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RESEARCH PAPER

Cannabinoid self-administration in rats: sex differences and the influence of ovarian function

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Background and purpose: We recently demonstrated the existence of strain differences in self-administration of the cannabinoid CB_1 receptor agonist WIN55,212-2 (WIN) by Long Evans (LE) and Lister Hooded (LH) but not Sprague-Dawley (SD) male rats. This follow-up study is aimed at verifying whether sex and ovarian hormones might also be critical factors in the initiation, retention and extinction of WIN self-administration.
Experimental approach: LE, LH and SD male and female rats, the latter either intact or bilaterally ovariectomized (OVX), were trained to self-administer WIN (12.5 $\mu g\ kg^{-1}$ per infusion) under a FR1 reinforcement schedule, using lever-pressing.
Key results: Data showed that contrary to the findings in SD rats, LE and LH rats developed robust cannabinoid intake, with rates of responding for WIN being constantly higher in intact females than in males ($+45$ and $+42\%$ for LE and LH strains, respectively). In comparison with intact females, OVX females of both strains acquired self-administration at lower rates, displaying slower acquisition, lower drug intake (-42 and -52% for LE and LH, respectively) and longer extinction.
Conclusions and implications: These findings provide the first evidence of significant sex differences in cannabinoid self-administration, females acquiring stable WIN intake at higher rates and more rapidly than males. Moreover, when compared to intact females, a lower percentage of LE and LH OVX rats acquired and maintained stable drug intake, suggesting that ovarian hormones might represent a critical factor in modulating the reinforcing effect of cannabinoids.
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Keywords: CB_1 receptor; WIN55,212-2; sex differences; strain; cannabinoid self-administration; ovarian cycle; addiction; behaviour

Abbreviations: LE, Long Evans; LH, Lister Hooded; OVX, ovariectomized; SA, self-administration; SD, Sprague–Dawley; THC, Δ9-tetrahydrocannabinol; WIN, WIN55,212-2 (R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]-pyrrolo [1,2,3-de]-1,4 benzoxazinyl]-(1-naphthalenyl)-methanone mesylate)

Introduction

Addiction is an extremely complex disorder in which genetic background, personal traits, environment, responses to stress and comorbidity act as major interacting elements (for comprehensive reviews see Brady and Randall, 1999; Lynch *et al*., 2002; Lynch, 2006). Evidence generated thus far indicates that biological responses, long-term effects and factors triggering drug abuse may differ considerably between the sexes, thus requiring different prevention and treatment strategies (Franconi *et al*., 2007; Niv and Hser, 2007). A marked increase in substance abuse and dependence has been observed in women (Ridenour *et al*., 2005; Lejuez *et al*., 2007); nevertheless, only recently have researchers started to focus on women and drug abuse disorders. A biological basis of sex differences is likely to underlie vulnerability to drug abuse as well as all phases of the addiction cycle, women being seemingly more prone than men to abuse and more responsive to the rewarding effects of addictive drugs (Roth *et al*., 2004; Lynch, 2006). For example, women are more sensitive than men to the physiological effects of alcohol (Walter *et al*., 2005), typically report a higher use of cocaine (Kosten *et al*., 1993; Hu *et al*., 2004), display shorter or less frequent periods of abstinence from nicotine (Bohadana *et al*., 2003) and have an earlier onset of heroin use (Chen *et al*., 1998).

Although to date in clinical research little appropriate attention has been paid to possible sex differences in smoking habits and drug taking, accumulating preclinical evidence suggests that men and women may differ in developing and maintaining drug self-administration (SA)

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behaviour (Hernandez-Avila *et al*., 2004; Roth *et al*., 2004). Indeed, sex differences in SA behaviour have been described in the acquisition of cocaine (Lynch *et al*., 2000; Hu *et al*., 2004), opioids (Lynch and Carroll, 1999), nicotine (Donny *et al*., 2000), phencyclidine (Carroll *et al*., 2000) and methamphetamine (Roth and Carroll, 2004) SA, as well as in the retention of such a behaviour (Klein *et al*., 1997; Almeida *et al*., 1998; Perkins *et al*., 1999; Vivian *et al*., 2001; Cicero *et al*., 2003; Carroll *et al*., 2005). Sex-dependent differences have been described during extinction of SA behaviour as well, that is, when response is no longer reinforced by contingent presentation of the drug (Perkins *et al*., 1999; Fuchs *et al*., 2005; Kippin *et al*., 2005; Lynch *et al*., 2005). Importantly, sex differences in drug abuse have also been detected in other behaviours predictive of, or connected to, reward and addiction, such as spontaneous locomotor activity (Krasnoff and Weston, 1976), conditioned place preference (Cirulli and Laviola, 2000), intracranial self-stimulation (Cohen and Lieblich, 1981) and ingestion of pleasurable/fat food (Ackroff and Sclafani, 2004; Geary, 2004), where women typically show increased level in motor activity, higher sensitivity to addictive drugs and enhanced reward incentive.

In the majority of animal models of reward and addiction, cannabinoids have proved to work as positive reinforcers and to sustain both conditioned and operant behaviours. Thus, cannabinoid CB_1 receptor agonists potentiate the rewarding effects of electrical brain stimulation (Lepore *et al*., 1996), induce conditioned place preference (Lepore *et al*., 1995), sustain intracerebral SA (Braida *et al*., 2001), and maintain intravenous SA behaviour in drug-naive mice (Martellotta *et al*., 1998), trained rats (Fattore *et al*., 2001; Spano *et al*., 2004) and monkeys (Justinova *et al*., 2003). Accordingly, cannabinoids enhance dopaminergic neurotransmission in the shell of the nucleus accumbens (Fadda *et al*., 2006) and increase the firing rate of dopaminergic neurons in brain limbic areas (French *et al*., 1997; Gessa *et al*., 1998).

