicated a significant effect of drug history

on training investigative time in adolescent-treated mice, with a CBD history increasing training investigation compared to a THC+CBD history ($p < .05$).

There were many differences in behavior during the test session in females. Drug*Age at Treatment ANOVAs revealed a trend towards an interaction on investigative time spent with the novel object ($p = .062$). Significant main effects of age at treatment were revealed for familiar, novel, and total investigative time and familiar average bout length (p's < .05), as well as a trend for number of novel bouts and percent of bouts that were novel (p's = .068 and .058, respectively). On all metrics, adult-treated females had higher levels of investigative activity. There were also persistent main effects of drug history on number of novel bouts and total test bouts (p's < .05), as well as a trend towards significant effects on total test investigation and familiar object investigation (p's = .066 and .057, respectively). Generally, CBD increased investigative behaviors compared to THC+CBD on the test day, although THC also increased number of novel bouts compared to THC+CBD (p's = .034-.069).

Examining drug history effects within each age group revealed that many effects in females' investigative activity during the test session were limited to an adolescent history of treatment (**Table 5**). In adolescent treated females, drug history significantly influenced total test investigation, familiar average bout length, and total number of test bouts (p's < .05) and trended towards influencing number of novel bouts ($p = .051$). CBD increased total test investigation ($p = .025$) and novel bouts ($p = .072$) compared to THC+CBD, as well as total investigative bouts ($p = .025$) compared to vehicle. Vehicle increased average familiar bout length compared to THC+CBD ($p = .034$). In adult-treated females there was a significant effect of drug history on average novel bout length, with vehicle increasing average novel bout length compared to THC ($p < .05$). There was also a trend towards an effect of drug history on average novel minus average familiar bout length ($p = .065$), but no differences between treatment groups trended towards or reached significance (p's > .075).

Table 6 displays group means ± standard error for investigative behaviors of female mice in the novel object task following a history of cannabinoid exposure. Displayed p values are for one-way ANOVAs assessing drug effects in each age group.

	Adolescents					Adults				
	Veh	**THC**	**CBD**	**THC+ CBD**	***p* value**	**Veh**	**THC**	**CBD**	**THC+ CBD**	***p* value**
Training Investigation (s)	51.2 ±4.04	74.0 ±9.46	82.9 ±7.22	60.7 ±5.23	***< .05***	77.4 ±7.25	74.5 ±4.33	67.0 ±8.38	55.2 ±10.03	*> .05*
Zone A:Zone B Training Time	0.98 ±0.07	1.00 ±0.07	1.01 ±0.06	1.03 ±0.08	*> .05*	0.95 ±0.11	1.10 ±0.15	1.08 ±0.16	1.05 ±0.10	*> .05*
Familiar Bouts	9.3 ±0.80	11.3 ±0.37	11.7 ±0.72	10.7 ±0.92	*> .05*	10.7 ±1.21	10.8 ±0.92	12.1 ±0.90	10.6 ±1.29	*> .05*
Novel Bouts	10.3 ±1.27	12.4 ±1.46	14.3 ±1.07	10.1 ±0.95	***= .051***	13.6 ±1.02	15.4 ±1.57	13.4 ±0.97	11.3 ±0.76	*> .05*
Total Bouts	19.6 ±1.72	23.8 ±1.62	26.0 ±1.42	20.8 ±1.36	***< .01***	24.2 ±1.79	26.2 ±2.30	25.5 ±1.36	21.9 ±1.81	*> .05*
% Novel Bouts	51.7 ±2.89	51.1 ±2.60	54.8 ±2.09	48.2 ±3.16	*> .05*	56.5 ±2.59	58.5 ±2.11	52.5 ±2.83	52.6 ±2.35	*> .05*
Avg. Familiar Bout (s)	1.07 ±0.14	0.86 ±0.08	1.01 ±0.09	0.65 ±0.10	***< .05***	1.08 ±0.08	1.19 ±0.14	1.18 ±0.14	1.23 ±0.09	*> .05*
Avg. Novel Bout (s)	1.82 ±0.50	1.34 ±0.21	1.54 ±0.20	1.47 ±0.30	*> .05*	2.01 ±0.18	1.36 ±0.13	1.50 ±0.16	1.46 ±0.16	***< .01***
Avg. Nov – Avg. Fam (s)	0.75 ±0.52	0.48 ±0.17	0.52 ±0.20	0..82 ±0.31	*> .05*	0.92 ±0.19	0.17 ±0.23	0.33 ±0.21	0.23 ±0.21	***= .065***
Familiar Investigation (s)	9.77 ±1.32	9.87 ±1.16	12.0 ±1.38	7.14 ±1.33	*> .05*	11.4 ±1.18	12.4 ±1.52	15.2 ±2.65	12.6 ±1.32	*> .05*
Novel Investigation (s)	15.0 ±2.54	15.8 ±2.36	21.4 ±2.66	13.7 ±2.21	*> .05*	27.5 ±3.30	21.8 ±3.91	19.4 ±1.74	16.8 ±2.44	*> .05*
Total Test Investigation (s)	24.8 ±2.80	25.7 ±3.36	33.4 ±3.20	20.9 ±2.63	***< .05***	38.8 ±4.10	34.2 ±4.64	34.6 ±3.79	29.4 ±3.13	*> .05*

Finally, novel object discrimination was evaluated. Females treated during adolescence with THC, CBD, and THC+CBD as well as females treated during adulthood with vehicle and THC demonstrated object discrimination to varying levels of significance (p's < .05). A Drug*Age at Treatment ANOVA revealed no significant main effects or interaction on discrimination index (p's > .05). One-way ANOVAs for each age group indicated a weak trend of drug history in adult-treated animals (p = .08), with a THC+CBD history significantly reducing object discrimination compared to a vehicle history (p < .05) (**Fig. 12F**).

3.3.4 Aged EPM

In males, a Drug*Age at Treatment ANOVA revealed no significant interaction or effect of drug history on time spent in the open arms of the EPM (p's > .05). There was a significant main effect of age at treatment; $F(3,69) = 5.45$, $p < .05$. One-way ANOVAs for each age group revealed no significant drug treatment effects (p's > .05) (**Fig. 13A**). In males, a Drug*Age at Treatment ANOVA revealed no significant interaction or main effect of drug on number of open arm entries in the EPM (p's > .05). There was a significant main effect of age; $F(1,69) = 4.474$, $p < .05$. Further, one-way ANOVAs revealed no effect of previous drug treatment in either age group (p's > .05) (**Fig. 13B**). Analyses of time spent in the open arm per each open arm entry revealed the same pattern, with a significant omnibus effect of age (p = .048), and no other significant effects (p's > .05) (data not shown).

