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Adolescent cannabinoid exposure attenuates adult female sexual motivation but does not alter adulthood CB_1R expression or estrous cyclicity

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ABSTRACT

Adolescence is a developmental period characterized by neuronal remodeling and the maturation of adult emotionality, reproductive behavior and social behavior. We examined whether chronic cannabinoid exposure in adolescent rats alters female sexual motivation, estrous cyclicity, sucrose preference, and CB_1R expression in adulthood. Female rats were administered with the synthetic cannabinoid agonist, CP-55,940 (0.4 mg/kg, intraperitoneal), daily during adolescent development (PND 35–45). In a subset of subjects, socio-sexual motivation was investigated in adulthood (PND 75–86) using a runway apparatus. Estrous cyclicity was tracked in adulthood via vaginal cytology and a single-mount test. A two-bottle sucrose preference test was also conducted to determine whether predicted changes in socio-sexual motivation might be linked to alterations in hedonic processing. CB_1R expression was examined in two separate subsets of subjects, one sacrificed following drug treatment (PND 46) and one before behavioral testing (PND 74). Drug treatment significantly decreased adult preference for a male conspecific (sexual motivation), as assessed by both Run Time and Proximity Time, but did not affect estrous cyclicity or sucrose preference. CP-55,940 treatment also induced immediate, but transient, decreases in CB_1R expression in the ventromedial nucleus of the hypothalamus and amygdala. Drug treatment did not affect CB_1R expression in the nucleus accumbens (core or shell) or globus pallidus at either time point. We suggest that the endocannabinoid system may play a role in the maturation of neuroendocrine axes and adult female reproductive behavior, and that chronic exposure to cannabinoids during adolescence disrupts these neurodevelopmental processes.

1. Introduction

Cannabis is the most commonly used illicit drug among adolescents in the United States (NIDA, 2010). Researchers have only recently begun to examine the possible long-term consequences of such experimentation from a neurodevelopmental standpoint. Adolescence is a developmental period characterized by neuronal remodeling and the maturation of adult emotionality and behavior (Sisk and Zehr, 2005). A growing body of rodent research demonstrates that chronic cannabinoid exposure during the adolescent period produces a wide variety of subtle, sex-dependent neurobehavioral alterations in adulthood (Realini et al., 2009; Rubino and Parolaro, 2008; Rubino et al., 2009; Viveros et al., 2005). For example, adolescent female rats given Δ^9-tetrahydrocannibinol (THC) for 11 days display enhanced behavioral despair in the forced swim test and reduced sucrose preference as adults, indicative of depressive emotionality (Rubino et al., 2008). The current study aims to expand this literature by examining the effects of adolescent cannabinoid exposure on adult socio-sexual motivation and estrous cyclicity in female rats.

Cannabinoid exposure during critical development periods can have a profound impact on neuroendocrine function, including that of the hypothalamic–pituitary–gonadal (HPG) axis. For example, prenatal THC exposure can significantly inhibit HPG development in both male and female rats, influencing subsequent release of both pituitary and gonadal hormones (Wenger et al., 1991). Moreover, perinatal cannabinoid exposure produces sex-dependent alterations in copulatory and socio-sexual approach behaviors in adult rodents (Dalterio and Bartke, 1979; Dalterio, 1980; Navarro et al., 1996). These early observations and others led to the remarkable finding that the endocannabinoid (eCB) system is involved in many stages of neuronal development, such as differentiation, migration, axon pathfinding and synaptogenesis (Harkany et al., 2008; Ramos et al., 2005). Perinatal cannabinoid exposure produces long-term neurobehavioral effects likely by interfering with normal eCB-mediated developmental processes. There is a relative paucity of research done on the long-term behavioral effects of cannabinoid exposure during *adolescence* on neuroendocrine function. Chronic prepubertal administration of THC delays vaginal opening (Wenger et al., 1988), and hypothalamic anandamide concentrations peak immediately before the onset of

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puberty (Wenger et al., 2002), suggesting that the eCB system helps regulate the emergence of puberty.

We have recently reviewed the complex, bi-directional interactions that exist between the eCB system and the HPG axis in mature animals (López, 2010). Endocannabinoid concentrations fluctuate across the estrous cycle (Bradshaw et al., 2006; González et al., 2000) and administration of estrogens to ovariectomized rats can alter brain cannabinoid receptor (CB_1R) expression and G protein coupling (Mize and Alper, 2000; Riebe et al., 2010; Rodríguez de Fonseca et al., 1994). Furthermore, acute administration of cannabinoid agonists and antagonists has been shown to significantly affect the expression of both male and female sexual behavior (Gorzalka et al., 2010). Our laboratory has found that acute administration of the CB_1R antagonist/inverse agonist, AM251, increased incentive sexual motivation in estrous female rats (López et al., 2009), and acute administration of the CB_1R agonist, CP-55,940, dose-dependently attenuated sexual motivation in estrous females (López et al., 2010). These observations have led us to hypothesize that the eCB system helps regulate the expression of female sexual motivation across the ovarian cycle.