A better understanding of sex differences in cannabis dependence may improve knowledge of the dynamics of marijuana smoking and enhance development of treatment regimens. Studies on this fundamental issue are urgently needed due to the current lack of evidence on a possible role of sex in cannabinoid reinforcement and behaviours related to reward. We recently showed that cannabinoid SA is significantly affected by strain and operant schedule, as Long Evans (LE) and Lister Hooded (LH), but not Sprague–Dawley (SD), male rats differentially acquired, maintained and extinguished SA of the cannabinoid CB_1 receptor agonist WIN55,212-2 (WIN; Deiana *et al*., 2007).

The present follow-up study was therefore undertaken to evaluate the hypothesis that similar to other drugs of abuse, acquisition, maintenance and extinction of cannabinoid SA may also be related to the sex of the animal.

To this purpose, male and female rats from the same strains (that is, SD, LE and LH) were trained to self-administer WIN as previously described (Fattore *et al*., 2001), and their performance was compared across all stages of cannabinoid SA. The reason for using the synthetic CB_1 receptor agonist WIN rather than the main psychoactive ingredient of marijuana, Δ9-tetrahydrocannabinol (THC), lies in the observation that THC failed to sustain drug-seeking and drug-taking behaviour in rodents (Mansbach *et al*., 1994; Fattore *et al*., 2002), while WIN has been reported to reliably develop SA behaviour in both drug-naive mice (Martellotta *et al*., 1998), LE and LH trained rats (Spano *et al*., 2004; Fadda *et al*., 2006), in a dose-related manner (Fattore *et al*., 2001) and under different schedules of reinforcement (Solinas *et al*., 2007) or response-like operanda (Deiana *et al*., 2007).

Following the finding of a more robust cannabinoid SA in females, we decided to examine the role of endogenous hormones by ovariectomizing female rats and comparing their responses with those of intact female rats. Results of this second part of the study indicated that ovarian hormones were a major factor underlying sex-related differences in cannabinoid SA behaviour.

Materials and methods

Animals

All experiments and animal procedures were approved by the Local Animal Care Committee and carried out in strict accordance with both the Guidelines for the Care and Use of Laboratory Animals (NIH) and the EC regulations for animal use in research (CEE no. 86/609). All efforts were made to minimize animal suffering and reduce the number of animals used. The health of rats was monitored daily by experimenters and periodically by a consultant veterinarian.

Drug-naive female and male LE, LH and SD rats (Harlan Nossan, Milan, Italy) weighing 250–300 g at the beginning of the study were used. All rats were housed four to a cage, with unlimited access to food and water until time of testing. Rats were allowed 7 days to acclimatize in a temperature-($\pm 21\,^{\circ}C$) and humidity (60%)-controlled room with a 12-h reversed light/dark cycle (dark on 0700 hours), and were handled once daily for approximately 10 min. Experimental procedure started 1 week after surgery and took place 5 days per week at the same time each day during the dark phase of the cycle (between 0900 and 1200 hours). From the first day of SA training onwards, rats were kept on a restricted diet, receiving a daily ration (20 g) of rat chow in the home cage at the end of each session, sufficient to maintain animals at approximately 85% of free-feeding body weight. Daily food amount was always fully consumed by animals, and no significant weight differences were observed among the experimental groups during the entire study.

Apparatus

Behavioural training and testing were conducted in 10 standard experimental chambers with lever-pressing as the test response ($29.5 \times 32.5 \times 23.5$ cm; Med Associates, St Albans, VT, USA) enclosed in a sound- and light-attenuating wooden box equipped with a fan for ventilation. A stainless-steel wall was mounted with two standard response retractable levers (each 4 cm wide), positioned 12 cm apart and 8 cm from the grid, and extending 1.5 cm into the box. A yellow stimulus light was located 5 cm above each lever, and a white house light, constantly illuminated during the

session except during infusion, was located on the opposite wall of the chamber. Intravenous infusions of WIN were delivered through a software-operated infusion pump (Med Associates) connected via an extra length of Silastic tubing to a single-channel swivel mounted on a counterbalanced arm to allow animals to move freely around the chamber, and a length of plastic tubing enclosed in a metal spring connecting the swivel to the catheter fitting on the animal's back. Infusion pumps were mounted outside each chamber.

Pressure of one lever, defined as *active*, resulted in: (1) extinction of the house light and illumination of the stimulus light above the active lever, which remained on for 5 s, (2) retraction of both levers for 20 s (time-out period, TO) and (3) activation of the infusion pump for 5.8 s, delivering 0.1-ml intravenous infusion of 12.5 $\mu g\,kg^{-1}$ WIN solution. On completion of the TO period, both levers were re-extended into the chamber, the stimulus light went out and the house light was switched on. Depressions on the other lever, defined as *inactive*, were not coupled to any succeeding event but were always recorded thus providing an index of basal activity levels. Assignment of the active (drug-paired) and inactive (not drug-paired) lever was counterbalanced between rats, remaining constant for each subject throughout the study. During each 2-h daily session, behavioural schedule programming, data collection (that is, the number of active and inactive lever-press) and storage were controlled by an IBM-compatible computer with Med-PC interface (Med Associates).