In females, a Drug*Age at Treatment ANOVA revealed no main effects on time spent in the open arms of the EPM (p's > .05). However, there was a significant interaction; $F(3,69) = 3.361$, $p < .05$. One-way ANOVAs assessing drug effects for each age group revealed no significant effects in adolescent-treated mice (p's > .05), but a significant effect of drug in adult-treated mice, with mice in the CBD group spending significantly less time in the open arms than the vehicle group (p < .05) (**Fig. 13C**). In females, a Drug*Age at Treatment ANOVA revealed no significant interaction or main effect of drug on number of open arm entries on the EPM (p's > .05). There was a significant main effect of age, with adolescent-treated mice making more entries; $F(1,70) = 5.165$, $p < .05$. A one-way ANOVA revealed a trend towards a significant effect in

adolescent-treated mice (p = .057), with THC treatment increasing the number of open arm entries compared to vehicle (p < .05). There was no significant effect of drug on open arm entries in adults (p > .05) (**Fig. 13C**). A one-way ANOVA assessing drug effects on time spent in the open arm per each open arm entry revealed no significant effect in adolescent-treated mice (p > .05). There was a significant drug history effect in adult-treated mice (p < .05), with THC reducing this metric compared to vehicle (data not shown).

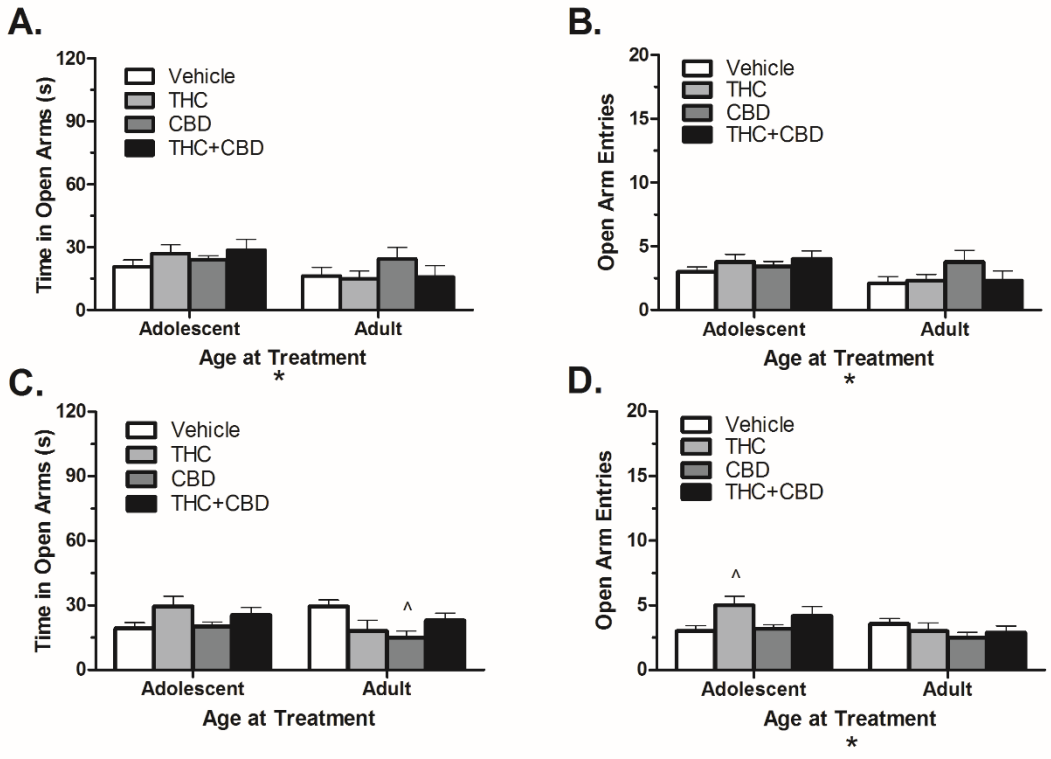

Figure 13 depicts time in the open arms and number of open arm entries for males (A, B) and females (C, D) during the EPM behavioral task following a history of cannabinoid treatment. Asterisk (*) indicates a significant main effect at p < .05. Carrot (^) indicates significantly different than respective vehicle group at p < .05. n's = 9-10.

3.3.5 Aged OF: Total Distance and Time

In males, a Drug*Age at Treatment ANOVA revealed no significant interaction or effect of previous drug treatment on total distance traveled in the open field (p > .05). There was a significant effect of age; $F(1,72) = 23.80$, p < .001. One-way ANOVAs indicated no significant effect of prior drug history in adolescent-treated mice, but a trend

towards an effect in adult-treated mice; (3,39) = 2.537, $p = .072$. Mice treated in adulthood with CBD traveled significantly more distance than their vehicle counterparts (p's < .05) (**Fig. 14A**). In males, a Drug*Age ANOVA revealed no significant interaction or main effect of previous drug history on total time spent moving in the open field (p's < .05). There was a significant effect of age $F(1,72) = 23.07$, $p < .001$. One-way ANOVAs indicated no significant effect of drug at either treatment age (p's > .05) (**Fig. 14B**).

In females, a Drug*Age at Treatment ANOVA revealed no significant interaction or main effect of drug on total distance traveled in the open field (p's > .05). There was a significant effect of age at treatment, with adolescent-treated mice traveling more; $F(3,71) = 11.07$, $p < .01$. One-way ANOVAs did not indicate a significant effect of drug history for either age group (p's > .05) (**Fig. 14C**). In females, a Drug*Age at Treatment ANOVA also revealed no significant interaction or main effect of drug on total time spent moving in the open field (p's > .05). There was a significant effect of age at treatment, with adolescent mice spending more time moving; $F(3,71) = 19.07$, $p < .001$. One-way ANOVAs did not indicate an effect of drug history in either age group (**Fig. 14D**).

