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Withdrawal from THC during Adolescence: Sex Differences in Locomotor Activity and Anxiety

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Abstract

Research suggests that the use and abuse of marijuana can be especially harmful if it occurs during adolescence, a period of vast developmental changes throughout the brain. Due to the localization of cannabinoid receptors within the limbic system and the established effects of cannabinoids on emotional states and anxiety levels of rats and humans, we studied the sex- and dose-related effects of Δ^9-tetrahydrocannabinol (THC, the main psychoactive component in marijuana) on behavior and anxiety during spontaneous withdrawal. Male and female Sprague Dawley rats were administered 2, 7.5 or 15 mg/kg THC or vehicle from postnatal day 35–41 (approximating mid-adolescence in humans). Locomotor activity and anxiety-related behaviors were measured during drug administration and abstinence. THC caused significant dose-dependent locomotor depression during drug administration. Locomotor depression initially abated upon drug cessation, but re-emerged by the end of the abstinence period and was greater in female than male rats. We found sensitization to the locomotor-depressing effects of THC in middle- and high-dose rats and the subsequent development of tolerance in high-dose rats. The high dose of THC increased anxiety-like behaviors while the low dose decreased anxiety-like behaviors during drug administration, with females more sensitive to the anxiogenic effects of THC than males. During abstinence, females were again especially sensitive to the anxiogenic effects of THC. This study demonstrates sexually-dimorphic effects of THC on anxiety-related behaviors and locomotor activity during and after THC administration during adolescence. This information may be useful in the development of therapeutic approaches for the treatment of marijuana withdrawal in adolescents.

Keywords

spontaneous withdrawal; tetrahydrocannabinol; sex differences; adolescence; puberty

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1. Introduction

Marijuana (cannabis sativa) use and abuse remains a serious problem worldwide [1]. Research concerning the adverse effects of marijuana is especially salient today with ongoing debates over its legalization [2] as well as increasing interest in its therapeutic potential. Δ^9-tetrahydrocannabinol (THC), the main psychoactive component in marijuana, is a ligand for pre-synaptically located, G-protein coupled CB1 receptors found primarily in the lungs, liver, kidneys and brain, and CB2 receptors found primarily in T-cells and macrophages [3]. CB1 receptor activation inhibits synaptic vesicle release and the adenylyl cyclase/PKA pathway [4;5;6;7]. By altering synaptic transmission at CB1 receptors located throughout the corticolimbic circuit [8;9;10;11] cannabinoids modulate emotion and anxiety. Exogenous cannabinoids (such as THC) lack the spatial and temporal acuity of endocannabinoids, which are released locally following excitation and then quickly degraded (see [12]). Therefore, acute cannabis use can produce altered emotional states and chronic use may disrupt the normal endocannabinoid regulation of emotion [13;14;15;16;17].

Chronic use can lead to tolerance to many of the behavioral effects of cannabinoids and this has been demonstrated in mice, rats and humans [18;19;20;21]. Following chronic use (or during drug deprivation or abstinence) withdrawal symptoms often develop [22]. However, a cannabis withdrawal syndrome has only recently been recognized [23;24;25] and there is still the common perception that cannabis withdrawal does not occur. Withdrawal in humans can occur within 24–48 hours of drug abstinence, peak within 2–6 days, and continue for 1–2 weeks depending upon the dose and length of exposure [22]. Symptoms include marijuana craving, anger and anxiety, depressed mood, loss of appetite and difficulty sleeping [22;26].

Although numerous studies have examined precipitated cannabis withdrawal by administering a CB1 receptor antagonist [27;28;29;30;31], few cannabis studies have been conducted using spontaneous withdrawal from THC. This may be due to difficulties in measuring robust withdrawal symptoms in a spontaneous withdrawal model [32;33;34], in part because of the long half-life of cannabinoids [35;36;37]. While hyperlocomotion and paw tremors have been identified as two somatic signs of precipitated withdrawal from THC [27; 30], humans do not typically undergo precipitated withdrawal. It may be difficult to extrapolate precipitated withdrawal results to the human population because the actions, pharmacokinetics and pharmacodynamics of the cannabinoid antagonist must be taken into account along with the effects of chronic exposure to the agonist.

Furthermore, the majority of pre-clinical research is conducted in adult male subjects despite research suggesting that there is increased vulnerability in the adolescent and/or female population. Past studies have demonstrated that an earlier age of onset of cannabis use, during mid-adolescence and especially during puberty, is associated with increased impairment including greater psychosis and cognitive and attentional deficits later in life [38;39;40;41]. Increased marijuana use during puberty may be especially damaging because the endogenous cannabinoid system is undergoing an array of developmental changes during adolescence that might make the brain vulnerable to developmental disruption by THC [42;43;44]. Additionally, there have been sex differences reported in cannabis research that

warrant further investigation (see [45]). For example, sex differences have been reported in the pharmacokinetics and pharmacodynamics of the drug and in its cognitive and behavioral effects; yet data on the sexually dimorphic effects of cannabinoids on anxiety and emotions during withdrawal is generally lacking (except see Rubino and Parolaro [46] for review).

In the present study, we address these issues by examining the sexually dimorphic effects of a low, middle or high dose of THC, the primary psychoactive component of marijuana as consumed by adolescents, on behavior and anxiety during drug administration and during spontaneous withdrawal during the periadolescent period.

2. Materials and Methods

2.1.1 Subjects

Experiment 1 and 2 subjects were male and female Sprague Dawley rats (Charles River, Wilmington, MA) housed in a 12-hour reverse light/dark cycle (lights out at 11am) with *ad libitum* access to food and water. Pups arrived in natural litters on postnatal day (PND) 14–15, were weaned on PND 21 and then housed in same-sex pairs. For each litter, THC was randomly assigned such that a portion of the rats received one of three doses of THC, or vehicle. Approximately 1/3-1/2 of the males from each litter and 1/3-1/2 of the females from each litter were assigned to the control group; the remaining rats were split between two of the three doses of THC. Behavioral testing occurred during the drug administration period and within 2 weeks after the last administration of THC, referred to as the drug abstinence period. Body weights were measured daily during drug administration before IP injection, and before all behavioral testing.

2.1.2 Treatment

Rats were dosed once daily via IP injection with either a low (2 mg/kg), middle (7.5 mg/kg), or a high (15 mg/kg) dose of Δ^9-THC (Research Triangle Park, North Carolina) in pluronic acid (Sigma-Aldrich, Inc., St. Louis, MO) /saline, or vehicle from PND 35–41, a period approximating mid-adolescence in humans. The low dose of THC (2 mg/kg) was selected for use because this dose had previously been shown to produce effects on locomotor activity [47], but does not produce catalepsy, as is demonstrated by Wiley *et al*, [48]. Additionally, previous research conducted in our laboratory (see supplemental material) indicated peak plasma levels after a chronic dose of 2 mg/kg THC during adolescence to be 50–100 ng/mL in females, and 40–60 ng/mL in males. Alternatively, human THC levels peak at about 140 ng/mL after inhalation of what is a relatively low dose of 34 mg marijuana; heavy chronic users may use several hundred mg per day (see [36]). Therefore, we can conclude that our dose of 2 mg/kg is indeed a low dose. The high dose of THC (15 mg/kg) was selected for use based upon the work of Wiley *et al*. [48] providing indication that this dose would produce effects different from those seen at 2 and 7.5 mg/kg THC, allowing us to successfully characterize a doseresponse curve. Additionally, spontaneous withdrawal from 15 mg/kg THC (administered twice daily for 6.5 days) was sufficient to cause alterations in neuronal firing rate similar to those found during precipitated withdrawal [27]. For the EPM study (Exp 2), the middle dose was excluded because we saw the greatest effects at the low and high doses and wanted to minimize animal usage.