We suggest that chronic cannabinoid exposure during adolescence may negatively impact the maturation of the HPG axis, its interconnections with the eCB system, and associated reproductive behaviors. The current experiment was designed to assess whether adolescent cannabinoid treatment alters the expression of sexual motivation in adult female rats. Realini et al. (2009) highlights the critical need for researchers to combine behavioral and neurochemical techniques to further our understanding of the long-term consequences of chronic cannabinoid use. As such, we explored the effects of chronic CP-55,940 on behavioral, physiological, and neurochemical indices of sexual motivation. Following a period of adolescent drug exposure, we allowed our subjects to mature to adulthood and then assessed their sexual motivation using an unconditioned approach methodology (the runway apparatus), which we have used successfully in previous studies. We also examined whether drug treatment affected subsequent estrous cyclicity. Hedonic processing was examined by measuring consumption of a naturally rewarding taste (the sucrose preference test). Finally, we examined both immediate and long-term alterations in CB_1R expression in various brain regions implicated in female reproductive behavior, including the hypothalamus, amygdala, and nucleus accumbens.

2. Method

2.1. Subjects

A total of 46 female and 4 male Long–Evans rats (Charles River Laboratories, Wilmington, MA) were used. All subjects were housed in plastic tubs with woodchip bedding within a temperature-controlled (23 ± 2 °C) vivarium maintained under a reverse 12:12 light–dark schedule (lights on 22:00–10:00 h). Food and water were available *ad libitum* and all subjects' cages were environmentally enriched with a Nylabone®. All experimental protocols were approved by the campus Institutional Animal Care and Use Committee (IACUC) in compliance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals*.

Forty-four females were 35 days old at the beginning of the experiment and were housed as pairs of one drug-treated and one vehicle-treated animal (except during the sucrose preference test, when all subjects were housed individually). Subjects were handled on four separate occasions by experimenters during the one-week period between arrival and treatment. Subjects were handled one or two times a week between chronic treatment and behavioral testing.

A subset of 6 single-housed animals (2 females and 4 males) served as either Goalbox targets or copulatory partners during the behavioral testing phase of the experiment. These subjects were 75–110 days old during behavioral testing. Target females were ovariectomized at Charles River Laboratory before arrival at our vivarium and were given at least 2 weeks of post-operative recovery time before involvement in any experimental procedures.

2.2. Drugs and exposure regimen

CP-55,940 (Tocris Biosciences, Ellisville, MO) was prepared in a vehicle of physiological saline, cremophor, and ethanol (18:1:1) as previously described (Braida et al., 2004). The drug solution was stored at −10 °C and utilized within 12 days of preparation. Subjects were randomly assigned to treatment condition and received daily intraperitoneal injections (between 13:00–15:00 h) of either CP-55,940 (0.4 mg/kg) or vehicle from postnatal day (PND) 35–45. This dosage regimen has been used previously by Biscaia et al. (2003) to explore the effects of adolescent CP-55,940 exposure on adult behavior and emotionality. We have also used it in pilot studies in our own laboratory. It is a relatively high dose (compared to those used in acute administration studies), designed to significantly and lastingly impact neurological systems whose development may be dependent upon endocannabinoid activity.

2.3. Apparatus

2.3.1. Runway

Behavioral testing was performed in two identical straight-arm runways. This apparatus has been previously utilized to assess sociosexual motivation in both male (López and Ettenberg, 2000, 2001, 2002; López et al., 1999) and female rats (López et al., 2007, 2009, 2010; Nofrey et al., 2008).

Each runway was comprised of a Startbox ($25 \times 25 \times 20$ cm), an Alley ($160 \times 10 \times 20$ cm), and a cylindrical Plexiglas Goalbox (50 cm diameter × 30 cm height). Two removable Plexiglas doors were located in the Alley and restricted access to the Alley from the Goalbox and the Startbox. A removable Plexiglas partition with thirty-five 1-cm diameter holes divided the Goalbox in half. This partition prevented physical contact but allowed visual, auditory, and olfactory cues to be passed between the subject and the target.

In each runway, three infrared (IR) photocell emitter-detector sensor pairs were connected to two electronic timers. The first sensor pair was located just outside the Startbox and was triggered when the subject first entered the alley. The second sensor pair was located within the Goalbox (15 cm from the entry) and was triggered when the subject's entire body entered the Goalbox. Timer #1 measured Run Time (RT), defined as the time elapsed between the first break of the Startbox sensor pair and the first break of the Goalbox sensor pair. A third sensor pair was located in the Alley (25 cm from the Goalbox entry). The Alley sensor pair became active after the first triggering of the Goalbox sensor pair. Timer #2 began counting when the Goalbox sensor pair was first broken. Timer #2 stopped counting when the subject trigged the Alley sensor pair and resumed counting when the subject re-triggered the Goalbox sensor pair. The amount of time Timer #2 counted across the 3 minute period following the first Goalbox entry was recorded as Proximity Time (PT).

2.3.2. Sex arenas

Single mount sex tests were performed with two identical sex arenas. The arenas were large Plexiglas cylinders (50 cm diameter × 30 cm height) with a wooden floor covered in wood chip bedding.

2.4. Procedure

A timeline of the exposure regimen and experimental procedures is displayed in Fig. 1.

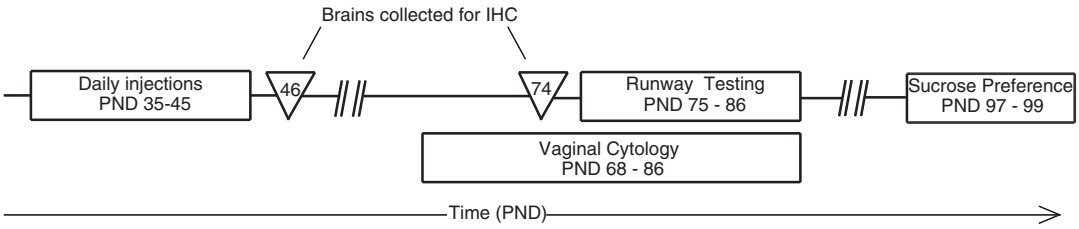

Fig. 1. A timeline of the experimental procedures.

