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Abstract: Estrous cycle-related fluctuations in delta-9-tetrahydrocannabinol (THC)-induced antinociception have been observed in the rat. The aim of this study was to determine which major ovarian hormone modulates the antinociceptive effects of *i.c.v.* THC, and whether hormone modulation of THC's behavioral effects could be due to changes in brain cannabinoid receptors (CB₁). Vehicle (oil) or hormones (estradiol or progesterone, or both) were administered to female rats on days 3 and 7 post-ovariectomy. On the morning or afternoon of day 8 or day 9, vehicle or THC (100 µg) was administered *i.c.v.* Paw pressure, tail withdrawal, locomotor activity and catalepsy tests were conducted over a 3-h period. Estradiol (with and without progesterone) enhanced THC-induced paw pressure antinociception only. Ovarian hormones time-dependently modulated CB₁ in brain structures that mediate antinociception and locomotor activity, but the changes observed in CB₁ did not parallel changes in behavior. However, the time course of CB₁ changes must be further elucidated to determine the functional relationship between receptor changes and antinociceptive sensitivity to THC.

Keywords: Hormone. Female. Cannabinoid. Antinociception, Locomotion, Radioligand binding

1. Introduction

Behavioral effects of cannabinoids are sexually dimorphic, with female rats being more sensitive than males to the reinforcing, antinociceptive, sedative and cognition-impairing effects of cannabinoids (Tseng and Craft, 2001; Romero et al., 2002; Cha et al., 2007; Fattore et al., 2007; Craft and Leitl, 2008; Wakley and Craft, 2011; Craft et al., 2012). Sex differences in antinociceptive response to cannabinoids such as delta-9-tetrahydrocannabinol (THC) may be explained by ovarian hormones. Estradiol (E2) enhanced systemic THC-induced antinociception in ovariectomized (OVX) females compared to OVX females without hormone replacement (Craft and Leitl, 2008). This enhancement was similar to that observed in gonadally intact females tested in estrus vs. those tested in diestrus (Craft and Leitl, 2008). Estrous stage also modulates antinociception following supraspinal THC administration: females in late proestrus showed greater paw pressure antinociception than females in estrus and males (Wakley and Craft, 2011). These studies demonstrate that ovarian hormones likely contribute to sex and estrous stage-related differences in cannabinoid antinociception.

Ovarian hormones also influence endocannabinoid signaling. E2 decreased pituitary cannabinoid receptor (CB₁) mRNA levels in OVX females (González et al., 2000). Similarly, acute E2 decreased Gα protein activation in the cortex and hippocampus of prepubescent

female rats (Mize and Alper, 2000). In the limbic forebrain, ovariectomy decreased, and E2 increased CBr density (Bonnin et al., 1993; Rodriguez De Fonseca et al., 1994). Similarly, ovariectomy decreased CBr density in amygdala but increased it in the hypothalamus and hippocampus; E2 reversed these changes (Riebe et al., 2010). The other major ovarian hormone, progesterone (P4), increased CBr density in the limbic forebrain and midbrain but decreased it in the striatum of OVX females; however, when P4 was given with E2, hypothalamic CBr density was increased (Rodriguez De Fonseca et al., 1994), suggesting that P4 can reverse E2's effects. Although hormone modulation of the brain endocannabinoid system is a likely mechanism underlying ovarian hormone enhancement of the behavioral effects of cannabinoids in females, hormone effects appear to be brain structure-dependent and hormone-specific.

Previous studies examining ovarian hormone modulation of the endocannabinoid system and the behavioral effects of cannabinoids have used hormone regimens that do not mimic natural hormone fluctuations in a gonadally intact female. Typically, a single hormone was administered, and effects were examined at a single time point thereafter (Bonnin et al., 1992, 1993; Rodriguez De Fonseca et al., 1994; Craft and Leitl, 2008; Kalbasi Anaraki et al., 2008; Riebe et al., 2010). During the 4- to 5-day estrous cycle of a rat, E2 and P4 peak in early and late proestrus, respectively (Smith et al., 1975; Feder, 1981; Freeman, 1988). Thus, in the present study a modified cyclic hormone regimen (Asarian and Geary, 2002) was used to determine which ovarian hormone is responsible for the enhanced supraspinal THC-induced antinociception previously observed in gonadally intact, late proestrous females (Wakley and Craft, 2011). The goals of the present research were to determine if: 1) E2, P4 or both are responsible for increased supraspinal THC-induced antinociception; and 2) cyclic ovarian hormones modulate CBr density/affinity in brain areas known to mediate antinociception.

2. Materials and methods

To examine cyclic ovarian hormone modulation of THC's effects in females, we measured the effect of *i.c.v.* THC on antinociception and motor behavior (*Experiment 1*), and on CBr density and affinity in brain structures known to mediate antinociception and motor behavior (*Experiment 2*). A third experiment was conducted to measure actual

serum hormone levels in females treated with the cyclic hormone regimen.

2.1. Subjects

Adult female Sprague–Dawley rats were used (60–100 days old, bred in-house from Taconic stock, Germantown, NY). *Ad libitum* access to food and water was provided except during surgery and testing. Rats were housed in a room with a 12:12 h light/dark cycle (lights on at 0600 h), maintained at $21 \pm 2^\circ\text{C}$. Pair-housing was implemented until surgery, after which rats were singly housed to avoid damage to the intracranial implant. This housing procedure was also implemented for rats used in Experiments 2 and 3. Animals were treated in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

2.2. Surgery

All rats were ovariectomized as described previously (Stoffel et al., 2003; Craft and Leitl, 2008). For *Experiment 1 only*, a guide cannula was also surgically implanted into the right lateral ventricle of all rats. First, s.c. chlordiazapoxide (3 mg/kg; Sigma Aldrich, St. Louis, MO) and pre-operative morphine (0.5 mg/kg; Sigma Aldrich, St. Louis, MO) were injected; 15 min later, rats were anesthetized with 90 mg/kg ketamine hydrochloride plus 10 mg/kg xylazine *i.p.* (Webster Veterinary Supply Inc., Kansas City, MO). A 22-gauge cannula (Plastics One, Roanoke, VA) was implanted into the right lateral ventricle (AP—1.3 mm; ML—1.8 mm; DV—3.4 mm) (Krivsky et al., 2006; Paxinos and Watson, 2007). Surgery duration was approximately 20 and 25 min for ovariectomy and cannula guide implantation, respectively. Post-operative morphine (2.0 mg/kg) then was administered. Behavioral testing (*Experiment 1*) or tissue and serum collection (*Experiments 2 and 3*) occurred 8–9 days after surgery.

