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# Male and Female Rats Differ in Brain Cannabinoid CB1 Receptor Density and Function and in Behavioural Traits Predisposing to Drug Addiction: Effect of Ovarian Hormones

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**Abstract:** Sex-dependent differences are frequently observed in the biological and behavioural effects of substances of abuse, including cannabis. We recently demonstrated a modulating effect of sex and oestrous cycle on cannabinoid-taking and seeking behaviours. Here, we investigated the influence of sex and oestrogen in the regulation of cannabinoid CB1 receptor density and function, measured by [$^3$H]CP55940 and CP55940-stimulated [$^{35}$S]GTPγS binding autoradiography, respectively, in the prefrontal cortex (Cg1 and Cg3), caudate-putamen, nucleus accumbens, amygdala and hippocampus of male and cycling female rats, as well as ovariectomised (OVX) rats and OVX rats primed with oestradiol (10 μg/rat) (OVX+E). CB1 receptor density was significantly lower in the prefrontal cortex and amygdala of cycling females than in males and in OVX females, a difference that appeared to be oestradiol-dependent, because it was no more evident in the OVX+E group. CP55940-stimulated [$^{35}$S]GTPγS binding was significantly higher in the Cg3 of OVX rats relative to cycling and OVX+E rats. No difference was observed in CB1 receptor density or function in any of the other brain areas analysed. Finally, sex and oestradiol were also found to affect motor activity, social behaviour and sensorimotor gating in rats tested in locomotor activity boxes, social interaction and prepulse inhibition tasks, respectively. Our findings provide biochemical evidence for sex- and hormone-dependent differences in the density and function of CB1 receptors in selected brain regions, and in behaviours associated with greater vulnerability to drug addiction, revealing a more vulnerable behavioural phenotype in female than in male rats.

**Keywords:** Sex difference, ovariectomy, oestrogen, CB1 receptor and [$^{35}$S]GTPγS binding, locomotor activity, social interaction, anxiety, prepulse inhibition.

## INTRODUCTION

A burgeoning body of research has shown that addictive drugs affect males and females differently, with the general pattern of sex differences being similar for most drugs of abuse. For example, female rodents display higher voluntary intake of alcohol [1, 2], caffeine [3], cocaine [4, 5], heroin [6], morphine [7, 8], and fentanyl [9] than males. The heightened response to stimulants shown by female rodents has been attributed to the dopamine-enhancing properties of oestrogen [10, 11], implying the possibility that females are inherently more sensitive than males to the rewarding properties of drugs of abuse, and therefore more biologically susceptible to developing drug addiction and dependence.

Cannabis is by far the most widely used illicit drug, consumed by 125–203 million people worldwide in 2009, corresponding to an annual prevalence rate of 2.8–4.5% [12]. Although cannabis withdrawal lacks clinical significance and is therefore not recognised in DSM-IV, marijuana users often display a withdrawal syndrome, and attempts to relieve withdrawal symptoms facilitate relapse to drug use during cessation attempts [13]. Importantly, marijuana-related cues increase self-reported craving and activate the reward brain pathway, including the ventral tegmental area (VTA), thalamus, anterior cingulate, insula, and amygdala [14]. According to the European Monitoring Centre for Drugs and Drug Addiction, more men than women use cannabis and attend drug treatment services in Europe [15]. In female smokers, increased marijuana use has been associated with premenstrual dysphoria, and women have reported significantly greater depression, anxiety, mood liability, anger, irritability and impaired social functioning [16].

Within the brain, cannabinoid CB1 receptors are differently expressed between males and females. Several studies have demonstrated that limbic brain regions, often referred to as the emotional brain, are particularly vulnerable to chronic marijuana use [17, 18]. Animal studies have confirmed that sex and ovarian hormones strongly influence the proclivity toward cannabinoid use [19-21]. Recently, it has been reported that repeated exposure to tetrahydrocannabinol (THC), the main psychoactive ingredient of marijuana, produces greater desensitization and down-regulation of CB1 receptors in the brain of adolescent female than male rats [22], demonstrating differential central actions of cannabinoids between the sexes. In support of a role of sex in the effects of THC on CB1 receptor level and CB1/G-protein coupling, it has been shown that THC exposure during adolescence significantly reduces CB1 receptor density and function in the amygdala, VTA and nucleus accumbens (Nac) of female rats, whereas in male rats it causes significant alterations in the amygdala and hippocampal formation [23]. Several years ago, we demonstrated that chronic intravenous self-administration of cannabinoid agonist alters, and in most cases increases, density and coupling of CB1 receptors in the reward-related brain of male Lister Hooded rats [24]. However, male rats exhibit slower acquisition of cannabinoid self-administration and less drug intake than females [25], and a lower response rate for the cannabinoid when exposed to acute drug and cue primings after extinction [26].

To date, CB1 receptor density and function in the male and female brain have been analysed in *post-mortem* studies on psychiatric or alcoholic patients [27-29], and in rodents exposed to cannabinoids prenatally [30], during adolescence [31, 22] and adulthood [22], or after subchronic treatment with antipsychotics [32].

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Besides *post-mortem* and animal data, only one study has been conducted thus far to assess the *in vivo* cerebral CB1 receptor distribution and its variation with healthy aging and sex, which has reported a region-dependent and gender-related up-regulation of CB1 receptors [33]. Specifically, by using positron emission tomography and a high-affinity, subtype-selective radioligand, it has been shown that binding to CB1 receptors increases with ageing in the basal ganglia, lateral temporal cortex and limbic system of women, whereas men show higher binding in clusters of the limbic system and cortico-striato-thalamic-cortical circuit [33]. Surprisingly, only a few animal studies have conducted a systematic comparison in adult drug-naïve males and females to investigate possible sex differences in brain CB1 receptor density and function [34-36]. Yet, sex and steroid influence on CB1 receptor density and function has not been evaluated in rat strains known to self-administer intravenous cannabinoids consistently [37-39], which would provide a biochemical basis for the enhanced susceptibility of females to self-administer cannabinoids.