2.4.1. Vaginal cytology

Beginning at PND 68, vaginal smears were obtained daily (between 1400 and 1800 h), as described by Maswood et al. (2008). Briefly, a cotton swab was dampened with deionized water, vaginal openings were swabbed and contents were smeared onto glass slides. Smears were obtained daily throughout runway testing (approximately 30 min after the runway trial) and cytology ended on the last day of runway testing. Proestrus was defined as smears possessing clusters of nucleated cells with some cornified epithelial cells and no leukocytes. Estrus was defined as smears with a majority of cornified cells, some nucleated cells and the absence of leukocytes. Diestrous I/II smears were defined by the presence of leukocytes.

2.4.2. Runway testing

Subjects were habituated to the runway apparatus on PND 73 and 74 (10 min each day). During habituation, all Plexiglas dividers were absent and subjects were allowed free access to all locations within the apparatus and no target was present. Behavioral testing began on PND 75. All procedures were performed under red-light illumination during the second third of the active phase of the animals' photoperiod.

Prior to initiating runway trials, the assigned target was placed within the Goalbox. The Plexiglas door separated the Alley from the Goalbox and the Goalbox partition was absent. This allowed the target to infuse the entire Goalbox area with his or her scent. After 10 min, the Goalbox partition was introduced, and the target was placed on the side of the partition farthest from the runway. The target remained in the Goalbox while all subjects were tested on a given day.

Two different nonestrous females and two intact males served as Goalbox targets. On a given test day, both runways contained targets of the same sex. Each subject ran one trial per day for 12 consecutive days. The order of target presentation across the 12 days was randomly determined, and every subject ran for each of the four targets three times across the course of testing.

At the start of an individual subject's trial, both removable Plexiglas doors were present in the runway. The subject was placed in the Goalbox on the opposite side of the partition from the target and given 2 min to investigate. The subject was then quickly transferred to the Startbox. The two doors were removed allowing the subject to move freely throughout the apparatus. Electronic timers connected to IR-sensor pairs (as described in Section 2.3.1) recorded the dependent variables. Run time (RT) is the time (sec) it takes the subject to enter the Goalbox following its first Startbox exit. Proximity time (PT) measures the amount of time (sec) the subject spends in the Goalbox in the 3 minute period after the first Goalbox entry. A low RT and a high PT are indicative of a high incentive-motivation towards the target.

At the conclusion of each trial, the subject was removed from the apparatus and returned to the vivarium. Any urine or feces in the apparatus was cleaned to prepare the apparatus for the next subject's trial. Subjects were run in the same order on each day of testing and habituation. The entire runway apparatus was cleaned with 20% ethanol after all animals were tested on a given day.

2.4.3. Single-mount sex tests

Approximately 30 min following a subject's runway trial, experimenters collected a vaginal smear to determine estrous status (see Section 2.4.1). In addition, a behavioral measure of estrus was obtained via a single-mount sex test, as described by Maswood et al. (2008). Subjects were individually placed into a sex arena with an adult, intact, sexually-experienced male. As with the runway tests, all single-mount tests were conducted under red-light illumination. Experimenters observed the receptive and proceptive behavior of the female. Specifically, receptivity was assessed by recording whether the female engaged in lordosis following a mount attempt by the male. This lordosis posture was rated on a scale from 0 to 3, with 0 being no lordosis and 3 being extreme. Subjects who exhibited lordosis at an intensity of 2 or 3 were classified as "estrous"; subjects scoring a 0 or 1 were classified as "nonestrous." Proceptivity was assessed by counting the number of hop-darts the female engaged in. Females categorized as nonestrous consistently did not engage in hop-darts. This single-mount test was ended following the first mount attempt by the male, and it should be emphasized that males never ejaculated during these tests. Experimenters were blind to the subject's experimental condition, runway performance, and vaginal smear results when rating the single-mount test.

2.4.4. Sucrose preference test

Subjects were moved to single housing on PND 88. After 1 week, subjects were habituated to the presence of a second 500 mL plastic bottle (filled with water) in their home cage. After 48 h of acclimation, one of the bottles from each cage was filled with sucrose solution (2% w/v). Fluid consumption from each bottle was measured over the next 72 h. The location of bottles was counter-balanced between groups and alternated sides in each cage every 24 h.

2.5. CB_1R immunohistochemistry

2.5.1. Preparation of tissue

Brain tissue was collected from drug and vehicle-treated animals at PND 46 and 74 (n = 6 per group) in preparation for CB_1R immunohistochemistry. Subjects were deeply anesthetized with pentobarbital sodium (150 mg/kg, intraperitoneal) and intracardially perfused with 4% paraformaldehyde/10 mM phosphate-buffered saline (PBS) at 4 °C. Brains were harvested and post-fixed in 10% sucrose/4% paraformaldehyde solution overnight at 4 °C and then were subsequently stored in 20% sucrose/PBS at 4 °C until tissue processing. Using a rotary microtome, 40 μm serial frozen sections were collected in PBS.

