

Chronic cannabinoid treatment in adolescence attenuates c-Fos expression in nucleus accumbens of adult estrous rats

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Chronic cannabinoid exposure during adolescence may negatively impact brain development and alter adult motivation and behavior. We present evidence that treatment with a cannabinoid agonist during adolescence attenuates estrous-mediated expression of c-Fos within the nucleus accumbens of female rats exposed to a male conspecific. Thirty-two female Long-Evans rats were administered either 0.4 mg/kg of CP-55,940 or vehicle on a daily basis between the ages of 35–45 days. When subjects reached adulthood (days 71–76), they were tested within an exposure paradigm designed to invoke sexual motivation without allowing for consummatory behavior. Female subjects were naturally-cycling; half were tested when in behavioral estrus (as determined by vaginal cytology) and half were tested outside of estrus. c-Fos expression was then quantified in multiple brain regions associated with female sexual motivation, in addition to two control regions. Analyses revealed that untreated females showed more c-Fos-positive neurons when estrous (versus non-estrous) within the medial preoptic area of the hypothalamus, the ventromedial hypothalamus, and the nucleus accumbens core and shell. Significant attenuation of this estrous effect was observed within the nucleus accumbens core and shell of drug-treated females. This suggests that adolescent cannabinoid exposure may negatively impact the development and/or function of mesolimbic circuitry. These findings build upon earlier research in our laboratory which indicated that chronic cannabinoid exposure during adolescence persistently attenuates the expression of sexual motivation in female rats and provide a potential neurobiological substrate for those behavioral deficits.

Keywords: CP-55,940; development; estrus; sexual motivation; incentives

Introduction

Adolescence is a developmental period characterized by increased risk-taking and enhanced reward salience (Steinberg, 2007). One common behavioral manifestation of these cognitive phenomena is substantial use of psychoactive substances and illicit drugs for recreational purposes; initial forays into drug use often begin during adolescence (Casey and Jones, 2010; Chen and Kandel, 1995; Kosterman et al., 2000). Such pharmacological explorations may carry enduring costs in psychological and neurological function. Our laboratory is particularly interested in the possible long-term consequences of adolescent cannabis use. Cannabis remains the most commonly-used illicit drug among adolescents

in the United States (NSDUH, 2010), and several epidemiological studies have shown that a significant number of young people go through a period of heavy cannabis use at some point in their lives (e.g., Johnston et al., 2009). Given the high prevalence of marijuana use among adolescents, the long-term cognitive and psychosocial consequences of cannabis consumption are of particular interest. Human studies have aimed to link adolescent cannabis abuse with increased risk of mental illness (for review, see Di Forti et al., 2007). Indeed, longitudinal studies have found that heavy cannabis intake during adolescence increases the risk of developing psychotic symptoms later in life (Rubino et al., 2012). However, additional

work is needed to establish a causal relationship between early cannabis use and increased risk mental illness.

Furthermore, a substantial body of recent research has indicated that the endogenous cannabinoid system (ECS) plays an important role in mediating brain development during puberty and adolescence (Viveros et al., 2012), and that cannabinoid intake during adolescence can increase the likelihood of developing certain mental illnesses due to disruption of normal developmental processes (Bossong and Niesink, 2010; Malone et al., 2010; Rubino and Parolaro, 2008; Schneider, 2008). Chronic treatment with Δ^9 -THC alters neurotransmitter receptor levels and lowers dendritic length and spine density in adult male rats pre-exposed during adolescence (Rubino et al., 2009). This study also reported impaired synaptic connectivity following adolescent cannabinoid treatment. Proteomic analysis within the hippocampus of adult rats pre-exposed to chronic adolescent Δ^9 -THC treatment revealed altered protein expression compared to untreated controls (Quinn et al., 2008). Given the extensive neuromodulatory reach of the ECS, heavy cannabis consumption has the potential to alter function in a variety of psychological and behavioral domains. Herein, we provide evidence that chronic adolescent cannabinoid exposure alters neuronal activity associated with behavioral estrus, potentially impacting the expression of sexual motivation.

Numerous studies have documented long-lasting neurobehavioral alterations following chronic, adolescent cannabinoid treatment in non-human subjects (Realini et al., 2009; Rubino and Parolaro, 2008; Viveros et al., 2005). Such effects are often sex-dependent, suggesting that gonadal hormones interact with the cannabinoid system during development (López, 2010; Viveros et al., 2011). Chronic adolescent cannabinoid exposure in rats has been associated with deficits in object recognition and social interaction (O'Shea et al., 2004, 2006; Schneider et al., 2008), spatial memory (Rubino et al., 2009), enhanced depressive behavior and anhedonia (Bambico et al., 2010; Realini et al., 2011; Rubino et al., 2008), disrupted pre-pulse inhibition (Schneider and Koch, 2003), and increased drug self-

administration (Ellgren et al., 2007; Higuera-Matas et al., 2008). Much of this research indicates that the adolescent brain is more susceptible to perturbation by cannabinoid treatment than the adult brain (Bambico et al., 2010; Quinn et al., 2008; Schneider, 2008). Trends in this literature also suggest that females may be more susceptible than males (Biscaia et al., 2003; Burston et al., 2010; but see Viveros et al., 2012, for a more nuanced discussion of sex differences).