Surgical procedures

Ovariectomy. At 9–10 weeks of age (approximately 200–225 g), female rats of the three strains ($n = 8$–10) underwent bilateral ovariectomy under deep anaesthesia with an i.p. injection of Equithesin (0.97 g pentobarbital, 2.1 g $MgSO_4$, 4.25 g chloral hydrate, 42.8 ml propylene glycol, 11.5 ml ethanol 90%; 0.5 $ml\,kg^{-1}$).

Ovariectomy was performed by means of a single ventral transverse incision along the abdominal wall muscles and a small incision through the muscles to separate subcutaneous tissues from the abdominal wall. The peritoneum was then punctured, the ovary was externalized from the abdominal wall and removed at the junction of the oviduct and the uterine body. The uterus with associated tissue was then returned to the abdomen; the same procedure was repeated on the other side. After bilateral removal of the gonads, the wound was closed with surgical sutures. A daily injection (s.c.) of Baytril (Bayer, 0.1 ml) was administered as a postoperative antibiotic for at least 5 days. Animals were left 3 extra weeks after gonadectomy for stabilization of gonadal and pituitary hormones (Peris *et al*., 1991) before undergoing catheter implantation surgery.

Implantation of intravenous catheters

Male (11–12 weeks old) and female (approximately 3 weeks after ovariectomy) rats were surgically implanted with a chronic indwelling catheter (CamCaths, Cambridge, UK) into the right jugular vein, as previously described (Fattore *et al*., 2001). Following surgery, each rat was individually housed and received daily injections of Baytril (0.1 ml, s.c.) for at least 7 days as postoperative treatment to prevent infection.

The patency of catheters was ensured daily before each SA session by flushing with 0.20 ml of a heparinized (1%), sterile saline solution. If a catheter was found to be not patent or damaged, a second catheter was implanted into the left jugular vein if possible, and testing resumed 7 days later.

Experimental procedure

For each rat strain, animals were divided into three groups: (1) gonadally intact males, (2) gonadally intact females and (3) ovariectomized (OVX) females.

Phase 1: acquisition

Self-administration training was initiated by providing access to WIN (12.5 $\mu g\,kg^{-1}$ per infusion) through lever-pressing, as previously described (Deiana *et al*., 2007). Animals were given a priming infusion in the experimental chamber before the start of each daily session. Acquisition training was carried out until steady baseline of drug intake was reached, typically within 21 days. Response was considered stable when animals displayed accurate discrimination between the active and the inactive lever with the number of active lever-presses not differing by more than 20% for 3 consecutive days.

Rats not meeting the acquisition criterion were excluded from the subsequent phases of the study. A percentage acquired variable was determined for each group by considering an animal as having acquired SA behaviour on displaying at least 12 active responses and less than 7 inactive responses over 3 consecutive days.

Phase 2: maintenance

Once animals had displayed accurate discrimination between the active and the inactive lever, no further priming infusion was administered throughout the rest of the study. Only rats developing a stable pattern of WIN intake were allowed to continue daily SA sessions for 5 extra days before introducing extinction conditions.

Phase 3: extinction

Responses to WIN were extinguished by replacing cannabinoid with sterile vehicle solution (1% Tween 80 in saline solution) allowing response to be recorded without drug consequences, leaving all other experimental parameters unchanged. Drug-reinforced behaviour was considered extinguished when response on the active lever was decreased by at least 85% for 3 consecutive days, occurring typically within 10 days for intact animals, but requiring longer periods for OVX group.

Statistical analysis

Lever-pressing activity during the three successive phases of the experiment (that is, acquisition, maintenance and

extinction) was first compared between intact females and males, and then between intact and OVX females by means of two-way ANOVA with s*ex* or s*train* as between-subject factor and *day* as within-subject factor. Once statistical significance was reached, the Bonferroni *post hoc* test was used for individual mean comparisons. With the exception of the initial days of the training period, response on the inactive lever remained minimal and was therefore not computed in data analysis. Differences with a $P<0.05$ value were considered as significant.

Drugs

WIN55,212-2 (Tocris, Bristol, UK) was dissolved in one drop of Tween 80 and diluted in sterile physiological saline solution, in a fixed volume of injection of 100 μl. To ensure sterility, drug solutions were filtered through 0.22-μm syringe filters prior to use. All antibiotics and anaesthetics were purchased as sterile solutions from a local distributor.

Results

Across SA training, four animals (one male LE, one LH intact female and two OVX LE rats) did not complete all phases of the study due to catheter blockade or disruption and were therefore not included in the statistical analysis, while one OVX LH rat did not fully recover from the second surgical procedure (catheter implantation).

Strain-related differences in SA behaviour

Analysis of variance revealed that male and female LE and LH, but not SD rats were capable of acquiring and retaining stable cannabinoid SA, as defined by significant differences ($P<0.01$) between responses in the active vs inactive lever. With the sole exception of the initial days of training (that is, beginning of the acquisition phase), responses on the inactive lever were negligible throughout the entire study and did not differ among groups (data not shown).

Table 1 illustrates the percentage of each group of rats meeting acquisition criteria for WIN self-administration. Intact LE and LH females acquired firm cannabinoid SA at higher rates than corresponding OVX and to an even greater extent compared to corresponding males, with intact LE females rising up to 87.5%.