2.5.2. Immunohistochemical staining

Every sixth section through the nucleus accumbens and a region of the forebrain containing hypothalamus, globus pallidus and amygdala was collected and processed for free-floating immunohistochemistry. Sections were washed in PBST (0.2% Triton-X-100/PBS) three times for 10 min each. This PBST wash was repeated after every step of the protocol and PBST was also used as the immunobuffer for both the primary and secondary antibodies. Sections were then incubated with the primary antibody (rabbit anti-CB_1-L15/0.1% BSA/PBST; 1:1000) for 20 h at 4 °C. The CB_1-L15 antibody recognizes the last 15 amino acids of the C-terminus of the CB_1R and was generously supplied by Dr. Ken Mackie (Indiana University). This particular antibody has been

shown to most accurately detect endogenous CB_1R expression compared to other available CB_1R antibodies (Grimsey et al., 2008). Sections were incubated with secondary antibody (biotinylated goat anti-rabbit IgG; 1:200; Vector Labs, Burlingame, CA, USA) for 20 h at 4 °C and subsequently incubated in an avidin–biotin complex (VECTASTAIN® Elite ABC kit, Vector Labs). The reaction was developed by staining with a VIP horseradish peroxidase substrate kit for 1 min (Vector Labs) and stopped by incubating sections in water for 5 min. Sections were mounted out of PBS onto gelatin-coated slides and coverslipped with DPX mounting media.

2.5.3. Semi-quantification of immunohistochemistry

Semi-quantification of CB_1R immunoreactivity at PND 46 and PND 74 was conducted, using a slight variation of a method that has been previously described (Boger et al., 2011). Briefly, images were captured with an Olympus BX60 microscope and Olympus DP70 videocamera system coupled with DP Controller and DP Manager software running on Microsoft Windows. The staining intensity of CB_1R-ir in the ventromedial nucleus of the hypothalamus (VMH), nucleus accumbens core (AccC) and shell (AccSH), amygdala, and external segment of the globus pallidus (GP) was subsequently determined using ImageJ software (Abramoff et al., 2004) to obtain mean density values. An outline estimating each brain region of interest was created and used to obtain measurements (see Fig. 2). Mean density measurements were taken from every section in the series of tissue for each brain region (usually about 6–8 sections per region). A measurement of background staining was subtracted from these density measurements, and the subsequent results were averaged to obtain one mean density value per brain region per animal.

3. Results

3.1. Estrous cyclicity

Estrous data collected between PND 68–86 were subjected to statistical analysis. Two subjects were excluded from analysis. One animal exhibited behavioral and cytological data that were greater than 2.5 standard deviations from the mean of the entire sample. This subject exhibited lordosis on 100% of testing days and exhibited diestrous smears on 12% of testing days. The second subject was excluded because she entered pseudopregnancy (displaying a diestrous pattern each day, with no change into estrous) that was likely induced by the vaginal cytology procedure.

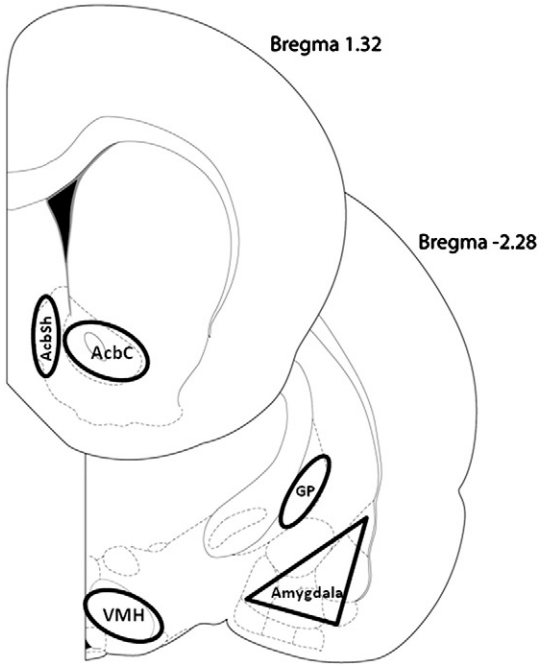

Fig. 2. Selection areas for immunohistochemical semi-quantification (modified from Paxinos and Watson, 2008); VMH = ventromedial hypothalamus, GP = globus pallidus, AcbSh = nucleus accumbens shell, AcbC = nucleus accumbens core.

Cytological data were used to calculate the percentage of days that each subject spent in diestrus (I/II), and the percentage of subjects that exhibited at least one abnormal cycle (as described in Goldman et al., 2007). An abnormal cycle was defined as cytological data of either >4 consecutive days of a proestrous or estrous smear or >6 consecutive days of diestrous I/II smears. In addition, results of the single-mount test were used to calculate the percentage of days that subjects showed evidence of receptivity (showing a lordosis rating of either 2 or 3 during the single-mount test). Means of these three variables were calculated for both controls and drug-treated subjects, and are displayed in Table 1.

An independent sample *t*-test (two-tailed) demonstrated there was no difference in the percent of days that animals exhibited diestrous I/II smears between groups ($t(16) = 1.09$, $p = 0.291$). There was no group difference in the percent of days that animals exhibited receptivity, as assessed by the single-mount test ($t(16) = 1.30$, $p = 0.213$). A χ^2 test for independence revealed that the percentage of subjects that exhibited at least one abnormal cycle did not differ between groups ($\chi^2(1) = 0.72$, $0.50 < p < 0.40$).

3.2. Runway

One additional subject was dropped from behavioral analyses because she showed signs of tail necrosis during the latter portion of the experiment.

The twelve days of runway data for each subject were divided into four cells that composed the within-subjects measures (nonestrous *vs.* estrous; male *vs.* female target). In cells where multiple trials of data were available, data were averaged. The aggregation of data from multiple testing days was necessary to fill as many cells with at least one value because subjects were only estrous on approximately one quarter of testing days (26.3% ± 0.93%). On average, 4.3 trials per subject were used to calculate a mean RT/PT when the subject was nonestrous. In comparison, only 1.5 trials per subject were available to calculate an estrous mean. This has the effect of making our estrous data less reliable than our nonestrous data.