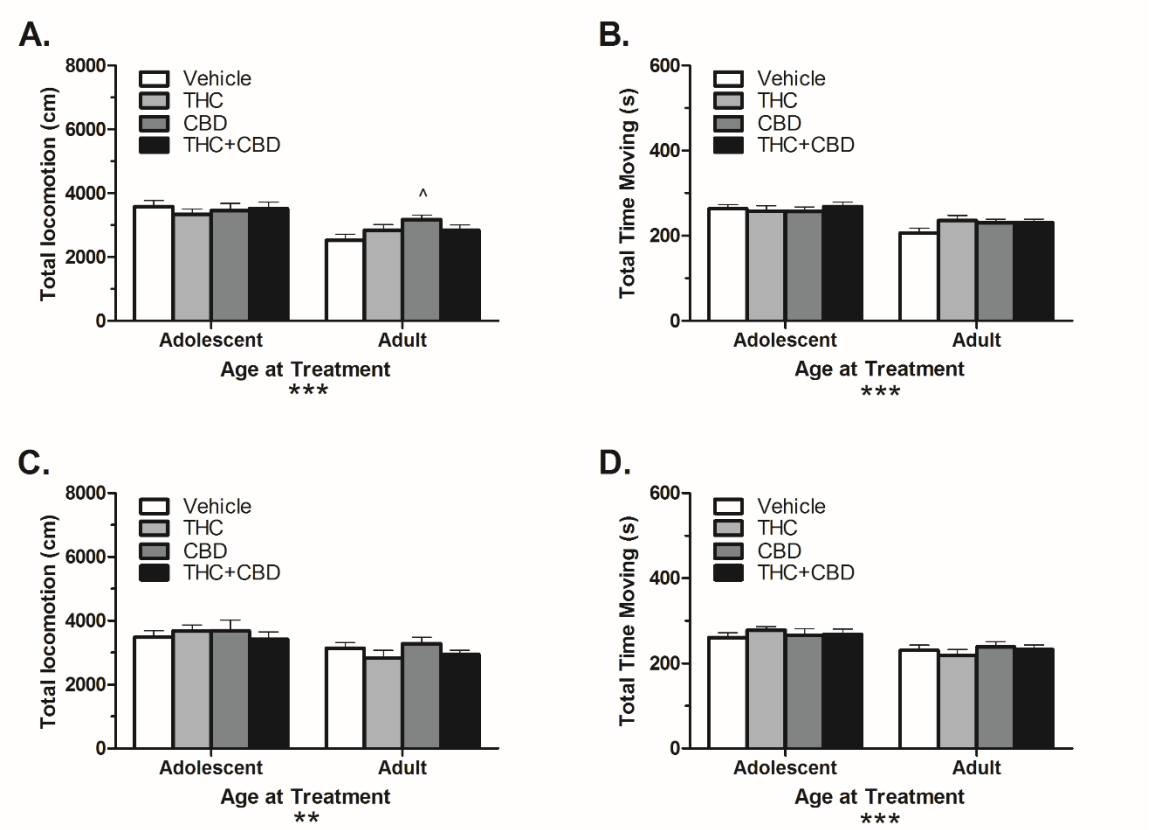

Figure 14 depicts the total distance and total time spent moving in the open field for males (A, B) and females (C, D) following a history of cannabinoid treatment. Asterisks indicate a main effect with significance of p < .01 (**) and p < .001 (***). Carrot (^) indicate significant compared to that group's control at p < .05. n's = 9-10 per group.

3.3.6 Acute OF: Center Distance and Time

In males, a Drug*Age at Treatment ANOVA revealed no significant interaction or main effects on distance traveled in the center of the open field (*p*'s > .05). One-way ANOVAs indicated no significant effect of drug in adolescent-treated mice ($p < .05$), but a trend towards a significant effect in adult-treated mice; $F(3,39) = 2.783$, $p = .055$. Mice treated in adulthood with CBD traveled significantly more distance in the center than of the OF than their vehicle counterparts (**Fig. 15A**). In males, there was no significant interaction of Drug*Age at Treatment or main effect of age on the amount of time spent moving in the center of the open field (*p*'s < .05). There was a significant effect of age at treatment; $F(3,72) = 6.17$, $p < .05$. One-way ANOVAs indicated no significant effect of previous drug history in either age group (*p*'s > .05) (**Fig. 15B**).

In females, a Drug*Age at Treatment ANOVA revealed no significant interaction or main effect of drug on distance traveled in the center of the open field (*p*'s > .05. There was a significant main effect of age, with adolescent-treated mice traveling more distance in the center; $F(3,71) = 6.588$, $p < .05$. One-way ANOVAs did not indicate a significant effect of drug in either age group (*p*'s > .05) (**Fig. 15C**). In females, a Drug*Age at Treatment ANOVA revealed no significant interaction or main effects on time spent moving in the center of the open field (*p*'s > .05). One-way ANOVAs did not indicate a significant effect of drug in either age group (*p*'s > .05) (**Fig. 15D**).

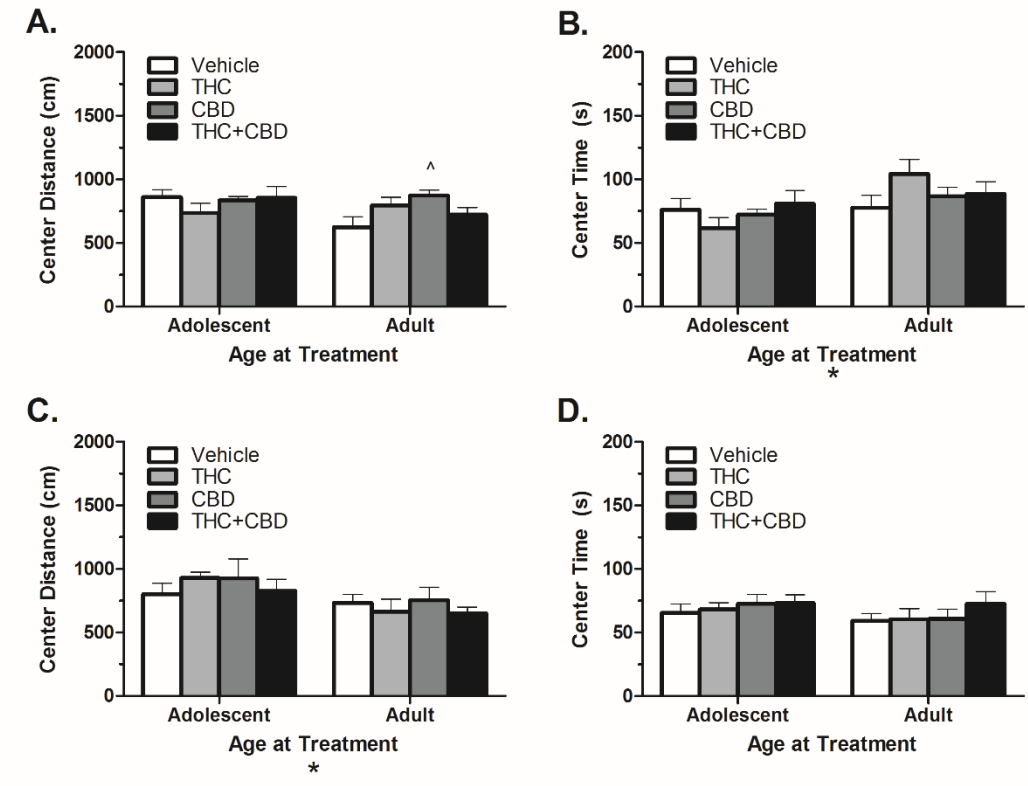

Figure 15 depicts the distance and time spent moving in the center of the open field for males (A, B) and females (C, D) following a history of cannabinoid treatment. Asterisk (*) indicates a main effect with significance of p < .05. Carrot (^) indicates significant compared to that group's control at p < .05. n's = 9-10 per group.