2.1.3 Experiment 1: Locomotor Activity and Time in the Center of the Accuscan Box

Locomotor activity was assessed using Accuscan equipment consisting of clear Plexiglas boxes (42 × 42 × 30 cm with no bedding) placed inside Versamax activity monitors (VMRXYZ16; Accuscan Instruments, Columbus, OH) that recorded the interruption of photobeams. Rats were allowed to acclimate to the testing room for at least 1 hour before the start of the trial. Both the testing room and Accuscan chambers were kept in near-darkness, with only red light illuminating the room. Animals were placed into the chambers immediately after injection with 2, 7.5 or 15 mg/kg THC or vehicle and movement was recorded for a total of one hour. Chambers were cleaned with a 30% ethanol solution after each trial. Total distance traveled in centimeters was calculated as a measure of locomotor activity, and time in the center of the box (greater than 2.5 cm from the wall) in seconds was calculated as a correlate for anxiety level. Percentage of total time spent in the center of the box was calculated with the following formula: (total time spent in the center of the box/ total time spent in the box) × 100. In rats, it has been shown that spending more time in the center of an open field is a behavior indicative of less anxiety (see [49]); therefore we used time spent in the center of the Accuscan box as an indicator of level of anxiety. Behaviors were assessed on days 35, 38 and 41 during the drug administration period and on days 42, 44, 47, 50, 53 and 56 during drug abstinence (Figure 1). Rats originated from 9 litters. Approximately 10 rats per litter were used, for a total of 88 rats (two rats were omitted from the study). 10 male and 10 female rats were given 2 mg/kg THC, 10 male and 10 female rats were given 7.5 mg/kg THC, 14 male and 14 female rats were given 15 mg/kg THC and 10 male and 10 female rats were given the vehicle. Rats were re-tested on each testing day of the drug administration/drug abstinence period in the same Accuscan recording chamber.

2.1.4 Experiment 2: Elevated Plus Maze

Elevated plus maze (EPM) equipment consisted of a black Plexiglas plus-shaped maze, elevated 55.9 cm from the floor. The maze had 2 closed arms and 2 open arms, each 50.8 cm long. The closed arms were enclosed on three sides by black Plexiglas walls 43.2 cm high, while the open arms were partially enclosed for 22.9 cm with a 6.4 cm high rail, and then continued without a rail for the remaining 27.9 cm. This design allows for analysis of time spent beyond the rail of the open arm vs. enclosed only by the short rail (combined in the present study due to lack of statistically significant difference), and has previously used by Guilinello and Smith [50] and Guilinello *et al.* [51]. At the intersection of the open and closed arms was the ‘center’ of the maze, a square 10.2 cm by 10.2 cm. Each arm of the maze was delineated by 3 grid lines. The test was conducted in near-darkness, with only red light illuminating the room.

Rats were allowed to acclimate to the testing room for at least 1 hour prior to the start of the test, and were kept in home cages until 10 seconds before the start of the trial session when they were placed in the center of the maze facing away from the experimenter. Each trial lasted for a total of 5 minutes. Entrance into an arm of the maze was counted if the rat crossed the grid line into the arm with all 4 legs, while entrance out of an arm was counted if the rat crossed the grid line out of the arm with 2 legs. The maze was cleaned between trials with a 30% ethanol solution. Rats were tested once on the EPM either on PND 42, 44 or on 56 (Figure 1).

Percentage of time spent in the open arm of the maze, the total number of head dips (unprotected + protected head dips) and the number of stretch-attend postures were calculated as correlates of anxiety level. An unprotected head dip occurred when the rat dipped its head towards the ground over the un-railed portion of the open arm, whereas a protected head dip occurred when the rat dipped its head over the side of the railed portion of the open arm. A stretch-attend posture occurred when the rats' hind legs were motionless but the rat stretched its body forward. Head dipping, an exploratory open arm behavior suggests less anxiety, while a stretch-attend posture, a risk –assessment behavior, suggests the animal is more anxious [52;53]. The number of grid crossings was calculated as a measure of locomotor activity. Additionally, each session was recorded on videotape for later analysis of somatic signs of withdrawal such as wet dog shakes and forepaw tremors. Rats originated from 29 litters (each with 10 pups), with a total of 81 male and 86 female rats assessed for plus maze behaviors. 26 female and 22 male rats were given the vehicle, 27 female and 23 male rats were given 2 mg/kg THC, and 27 female and 26 male rats were given 15 mg/kg THC and included in the analysis.

All EPM behavioral testing took place between the hours of 12 and 3:30pm in a darkened room, in order to control for time of day effects reported for HPA axis responses on stress [54] and maximize time on the open arm for all groups. Estrous cycle was determined via vaginal cytology post-experiment.

3. Statistical Analysis

Data were analyzed by SAS Statistical Software, v. 9.2 (SAS Institute Inc., Cary, NC) and Systat Statistical Software, v. 13. Since this was a split litter design, litter is the statistical unit. For all tests, a significance level of $p<0.05$ was used; $p<0.1$ was considered a trend towards significance.

3.1.1 Total Distance Traveled Analysis

A mixed linear model was constructed with distance traveled as the dependent variable (data were power-transformed to stabilize variance). Two separate analyses were done: one for PND 35, 38, and 41; one for PND 42, 44, 47, 50, 53, and 56. Fixed factors were day, sex, dose and their mutual interactions. Litter was a random factor. A heterogeneous compound symmetry structure (allowing for unequal variance amongst samples) was used to model intra-subject correlation and heteroskedasticity (occurring when variables have unequal variances) across observation days. Due to unequal variances and missing data, Satterthwaite corrections to denominator degrees of freedom were applied; however, as data were essentially balanced, corrections were minimal. Model residuals were inspected for skew and for outliers.

3.1.2 Time in the Center of the Box and Elevated Plus Maze Analysis

For all other experiments missing data were minimal; therefore, an analysis of variance (ANOVA) was used followed by post-hoc Dunnett's tests to compare the behavior of THC-treated rats to controls. Post-hoc t-tests were also conduced where appropriate, to compare male and female rats to each other and across age. Dependent variables were the individual

behaviors being assessed. Fixed factors were day of testing, sex, dose and their mutual interactions. Litter was a random factor. Model residuals were inspected for skew and for outliers.

4. Results

4.1.1 Experiment 1a: Locomotor Activity

Total distance traveled in the Accuscan box differed significantly in a dose-dependent manner during the drug administration period and in a sex- and dose-dependent manner during the drug abstinence period. Locomotor activity was assessed in 12 5-minute time blocks and combined to determine the mean locomotor activity across the hour for each subject.

4.1.2 Locomotor Activity During the Drug Administration Period

Locomotor activity was depressed in a dose-dependent manner by THC, and remained depressed in both male and female THC-treated rats compared to controls across the entire hour of testing (data not shown).

Figure 2 shows the mean total distance traveled for the testing hour collapsed across sex during drug administration. ANOVA revealed a significant main effect of dose ($F[3,78]=45.96$, $p<0.001$) and test day ($F[2,120]=8.61$, $p<0.001$). There was also a significant dose by day interaction ($F[6,135]=10.32$, $p<0.001$). There were no significant litter effects ($Z=0$, $p=1.000$). That is, litter did not significantly contribute to the variance of the dependent measure. There was no significant main effect of sex nor any significant sex by dose interactions. On the first day of drug administration, PND 35, only the high dose of 15 mg/kg caused significant locomotor depression compared to controls. However, on days 38 and 41, all three doses of THC caused significant locomotor depression compared to the control.

We found evidence of the development of sensitization to the locomotor-depressing effects of THC in mid- and high-dose rats. Control rat locomotor activity increased significantly between PND 35 and PND 38 (Fig. 2, #). In contrast, rats dosed with 7.5 mg/kg and 15 mg/kg THC showed a significant decrease in locomotor activity between PND 35 and 38 (Fig. 2, #). This suggests that there was sensitization to the locomotor-depressing effects of THC in mid- and high-dose rats between PND 35 and 38. There was also evidence of the subsequent development of tolerance to the locomotor-depressing effects of THC in rats dosed with 15 mg/kg. While THC initially caused a significant decrease in locomotor activity in high-dose rats from PND 35 to 38, locomotor activity of high-dose rats increased significantly from PND 38 to 41 (Fig. 2, #), while the locomotor activity of control rats did not significantly change. Therefore, although the locomotor activity levels of high-dose rats did not return to control levels, there is evidence of the development of tolerance in these rats.