3.2.1. Run time

A 2(treatment) × 2(estrous status) × 2(target) mixed design analysis of variance (ANOVA) on Run Time (RT) revealed a significant interaction between target and treatment ($F(1,10) = 10.906$, $p = 0.008$). Control subjects ran faster for the male target (17.1 ± 2.8; mean seconds ± SEM) than the female target (23.8 ± 2.4), whereas drug-treated subjects exhibited the inverse pattern and ran faster for the female target (20.0 ± 3.80) compared to the male target (26.6 ± 3.8). We conducted a post-hoc analysis via two repeated measures ANOVA's and utilizing the Šidák–Bonferroni correction (with a family-wise $\alpha = 0.05$), to assess

Table 1
Estrous cyclicity data for both controls and drug-treated subjects. Displays the mean (± SEM) percent of days that subjects exhibited diestrous I/II smears, and the percent of days subjects exhibited behavioral estrus. The percent of subjects exhibiting ≥1 abnormal cycle is also indicated. No significant differences were observed between treatment groups.

	% days diestrus I/II	% days behavioral estrus	% subjects exhibiting ≥1 abnormal cycle
Control	45.17 (±2.67)	27.63 (±1.42)	25
CP55,940	49.67 (±3.00)	25.26 (±1.17)	10

whether these within-subject differences were statistically significant. Control subjects did show a significant effect of target ($F(1,4)=11.888$, $p=0.026$), while drug-treated subjects did not ($F(1,6)=4.658$, $p=0.074$). Fig. 3A and B display the mean RT's for subjects in the control group and drug-treatment group, respectively, across both estrous status conditions and for both goalbox targets. It should be noted that there was neither a significant main effect of estrous status on RT, nor any significant interactions involving estrous status.

3.2.2. Proximity time

A 2(treatment)×2(estrous status)×2(target) mixed design ANOVA compared Proximity Time (PT) across all conditions. A significant main effect of target ($F(1,10)=7.605$, $p=0.02$) demonstrated that PT's for the male target (91.7±6.6) were significantly higher than PT's for the female target (74.3±3.8). A significant main effect of estrous status ($F(1,10)=17.37$, $p=0.002$) demonstrated that PT's were higher when subjects were estrous (95.9±6.1) compared to nonestrous (70.1±3.6). There were no significant interactions.

Although we did not see a significant main effect of treatment ($F(1,10)=0.583$, $p=0.46$), or a treatment by target interaction ($F(1,10)=0.772$, $p=0.40$), close inspection of the PT data did suggest a more subtle effect of cannabinoid treatment on subject motivation. Fig. 4A and B display the control and drug-treated subjects' PT's, respectively, as mediated by both estrous status and goalbox target. Drug-treated subjects are showing an attenuation of motivation to maintain close proximity to a male target when they are estrous, when compared to non-treated controls. To explore this issue using a more powerful statistical approach, we conducted two fully-within 2(estrous status)×2(target) ANOVA's: one on the PT data for control subjects (Fig. 4A) and one on the PT data for drug-treated subjects (Fig. 4B). For controls, there was a main effect of estrous status ($F(1,4)=7.81$, $p=0.049$), such that PT's were higher when subjects were estrous (104.3±10.4) compared to when they were nonestrous (69.5±5.2). Furthermore, there was a main effect of target ($F(1,4)=26.32$, $p=0.007$), such that control subjects expressed higher PT's for male targets (99.0±11.4) than for female targets (74.8±6.5). In contrast, the same analysis on drug-treated animals revealed only a main effect of estrous status ($F(1,6)=8.64$, $p=0.026$), again with estrous subjects expressing higher PT's (89.9±7.2) than nonestrous subjects (70.6±5.2). There was no main effect of target ($F(1,6)=1.39$, $p=0.283$); drug-treated subjects did not show greater motivation to maintain close proximity to male targets (86.5±8.1) than female targets (74.0±4.7). These analyses suggest that while controls exhibited an overall preference for the male target, drug-treated subjects did not.

3.3. Sucrose preference

Sucrose preference was defined as the percentage of sucrose solution consumed relative to total fluid intake across the 72 h of availability. An independent sample, two-tailed *t*-test ($\alpha=0.05$) indicated that there was no significant difference in sucrose preference between control (76.4±7.9) and drug-treated (75.5±9.3) subjects.

3.4. CB_1 receptor expression

Within the brain regions assessed, the most intense immunostaining was observed in the GP, while CB_1R immunoreactivity was more modest elsewhere. CB_1R immunoreactivity predominated in neuronal fibers. A representative immunopositive result from the GP of a vehicle-treated adult rat is displayed in Fig. 5. Table 2 summarizes the results of our immunohistochemical analysis of CB_1R expression across all treatment groups. We modeled our statistical approach after Rubino et al. (2008), who conducted a similar analysis on the effects of adolescent THC exposure on CB_1R density, assessed via autoradiography. Independent sample, two-tailed *t*-tests ($\alpha=0.05$) were used to compare mean CB_1R expression within each brain region between control subjects and CP55,940-treated subjects. This was done for both adolescents and adults. We did not employ a 2(treatment)×2(age) ANOVA because none of our *a priori* hypotheses were concerned with the effects of age upon CB_1R expression.

Within the VMH, CB_1R expression was significantly lower (81.6±1.7) in drug-treated adolescents compared to control adolescents (87.7±1.8); $t(10)=2.5$, $p=0.03$. There was no significant effect of drug treatment on adult VMH ($t(10)=0.20$, $p=0.84$).

