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Ovarian hormones and chronic administration during adolescence modify the discriminative stimulus effects of delta-9-tetrahydrocannabinol (Δ^9 -THC) in adult female rats

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Abstract

Marijuana abuse during adolescence may alter its abuse liability during adulthood by modifying the interoceptive (discriminative) stimuli produced, especially in females due to an interaction with ovarian hormones. To examine this possibility, either gonadally intact or ovariectomized (OVX) female rats received 40 intraperitoneal injections of saline or 5.6 mg/kg of Δ^9 -THC daily during adolescence, yielding 4 experimental groups (intact/saline, intact/ Δ^9 -THC, OVX/saline, and OVX/ Δ^9 -THC). These groups were then trained to discriminate Δ^9 -THC (0.32–3.2 mg/kg) from saline under a fixed-ratio (FR) 20 schedule of food presentation. After a training dose was established for the subjects in each group, varying doses of Δ^9 -THC were substituted for the training dose to obtain dose-effect (generalization) curves for drug-lever responding and response rate. The results showed that: 1) the OVX/saline group had a substantially higher mean response rate under control conditions than the other three groups, 2) both OVX groups had higher percentages of THC-lever responding than the intact groups at doses of Δ^9 -THC lower than the training dose, and 3) the OVX/ Δ^9 -THC group was significantly less sensitive to the rate-decreasing effects of Δ^9 -THC compared to other groups. Furthermore, at sacrifice, western blot analyses indicated that chronic Δ^9 -THC in OVX and intact females decreased cannabinoid type-1 receptor (CB1R) levels in striatum, and decreased phosphorylation of cyclic adenosine monophosphate response element binding protein (p-CREB) in intact and OVX females in hippocampus. In contrast to hippocampus, chronic Δ^9 -THC only decreased p-CREB in the OVX group in striatum. Extracellular signal-regulated kinase (ERK) was not significantly affected by either hormone status or chronic Δ^9 -THC. In summary, these data in female rats suggest that cannabinoid abuse by adolescent human females could alter their subsequent responsiveness to cannabinoids as adults and have serious consequences for brain development.

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1. Introduction

Experience with the effects of marijuana during adolescence might contribute to its abuse in adults by permanently altering the perception or composition of the interoceptive stimuli produced, as these stimuli comprise the subjective experience induced by this drug. Furthermore, as stated by Holtzman (1990), “the qualitative nature of the subjective effects that a drug produces is a principal determinant of the abuse potential of that drug,” p. 193. One experimental method for investigating the subjective effects of a drug is to have a specific dose of that drug serve as discriminative stimuli for responding in a discrimination procedure (Balster and Prescott, 1992; Jarbe et al., 1989). A powerful aspect of this methodology is that once the subjective effects of a drug are conditioned to serve reliably as discriminative stimuli, the experimenter has a pharmacologically-specific behavioral model for studying the components of a drug’s actions, which generally reflect events at the neuronal level (Balster and Prescott, 1992; Holtzman, 1990).

To test the hypothesis that the discriminative stimulus effects may differ substantially between Δ^9 -THC-experienced and Δ^9 -THC-naïve individuals, we established Δ^9 -THC as a discriminative stimulus in adult female rats that had received it chronically as adolescents. By ovariectomizing these females during adolescence, the role of ovarian hormones in the long-term effects of Δ^9 -THC could also be examined as a systematic replication of the recent literature showing that ovarian hormones and cannabinoids likely interact at multiple levels (Gonzalez et al., 2000; Mize and Alper, 2000; Rodriguez et al., 1994; Winsauer et al., 2011). For example, Rodriguez et al. (1994) demonstrated that the presence or absence of sex steroids in rats differentially affected the density and/or affinity of cannabinoid receptors in distinct brain areas such as the striatum. In this area, cannabinoid receptor affinity was increased after ovariectomy (OVX), suggesting that ovarian hormones might constitutively inhibit cannabinoid binding in this area. Consistent with this possibility, 17β -estradiol administration in OVX rats was shown to significantly decrease GTP γ S binding or the coupling of cannabinoid type-1 receptors (CB1R) to signal transduction pathways in the cortex and hippocampus (Mize and Alper, 2000), and estradiol in OVX rats significantly lowered mRNA levels for CB1R in the anterior pituitary gland compared to OVX females without estradiol administration (Gonzalez et al., 2000). However, the interaction of the cannabinoids and the hormone status of a female may depend on the age of the female, duration of the chronic administration, and the brain region. As an example of this, Winsauer et al. (2011) recently demonstrated that intact female rats had higher CB1R levels in the hippocampus after chronic, adolescent, Δ^9 -THC administration than OVX females, suggesting that ovarian hormones are also capable of enhancing components of cannabinoid signaling.