2.3. Cyclic hormone administration

Estradiol-3-benzoate (E2) and progesterone (P4; Steraloids, Newport, RI) were dissolved in safflower oil, which served as the vehicle for both hormones. Vehicle and hormones were administered in a 0.1-mL volume. In all experiments, on post-surgery days 3 and 7 at

0700 h, either safflower oil (vehicle control) or E2 (2 μg) was administered s.c. to each female. Ten hours later, a second s.c. injection of either safflower oil or P4 (500 μg) was administered (see Fig. 1). This cyclic hormone regimen was based on one used by Asarian and Geary (Asarian and Geary, 2002) to mimic the natural fluctuation of E2 in gonadally intact, cycling females; the regimen was modified to include a P4 injection to also mimic fluctuating P4 levels found in cycling females. P4 was administered 10 h after E2 based on previous research showing that P4 levels peak 8–12 h after E2 levels in gonadally intact, cycling female rats. (Smith et al., 1975; Feder, 1981; Freeman, 1988; Haim et al., 2003) Rats in *Experiment 1* were randomly assigned to one of four hormone conditions (oil + oil, oil + P4, E2 + oil, or E2 + P4) and rats in *Experiment 2* were randomly assigned to one of two hormone conditions (oil + oil or E2 + P4) based on results from *Experiment 1*. All rats in *Experiment 3* were treated with both E2 and P4.

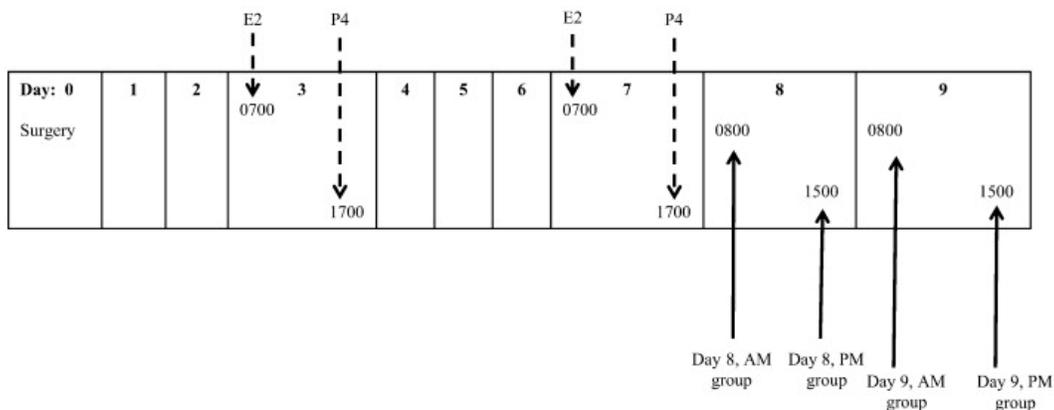


Fig. 1. Schematic representation of procedure for behavioral experiment (*Experiment 1*). On post-surgery days 3 and 7, females were given s.c. injections (dashed arrows) of oil or estradiol (E2) at 0700 and then 10 h later (1700) given oil or progesterone (P4). On days 8 and 9, separate groups of females were tested at 0800 and 1500 (solid arrows).

2.4. *Experiment 1: Cyclic ovarian hormone modulation of ICV THC antinociception and motor behavior*

Once two cycles of hormones were given (post-surgery days 3 and 7), behavioral testing was conducted at one of three time points following the last hormone (or oil) injection (see below). Each rat was tested in only one hormone condition, with either vehicle or THC, at one of the three time points. Rats were tested starting at 15 h (0800 h

on post-surgery day 8: "day 8, AM"), or 22 h (1500 h on post-surgery day 8: "day 8, PM"), or 39 h (0800 h on post-surgery day 9: "day 9, AM") after the last hormone (or oil) injection (see Fig. 1). These time points were chosen to capture the onset and offset of hormonal modulation of supraspinal THC-induced antinociception, based on a pilot study. There were 12–13 rats in each vehicle-treatment group and 15–16 rats in each THC-treatment group (day, dose, hormone condition) that were included in the final analysis. Baseline latency to respond was obtained by testing each subject on the tail withdrawal and paw pressure assays in that sequence, three times. For the tail withdrawal assay, the distal 5 cm of the tail was submerged in a 50 ± 0.5 °C warm water bath (2.5-L; Precision Scientifics Inc., Winchester, VA) and latency to withdraw the tail was measured to the nearest 0.01 s using a stopwatch. A cutoff of 20 s was used to avoid tissue damage. Paw pressure antinociception was assessed using an Analgesy-meter (Ugo-Basile, Varese, Italy). The pressure on the paw began at 30 g and increased at a constant rate of 48 g/s to a maximum of 990 g (20 s). Latency to withdraw or attempt to withdraw the hindpaw was measured to the nearest 0.1 s and a cutoff of 20 s was used.

After baseline testing, vehicle (1:1:8 ethanol/cremophor/saline) or 100 µg THC (National Institute on Drug Abuse, Bethesda, MD) was administered *i.c.v.* in a volume of 5 µL, at a rate of 20 µL/min; the injector was left in place for 1 min following infusion to reduce backflow up the cannula. Only one dose of THC was examined due to solubility limitations. Rats were then tested for tail withdrawal and paw pressure antinociception at 5, 15, 30, 60, 120 and 180 min post-injection. Horizontal locomotor activity was measured using a photobeam apparatus (Opto-varimex, Columbus Instruments, Columbus, OH) in which 15 photobeams (spaced 2.5 cm apart and 8 cm above the cage floor) cross the width of a 20 × 40 × 23-cm clear Plexiglas rodent cage. Locomotor activity was measured as the number of photobeams broken in a 5-min period, beginning immediately after nociceptive testing at 15, 30, 60, 120 and 180 min post-injection. Catalepsy (cutoff of 12 s) was assessed at 15 and 30 min post-injection only, to avoid practice effects. For the catalepsy test, the rat's forepaws were placed on a bar (ring stand with a 1.5-cm diameter horizontal bar set at approximately 12 cm above the table surface) and latency to remove both paws from the bar or jump onto

the bar was recorded to the nearest 0.01 s. Rats were returned to their home cage between testing periods.

Five to 10 min prior to the first baseline test and immediately after all behavioral testing was complete, a vaginal cell sample was obtained from each female via lavage to verify hormone manipulation. Proestrus (Pro) was identified by the predominance (approximately 75% or more of cells in the sample) of nucleated epithelial cells; proestrus to estrus (P/E, sometimes referred to as "late proestrus") (Bradshaw et al., 2006) was identified by approximately equal proportions of nucleated and cornified epithelial cells; estrus (Est) was identified by the presence of dense sheets of cornified epithelial cells; and diestrus (Diest) was identified by scattered nucleated and cornified epithelial cells and leukocytes (diestrus-1) or a relative lack of any cells (diestrus-2) (Freeman, 1988).

