

THEMED ISSUE: CANNABINOIDS

RESEARCH PAPER

Drug- and cue-induced reinstatement of cannabinoid-seeking behaviour in male and female rats: influence of ovarian hormones

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Background and purpose: Animal and human studies have shown that sex and hormones are key factors in modulating addiction. Previously, we have demonstrated that self-administration of the cannabinoid CB₁ receptor agonist WIN55,212-2 (WIN; 12.5 µg·kg⁻¹ per infusion) is dependent on sex, intact female rats being more sensitive than males to the reinforcing properties of cannabinoids, and on the oestrous cycle, ovariectomized (OVX) females being less responsive than intact females.

Experimental approach: This follow-up study investigated whether sex and ovarian function also affect reinstatement of cannabinoid-seeking in rats after exposure to drug or cue priming.

Key results: After priming with 0.15 or 0.3 mg·kg⁻¹ WIN, intact female rats exhibited stronger reinstatement than males and OVX females. Responses of intact female rats were higher than those of male and OVX rats even after priming with a drug-associated visual (Light) or auditory (Tone) cue, or a WIN + Light combination. However, latency to the first response did not differ between intact and OVX female rats, and males showed the longest latency to initiate lever-pressing activity.

Conclusions and implications: Our study provides compelling evidence for a pivotal role of sex and the oestrous cycle in modulating cannabinoid-seeking, with ovariectomy diminishing drug and cue-induced reinstatement. However, it is possible that sex differences during self-administration training are responsible for sex differences in reinstatement. Finding that not only drug primings but also acute exposure to drug-associated cues can reinstate responding in rats could have significant implications for the development of pharmacological and behavioural treatments of abstinent female and male marijuana smokers.

British Journal of Pharmacology (2010) **160**, 724–735; doi:10.1111/j.1476-5381.2010.00734.x

This article is part of a themed issue on Cannabinoids. To view the editorial for this themed issue visit <http://dx.doi.org/10.1111/j.1476-5381.2010.00831.x>

Keywords: WIN55,212-2; sex-dependent differences; relapse; cannabinoid-seeking behaviour; reinstatement; cannabinoid self-administration; drug priming; cue priming; ovariectomy; gonadal hormones

Abbreviations: CB₁, type 1 cannabinoid receptor; EXT, extinction; ONDCP, Office of National Drug Control Policy; OVX, ovariectomized female rats; SAL, saline; UNODC, United Nations Office on Drugs and Crime; WIN, R(+)-[2,3-dihydro-5-methyl-3-(morpholinyl)-methyl]-pyrrolo(1,2,3-de)-1,4-benzoxazinyl-(1-naphthalenyl)-methanone mesylate salt

Introduction

Cannabis dependence is becoming a problem of great concern because of its increasing use (UNODC, 2007), its concurrent

consumption with other drugs of abuse (Groß *et al.*, 2009) and its co-morbidity with anxiety and mood disorders (Dorand *et al.*, 2008). Marijuana withdrawal syndrome has been acknowledged widely, not only in laboratory animals (Lichtman and Martin, 2002) but also in humans (Haney *et al.*, 2004). Importantly, a cluster of withdrawal symptoms typically is experienced after cessation of regular marijuana smoking by adults (Budney *et al.*, 1999) and adolescents (Vandrey *et al.*, 2005), who are seeking treatment for marijuana dependence, as well as in non-treatment-seeking adult

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Received 13 November 2009; revised 28 January 2010; accepted 7 February 2010

cannabis users (Copersino *et al.*, 2006). Drug abstinence is believed to contribute substantially to relapse and marijuana seems to be no exception (Cornelius *et al.*, 2008). Frequent cannabis users perceive that withdrawal symptoms negatively affect their intention and capability to quit (Budney *et al.*, 2008), although the severity of withdrawal symptoms seems not to predict relapse in smokers who seek treatment for marijuana dependence (Arendt *et al.*, 2007). Several promising treatment approaches are being tested for cannabis use disorders (comprehensively reviewed in Elkashef *et al.*, 2008; Vandrey and Haney, 2009) but very limited data exist on the mechanisms that regulate marijuana withdrawal, craving and relapse, three main features that define human cannabis addiction.

Evidence is accumulating to show that the initiation of drug use generally is triggered by factors that may differ between men and women (Wasilow-Mueller and Erickson, 2001). Dependence, craving and propensity to relapse after abstinence may develop differently between the sexes, and require distinct treatment approaches (Fattore *et al.*, 2008a; 2009b). For example, epidemiological analyses have demonstrated that girls are now surpassing boys in their rates of initiation of cannabis use (McCreary Centre Society, 2003; ONDCP, 2006), a phenomenon with potentially alarming consequences because frequent cannabis use in young girls predicts later depression and anxiety (Patton *et al.*, 2002). Accordingly, frequent and heavy cannabis use in female students is more likely to correlate with poor mental health (Tu *et al.*, 2008). Among the factors that determine vulnerability to cannabis dependence, genetic and biological factors, such as animal strains (Deiana *et al.*, 2007), sex and ovarian hormones (Fattore *et al.*, 2007b), recently have been identified by preclinical studies. Unfortunately, the study of sex-dependent differences in cannabis consumption in humans and laboratory animals generally has been neglected (Fattore and Frata, 2010), and there have been very few studies on possible sex-related differences in relapse to cannabis use (Fattore *et al.*, 2007a,c).

In virtue of their ability to acquire higher incentive salience during drug use, a variety of drug-associated stimuli have been found to induce conditioned physiological responses, which in turn lead to craving and renewed drug-taking behaviour (Mezinskas *et al.*, 2001). Abstinent people usually are very responsive to external stimuli previously associated with drug experience, especially to visual cues that can capture attention and activate drug-specific memory traces (Flagel *et al.*, 2009). Also, marijuana-abstinent smokers are particularly vulnerable to drug-associated stimuli and clear cue-elicited craving for marijuana recently has been documented in 3-day abstinent subjects (Filbey *et al.*, 2009). Enhanced reactivity to marijuana-related cues has been found in virtual reality cannabis environments, where subjective craving and attention to cues are increased significantly compared with the virtual reality neutral environments (Bordnick *et al.*, 2009). Similarly, higher arousal after exposure to drug-related visual stimuli recently has been assessed in a cue-reactivity paradigm that measured psychophysiological parameters in chronic heavy cannabis users (Wöfling *et al.*, 2008). In keeping with this, higher levels of craving have been observed during marijuana cue presentations also in adolescents and young adults with cannabis use disorders (Gray *et al.*, 2008).

In animals, the reinstatement of cannabinoid-seeking behaviour has been shown to be specifically affected by pharmacological manipulation of the endogenous endocannabinoid and opioid systems, because it can be provoked by exposure to priming with the CB₁ receptor agonist WIN55,212-2 (WIN) or heroin but not cocaine (Fattore *et al.*, 2004; 2005a; Spano *et al.*, 2004). However, although environmental stimuli associated with drug-taking are known to elicit drug craving and increase the likelihood of relapse in humans, the role played by drug-associated stimuli in reinstating cannabinoid-seeking behaviour in animals remains to be ascertained. Thus, no information is available currently on the role played by drug-associated cues on the propensity to resume cannabinoid-seeking behaviour in laboratory animals (Fattore *et al.*, 2008b).