In addition to addressing our hypothesis, the present study also sought to determine whether chronic Δ^9 -THC administration and OVX during adolescence affected molecular mechanisms beyond GTP γ S binding (Breivogel et al., 1999; Mize and Alper, 2000; Rubino et al., 2000; Winsauer et al., 2011). Two important targets may be the phosphorylation status or activation of cAMP response-element binding protein (CREB) and extracellular signal-regulated kinase (ERK). Chronic Δ^9 -THC, for example, has been shown to markedly attenuate the phosphorylation of CREB in hippocampus (Fan et al., 2010) and cerebellum (Casu et al., 2005) and this effect was mediated by CB1R in both areas. Furthermore, chronic Δ^9 -THC during adolescence produced a sex-dependent decrease in CREB phosphorylation (p-CREB) in the hippocampus and prefrontal cortex of adult females, but not males (Rubino et al., 2008). CREB is also important as a transcription factor for the production of brain-derived neurotrophic factor (BDNF), which is thought to play a key role in synaptic plasticity, as well as long-term potentiation (LTP) and long-term depression (LTD). ERK, on the other hand, can be activated in the hippocampus of mice after acute Δ^9 -

THC administration (Derkinderen et al., 2003) and ERK inhibition has been shown to block a Δ^9 -THC-induced conditioned place preference (Valjent et al., 2001). Finally, Rubino et al. (2006) have hypothesized that the main role of Δ^9 -THC-induced ERK phosphorylation (p-ERK) in the striatum and cerebellum (as opposed to other areas such as the hippocampus) is the modulation of trafficking proteins involved in CB1R activity. This hypothesis would also be consistent with data from Filipeanu et al. (2011) showing that the over expression of AHA1, a co-chaperone of heat shock protein (HSP) 90, enhanced CB1R levels at the plasma membrane and the effects of CB1R stimulation on ERK 1/2 phosphorylation. Thus, in female rats that have been actively discriminating Δ^9 -THC from saline, characterizing these regulators of cannabinoid signaling after chronic Δ^9 -THC could be extraordinarily helpful in unraveling the molecular mechanisms that underlie its discriminative stimulus effects.

2. Materials and Methods

2.1 Subjects

A total of 36 female Long-Evans rats, purchased in three separate cohorts of 12, served as subjects for this experiment. Each cohort was purchased from the same commercial vendor (Harlan Sprague Dawley, Indianapolis, IN) as pups and arrived at the Animal Care facility on postnatal day (PD) 21. Following their arrival, the pups were group housed and provided a standard diet of rodent chow (Rodent Diet 5001, PMI Inc. St. Louis, MO) and water *ad libitum* until PD 30 when all the subjects were either ovariectomized or underwent a sham surgery (see Figure 1). After these procedures, the subjects were individually housed in polypropylene plastic cages with hardwood chip bedding and nestlets (Ancare, Bellmore, NY) to allow for recovery. Food restriction was also instituted at this time to maintain the compatibility of the treated groups; in this case, subjects were maintained at approximately 90% of their free-feeding weights while allowing for a gain of 5 grams per week to control for normal growth.

Throughout testing, the colony room was maintained at $21 \pm 2^\circ \text{C}$ with $50 \pm 10\%$ relative humidity on a 14L:10D light/dark cycle (lights on 06:00 h, lights off 20:00 h). The subjects used in these studies were maintained in accordance with the Institutional Animal Care and Use Committee, Louisiana State University Health Sciences Center, and in compliance with the recommendations of the National Research Council in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996).

2.2 Adolescent ovariectomy

Subjects were ovariectomized while under general anesthesia and as previously described (Winsauer et al., 2011). Ovariectomy was also confirmed at the end of the study by visual inspection and uterine weight.

2.3 Adolescent administration of saline or Δ^9 -THC

From PD 35 to PD 75 (i.e., the beginning of adolescence to sexual maturity for most rats as indicated by Waynforth, 1992), both the OVX and intact (sham surgery) subjects received a single injection of either 5.6 mg/kg of Δ^9 -THC or saline intraperitoneally (i.p.) at the same time each day, yielding 4 experimental groups among each cohort of 12 with respect to hormone status and chronic Δ^9 -THC administration (i.e., intact/saline, intact/ Δ^9 -THC, OVX/saline and OVX/ Δ^9 -THC). The Δ^9 -THC was obtained from National Institute on Drug Abuse (Research Technical Branch, Rockville, MD), and prepared as described previously (Winsauer et al., 2011). When needed, aliquots of Δ^9 -THC were reconstituted for injection as an emulsion using ethanol, emulphor (Alkamuls EL-620, Rhodia, Inc., Cranbury, NJ), and saline in a proportion of 1:1:18. Saline alone rather than Δ^9 -THC vehicle was selected as the control for the chronic injections to reduce the possibility of i.p. irritation

from repeatedly administering this emulsion, although this vehicle has not been found to produce this problem (unpublished observations). The volume for both saline and Δ^9 -THC injections was 0.1 ml/100 g body weight.

2.4 Apparatus

Twelve operant test chambers (6 from MED Associates, Inc., St. Albans, VT, and 6 from BRS/Foringer, Beltsville, MD) enclosed within sound-attenuating cubicles were used to conduct the experiments. Each chamber was equipped with a houselight, pellet trough, pellet dispenser, and two response levers with stimulus lights located above each lever. White noise was present in each chamber to mask extraneous noise and a fan provided ventilation. Data were collected using MED-PC for Windows, Version IV (MED Associates, Inc., St. Albans, VT).