Following the last vaginal lavage, rats were euthanized and then decapitated. Giemsa dye (~ 0.10 mL, Sigma-Aldrich, St. Louis, MO) was injected into the cannula and the brain was extracted and sectioned to verify cannula placement. Uteri were harvested, fixed in 10% formalin, and later trimmed and weighed.

2.5. Experiment 2: Membrane preparation and [³H]SR141716A binding assays

Based on the results from the behavioral experiment (*Experiment 1*), tissue for the radioligand binding study was taken from separate groups of oil + oil-treated and E2 + P4-treated rats, either 15 h (0800 h on post-surgery day 8: "day 8, AM"—when hormone enhancement of THC's effect was first observed) or 46 h (1500 h on post-surgery day 9: "day 9, PM"—when hormone enhancement of THC's effect was no longer evident) after the last oil or hormone injection. Brains were quickly removed and placed on ice. The caudate putamen, hypothalamus, amygdala, periaqueductal gray (PAG) and cerebellum were immediately dissected. These structures were chosen because they have been shown to mediate cannabinoid antinociception (Lichtman et al., 1996; Martin et al., 1999) and motoric effects (Gough and Olley, 1977; Dar, 2000; Shi et al., 2005). Tissues were frozen in liquid nitrogen and stored at – 80 °C until protein estimation and binding was conducted.

Brain tissues were thawed and homogenized in 700 μ L TME buffer (28 mM Tris-HCl, 22 mM Tris Base, 3 mM MgCl₂, 1 mM EDTA, pH 7.4). PAG tissue was pooled from two females in the same condition in order to obtain sufficient protein concentration for each assay. Homogenates were centrifuged twice at 15,000g for 25 min at 4°C and the remaining pellet was re-suspended in the same volume of TME buffer. Protein concentrations were determined by the Pierce BCA method (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin (BSA) as a standard.

Saturation binding analysis was conducted by incubating 25 μ g membrane protein with 0.1–10 nM [³H]SR141716A (Perkin Elmer, Waltham, MA, USA) in TME buffer containing 0.5 g/L fatty acid free BSA in a final volume of 0.5 mL (Kearn et al., 1999; Breivogel, 2006; Wiley et al., 2008). Non-specific binding was determined by the presence of 1 μ M of unlabeled SR141716A (National Institute on Drug Abuse, Bethesda, MD, USA). Assays were conducted in duplicate and incubated at 30°C for 60 min (with gentle agitation). Using a harvester (Brandel, Gaithersburg, MD, USA), incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters that had been soaked in TME buffer containing 5 g/L fatty acid free BSA. Filters were washed three times with ice-cold TME buffer containing 2 g/L fatty acid free BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 60% efficiency for ³H after extraction of the filters in 3 mL of scintillation fluid (EcoScint, National Diagnostics, Atlanta, GA, USA). B_{max} (maximal binding site density) and K_D (equilibrium dissociation constant) were determined by nonlinear curve fitting of data to the one-site binding equation (Sigma Plot Version 11.0).

2.6. Experiment 3: Radioimmunoassays to determine serum hormone levels

To examine E2 and P4 serum levels at various time points after injection, a separate group of females was treated with two cycles of E2 + P4 as described above. Trunk blood was taken from 6–8 rats/time point, at the same times that behavioral testing was conducted (15, 22, 39, and 46 h after the last hormone/oil injection), plus at several earlier time points (4 and 6 h after the last E2 and P4 injections), to capture peak hormone levels. Blood samples were

centrifuged for 20 min at 2000 rpm at 4°C; serum was removed and stored at – 80°C until analysis. Hormone levels were determined via double antibody radioimmunoassay kits (estradiol (Cat # KE2D1) and progesterone (Cat # TKPG1): Siemens Healthcare Diagnostics, Los Angeles, CA, USA). Manufacturer's instructions were followed except for the E2 assay, in which samples and standards were extracted twice using methyl tert butyl ether (done in duplicate); after the ether evaporated, the antibody was added to the extraction tubes, which were incubated for 24 h at 4°C, followed by addition of the tracer and then overnight incubation.

2.7. Data analysis

In *Experiment 1*, only data from rats determined to have accurate cannula placements were included in analyses ($N = 19$ were removed). If post-injection backflow of fluid (possibly vehicle or THC) from the cannula was observed and less than 40% antinociception was observed on either nociceptive assay, all data from that rat were removed before any analysis ($N = 2$ from oil + oil group; $N = 7$ from E2 + oil group; $N = 6$ from oil + P4 group; $N = 3$ from E2 + P4 group). Vaginal samples from three females treated with oil + oil or oil + P4 indicated that they were in proestrus or estrus during testing, suggesting that an ovary was not fully removed; all data from these rats were removed before analysis. Baseline nociceptive latencies for each rat on the tail withdrawal and paw pressure tests were calculated as the mean of the three pre-injection trials. Individual nociceptive response latencies following vehicle or drug administration were converted to % maximum possible effect (%MPE): $(\text{drug latency} - \text{baseline latency}) / (\text{cutoff latency} - \text{baseline latency}) \times 100$. Time course %MPE data for both tail withdrawal and paw pressure were converted to area-under-the-curve (AUC) values using the trapezoidal rule. Because there were differences in locomotor activity across days in vehicle-treated rats, locomotor activity data in THC-treated rats were converted to % control, on each test day: $(\# \text{ photobeam breaks in THC-treated rat} / \text{mean } \# \text{ photobeam breaks in same-hormone, same-day, vehicle-treated control group}) \times 100$. Percent of control locomotor activity data were also converted to AUC values. A catalepsy score for each rat was obtained by averaging response latencies at 15 and 30 min post-injection. Some rats displayed behaviors that were incompatible with catalepsy (i.e., barrel

rolling, horizontal rocking, loss of muscle tone, lateral head movement or alternating forepaw movements) at one time point; for those rats a single catalepsy score was used for analysis. Because there were significant differences in catalepsy scores among vehicle-treated rats in the different hormone groups, catalepsy scores were converted to difference scores (catalepsy score for each THC-treated rat – mean catalepsy score for vehicle-treated controls in the same hormone group) before analysis. To control for differences in uterine weight that were due to differences in body weight, uterine weights (g) were divided by the rat's body weight in kg, before analysis.

For *Experiment 1*, antinociception AUC values and uterine weight were analyzed using a four-way ANOVA: day (3 levels), THC dose (2 levels), E2 (2 levels), and P4 (2 levels). Locomotor AUC values and catalepsy data were analyzed using a three-way ANOVA: day (3 levels), E2 (2 levels), and P4 (2 levels). Post-hoc comparisons were conducted using Bonferroni for comparison across days or two-way ANOVA for comparisons across hormone groups at different time points.