There is also substantial evidence that cannabinoids affect the expression of reproductive behavior in both males and females (for reviews, see Gorzalka et al., 2010; López, 2010). Our laboratory has shown that acute cannabinoid administration inhibits sexual incentive-motivation in female rats, as assessed by behavior in a straight-arm runway (López et al., 2010). The straight-arm runway apparatus utilizes an unconditioned approach methodology in which a subject's run time and proximity time to a conspecific serves as a measure of socio-sexual motivation. Administration of the cannabinoid receptor antagonist/inverse agonist, AM251, has the opposite effect of stimulating sexual motivation, proceptive displays, and lordosis (López et al., 2009). Moreover, we recently demonstrated that chronic treatment with a cannabinoid agonist (CP-55,940) during adolescence (post-natal days, PND, 35-45) elicits a significant attenuation of sexual motivation in estrous female rats when they were tested as adults (Chadwick et al., 2011). CP-55,940 is synthetic analogue of Δ^9 -THC, the main psychoactive compound of the marijuana plant, and acts as a potent agonist of the CB₁ receptor (CB₁R), the primary cannabinoid receptor in the nervous system (Sim-Selly, 2003). The behavioral and neurodevelopmental effects of exposure to cannabinoids are triggered by CB₁ receptor activation. Synthetic Δ^9 -THC analogues such as CP-55,940 are used widely in functional assays of the cannabinoid system. We also assessed CB₁ receptor (CB₁R) levels following adolescent treatment, but did not observe alterations that persisted into adulthood in CB₁R in brain regions associated with the normal expression of female sexual behavior (ventromedial hypothalamus, amygdala, nucleus accumbens core and shell). It is possible that

adolescent exposure to high cannabinoid doses alters the development of neural pathways linked to sexual motivation, thus influencing later adult behavior in ways that are independent of CB₁R downregulation.

The underlying mechanisms by which exogenous cannabinoids negatively impact the development and expression of emotional behavior, including sex, remain unclear. The ECS is implicated in many fundamental aspects of neuronal development, including differentiation, migration, axon pathfinding, fasciculation, and synaptogenesis (Harkany et al., 2008; Viveros et al., 2012). Therefore, exogenous intake of cannabinoids during adolescence may affect neuronal development in a variety of ways and in various locations. For instance, Rubino et al. (2008) have shown that chronic cannabinoid exposure during adolescence induces significant and persistent reductions in both CB₁R binding and CB₁R/G-protein coupling in the amygdala, ventral tegmental area (VTA), and nucleus accumbens (NAc) of female rats – all brain regions relevant to motivation and reward processing. Adolescent cannabinoid exposure may also negatively impact the function and development of neuroendocrine pathways, such as the hypothalamic-pituitary-gonadal (HPG) axis, that regulate various behavioral systems, including mating (López, 2010). In the current study, we examined how cannabinoid treatment impacts neuronal activity during behavioral estrus, when female rats experience a significant increase in sexual motivation. Female subjects were treated with either CP-55,940 or vehicle during adolescence and then allowed to mature to adulthood. They were then exposed to an adult male conspecific in an apparatus that prevented copulation; half were tested while in a non-estrous state and half were tested while estrous. Neuronal activity induced by these experimental conditions was assessed via quantification of c-Fos-positive cells in several brain regions associated with female sexual motivation: ventromedial hypothalamus (VMH), medial preoptic area (MPA), and nucleus accumbens core and shell (NAcC, NAcSh). We also included two control brain regions: 1) the posterodorsal medial amygdala (MePD), which is involved in female sexual behavior but

primarily processes mating stimulation (such as vaginocervical and clitoral; Cameron and Erskine, 2003; Parada et al., 2010), and 2) the piriform cortex (PirCtx), which does not generally respond to the presence of unconditioned sexual incentives (Kippin et al., 2003). In the current paradigm, exposure to a male conspecific was not expected to influence activity within the MePD or PirCtx. Furthermore, analysis of these control brain regions provided a baseline measure of neuronal activity that should not be affected by drug treatment or by estrous status.

The c-fos gene is a member of a family of immediate-early genes, which are the first genes expressed in response to extracellular stimuli and lead to the subsequent activation of cells (Herrera and Robertson, 1996). In neurons, the c-fos gene is transcribed within minutes of the application of a stimulus, and the expression of the c-fos protein product (c-Fos) reaches peak levels at 45 minutes (Muller et al., 1984). Immunohistochemical analysis with an antibody directed against c-Fos has become a standard technique to identify neurons that have become activated in response to a specific stimulus (Krukoff, 1999). Fos-immunoreactivity (IR) has been used successfully to map brain regions associated with sexual motivation. For example, Kippin et al. (2003) used c-Fos-IR to explore brain regions that become active in male rats when they are exposed to either estrous female odors or sexually conditioned neutral odors. López and Ettenberg (2002) similarly measured c-Fos when comparing neuronal activation between sexually-naïve and sexually-experienced males exposed to an estrous female. Female sexuality has also been explored via Fos-IR (Coolen et al., 1996; Coria-Avila and Pfau, 2007; Parada et al., 2010; Paredes-Ramos et al., 2011; Polston and Erskine, 1995), although rarely with a specific focus on incentive-motivation (versus consummatory behavior and reward). One goal of the current experiment was to identify changes in neural activity associated with behavioral estrus that may be correlated with the perception of male incentives and the generation of sexual motivation.