Conversely, SD rats did not acquire stable operant behaviour, specifically, few SD female rats (two intact and one OVX rats) showed swinging response to WIN but never stabilized drug intake or discrimination between active and inactive levers, while male SD rats did not initiate response at all.

Table 1 Percentage of male and female, intact and ovariectomized (OVX) Lister Hooded (LH), Long Evans (LE) and Sprague–Dawley (SD) rats that acquired stable WIN55,212-2 self-administration

	LE (%)	LH (%)	SD (%)
Male	66.6	60	0
Intact female	87.5	75	0
OVX female	77.7	70	0

A minimum of 12 responses over 3 consecutive days during the second or third week of training was taken as the 'acquisition' criterion.

Notably, while male and female LE and LH rats proved capable of compensating following changes to the available unit dose of WIN, SD did not develop SA behaviour even subsequent to presentation of lower (6.25 $\mu g\,kg^{-1}$ per infusion) or higher (25 and 50 $\mu g\,kg^{-1}$ per infusion) doses of WIN (data not shown).

Sex-related differences: female versus male rats

Acquisition. Figure 1 shows the mean (±s.e.mean) number of active responses over 25 days of SA of the cannabinoid WIN, in LE (left panel) and LH (right panel) female and male rats.

In the LE strain, females displayed a significantly ($P<0.01$) faster acquisition of cannabinoid SA and maintained robust response rates throughout the training period. *Post hoc* comparisons indicated that, from day 13 onwards, differences in the response rates between females and males became more pronounced, as females gradually increased the number of active lever-presses at a significantly ($P<0.001$) higher level than males (+44%). However, by the end of the third week of training, males had also reached stable rate of SA, although at a lower rate than females (mean (±s.e.mean) over the last 5 days of training: 18±0.3 and 30±0.3, respectively). Two-way ANOVA revealed a significant effect of sex ($F(1,300) = 144.70$; $P<0.0001$), day ($F(24,300) = 19.83$; $P<0.0001$) and day × sex interaction ($F(24,300) = 2.2$; $P<0.001$).

With respect to the LH strain, no statistically significant differences in the acquisition rate of cannabinoid SA between females and males were found during the first 2 weeks of training ($P>0.05$), as the mean number of active responses did not differ significantly. However, by day 13, LH female rats displayed a significant ($P<0.01$) upward shift (+39%) in active responses with respect to males (Figure 1). Two-way ANOVA revealed a significant effect of sex ($F(1,300) = 112.79$; $P<0.0001$), day ($F(24,300) = 12.42$; $P<0.0001$) and day × sex interaction ($F(24,300) = 4.02$; $P<0.001$).

Notably, when comparing females from the two strains, two-way ANOVA highlighted significant differences by day ($F(24,300) = 22.24$; $P<0.001$), LE females acquiring WIN self-administration faster than LH females, but not strain or strain × day interaction, as both strains displayed very similar levels of response rate (29.4±2.4 and 29.9±1.9, respectively). Both male and female LE and LH increased lever-pressing activity over time on the active but not the inactive lever (data not shown), thus indicating that increase in response was solely a result of cannabinoid-seeking behaviour.

Maintenance. Results from the 5-day maintenance phase of cannabinoid SA are given in Figure 2 for males and females from both strains. Relative to male LE rats, LE females displayed a markedly higher rate of response (+45%), the mean total amount of self-administered cannabinoid during the 2-h session being equal to 245±0.6 and

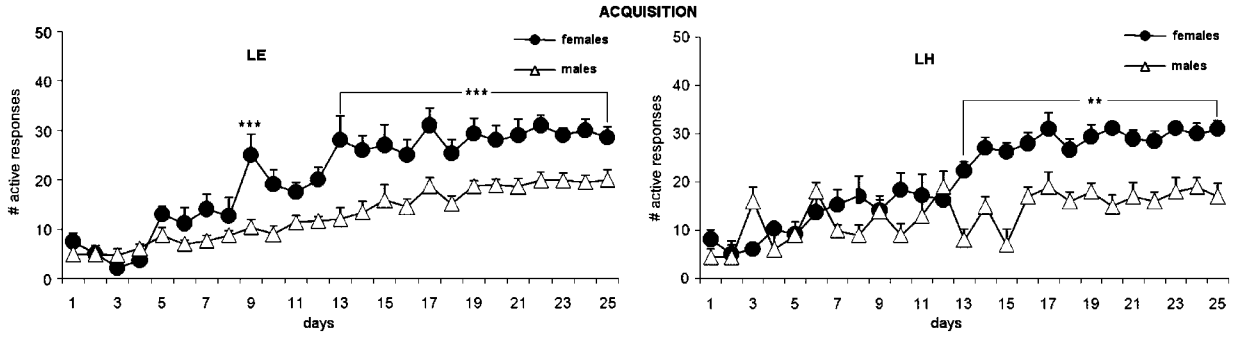

Figure 1 Acquisition of WIN55,212-2 (WIN) self-administration in Long Evans (LE; left panel) and Lister Hooded (LH; right panel) female and male rats. Values are expressed as the number of active lever-presses (mean ± s.e.mean) during each daily 2-h session. Each group included seven animals. ***$P<0.001$ and **$P<0.01$ vs corresponding values for males.