For *Experiment 2*, B_{\max} and K_D values for each brain structure were analyzed using a two-way ANOVA: day (2 levels) and hormone treatment (2 levels). Post-hoc comparisons were conducted using t-tests to compare across day and hormone treatment. For *Experiment 3*, a one-way ANOVA was used to compare serum levels of each hormone over time. Significance level was $P \leq 0.05$ for all statistical tests.

3. Results

3.1. Experiment 1: Cyclic ovarian hormone modulation of THC's effects on antinociception and motor behavior

3.1.1. Baseline (non-drug) responding

Average baseline tail withdrawal latency across all groups was 5.53 ± 0.04 s. Baseline tail withdrawal latency significantly differed between rats tested on different days (data not shown; $F(2, 133) = 3.69$, $P < 0.05$): tail withdrawal latencies in rats tested on day 8, PM (5.69 ± 0.12) were higher than those tested on day 9, AM (5.24 ± 0.12) ($P < 0.05$). However, when tail withdrawal latencies

were compared across the 180-min test period in rats given vehicle (1:1:8 ethanol/cremophor/saline), latency did not differ significantly among the four hormone groups on any of the test days. Average baseline paw pressure latency across all groups was 4.31 ± 0.04 s and did not differ significantly among rats tested on the different days or given different hormones. Additionally, paw pressure latency did not differ in vehicle-treated rats across the 180-min test period regardless of hormone group or test day.

3.1.2. Antinociception

Fig. 2A shows *i.c.v.* THC-induced tail withdrawal antinociception in OVX females treated with oil, E2, P4, or both hormones, and tested 15, 22, or 39 h after the last hormone or oil injection (i.e., on post-surgery day 8, AM, day 8, PM or day 9, AM). THC produced tail withdrawal antinociception (THC dose: $F(1, 310) = 246.74$, $P < 0.001$). Neither hormone, given alone or in combination, altered THC-induced tail withdrawal antinociception on any of the test days examined, nor were any significant interactions observed. Although P4 appeared to blunt THC-induced tail withdrawal antinociception on day 8, AM (Fig. 2A), this effect was not statistically significant (P4 \times THC dose \times day: $F(2, 310) = 0.55$, ns). Fig. 2B shows that *i.c.v.* THC produced paw pressure antinociception (THC dose: $F(1, 310) = 250.03$, $P < 0.001$). E2 significantly enhanced THC's antinociceptive effect (E2 \times THC dose: $F(1, 310) = 4.33$, $P < 0.05$), with no significant day or P4 interactions (E2 \times THC dose \times day: $F(2, 310) = 0.17$, ns; E2 \times P4 \times THC dose \times day: $F(2, 310) = 0.98$, ns).

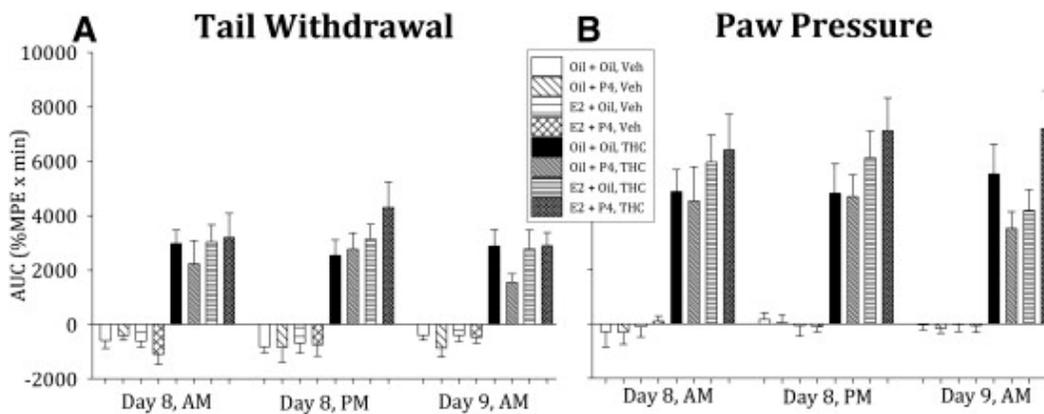


Fig. 2. Area-under-the-curve (AUC) values for *i.c.v.* THC-induced tail withdrawal (A) and paw pressure (B) antinociception in cyclic hormone groups tested 15, 22, or 39 h after the last hormone (or oil) injection (i.e., on post-surgery day 8, AM, day 8, PM or

day 9, AM). Each bar is the mean \pm 1 SEM of 12–16 rats. When collapsed across days and E2 group (E2 alone and E2 + P4), E2 significantly enhanced paw pressure antinociceptive effect of THC ($P < 0.05$).

3.1.3. Locomotor activity

Locomotor activity in vehicle-treated rats differed among groups tested at each day (data not shown; day: $F(2, 310) = 8.43$, $P < 0.001$). Specifically, locomotor activity was significantly lower on day 8, PM than on day 8, AM ($P < 0.001$) and on day 9, AM ($P < 0.05$). Neither hormone significantly altered locomotor activity AUC values on any test day, nor were any significant interactions observed (Fig. 3A; E2 \times P4 \times day: $F(2, 177) = 0.20$, ns; P4 \times day: $F(2, 177) = 1.15$, ns).

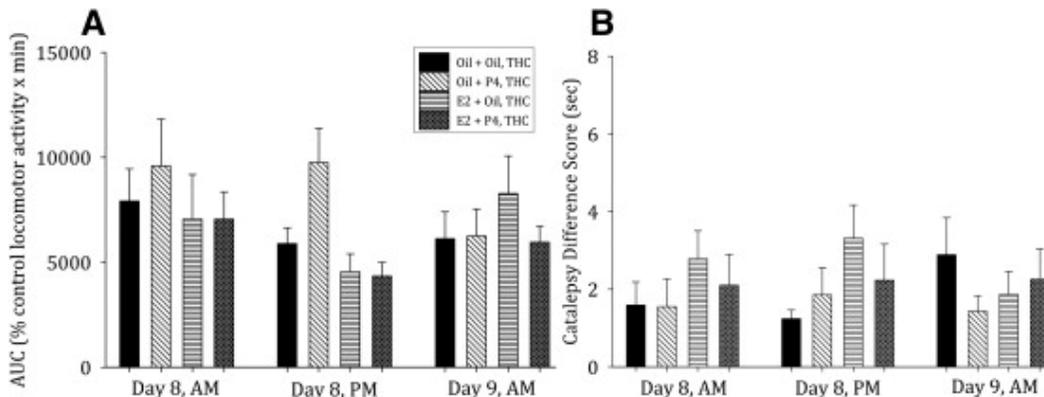


Fig. 3. Area-under-the-curve (AUC) values for *i.c.v.* THC-induced locomotor suppression (A) and catalepsy difference scores 15–30 min after *i.c.v.* THC (B) in cyclic hormone groups tested 15, 22, or 39 h after the last hormone (or oil) injection (i.e., on post-surgery day 8, AM, day 8, PM or day 9, AM). Locomotor data are presented as the percentage of same-hormone group, vehicle-treated controls. Catalepsy data are presented as the difference scores between THC-treated rats and vehicle-treated controls (see Data Analysis section). Each bar is the mean \pm 1 SEM of 12–16 rats.