4.1.3 Locomotor Activity during the Drug Abstinence Period

Within each testing session, the locomotor activity of all rats decreased (data not shown). However, the locomotor activity of THC-treated rats tended to stay depressed across the entire testing hour compared to controls. There was a sexually-dimorphic pattern of THC-induced locomotor depression (Fig. 3 and insets). Analysis of variance revealed a significant main effect of day (F[5,208]=31.96, p<0.001), a significant sex by day interaction (F[5,207]=4.82, p<0.001) and dose by day interaction (F[15,274]=3.39, p<0.001). There was also a significant main effect of sex (F[1,70]=8.31, p=0.005). There were no significant litter effects (Z=0.64, p=0.261).

Post-hoc Dunnett's tests (used to compare THC-treated rats to controls) indicated significant locomotor depression in female THC-treated rats more frequently during the drug abstinence period than in male THC-treated rats. For example, on PND 42, the first day of drug abstinence, significant locomotor depression was seen in all three THC dose groups of female rats, while there was no significant locomotor depression seen in male THC-treated rats. On PND 50 and 53, locomotor depression was seen in both the low and high dose groups of female rats; there was no significant THC-induced locomotor depression in male rats on these days. However, on PND 56 both low-and high-dose groups of male rats showed significantly reduced locomotor activity compared to controls, while female low- and high-dose rats only showed a trend towards significant locomotor depression. Collapsed across sex, there were significant differences among dose groups on PND 50 and 56 (p=0.048 and p=0.018). On PND 50, significant locomotor depression was seen in the 2 mg/kg and 15 mg/kg THC groups compared to the control, while on PND 56 all three doses of THC caused significant locomotor depression compared to the control. Activity in the control group gradually increased from PND 42 to 56.

4.2.1 Experiment 1b: Time in the Center of the Accuscan Box

Overall, for time spent in the center of the box during drug administration and drug abstinence combined, there was no significant litter effect (Z=1.26, p=0.104).

4.2.2 Time in the Center of the Box during the Drug Administration Period

Data were analyzed as the percentage of time spent in the center of the Accuscan box (Fig. 4). A two-way ANOVA revealed a significant main effect of test day (F[2,82]=7.559, p=0.001) and a significant sex by test day interaction (F[2,82]=4.410, p=0.014). There was a significant main effect of dose on PND 35 (F[3,82]=6.570, p=0.001) and a significant main effect of dose (F[3,84]=5.019, p=0.003) and sex on PND 38 (F[1,86]=7.271, p=0.009) and 41 (F[1,85]=3.976, p=0.050).

Post-hoc Dunnett's tests revealed that on PND 35, rats administered the low dose of 2 mg/kg THC spent significantly more time in the center of the box than control rats (p=0.002), indicating an anxiolytic-like effect of THC. Conversely, on PND 38, rats administered the high dose of 15 mg/kg spent significantly less time in the center of the box than control rats (p=0.014), indicating an anxiogenic-like effect of THC.

Post-hoc Dunnett's tests revealed that the effects of THC during drug administration on anxiety-like behaviors were sexually dimorphic (Fig. 4 insets). On PND 35, low-dose male rats spent significantly more time in the center of the box than controls ($p<0.05$), with no significant differences in female rats. In contrast, although on PND 38 both male and female high dose rats showed a trend towards spending less time in the center of the Accuscan box than controls, on PND 41, only female high-dose rats showed a trend towards spending less time in the center of the box than controls ($p<0.1$). These results indicate that females were especially sensitive to the anxiogenic properties of THC (on PND 41) while males were more sensitive to the anxiolytic properties (on PND 35).

4.2.3 Time in the Center of the Box during the Drug Abstinence Period

There were no significant main effects of dose from PND 42 through PND 53 of the drug abstinence period (data not shown). A two-way ANOVA revealed a significant main effect of test day ($F[5,58]=4.772$, $p<0.001$), and a significant day by dose interaction ($F[15,48]=1.785$, $p=0.036$). The percentage of time spent in the center of the box by male and female control rats during drug abstinence increased overall with age, consistent with increased exploration in rats with age [50].

However, Dunnett's tests indicated that on PND 56 (collapsed across sex), rats that had been administered the 15 mg/kg high dose spent significantly less time in the center of the box than controls ($p=0.033$), suggesting that this dose had long-term effects on anxiety-like behavior in males and females (data not shown).

4.3 Experiment 2A: Elevated Plus Maze Behaviors (Drug Abstinence Period)

4.3.1 Percentage of Time Spent in the Open Arm—Figure 5 depicts the time spent in the open arm of the EPM across the three testing days during drug abstinence. Results were analyzed according to percentage of time spent in the open arm of the EPM. There was no significant difference between time spent within the short rail of the open arm and time spent beyond the rail, therefore these were combined during analysis of time spent in the open arm. A two-way ANOVA revealed a significant main effect of sex ($F[1,150]=11.099$, $p=0.001$), a significant sex by dose interaction ($F[2, 149]=4.505$, $p=0.013$) and a significant sex by dose by day interaction ($F[4, 148]=2.555$, $p=0.042$).

Post-hoc Dunnett's tests showed that on PND 42, both female low- and high-dose groups spent significantly less time in the open arm than female controls ($p=0.001$ and $p=0.017$, respectively), suggesting an anxiogenic effect of these doses in females. In contrast to this, male high-dose rats spent significantly more time in the open arm than male controls ($p=0.007$) on PND 42, suggesting an anxiolytic effect of this dose in males. On both PND 44 and 56 there were no significant differences between male controls and male THC rats, nor female controls and female THC groups. However, in general, females spent more time on the open arm than males.

In order to determine if time spent in the open arm was correlated with stage of estrous cycle, a Pearson's correlation coefficient was calculated. Overall, no significant correlation between estrous cycle and percent time spent in the open arm was found. (Data not shown)

4.3.2 Total Number of Grid Crossings - Locomotor Activity—We found significant dose- and sex-dependent alterations in the number of grid crossings, a measure of locomotor activity on the EPM during withdrawal. On PND 42 (Fig. 6), a two-way ANOVA revealed a significant main effect of dose (F[2, 44]=5.393, p=0.008). Collapsed across sex, both the low and high dose of THC significantly decreased the number of grid crossings compared to the control. Post-hoc Dunnett's tests indicated that this effect was driven by female treated rats making significantly fewer grid crossings than female controls on PND 42 (Fig. 6A inset). On PND 44 and PND 56 there were no significant differences found in the number of grid crossings between male and female THC-treated rats and controls.

A Pearson's correlation coefficient was computed to determine if the decrease in locomotor activity (number of grid crossings) contributed to the decrease in open arm time in female THC-treated rats on PND 42. No significant correlation was found between the number of grid crossings made by female treated rats and the time spent on the open arm by female treated rats, suggesting that the decrease in open arm time was not simply due to decreased locomotor activity.

4.3.3 Total Number of Head Dips and Stretches—The total number of head dips suggested that THC had anxiolytic-like effects in males, and anxiogenic-like effects in females (Fig. 7). ANOVA indicated that there was a significant sex by dose by day interaction (F[4,138]=7.525, p<0.001). Female control rats made more head dips than male control rats on all three testing days (significant only on PND 42, p<0.01).