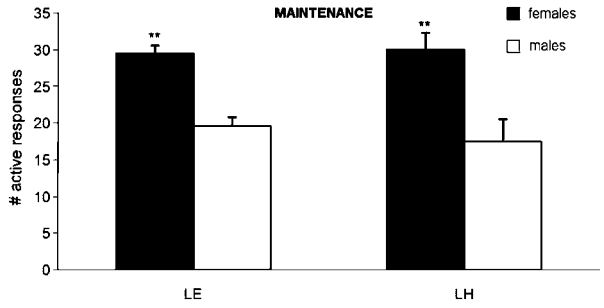

Figure 2 Maintenance of WIN55,212-2 (WIN) self-administration in Long Evans (LE; left panel) and Lister Hooded (LH; right panel) female and male rats. Each bar represents the number of active lever-presses (mean ± s.e.mean) during each daily 2-h session, over the 5 days of this phase. Each group included seven animals. **$P<0.01$ vs corresponding values for males.

$368.75 \pm 0.3\,\mu g\,kg^{-1}$, respectively. Similarly, LH females stabilized their response rate at a higher percentage (+42%) than corresponding males, and the mean WIN intake was 366.2 ± 0.6 and $217.5 \pm 0.3\,\mu g\,kg^{-1}$, respectively.

Two-way ANOVA revealed an overall significant main effect of sex ($F(1,24) = 28.62$; $P<0.0001$) but not strain or day × strain interaction (P = NS), as response rates for female LE and LH rats did not differ significantly.

Extinction. In both LE and LH females and males, removal of WIN reinforcement resulted in a gradual decrease in response on the previously active lever (Figure 3). The mean response rates over the last 3 days of the maintenance phase of cannabinoid SA are also shown in this figure, as basal values (on the extreme left). As shown in Figure 3, LE but not LH females reacted to saline substitution by increasing responses for the first day of the extinction phase, compared to the corresponding basal levels, although soon afterwards their responses decreased in a manner not dissimilar to their male counterparts. On the last day (tenth day) of the extinction phase, the mean active responses did not differ between males and females. Two-way ANOVA indicated significant differences by sex ($F(1,120) = 10.75$; $P<0.001$), day ($F(9,120) = 42.06$; $P<0.0001$) and sex × day interaction ($F(9,120) = 5.12$; $P<0.0001$). *Post hoc* analysis confirmed significant sex differences ($P<0.001$) on day 1.

For the LH strain, neither males nor females increased responses during the extinction phase. However, extinction was faster for males, with LH females retaining higher rates of response during the first 5 days of extinction. From day 6, no significant differences were observed between female and male LH rats. Two-way ANOVA indicated significant differences by sex ($F(1,120) = 119.60$; $P<0.0001$), day ($F(9,120) = 34.88$; $P<0.0001$) and sex × day interaction ($F(9,120) = 8.01$; $P<0.0001$). In the LH rats, *post hoc* analysis confirmed significant sex differences ($P<0.001$) on days 1–5.

Thus, although LE and LH females started extinction from similar levels of responses, LH females displayed more persistent response activity on the early days of extinction than LE females, while no significant strain differences were found at the end of extinction. Two-way ANOVA revealed strain ($F(1,120) = 4.36$; $P<0.05$), day ($F(9,120) = 59.52$; $P<0.0001$) and strain × day interaction ($F(9,120) = 2.37$; $P<0.05$).

Effect of ovariectomy: intact versus OVX females

Acquisition. The effects of ovariectomy in LE and LH females on the responses (mean number of active lever-presses per session) in our SA model are shown in Figure 4. Relative to those in intact females, lower levels of SA behaviour were typically acquired in OVX rats of both strains. In particular, LE OVX showed a more variable response during the initial period of training, responding on days 1–12 showing a higher number of active lever-presses than intact females. However, from day 14, LE OVX decreased the number of active responses and stabilized responses at a lower level, almost half that of the intact counterparts on the last day of acquisition. Two-way ANOVA revealed a significant effect of OVX ($F(1,300) = 4.56$; $P<0.05$), day ($F(24,300) = 7.13$; $P<0.0001$) and OVX × day interaction ($F(24,300) = 5.16$; $P<0.0001$).

Conversely, acquisition curves of intact and OVX females of the LH strain overlapped during the initial 2 weeks of acquisition, the level of response being rather similar up to day 13 (Figure 4). However, after day 14, intact LH females steadily increased their responses and developed a stable, higher cannabinoid SA response, while the LH OVX retained the lower response rate during the remaining part of

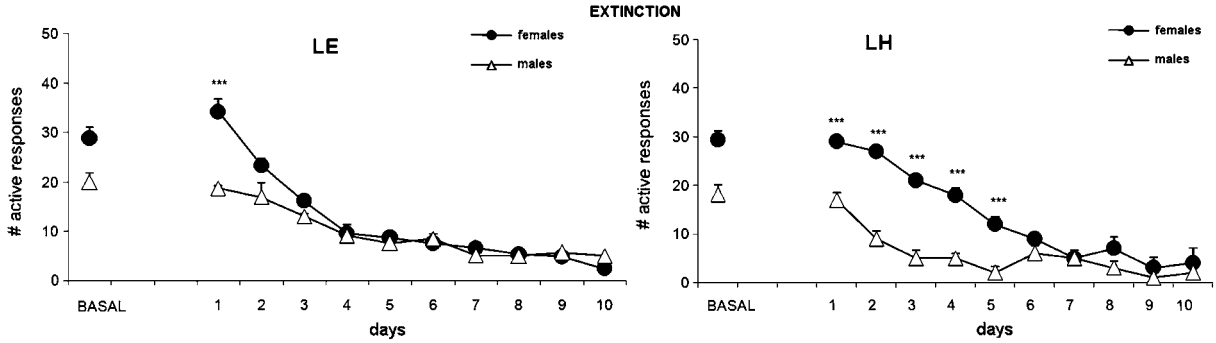

Figure 3 Extinction of WIN55,212-2 (WIN) self-administration in Long Evans (LE; left panel) and Lister Hooded (LH; right panel) female and male rats. Values are expressed as the number of active lever-presses (mean ± s.e.mean) during each daily 2-h session. BASAL = mean value of active responses during the last 3 days of cannabinoid intake (end of the maintenance phase). Each group included seven animals. ***$P<0.001$ vs corresponding values for males.