The first aim of the present study was therefore to investigate the influences of sex on regulation of CB1 receptor density and function, measured by quantitative autoradiographic binding studies with [$^3$H]CP55940 and CP55940-stimulated [$^{35}$S]GTPγS binding autoradiography, respectively, in selected brain areas of male and cycling female Lister Hooded rats. Moreover, since oestrogen has been recently found to affect limbic cannabinoid receptor binding [36] and produce long-term effects on operant learning and performance tasks [40], we also evaluated CB1 receptor density and function in brain regions involved in emotional and cognitive functions, that is, prefrontal cortex (Cg1 and Cg3), caudate-putamen (CPu), Nac core (Nac Core) and shell (Nac Shell), amygdala and hippocampus, in ovariectomised rats (OVX), and in OVX rats acutely primed with subcutaneous (sc) injection of β-oestradiol 3-benzoate (10 μg/rat) (OVX+E), that is, following oestradiol replacement.

Previous studies have identified several vulnerability factors that contribute to individual variation in the risk of addiction, ranging from genetic susceptibility to social and cultural characteristics. In laboratory animals, enhanced spontaneous motor activity in a novel environment [41, 42] and an increased level of anxiety [43, 44] have been demonstrated to be important factors that enhance vulnerability to initiate drug self-administration and accelerate the rate of acquisition of drug-reinforced responses. Importantly, sex differences have been detected in these vulnerable behavioural traits, with female animals and humans displaying more cocaine-induced locomotor activation and stronger behavioural sensitization to psychostimulants than males [45-47]. Notably, such a hyperactive/hyper-responsive behavioural phenotype in females persists in rat strains selected for low levels of novelty-induced locomotion (Low Responders, LR), as LR-bred males are significantly less active than LR-bred females [48]. In humans, individuals with anxiety disorders shift from regular drinking to alcohol dependence more rapidly than do individuals without anxiety disorders [44], and young marijuana users with social anxiety disorder engage in more frequent drug use [49]. Gender differences in problematic cannabis use among individuals with social anxiety has been described [50], with young women appearing particularly vulnerable to the social impairments associated with high social anxiety [51].

Thus, a second aim of the present study was to test the hypothesis that in the same strain of rats sex and oestradiol may also affect these core behavioural traits that contribute to individual variation in the propensity to abuse drugs. For this purpose, we compared motor activity and social anxiety of male, cycling female, OVX and OVX+E Lister Hooded rats by using motor activity and social interaction tests, respectively.

Finally, altered baseline sensorimotor gating and information processing are associated with vulnerability to drug dependence [52, 53]. Several drugs of abuse, including cannabis, have been consistently found to modulate the prepulse inhibition (PPI) of the acoustic startle reflex [54-58], a sensorimotor gating task in which a low-intensity acoustic stimulus presented prior to a high-intensity, startle-eliciting stimulus can attenuate the acoustic startle response [59]. Individuals with schizophrenia show impaired baseline PPI and attention modulation of PPI, and are at increased risk for developing substance abuse disorders. Among the schizophrenic population, the rate of cannabis use disorders is significantly higher among men than women [60-62]. Although men are more socially isolated and dysfunctional, schizophrenic women are associated with better social functioning in that they are generally more active and integrated [63]. Notably, sexually dimorphic effects of cannabinoid agonists on the PPI of the acoustic startle reflex [64] and on anxiety-related behaviour and motor activity have recently been described in rats [65]. In light of this, we assessed the moderating effect of sex and oestradiol on PPI and sensorimotor gating in male, cycling female, OVX and OVX+E rats.

## MATERIALS AND METHODS

### Animals

Adult Lister Hooded rats of both sexes (males 275±25 g, females 210±25 g; Harlan-Nossan, Italy) were used. Animals were housed 4/cage on a reversed 12/12 hours light/dark cycle (lights on from 7:00 pm), with constant room temperature (21±2 °C) and humidity (60%), and with free access to water and food. All experiments were approved by the local Animal Care Committee and carried out in strict accordance with the E.C. Regulations for Animal Use in Research (CEE No. 86/609).

### Drugs

Forty-eight hours prior to behavioural testing or brain collection for biochemical studies, 4 different groups of OVX rats (1 for biochemical studies, 3 for behavioural testing) received a 0.1 ml s.c. injection of 10 μg β-oestradiol 3-benzoate (Sigma-Aldrich, Italy) dissolved in peanut oil. The oestradiol treatment protocol was based on an established protocol showing that it elicits a wide range of behavioural effects in rodents [66-69].

### Surgery

Under isoflurane anaesthesia, ovaries were accessed aseptically through a small ventral incision followed by two cuts through the muscle layer of the abdominal cavity exposing the ovaries. Parovarian fatty tissue was identified and pulled out; each oviduct was first ligated at its junction with the uterine body, and then removed with the associated ovary. The uterus with associated tissue was then returned to the abdomen. Following bilateral ovariectomy, the skin incision was sutured and covered with a local antiseptic, and the animal was given antibiotic treatment (0.1 ml s.c., Baytrill; Bayer) for 3 days. To minimise wound damage, operated animals were isolated from the rest of the group for 2 days, after which they were housed together in cages of four. Animals were left a minimum of 3 extra weeks after ovariectomy for stabilisation of gonadal and pituitary hormones before starting behavioural testing [70]. All antibiotics and anaesthetics were purchased as sterile solutions from local distributors.

### Binding Studies

#### *Brain Tissue Preparation*

Rats were sacrificed by rapid decapitation, and brains were rapidly removed, immediately immersed in isopentane and then stored at –80 °C until sectioning for autoradiographic studies.