2.5 Training procedure

On PD 76 (beginning of adulthood), all of the treatment groups were trained to discriminate Δ^9 -THC from saline while responding under a fixed-ratio (FR) 20 schedule of food presentation. During the initial training sessions, the stimulus lights above both levers and the house light were illuminated, and a response on either lever dispensed a 45-mg food pellet (Test Diet, Richmond, IN). When subjects were responding reliably on at least one lever under this continuous reinforcement (CRF) schedule, they were then shaped to respond on only one lever per session by alternating the stimulus and lever that dispensed the pellets each session in a mixed order. The response requirement on each lever was also increased gradually during these training sessions until subjects responded under a FR-20 schedule. When responding on both levers stabilized under the FR-20 schedule, the stimuli over the levers were eliminated and injections of either saline or Δ^9 -THC were initiated. More specifically, saline or Δ^9 -THC was administered i.p. 20 min before the start of the session, which began with a 10-min timeout. The 10-min timeout period was followed by a 30-min period during which the houselight was illuminated and responding under the FR-20 schedule resulted in food presentation. Injections occurred in a fixed sequence that was repeated throughout the experiment (Δ^9 -THC [T], Saline [S], S, T, S, T, S, T, S, T, S) and each injection served as the discriminative stimulus for the appropriate lever (i.e., saline served as the discriminative stimulus for responding on one lever, whereas Δ^9 -THC served as the discriminative stimulus for responding on the other lever). For half of the rats in each adolescent-treated group, responding on the left lever resulted in food presentation following the administration of Δ^9 -THC, and responding on the right lever resulted in food presentation following the administration of saline; the lever designations were reversed for the other half of the subjects. Responding on the incorrect lever (i.e., the saline-associated lever following administration of Δ^9 -THC or the Δ^9 -THC-associated lever following administration of saline) had only one programmed consequence, which was resetting the ratio requirement on the correct lever. Training continued until subjects met two criteria on 9 of 10 consecutive days: 1) greater than 90% responding on the appropriate lever, and 2) less than 20 responses on the incorrect lever prior to the first reinforcement.

In all subjects, the initial training dose of Δ^9 -THC was 1 mg/kg; however, if this dose had an inordinately large effect on response rate, the training dose was decreased in quarter-log unit increments until a dose was reached that did not decrease the overall rate of responding. If the 1-mg/kg dose did not decrease response rate, and there was no evidence the subject was discriminating between injections of saline and Δ^9 -THC, the training dose was increased in quarter-log unit increments until the training criteria were met. In a few instances, the training dose was also increased after a subject had met the training criteria, because the training dose was not accurately discriminated under test conditions (see below).

Test sessions with other doses of Δ^9 -THC commenced after the subjects met the training criteria.

2.6 Test sessions

Test sessions were conducted similarly to daily training sessions; however, 20 responses on either lever resulted in presentation of a food pellet. Substitution tests with different doses of Δ^9 -THC were conducted during these sessions to determine dose-effect curves for Δ^9 -THC. The discriminative stimulus effects of saline and Δ^9 -THC vehicle were also assessed routinely under test conditions. After substitution tests, subjects were always required to meet the training criteria for 3 consecutive days.

2.7 Western blot analyses

When the Δ^9 -THC dose-effect curves for the subjects in each experimental group were completed, each gonadally intact female was sacrificed with one OVX female from the respective adolescent treatment groups (saline or Δ^9 -THC) when the intact member of the pair was in proestrus to control for the effects of circulating levels of endogenous hormones across the estrous cycle. After sacrifice, the brains were rapidly removed and stored at -80°C . The striatum and hippocampus were separated from the other brain areas as described by Glowinski and Iversen (1966) prior to any biochemical analyses, and the levels of CB1R, p-CREB, and pERK in these areas were determined by western blotting of brain homogenate as described by Filipeanu et al. (2004, 2011). The sources for the antibodies were as follows: CB1R was from Cayman Chemical Company (Ann Arbor, MI), p-ERK 1/2, total-ERK 1/2 and β -actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and p-CREB and total-CREB were from Cell Signaling Technology, Incorporated (Danvers, MA).

2.8 Data analyses

To determine if there were significant differences among the groups in either the training dose that was required to establish the discrimination or the number of training days, two-way ANOVA tests were conducted on these data with hormonal status and adolescent drug treatment serving as factors. The data for each session were expressed as the percentage of responses on the lever for which Δ^9 -THC was serving as a discriminative stimulus (i.e., " Δ^9 -THC-lever" responding) and overall response rate in responses per second. Group data for each variable were expressed as a grand mean and standard error of the mean (SEM) that was tabulated by averaging the mean data for each subject; however, due to individual differences in potency of Δ^9 -THC's rate-decreasing effects (particularly at the larger doses) not all doses were studied in all subjects, which led to differences in the number of subjects represented by each data point. Although this is not unusual in drug discrimination studies, differing numbers of subjects for certain drug doses can complicate the statistical analyses, particularly for repeated measures. In the present study, two-way ANOVA tests were used to analyze Δ^9 -THC-lever responding and response rate within the intact and OVX groups, with chronic treatment serving as a between subjects factor and dose of Δ^9 -THC serving as a repeated measure. In cases where there were missing values, such as for Δ^9 -THC-lever responding, the statistical software defaults to a General Linear Model of analysis whereby the sum of squares is estimated to produce a marginal sum of squares (SigmaPlot Software, SYSTAT Software, Inc. Point Richmond, CA). One advantage of this model is that it does not eliminate the assumption of a possible interaction between factors. Typically, full substitution in drug discrimination studies is considered to be 80% drug-lever responding or more, whereas a 20% increase or decrease from control is considered to be an effect on response rate. The percentage of Δ^9 -THC-lever responses was not included in the analyses when the response rate was less than 0.08 responses/sec. To further characterize and compare the dose-effect curves for Δ^9 -THC-lever responding, the doses that generated a