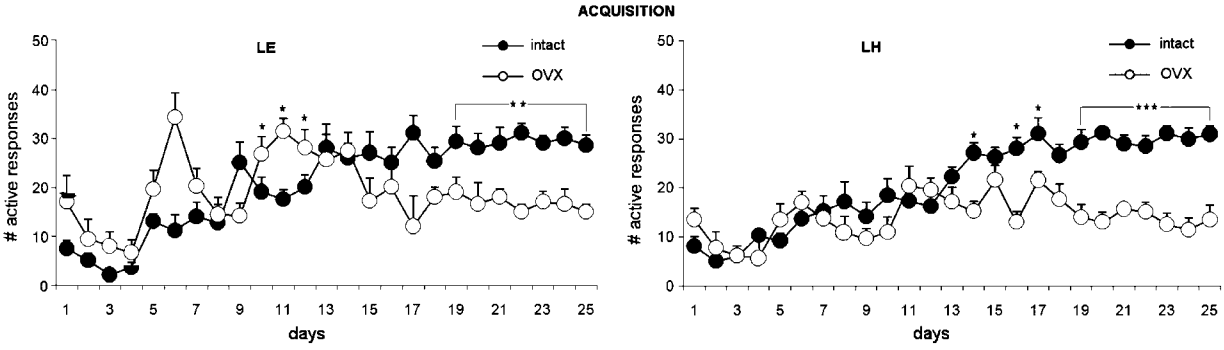

Figure 4 Acquisition of WIN55,212-2 (WIN) self-administration in Long Evans (LE; left panel) and Lister Hooded (LH; right panel) intact and ovariectomized (OVX) female rats. Values are expressed as the number of active lever-presses (mean ± s.e.mean) during each daily 2-h session. Each group included seven animals. ***$P<0.001$, **$P<0.01$ and *$P<0.05$ vs corresponding values for intact females.

training. On the last day of training, the OVX rats were responding at less than half the rate of the intact females. Analysis by two-way ANOVA detected a significant effect of OVX ($F(1,300)=87.42$; $P<0.0001$), day ($F(24,300)=9.76$; $P<0.0001$) and OVX × day interaction ($F(24,300)=4.75$; $P<0.0001$). *Post hoc* analysis confirmed significant differences ($P<0.001$) between intact and OVX from day 14 onward.

When the numbers of responses of OVX females of both strains were compared, two-way ANOVA revealed main effects of strain ($F(1,300)=25.59$; $P<0.0001$), day ($F(25,300)=4.72$; $P<0.0001$) and strain × day interaction ($F(24,300)=1.56$; $P<0.05$). However, mean response rates for the last 3 days of maintenance (basal values in Figure 6), were similar for the two OVX groups.

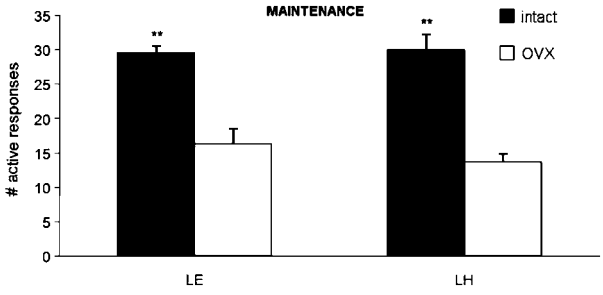

Figure 5 Maintenance of WIN55,212-2 (WIN) self-administration in Long Evans (LE; left panel) and Lister Hooded (LH; right panel) intact and ovariectomized (OVX) female rats. Each bar represents the number of active lever-presses (mean ± s.e.mean) during each daily 2-h session, over 5 consecutive days. Each group included seven animals. **$P<0.01$ vs corresponding values for intact females.

Maintenance. Figure 5 illustrates the mean number of active responses during the 5-day maintenance phase of cannabinoid SA in LE (left panel) and LH (right panel) intact and OVX female rats.

In the LE strain, OVX females stabilized their responses at significantly lower levels (−42%) with respect to intact females and the corresponding total amount of cannabinoid intake was 212.5 ± 0. 2 and 368.7 ± 0.3 μg kg^{-1}, respectively. Similarly, LH OVX females maintained a significantly ($P<0.001$) lower response rate (−52%) than intact females and the mean cumulative amount of self-administered cannabinoid was 175 ± 0.2 and 366.2 ± 0.6 μg kg^{-1}, respectively. Two-way ANOVA revealed a significant main effect of OVX ($F(1,24)=67.74$; $P<0.0001$), but not strain or OVX × strain interaction (P = NS), as the response rate did not significantly differ between LE and LH rats.