We predicted that among untreated controls, estrous females would show greater

numbers of c-Fos-positive (c-Fos+) cells within the VMH, MPA, NAcC, and NAcSh compared to non-estrous females. Furthermore, we hypothesized that drug-treated subjects would show a significant attenuation of this estrous effect – providing evidence that adolescent cannabinoid exposure disrupts subsequent neural regulation of female sexual motivation in adulthood.

Materials and Methods

All experimental protocols were approved by the Skidmore College Institutional Animal Care and Use Committee (IACUC) in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

Subjects

A total of 32 female and 2 male Long-Evans rats (Charles River Laboratories, Wilmington, MA) were used. Females were 25 days old upon arrival and were housed in pairs. Males were single-housed. The males were used as target stimuli during the exposure test, and were approximately 70 days old at the start of exposure testing. All subjects were housed in plastic tubs with wood chip 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® chew toy. Animals were handled by experimenters on a daily basis for five consecutive days after arrival in the laboratory and prior to any experimental procedures.

Apparatus: Exposure Chamber

Female subjects were exposed to a male conspecific on test day within a specifically designed exposure chamber. Exposure tests occurred in a modified version of the chamber apparatus used by López and Ettenberg (2002). The exposure chamber was a large Plexiglas cylinder (50 cm diameter x 30 cm height) with a floor covered in wood chip bedding. The bedding was taken from the homecage of an

adult male (male subject A) and therefore contained some male urine and scent. This was done to provide the female subject with a potent stimulus (in addition to the physical presence of a different male) that would activate brain regions involved in the processing of sexual incentives. A divider was placed within the chamber to separate the female subject from the male target and prevent physical interaction. Physical contact was prevented to ensure that neuronal activity was a result of incentive-motivation and not from copulatory behavior. The divider was composed of two layers of wire-mesh, separated by 3.5 cm, stapled to a wooden frame. Holes within the wire-mesh allowed animals to see, smell, and hear one another within the apparatus, but did not allow for physical contact.

Procedure

Adolescent Cannabinoid Exposure

CP-55,940 (Tocris Biosciences, Ellisville, MO) was prepared in a vehicle of physiological saline, cremophor, and ethanol (18:1:1). The drug solution was stored at -10°C and utilized within 7 days of preparation. Subjects were randomly assigned to treatment condition and received daily intraperitoneal injections (between 13:00–16:00h) of either CP-55,940 (0.4 mg/kg) or vehicle from PND 35 – 45. While there is some variation in how researchers define “adolescence” in rats, PND 35–45 is a commonly used treatment window in chronic exposure experiments (e.g., Biscaia et al., 2003; Rubino et al., 2008). We used this same treatment period and dosage regimen when examining the long-term impact of adolescent cannabinoid exposure on adult female sexual motivation and estrous cyclicity (Chadwick et al., 2011).

Vaginal Cytology

The rat estrous cycle is typically four or five days in length and divided into four stages: proestrus, estrus, metestrus, and diestrus (Nelson, 2011). Behavioral estrus, which is defined by increases in female sexual receptivity and motivation, begins on the afternoon of proestrus and extends into

estrus. Beginning at PND 60, vaginal smears were obtained daily (between 10:00-12:00h), as described by Maswood et al. (2008). Briefly, a cotton swab was dampened with deionized water, vaginal openings were swabbed, and the contents were smeared onto glass slides. Determination of estrus cycle phase was based upon the relative proportions of nucleated cells, cornified epithelial cells, and leukocytes (Goldman et al., 2007; Marcondes et al., 2002). Proestrous smears possessed clusters of nucleated cells with some cornified epithelial cells and no leukocytes. Estrous smears possessed a majority of cornified cells, some nucleated cells, and few, if any, leukocytes. Metestrus smears consisted of approximately the same proportion of leukocytes, cornified, and nucleated cells. Diestrus smears primarily consisted of leukocytes. Transitional states were also included in this identification procedure. For example, early proestrus (diestrus – proestrus) was distinguished from late proestrus (proestrus – estrus). In addition, previous estrous history (i.e., the mean cycle length of that particular subject, the vaginal smear from the previous day) was used to guide categorization, especially with regard to transitional states.

Exposure Test

Starting on PND 68, all female subjects and one male target (male subject B) were individually habituated to the exposure apparatus for 10 min/day for three consecutive days. During each day's habituation sessions, the male target was always the last animal placed within the apparatus.

Exposure testing occurred between PND 71 and 76. On each day, experimenters conducted vaginal cytology early in the day (between 9:00-11:00h) to determine the current estrous status of each female. Cytology was conducted several hours before exposure testing, so that the vaginal stimulation of this procedure would not confound the later assessment of estrous-mediated c-Fos expression. Subjects were then chosen to participate in that day's exposure tests depending upon their status. Non-estrous (NE) females included those who displayed metestrus, diestrus, or early proestrous vaginal smears. Estrous (EST) females included those who showed mid/late

proestrous or estrous smears. Each day, an attempt was made to test at least one non-estrous and one estrous female within both the control and drug-treated groups.