The only significant THC-induced alteration in the number of head dips was found on PND 42. Post-hoc Dunnett's tests revealed that female low-dose rats made significantly fewer head dips than female controls (p=0.006), and that female high-dose rats showed a trend towards making significantly fewer head dips than female controls (p=0.056), an indication of anxiogenic effects of THC in the females. Conversely, male low-dose rats made significantly more head dips than male controls (p=0.008), an anxiolytic-like effect. No dose-related effects were found on PND 44 or 56.

The only significant dose-related difference in the number of stretch-attend postures was found on PND 44, where a two-way ANOVA revealed a significant main effect of dose (F[2,51]=3.577, p=0.036) as well as a significant dose by sex interaction (F[2,51]=4.119, p=0.022). Post-hoc Dunnett's tests revealed that female low-dose rats made a greater number of stretch-attend postures than female controls (Data Not Shown).

5. Discussion

In the present study, we looked at the effects of low, middle and high doses of THC on locomotor activity and anxiety-related behaviors during drug administration and during a subsequent drug abstinence period. Our main goal was to determine the time-course of spontaneous withdrawal and to examine sex- and dose-related differences within the drug abstinence period. Results indicate that spontaneous withdrawal is substantially different from precipitated withdrawal and that measures of anxiety-related behavior support a physiological withdrawal that is not manifested in hyperlocomotion. Withdrawal is not the

same in males and females since it is associated with sexually dimorphic changes in anxiety-related behaviors.

5.1.1 Locomotor Activity during Drug Administration and Withdrawal

The Accuscan recording chamber is an open field environment enabling free exploration. In general, the locomotor activity of control rats increased with age across the drug administration period, a reflection of increased exposure to the testing environment as well as increased exploration that occurs in rats during adolescence [55]. Based upon previous results examining the effects of chronic 2 mg/kg THC on locomotor activity in early and late adolescence (PND 21–40 and 41–60; [44]), we hypothesized that THC administration would significantly reduce locomotor activity in both male and female rats. All three doses of THC caused significant dose-dependent locomotor depression during drug administration. Acute THC-induced locomotor depression has also been widely reported elsewhere using a variety of different doses of THC [56;57]. However, while the vast majority of studies report locomotor depression after cannabinoid administration, increased locomotor activity after cannabinoid administration has also been previously reported, often at low doses of THC [58]. Such hyperlocomotion may be caused by differences in experimental procedure such as species of animal used, prior handling and type of locomotor activity box in addition to differences in age of animal at testing.

The largest alterations in locomotor activity during drug abstinence were seen in female THC-treated rats. In both experiment 1 and experiment 2, female rats that had been treated with THC showed greater locomotor depression than male THC-treated rats. Few studies have examined the sex differences of chronic THC exposure on locomotor activity during a withdrawal period, especially during adolescence. However, while intracerebroventricular THC decreased locomotion in both male and female rats, only male rats showed hyperlocomotion at 4 hours following the injection [59], providing evidence that THC may affect locomotor activity differently in male and female rats. Wiley *et al.* [48] also found that both male and female rats showed significant locomotor depression 24 hours following 10 days of chronic exposure to THC. Differences in age at drug administration/testing may account for these differential effects. The averseness of the testing paradigm may also account for differences in locomotor activity between testing paradigms, as bright light (highly aversive) has been demonstrated to impact locomotor activity in CB1 receptor knockout mice [60;61;62]. In the present study, nonaversive low lighting conditions were used; locomotor activity may have been different with greater test-paradigm averseness. Importantly, we found neither signs of hyperlocomotion nor any significant head shaking, paw tremors or wet-dog shakes as is commonly seen in studies of precipitated withdrawal [27;63]. Possibly, the use of a greater dose, a more potent CB1 receptor agonist, a longer dosing period or a different level of test averseness may have produced these somatic signs of withdrawal.

The endocannabinoid system plays a crucial role in regulating motor control due to the large number of cannabinoid receptors located throughout the basal ganglia. The basal ganglia integrate sensory and decision-making information, and are involved in selecting, filtering, and initiating motor programs such as those involving locomotion [64]. Information from

the cortex is first relayed to the dorsal striatum, which either projects directly to the substantia nigra or internal globus pallidus (the direct pathway), or projects to the external globus pallidus, through the subthalamic nucleus, and then to the substantia nigra (the indirect pathway). The substantia nigra then sends inhibitory projections to the thalamus. CB1 receptors are located on glutamatergic cortical projection neurons synapsing on striatal medium spiny neurons [65;66] and on the presynaptic terminals of GABAergic striatal medium spiny neurons synapsing in the internal globus pallidus and the substantia nigra [67;68;69;70]. Increased excitation of the indirect pathway is primarily responsible for decreasing movement, while increased excitation of the direct pathway increases movement. However, CB1 receptor activation in these pathways has the opposite effect; in the indirect pathway, CB1 receptor activation can increase locomotor activity, while in the direct pathway CB1 receptor activation can decrease locomotor activity [71].

Endocannabinoids are synthesized in the basal ganglia upon depolarization of post-synaptic neurons, and act through retrograde signaling to inhibit both excitatory and inhibitory neurotransmission [72;73;74]. It is the balance of cannabinoid signaling at excitatory and inhibitory synapses in both the direct and indirect pathways that determines whether or not locomotor activity will be increased or decreased; activation of CB1 receptors in the indirect pathway can cause increased locomotor activity, while activation of CB1 receptors in the direct pathway can inhibit locomotor activity [71]. As a result, the administration of exogenous cannabinoids such as THC can heavily impact motor functioning and locomotion, as seen in the present study. Interestingly, the greatest effects on locomotor activity were seen with the low and high dose of THC, but not the middle dose. It is possible that the low dose of THC may have been sufficient to activate CB1 receptors in the indirect pathway, while the high dose interacted with CB1 receptors in the direct pathway; the middle dose may have struck a balance between the two, resulting in no significant change in locomotor activity.

No significant sex differences were found in the effects of THC on locomotor activity during the dosing period. As demonstrated by previous results [47], the locomotor system may be equally sensitive during THC administration in males and females at this age. In contrast, the sexually differential effects of THC during drug abstinence may be caused by greater CB1 receptor desensitization and down-regulation in the striatum and PFC of adolescent female rats compared to male rats after chronic THC administration [75;76], causing a potential decrease in CB1 receptor activation by endocannabinoids in female rats during drug abstinence. Further research must be conducted to determine the specific subpopulations of neurons in these areas that are potentially down-regulated.

Locomotor depression re-emerged in experiment 1 in treated rats by PND 50, and was also present on PND 56. On the other hand, there were no long term effects on grid crossings in experiment 2 in either sex. The long-term locomotor depression seen in male and female rats in Experiment 1 was an unexpected result. Pilot data from our own laboratory showed no evidence of long-term locomotor depression in rats administered 2 mg/kg THC from PND 41–60 (mid-late adolescence), and then tested in adulthood at approximately PND 75 (unpublished results). However, the difference in age of administration and length of dosing interval are most likely the cause of this discrepancy. Similarly, Amal *et al.* [77] and Rubino

et al. [75] found no evidence of locomotor depression 3–4 weeks after the last dose of THC in adult male and female rats. Since behavioral assessment in the present study was not continued past PND 56, we do not know if the locomotor depression was an isolated finding or the beginning of a trend. It may be that treated rats habituated to the activity chamber following repeated exposure more so than did the controls. Importantly, we found no effect of litter origin on locomotor activity, a factor that has previously been reported to alter behavior [78].

5.1.2 Tolerance and Sensitization during Drug Administration

We found the development of tolerance to the locomotor-depressing effects of THC in Experiment 1 rats administered the high dose of 15 mg/kg THC (males and females combined) during the latter part of the dosing period. No tolerance was seen with the low and middle doses of THC. Tolerance has previously been demonstrated in both pre-clinical and clinical settings to many of the behavioral effects of cannabinoids [19;79;80], and may be produced through receptor desensitization, internalization and down-regulation [81;82;83;84]. The low and middle doses of 2 and 7.5 mg/kg THC may not have been adequate to produce maximum CB1 receptor downregulation or internalization.