3.1.4. Catalepsy

Average time spent on the bar for *i.c.v.* vehicle-treated females in the oil + oil, oil + P4, E2 + oil and E2 + P4 groups across all days was 1.10 ± 0.09 , 1.16 ± 0.09 , 1.33 ± 0.13 and 1.50 ± 0.16 s, respectively. E2-induced increases in time spent on the bar were significant in *i.c.v.* vehicle-treated females (Fig. 3B; E2: $F(1, 120) = 4.45$, $P < 0.05$). Fig. 3B shows the catalepsy difference scores for THC-treated females. Neither hormone, given alone or in combination, significantly altered THC-induced catalepsy on any day,

nor were there any significant interactions. It should be noted that other motoric effects of THC were observed in some rats: barrel-rolling or involuntary spinning of the whole body (Kelly et al., 1977; Wakley and Craft, 2011), and/or horizontal rocking and loss of muscle tone were observed in 33% of rats given THC (Table 1). Although barrel rolling was observed in 25% of the E2 + P4-, THC-treated rats, approximately 18% of the oil + oil-, THC-treated rats displayed the same behavior.

Table 1. Percent of THC-treated rats showing various motoric effects during the 15- and/or 30-min post-injection catalepsy measurements (# rats showing behavior/total # rats tested), and catalepsy score (mean \pm 1 SEM, in seconds).

Behavior	Oil + Oil	Oil + P4	E2 + Oil	E2 + P4
Loss of muscle tone ^a	4.3% (2/47)	10.9% (5/46)	10.4% (5/48)	8.3% (4/48)
Horizontal rocking ^b	8.5% (4/47)	4.3% (2/46)	18.8% (9/48)	2.1% (1/48)
Barrel-rolling ^b	19.0% (9/47)	6.5% (3/46)	10.4% (5/48)	25.0% (12/48)
Catalepsy ^b	2.83 \pm 0.33 (46/47)	2.81 \pm 0.39 (46/46)	3.98 \pm 0.41 (46/48)	3.66 \pm 0.39 (46/48)

^aRat slipped off bar immediately after forepaws were placed on bar.

^bBehaviors are not mutually exclusive; i.e., a rat could show both loss of muscle tone and barrel-rolling behavior.

3.1.5. Supraspinal effects of THC 46 h after the last hormone injection (day 9, PM)

Because enhancement of paw pressure antinociception persisted out to day 9, AM, in E2 + P4-treated females (Fig. 2B), separate groups of females were treated with oil + oil or E2 + P4 and then were tested 46 h after the last hormone injection (day 9, PM) to determine if this enhancement waned. On day 9, PM, tail withdrawal and paw pressure AUC values were comparable between oil + oil and E2 + P4 rats (data not shown; hormone \times THC dose: $F(1, 52) = 0.85$, ns for tail withdrawal; hormone \times THC dose: $F(1, 52) = 0.05$, ns for paw pressure). Similarly, no hormone effects on THC-induced locomotor suppression or catalepsy were observed (data not shown; hormone \times THC dose: $F(1, 52) = 0.01$, ns for locomotor activity; hormone \times THC dose: $F(1, 52) = 0.75$, ns for catalepsy). However, uterine weights of E2 + P4-treated females were still significantly increased compared to oil + oil-treated rats on day 9, PM (data not shown; hormone: $F(1, 52) = 210.81$, $P < 0.001$). Finally, on day 9,

PM, 54% of E2 + P4-treated rats were in Pro, 32% were in Est, and the remaining 14% were in P/E.

3.1.6. Vaginal cytology

Vaginal cytology was assessed both before and after the 3-h test period to obtain additional information regarding hormone status. Fourteen percent of females did not show the same vaginal cytology from pre-test to post-test and in these cases the pre-test cytology was used. Table 2 shows the percent of rats in each estrous stage in each hormone group across different test days. Oil-treated females (oil + oil) and P4-treated females (oil + P4) were all in the expected stage: 100% of all samples were diestrus (Diest). In contrast, approximately 75% of E2-treated rats (E2 + oil) were in proestrus (Pro) on day 8 (both AM and PM) with the remaining rats in either proestrus/estrus (P/E) or estrus (Est). On day 9, AM, E2-treated rats were predominately in Est (79%) with only 14% of samples showing vaginal cytology indicative of Pro. A majority of the rats (61%) treated with both E2 and P4 were in Pro on day 8, AM, with the remaining in either P/E or Est. However, only 21% of E2 + P4-treated rats tested on day 8, PM were in Pro; 50% were in Est. On day 9, AM, 78% of E2 + P4-treated rats were in either Pro or Est.

Table 2. Pre-test estrous stage of rats in each hormone group, as % of rats showing vaginal cytology indicative of proestrus (Pro), proestrus/estrus (P/E), estrus (Est), or diestrus (Diest) (N = 28–29 rats/hormone group/day).

Hormone treatment	Day 8, AM				Day 8, PM				Day 9, AM			
	Pro	P/E	Est	Diest	Pro	P/E	Est	Diest	Pro	P/E	Est	Diest
Oil + Oil	0	0	0	100	0	0	0	100	0	0	0	100
Oil + P4	0	0	0	100	0	0	0	100	0	0	0	100
E2 + Oil	75	14	11	0	72	14	14	0	14	4	82	0
E2 + P4	61	18	21	0	21	11	68	0	39	14	47	0

3.1.7. Uterine weight

Fig. 4 shows that E2 significantly increased uterine weight (E2: $F(1, 310) = 1611.37, P < 0.001$). P4 attenuated E2's effect on uterine weight (E2 × P4: $F(1, 310) = 13.01, P < 0.001$). Females given THC also had lower uterine weights compared to those given vehicle (THC dose: $F(1, 310) = 4.04, P < 0.05$).