This study was undertaken to investigate the reinstatement of cannabinoid-seeking behaviour triggered by drug and cue priming in male and female rats, either intact or ovariectomized (OVX), with two final goals: (i) to detect similarities (i.e. vulnerability to drug-associated cues) that can help to identify factors that render marijuana users at risk of relapse, and (ii) to recognize differences that can provide the basis for sex-specific treatments.

Methods

Animals

All animal care and experimental procedures were approved by the local animal care committee and followed the EU regulations for animal use in research (CEE n°86/609). Male and female Lister Hooded rats (Harlan, UK), initially weighing 250–275 g, were housed in groups of four with food and water available *ad libitum*, in a room maintained on an inverted 12 h light/dark cycle (light from 7 PM) at 21 ± 2°C and 60% humidity. All animals were handled daily for 7 days prior to surgery to diminish stress associated with handling.

Treatment procedures

For self-administration training, WIN was dissolved in one drop of Tween 80, and diluted in heparinized (1%) sterile saline (SAL) solution (volume of injection: 0.1 mL). To ensure sterility, drug solutions were filtered through 22 µm syringe filters before use. For reinstatement testing, WIN was prepared freshly at a dose of 0.15 or 0.3 mg·kg⁻¹ and administered intraperitoneally (i.p.) at a volume of injection of 5 mL·kg⁻¹ 10 min before starting the session. As a control study, one group was primed with SAL and a different one with the vehicle (VEH) of the cannabinoid (2 mL per rat, i.p., 10 min before).

Surgery

Rats were implanted with chronically indwelling catheters (CamCaths, Ely, UK) into the right jugular vein under deep anaesthesia with Equithesin (0.97 g pentobarbital, 2.1 g magnesium sulphate, 4.25 g chloral hydrate, 42.8 mL propylene glycol, 11.5 mL ethanol 90%, 5 mL·kg⁻¹, i.p.; this mixture was made up just before use). Catheters were passed under the skin and threaded out of an incision made on the back. Rats

were allowed to recover individually for a minimum of 6 days and received daily subcutaneous injections of antibiotic (0.1 mL Baytrill, Bayer, Milan, Italy) during the first 5 days after surgery. Once recovered from surgery, a food supply of 20 g·day⁻¹ rat chow was maintained, given at the end of the session with *ad libitum* access to water. Before starting each self-administration session, the catheter was connected to a single-guide cannula (Plastics One, Roanoke, VA, USA) and attached to an infusion harness (Instech Laboratories, Plymouth Meeting, PA, USA). At the end of each daily session, catheters were flushed with heparinized SAL sterile solution (20 USP mL⁻¹) to maintain patency.

Apparatus

Twelve standard operant conditioning chambers (29.5 × 32.5 × 23.5 cm) were enclosed in ventilated sound-attenuating cubicles that were provided with fans to mask outside noise (Medical Associates, St. Albans, VT, USA). As previously described (Fattore *et al.*, 2007d), on the front wall of each chamber, there were two response levers; a white light stimulus (cue light) was placed between them, while a red house light was located on the opposite (back) wall. Chambers were equipped with syringe pump systems that consisted of an infusion pump (Medical Associates) with a 5 mL syringe connected by a single channel 22 gauge swivel and Teflon tubing. The infusion pump apparatus was located within the sound-attenuating cubicles that housed the operant chamber. The tubing was in turn connected to the catheter system of the animal to deliver drug solutions intravenously contingent with each active lever press. Each downward pressure of one lever (defined as active) resulted in: (i) a contingent 100 µL infusion of WIN (12.5 µg·kg⁻¹ per infusion) solution delivered through the catheter over 5 s; (ii) illumination of the cue light for 5 s; and (iii) initiation of a 15 s timeout period during which both levers were retracted and the house light was turned off. After the timeout period, both levers were re-extended into the chamber, the stimulus light went out and the house light was switched on.

The depression of the other lever (defined as inactive) was tabulated but had no programmed consequence. The assignment of the active (drug-paired) and inactive (no drug-paired) levers was counterbalanced between rats and remained constant for each subject throughout the study. Experimental parameters, such as schedules of reinforcements, timeout period, volume and speed of infusion, were programmed using a software package (Medical Associates) installed on a computer.

Experimental procedure

Cannabinoid self-administration occurred during the dark phase of the cycle under a continuous (FR-1) schedule of reinforcement as previously described (Fattore *et al.*, 2001). The study included three different phases: (i) WIN self-administration training, during which animals were trained to press a lever to obtain intravenous infusions of the drug; (ii) extinction (EXT), during which the WIN solution was replaced by SAL solution, so that active lever pressing was no longer reinforced; and (iii) reinstatement testing, when

response continued to be not reinforced and animals received drug and/or cue priming before starting the session. All sessions lasted 2 h and were conducted 6 days per week.

Throughout each phase of the study, locomotor activity was monitored within the operant chambers through two series of four photocells each located at regular 3.5 cm intervals above the cage floor. The number of photocell beam breaks was recorded and served as a measure of general horizontal locomotor activity, and in particular to assess potential non-specific changes in activity induced by the experimental manipulation as previously described (Fattore *et al.*, 2009c).

Cannabinoid self-administration training. Each session began with the insertion of both levers and illumination of the red house light, which remained on for the entire session. At the end of each session, the house light was turned off and both levers retracted. Animals needed 15–20 days to acquire steady cannabinoid intake. The criteria for the acquisition of stable cannabinoid self-administration behaviour were as follows: (i) animals displayed four consecutive days of firm response within ±20% of variation from the mean number of reinforcers obtained; (ii) a minimum of 16 drug infusions per session; and (iii) ≤6 responses made on the inactive lever. When stability was acquired, training was continued for an extra 7 days before switching to the next phase, that is, EXT.

Extinction. After the last conditioning session, rats were subjected to daily EXT sessions during which they were placed into the box, and attached to the drug tether. The house light and ventilation fan were on, levers were extended into the box, but the drug-associated light (visual cue), as well as the infusion pump and the lever retraction system (the noise of which represents the auditory cue) were switched off (Chauvet *et al.*, 2009). Thus, responses on the active lever had no programmed consequences during the EXT training. However, responses on the active and inactive lever were counted by the software. EXT training proceeded over a 3 week period. Drug-reinforced behaviour was considered extinguished when the number of active lever presses was ≤8 and the total number of lever responses (active + inactive) in a single test session was <12 (Fattore *et al.*, 2009a).