In contrast to the tolerance at high doses, we found sensitization to the locomotor-depressing effects of THC in rats administered the 7.5 mg/kg middle dose and, initially, in high-dose rats. Sensitization describes an increase in drug effect with repeated administration, and may be an important initial step in addiction [85]. Sensitization to THC has been previously demonstrated in rodents [86;87] and may be attributed in part to increased dopamine levels and dendritic arborization in the nucleus accumbens shell and core [87;88]. Sensitization may also be the result of the accumulation of THC in the brain. Due to the long half-life of THC [35], increased levels of THC or its metabolites may be present by the third day of dosing in this study (PND 38), leading to increased effects on locomotor activity.

5.1.3 Anxiety-Related Measures during Drug Administration and Withdrawal

Results from experiment 1 indicate that the low dose of 2 mg/kg THC decreased anxiety-like behaviors while the high dose of 15 mg/kg THC increased anxiety-like behaviors as assessed by center time in the Accuscan box during drug administration. These results are consistent with previous studies reporting that lower doses of THC are anxiolytic while higher doses are anxiogenic [89;90]. In humans, low doses of THC also decrease anxiety and increase ratings of ‘high’ and ‘euphoria’, while high doses produce greater anxiety, irritability and paranoia [13;14;91]. Additionally, during withdrawal, these effects were sexually differential; THC exposure increased anxiety-like behavior in females and decreased anxiety-like behavior in males on the EPM.

Interestingly, female control rats spent significantly more time in the open arm than male controls on PND 42. Female control rats also made significantly more head dips than male control rats on all three testing days. Increased time spent in the open arm and the greater number of head dips in control females most likely reflects both the increase in exploration and risk-taking with age and the earlier onset of puberty in females [55].

We examined the possibility that the changes in time spent on the open arm of the EPM were simply due to decreases in locomotor activity (the number of grid crossings). We found no significant correlation between open arm time and the number of grid crossings for female low- and high-dose rats on PND 42. Therefore, the decrease in open arm time in female THC-treated rats on PND 42 was not likely due to decreased locomotor activity.

The endocannabinoid system is essential in the regulation of stress [92;93;94] and the localization of CB1 receptors throughout the corticolimbic circuit can account for the paradoxical effects of THC administration on anxiety and emotion seen in the present study. CB1 receptors are located on both glutamatergic and GABAergic neurons within the amygdala, and activation of CB1 receptors has been shown to decrease both excitatory and inhibitory neurotransmission [95]. CB1 receptors are also located in the PFC on both GABAergic and glutamatergic cells (see [96]) and are found throughout the hippocampus [97], primarily on GABAergic synapses [98]. The PFC is important in executive control of emotional behavior, while the hippocampus is important in the consolidation and retrieval of memories with an emotional weight [3]. The amygdala makes reciprocal connections with both the PFC and the hippocampus, and it is the balance of CB1 receptor-mediated excitatory and inhibitory transmission that maintains normal levels of emotion/anxiety [91;94]. The increase and decrease in anxiety-like behaviors seen in this study during drug administration might be produced by differential corticolimbic CB1 receptor activation by THC. Furthermore, alterations in endocannabinoid levels after chronic cannabinoid administration [75;99] may account for changes in anxiety-related behaviors seen during drug abstinence. Cannabinoid withdrawal has also been associated with increased levels of corticotropin-releasing hormone in the amygdala [100;101], which is likely associated with changes in anxiety.

A key finding of the present study is that THC exposure increased anxiety-like behavior in females and decreased anxiety-like behavior in males early in withdrawal (on the EPM). CB1 receptor levels in the limbic system are modulated by estrogen in a sexually-dimorphic manner. Riebe *et al.* [102] found that CB1 receptor binding site density was reduced in the hypothalamus and increased in the amygdala of normal adult female rats and ovariectomized females receiving estradiol compared to ovariectomized female rats and male rats. Estrogens in female rats in the present study may have altered the density and distribution of CB1 receptors in the amygdala and hypothalamus, thereby altering CB1 receptor binding by THC during drug administration and by endocannabinoids during withdrawal. Further research concerning the exact population of neurons in which CB1 receptors are affected by estrogen must be conducted, for example by use of mice that are CB1 receptor-deficient in certain neuronal populations (for example, principal cells, GABAergic cells or dopamine D1-expressing neurons; as has been investigated by Monory *et al.* [103]). It has also been shown that the stage of estrous cycle can impact the endocannabinoid system [104]; however, we found no correlation between stage of the estrous cycle and behavior.

Sex differences in the pharmacokinetic and pharmacodynamic processing of THC may contribute to the increased anxiety-like behaviors in females. Previous data suggest that peak plasma levels of both Δ^9-THC and 9-Carboxy-THC, an inactive metabolite, are greater in adolescent females than males (see supplemental material). Therefore, there may be greater

residual THC in female rats compared to male rats during the drug administration and drug abstinence period, increasing anxiety-like behaviors in females. Additionally, adult female rats metabolize Δ^9-THC preferentially to 11-hydroxy-Δ^9-THC (a form equivalent or greater than Δ^9-THC in its potency), while adult male rats metabolize Δ^9-THC to a number of different compounds [105], a factor that may also contribute to the greater behavioral effects seen in females rats in the present study.

A further possibility is that increased levels of the neurosteroid, allopregnanolone, contributed to increased female sensitivity to the anxiogenic effects THC. There is evidence that THC administration can increase cortical levels of allopregnanolone in rats [106]. Shen *et al*. [107] demonstrated that increased levels of allopregnanolone in pubertal females leads to increased anxiety-like behaviors. In our own lab, we found elevated levels of allopregnanolone in female rats, control and treated rats combined, compared to male rats, control and treated rats combined (see supplemental material). While there were no treatment differences, this may have contributed to a vulnerability to greater anxiety-like behaviors in female rats compared to males in general.

It is important to note that THC was administered during puberty (typically occurring about PND 35 in female rats and PND 39 in male rats [55; 108;109;110]. Puberty is a period that has been shown to be especially sensitive to the effects of THC [40;111;112]. During the pubertal period, the endogenous cannabinoid system is undergoing an array of developmental changes; for example, the density of cannabinoid receptors in the limbic forebrain, striatum and ventral mesencephalon increases until around PND 40, and then subsequently decreases until reaching adult receptor levels by PND 60 [42]. In the present study, the THC administration period from PND 35–41 coincided with puberty in females but only partially overlapped with the start of puberty in males. This discrepancy may contribute to the sex differences seen locomotor activity and anxiety-like behavior. Importantly, puberty often occurs earlier in human female adolescents than male adolescents (see [55]). Additionally, similar developmental changes in the endocannabinoid system occur in the human brain from the prenatal period to adulthood [43].

There was some indication of long-term effects of THC on anxiety-like behavior. In experiment 1, rats administered the high dose of THC spent significantly less time in the center of the Accuscan box on PND 56, suggesting a long-term anxiogenic effect of this dose. In contrast, there were no long-term effects seen in EPM behavior on PND 56. There was no correlation between locomotor activity and time in the center of the Accuscan box on PND 56, suggesting that this anxiogenic effect was not likely due to reduced locomotion. Differences in the anxiolytic/anxiogenic properties of the two paradigms (Accuscan box and EPM) might explain these results. The EPM may have been more sensitive to changes in anxiety-related behaviors as it was a novel environment and may have produced more fear and anxiety compared to the Accuscan box since rats were repeatedly placed inside the Accuscan box over the course of the 21 day experiment.

6. Implications

The current study has several important implications for both pre-clinical and clinical research. This study has further demonstrated that the behavioral measures of precipitated withdrawal and spontaneous withdrawal are not equivalent. While precipitated withdrawal produces robust symptoms, the more subtle symptoms of spontaneous withdrawal can be measured and may be more human-relevant.