#### *[$^3$H](-)-CP55940 Autoradiography*

Coronal sections 12-16 μm thick were prepared with a cryostat at –20 °C, thaw mounted onto Superfrost Plus slides (BDH, Lutterworth, UK) and stored with desiccant at –20 °C until use. Brain regions selected for analysis were the following, according to the

Atlas of Paxinos and Watson [71]: Cg3 and Cg1 (AP: +3.2), CPu, Nac Core and Nac Shell (AP: +1.60), CA1, CA2 and CA3 fields of hippocampus, dentate gyrus (DG) of hippocampus, and amygdala (AMY) (AP: from –2.14 to –3.14), VTA (AP: –4.80) (Fig. **1**). Adjacent sections to those used for autoradiography were collected and stained with Neutral Red to facilitate the identification of the selected brain areas. [$^3$H](-)-CP55940 binding autoradiography was performed as previously described [72]. Briefly, tissue slides were incubated at 37 °C for 2.5 hours in 50 mM Tris-HCl (pH 7.4) containing 5% bovine serum albumin (BSA) and 10 nM [$^3$H]CP55940 (specific activity, 139.6 Ci/mmol; Perkin Elmer, Boston, MA, USA). Non-specific binding was determined in adjacent brain sections in the presence of 10 μM unlabelled CP55940. Following incubation, tissue slides were rinsed twice at 4 °C for 2 hours in ice-cold Tris-HCl buffer (50 mM, pH 7.4) with 1% BSA, once (5 min) with 50 mM Tris-HCl, dipped in ice-cold deionised water and then air-dried.

### *CP55940-stimulated [$^{35}$S]GTPγS Binding in Autoradiography*

[$^{35}$S]GTPγS binding in autoradiography was performed as previously described [31]. Briefly, tissue slices were preincubated in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, 0.1% BSA, pH 7.4) at 25 °C for 10 min and preincubated further with 3 mM GDP for 10 min in assay buffer at 25 °C. Agonist-stimulated activity was determined by incubating tissue slices for 2 hours at 25 °C with 5 μM CP55940 in fresh buffer containing 0.04 nM [$^{35}$S]GTPγS and 3 mM GDP. Basal activity was assayed in the absence of CP55940 and in the presence of 3 mM GDP. Non-specific binding was determined in adjacent brain sections in the presence of 10 μM unlabelled GTPγS. Following incubation, tissue slices were rinsed twice in ice-cold Tris-HCl buffer (50 mM, pH 7.4) and once in deionised water, and air-dried.

### *Image Analysis*

Dried tissue sections and slide-mounted [$^3$H]micro-scales standards (RPA 501 and 505; Amersham, USA) for [$^3$H](-)-CP55940 or [$^{14}$C]micro-scales standards (RPA 504 and 511; Amersham, USA) for [$^{35}$S]GTPγS binding autoradiography were placed in a Fujifilm BAS cassette with a BAS-5000 imaging plate. The resulting images were analysed with the Fujifilm-BAS 5000 imaging system (AIDA, Raytest, Wilmington, NC, USA), and optical densities (ODs) were transformed into levels of bound radioactivity (fmol/mg protein) with gray values generated by co-exposed [$^3$H] or [$^{14}$C] standards. CP55940-stimulated [$^{35}$S]GTPγS activity in brain sections was calculated by subtracting the OD in basal sections (incubated with GDP alone) from that of agonist-stimulated sections; results were expressed as percentage basal activity.

### *Statistical Analysis*

With regard to binding studies, data corresponding to each receptor and CP55940-stimulated GTPγS binding were expressed as mean ± SEM. Statistical differences between males and females were assessed by Student's *t*-test; data comparing cycling females, OVX and OVX+E rats were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test for *post hoc* comparisons.

## Behavioural Studies

To avoid exposure to prior tests influencing performance in subsequent tasks, a separate group of rats was used in each experiment. All behavioural tests were conducted during the dark phase of the light-dark cycle.

### *Motor Activity*

We tested the moderating effects of sex and oestradiol on spontaneous locomotor activity in male, female, OVX and OVX+E rats (n=8/group) using the Digiscan Animal Activity Analyser (Omnitech Electronics, USA). Each operant cage (42×30×60 cm) had