Reinstatement testing. The conditions during reinstatement testing were similar to those of EXT, as the house light and ventilation fan were on, and the animals were placed in the operant chamber environment, attached to the drug tether, and allowed to press the levers. The stimulus complex (i.e. cue light, noise of the infusion pump and lever retraction system) remained deactivated. Animals were exposed for 5 s to the visual cue (i.e. cue light) and/or the auditory stimulus complex (i.e. infusion pump + lever retraction system) only immediately before the corresponding cue-induced reinstatement test sessions. Three days prior to testing, rats were given daily SAL injections (2 mL·kg⁻¹, i.p.) as habituation for future pharmacological treatments. Exposure to drug priming (0.15 or 0.3 mg·kg⁻¹ WIN, i.p.), cues (either visual and/or auditory), drug + cue combination and control priming injections (SAL or drug VEH, 2 mL·kg⁻¹, i.p.) occurred in a counterbalanced

manner. For each subject, reinstating test sessions were conducted every fourth day of EXT training (to assess carryover effects of priming) for a total of three sessions per animal. The two priming doses of WIN were chosen based on previous studies that demonstrated their efficacy in reinstating heroin (Fattore *et al.*, 2003; 2005b) and cannabinoid-seeking (Spano *et al.*, 2004) behaviour in rats. The order of presentation of different test drugs varied between animals, and each treatment group included six animals. Non-reinforced responding on the active lever was interpreted to reflect drug-seeking behaviour, while responding on the inactive lever was used as a measure of general (non-directed) activity and response generalization.

Statistics

The number of active and inactive lever presses were recorded for each session, as well as the time elapsing from the beginning of the session and the first active lever press (i.e. response latency). Data from self-administration training (baseline) were first compared with EXT by using two-way ANOVA followed by *post hoc* Bonferroni test. Mean active responses after drug primings (i.e. SAL, VEH, WIN 0.15 and 0.3) were compared with those recorded during EXT and analysed by two-way ANOVA followed by *post hoc* Bonferroni test. Similarly, mean active responses after primings with the drug-associated visual and auditory cues, that is, Light and Tone, along with SAL and VEH primings, were compared with EXT and analysed by two-way ANOVA followed by *post hoc* Bonferroni test. The two factors taken into consideration were (i) sex (male vs. intact female, and intact vs. OVX females) and (ii) treatment (drug vs. cue primings). Then, mean active response of each group after a drug + cue combination priming was compared with that recorded after corresponding drug priming alone and cue priming alone, and data were analysed by two-way ANOVA followed by *post hoc* Bonferroni test.

The mean response latency following either drug (i.e. SAL, VEH, WIN 0.15 and 0.3), cue (i.e. Light and Tone) and drug + cue combination priming was analysed by two-way ANOVA followed by *post hoc* Bonferroni test. Mean response latencies after drug + cue combination priming were compared with the corresponding drug priming alone and cue priming alone, and were analysed by two-way ANOVA followed by *post hoc* Bonferroni test.

Moreover, the mean number of active responses within each treatment group, that is, males, intact females and OVX, was compared by one-way ANOVA followed by the Newman-Keuls as a *post hoc* test. $P \leq 0.05$ was considered significant.

Materials

WIN55,212-2 (WIN; R(+)-[2,3-dihydro-5-methyl-3-(morpholinyl)-methyl]-pyrrolo(1,2,3-de)-1,4-benzoxazinyl-(1-naphthalenyl)-methanone mesylate salt) was obtained from Tocris, (Bristol, UK) and the pentobarbital and chloral hydrate for the Equithesin from Sigma (Milan, Italy). Drug and receptor nomenclature follows Alexander *et al.* (2009).

Results

Sex differences in drug- and cue-induced reinstatement of cannabinoid-seeking behaviour

Figure 1 shows the effect of WIN, cue and drug + cue combination priming on the reinstatement of extinguished cannabinoid-seeking behaviour in Lister Hooded rats. Male and intact female rats readily acquired and maintained WIN self-administration, with females exhibiting significantly higher (+47%, $P < 0.0001$) daily cannabinoid intake compared with males (baseline). In particular, the mean (\pm SEM) active response over the last 3 days of cannabinoid self-administration training in male and female rats was 19.5 ± 0.8 and 28.3 ± 1.8 respectively. Subsequently, when SAL was substituted for WIN over 21 consecutive days, both groups gradually decreased the rate of active lever pressing until reaching the established criterion for EXT, that is, ≤ 8 active responses over 5 consecutive days. Two-way ANOVA revealed a significant effect of treatment [$F(1,20) = 350.37$; $P < 0.0001$], sex [$F(1,20) = 22.45$; $P < 0.0001$] and treatment \times sex interaction [$F(1,20) = 14.25$; $P < 0.001$].

An acute priming injection with either SAL or the cannabinoid VEH had no effect on operant response, and mean active lever pressing of both groups did not differ significantly from those shown during previous EXT (Figure 1). However, acute priming with the CB₁ receptor agonist WIN significantly restored the extinguished drug-seeking behaviour selectively on the previously drug-paired (active) lever. Two-way ANOVA showed a significant effect of treatment [$F(4,50) = 223.95$; $P < 0.0001$], sex [$F(1,50) = 12.79$; $P < 0.001$] and a treatment \times sex interaction [$F(4,50) = 6.05$; $P < 0.001$]. Specifically, at both doses tested (0.15 and 0.3 mg·kg⁻¹, i.p.), WIN promptly reinstated responding in male and female rats, with the latter showing a significantly higher lever pressing activity than the former (0.15 mg·kg⁻¹ WIN: $P < 0.01$; 0.3 mg·kg⁻¹ WIN: $P < 0.05$). As compared with EXT, when primed with the lowest dose of WIN, males and females increased their active responding by 81 and 95%, respectively, while following priming with the higher dose of WIN they increased active responding by 126 and 130% respectively. Notably, WIN priming reinstated extinguished responding in a dose-related manner, and at a level that was significantly higher than during cannabinoid self-administration (baseline), as demonstrated by the significant effect of treatment [$F(2,30) = 67.39$; $P < 0.001$], sex [$F(1,30) = 23.09$; $P < 0.001$], but not of treatment \times sex interaction [$F(1,20) = 0.76$; $P = ns$].

We verified also the effect of acute exposure to a drug-associated visual (Light) or auditory (Tone) cue on the reinstatement of extinguished cannabinoid-seeking behaviour. Figure 1 shows that, similar to drug priming, intact female rats showed higher responding than males after presentation of the drug-paired cue light or cue tone. Two-way ANOVA highlighted a significant effect of cue [$F(4,50) = 83.46$; $P < 0.0001$], sex [$F(1,50) = 23.68$; $P < 0.0001$] and cue \times sex interaction [$F(4,50) = 11.33$; $P < 0.0001$]. As compared with EXT, when exposed to the cue Light, males and females increased their active responding by 43 and 63%, respectively, while following a Tone priming, they increased active responding by 39 and 59%, respectively. However, contrary to the effects of drug priming, drug-associated cues reinstated response in