Furthermore, the present study highlights the importance of examining sex differences in THC effects. We found opposite effects in male and female rats in anxiety-related behaviors. Currently, few pre-clinical and clinical studies examine cannabinoid effects in female animals, especially in adolescence and concurrently with male animals. Future pre-clinical research is needed to further examine sex differences in chronic cannabinoid administration and subsequent withdrawal.

7. Limitations

It is important to note that while we are administering THC in our research, street marijuana consists of numerous cannabinoid compounds in addition to THC including cannabidiol and cannabigerol. These compounds have been shown to interact with each other, and with tobacco and nicotine found in blunts, to produce effects that will not occur with THC alone. For example, cannabidiol has been shown to potentiate catalepsy, hypothermia, hypoactivity and impairment in spatial memory after THC exposure [113,114]. Alternatively, cannabidiol attenuates THC-induced increases in anxiety [115,116]. Therefore, developmental exposure to cannabidiol could certainly act in conjunction with the THC found in marijuana to produce effects other than those seen in the present study. Furthermore, the pharmacokinetics of different cannabinoid compounds are not equivalent (see [36]). However, it is nearly impossible to study the interactions of all of the compounds in marijuana at one time. Additionally, in clinical studies, it is impossible to know the duration and level of prior cannabis exposure, as individuals typically over- or under-report their cannabis use. Thus, in the present study, THC is used alone for simplicity of experimental design and because it is important to examine its effects before studying marijuana containing various other cannabinoids. In addition, THC administration is a preferred model for human marijuana use compared to the administration of synthetic agonists which possess unique pharmacologic characteristics.

Anther potential limitation of this study is the shipping of the pups with dams at PND 14–15. Several rodent studies have shown that shipping is a stressor [117;118;119] and this may have impacted the rats in our study. However, as all rats were subject to the same shipping practices and individual litters were divided into vehicle and THC-injected cohorts, all rats would have experienced the same level of stress. Additionally, pups were allowed to acclimate to our animal facility for 20–21 days prior to any experimental procedures.

8. Conclusion

This study adds to the growing body of evidence demonstrating that the long-term effects of THC exposure are sexually dimorphic in adolescent rats. Furthermore, the results of the

present study indicate that behavioral measures of a spontaneous withdrawal paradigm are manifested more in changes in anxiety-like behaviors rather than in hyperlocomotion and somatic signs of withdrawal, as seen in precipitated withdrawal. Additionally, females appear to be more sensitive to increased anxiety-like behavior during the drug administration and abstinence period than males. In conclusion, the results of the present study draw attention to the importance of studying cannabinoid withdrawal in both the adolescent and female population, and may help in the development of sex-specific therapeutics for cannabinoid withdrawal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research Highlights

Withdrawal from chronic THC during adolescence in the rat

Differential effects in males and females on anxiety

Implications for treatment for marijuana withdrawal

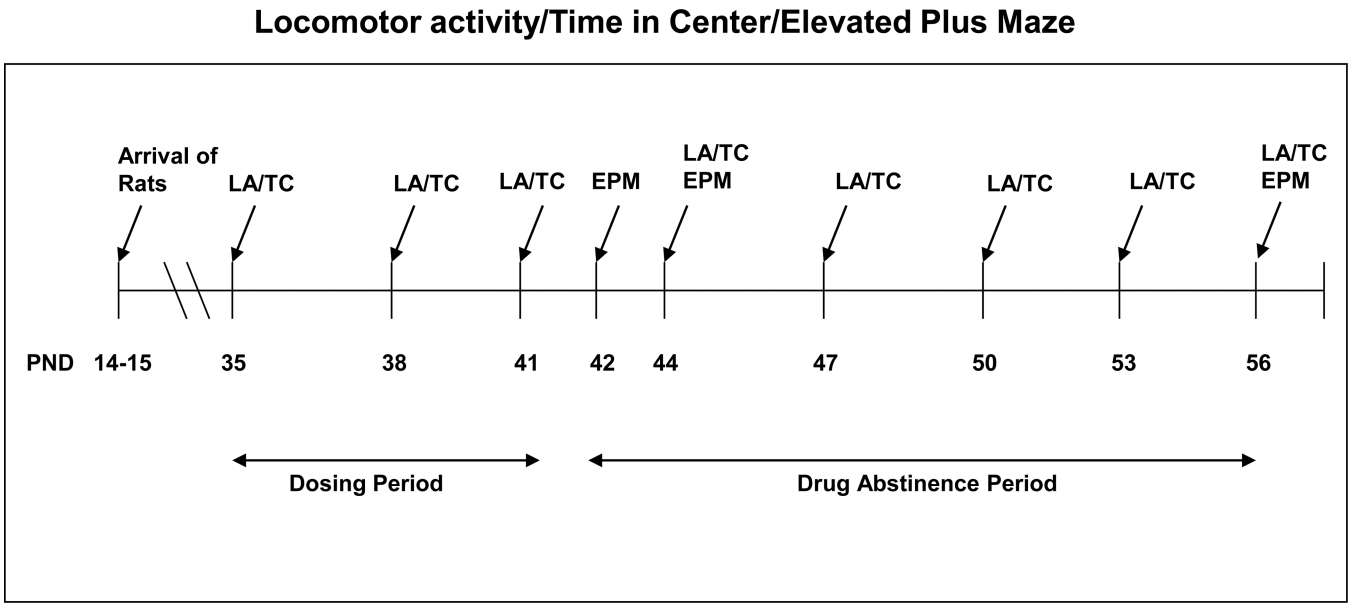

Figure 1.

Timeline of Experiments. LA/TC, locomotor activity/time in the center of the Accuscan box. EPM, elevated plus maze.

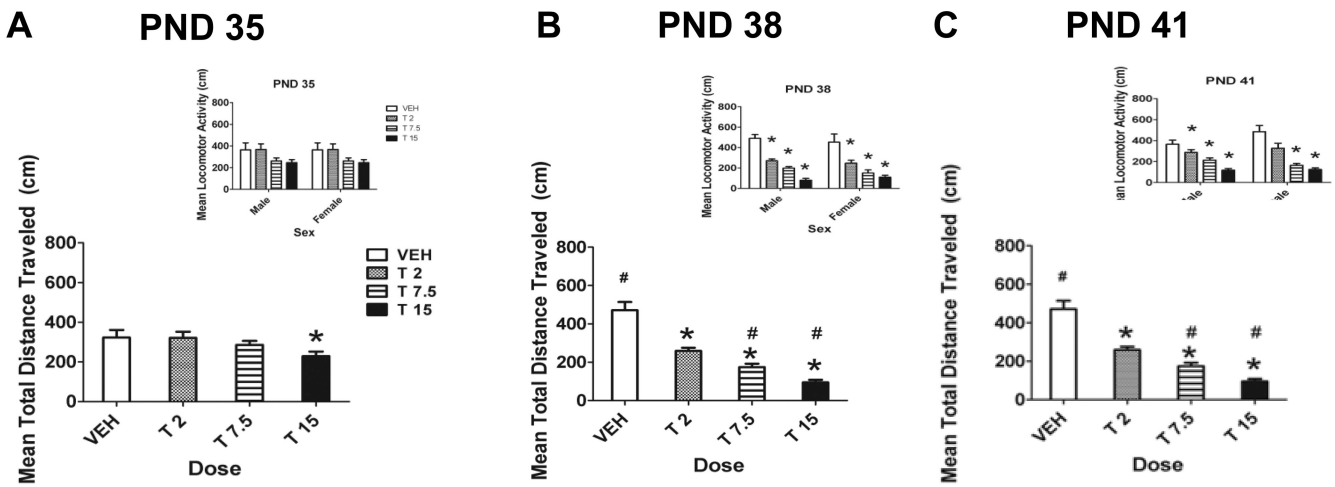

Figure 2.