3.3.7 Acute OF: Percent of Distance and Time in the Center

To analyze whether measures of activity in the center of the open field reflected similar patterns to total distance and time the percent of total distance and total time spent

moving in the center [(center activity/total activity)*100] was analyzed. In males, a Drug*Age at Treatment ANOVA indicated no significant interaction or main effect of previous drug treatment on percent of distance travelled in the center (*p*'s > .05). There was a significant main effect of age at treatment; $F(1,72) = 5.087$, $p < .05$. One-way ANOVAs indicated no significant effect of prior drug history for either age group (*p*'s > .05) (**Fig. 16A**). In males, A Drug*Age at Treatment ANOVA revealed no significant interaction or main effect of drug history on percent of total time spent in the center (*p*'s > .05). There was a significant main effect of age; $F(1,72) = 16.96$, $p < .001$. One-way ANOVAs indicated no significant effect of drug in adolescent- or adult-treated mice (*p*'s > .05) (**Fig. 16B**).

In females, a Drug*Age at Treatment ANOVA indicated no significant interaction or main effects on percent of distance travelled in the center (*p*'s > .05). One-way ANOVAs indicated no significant drug history effects in either age group (*p*'s > .05) (**Fig. 16C**). A Drug*Age at Treatment ANOVA also indicated no significant interaction or main effects on percent of total time spent in the center of the open field (*p*'s > .05). One-way ANOVAs indicated no significant drug history effects in either age group (*p*'s > .05) (**Fig. 16D**).

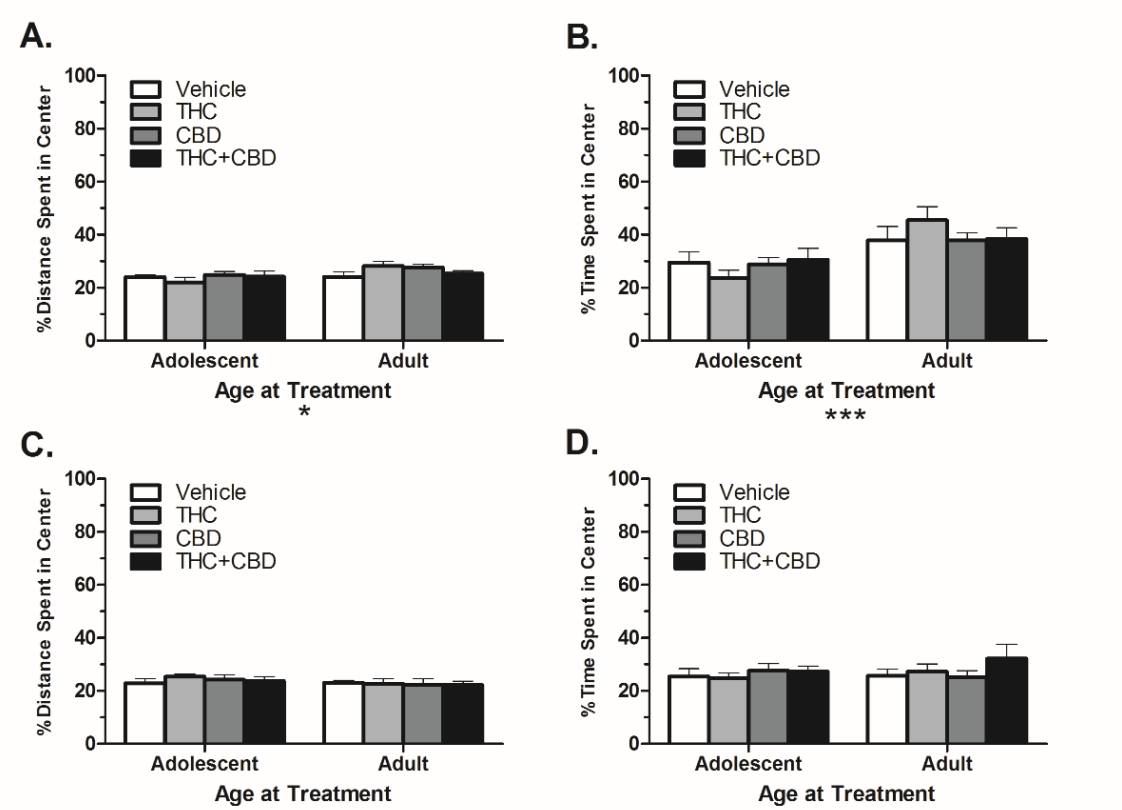

Figure 16 depicts the percent of distance and time spent moving in the center of the open field for males (A, B) and females (C, D) following a history of cannabinoid treatment. Asterisks indicates a main effect with significance of $p < .05$ (*) or $p < .001$ (***). n's = 9-10 per group.

3.4 Combined Results

Results of the 10 mg/kg THC, 20 mg/kg CBD, and THC+CBD are shown in Table 7 for comparison. EPM and OF were conducted following the first acute injection in single-housed mice for Aim 1, the second acute injection in pair-housed mice in Aim 2, and under no injection following a history of eight injections in Aim 3. NOR was conducted following the first acute injection in pair-housed mice in Aim 2 and under no injection following a history of eight injections in Aim 3.

Table 7 indicates the results of 10 mg/kg THC, 20 mg/kg CBD, or their combination on activity in the open arms in the EPM, total distance travelled in the OF, and object discrimination index in NOR for each aim. Minus signs (-) indicate significantly reduced compared to respective vehicle at $p < .05$ (-), $p < .01$ (--), and $p < .001$ (---). A plus sign indicates increased compared to respective vehicle at $p > .075$. ns indicates not significant.