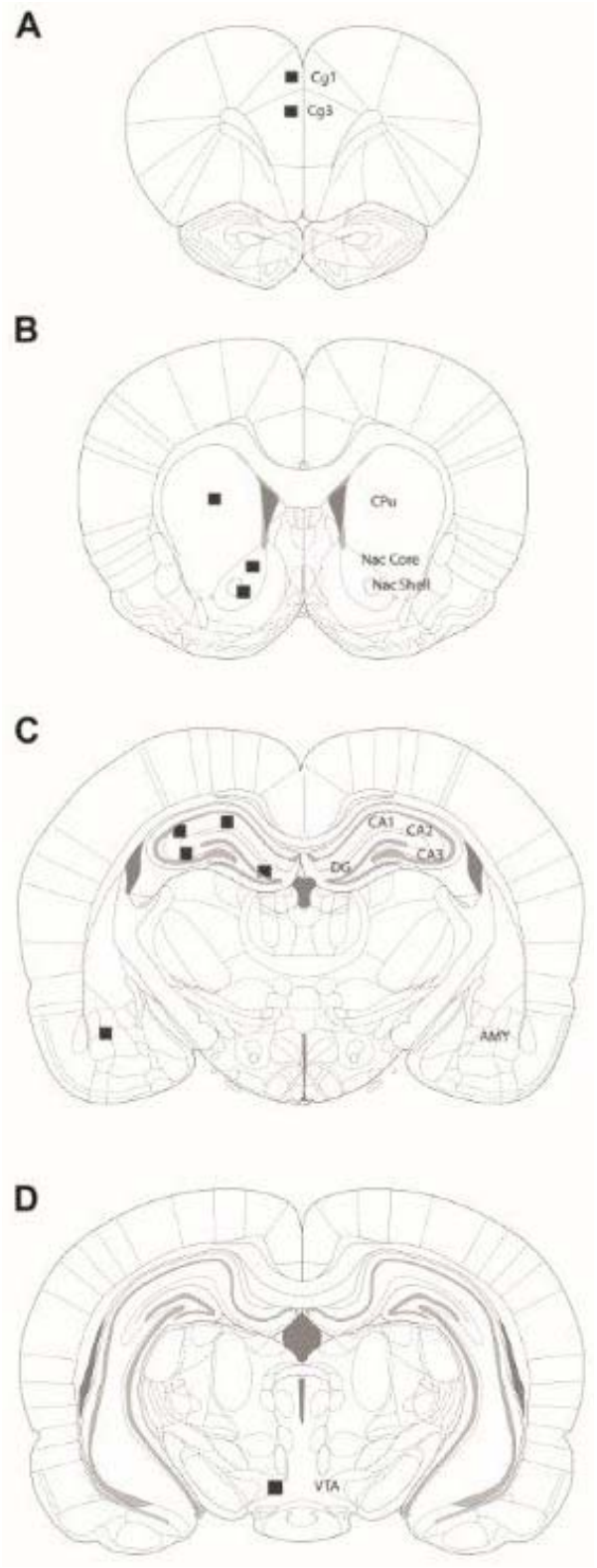


**Fig. (1).** Schematic redrawing based on the Atlas of Paxinos and Watson [71] showing the prefrontal cortex (cingulate cortex areas 3 and 1, Cg3 and Cg1) (**A**); caudate-putamen (CPu), nucleus accumbens core (NAc Core) and shell (Nac Shell) (**B**); CA1, CA2 and CA3 fields of Ammon's horn, gyrus dentate (DG) of hippocampus and amygdala (AMY) (**C**); ventral tegmental area, VTA (**D**). Boxed areas indicate the approximate site of the regions analyzed.

two sets of 16 photocells located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 2 cm above the cage floor, and a further set of 16 horizontal beams whose height could be adapted to the size of the animals. Locomotor activity was performed in a dark room dimly illuminated by a red lamp. Rats were placed individually into the box and allowed to explore the novel environment over a 60-min session, during which the following behavioural parameters were measured:

*Horizontal activity*: the total number of beam interruptions that occurred in the horizontal sensors;

*Vertical activity*: the total number of beam interruptions that occurred in the vertical sensors, that is, the number of times the animal rose onto its hind legs with the front limbs either against the wall or freely in the air (number of rearing episodes);

*Total distance (cm)*: the horizontal distance travelled by the animal during the 60-min session (dependent on animal's path).

At the end of the session, animals were gently removed from the plexiglass motility cages, and then returned to the same cage. The arena was wiped out with $H_2O_2$ between sessions to prevent olfactory cues. Motor activity was determined as total number of photo-beam breaks (activity counts) and recorded for 60 min. Counts were referred to 10-min time intervals.

### *Social Interaction*

The testing arena consisted of a plexiglass box (60×60×42.5 cm) placed in a room under dim-light conditions. Male, female, OVX and OVX+E rats (n=8/group) were individually habituated to the experimental cage for 10 min on each of the 2 days before testing. On the test day, animals were socially isolated for 4 h before testing. The experimental animal was placed into the arena with a novel unfamiliar conspecific rat of the same sex and not differing more than 20g in body weight. As previously described [73], animals were allowed to interact for 10 min, during which, time spent by the experimental rat in social interactions (sniffing, following or grooming the partner, boxing and wrestling) as well as the number of social contacts were monitored. The box was cleaned with $H_2O_2$ after each experimental session.

### *PPI and Sensorimotor Gating*

The apparatus for the detection of the startle reflexes (Med Associates, St Albans, USA) consisted of four standard cages, each placed inside a sound-attenuated and ventilated chamber. Each startle cage was a non-restrictive Plexiglas cylinder 9 cm in diameter mounted on a piezoelectric accelerometer platform connected to an analogue-digital converter. Background noise and acoustic bursts were conveyed by two separate speakers, placed in proximity to the startle cage, in order to produce a variation in sound within 1 dB. Both speakers and startle cages were connected to a main PC, which detected and analysed all chamber variables with custom software. Before each testing session, acoustic stimuli and mechanical responses were calibrated via specific devices supplied by Med Associates.

Test for PPI and sensorimotor gating were conducted as previously described [73] in male, female, OVX and OVX+E rats (n=11-15/group). Briefly, on the test day, each rat was placed in the experimental cage for a 5 min acclimatization period with a 70 dB white noise background, which continued for the remainder of the session. The session consisted of three consecutive blocks of trials. Differently from the first and third block, which consisted of 5 pulse-alone trials of 40 ms at 115 dB, the second block (*test block*) displayed a pseudorandom sequence of 50 trials, including 12 pulse-alone trials, 30 trials of pulse preceded by 73, 76 or 82 dB pre-pulses (10 for each level of prepulse loudness), and 8 no-stimulus trials, where the only background noise was delivered. Intertrial intervals (ITIs) were selected randomly between 10 and 15 sec. The percentage (%) PPI was calculated based only on the values relative to the second block, and using the following formula: 100 – [(mean startle amplitude for pre-pulse + pulse trials/mean startle amplitude for pulse-alone trials) × 100].

### *Statistical Analysis*

For behavioural studies, statistical differences between males and females were assessed by Student's *t*-test, whereas data comparison among cycling females, OVX and OVX+E rats was performed by one-way ANOVA followed by Newman-Keuls test for *post hoc* comparisons.

## RESULTS

### Binding Studies

### *Effects of Sex and Oestrogen on CB1 Receptor Density and Function*

As shown in Table **1**, cycling females revealed a significantly ($p<0.05$) lower levels of CB1 receptors than males in the Cg1 (–30%, $t_7=2.649$), Cg3 (–28%, $t_7=2.304$) and amygdala (–27%, $t_7=2.458$), whereas no sex-dependent differences were found in CB1 receptor function. No sex differences were observed in either CB1 receptor levels or CP55940-stimulated [$^{35}$S]GTPγS binding in any of the other brain areas analysed.