Exposure testing began around 13:00h and continued until all of that day's animals had been tested and subsequently sacrificed. The order in which animals were tested was determined randomly. The male target (male subject A) was placed into the exposure chamber 10 min before the test began. Male subject A was first given access to the female side of the chamber (without the subject present), such that his scent permeated the apparatus and bedding. After 10 min, Male subject A was confined to the opposite side of the chamber. The subject was then placed on the female side of the chamber, which initiated the exposure test. During this period, an experimenter in another room observed the animals through a live video feed on a computer monitor to ensure that no physical contact occurred. After a 15 min exposure period, the subject was immediately removed from the apparatus and returned to her homecage. Exactly one hour after conclusion of the exposure test, the subject was perfused in preparation for immunohistochemical processing.

After each exposure test, the apparatus was cleaned: the plexiglass wall of the arena was lifted from the base of the apparatus, wood chip bedding and excrement was disposed of and the floor and walls of the chamber were wiped down with a 20% ethanol solution. After the base and wall of the arena had dried completely, the apparatus was reconstructed and fresh bedding was added, and male subject A was again given access to the female side of the chamber for 10 min prior to the next exposure test.

c-Fos Immunohistochemistry

Tissue Preparation and Staining

One hour after completion of the exposure test, subjects were deeply anesthetized with pentobarbital sodium (150mg/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 sliding microtome, 40 µm frozen coronal sections were cut serially through a region of the forebrain extending from Bregma 2.52 to -4.20 and collected in PBS.

Every sixth section was selected from a region of tissue extending from Bregma 2.28 through -0.84, which contained nucleus accumbens core and shell (NAcC, NAcSh), medial preoptic area (MPA) and piriform cortex (PirCtx), and from a second set of tissue that extended from Bregma -1.80 through -3.48, which contained ventromedial hypothalamus (VMH) and posterodorsal medial amygdala (MePD). Each set of tissue was processed separately. Sections were first incubated in 0.3% hydrogen peroxide to quench endogenous peroxidase activity. Sections were then washed in PBS three times for 10 minutes each. Tissue was blocked in 10% normal goat serum (NGS)/PBST (0.2% Triton-X-100/PBS) to prevent non-specific binding. Sections were then incubated with the primary antibody (rabbit anti-c-Fos/3% NGS/PBST; 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA) for 20 hours at room temperature. After three 10-minute washes in PBST, sections were incubated with secondary antibody (biotinylated goat anti-rabbit IgG; 1:500; Vector Labs, Burlingame, CA) for 2 hours at room temperature. Following another set of three 10-minute washes in PBST, sections were incubated in an avidin-biotin complex (VECTASTAIN® Elite ABC kit, Vector Labs). The reaction was developed using a VIP horseradish peroxidase substrate kit for 1.5 minutes (Vector Labs) and stopped by incubating sections in water for 5 minutes. Sections were mounted out of PBS onto gelatin-coated slides and coverslipped with DPX mounting media.

Quantification

Quantification of c-Fos-positive cells was conducted by experimenters blind to the as cells with dark purple staining, a clear cell wall, and a visible nucleus. A Leica CME microscope was equipped with an eyepiece reticle that divided the field of view into a 20 x 20 grid (0.25 mm²). The grid was centered on the region

of interest (ROI) at 10X magnification and a count of the total number of c-Fos+ cells within the grid was performed at 40X magnification. For each ROI, c-Fos+ cells were counted bilaterally from three sections spaced approximately 240µm apart, allowing for the collection of six data points per animal. To assist with determination of the anatomical boundaries and grid placement within our ROI, Nissl staining was performed on a set of sections anatomically adjacent to the tissue processed for c-Fos immunohistochemistry. Representative grid placement within the six measured regions is illustrated below in Figure 1.

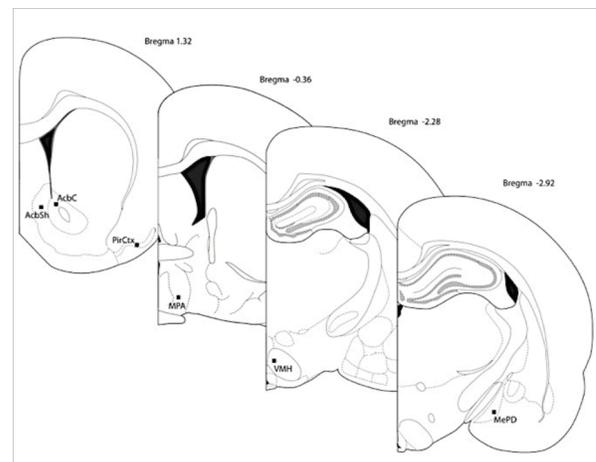


Figure 1: Selection areas and representative grid placement for quantification of c-Fos expression (modified from Paxinos and Watson, 2008); MPA=medial preoptic area, VMH=ventromedial hypothalamus, NAcSh=nucleus accumbens shell, NAcC=nucleus accumbens core, MePD=posterodorsal medial amygdala, PirCtx=piriform cortex. Expression of c-Fos compared across treatment group (drug/vehicle) and estrous phase (EST/non-EST). Exposure of estrous females to male conspecific elicited an increase in c-Fos expression that was absent in subjects tested while non-estrous. Subjects treated with cannabinoids in adolescence exhibited reduced levels of c-Fos expression, with the strongest effects observed in the NAc.