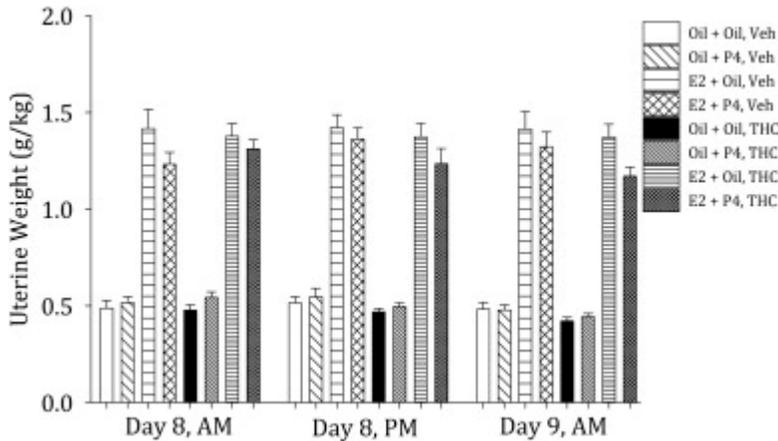


Fig. 4. Uterine weight (g/kg body weight) of cyclic hormone-treated OVX rats tested 15, 22, or 39 h after the last hormone (or oil) injection (i.e., on post-surgery day 8, AM, day 8, PM or day 9, AM). Each bar is the mean \pm 1 SEM of 12–16 rats.

3.2. Experiment 2: Cyclic ovarian hormone modulation of CBr receptor binding and affinity

Fig. 5 shows B_{\max} values and Table 3 shows K_D values, for oil- vs. hormone-treated groups when brain tissue was harvested on day 8, AM, or day 9, PM. These two time points were chosen as the earliest time at which hormone enhanced THC-induced antinociception and a later time point at which hormone had no effect on THC-induced antinociception in *Experiment 1*. Fig. 5A shows that in the PAG, E2 + P4 treatment increased B_{\max} on day 9, PM compared to oil-treated controls (hormone \times day: $F(1, 26) = 4.40, P < 0.05$). Additionally, B_{\max} was decreased in day 9, PM oil-treated controls compared to day 8, AM oil-treated controls ($t(14) = 2.65, P < 0.05$). In the PAG, changes in B_{\max} were not accompanied by changes in K_D values (Table 3; hormone \times day: $F(1, 26) = 0.33, ns$). Conversely, in the amygdala, neither day nor hormone treatment significantly affected B_{\max} or K_D values (Fig. 5B and Table 3; all $P_s > 0.05$).

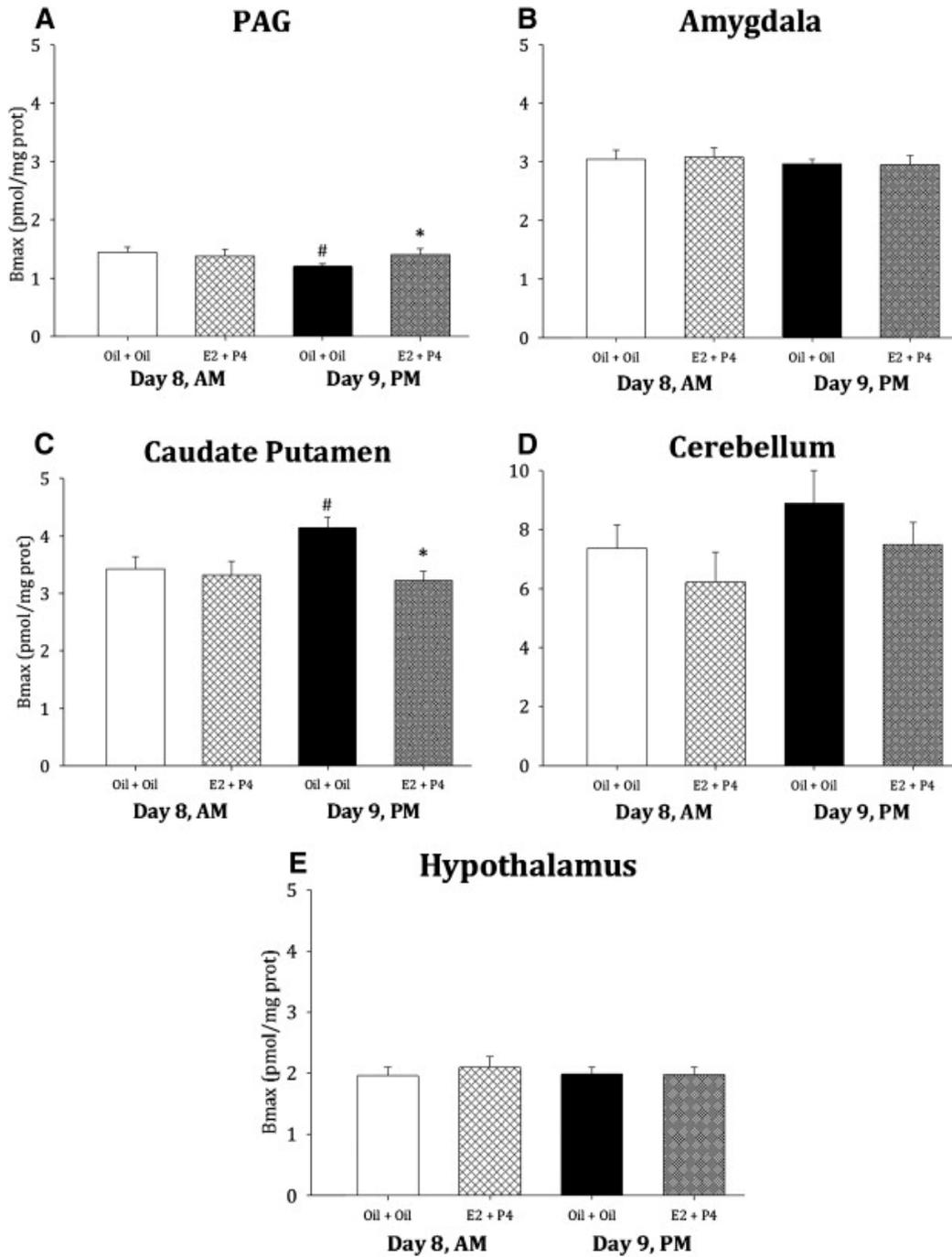


Fig. 5. Maximum binding capacity (Bmax) of cannabinoid receptors (CB₁) in the periaqueductal gray (A), amygdala (B), caudate putamen (C), cerebellum (D) and hypothalamus (E) of OVX female rats treated with cyclic oil + oil or E2 + P4. Brain tissue was harvested 15 h (day 8, AM) or 46 h (day 9, PM) after the last oil or hormone injection. Each bar is the mean \pm 1 SEM of 6–9 rats/hormone group. *Significantly different from same-day, oil-treated females, $P < 0.05$. #Significantly different from oil-treated day 8, AM

females, $P < 0.05$. Note: The y-axis scale of cerebellum (D) is larger than other brain regions due to high density of CBr found in this structure.