			Males		**Females**	
Behavior	Drug	Aim	Ado	Adult	Ado	Adult
EPM	THC	1	ns	---	Ns	--
		2	ns	ns	Ns	ns
		3	ns	ns	Ns	ns
	CBD	1	ns	ns	Ns	ns
		2	ns	ns	Ns	ns
		3	ns	ns	Ns	-
	THC+CBD	2	ns	-	Ns	ns
		3	ns	ns	Ns	ns
OF	THC	1	ns	--	--	---
		2	--	-	-	-
		3	ns	ns	Ns	ns
	CBD	1	ns	ns	Ns	ns
		2	ns	ns	Ns	ns
		3	ns	+	Ns	ns
	THC+CBD	2	-	---	Ns	---
		3	ns	ns	Ns	ns
NOR	THC	2	+	ns	Ns	ns
		3	ns	ns	Ns	ns
	CBD	2	ns	ns	Ns	ns
		3	ns	ns	Ns	ns
	THC+CBD	2	ns	ns	Ns	ns
		3	ns	ns	Ns	-

4 DISCUSSION

The current studies demonstrate a comprehensive set of experiments examining age- and sex-effects of cannabinoid administration on acute and long-term behaviors. Although many significant acute actions of cannabinoids were demonstrated, there were minimal long-term effects associated with repeated drug administration across age and sex.

4.1 THC & CBD Dose Responses

The first aim of the current studies was to assess a dose-response to acute THC and CBD pretreatment on anxiety in the EPM and OF activity. Based on previous studies (e.g. Onaivi et al., 1990; Guimares et al., 1990; Kasten et al., under review) a divergent anxiogenic and anxiolytic effect of THC and CBD, respectively, for each age group was hypothesized. THC was expected to show a dose-dependent sedative profile, whereas CBD was not expected to have locomotor effects. The results demonstrated a strong dose-dependent anxiogenic effect of THC in adult males and females, with no significant effects in adolescents. CBD did not produce an anxiolytic profile (**Figs. 2, 3)**. However, the 10 mg/kg dose of THC effectively reduced locomotion in all mice but the adolescent males, which displayed an insignificant total activity reduction of 31.8% (**Fig. 4**). This indicates that THC was pharmacologically active in adolescent mice, but did not alter anxiety-like activity. Interestingly, CBD treatment produced a dose-dependent effect on activity in adult males, with the low 5 mg/kg dose significantly reducing activity (**Fig. 4C**).

One potential explanation for the lack of cannabinoid effects on adolescent EPM behavior is the lower level of *in vivo* CB1R binding in the adolescent brain in gray matter areas, including the amygdala (Verdurand et al., 2011). As previously discussed, THC may work as an antagonist at areas with lower CB1R expression (Pertwee, 2005), and reduced CB1R function in the amygdala may blunt the ability of THC to induce anxiety-like behavior. A secondary possibility is that the differences in control behavior influenced the ability of cannabinoids to exert behavioral effects. In both the EPM and

the OF, male and female mice administered THC demonstrated similar time in the open arms and time spent moving regardless of age. However, as demonstrated in the CBD dose response data, their baseline behavior differed (**Figs. 2, 4**). This difference is particularly striking between adolescent and adult males in the EPM, wherein adolescent males given vehicle displayed much less time in the open arms than their adult counterparts. If THC was unable to elicit an effect in adolescent mice due to differences in baseline behavior, a higher dose of THC may be effective. However, if the difference is due to less availability of functional receptors, a higher dose of THC may still be ineffective at eliciting a behavioral response.

4.2 Developmental Cannabinoid Effects: EPM and OF

Although the acute effects of THC and CBD were tested in Aim 1, mice in Aim 2 were also tested for acute effects of cannabinoids on EPM and OF activity to include the effects of the combination THC+CBD dose. Only adult males showed a significant anxiogenic response to THC+CBD (**Fig. 7A**). Notably, THC did not produce a significant anxiogenic effect in adults following acute administration in Aim 2, whereas it did in Aim 1 (**Figs. 2, 7**). Two major differences exist between procedures in Aims 1 and 2. Mice were single-housed and received their first injection prior to the test in Aim 1. In Aim 2, mice were pair-housed and were receiving their second drug injection, as their first injection took place following NOR training. The interpretation of rapid tolerance to cannabinoid injection in the EPM and influence of housing can be supported. Onaivi et al. (1990) demonstrated that on the 5th day of THC injections there was no anxiogenic response to 10 mg/kg THC in ICR mice, although a strong anxiogenic effect was seen following one acute administration. Our previous work has demonstrated that, upon second injection with 10 mg/kg THC, single-housed adult male mice show an anxiogenic response in the EPM (Kasten et al., under review). As such, the interpretation of the acute effects of THC+CBD becomes unclear. If mice had been tested following the first injection of THC+CBD it is possible that more groups would have shown an anxiogenic response and that the response to THC+CBD may have been greater than to THC alone.

Although the anxiogenic effects of THC in the EPM were attenuated following a second THC administration, locomotor effects persisted (**Fig. 8**). THC reduced distance

traveled in all groups, whereas THC+CBD reduced distance traveled in all groups but adolescent females. As adolescent males did not demonstrate a locomotor depressant effect following one dose of THC (**Fig. 4**), this may indicate that locomotor depression may develop over repeated THC injections. These results, paired with those of the EPM, suggest both tolerance and sensitivity to repeated THC injections in different behavioral assays in the same mice. Acute THC+CBD also elicited a reduction in locomotor activity in all groups but the adolescent females (**Fig. 8**).

Effects on time spent moving paired with total distance traveled indicates that cannabinoids differentially affect the minutia of movement in a sex- and age-specific manner. THC administration also reduced time spent moving in both adolescent and adult male mice, potentially indicating that it had minimal effects on acceleration. Female mice, which traveled less distance, spent the same amount of time moving as their vehicle counterparts. This indicates a slower pace of movement. Only adults were sensitive to THC+CBD's effects on time spent moving, indicating that when adults initiated movement they were able to move at a pace similar to their vehicle counterparts (**Fig. 8**).