50% increase in Δ^9 -THC-lever responding (ED_{50} s) were estimated. The ED_{50} values were determined by linear regression using two or more data points that reflected the ascending portion of the individual dose-effect curves. Differences in the slopes of the linear regressions were also reported in tabular form (Tables 1 and 2), and both the ED_{50} values and slopes were considered significantly different from control when the mean fell outside of the 95% confidence interval for the respective intact group.

3. Results

Of the 36 subjects, 8 were excluded from the analyses as 5 had complications associated with their hormone status (e.g., partial ovariectomy), 2 were never able to successfully meet the training criterion, and 1 had to be euthanized due to illness unrelated to the experiment. For the remaining 28 subjects that comprised the 4 experimental groups, the Δ^9 -THC discrimination was successfully acquired and there were no significant main effects or significant interactions among the four groups for either the training dose that was required to establish the discrimination (hormone status: $F(1,24)=1.22$, $p=0.28$; chronic treatment: $F(1,24)=0.29$, $p=0.60$; interaction: $F(1,24)=0.27$, $p=0.61$) or the mean number of training days that were required to meet the training criterion (hormone status: $F(1,24)=0.40$, $p=0.53$; chronic treatment: $F(1,24)=0.475$, $p=0.50$; interaction: $F(1,24)=0.29$, $p=0.59$). The mean and SEM for the number of training days was 52.25 ± 9.06 for the intact/saline group, 71.43 ± 23.43 for the intact/ Δ^9 -THC group, 50.86 ± 11.42 for the OVX/saline group, and 53.17 ± 7.16 for the OVX/ Δ^9 -THC group. Table 1 shows the training doses at which the subjects from each group reliably discriminated between saline and Δ^9 -THC.

As shown in the upper left-hand panel of Figure 2, substituting increasing doses of Δ^9 -THC (0.18–5.6 mg/kg) produced dose-dependent increases in Δ^9 -THC-lever responding compared to saline substitution in all of the adolescent-treated groups; however, there were notable differences between the intact and OVX groups in Δ^9 -THC-lever responding and the overall response rate. In intact females, increasing doses of Δ^9 -THC produced a marked transition between saline-lever responding at low doses (0.18–0.32 mg/kg) and Δ^9 -THC-lever responding at high doses (0.56–5.6 mg/kg), irrespective of their adolescent treatment (Δ^9 -THC or saline). This observation was supported statistically, as there was no significant effect of chronic treatment ($F(1,85)=0.34$, $p>0.05$) and no significant interaction between chronic treatment and the doses that produced Δ^9 -THC-lever responding ($F(7,85)=0.72$, $p>0.05$). There was, however, a main effect of dose ($F(7,85)=43.33$, $p<0.001$), and post hoc analyses indicated that 0.32–5.6 mg/kg produced Δ^9 -THC-lever responding that was significantly different from Δ^9 -THC-lever responding after saline administration (i.e., control injections).

Increasing doses of Δ^9 -THC in OVX females (upper right-hand panel) also dose-dependently increased Δ^9 -THC-lever responding irrespective of their chronic treatment during adolescence (i.e., Δ^9 -THC or saline). This was also verified statistically in that there was only a main effect of dose ($F(7,73)=15.08$, $p<0.001$), with no effect of chronic treatment ($F(1,73)=0.09$, $p>0.05$) and no significant interaction between these factors ($F(7,73)=1.38$, $p>0.05$). Unlike the effects of Δ^9 -THC in the intact females, however, even the lowest dose tested (0.18 mg/kg) produced Δ^9 -THC-lever responding that was significantly different than responding after saline administration ($p<0.05$) and successive doses increased Δ^9 -THC-lever responding in a more graded manner. This difference in the dose-effect curves between hormone groups is also reflected in the significant difference in the slopes of curves, as the mean slope for the OVX/saline group fell outside of the 95% confidence interval for the intact/saline group, and the mean slope for the OVX/THC group fell outside of the 95% confidence interval for the intact/THC group (see Tables 1 and 2). No significant difference in the ED_{50} s was evident as the mean values the different groups were similar. For example,

the ED50s for the intact groups were 0.53 and 0.41 mg/kg for the saline- and Δ^9 -THC-treated groups, respectively, whereas the ED50s for the OVX groups were 0.46 and 0.39 mg/kg, respectively.