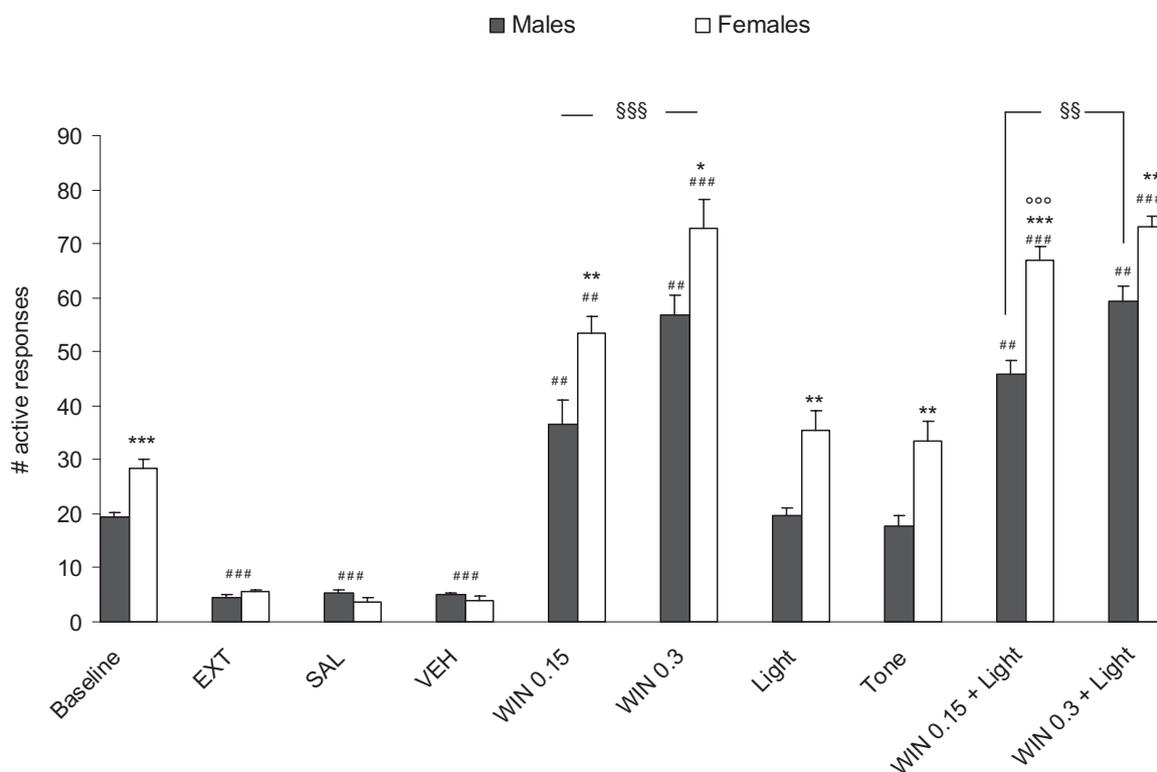


Figure 1 Reinstatement of cannabinoid-seeking behaviour following prolonged (3 weeks) extinction in male and female rats after acute priming with WIN, drug-associated cues or drug + cue combination. Each bar represents the mean \pm SEM of active lever presses over the last 3 consecutive days of cannabinoid self-administration (baseline), over the last 5 consecutive sessions of the extinction training (EXT), and during the reinstatement test sessions following saline (SAL), cannabinoid vehicle (VEH), WIN (0.15 and 0.3 mg·kg⁻¹, i.p.) acute priming injections, or after exposure to a drug-associated visual (Light) or auditory (Tone) cue, or WIN + Light combination ($n = 6$ per group). Drug doses are expressed as mg·kg⁻¹. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ versus male rats; ### $P < 0.001$ and ## $P < 0.01$ versus corresponding Baseline; \$\$\$ $P < 0.001$ between priming with the two doses of WIN; °°° $P < 0.001$ versus corresponding single drug priming.

male and female rats at a level that was not significantly different from that shown during cannabinoid self-administration (baseline). Moreover, in both males and females, the effect on responding induced by the simultaneous exposure to both cues, that is, the light + tone combination priming, was not significantly different from that induced by each cue alone (data not shown), suggestive of no additive nor synergic effect of the drug-associated cues on cannabinoid-seeking reinstatement.

Conversely, when the exposure to the cue Light was combined with the lowest dose of WIN (0.15 mg·kg⁻¹), female rats showed higher responding than males (Figure 1). Two-way ANOVA indicated a significant effect of the drug + cue combination [$F(5,60) = 211.73$; $P < 0.0001$], sex [$F(1,60) = 43.67$; $P < 0.0001$] and drug + cue combination \times sex interaction [$F(5,60) = 10.67$; $P < 0.0001$]. Similarly, for the higher dose of WIN tested (0.3 mg·kg⁻¹), two-way ANOVA showed a significant effect of the drug + cue combination [$F(5,60) = 298.27$; $P < 0.0001$], sex [$F(1,60) = 25.99$; $P < 0.0001$] and drug+cue combination \times sex interaction [$F(5,60) = 6.38$; $P < 0.0001$]. As compared with EXT, responding of both males and females increased significantly by 102 and 120%, respectively, after WIN 0.15 + Light combination priming, and by 132 and 131%, respectively, following WIN 0.3 + Light combination. Importantly, the combination of the cue Light with WIN

0.15 mg·kg⁻¹, but not WIN 0.3 mg·kg⁻¹, was more effective than the drug priming alone in reinstating active responding ($P < 0.001$), but only in the females. Yet, the dose-dependent effect of WIN primings was still evident in the male ($P < 0.01$ between the two doses of WIN) but not in the female group ($P = ns$).

The lack of alterations in the mean number of inactive lever presses throughout the reinstatement test sessions indicated that rats retained good discrimination between the drug-paired (active) and not drug-paired (inactive) levers, suggesting that the results were not influenced by non-specific effects, that is, generalization (see patterns of responding below).

Effect of OVX on drug- and cue-induced reinstatement of cannabinoid-seeking behaviour

Figure 2 illustrates the effect of acute priming of WIN, cue and drug + cue combination priming on the reinstatement of extinguished cannabinoid-seeking behaviour in female rats. OVX and intact females readily acquired and maintained stable active responding but intact females displayed a significantly higher (+58%, $P < 0.001$) response rate compared with their OVX counterparts. In particular, the mean (\pm SEM) active responses over the last 3 days of cannabinoid self-