Mean locomotor activity during drug administration. Total distance traveled (cm) for 1 hour collapsed across sex on PND 35 (A), PND 38 (B) and PND 41 (C) during the drug administration period. There were significant main effects of dose and test day ($p<0.05$). Insets illustrate the data by sexes.* Denotes significant difference from control and # denotes significant difference from same dose group on the previous testing day, $p<0.05$. n = 9–14/group

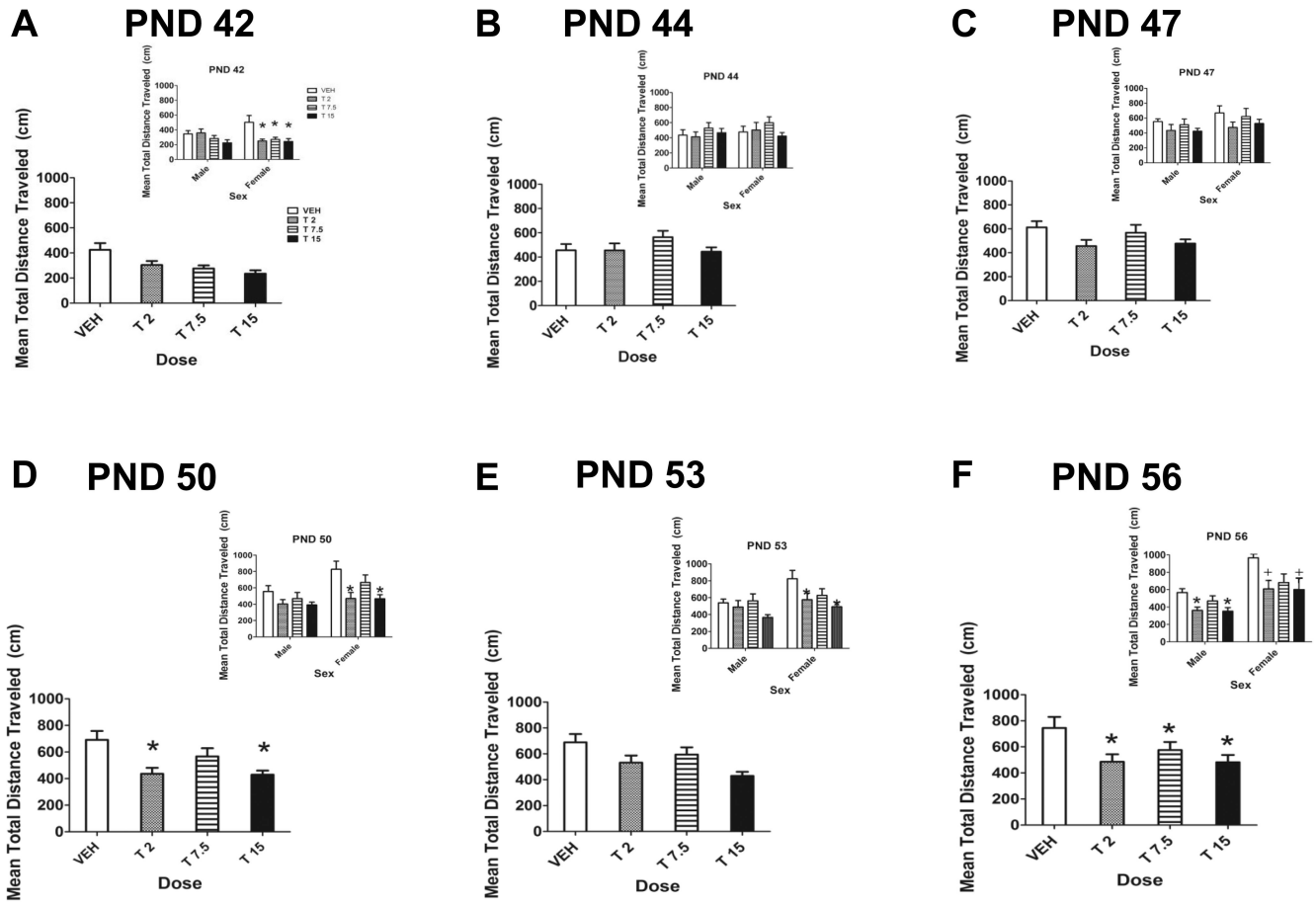

Figure 3.

Mean locomotor activity during drug abstinence. Total distance traveled (cm) for 1 hour collapsed across sex on PND 42 (A), 44 (B), 47 (C), 50 (D), 53 (E) and 56 (F). There was a main effect of dose on PND 50 and 56 ($p<0.05$). Insets depict locomotor activity of male and female rats on PND 42 (A), 44 (B), 47 (C), 50 (D), 53 (E) and 56 (F). * denotes significant difference from control (Dunnett's), $p<0.05$. + denotes a trend towards a significant difference from control, $0.05<p<0.1$. n = 9–14/group.

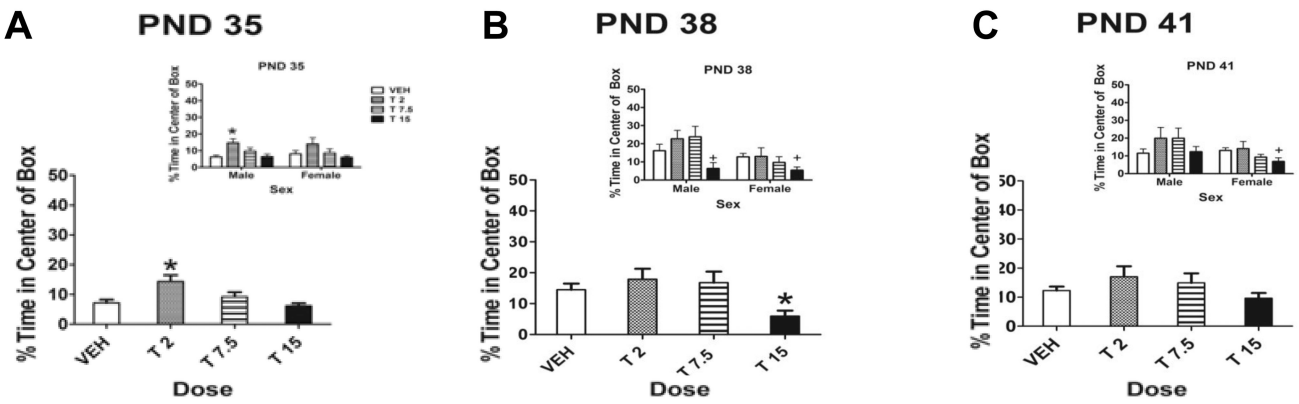

Figure 4.

Percentage of time spent in the center of the Accuscan box. Data are for male and female rats (insets) and collapsed across sex on PND 35 (A), PND 38 (B) and PND 41 (C) during the dosing period. There was a significant main effect of dose on PND 35, a significant main effect of dose and sex on PND 38 and a significant main effect of sex on PND 41 ($p<0.05$). Insets depict percentage of time spent in the center of the box in male and female rats on PND 35 (A), PND 38 (B) and PND 41 (C). Note that the maximum of the y-axis is 50%. * denotes significant difference from controls, $p<0.05$. + indicates trend towards significant difference from control, $0.05< p<0.10$. n = 9–14/group