In terms of the overall response rate for the intact groups (lower left-hand panel of Figure 2), mean response rate during control (saline or vehicle substitution) sessions generally reflected mean response rate during their training sessions. The mean response rate for the intact/saline group was 1.47 ± 0.27 responses per second, whereas the mean response rate for the intact/ Δ^9 -THC group was 1.11 ± 0.15 responses per second. When compared to these mean values, increasing doses of Δ^9 -THC produced similar dose-dependent decreases in responding in both groups of intact females ($F(7,89)=15.19$, $p<0.001$), which indicates that the rate-decreasing effects of Δ^9 -THC were not altered by chronic treatment during adolescence ($F(1,89)=0.04$, $p>0.05$) and that there was no interaction between the dose of Δ^9 -THC and chronic adolescent treatment ($F(7,89)=1.87$, $p>0.05$). Further, because there was no interaction, the effects of dose were examined with the hormone groups combined and this post hoc analysis indicated that both the 3.2- and 5.6-mg/kg doses produced significant differences from the control rate of responding.

Compared to the mean response rates for the intact groups, which were similar under baseline conditions, the saline- and Δ^9 -THC-treated OVX groups had very different mean rates of responding during training sessions and after saline substitution (see lower right-hand panel of Figure 2). The mean and SEM for the OVX/saline group was 2.14 ± 0.22 responses per second, whereas the mean and SEM for the OVX/ Δ^9 -THC group was 0.97 ± 0.08 responses per second. This difference in response rate between the two OVX groups was verified statistically by a two-way ANOVA that indicated there was a significant effect of chronic treatment ($F(1,75)=10.43$, $p=0.008$), a significant effect of dose ($F(7,75)=11.86$, $p<0.001$), and a significant interaction between chronic treatment and the dose of Δ^9 -THC administered ($F(7,75)=4.09$, $p<0.001$). Furthermore, post hoc analyses indicated that the 3.2- and 5.6-mg/kg doses were significantly different from control in the OVX saline group, but none of the doses of Δ^9 -THC had an effect on response rate in the OVX/ Δ^9 -THC group.

Figure 3 shows CB1R protein levels in both hippocampus and striatum as determined by Western blot, which detected CB1R as a major band of approximately 50 kDa. When normalized to β -actin, an internal control for the overall amount of protein, significant differences in CB1R levels were not apparent among the different treatment groups in hippocampus (hormone status: $F(1,8)=3.59$, $p=0.095$; chronic treatment: $F(1,8)=2.89$, $p=0.13$; interaction: $F(1,8)=4.35$, $p=0.071$), but they were apparent in striatum. More specifically, there was a significant effect of chronic treatment ($F(1,8)=8.75$, $p=0.018$), but no significant effect of hormone status ($F(1,8)=0.28$, $p>0.05$) and no significant interaction between hormone status and chronic treatment ($F(1,8)=2.63$, $p>0.05$).

The effects of chronic adolescent treatment and hormone status on levels of p-CREB relative to the levels of total CREB (t-CREB) are shown in Figure 4. In contrast to CB1R levels, p-CREB levels in both the hippocampus and striatum were affected significantly, but in different ways. Chronic Δ^9 -THC during adolescence significantly decreased p-CREB levels in hippocampus ($F(1,8)=10.85$, $p=0.011$), whereas there was no effect of hormone status ($F(1,8)=0.6$, $p>0.05$) and no significant interaction between chronic treatment and hormone status in this area ($F(1,8)=0.02$, $p>0.05$). In striatum, there was a significant interaction between chronic adolescent treatment and hormone status ($F(1,8)=22.67$, $p<0.001$), and main effects of chronic treatment ($F(1,8)=7.84$, $p=0.02$) and hormone status ($F(1,8)=20.32$, $p=0.002$). Subsequent post hoc analyses that included a one-way ANOVA to compare the different experimental groups with the control group (intact/saline) indicated that p-CREB levels in the OVX/saline group were significantly larger than levels in the control group

($F(3,8)=16.94$, $p<0.001$); however, neither group that received chronic Δ^9 -THC had p-CREB levels that significantly differed from control.

Figure 5 shows the levels of p-ERK relative to the levels of total ERK (t-ERK) in both hippocampus and striatum where there were no significant effects of chronic treatment (hippocampus: $F(1,8)=0.00$, $p>0.05$; striatum: $F(1,8)=0.11$, $p>0.05$) or hormone status (hippocampus: $F(1,8)=3.93$, $p>0.05$; striatum: $F(1,8)=2.88$, $p>0.05$), and no significant interaction between these treatments (hippocampus: $F(1,8)=0.32$, $p>0.05$; striatum: $F(1,8)=2.53$, $p>0.05$).

4. Discussion

The main finding from the present study involving female rats was that chronic Δ^9 -THC during adolescence significantly affects both its discriminative stimulus and rate-decreasing effects for extended periods of time after the cessation of chronic administration (i.e., into adulthood). Furthermore, the effects on these two behavioral measures occurred concomitantly with effects on CB1R density and CREB phosphorylation in specific brain areas. These data complement and extend data in the literature suggesting that chronic Δ^9 -THC during adolescence produces persistent behavioral and pharmacodynamic effects (Fehr et al., 1976; O'Shea et al., 2006; Quinn et al., 2008), and data indicating that these effects may be dependent upon hormone status in female rats (Gonzalez et al., 2000; Rubino et al., 2008; Winsauer et al., 2011).