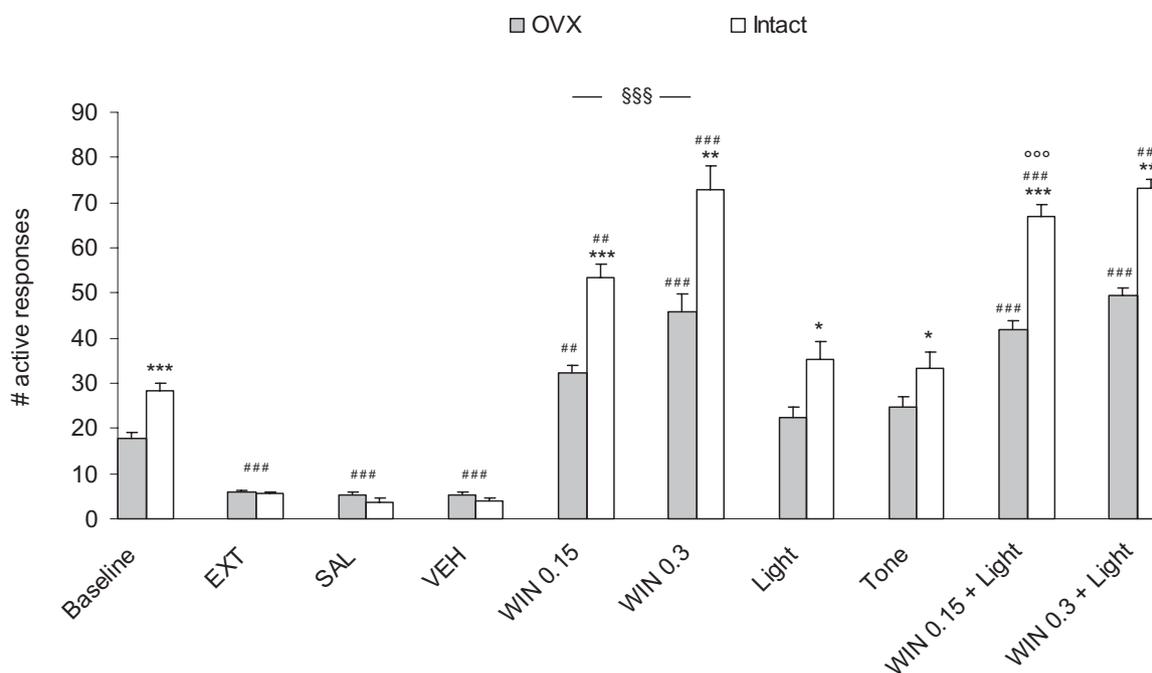


Figure 2 Reinstatement of cannabinoid-seeking behaviour following prolonged (3 weeks) extinction in ovariectomized (OVX) and intact female rats after acute priming with WIN, drug-associated cues or drug + cue combination. Each bar represents the mean \pm SEM of active lever presses over the last 3 consecutive days of cannabinoid self-administration (baseline), over the last 5 consecutive sessions of the extinction training (EXT), and during the reinstatement test sessions following saline (SAL), cannabinoid vehicle (VEH), WIN (0.15 and 0.3 mg·kg⁻¹, i.p.) acute priming injections, or after exposure to a drug-associated visual (Light) or auditory (Tone) cue, or WIN + Light combination ($n = 6$ per group). Drug doses are expressed as mg·kg⁻¹. *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ versus OVX female rats; ### $P < 0.001$ and ## $P < 0.01$ versus Baseline; \$\$\$ $P < 0.001$ between priming with the two doses of WIN; °°° $P < 0.001$ versus corresponding single drug priming.

administration training in OVX and intact female rats was 17.8 ± 1.3 and 28.3 ± 1.8 respectively (baseline). Substitution of saline for WIN over 21 consecutive days led both female groups to attain the extinction criterion, that is, ≤ 8 active lever presses for 5 consecutive days. Two-way ANOVA detected a significant effect of treatment [$F(1,20) = 242.43$; $P < 0.0001$], sex [$F(1,20) = 17.70$; $P < 0.001$] and treatment \times sex interaction [$F(1,20) = 25.93$; $P < 0.0001$].

No significant difference was observed in the mean (\pm SEM) number of active responses between EXT and reinstatement test sessions when animals received SAL or cannabinoid VEH priming. On the contrary, as shown in Figure 2, non-contingent, non-reinforced priming with the previously self-administered drug selectively reinstated active responses at a level significantly higher than that for previous training (baseline). Two-way ANOVA revealed a significant effect of treatment [$F(2,30) = 64.53$; $P < 0.0001$], ovariectomy [$F(1,30) = 55.87$; $P < 0.0001$] and a treatment \times ovariectomy interaction [$F(2,30) = 3.42$; $P < 0.05$]. Specifically, at both doses tested (0.15 and 0.3 mg·kg⁻¹, i.p.), WIN promptly reinstated active lever pressing in both groups, with intact female rats showing a significantly higher response rate than the OVX counterparts (WIN 0.15 mg·kg⁻¹: $P < 0.001$; WIN 0.3 mg·kg⁻¹: $P < 0.0001$). As compared with EXT, when primed with the lowest dose of WIN, OVX and intact females increased their active responding by 54 and 94%, respectively, while following priming with the higher dose of WIN, they enhanced active responding by 72 and 128% respectively. Remarkably, the dose-dependent effect of WIN priming was still evident in both female groups ($P < 0.0001$ between the two doses of WIN).

Moreover, when primed with a visual (Light) or an auditory (Tone) drug-associated cue, intact female rats reinstated active responses at a level significantly higher than those of their OVX counterparts (Figure 2). Two-way ANOVA indicated a significant effect of cue [$F(5,60) = 89.33$; $P < 0.0001$] and cue \times ovariectomy interaction [$F(5,60) = 10.80$; $P < 0.0001$], but not ovariectomy effect [$F(1,60) = 0$; $P = \text{ns}$]. As compared with EXT, when exposed to the cue Light, OVX and intact females increased their active responding by 37 and 62%, respectively, while following a Tone priming, they augmented active responding by 41 and 58% respectively. Similar to the male and intact female rats, in the OVX group, drug-associated cues reinstated responses at a level that was not significantly different from that shown during cannabinoid self-administration (baseline). In addition, even in the OVX females the effect on responding induced by the simultaneous exposure to both cues, that is, the Light + Tone combination priming, was not significantly different from that induced by each cue alone (data not shown).

On the other hand, when the exposure to the cue light was combined with the lowest dose of WIN (0.15 mg·kg⁻¹), intact females showed higher responding than OVX females (Figure 2). Two-way ANOVA showed a significant effect of ovariectomy [$F(1,60) = 61.70$; $P < 0.0001$], drug + cue combination [$F(5,60) = 242.31$; $P < 0.0001$] and drug + cue combination \times ovariectomy interaction [$F(5,60) = 18.76$; $P < 0.0001$]. Similarly, for the higher dose of WIN tested (0.3 mg·kg⁻¹), two-way ANOVA indicated a significant effect of ovariectomy [$F(1,60) = 47.35$; $P < 0.0001$], drug + cue combination [$F(5,60) = 242.07$; $P < 0.0001$] and drug + cue combination \times ovariectomy inter-