Statistical Analysis

A 2 (treatment: drug vs. vehicle) x 2 (estrous status: NE vs. EST) analysis of variance (ANOVA) was conducted on the mean c-Fos+ cell values for each region. When a significant interaction between treatment and estrous status was discovered, we conducted a limited number of post-hoc *t*-tests to decompose the interaction. These *t*-tests (one-tailed, two-

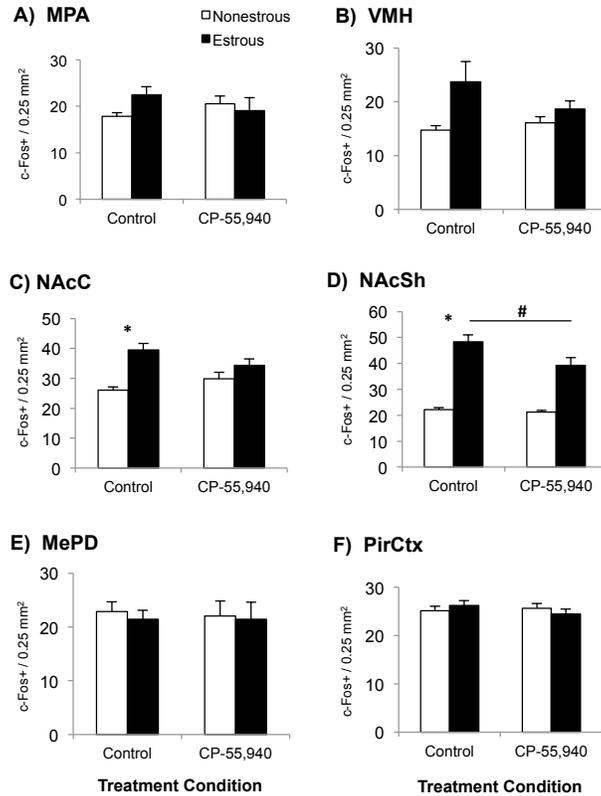


Figure 2: Mean (\pm SEM) numbers c-Fos+ cells within a 0.25 mm² area for each of the six brain regions examined, as influenced by estrous status (non-estrous versus estrous) and treatment condition during adolescence (control versus CP-55,940). Panels A–F represent means from the (a) medial preoptic area, (b) ventromedial hypothalamus, (c) nucleus accumbens core, (d) nucleus accumbens shell, (e) posterodorsal medial amygdala, and (f) piriform cortex, respectively. White bars are non-estrous subjects and black bars are estrous subjects. * indicates a significant difference between group means across estrous status; # indicates a significant difference between group means across drug treatment conditions, as assessed by post-hoc comparisons (one-tailed, two-sample Student's *t*-tests; $\alpha=0.025$).

sample) were conducted to test the central hypotheses of this experiment. First, we compared mean c-Fos+ cells between the vehicle-NE and vehicle-EST groups (for each brain region), to assess whether estrous females displayed greater neuronal activation compared to non-estrous females. Second, we compared mean c-Fos+ cells between the vehicle-EST and drug-EST groups, predicting that drug-EST subjects would show altered levels of c-Fos+ cells compared to vehicle-EST subjects. We used the Bonferroni correction to adjust our alpha level for conducting multiple *t*-tests ($\alpha = 0.025$).

Results

A total of 3 female subjects were euthanized prior to exposure testing because

they became sick during the course of the experiment and could not proceed. Two additional females were dropped from statistical analysis because inadequate perfusion resulted in brain tissue that was not suitable for immunohistochemical staining. Of the remaining 27 subjects, there were 7 each in the vehicle-NE, vehicle-EST, and drug-NE groups, and 6 subjects remaining in the drug-EST group. After histochemical processing, some brain tissue was not intact enough to provide reliable counts of c-Fos+ cells. Therefore, the analyses reported here are based upon group sizes that range from 5-7 subjects/group.

Figure 2 (A-F) shows the mean (\pm SEM) number of c-Fos+ cells for each brain region.

Hypothalamus: MPA and VMH

For the MPA, there was no significant main effect of treatment, no effect of estrous status, and no interaction ($F(1,21) = 2.70, p > 0.05$).

For VMH, EST females showed a significantly greater number of c-Fos+ cells compared to NE females. There was neither a main effect of drug nor a significant interaction ($F(1,18) = 1.93, p > 0.05$) within the VMH.

Nucleus Accumbens: core and shell

Within the NAcC, EST females showed significantly greater numbers of c-Fos+ cells compared to NE females ($F(1,23) = 21.56, p < 0.001$). There was also a significant interaction between treatment and estrous status ($F(1,23) = 5.54, p = 0.03$). Post-hoc tests revealed that vehicle-EST subjects showed significantly more c-Fos+ cells (39.6 ± 2.1) compared to vehicle-NE subjects ($26.1 \pm 1.1; t(12) = 5.7, p < 0.001$). Levels of c-Fos+ cells were not significantly different between vehicle-EST subjects and drug-EST subjects ($34.3 \pm 2.2; t(11) = 1.7, p > 0.05$).