Extinction. The mean active responses for female rats, over the last 3 days of cannabinoid SA (basal) in the maintenance phase are shown in Figure 6. When saline replaced WIN, the mean numbers of active responses on the first day of

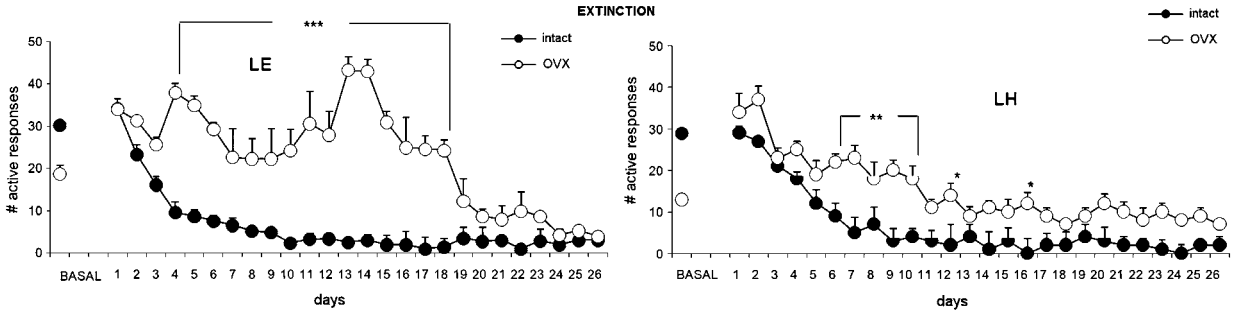

Figure 6 Extinction of WIN55,212-2 (WIN) self-administration in Long Evans (LE; left panel) and Lister Hooded (LH; right panel) intact and ovariectomized (OVX) female rats. Values are expressed as the number of active lever-presses (mean ± s.e.mean) during each daily 2-h session. BASAL = the number of active lever-presses (mean ± s.e.mean) during each daily 2-h session over the last 3 days of cannabinoid intake (end of the maintenance phase). Each group included seven animals. ***$P<0.001$, **$P<0.01$ and *$P<0.05$ vs corresponding values for intact females.

extinction were markedly increased in the OVX rats from either strain. Figure 6 also shows how OVX females of both strains exhibited longer extinction with a persistently higher rate of response than the corresponding intact females. Indeed, LE OVX females took at least 3 weeks to achieve complete extinction of SA for WIN, although starting (day 1 of extinction) from similar response levels to those of their intact counterparts (Figure 6). For this strain, two-way ANOVA revealed a significant main effect of OVX ($F(1,312) = 303.94$; $P<0.0001$), day ($F(25,312) = 10.71$; $P<0.0001$) and OVX × day interaction ($F(25,312) = 5.64$; $P<0.0001$).

In the LH females, the OVX group exhibited generally higher response rates than the intact group until the last day of extinction. Two-way ANOVA revealed a significant main effect of OVX ($F(1,312) = 135.13$; $P<0.0001$), day ($F(25,312) = 17.20$; $P<0.0001$) but not OVX × day interaction.

Although the basal values (last 3 days of maintenance) for both LE and LH females were similar, two-way ANOVA revealed differences in extinction according to strain ($F(1,312) = 58.17$; $P<0.0001$), day ($F(25,312) = 10.98$; $P<0.0001$) and strain × day interaction ($F(25,312) = 4.53$; $P<0.0001$).

Discussion

The results of the present study (1) extend to female rats the notion of strain-dependent differences in voluntary cannabinoid intake, (2) reveal an important role of sex in cannabinoid SA and (3) point to ovarian hormones as key factors in modulating the reinforcing effects of cannabinoids.

In line with our earlier study evaluating strain- and schedule-dependent differences in males (Deiana *et al*., 2007), we further demonstrate here the inability of female rats of the SD strain to develop cannabinoid SA behaviour, thus confirming a lack of sensitivity to cannabinoid-rewarding effects in this particular rat strain. On the contrary, males and females of both LE and LH strains developed firm cannabinoid SA, although with different timing and level of response. By acquiring stable WIN intake at a higher rate over a shorter period of time than corresponding male rats, females appear more susceptible to cannabinoid-induced reward, in line with clinical observations reporting women as more vulnerable than men during the transition periods from opportunity to use and drug abuse (Brady and Randall, 1999; Randall *et al*., 1999). Likewise, the higher level of WIN intake reached and maintained by females is in line with clinical observations that women are more sensitive than men to the addictive properties of drugs (Lynch *et al*., 2002). Accordingly, female rats seem to be more reactive than male rats to saline substitution, as they promptly increased their active response on the very first days of extinction, although such a response was also dependent on strain.

The present findings are consistent with preliminary reports of cannabinoid effects in human users, which showed that although no apparent sex differences are present in impulsivity (McDonald *et al*., 2003) nor intoxication or plasma THC levels after smoking marijuana or THC cigarettes (Miller *et al*., 1983; Mathew *et al*., 2003), whenever sex differences are found, females usually appear more sensitive than males to cannabinoids (Craft, 2005). Accordingly, expression of CB_1 cannabinoid receptors and their gene transcripts in blood cells of drug-naive human volunteers was found to be greater in women than in men (Onaivi *et al*., 1999).

Unfortunately, the role of sex in cannabinoid reinforcement or conditioning processes has been, to date, very poorly investigated and behavioural comparisons between females and males are also lacking in other important reward-related behavioural paradigms, such as intracranial self-stimulation or place-preference studies. The only indirect evidence available at present on possible sex differences in cannabinoid reinforcement is the finding that female mice lacking the enzyme fatty acid amidohydrolase (FAAH knockout (FAAH-KO)), therefore severely impaired in their ability to degrade the endogenous ligand anandamide, show increased ethanol consumption and preference and decreased ethanol sensitivity, compared to FAAH-KO males (Basavarajappa *et al*., 2006).