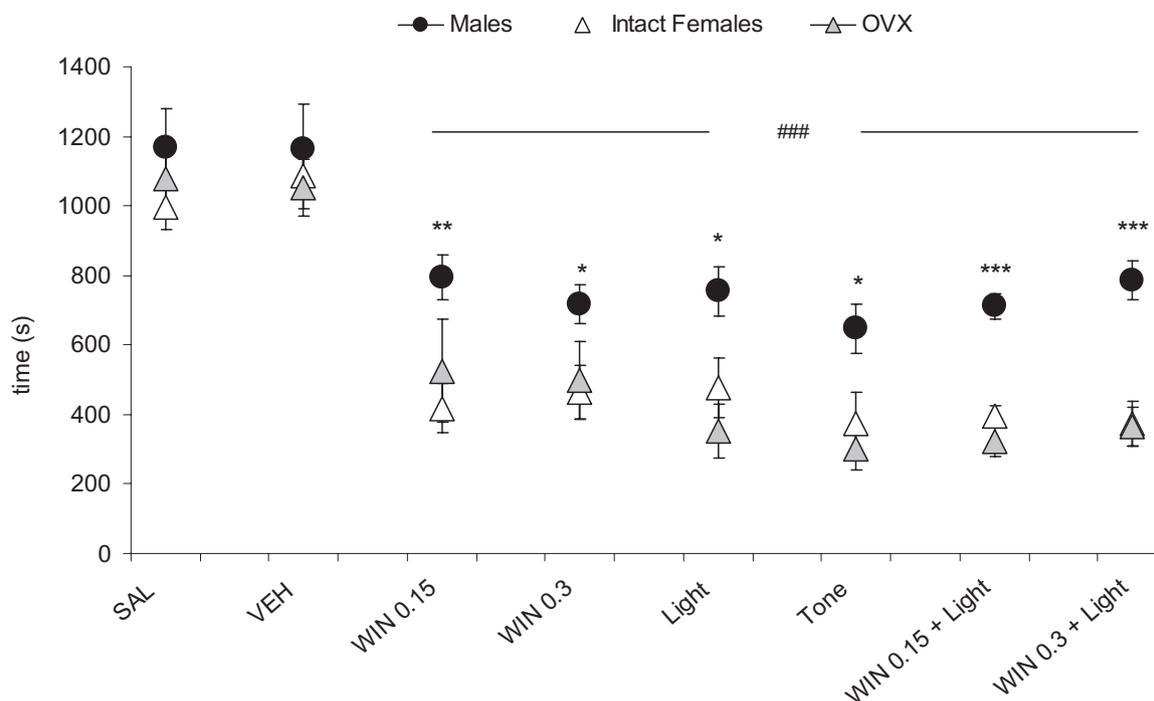


Figure 3 Response latency to the first active response in male, ovariectomized (OVX) and intact female rats after acute priming with WIN, drug-associated cues or drug + cue combination. Each point represents the mean \pm SEM of the time elapsed from the beginning of the test session and the first active lever press ($n = 6$ per group). Drug doses are expressed as $\text{mg}\cdot\text{kg}^{-1}$ (i.p.). ### $P < 0.001$ versus saline (SAL) and cannabinoid vehicle (VEH) groups; *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ versus female groups.

action [$F(5,60) = 14.41$; $P < 0.0001$]. As compared with EXT, responding of both OVX and intact females increased significantly by 70 and 118%, respectively, after WIN 0.15 + Light combination priming, and by 82.5 and 129%, respectively, following WIN 0.3 + Light combination. In contrast to the effects of drug priming alone, the dose-dependent effect of WIN priming was no more evident after drug + cue combination priming.

Apart from the initial days of training, only minimal responses were observed on the inactive lever throughout the entire period of testing, thus confirming the specificity of the effect of the priming (data not shown).

Effect of sex and ovariectomy on the latency to the first active response after drug and cue priming

Response latency was defined as the time elapsed from the commencement of the experimental sessions until the first lever press. The effect of sex and ovariectomy on the mean latency (\pm SEM) to active response after drug, cues and drug + cue combination primings is shown in Figure 3. Overall, all primings decreased the response latency compared with that in the control groups primed with SAL or cannabinoid VEH ($P < 0.001$). Moreover, in all groups, WIN and cue primings, either alone or in combination, did not affect the latency for initiating response on the inactive lever compared with SAL or VEH primings (data not shown).

Pairwise comparisons indicated that male rats exhibited a significantly longer response latency on the active lever than

females, regardless of whether they were primed with the drug (0.15 $\text{mg}\cdot\text{kg}^{-1}$ WIN: $P < 0.01$; 0.3 $\text{mg}\cdot\text{kg}^{-1}$ WIN: $P < 0.05$), or the drug-associated cues ($P < 0.05$), or the drug + cue combination ($P < 0.001$), as shown in Figure 3. Specifically, when comparing the effect of drug priming in male and intact female rats, two-way ANOVA revealed a significant effect of treatment [$F(3,40) = 22.48$; $P < 0.0001$], sex [$F(1,40) = 12.73$; $P < 0.001$], but not treatment \times sex interaction [$F(3,40) = 1.08$; $P = \text{ns}$]. Likewise, following cue priming, two-way ANOVA indicated a significant effect of cue [$F(3,40) = 23.22$; $P < 0.0001$] and sex [$F(1,40) = 9.33$; $P < 0.01$], but not cue \times sex interaction [$F(3,40) = 0.54$; $P = \text{ns}$]. Finally, after WIN 0.15 + Light combination priming, two-way ANOVA showed a significant effect of sex [$F(1,30) = 39.56$; $P < 0.0001$], but not a drug + cue combination effect [$F(2,30) = 0.57$; $P = \text{ns}$] nor a drug + cue combination \times sex interaction [$F(2,30) = 0.7279$; $P = \text{ns}$]. Similarly, after WIN 0.3 + Light combination priming, two-way ANOVA indicated a significant effect of sex [$F(1,30) = 30.85$; $P < 0.0001$], but not a drug + cue combination effect [$F(2,30) = 0.14$; $P = \text{ns}$] nor a drug + cue \times sex interaction [$F(2,30) = 0.4719$; $P = \text{ns}$].

Latency in the primed OVX female rats did not differ from that in their intact counterparts, as two-way ANOVA detected no significant effect of ovariectomy [$F(1,40) = 0.48$; $P = \text{ns}$] or treatment \times ovariectomy interaction [$F(3,40) = 0.22$; $P = \text{ns}$]. Unexpectedly, no significant differences were found for the latency to initiate active response between OVX and intact female rats following any priming, in contrast to the higher number of active lever presses made by intact females (Figure 2).