Within NAcSh, treated females showed significantly fewer c-Fos+ cells compared to control females ($F(1,23) = 6.50, p = 0.02$). Comparison across estrus status in the AchSh revealed that EST females revealed significantly higher numbers of c-Fos+ cells compared to NE females ($F(1,23) = 124.88, p < 0.001$). Furthermore, there was a significant interaction between treatment and estrous status ($F(1,23) = 4.32, p = 0.05$). Vehicle-EST subjects expressed more c-Fos+ cells (48.4 ± 2.6) than both vehicle-NE subjects ($22.2 \pm 0.8; t(12) = 9.6, p < 0.001$) and drug-EST subjects ($39.2 \pm 3.1; t(11) = 2.3, p = 0.02$). Figure 3 presents photomicrographs of representative c-Fos immunoreactivity (Fos-IR) within the nucleus accumbens (including both core and shell subregions) of subjects in the four experimental conditions. Drug-treated animals had greater Fos-IR than untreated controls, and Fos-IR was greater in estrous compared to non-estrous subjects. The vehicle-EST group showed the greatest degree of Fos-IR.

Amygdala: MePD

Analysis of c-Fos+ cells in the MePD

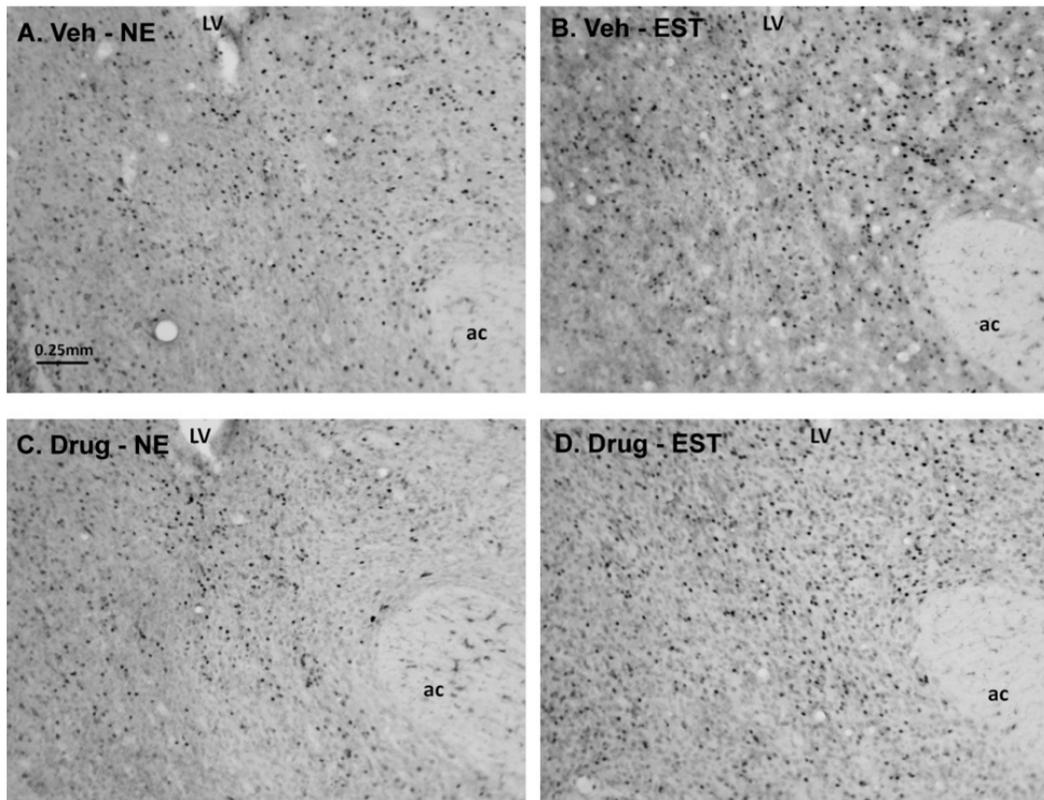


Figure 3: Photomicrographs (25x magnification) of representative c-Fos expression within the general area of the nucleus accumbens (including core and shell subregions). Panels A and B are from control subjects, while C and D are from drug-treated subjects. Panels A and C are from non-estrous subjects, and panels B and D are from estrous subjects. ac=anterior commissure, LV=lateral ventricle.

revealed no significant effect of treatment or estrous status, or an interaction ($F(1,16) = 0.12$, $p > 0.05$).

Piriform Cortex

There were no significant main effects of treatment or estrous status within PirCtx, and no significant interaction ($F(1,23) = 1.19$, $p > 0.05$).

Discussion

The current experiment provides evidence that adolescent cannabinoid exposure has a persistent detrimental effect on certain neural pathways that process sexual incentives during behavioral estrus. We measured expression of c-Fos, a marker of neuronal activation, in female subjects following a brief period of exposure to a male conspecific. Half the subjects had been administered a relatively high dose of CP-55,940 for 11 days during adolescence (PND 35-45), while the remainder served as untreated controls. Among controls subjects we observed greater c-Fos expression in estrous females compared to non-estrous females within the following brain regions: the VMH, NAcC, and NAcSh. Furthermore, cannabinoid pretreatment during adolescence had a significant impact on estrous-mediated c-Fos expression within the NAcSh. Drug-treated estrous females displayed fewer c-Fos+ cells in this brain region compared to untreated estrous females.