Similarly, within the amygdala, drug-treated adolescents expressed less CB_1R (44.4±1.3) compared to controls (51.2±1.5); $t(10)=3.5$, $p=0.006$. Again, this difference was not present upon adulthood ($t(10)=1.20$, $p=0.26$).

There were no significant effects of drug treatment on CB_1R expression in AccC, AccSh, or GP, regardless of subject age.

4. Discussion

Acute treatment with cannabinoid ligands is known to modulate male and female sexual behavior in a sex-steroid dependent manner (Gorzalka et al., 2010; López, 2010). Furthermore, adolescent exposure to cannabinoid agonists produces a variety of neurobehavioral alterations that persist into adulthood (Realini et al., 2009; Rubino and Parolaro, 2008; Viveros et al., 2005). We are the first to report that repeated adolescent exposure to the synthetic cannabinoid agonist,

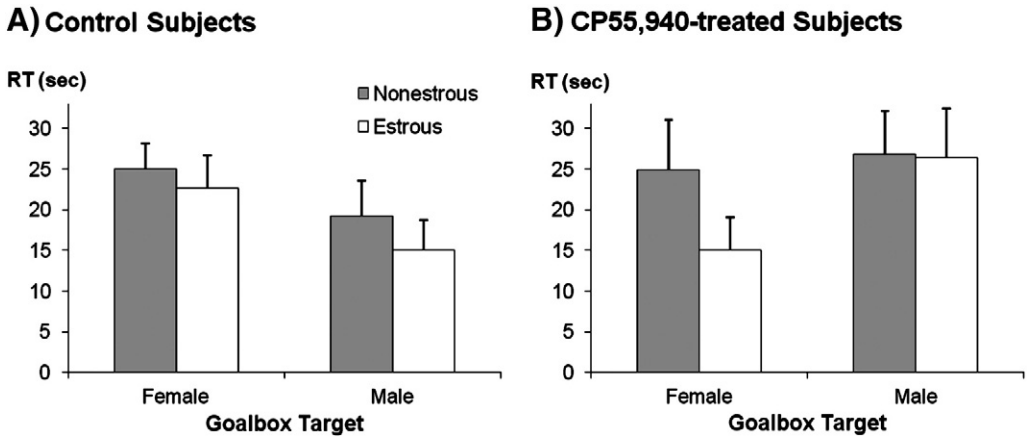

Fig. 3. Mean (±SEM) Run Times for subjects in the control condition (A) and those given chronic CP-55,940-treatment during adolescence (B). Subjects ran for a female and male target, under both nonestrous and estrous conditions. A significant interaction indicated that control subjects ran significantly faster for the male target than the female (collapsed across estrous conditions), whereas the CP-55,940-treated subjects did not show a significant preference for either target.

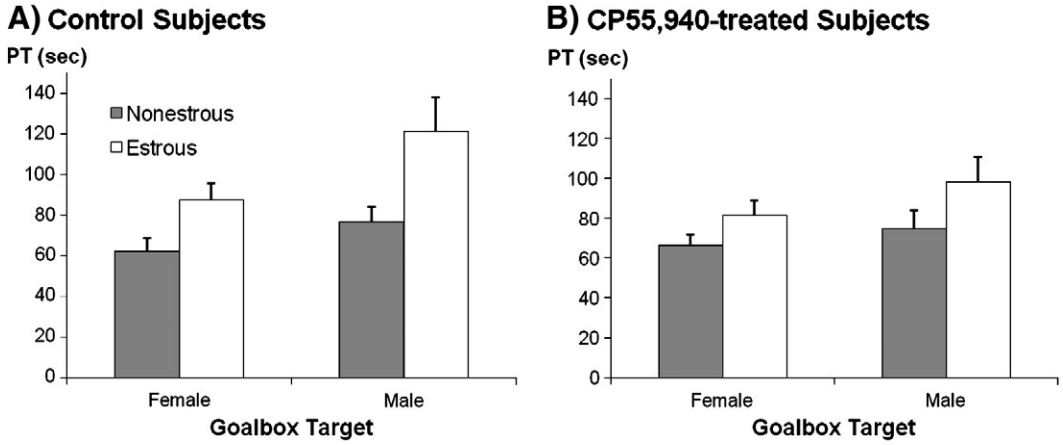

Fig. 4. Mean (±SEM) Proximity Times for subjects in the control condition (A) and those given chronic CP-55,940-treatment during adolescence (B). Controls exhibited a significant preference for the male target over the female target (collapsed across estrous conditions), whereas CP-55,940-treated subjects exhibited no such preference.

CP-55,940, decreases sexual motivation in adult female rats. Decreased motivation was detected in both initial approach behavior and proximity-maintenance behavior. Experimental alterations in approach behavior (Run Time) were particularly robust. Control subjects displayed a significant preference for male targets over female targets, which we interpret as a behavioral manifestation of sexual motivation. This interpretation is bolstered by our finding that control females ran fastest both when the goalbox contained a male and they were estrous (RT = 15.0 ± 3.7; see Fig. 3). Drug-treated subjects, in contrast, showed no significant preference for a male target, which we interpret as an attenuation of sexual motivation. Alterations in proximity behavior were more subtle. Controls exhibited a significant preference for a male target *versus* a female (Fig. 4A), while drug-treated animals failed to show a preference for either target (Fig. 4B). These data suggest that cannabinoid treatment during adolescence disrupted the expression of normal adult sexual preference, specifically decreasing female sexual motivation for a male conspecific.