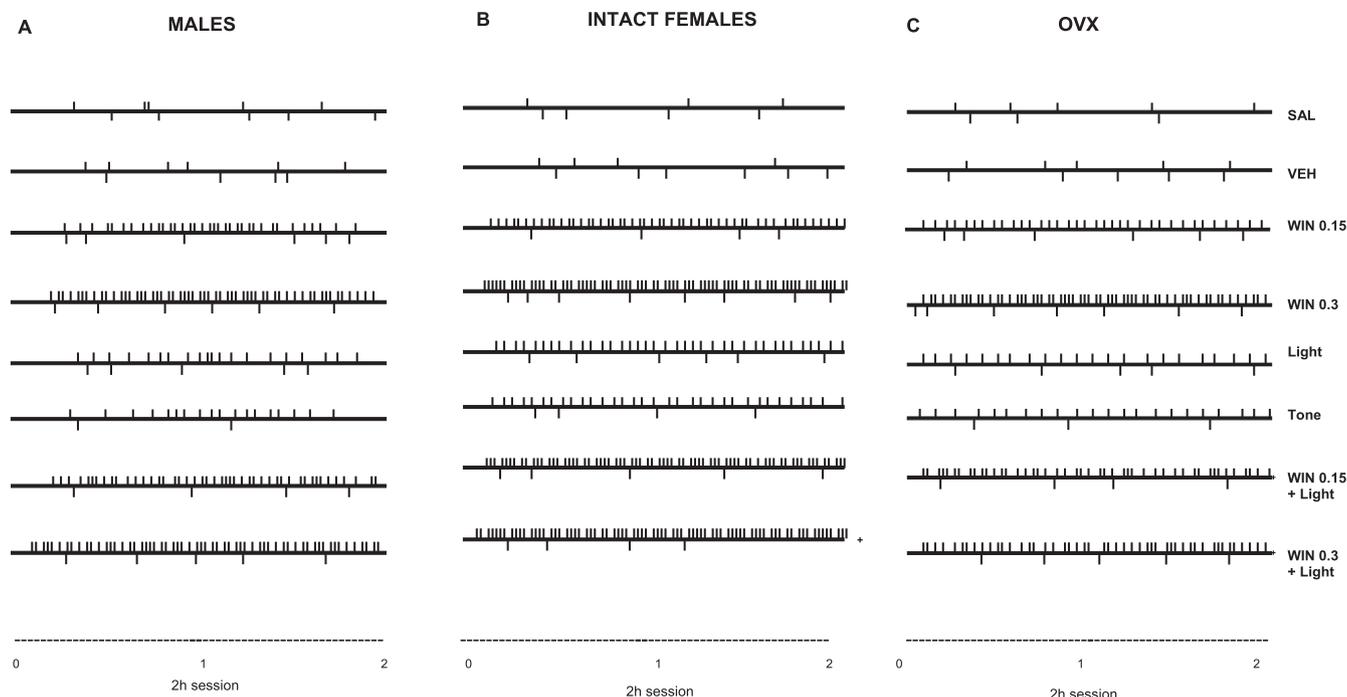


Figure 4 Representative event recordings of individual patterns in male (A), intact female (B) and ovariectomized (OVX) (C) rats during reinstatement test sessions after saline (SAL), cannabinoid vehicle (VEH), WIN (0.15 and 0.3 mg·kg⁻¹, i.p.) acute priming injections, and after exposure to drug-associated visual (Light) and auditory (Tone) cues, or drug + cue combination priming. Drug doses are expressed as mg·kg⁻¹. Each small vertical deflection above and below the horizontal line represents, respectively, every individual response on the active and inactive lever during the 2 h reinstatement session.

Effect of sex and ovariectomy on response patterns during drug- and cue-induced reinstatement of cannabinoid-seeking behaviour

A closer examination of the response pattern of each rat tested better emphasized the sex-dependent differences in the reinstatement of cannabinoid-seeking behaviour following the different primings. Figure 4 shows representative examples of response patterns in abstinent male (A), intact female (B) or OVX female (C) rats after priming with SAL, cannabinoid VEH, WIN (0.15 and 0.3 mg·kg⁻¹ i.p.), drug-associated cues (light and tone) and WIN + Light combination. Two common traits were apparent among the three experimental groups: (i) rats made only a few lever presses on either the active or inactive lever when primed with SAL or cannabinoid VEH; and (ii) after an initial period of no response, there was an accelerated response rate that continued over the session. However, male rats typically tended to commence responding later than females after acute drug priming or exposure to a drug-associated cue, while no differences were observed between intact and OVX females.

Specifically, male rats generally showed a marked delay in the onset of response and a pattern of response that was fairly evenly distributed across the remainder of the session, in line with our previous observations (Spano *et al.*, 2004). In this group, maximal lever pressing activity occurred at approximately the middle of the session, with only a few erratic bursts of response. On the other hand, drug and cue priming produced distribution patterns of response rather similar in intact and OVX female rats, which was characterized instead by an earlier onset and longer duration of response, as both groups initiated lever pressing after comparably small periods

of time, and continued activity until the end of the 2 h session.

However, all drug- and cue-primed groups displayed a very low number of responses on the inactive lever throughout all the experiments, which indicated that the operant behaviour of animals was oriented specifically towards the previously drug-associated lever.

Locomotor activity

In line with previous observations (Fattore *et al.*, 2003; 2005b), locomotor activity during the reinstatement test sessions (mean \pm SEM of photocell beam breaks) was not altered in male and female rats by acute cannabinoid administration (data not shown), thus ensuring the absence of any non-specific effect on response. Similarly, acute exposure to visual and auditory cues or drug + cue combination priming did not affect spontaneous motor activity in any group. However, throughout the entire study, that is, during self-administration training, extinction, and reinstatement test sessions, intact and OVX female rats exhibited slightly higher spontaneous locomotor activity than males, although ANOVA did not reveal significant interactions (data not shown).

Discussion

The present study demonstrated that male and female rats responded differently to drug and drug-associated cue priming after extinction of cannabinoid self-administration,

thus providing the first evidence of a sex-dependent difference in the reinstatement of cannabinoid-seeking behaviour. We also showed that ovarian hormones are likely to be key players in the modulation of relapse to cannabinoids, as OVX rats were less responsive than intact females to drug-priming injections and exposure to drug-associated cues. These findings are consistent with our earlier study that showed that female rats acquire stable cannabinoid self-administration behaviour at a higher rate and over a shorter period of time than their male counterparts (Fattore *et al.*, 2007c). Actually, the sex-dependent differences in cannabinoid intake may account, at least in part, for the different level of responding reinstated by drug and cue-primed male and female rats after extinction.

To date, only a small number of animal studies have investigated sex differences in the reinstatement of extinguished drug-seeking behaviour, mainly focusing on nicotine (Perkins *et al.*, 1999; Donny *et al.*, 2000), cocaine (Fuchs *et al.*, 2005; Kippin *et al.*, 2005) and alcohol (Juarez *et al.*, 1993; Middaugh *et al.*, 1999). Broadly speaking, these studies converge in showing females as more susceptible than males to the rewarding properties of drugs of abuse and more inclined to relapse (reviewed in Fattore *et al.*, 2009b), in agreement with human studies that have shown that women have a higher risk of craving and relapse than men (Fattore *et al.*, 2008a), probably because of higher sensitivity to stressful events or depression (Wasilow-Mueller and Erickson, 2001; Weinberger *et al.*, 2009).

In our study, intact female rats showed a significantly greater reinstatement of responding than males when primed with cannabinoid and/or drug-associated cues. This not necessarily implies that females have a higher risk of relapse than males, as differences in the reinstated responding between the two groups were comparable with that showed during cannabinoid self-administration. That is, mean active responses were 1.45 times higher in female than in male rats during self-administration training, and 1.45 and 1.28 times higher after WIN 0.15 and WIN 0.3 primings respectively. Even in the female population, the dose-dependent effect of WIN primings previously described in male abstinent rats is noticeable (Fattore *et al.*, 2003). More importantly, the present study provides the first preclinical evidence of the ability of a drug-associated stimulus (either a visual or auditory cue) to reinstate extinguished cannabinoid-seeking behaviour in rats, in line with recent studies that have demonstrated the possibility of experimentally eliciting cue reactivity among adolescents and young adults with cannabis use disorders (Gray *et al.*, 2008; Bordnick *et al.*, 2009).