We did not observe any alteration in c-Fos expression within the posterodorsal medial amygdala (MePD) or piriform cortex (PirCtx) due to estrous status or drug treatment. The MePD was included because it is a brain region that becomes active in females during copulation and seems to be linked to the processing of genital stimulation (Cameron and Erskine, 2003; Parada et al., 2010). As such, while it is part of female sexual circuitry, its activity should not be influenced by the mere presence of a male conspecific. The PirCtx is not a brain region directly associated with female sexual motivation. Interestingly, Kippin et al. (2003) found that while female estrous bedding had no impact on Fos-IR within the

PirCtx of male rats, a sexually-conditioned odor did. Similarly, Coria-Avila and Pfaus (2007) noted increased Fos-IR in the PirCtx of female rats presented with an odor associated with paced mating. As such, the PirCtx is likely involved in higher-level processing of olfactory information, including stimuli that have become associated with natural incentives through learning processes. The lack of estrous effects in these brain regions suggests that the estrous-mediated increases in c-Fos expression within the current experiment reflect the processing of unconditioned sexual incentives.

One interpretation of these results is that adolescent cannabinoid exposure disrupts the development of neuroendocrine systems associated with the regulation of behavioral estrus. The generation of female sexual behavior in the rat is dependent upon the activity of both estradiol and progesterone within brain circuitry that includes the MPA and VMH (Nelson, 2011). In estrous females, enhanced c-Fos expression within these hypothalamic areas may be reflective of hormone-induced neural activity. Estrogen and progesterone can also modulate the activity of the NAc across the estrous cycle (Becker, 1999). The capacity for cannabinoids to interfere with the normal activity of the HPG axis has long been recognized (Biswas et al., 1976), but it is not known whether chronic cannabinoid administration during adolescence causes a persistent change in the release of HPG hormones or the distribution, density, and/or functional properties of estrogen and progesterone receptors. Notably, Higuera-Matas et al. (2009) failed to see an effect of chronic CP-55,940 (administered during early adolescence) on subsequent estradiol levels in adult female rats. In our previous work (Chadwick et al., 2011), we did not see a significant effect of adolescent cannabinoid treatment on estrous cyclicity. With respect to the current data, it is perhaps relevant that we did not observe a significant difference in numbers of c-Fos+ neurons between treated and control females in the two brain regions that possess especially high levels of estrogen and progesterone receptors: the MPA and VMH. Therefore, adolescent cannabinoid exposure is not prominently interfering with the function of

hypothalamic nuclei that regulate female sexual behavior.

That the effects of our cannabinoid treatment were strongest within the NAc points to specific disruption of the mesolimbic pathway. The mesolimbic pathway, which projects from the VTA to the NAc, mediates motivational processes that subsume a variety of behavioral domains, including copulatory behavior and the desire to ingest drugs of abuse (e.g., Phillips et al., 2008). Female rats experience dopamine release within the NAc during paced mating (Becker et al., 2001; Jenkins and Becker, 2003), and more pertinent to the current research, when they are exposed to a male rat located behind a wire-mesh screen (Pfaus et al., 1995). We observed more c-Fos+ cells in both the NAcC and NAcSh of estrous females exposed to a male conspecific, compared to c-Fos+ cells in non-estrous females. These data support the premise that the perception of male sexual incentives activates the mesolimbic dopamine pathway, and that NAc activity is involved in the generation of female sexual motivation (Pfaus, 2009).

c-Fos expression was more heavily affected in the NAcSh than in NAcC. Differences in the connectivity of the accumbens subregions may provide a partial explanation for this finding. The core and shell of the NAc receive input from separate cortical areas (Brog et al., 1993) and have distinctive projection patterns (Heimer et al., 1991). The core projects to areas such as the substantia nigra, globus pallidus and subthalamic nucleus, which are involved with voluntary motor function, whereas the shell sends projections to limbic components such as the hypothalamus and amygdala (Zahm and Brog, 1992). The NAcSh may therefore be more closely associated with a purely motivational state while NAcC activation is associated with motor output (Balfour et al., 2004). Applying this idea to the current paradigm provides a potential explanation for the patterns of c-Fos expression we observed within the NAc. The barrier in the arena prevented physical interaction between the subject and target. The subjects were not able to partake in the motor behavior necessary to translate motivation into copulation, therefore increased core activation did not occur.

Adolescent cannabinoid treatment reduced neuronal activity within the NAcSh of estrous females exposed to a male. This finding could reflect reduced incentive-mediated dopaminergic activity within the NAc of treated females, which in turn could be responsible for an attenuation of sexual motivation (Chadwick et al., 2011). Lesions of the NAc in female rats dramatically increase approach latencies towards males (Jenkins and Becker, 2001), and drugs that reduce or block dopaminergic activity tend to inhibit the expression of sexual behavior in both female rats and women (Pfaus, 2009).