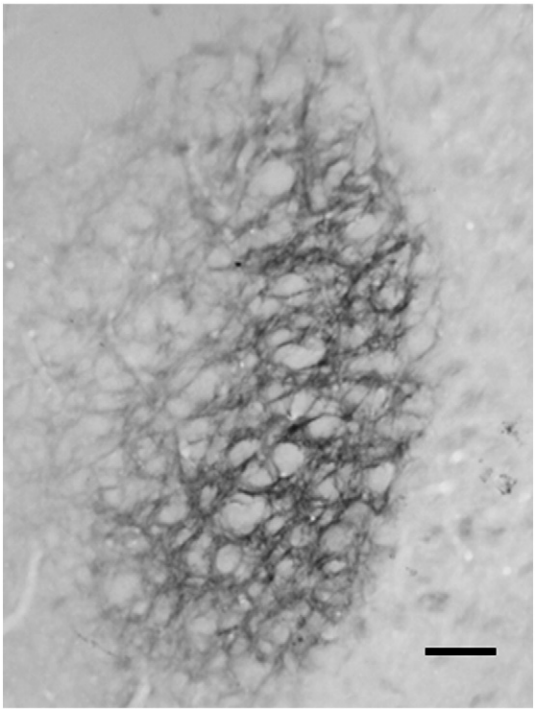

Fig. 5. A representative photomicrograph illustrates characteristic CB_1R immunoreactivity in the globus pallidus of a vehicle-treated, adult female rat. Scale bar = 200 μm.

Among our control subjects, "estrous" effects on sexual motivation were relatively weak. For instance, in control subjects we would expect females to express significantly lower RT's for a male target when in estrus compared to when not in estrus. Indeed, such a difference has previously been observed in ovariectomized females given exogenous hormone treatment to induce behavioral estrus (Nofrey et al., 2008). We did see the expected pattern in our results, whereby subjects expressed lower RT's and higher PT's for a male target when estrous *vs.* nonestrous; however, the differences in RT were non-significant. There are a number of possible explanations for this. Most importantly, we were not able to collect a great deal of estrous data in this experiment. As expected of intact, naturally-cycling females, subjects were estrous on only 26% of test trials. When dividing this across the four test conditions (nonestrous/estrous, female/male target), we often found that a subject was only providing a single data point for the estrous — male target condition. In contrast, nonestrous data were typically an average of several trials. As such, there was less inherent reliability in our estrous data, making comparisons between nonestrous and estrous more difficult. Secondly, we have noted in previous publications (López et al., 2007, 2009) that RT, as a dependent variable, tends to demonstrate greater within-group variance when compared to PT. Perhaps this is because RT can be easily influenced by disruptive stimuli that occur at the start of a runway trial or transient emotional states, such as disorientation. Even with these caveats, however, we believe that both RT and PT are valid measures of socio-sexual motivation. The robust differences we observed in subject motivation to approach male *versus* female targets are supportive of this conclusion.

Previous research has indicated that chronic adolescent cannabinoid treatment increases social anxiety (O'Shea et al., 2004, 2006). We did not see a significant effect of drug treatment on motivation to approach and maintain close proximity to another female (our social control). However, our behavioral model is quite different from the social interaction test (SIT) used in previous research. More importantly, our results indicate that the observed changes in sexual

Table 2
Mean (±SEM) CB_1R expression within VMH, amygdala, AcbC, AcbSh, and GP (*significantly different from age-matched controls, $p<0.05$, using an independent sample, two-tailed *t*-test).

Brain region	Juvenile-control	Juvenile-CP55,940	Adult-control	Adult-CP55,940
VMH	87.7 (±1.8)	81.6 (±1.7)*	83.6 (±2.6)	84.2 (±1.6)
Amygdala	51.2 (±1.5)	44.4 (±1.3)*	54.0 (±2.4)	51.0 (±0.7)
AcbC	69.1 (±1.9)	71.9 (±3.2)	73.0 (±4.3)	71.8 (±2.9)
AcbSh	77.2 (±2.4)	82.8 (±2.0)	79.0 (±3.0)	82.9 (±5.5)
GP	111.4 (±3.3)	111.3 (±3.2)	103.1 (±3.0)	101.6 (±3.2)

motivation for a male target cannot be explained via a reduction in social motivation.

We did not observe any treatment-induced changes in sucrose preference. This suggests that chronic adolescent exposure to CP-55,940 does not significantly affect reward processing. This also indicates that the reduction in sexual motivation seen in our subjects was not mediated by a more global anhedonic state. There is some conflict in the literature over whether chronic adolescent cannabinoid treatment affects reward processing. Rubino et al. (2008) reported a significant decrease in adulthood sucrose preference in both male and females that had been treated with THC during adolescence. However, other laboratories have recently reported that a treatment regimen of CP-55,940, similar to the one used in the current study, did not affect sucrose preference in adult males (Medina et al., 2010).

Endocannabinoids are suspected to exert a significant modulatory effect on neuroendocrine function, and CB_1R's are believed to be involved in the feedback control of gonadotropin releasing hormone (Gammon et al., 2005; Maccarrone and Wenger, 2005). As such, we compared estrous cyclicity between treated and untreated females, hypothesizing that treated females would show abnormal periodicity of behavioral estrus. Interestingly, treatment did not affect estrous cyclicity, as assessed by both cytological and behavioral data (Table 1). This suggests that the observed reduction in sexual motivation was not mediated by a large-scale disruption in HPG-axis function. In future experiments, it would be helpful to directly assess pituitary endocrine output (via plasma assays of lutenizing hormone, for example) following chronic adolescent cannabinoid exposure, given the substantial evidence that the eCB system is implicated in both hypothalamic and pituitary function (reviewed in López, 2010).