Activity spent in the center of an open field, known as thigmotaxis, is commonly used to quantify anxiety-like behavior. Although details on absolute center distance and time spent in the center of the field are given in **Figure 9**, a better way to measure center activity is as a percentage of total behavior. Cannabinoid administration had a minimal effect on percent of total time and distance in the open field that was spent in the center by adolescent mice (**Fig. 10**). A second injection of THC+CBD reduced percent of center distance in adolescent females, while the injection of THC alone appeared to reduce percent of center time in adolescent males. The effect in adolescent males is not significant, likely due to high levels of variability in the vehicle mice. Conversely, adult mice showed a strong anxiogenic profile in this metric. Both THC and THC+CBD reduced percent distance and time spent in the center of the open field, with CBD also reducing percent center time in the adult females. These results more closely resemble the anxiogenic effect in adults on the EPM following one dose of THC (**Fig. 2**). Again, this questions whether the change in anxiogenic sensitivity in the EPM between Aims 1 and 2 is due to housing conditions or rapid tolerance. While it is tempting to assert that anxiogenic activity should be consistent between the EPM and center metrics in the OF, a

recent meta-analysis by Mohammad et al. (2016) indicates that these two tasks do not reliably reproduce one another, and should not be interpreted as reflecting the same behavioral motivation. Interestingly, Todd & Arnold (2016) demonstrated that an acute 1:1 THC:CBD injection rescues avoidance of the center of the open field in adult B6 male mice compared to those given 10 mg/kg of THC alone. The current study may therefore demonstrate a sensitivity to repeated THC+CBD injection, or that a 1:2 THC:CBD injection produces altered behavioral effects compared to a 1:1 ratio.

Long-lasting effects of cannabinoid exposure were minimal. Interestingly, an adult history of CBD resulted in anxiogenesis in females, whereas an adolescent history of THC in females increased the number of open arm entries. However, this change in arm entries did not translate to more time spent in the open arms nor did it significantly alter the amount of time per open arm entry (**Fig. 13**). The current null results may be due to the ability of pair-housing to alter anxiety-like behavior in B6 mice. The direction of this effect appears to be sensitive to the timing of isolation, with isolation throughout adolescence resulting in an anxiolytic phenotype (Voikar et al., 2005; Lopez & Laber, 2015), whereas isolation in adulthood results in an anxiogenic phenotype compared to sustained group-housing (Demuyser et al., 2016). Our previous work in single-housed mice also demonstrated minimal long-lasting effects of THC exposure, only finding that repeated exposure in males during adulthood lead to significantly more percent of distance traveled in the center of the open field (Kasten et al., under review). This anxiolytic phenotype is in direct opposition to the anxiogenic phenotype demonstrated by Demuyser et al., (2016). Although Demuyser et al. (2016) used B6 mice, these mice were sourced from a different vendor. They also were not singly-housed until 3 months of age, compared to the young-adult stage of 2 months in our previous work.

4.3 Object Recognition

Previous studies using a range of THC doses have not demonstrated an acute effect on object memory (Ciccocioppo et al., 2002; Swartwelder et al., 2012; Kasten et al., under review) and acute effects of CBD or THC+CBD have not been reported. As hypothesized, all mice but adolescent males significantly discriminated the novel object when injected with vehicle post-training (**Fig. 6**). These objects were specifically chosen

for their ability to produce significant discrimination under naïve conditions (**Fig. 5**), suggesting that adolescent male memory may be particularly sensitive to a single stressor. Interestingly, THC administration significantly rescued the injection effect seen in adolescent male mice and the CBD group also significantly discriminated, whereas adult male mice only showed significant object discrimination following the vehicle injection (**Fig. 6C**). Females did not display a similar stark age-effect of injection or cannabinoid action as the males (**Fig. 6E**). Although it has been suggested that more time spent with the objects during training may indicate better performance in the test session (Cohen & Stackman, 2015), we found no strong evidence supporting this relationship (**Fig. 6**).

The effects of cannabinoid history were tested 23 days following the last of eight injections. Based on prior research it was hypothesized that mice with an adolescent history of THC would show impaired object recognition (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012; Kasten et al., under review), whereas addition of CBD to THC would rescue this deficit (Fagherazzi et al., 2012; Cadoni et al., 2013; Campos et al., 2015; Gomes et al., 2015). Our hypothesis was not supported, as males and females treated with THC during adolescence significantly discriminated the novel object following a period of drug removal. Although six injections were sufficient to impair object memory in our previous study (Kasten et al., under review), the use of pair-housing may reduce susceptibility to THC's impairing effects (Voikar et al., 2005). A more frequent dosing regimen over the same age period may have resulting in previously seen deficits, such as the every-day dosing paradigm used in rat studies (Quinn et al., 2008; Realini et al., 2011; Zamberletti et al., 2012).

Only two studies have used adult controls to observe whether the effects of THC treatment on object memory are specific to adolescent administration. Quinn et al. (2008) found no effect of adult THC treatment on later object memory, whereas our previous findings demonstrated that an adult history of THC rescued a significant impairment in object memory seen in vehicle-treated male mice (Kasten et al., under review). However, the current study found no major differences between treatment groups in adult-treated males. Conversely, the adult-treated females showed a step-wise response to cannabinoid treatment, with the vehicle group showing very strong object discrimination. The females that received THC+CBD during adulthood demonstrated significantly impaired object

discrimination compared to the vehicle group. The THC and THC+CBD adult-treated females had training investigation times that were significantly positively correlated with discrimination index (**Fig. 12 E, F**), indicating that increased exploration during training facilitated object memory in the test session and that previous THC exposure in this group may require more cognitive effort to successfully complete a task. This interpretation is supported by findings in the human visual paired-comparison task, which indicate that impaired visual recognition in high-risk infants can be bolstered by increasing the length of time to familiarize with an object (Burbacher & Grant, 2012).

The current study did not replicate our previous findings that an adolescent history of THC disrupts object discrimination, whereas an adult history rescues discrimination in male B6 mice. Importantly, the previous study used single-housed mice, whereas the current study employed pair-housed mice. Daily increasing THC administration from PND35-45 of adolescence results in a pro-inflammatory shift in the hippocampus during adulthood (Zamberletti et al., 2015). A simple saline injection also results in increased inflammatory response, which can be interpreted as a sign of stress (Freiman et al., 2016). Stress, inflammation, and prolonged single-housing have all been shown to reduce performance in the NOR task (Võikar et al., 2005; Carey et al., 2009; Fishbein-Kaminietsky et al., 2014). Importantly, some studies using varying models have shown that group-housing mediates the severity of outcomes such as stroke morbidity and Alzheimer symptomology while also normalizing inflammatory responses (Karelina et al, 2009; Iseri et al., 2010; Huang et al., 2015). The presence of CB1Rs on noradrenergic cells is necessary for stress-induced impairment of the NOR task (Busquets-Garcia et al., 2016), thereby indicating a role of cannabinoids in this pathway. In our previous study, the ability of THC to mediate object discrimination may have hinged on the presence of isolation housing, injection stress, and neuroinflammatory response. If alterations in neuroinflammation are critical for THC impairment or rescue, and pair-housing mediates neuroinflammation, then our current findings in males and females administered vehicle and THC would be expected. To examine the role of housing and inflammatory processes, the same study could be completed with paired and single-housed mice. Using lipopolysaccharide (LPS), the inflammatory response pathways could be activated prior to the NOR task and/or tissue collection to evaluate whether a history of cannabinoid

treatment and isolation housing differentially affects behavior and inflammatory cascades following systemic insult.