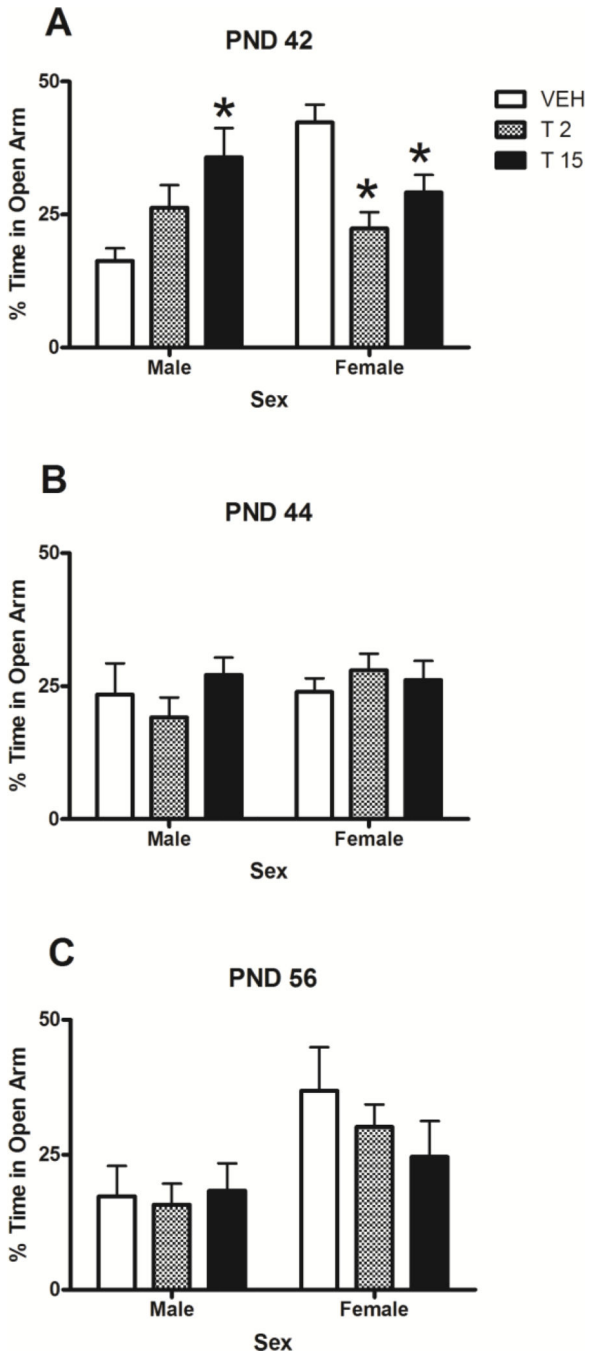

Figure 5.

Percentage of time spent in the open arm of the EPM during drug abstinence on PND 42, 44 and 56. On PND 42, there was a significant sex by dose interaction ($p<0.05$). Note that the scale is out of 50%. * denotes significant difference from same-sex control and # denotes significant difference from male control ($p<0.05$). n = 7–9/group.

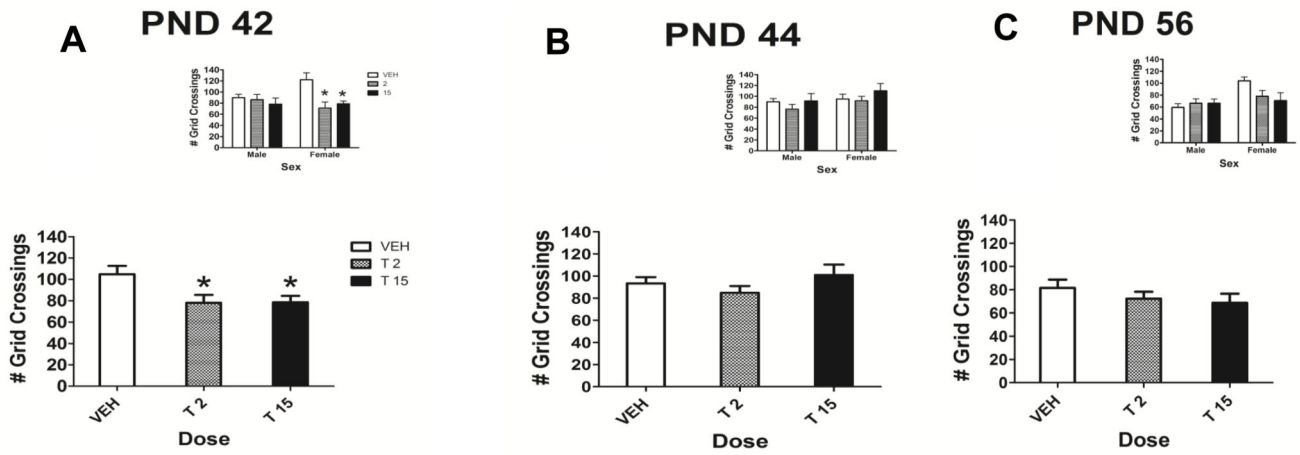

Figure 6.

Total number of grid crossings during drug abstinence collapsed across sex on PND 42 (A), PND 44 (B) and PND 56 (C), the spontaneous withdrawal period. On PND 42, there was a main effect of dose, ($p<0.05$). Insets depict total number of grid crossings on PND 42, 44 and 56 for male and female rats. * denotes significant difference from control, $p<0.05$. n = 7–9/group.

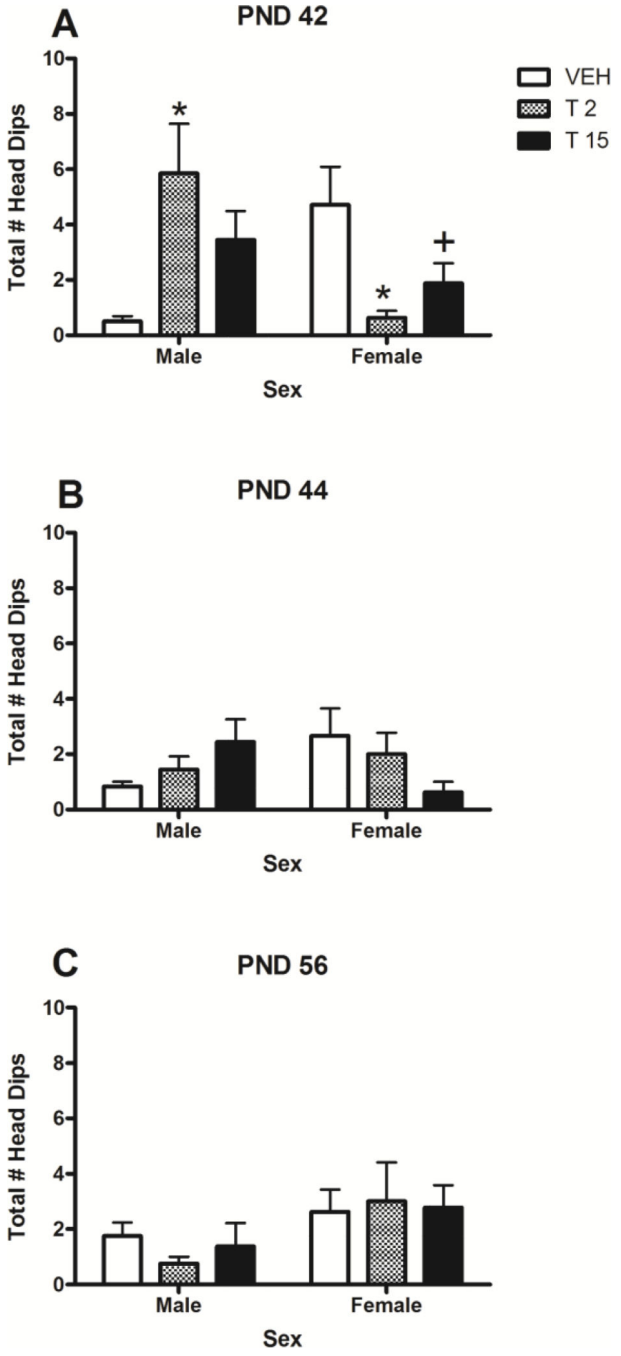

Figure 7.

Total number of head dips for male and female rats on the elevated plus maze on PND 42 (A), PND 44 (B) and PND 56 (C). There was a significant sex by dose by day interaction on PND 42 ($p<0.05$). * denotes significant difference from controls, $p<0.05$. + denotes trend towards a significant difference from controls, $p<0.10$. n = 7–9/group.