Although both Δ^9 -THC and estradiol have been shown to affect learning and memory individually in mammals (Daniel, 2006; Lichtman and Martin, 1996; Winsauer et al., 1999), there is remarkably little data regarding the interaction of these ligands (Daniel et al., 2002), and even less data on the possibility that their interactive effects may be age dependent. Because our laboratory had already investigated the interactive effects of these substances on acquisition and performance behavior (Daniel et al., 2002; Winsauer et al., 2011), this investigation focused on how they might interact to alter the discriminative stimulus effects of Δ^9 -THC. To that end, adolescent female rats in this study received the same chronic regimen of Δ^9 -THC as that used in Winsauer et al. (2011), and were trained to discriminate Δ^9 -THC from saline immediately afterward. Given that the dose used to establish a Δ^9 -THC discrimination can influence the extent to which subsequent test doses (or test drugs) generalize to the conditioned subjective effects (Jarbe et al., 1998), training dose was incorporated as a dependent variable. Another reason for using training dose as a dependent measure was that there was no substantial drug-free period after chronic administration for behavioral training as in the prior study, and females continued to receive Δ^9 -THC 2–3 times per week while acquiring the discrimination. Though allowing training dose to vary among subjects is somewhat unusual in drug discrimination procedures, there was the possibility that selecting a predetermined training dose might have overshadowed or eliminated any differences in sensitivity that resulted from the adolescent treatments. This concern was unfounded, however, as the dose of Δ^9 -THC that produced stable discrimination behavior did not differ by more than two fold in the vast majority of subjects (Table 1), and varied similarly across the treatment groups.

While dose-dependent increases in Δ^9 -THC-lever responding have been shown previously for rats (Alici and Appel, 2004; Jarbe et al., 1998; Wiley et al., 1995), pigeons (Mansbach et al., 1996), and non human primates (Gold et al., 1992; Wiley et al., 1993b; Wiley et al., 1995), an increase in Δ^9 -THC-lever responding at doses lower than the training dose in the OVX groups relative to the intact groups has not been shown previously. Typically, dose-effect functions for drug-lever responding in drug discrimination procedures are quantal in nature (Colpaert et al., 1976); that is, intermediate levels of responding (greater than 20%

and less than 80%) usually reflect differences among subjects rather than intermediate levels of responding by individual subjects. This was also true in the present study as the small, graded increases in Δ^9 -THC-lever responding in both of the OVX groups did not reflect intermediate levels of responding by individual subjects, but rather an increased variability among subjects in the lowest dose that was reliably discriminated as similar to the training dose, and in the variability produced by a given dose in those subjects. As a result, the slopes of the dose-effect curves were significantly different for the OVX and intact groups, and this difference reflected the increased variability in the discriminative stimulus effects of Δ^9 -THC in OVX female rats.

With respect to response rate, the present results were consistent with a previous study by Winsauer et al. (2011) showing that the OVX/ Δ^9 -THC group was the least sensitive to the rate-decreasing effects of Δ^9 -THC in adulthood. More specifically, 3.2 and 5.6 mg/kg decreased response rate in the intact groups and the OVX/saline group, but not in the OVX/ Δ^9 -THC group. An effect that was not observed previously was the significant difference in response rates under control conditions between the two OVX groups. In fact, in the previous adolescent study, there was a much larger difference between the mean baseline response rates for the intact groups than the OVX groups, and the two OVX groups had significantly higher response rates than the intact groups. To what extent the differences between studies can be explained by the differences in Δ^9 -THC administration (continued subchronic administration immediately after chronic administration versus an extend drug free period after chronic administration), the behavioral task (acquisition and performance versus a conditional discrimination), or differences in signaling are unknown and need to be investigated further.

There were also differences between studies in the effects of adolescent Δ^9 -THC on CB1R levels, which were surprising given that the same chronic regimen of Δ^9 -THC was used. In the present study, chronic Δ^9 -THC during adolescence did not alter adult CB1R levels in the hippocampus, but reduced them significantly regardless of hormone status in the striatum. This result contrasts with our previous study where CB1R were decreased significantly in both OVX groups in striatum and increased selectively in the intact/ Δ^9 -THC group. Interestingly, those effects were fairly consistent with the behavioral effects that were observed. In the present study, for example, the decrease in CB1R levels in the striatum could explain the lack of a rate-decreasing effect in the OVX/ Δ^9 -THC group, if a strong relationship between response rate in a discrimination procedure and striatum-mediated motor activity exists. Why the same insensitivity to Δ^9 -THC's rate-decreasing effects was not obtained in intact/ Δ^9 -THC group after CB1R levels were reduced remains to be investigated. One possibility could be that the significantly higher response rate under control conditions in this group was more susceptible to the rate-decreasing effects of Δ^9 -THC.