A major unexpected finding is the significant impairment in females administered THC+CBD in adulthood. The hypothalamic-pituitary-adrenal axis (HPA-axis) both influences and is influenced by the hypothalamo-pituitary-gonadal axis (HPG-axis) via cross-talk of sex steroids and glucocorticoids (Viau, 2002). Stress disrupts function of both the HPA- and HPG-axis, in part by blocking sex steroid synthesis and release (Viau, 2002; Lee & Sawa, 2014). Differing levels of testosterone and estrogen in males and females also plays a role in stress response. Intrinsic differences in hormone levels exist from animal to animal (Viau, 2002), which may mediate the individual effects of cannabinoids and stress, thereby contributing to the large amounts of variance seen in the novel object task even following a period of no injection (**Fig. 12**). Overall, disruptions induced by acute stress are lesser in adult males, in part due to the ability of testosterone to mediate neurochemical and behavioral markers of stress response (Viau, 2002; Fenchel et al., 2015). Chronic stress may disrupt normal processes of the HPA-axis resulting in neuropsychiatric disorders (Lee & Sawa, 2014), potentially through interaction with the HPG-axis. Females have been shown to be resistant to the effects of repeated restraint stress on electrophysiological activity, receptor expression, and dendritic morphology (McLaughlin et al., 2009; Wei et al., 2014; but see Garrett & Wellman, 2009). However, the lack of physiological changes may actually reflect an abnormal response wherein cellular compensation does not occur following stress, thereby impairing long-term recovery. Bollinger et al. (2016) provide some evidence of impaired long-term recovery in females by demonstrating a suppression of microglial activity following chronic stress, whereas males show normalized levels. This sex difference reinforces the role of inflammatory processes in stress as well as their potential to influence cellular and behavioral responses to cannabinoids.

HPA- by HPG-axis may also contribute to the age differences observed herein. Based on the knowledge that sexual- and HPA-axis development occurs during the adolescent period, as well as the adolescent HPA-axis being hypersensitive to stress (Burke & Miczek, 2014), it may be expected that the current study would demonstrate acute and long-lasting effects of cannabinoids in mice treated during adolescence. As discussed

previously, in adolescent males acute THC rescues impairment in object discrimination following a vehicle injection, possibly due to injection stress. Acute vehicle does not obstruct object discrimination in adolescent females or adults of either sex, which may be indicative of an interaction of HPA-axis development and levels of testosterone (**Fig. 6**). Yet, object discrimination following a period with no drug is not affected by whether injections occurred during adolescence or adulthood in males. In females, mice with an adolescent history of vehicle injections appear to have impaired object discrimination compared to their adult-treated counterparts, and cannabinoid treatment during adulthood alters this relationship (**Fig. 12E**).

The step-wise impairment induced by cannabinoids in adult-treated females may be due to changes in estrogen and the 5HT1a receptor system induced by the effects of chronic stress on a fully-developed HPA-axis (Toufexis et al., 2014). Females treated with vehicle in adulthood show significant novel object discrimination which is not present in their counterparts treated with CBD, and is significantly reduced in those treated with THC+CBD. As CBD is known to exert effects via action on the 5HT1a receptor system (e.g. Russo et al., 2005; Campos et al., 2012), it may be expected that alterations in this developed system due to stress, steroid response, and repeated drug treatment may result in long-term impairment. Addition of THC may reduce the metabolic rate of CBD (Stout & Cinimo, 2014), thereby increasing the time of action of CBD at 5HT1a receptors.

5HT1a plays an important role in hippocampal memory tasks. Postsynaptic 5HT1a receptor activation in areas such as the hippocampus results in facilitation of serotonin transmission, whereas heteroreceptor activation in the raphe nucleus results in suppression of serotonin transmission (Glikmann-Johnston et al., 2015). Although 5HT1a receptors are well-documented as undergoing rapid changes in early postnatal development, it is unclear whether functional pre- and postsynaptic differences persist into the developmental period investigated in the current study (Altieri et al., 2013), although it is known that estrogen decreases heteroreceptor and increases postsynaptic receptor expression, but stress reduces estrogen release in fully-developed females (Toufexis et al., 2014). As such, it may be speculated that repeated stress in adulthood increases hetero- and decreases postsynaptic receptor expression, shifting the system

towards one of increased serotonin transmission suppression when 5HT1a receptors are activated by CBD (**Fig. 17**). Conversely, the adolescent brain may be undergoing rapid developments in this system, which makes it less susceptible to long-term consequences of repeated exposure. The role of 5HT1a receptors in this phenomena could be investigated using pharmacological or neurochemical techniques such as concurrently administering the 5HT1a antagonist WAY-100,135, employing conditional knockout of 5HT1a receptors over the course of cannabinoid treatment, using electrophysiological techniques to observe whether cannabinoid administration alters the electrical activity of neurons containing hetero- or postsynaptic 5HT1a receptors, and using *in situ* hybridization to quantify functional binding of these receptors.