While this experimental work points to accumbens dopamine as an important mediator of sexual motivation and/or reward, there is conflicting evidence. In a review of dopamine's pharmacological effect on sexual behavior, Paredes and Agmo (2004) argue that dopamine is critical for the generation of motor function and for general increases in arousal. For this reason, motor effects of dopamine manipulation may be incorrectly interpreted as changes in certain observable motivational and copulatory behaviors. Increased activity in the dopamine system that was initially attributed to incentive motivation may instead simply reflect general increases in arousal. Changes observed in the NAc activity noted in the current experiment may not indicate sexual motivation, but rather reflect a general increase in arousal when presented with the male stimulus. Moreover, adolescent cannabinoid administration may have disrupted this arousal system.

It is possible that repeated activation of central cannabinoid receptors by exogenous agonists during adolescence disrupts the normal developmental trajectory of the mesolimbic pathway. It is known that cannabinoids, both endogenous and exogenous, impact mesolimbic activity via regulation of excitatory and inhibitory inputs to dopaminergic neurons (Gessa et al., 1998; Szabo et al., 2002; for a review, see Gardner, 2005). The mesolimbic pathway displays significant developmental change throughout adolescence (for a review, see Spear, 2000). For example, there appears to be an adolescent peak of the two primary dopamine receptors in the CNS, the D₁ and D₂ receptors, in striatal and mesolimbic regions, followed by a substantial decline due to neural

pruning. Furthermore, there are dynamic alterations in endocannabinoid function within the NAc during adolescence that participate in the development of normal reward processing and emotional behavior (Ellgren et al., 2008). Thus, cannabinoid disruption of mesolimbic development during adolescence seems plausible.

Several studies have provided direct evidence that cannabinoid exposure interferes with proper development of mesolimbic circuitry. Pistis et al. (2004) used single-unit extracellular recordings to assess the response properties of mesolimbic dopamine neurons following adolescent treatment with the cannabinoid agonist, WIN55212-2 (WIN). Two weeks following treatment, male rats were challenged with WIN, morphine, amphetamine, or cocaine. In pretreated subjects, there was significant evidence of reduced neuronal responsiveness to all four drugs (i.e., tolerance and cross-tolerance), suggesting that adolescent cannabinoid exposure induces long-lasting deficits in the reward-processing of mesolimbic circuitry. More recently, Higuera-Matas et al. (2010) noted that adolescent female rats treated with CP-55,940 experienced a persistent (extending into adulthood) upregulation of striatal dopamine transporter (DAT), the transmembrane protein responsible for dopamine reuptake. This long-term upregulation of DAT may reflect enhanced basal dopaminergic activity and be responsible for increased rates of cocaine self-administration. Finally, Wegener and Koch (2009) treated male rats with WIN for 25 days during adolescence and puberty, and later assessed baseline c-Fos-IR within a variety of brain regions, including the NAc. They noted higher c-Fos-IR in the NAcC of pretreated subjects (compared to controls) in response to a vehicle injection, indicative of a persistent alteration in neuronal excitability of mesolimbic circuitry.

In summary, we present evidence that treatment with a potent cannabinoid agonist during adolescence attenuates estrous-mediated expression of c-Fos within the nucleus accumbens shell in response to a male conspecific. Given the suspected role of mesolimbic dopamine in generating female sexual incentive-motivation, we interpret this

finding to be a potential neurobiological correlate of reduced sexual interest. In our previous behavioral work (Chadwick et al., 2011), we established that an identical treatment regimen significantly attenuated sexual motivation in adult females. The current data extend those findings and provide possible new avenues for subsequent research. In particular, we hypothesize that cannabinoid exposure during adolescence has the potential to impair the development and function of the mesolimbic dopamine pathway, and thus reduce the motivational value and reward salience of a variety of external stimuli, including natural rewards like potential mates.

It is important to highlight some interpretive limitations of our work. In interpreting our findings, it was assumed that alterations in NAc function are 1) related to dopamine activity, and 2) associated with the generation of sexual motivation. It should be noted that we did not directly assess either of these two phenomena in the current study. Expression of c-Fos was instead used as an indirect measure of general neuronal activity within brain regions thought to be associated with sexual motivation. Future research must aim to explore how chronic adolescent cannabinoid treatment influences the development of dopamine pathways, as well as dopamine function in the adult female brain.

The ECS mediates neuronal maturation throughout adolescence, and these processes are vulnerable to disruption in young cannabis users. In addition to the neuropsychological and behavioral impairments associated with early cannabis abuse, adults with a history of previous cannabis use face an increased risk of developing psychiatric disorders (Rubino et al., 2012). Adolescent cannabis abusers can develop amotivational syndrome, a psychiatric disorder characterized by personality and neurocognitive changes in which an individual is less motivated to engage in the world and seek reward (Kogan and Mechoulam, 2007). It is also possible that the symptoms of amotivational syndrome influence sexual desire; females who abuse marijuana at an early age may be more likely to suffer from reduced libido and socio-sexual motivation as adults. More evidence is needed to

establish a link between early cannabis use and impaired sexual desire in adulthood.

Acknowledgements

The authors would like to thank Carolyn O'Connor, Sarah Minney, and Jeffery Forte for their help in collecting data. Thanks to Flip Phillips for his expertise in image analysis. This research was partially funded by Skidmore College's Summer Faculty/Student Research Program.

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