Unlike the effects of chronic Δ^9 -THC on CB1R levels, which were limited to the striatum, chronic Δ^9 -THC significantly decreased p-CREB in the hippocampus regardless of hormone status, and significantly interacted with hormone status in the striatum (i.e., p-CREB was increased in the OVX/saline group whereas no increase occurred in the OVX/ Δ^9 -THC group). Though acute and chronic Δ^9 -THC administration have been reported to produce different effects on p-CREB (e.g., Casu et al., 2005; Fan et al., 2010; Rubino et al., 2006), chronic Δ^9 -THC administration for 7 days or more has been shown to decrease p-CREB in the cerebellum (Casu et al., 2005) and hippocampus (Fan et al., 2010) of rodents. Δ^9 -THC has also been shown to disrupt hippocampally-mediated LTP and LTD (Fan et al., 2010; Misner and Sullivan, 1999), as well as many behavioral tasks that are mediated by the hippocampus (Deadwyler et al., 1995; Hampson and Deadwyler, 2000; Heyser et al., 1993; Lichtman et al., 1995). Moreover, antagonism studies with the CB-1 antagonist,

SR141716A, have demonstrated that most of these Δ^9 -THC-induced behavioral effects (Hampson and Deadwyler, 2000; Lichtman and Martin, 1996), and the Δ^9 -THC-induced effects on p-CREB (Fan et al., 2010), are mediated by CB1R. One of the important downstream proteins modulated by CREB-dependent transcription is the neurotrophin BDNF, which plays a key role in activity-dependent neuronal plasticity in the adult brain. Provided p-CREB served a similar role in this study, the present data suggest that chronic Δ^9 -THC may also decrease neuronal plasticity during adolescence (Rubino et al., 2009), and reciprocally implicate the decrease in p-CREB as a contributing factor.

Interestingly, the levels of striatal p-CREB in the four treatment groups closely matched the differences in baseline response rates for these groups; that is, p-CREB and the baseline response rate was significantly higher for the OVX/saline group than for the other three groups. In addition, if chronic Δ^9 -THC decreases p-CREB in certain brain areas as reported previously (Casu et al., 2005; Fan et al., 2010), this would explain why the OVX/THC group had a p-CREB level and response rate similar to intact groups. Unfortunately, the increase in p-CREB in the OVX/saline group would not be consistent with a fairly large literature showing that estradiol administration in OVX females, as opposed to OVX alone, generally increases p-CREB in many brain regions (Carlstrom et al., 2001; Fan et al., 2008; Sharma et al., 2007). Though this raises a number of interesting issues concerning the effects of adolescent OVX on CREB-dependent transcription in various brain regions, the results obtained from hippocampus and striatum do support data in the literature indicating that these areas are differentially affected by hormone status and chronic Δ^9 -THC, and that desensitization of cannabinoid signaling in these areas may have different time courses (McKinney et al., 2008; Romero et al., 1998), and initiate different signaling cascades (Rubino et al., 2006). In general, there is a consensus that decreases in CBR uncoupling or desensitization likely precede receptor downregulation, and both of these phenomenon precede changes in mRNA expression (Gonzalez et al., 2005).

Finally, unlike the levels of CB1R and p-CREB, levels of p-ERK 1/2 were not significantly affected by OVX, chronic treatment, or their interaction. This was surprising given the hypothesized role for ERK in the modulation of CB1R trafficking in the striatum and cerebellum (Derkinderen et al., 2003; Valjent et al., 2001), and its suspected role in receptor desensitization during the development of physiological and behavioral tolerance (Daigle et al., 2008; Rubino et al., 2006). Possible reasons for the lack of an effect on ERK 1/2 include the fact that chronic Δ^9 -THC had ceased much earlier in the study (i.e., during adolescence) and that tolerance to the discriminative stimulus effects of most drugs, including Δ^9 -THC, does not seem to readily develop (Wiley et al., 1993a).

In summary, chronic Δ^9 -THC during adolescence produced effects alone and in combination with ovarian hormone status that altered the discriminative stimulus of Δ^9 -THC and its rate-decreasing effects in adult female rats. These behavioral effects were also concomitant with persistent alterations in CB1R levels in the striatum, and p-CREB levels in both the striatum and hippocampus as measured by western blot analyses. No statistically significant effects were obtained for levels of p-ERK 1/2. Together, both the long-term behavioral and pharmacodynamic effects of adolescent Δ^9 -THC administration in female rats suggests that cannabinoid abuse by adolescent human females likely affects their subsequent responsiveness to cannabinoids as adults and could have serious consequences for brain development.

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Highlights

- Animal model to assess the relationship between adolescent and adult drug abuse.
- Discriminative stimuli of delta-9-tetrahydrocannabinol (THC) were assessed.
- Ovarian hormones (OVX) modified effects of chronic THC in adolescent female rats.
- Adolescent OVX and chronic THC affected the molecular protein CREB, but not ERK.
- OVX and chronic THC differentially affected the striatum and hippocampus.

Fig. 1.
Timeline of manipulations for the subjects in each experimental group.

Fig. 2.