As observed with drug priming, after exposure to a drug-associated cue, male and female rats displayed similar increases in the mean active responding, as lever pressing activity in females was 1.79 and 1.87 times higher than in males after exposure to a visual or auditory cue, respectively. Our study therefore strengthens the notion that cue-elicited craving for marijuana is a reliable and valid phenomenon, comparable with cue-elicited craving for other drugs of abuse. In support of this, a very elegant study has recently shown for the first time that marijuana visual and tactile cues are able to increase activation in reward-related brain areas, including the ventral tegmental area, thalamus and amygdala of abstinent marijuana users (Filbey *et al.*, 2009).

Many factors are thought to play a part in the sex-dependent differences frequently detected in drug addictive behaviours and relapse, including genetic, socio-cultural and environmental factors, as well as brain sexual dimorphism, pharmacokinetics and pharmacodynamics (Thomasson, 1995; Perkins *et al.*, 1999; Milesi-Hallé *et al.*, 2005; Angelopoulou *et al.*, 2006). The prominent role played by gonadal hormones in craving and drug addiction is unambiguous, as oestrogens and progesterone not only influence ovulation and reproduction, but they also affect hedonic reward (Wise, 1998), incentive motivational aspects of drug-seeking (Robinson and Berridge, 2008) and a large number of emotional and neurocognitive functions (Fink *et al.*, 1998). An increasing number of studies have revealed that circulating hormone levels during the oestrous cycle may have a considerable impact on drug intake and relapse (Sinha *et al.*, 2007; Fox *et al.*, 2008). In line with this, ovarian hormones have been shown to be a key factor that underlies cannabinoid intake in rats (Fattore *et al.*, 2007c). In humans, the menstrual cycle and ovulation can modulate response to marijuana (Mello and Mendelson, 1985; Jukic *et al.*, 2007), while marijuana and Δ^9 -tetrahydrocannabinol may in turn affect the menstrual cycle of regularly cycling rhesus monkeys (Asch *et al.*, 1981; Almirez *et al.*, 1983). Thus, it is possible that, in our study, the oestrous cycle contributed to the increased response observed in the intact female group during reinstatement testing.

Once more, differences in mean active responses of intact and OVX females were similar during cannabinoid self-administration and reinstatement test sessions, being 1.58 times higher in intact females during cannabinoid intake, 1.65 and 1.59 times higher after WIN 0.15 and WIN 0.3 primings, respectively, and 1.58 and 1.35 times higher after exposure to a visual or auditory cue respectively. Note that when primed with a combination of WIN 0.15 mg·kg⁻¹ and the cue light, only intact females increased their responding with respect to that showed after the corresponding drug priming alone, thus showing greater vulnerability to relapse when exposed to the two types of stimuli simultaneously. Intriguingly, the higher dose of WIN tested in our study, 0.3 mg·kg⁻¹, is likely to produce a ceiling effect on reinstatement, as responding did not increase further in male and female animals, either intact or OVX, when such a priming was co-administered with the cue light.

The major findings of our study are therefore threefold: (i) reliable cannabinoid-seeking behaviour in abstinent rats can be elicited not only by drug priming (Spano *et al.*, 2004), but also by drug-associated cue priming; (ii) sex significantly affects not only cannabinoid intake (Fattore *et al.*, 2007c), but also drug- and cue-induced reinstatement of cannabinoid-seeking behaviour; and (iii) ovarian hormones crucially modulate not only cannabinoid self-administration, but also reinstatement of cannabinoid-seeking behaviour. Our data also demonstrated that drug priming was a stronger trigger of the reinstatement of cannabinoid-seeking behaviour than cue priming, because in all groups, response was significantly higher after priming with the cannabinoid than with visual or auditory cues. This is in agreement with previous studies that have shown greater reinstatement of cocaine-seeking (Feltenstein *et al.*, 2009; Kumaresan *et al.*, 2009) and heroin-

seeking (See, 2009) behaviour after drug priming than after cue exposure, although the opposite has been reported for nicotine-seeking behaviour (LeSage *et al.*, 2004; Dravolina *et al.*, 2007).

Finally, finding that response latency was not significantly different between the two groups of female rats, despite the significantly higher mean number of active responses in the intact compared with the OVX rats, is a point of concern. However, a closer analysis of the individual patterns of response in these animals may help to explain the apparent inconsistency. In fact, while response latency was similar in intact and OVX female rats, intact females showed shorter inter-response pauses than their OVX counterpart, thus reaching a higher number of responses during the 2 h session. This suggests that response latency is likely to reflect changes in general activity (i.e. arousal, behavioural activation) rather than incentive motivation for the drug, in line with motor activity data that showed that both groups of females typically were more active than males. If so, intact and OVX female rats would be expected to display a similar shorter response latency than males because of their higher motor activity, while greater accumulation of active lever presses by intact female rats would reflect greater sensitivity to cannabinoid-positive reinforcing effects.

By showing that male and intact and OVX female rats behave differently in response to drug or cue priming, our study adds further to understanding the intricate relationship among cannabinoids, gonadal hormones and drug-seeking behaviour (López, 2009). Sex-dependent differences in cannabinoid addiction have been corroborated by recent epidemiological observations (ONDCP, 2006; Tu *et al.*, 2008), while hormonal modulation of cannabinoid-seeking reinstatement may be ascribed, at least in part, to the potent anxiolytic effects of oestrogen (Hill *et al.*, 2007). The finding that female rats with blunted ovarian function react to drug and cue priming in a manner comparable with that of male rats leads to the hypothesis that ovarian hormones rather than sex *per se* facilitate relapse to cannabinoids, by enhancing the perception of the rewarding properties of cannabinoid agonists (Fattore *et al.*, 2007b) and/or facilitating formation of learned association between the effect of the drug and discriminative stimuli (Daniel *et al.*, 2002). This cannabinoid extinction/reinstatement animal model of relapse has therefore confirmed its usefulness for studying central mechanisms regulating marijuana abuse and relapse.

Conclusions

The present study extends previous findings on the crucial role played by sex and ovarian hormones in cannabinoid self-administration, by demonstrating the existence of sex-dependent differences in the reinstatement of cannabinoid-seeking behaviour. At the same time, our study substantiates the utility of the extinction/reinstatement procedure as a model to unravel sex-dependent differences in cue-induced relapse to cannabinoid.

There are three clear targets for new research. First, to investigate whether male and female rats respond differently to acute stress, which is well known to trigger relapse to most

drugs of abuse, even after a prolonged period of drug abstinence (Shaham *et al.*, 2000). Second, to verify whether females are more vulnerable to relapse to cannabinoid-seeking by evaluating whether stimuli that are sub-threshold for males (i.e. lower WIN priming doses, personal communication) can be effective in females. And, lastly, to conduct hormone manipulations in OVX female rats to assess the specific role of oestrogen in cannabinoid self-administration and relapse.

Acknowledgement

The authors are grateful to Mrs Barbara Tuveri for the technical assistance.

Conflict of interest

None.

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