Chronic cannabinoid exposure induced decreases in CB_1R expression in a transient, region-dependent manner. Treatment induced short-term (PND 46) decreases in CB_1R expression in the VMH and amygdala, but not in the GP or NAc. We did not observe long-term (PND 74) changes in CB_1R expression in any of our measured brain regions. To our knowledge only one other published report has examined CB_1R expression following a similar adolescent exposure regime in females (Rubino et al., 2008). In that study, CB_1R binding densities were measured immediately and long-term via [^{3}H]CP-55,940 autoradiography following adolescent exposure to THC. The changes reported here are consistent with those reported by Rubino et al. (2008) in the hypothalamus and GP. In the amygdala, the immediate decreases in CB_1R expression we observed were consistent with findings of the Rubino et al. study (2008); however, we failed to detect the long-term down-regulation observed by their group. Rubino et al. (2008) also reported significant long-term, but not immediate, down regulation of CB_1R density in the nucleus accumbens, whereas we observed no drug effects on this brain region (either core or shell). One previous study that examined CB_1R protein levels via Western blotting following a prolonged exposure regimen to THC (30 days) and a longer period of abstinence (185 days) did not detect drug effects in the striatum (Winsauer et al., 2011). These disparities may be due to the use of different cannabinoid ligands (THC *vs.* CP-55,950), differences in sensitivity between techniques, and/or differences in the specific population of CB_1R measured via particular assays (membrane-expressed, intracellular, or both). In the present study, the inclusion of Triton-X-100 in immunobuffers was necessary to achieve adequate antibody penetration; however, the addition of this detergent causes a degree of membrane perturbation, which limits the ability to make an absolute distinction between membrane-bound and intracellular populations of the CB_1R. Despite this limitation, our data highlight a meaningful decrease in CB_1R expression after chronic cannabinoid exposure.

The presence of long-term behavioral alteration in the absence of persistent changes in CB_1R expression has multiple interpretations. The nucleus accumbens, VMH and amygdala are known to regulate various aspects of female reproductive behavior (Mong and Pfaff, 2004; Pfaus, 2009; Spiteri et al., 2010) and these regions are known to possess CB_1R's (Mackie, 2005). Despite this, one possible interpretation of our finding is that these structures are not the loci of cannabinoid-induced alterations in female sexual motivation.

Another possibility is that a process independent of receptor down-regulation may cause the observed behavioral changes. There is evidence that the development of tolerance to CP-55,940 involves mechanisms beyond the down-regulation of CB_1R (Fan et al., 1996). CB_1R's show decreased CB_1R G protein coupling following chronic adolescent cannabinoid exposure. Changes in CB_1R G protein coupling have even been observed when receptor-binding densities are not altered (Childers, 2006; Rubino et al., 2000, 2008). This suggests that receptor desensitization dynamics could mediate long-term behavioral changes. However, based upon results previously collected in our laboratory, we might expect that attenuated CB_1R function would be associated with *enhanced* female sexual motivation. We have previously shown that acute administration of a cannabinoid agonist to adult, estrous females attenuates sexual motivation (López et al., 2010), and administration of a cannabinoid antagonist increases female sexual motivation (López et al., 2009). This pattern of results suggests that endocannabinoids normally serve to inhibit female sexual motivation. There is some evidence that a cyclic reduction in eCB activity may stimulate behavioral estrus via disinhibition (reviewed in López, 2010). Therefore, adolescent cannabinoid exposure likely affects adult sexual motivation in a less obvious fashion that does not involve reduced cannabinoid inhibition of sexual motivation circuitry.

One intriguing possibility is that our treatment regimen affected eCB tone during adolescent brain development. Adolescence is an important development phase characterized by remodeling of neuronal circuits that are critical for the maturation of adult emotionality and reproductive behavior (Sisk and Zehr, 2005). Endocannabinoids and gonadal hormones are known to reciprocally regulate each other, and it has been reported that estrogen can recruit the eCB system to modulate emotionality (Hill et al., 2007; López, 2010). Gonadal hormones are believed to be involved in neuronal remodeling during adolescence that is associated with the development of adult reproductive behavior (Schulz et al., 2009). eCBs and CB_1R signaling has been implicated in the development and regulation of long term synaptic plasticity believed to play a role in emotional responses (Harkany et al., 2008; Mackie, 2008; Ramos et al., 2005; Viveros et al., 2007). Perhaps gonadal hormones and the eCB system work in symphony during adolescent development to remodel neural circuits involved in regulating the emotional and motivational response to sexually relevant stimuli. Chronic exposure to a cannabinoid agonist during this time might detrimentally impact these developmental processes.

In summary, adolescent cannabinoid exposure decreases adult female sexual motivation, as assessed by both approach behavior and proximity-maintenance. This finding adds to a growing body of research which indicates that heavy cannabinoid use during adolescence may have subtle, but potentially profound, long-term consequences on adult emotionality, motivation, and behavior (Rubino and Parolaro, 2008). Such changes in behavior could be mediated by alterations in neuronal and neuroendocrine development. In the current study, cannabinoid-treated animals displayed altered CB_1R expression in the amygdala and VMH shortly after drug exposure, but this down-regulation was transient and adult CB_1R expression was comparable between drug-treated and control animals. Drug treatment also did not detrimentally affect estrous cyclicity. Future investigation is required to elucidate the mechanism by which adolescent cannabinoid exposure affects adult sexual motivation.

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