Table 3. Binding affinity of [³H]SR141716A (nM, mean ± 1 SEM) in OVX female rats treated with cyclic oil + oil or E2 + P4.

Day	Hormone group	Brain structure				
		Caudate Putamen	Amygdala	Hypothalamus	PAG	Cerebellum
Day 8, AM	Oil + Oil	0.45 ± 0.04	0.39 ± 0.02	0.36 ± 0.03	0.39 ± 0.04	0.54 ± 0.06
	E2 + P4	0.35 ± 0.04	0.38 ± 0.04	0.45 ± 0.09	0.39 ± 0.06	0.41 ± 0.04
Day 9, PM	Oil + Oil	0.54 ± 0.06*	0.40 ± 0.04	0.43 ± 0.05	0.34 ± 0.03	0.62 ± 0.07
	E2 + P4	0.45 ± 0.03**	0.48 ± 0.06	0.40 ± 0.05	0.42 ± 0.05	0.54 ± 0.06

Brain tissue collection occurred at 15 h (day 8, AM) or 46 h (day 9, PM) after the last oil or hormone injection. Each bar is the mean ± 1 SEM of 6–9 rats/hormone group.

*Significantly different from oil-treated day 8, AM females, $P < 0.05$.

**Significantly different from same-day, oil-treated females, $P < 0.05$.

Fig. 5C shows that in the caudate putamen, E2 + P4 treatment decreased B_{max} compared to oil-treated controls only on day 9, PM (hormone × day: $F(1, 27) = 4.45$, $P < 0.05$). Unexpectedly, B_{max} was increased in oil-treated day 9, PM females compared to oil-treated day 8, AM females ($t(13) = 2.61$, $P < 0.05$). In the caudate putamen, E2 + P4 treatment also decreased K_D compared to oil treatment (hormone: $F(1, 27) = 6.39$, $P < 0.05$), and K_D values were decreased on day 8, AM compared to day 9, PM (Table 3; day: $F(1, 27) = 6.06$, $P < 0.05$). In the cerebellum, E2 + P4 treatment appeared to decrease B_{max} ; however, this effect was not significant (Fig. 5D; hormone: $F(1, 24) = 2.21$, $P = 0.15$). Neither day nor hormone treatment significantly altered K_D (all $P_s > 0.05$). Lastly, Fig. 5E and Table 3 show that in the hypothalamus, day and hormone treatment did not alter B_{max} or K_D values (all $P_s > 0.05$).

3.3. Experiment 3: Serum levels of E2 and P4 using a cyclic hormone regimen

Fig. 6 shows that serum E2 reached peak levels by 16 h after the s.c. E2 injection given on day 7 at 0700 h, and levels were low by 25 h after injection (time: $F(7,47) = 2.99$, $P < 0.05$). Serum P4 reached peak levels by 4 h after the s.c. P4 injection given on day 7 at

1700 h, and levels declined over the next 18 h (Fig. 6; time: $F(7,49) = 17.96, P < 0.001$).

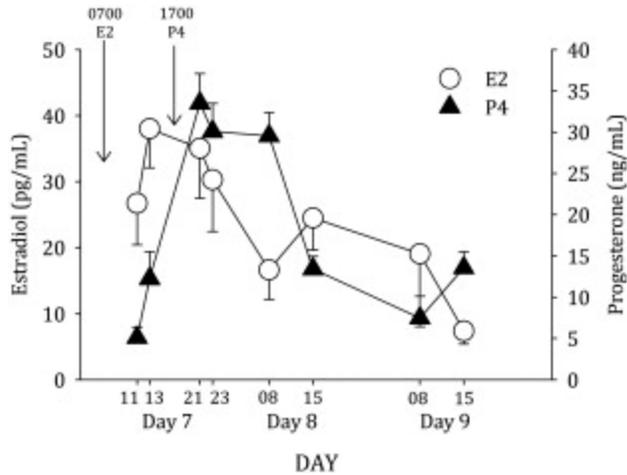


Fig. 6. Serum levels of estradiol (E2; pg/mL) and progesterone (P4; ng/mL) at various points following hormone injections given on post-OVX day 7. Each point is the mean \pm 1 SEM of 6 rats.

4. Discussion

The present study demonstrates that ovarian hormones alter sensitivity to supraspinal cannabinoid antinociception in female rats. In OVX females, E2 enhanced *i.c.v.* THC-induced mechanical antinociception, whether E2 was given alone or with P4. THC-induced antinociception was enhanced approximately 24–48 h after hormone injection. Ovarian hormones did not significantly modulate THC-induced locomotor suppression or catalepsy, and therefore enhanced antinociception does not appear to be simply due to enhanced motoric effects. Ovarian hormones also altered CBr density in PAG and striatum and CBr affinity in striatum, but these changes did not parallel behavior in the predicted manner.

The first finding, E2 enhancement of THC-induced mechanical antinociception, corroborates previous work from our laboratory. We first reported E2 enhancement of THC-induced antinociception following systemic THC administration in OVX female rats (Craft and Leitl, 2008). This hormone enhancement was similar to that observed in estrous compared to diestrous females receiving *i.p.* THC (Craft and Leitl, 2008), and in P/E compared to estrous females and males receiving *i.c.v.* THC (Wakley and Craft, 2011). The present study provides further evidence that E2 can act supraspinally to enhance

females' response to THC. Together, these studies indicate that sex and estrous stage differences in cannabinoid antinociception are due to E2 in females.

In the present study, E2 enhanced only THC-induced mechanical antinociception. In all of our studies, THC-induced mechanical antinociception appears to be more sensitive than THC-induced thermal antinociception to ovarian hormone modulation (Tseng and Craft, 2001; Craft and Leidl, 2008; Wakley and Craft, 2011; Craft et al., 2012). E2 modulation of cannabinoid antinociception on one nociceptive test but not the other may reflect differences in the extent to which each nociceptive response engages spinal vs. supraspinal mechanisms. THC-induced tail withdrawal antinociception has been shown to be more spinally than supraspinally mediated (Smith and Martin, 1992). There is no direct evidence that the paw withdrawal response is supraspinally mediated, however, the postural adjustments required for this response likely involve supraspinal components (e.g., cerebellum, basal ganglia). Therefore, it is possible that E2 enhancement of THC-induced antinociception was greater on the paw pressure than on the tail withdrawal assay due to a larger contribution of supraspinal mechanisms in the paw withdrawal response, and greater cannabinoid and/or E2 effects supraspinally than spinally. In the future, it will be important to determine whether E2 enhancement of cannabinoid antinociception can also occur at the spinal level, and whether this phenomenon is sensory modality-specific.