In the present study, findings of faster acquisition and higher levels of cannabinoid intake in females are in line with those from SA studies conducted with cocaine (Hu *et al*., 2004), heroin (Lynch and Carroll, 1999), methamphetamine (Roth and Carroll, 2004), nicotine (Donny *et al*., 2000) and phencyclidine (Carroll *et al*., 2000). Accordingly, female rats acquire cocaine- and morphine-conditioned

place preference in fewer sessions and at lower doses than males (Randall *et al*., 1998; Russo *et al*., 2003), show higher spontaneous activity level (Tropp and Markus, 2001) and explore more than males (Ray and Hansen, 2004), although no sex differences were found in the threshold for electrical brain self-stimulation (Stratmann and Craft, 1997; Craft *et al*., 2001).

Drug seeking is composed of several components, including incentive motivational, pleasurable hedonic and conditioning effects, and males and females may differ in one or more of these components. Although in the case of cannabinoids very little is known on the magnitude of impact of each of these three components, this study seems to suggest greater hedonic reinforcement for females, as response to WIN was stabilized at higher levels than males. Greater sensitivity to cannabinoids could therefore explain enhanced WIN self-administration in females. However, sex differences may also be involved in dictating the strength of the association between non-pharmacological stimuli and the drug itself, that is conditioned responses. Thus, faster acquisition and higher WIN intake in females may be ascribed, at least in part, to the higher impact of the stimulus light associated with cannabinoid delivery during SA training. This hypothesis is corroborated by evidence of greater conditioned reinforcement of nicotine, another widely smoked drug, in the female population (Perkins *et al*., 1999).

Besides SA behaviour, sex differences may be implicated in other pharmacological cannabinoid effects, some of which are pleasurable and hence reinforcing, such as anxiolytic effects, attenuation of pain perception and mood amelioration (Ameri, 1999). It is moreover possible that characteristics of factors closely associated with sex, rather than sex *per se*, may underlie the differential response to cannabinoids, among which crucially important is the distribution of central cannabinoid receptors (Rodriguez de Fonseca *et al*., 1994) or functional organization of reward-related brain areas (Gu *et al*., 2003). In support of the latter, female and male brains have been shown to differ substantially, differences beginning early during development and continuing throughout the lifespan (Emmerson-Hanover *et al*., 1994; Becker *et al*., 2005). Finally, sex differences in cannabinoid metabolism and disposition (that is, metabolic rate and % body fat) could also provide, at least in part, a possible explanation for differences in cannabinoid-reinforced operant behaviour. Although no studies have investigated, so far, the sex-related differences in WIN-induced behavioural effects, it is known that THC may be differentially metabolized to active and inactive metabolites in male or female rodents (Narimatsu *et al*., 1991), suggesting that the higher levels of active THC metabolites in females may contribute to the greater behavioural effects of THC (Tseng *et al*., 2004).

As oestrogen has been proposed to provide the biological basis for sex differences in behavioural responses to many drugs of abuse (Lynch *et al*., 2002; Carroll *et al*., 2004; Festa and Quinones-Jenab, 2004; Lynch, 2006), in the second part of this study, we compared operant behaviour of intact and oestrogen-deprived females during all three phases of cannabinoid SA. This comparison allowed the identification of ovarian function as a major determinant of underlying sex differences, as intact female rats performed better across all SA phases. Indeed, OVX females not only showed slower acquisition and lower WIN intake, but they also displayed higher and longer lasting responses, even in the absence of the drug (that is, under extinction conditions). Intriguingly, in both LE and LH females ovariectomy decreased WIN intake to levels not significantly different from those shown by males, suggesting that the ovarian hormones play a crucial role in these responses to cannabinoids. By translating these findings to the human situation, it could be argued that pre-pubescent and post-menopausal women might be less vulnerable to cannabinoid reinforcing effects compared to the remaining female population, although no studies have been conducted so far on such an issue.

As oestrogen receptors are often found in dopamine-rich areas (Kritzer, 1997; Creutz and Kritzer, 2002), the slower decline of responses in OVX females during extinction suggests that hormone-induced changes in receptor functions may be capable of influencing cannabinoid sensitivity. Fluctuations in these hormones have been reported to alter brain dopamine activity or dopamine-related behaviours. Thus, ovarian hormones could influence cannabinoid responsiveness and reinforcement by acting on dopamine receptors located on CB_1-containing neurons, particularly those on the mesolimbic dopaminergic system.

In conclusion, the present study provides evidence that cannabinoid SA is significantly influenced by strain, sex and ovarian function, such differences being manifested across all stages of dependence, from acquisition to extinction of drug intake. Whatever the precise role of ovarian hormones in drug SA behaviour, the female's reproductive status and ovarian cycle should be taken into serious consideration when studying sex differences in brain functions and behaviour. Given the significant therapeutic potential of cannabinoid antagonists, as well as the widespread recreational use of CB_1 agonists, it will prove fundamental to determine the functional significance of mechanisms underlying sex differences in the behavioural effects of cannabinoids. Future studies will be therefore directed to assess in male and female rats the exact role of steroid hormones in cannabinoid reinforcement.

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Conflict of interest

The authors state no conflict of interest.

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