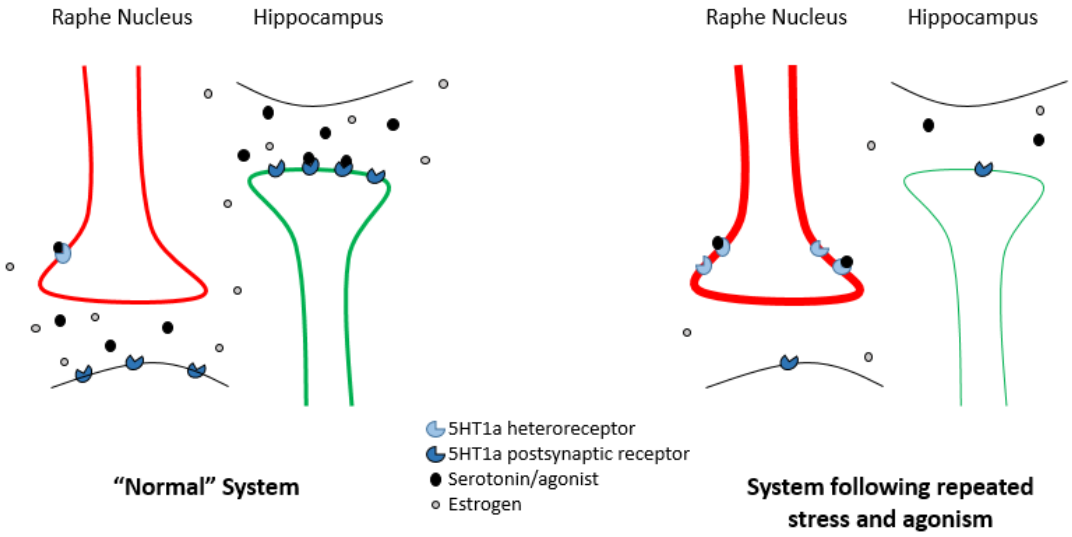

Figure 17 depicts the proposed mechanism of loss of object memory following exposure to CBD and THC+CBD during adulthood in females. Estrogen, which is decreased following repeated stress, mediates the expression of 5HT1a receptors. Following stress, expression of 5HT1a heteroreceptors is increased, leading to suppression of serotonergic activity. Conversely, postsynaptic expression of 5HT1a receptors in the hippocampus, which play an important role in memory, is decreased. 5HT1a receptors are a binding site of CBD.

The intersection of cannabinoids, stress, and sex is especially important for considering the effects of long-term cannabinoid use. These results detail a sex-dependent effect on object recognition, potentially due to the role of CB1Rs located on noradrenergic neurons in memory (Busquets-Garcia et al., 2016), and how the serotonergic receptor system mediates stress via the noradrenergic system (Leonard,

2005). This result may indicate that adult females are more sensitive to the adverse effects of cannabinoid use. Of particular interest is the small increased risk of developing a depressive disorder associated with cannabis use (National Academies of Sciences, 2017). Homozygosity of the G-1019 allele, located on the 5HT1a functional promotor, is associated with increased incidences of major depression, treatment-resistance to antidepressants, and increased binding potential (Lemonde et al., 2003; 2004; Parsey et al., 2006). The brains of individuals with major depression who committed suicide have increased levels of 5HT1a autoreceptors compared to individuals without a psychiatric diagnosis who did not commit suicide (Stockmeier et al., 1998). The proposed mechanism of impaired object memory following THC+CBD in the current study is an increase of 5HT1a autoreceptors in females treated during adulthood (**Figure 17**). Females are diagnosed with major depression at twice the rates of males and also have higher rates of seasonal, anxious, and atypical depressions (Grigoriadis & Robinson, 2007). This gender disparity may be heightened by use of cannabinoids including CBD, which may alter serotonergic function leading to increased rates or severity of depressive disorders. Use of CBD by females may also contribute to development of other disorders that are associated with decreased 5HT1a binding, such as panic and social anxiety disorders (Martin et al., 2009). As such, CBD may not exhibit a non-psychoactive profile following repeated use in females.

4.4 Conclusion

The current studies demonstrate a comprehensive set of experiments examining age- and sex-effects of cannabinoid administration on acute and long-term behaviors. Although many significant acute actions of cannabinoids were demonstrated, there were minimal long-term effects associated with repeated drug administration across age and sex. Surprisingly, acute administration of THC+CBD resulted in behavioral deficits, potentially due to the ability of administration of two or more cannabinoids to prolong metabolism and drug availability (Klein et al., 2011; Stout & Cinimo, 2014). THC+CBD administration also resulted in the only long-lasting effect of cannabinoids, wherein females repeatedly treated in adulthood demonstrated impaired object memory. This impairment is potentially due to CBD's actions at 5HT1a receptors. Although CBD is

generally considered not to have a psychoactive profile (Pertwee, 2008), the current results indicate that females may have a different sensitivity to CBD due to its actions at 5HT1a receptors. In females, stress, hormones, and 5HT1a activation may be more likely to contribute to negative outcomes of cannabinoid usage, such as impaired cognition or increases in susceptibility for major depression.

The findings that THC+CBD resulted in increased impairment were in conflict with the hypotheses that combining THC+CBD would result in reduced impairment. Concerning medical and recreational use, this may indicate that higher concentrations of CBD with lower concentrations of THC serve to extend moderate and beneficial effects of THC administration. However, at a higher ratio, such as the 1:2 ratio used in the current studies, CBD may enhance and prolong the negative effects of THC use. A range of THC:CBD ratios should be investigated to fully understand how their pharmacological interaction affects behavior. The minimal long-lasting effects of cannabinoid injections can be positively interpreted, as they suggest that both male and female mice demonstrate a relative robustness against cannabinoid use, regardless of whether exposure occurs during adolescence or adulthood. This may indicate that cannabinoids are more suitable for long-term medical treatment and may be more appropriate as an intervention for diseases that occur during childhood. However, only eight injections were given in the current study, and the adolescent treatment regimen ended at PND45. PND45 is roughly equivalent to 18 years of age in humans (Lee & Gorzalka, 2012), which is the same period of age when self-reports of past-month cannabis use nearly triples (Azofeifa et al., 2016). Therefore, the current studies may not represent the trajectory of behavioral outcomes following actual medical or recreational cannabinoid usage.

Although the current studies suggest that a period of repeated cannabinoid administration results in minimal detrimental effects, the choice of behaviors must be considered. A recent review by the National Academies of Sciences (2017) reported that there is moderate evidence of cognitive impairment following acute cannabinoid use and limited evidence of long-lasting cognitive impairment following abstinence. There is also limited evidence of a relationship between development of non-social anxiety disorders and cannabis use, although anxiety-like and sedative responses should be monitored. Although the current behaviors were chosen based on previous literature and findings in

our own lab which suggested that cannabinoid treatment results in deficits in object memory and unconditioned anxiety, it is possible that the role cannabinoid use plays in these impairments is more limited than initially expected. One major factor that may have contributed to susceptibility is our lab's previous use of single-housing, which was changed to pair-housing for the current study. Only single doses of THC, CBD, and THC+CBD were chosen, when these behaviors may show a dose-range response that differs between age and sex. Using a different injection timeline, such as daily injections, may also result in different effects than seen in the current studies. Each of these variables may be impacted by the roles of inflammatory pathways, stress, and hormonal influence, which are not well-characterized. The use of preclinical behavioral assays that are analogs to the conditions that the National Academies of Sciences have more strongly associated with cannabinoid use - such as development of other substance use disorders, social anxiety, depressive symptomology, and psychoses – may reveal more effects than the behavioral assays chosen herein. Lab animals do not encounter the repeated insults experienced by humans, ranging from every day stress of working and raising a family to diagnosis of a severe illness. These stressors alter the other systems proposed to play a role in response to cannabinoids. Therefore, the application of these results should be cautiously interpreted.

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