This is the first study to examine the effects of ovarian hormones on CBr density and affinity in the PAG, a region of the brain involved in cannabinoid-mediated antinociception (Martin et al., 1995, 1999; Lichtman et al., 1996). We found a significant increase in receptor density on day 9 PM but not on day 8 AM. Thus, hormone-induced increases in CBr density did not parallel hormone-induced increases in behavioral sensitivity to THC. Further work will be necessary to fully characterize the time course of hormone-induced brain CBr changes, so that the temporal relationship between enhanced antinociception and CBr can be more precisely compared. Also, the effects of E2 alone on binding parameters need to be determined, as binding results from the present study are limited by examining only the E2 + P4-treated group. Finally, it is possible that a change in CBr density is not responsible or only partly responsible for

E2 enhancement of THC effect. Given the importance of PAG signaling in the antinociceptive effect of THC (Vaughan et al., 2000), it will be important to carefully examine the ability of ovarian hormones to upregulate CBr in this region.

We also found significant ovarian hormone-dependent changes in binding parameters in the striatum. Interestingly, especially given the lack of significant interaction between hormones and THC on locomotor behavior, we found opposing changes in striatal binding parameters, with hormone decreasing CBr density but increasing affinity. These changes may be functionally related, with a change in one variable causing a subsequent response in the other, resulting in stable cannabinoid signaling. The fact that this ovarian hormone regimen produced both an increase and decrease in B_{max} in different brain regions indicates that multiple regulatory pathways may be involved. Finally, we found no changes in CBr density or affinity in the hypothalamus, amygdala or cerebellum.

Our methodology differs from previous investigations of the role of ovarian hormones on the regulation of CBr signaling. This is the first study to demonstrate that a cyclic hormone regimen (to mimic normal hormone fluctuation) produced changes in CBr density and affinity and that CBr density changes over time following ovariectomy. Although the effects of chronic and acute E2 or acute P4 on CBr in different brain regions have been examined previously (Bonnin et al., 1992, 1993; Rodriguez De Fonseca et al., 1994; Riebe et al., 2010), only one has examined the combined effects of both ovarian hormones: E2 + P4 given simultaneously increased CBr density compared to oil-treated controls, but only in the hypothalamus; no significant differences between the hormone group and oil-treated controls were observed in any other brain region (Rodriguez De Fonseca et al., 1994). Furthermore, in the present study, E2 + P4 increased binding affinity in the caudate putamen, whereas (Rodriguez de Fonseca and colleagues 1994) did not find group differences in affinity in this brain region or any other. We utilized a cyclic hormone regimen (2 cycles of 2 μg E2/rat and 500 μg P4/rat, with E2 and P4 given 10 h apart) with tissue collection at 15- and 46-h after the last hormone injection, whereas previous work used lower (0.02–0.03 μg E2/rat and 38–50 μg P4/rat) or higher (10 μg E2/rat) hormone doses; single or concomitant hormone injections; or a shorter pretreatment time, with tissue

collection occurring either 1 or 48 h after hormone injections (Rodriguez De Fonseca et al., 1994; Riebe et al., 2010). Taken together, the present and previous results suggest that ovarian hormones regulate expression of cannabinoid receptors in a highly time- and region-specific manner.

Ovarian hormones could also enhance THC-induced antinociception through other mechanisms. For example, E2 may modulate cannabinoid effects through regulating N-type voltage-gated calcium channels (Lee et al., 2002; Adams et al., 2010) or other signaling mechanisms in the descending pain pathway to produce enhancement of THC antinociception. E2 has also been found to potentiate the presynaptic inhibition of GABA produced by WIN55,212 in proopiomelanocortin neurons of the arcuate nucleus (Nguyen and Wagner, 2006), although the precise mechanism of this modulation remains unknown. If E2 produces a similar effect in the PAG, inhibiting the release of GABA such that output neurons are disinhibited, antinociception would be enhanced.

Cyclic administration of E2 and P4 was used in the present study to mimic the normal hormone fluctuation of a gonadally intact cycling female rat. Although the time course of serum hormone levels was similar to that observed in cycling females, reproductive tissue indices revealed that this hormone regimen still was not precisely modeling a normally cycling female. Normally cycling females show peak levels of E2 and P4 between 40–45 pg/mL and 50–55 ng/mL, respectively, with E2 and P4 peaks approximately 8–12 h apart (Smith et al., 1975; Haim et al., 2003) – which is similar to what we observed in the present study. However, uterine weights and vaginal cytology did not show the progressive, rapid changes that are characteristic of cycling females post-ovulation, but instead were indicative of a lingering E2 presence. Additionally, E2 enhancement of THC-induced antinociception in OVX females was prolonged compared to that observed in intact cycling females, occurring up to 49 h after the last E2 injection. In normal cycling females, the transition from late proestrus to estrus occurs in approximately 4 h (Feder, 1981) and enhancement of THC-induced antinociception in late proestrous females was absent in estrous females (Wakley and Craft, 2011). The prolonged effects of E2 on antinociception and on reproductive tissue were likely due to the use of estradiol-3-benzoate, which is longer-

acting than 17 β -estradiol (Becker et al., 2005). Consequently, 17 β -estradiol should be used to more accurately mimic natural cycling in females, as this would allow for more accurate characterization ovarian hormone modulation of drug sensitivity.

In conclusion, the present study shows that the ovarian hormone E2 enhances female rats' sensitivity to supraspinal cannabinoid-induced antinociception. Using a cyclic hormone regimen, we found that E2 alone and in combination with P4 enhanced mechanical antinociception produced by *i.c.v.* THC, when THC's effects were measured approximately one day after E2 administration. Therefore, previously observed enhancement of *i.c.v.* THC in proestrous–estrous females is likely due to estradiol modulation of supraspinal mechanisms. Ovarian hormones did not modulate THC-induced locomotor suppression or catalepsy, and therefore hormone effects on motor function do not appear to explain the observed enhancement of antinociception. Ovarian hormones time-dependently modulated CBr in brain structures that mediate antinociception and locomotor activity, but the changes observed in CBr did not parallel changes in behavior. Definitive causality between the receptor changes and enhanced sensitivity to THC cannot be ruled out since CBr density was only measured at the beginning and after the period of enhanced antinociception, rather than across the entire time course. Examination of hormone effects on downstream signaling of CBr may yield additional mechanistic explanations for hormone modulation of supraspinal cannabinoid effects.

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