We investigated whether the density of CB1 receptors was under sex steroid-dependent regulation by examining CB1 receptor expression in the same brain areas of cycling females, OVX females and OVX+E rats. As shown in (Fig. **2A**), one-way ANOVA revealed a significant difference among these three groups in the Cg1 ($F_{(2,13)}=5.743$, $p<0.05$), Cg3 ($F_{(2,14)}=4.458$, $p<0.05$) and amygdala ($F_{(2,13)}=18.13$, $p<0.001$). *Post hoc* analysis showed that, in the Cg1 and Cg3, OVX rats exhibited higher levels of CB1 receptor binding than either cycling females (+62 and +54%, respectively for Cg1 and Cg3) or OVX+E rats (+54 % increase for both Cg1 and Cg3) (Fig. **2A**). Similarly, CB1 receptor levels within the amygdala were significantly higher in OVX than in both cycling (+39%, $p<0.001$) and OVX+E (+69%, $p<0.01$) rats (Fig. **2A**).

To determine whether the changes in CB1 receptor density were associated with G-protein-mediated CB1 receptor function, CP55940-stimulated [$^{35}$S]GTPγS binding was performed in the same brain areas of all groups. Basal levels of [$^{35}$S]GTPγS binding did not significantly differ between males, cycling females, OVX, and OVX+E rats in any region analysed (data not shown). Conversely, one-way ANOVA revealed a significant difference among cycling, OVX and OVX+E rats in Cg3 ($F_{(2,12)}=9.016$, $p<0.01$), CB1 receptor-G protein coupling being significantly higher in the Cg3 of OVX than in cycling (+14%, $p<0.05$) and OVX+E (+25%, $p<0.01$) rats (Fig. **2B**). No significant differences in CP55940-stimulated GTPγS binding were found in the Cg1 and the amygdala (Fig. **2B**).

### Behavioural Studies

### *Effects of Sex and Oestrogen on Spontaneous Motor Activity*

Fig. (**3**) shows motor activity in Lister Hooded rats as measured in the Omnitech Digiscan cages during the 60-min session. As shown in panels A, the cumulative horizontal activity of female rats at the end of the session was significantly higher ($p<0.01$) than that of males (*left*), revealing a more elevated motor activity profile. When comparing cycling females with OVX and OVX+E rats (*right*), one-way ANOVA revealed that ovariectomy and oestradiol replacement in OVX rats significantly reduced and enhanced, respectively, horizontal motor activity ($F_{(2,23)}=9.878$, $p<0.05$). Similarly, cumulative vertical activity of female rats at the end of the session (Fig. **3B**, *left*) was significantly higher than in male rats ($p<0.01$). However, ovariectomy induced only a slight (not significant) decrease in vertical activity (Fig. **3B**, *right*), whereas oestradiol replacement in OVX rats confirmed its enhancing effect on this motor parameter ($F_{(2,23)}=18.71$, $p<0.01$). Cumulative distance trav-

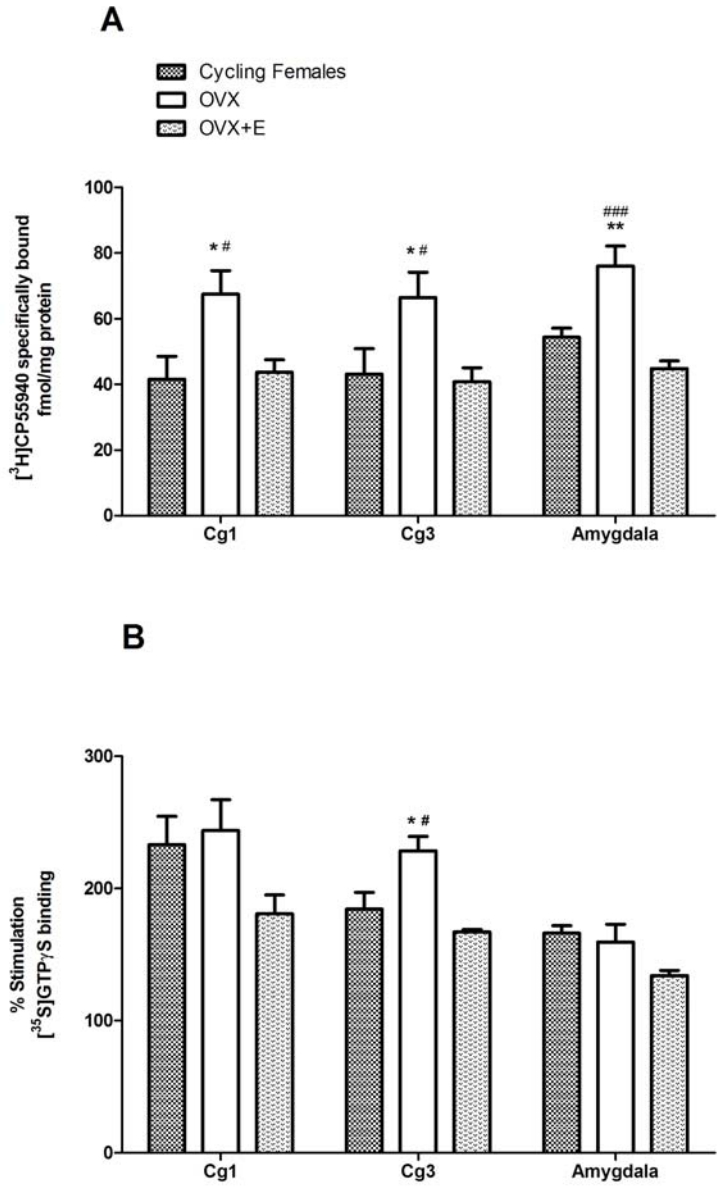


**Fig. (2).** Effect of oestrogen on CB1 receptor density (**A**) and on CP55940-stimulated [$^{35}$S]GTPγS binding (**B**) in the Cg1, Cg3, and amygdala. Data are expressed as mean ± SEM of density reading (six tissue sections for each brain area per animal) expressed as fmol/mg protein of [$^{3}$H]CP55940 specific bound (**A**) and as percentage of stimulation of basal binding (**B**). *p<0.05 and **p<0.01 vs cycling females; $^{\#}$p<0.05 and $^{\#\#}$p<0.01 vs OVX+E (one-way ANOVA followed by Newman-Keuls test).

elled by male and female rats during the 60-min session is shown in (Fig. **3C**). In line with horizontal activity data, distance travelled was significantly longer (p<0.001) in female than in male rats (*left*), with OVX and OVX+E rats respectively decreasing and increasing distance travelled (*right*) ($F_{(2,23)}$=72.54, p<0.001).