Effects of Δ^9 -THC on the percentage of Δ^9 -THC-lever responding (upper panels) and overall response rate (lower panels) in adult female rats that received chronic Δ^9 -THC (intact/ Δ^9 -THC), ovariectomy (OVX/saline), or both (OVX/ Δ^9 -THC) during adolescence. The control group received a sham surgery and chronic saline during adolescence (intact/saline). Subjects in each group discriminated different training doses while responding under a FR-20 schedule of food reinforcement (see Table 1). Doses of Δ^9 -THC were administered i.p. 20 min before the start of the session, which began with a 10-min timeout. Data points and vertical lines above "C" in each panel represent the grand mean and SEM for each group, which was calculated from the means for 4 to 11 injections of saline or vehicle for each subject in that group. The data points and vertical lines in the dose-effect curves for each group in each panel also represent a grand mean and SEM. The grand mean and SEM for each dose was calculated from the means for 1 to 5 determinations of that dose in each subject. Numerical values in parentheses and adjacent to a data point indicate the number of subjects represented by that point when it differed from the total number of subjects for that group. Asterisks along with a bracket indicate doses of Δ^9 -THC that were significantly different from saline administration (control) for both chronically-treated groups as there was neither a main effect of chronic treatment nor an interaction. Asterisks without a bracket indicate doses of Δ^9 -THC that were significantly different from saline administration (control) for a particular group. A cross with a bracket indicates the only dependent measure for which there was a significant main effect of chronic treatment.

Fig. 3.

Panel A shows CB1R protein expression in the hippocampus and striatum of adult female rats that received chronic Δ^9 -THC (intact/ Δ^9 -THC), ovariectomy (OVX/saline), or both (OVX/ Δ^9 -THC) during adolescence. The control group received a sham surgery and chronic saline during adolescence (intact/saline). The bar graphs in panel B show the quantification of CB1R levels when they are normalized to β -actin in the hippocampus and striatum. The letters in the bar graph for striatum represent the significant ($p < 0.05$) main effect of Δ^9 -THC as indicated by a two-way ANOVA and Holm-Sidak post hoc tests (i.e., a's are significantly different from b's, but not from each other).

Fig. 4.

Panel A shows the levels of p-CREB and t-CREB in the hippocampus and striatum of adult female rats that received chronic Δ^9 -THC (intact/ Δ^9 -THC), ovariectomy (OVX/saline), or both (OVX/ Δ^9 -THC) during adolescence. The control group received a sham surgery and chronic saline during adolescence (intact/saline). The bar graphs in panel B show the quantification of p-CREB in the hippocampus and striatum as a ratio of p-CREB to t-CREB. Letters in the left-hand graph for the hippocampus represent the significant ($p < 0.05$) main effect of Δ^9 -THC as indicated by a two-way ANOVA and Holm-Sidak post hoc tests. The asterisk in the right-hand graph for the striatum represents the significant difference from control (intact/Saline), which was established by a post hoc test conducted after a significant interaction was obtained with a two-way ANOVA.

Fig. 5.

Panel A shows the levels of p-ERK 1/2 and t-ERK 1/2 in the hippocampus and striatum of adult female rats that received chronic Δ^9 -THC (intact/ Δ^9 -THC), ovariectomy (OVX/saline), or both (OVX/ Δ^9 -THC) during adolescence. The control group received a sham surgery and chronic saline during adolescence (intact/saline). The bar graphs in panel B show the quantification of p-ERK in the hippocampus and striatum as a ratio of p-ERK to t-ERK. A two-way ANOVA indicated there were no significant main effects of OVX or chronic Δ^9 -THC, and no significant interaction between these two factors.

Table 1

Training dose, as well as the ED₅₀ and slope of the dose-effect curve for the percentage of drug-lever responding, for the intact females in each chronically-treated group.

Subj.	Intact/Saline			Intact/THC			
	TD	ED ₅₀	slope	Subj.	TD	ED ₅₀	slope
595	1	0.42	99.72	584	1.8	0.24	100
597	1.8	1.8	47.78	599	1	0.43	91.47
722	1.8	0.32	49.99	609	1.8	0.62	49.65
734	1.8	0.19	56.54	723	1.8	0.35	23.75
736	1.8	0.44	28.85	738	1.8	0.42	98.97
824	1	0.29	17.61	825	1	<0.10	
836	1	0.42	99.81	840	0.56	0.42	97.74
838	1	0.32	49.95				
Mean		0.53	56.28			0.41	76.93
95% CI		0.09–0.96	31.44–81.12			0.28–0.54	42.97–110.8

Table 2

Training dose, as well as the ED₅₀ and slope of the dose-effect curve for the percentage of drug-lever responding, for the OVX females in each chronically-treated group.

OVX/Saline				OVX/THC			
Subj.	TD	ED ₅₀	slope	Subj.	TD	ED ₅₀	slope
580	1.8	<0.1		598	3.2	0.59	49.57
592	1.8	0.19	49.84	608	1.8	<0.1	
719	3.2	0.57	19.96	737	1.8	0.55	49.49
731	3.2	0.75	29.98	828	1	0.47	21.27
821	1	0.34	23.43	839	0.56	0.17	49.99
833	1	<0.1		849	1	0.18	25.22
845	1	<0.1					
Mean		0.46	30.80 [*]			0.39	39.11 [†]
95% CI		0.07–0.86	9.55–52.05			0.14–0.64	21.04–57.18

<indicates that the ED₅₀ could not be calculated because it was likely below the lowest dose tested; therefore, these subjects were not included in the mean value presented.

^{*} indicates a significant difference from the confidence interval for the control group (i.e., OVX/saline)

[†] indicates a significant difference from the confidence interval for the respective hormone condition (i.e., intact/ Δ^9 -THC)