### *Effects of Sex and Oestrogen on Sociability*

Fig. (**4**) illustrates findings from the social interaction test in male and cycling female rats as well as in OVX and OVX+E rats. When comparing performances of male and cycling female rats (Fig. **4A**, *left*), statistical analysis revealed that females spent significantly less time (p<0.01) than males in interacting with the age- and sex-matched partner, thus revealing an enhanced social anxiety level which, however, seems not to depend upon ovarian hormones, because neither ovariectomy nor oestradiol replacement in OVX rats had a significant effect on the time spent interacting with the partner (Fig. **4A**, *right*) ($F_{(2,23)}$=0.3025, p>0.05, one-way ANOVA). Conversely, cycling female rats displayed a significantly (p<0.001) higher number of social contacts than males (Fig. **4b**, *left*), suggesting a more anxiety-like basal state than the male littermates had. Notably, the number of contacts did not differ significantly among the three female groups (Fig. **4b**, *right*) ($F_{(2,23)}$=0.889, p>0.05).

### *Effects of Sex and Oestrogen on PPI and Acoustic Startle Reflex (ASR)*

We examined the impact of sex and ovarian hormones on PPI of the startle reflex, the disruption of which is widely utilised as a model of perceptual distortion [74]. Statistical analyses revealed a significant effect of sex on basal PPI (Fig. **5A**, *left*), with males exhibiting significantly (p<0.001) more robust PPI than females. One-way ANOVA also revealed a significant effect of ovarian hormones on PPI ($F_{(2,37)}$=7.372, p<0.01), which was enhanced fol-

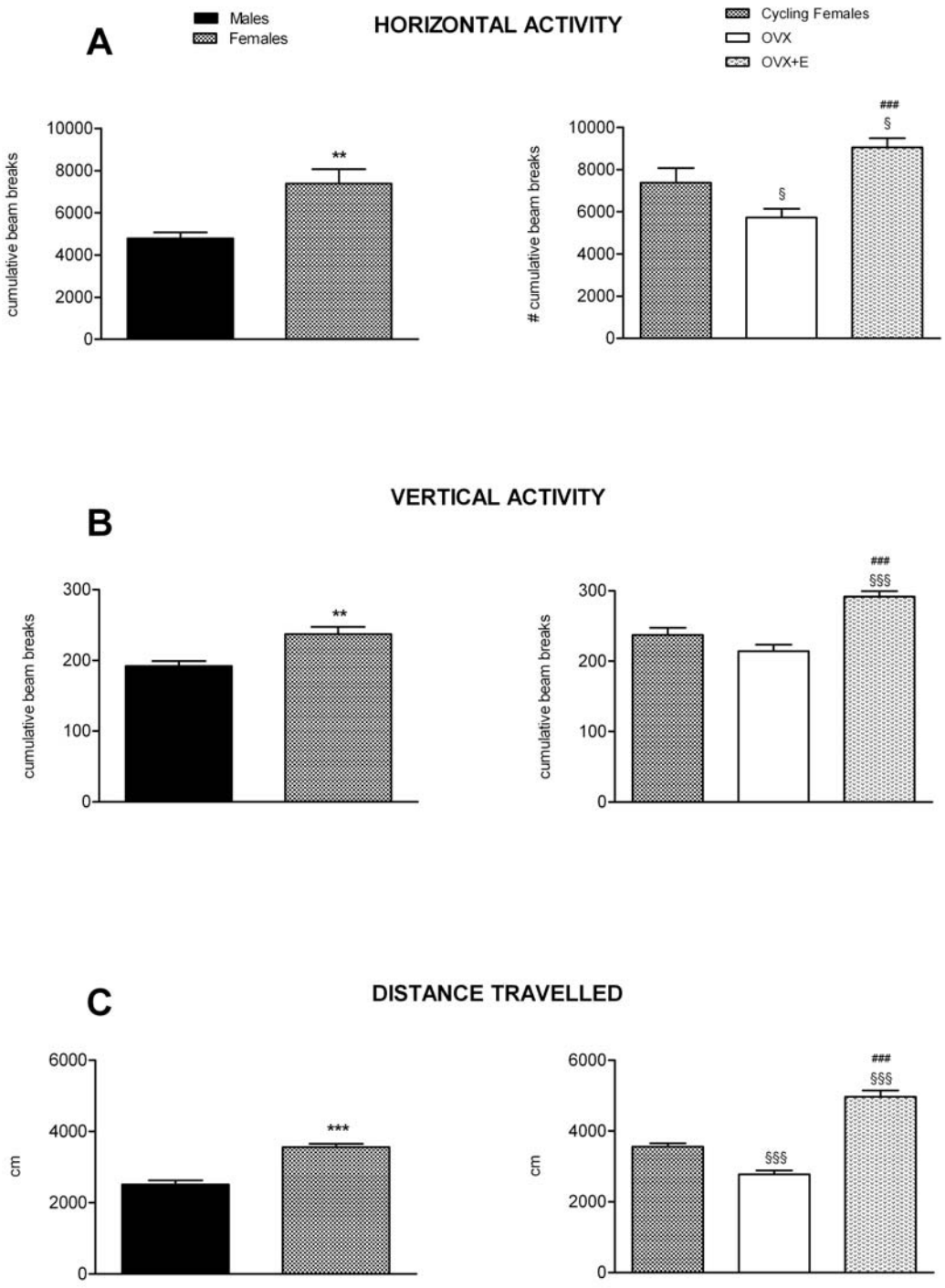


**Fig. (3).** Effect of sex (*left*) and ovarian hormones (*right*) on the spontaneous motor activity, that is, horizontal (**A**) and vertical (**B**) activity, and distance travelled (**C**). Each value represents the mean ± SEM of total locomotor activity during 60 min of observation (n=8/group). **p<0.01 and ***p<0.001 vs males (Student *t*-test); $^{§}$p<0.05, $^{§§}$p<0.01 and $^{§§§}$p<0.001 vs cycling females, $^{###}$p<0.001 vs OVX (one-way ANOVA followed by Newman-Keuls test).

lowing ovariectomy, and reverted by oestradiol replacement to the level of cycling females (Fig. **5A**, *right*).

A significant effect of sex was also found in the baseline startle amplitude (Fig. **5B**, *left*), with cycling females showing significantly (p<0.001) lower ASR than males, confirming that male and female rats significantly differ in terms of basic auditory function. Notably, ovarian hormones seem not to play a significant role in vigilance and reactivity to sensory stimuli, as neither ovariectomy nor oestradiol replacement affected startle amplitude (Fig. **5B**, *right*) or latency (data not shown).

When analysing habituation, that is, baseline activity level in the acoustic startle chambers in response to the background level of noise (during the acclimation period) and the presentation of pulses (during the test session), we found that a greater response typically occurred at the highest intensity tested in all groups of rats, but did not detect a significant sex × time or a hormonal status x time inter-

**Table 1. CB1 Receptor (CB1R) Density and CP55940-stimulated [$^{35}$S]GTPγS Binding in Selected Brain Areas of Males and Cycling Females.**

| | Males | | Cycling Females | |
|---|---|---|---|---|
| Brain areas | *CB1R density (fmol/mg prot)* | *GTPγS binding (% stimulation)* | *CB1R density (fmol/mg prot)* | *GTPγS binding (% stimulation)* |
| Cg1 | 52.82 ± 2.9 (5) | 232.0 ± 15.3 (5) | 36.83 ± 5.7 (4)* | 233.2 ± 21.4 (5) |
| Cg3 | 57.91 ± 3.2 (5) | 188.1 ± 15.0 (5) | 41.53 ± 6.9 (4)* | 184.4 ± 12.7 (5) |
| Amygdala | 74.74 ± 7.0 (5) | 168.8 ± 12.8 (5) | 54.28 ± 2.8 (4)* | 166.1 ± 5.8 (4) |
| Nac Core | 52.14 ± 6.3 (5) | 141.7 ± 5.9 (5) | 50.20 ± 7.1 (5) | 168.3 ± 7.3 (5) |
| Nac Shell | 53.26 ± 6.0 (5) | 148.7 ± 7.9 (4) | 54.65 ± 4.7 (5) | 160.8 ± 6.5 (5) |
| CPu | 83.60 ± 10.4 (5) | 170.7 ± 10.2 (5) | 82.27 ± 7.2 (5) | 172.5 ± 9.5 (5) |
| VTA | 40.52 ± 4.5 (4) | 129.1 ± 7.9 (4) | 40.16 ± 4.8 (5) | 132.1 ± 4.3 (3) |
| CA1 | 108.9 ± 5.4 (3) | 190.2 ± 14.5 (5) | 104.2 ± 4.0 (4) | 173.8 ± 12.4 (4) |
| CA2 | 101.8 ± 4.6 (4) | 191.5 ± 16.4 (5) | 87.82 ± 5.9 (4) | 172.2 ± 10.9 (4) |
| CA3 | 102.5 ± 7.7 (4) | 178.8 ± 14.6 (5) | 96.83 ± 4.6 (4) | 163.5 ± 13.8 (4) |
| DG | 91.82 ± 8.9 (3) | 174.0 ± 17.11 (5) | 95.85 ± 5.5 (4) | 160.2 ± 10.0 (4) |

Values are the mean ± SEM of density reading (six tissue sections for each brain area per animal), expressed as fmol/mg protein and as percent of stimulation of basal binding for CB1R density and CP55940 stimulated [$^{35}$S]GTPγS binding, respectively. In parentheses is indicated the number of animals per experimental group. Abbreviations: Cg1, cingulated area 1; Cg3, cingulated area 3; Nac Core, nucleus accumbens core; Nac Shell, nucleus accumbens shell; CPu, caudate-putamen; VTA, ventral tegmental area; CA1-CA3, CA1-CA2-CA3 fields of Ammon's horn; DG, dentate gyrus of hippocampus. *p<0.05 versus corresponding males (Student *t*-test).

action among experimental groups (data not shown), suggesting that both sexes had clear and similar within-session habituation that was unaffected by ovarian hormones.

## DISCUSSION

Although drug addiction is more prevalent in men than women, the gap is narrowing rapidly leading to assume that it may simply reflect earlier disparities in opportunity rather than vulnerability [75, 76]. To strengthen this idea, women tend to increase their rate of drug consumption more quickly than men, are more likely to relapse, and report more pronounced cravings and subjective drug effects. Several years ago we demonstrated that female rats self-administer higher amount of cannabinoid than males, take longer to extinguish cannabinoid-seeking behaviour, and show a higher risk of relapse, and that ovarian hormones are crucial modulators of cannabinoid-taking and seeking behaviours [25, 26].

In this follow-up study, the first aim was to investigate whether different expression and function of the brain CB1 receptors among sexes might account, at least partly, for the documented enhanced vulnerability of females to the rewarding properties of cannabinoids. There is considerable variation in individual vulnerability to addiction that may be related to behavioural endophenotype. High novelty-induced locomotor response and lower social behaviour have been posited as predisposing traits enhancing susceptibility to addiction [49, 77]. Moreover, substance abuse comorbidity has been frequently described in schizophrenic patients [78], which may reflect the impact of the neuropathology of schizophrenia on the neural circuitry underlying drug reward and reinforcement [79]. A second aim of the present study was therefore to ascertain whether sex differences also exist in motor parameters, social anxiety and PPI of the ASR in male and female rats, and whether ovarian hormones may have a role in modulating these behaviours.

### Biochemical Findings

The first relevant finding of the present study was the occurrence of sex-dependent differences in CB1 receptor levels between male and cycling female rats, with the latter showing lower CB1 receptor density in the prefrontal cortex (Cg1 and Cg3) and amygdala. In these same brain areas, ovariectomy increased CB1 receptor expression, whereas replacement with oestradiol markedly reduced the amount of CB1 receptors to the levels found in cycling females. These findings suggest that ovarian hormones might negatively regulate the density of CB1 receptors in brain areas involved in cognition and emotional processing.

Thus far, the few studies that have examined the role of sex and/or oestrogen on CB1 receptor expression and function have provided conflicting results, mostly depending on the specific brain area considered and showing reduced, increased or no effect [22, 31, 34, 88]. Importantly, almost all studies have not investigated the effects of sex and oestrogen on CB1 receptor and G-protein activity in the same brain region. Many subregions of the prefrontal cortex, and in particular the anterior cingulate cortex (Cg1,2) and the prelimbic cortex (Cg3), are part of the neural network that mediates executive control, governing behavioural inhibition, implementation of control, and decision making [81]. Relevant to cannabinoid addiction, these brain regions are greatly affected by marijuana exposure [14, 18, 82]. To the best of our knowledge, there is only one study that has addressed possible differences in cannabinoid receptor binding and function of male and female adult rats in the prefrontal cortex, which has, however, reported no sex-dependent differences in the number of CB1 receptors number or their activation [22]. The divergence between our findings and those of Burston might lie in the different assay used, because we used autoradiographic analysis instead of radioligand binding and agonist-stimulated [$^{35}$S]GTPγS binding in whole cortex homogenates.

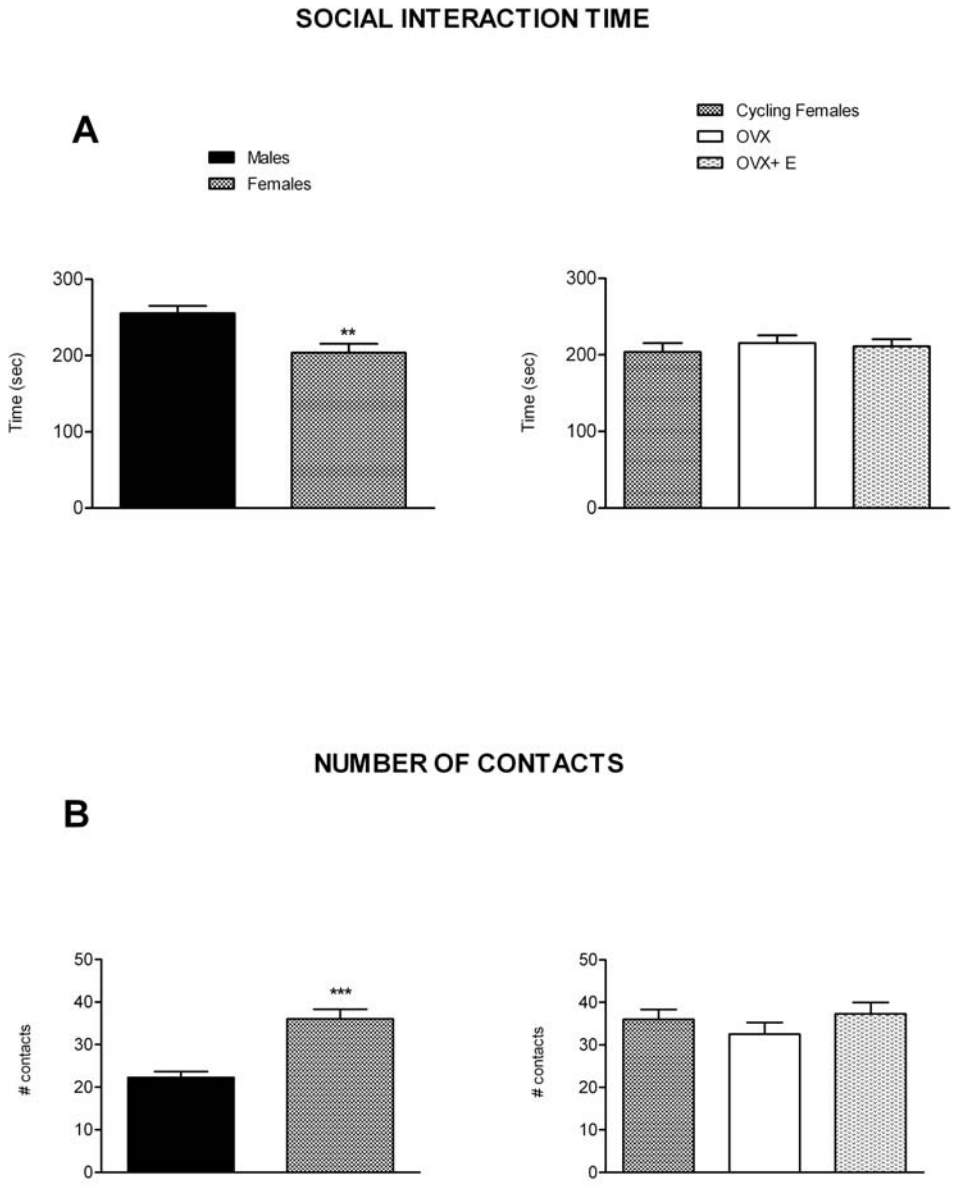


**Fig. (4).** Effect of sex (*left*) and ovarian hormones (*right*) on time (sec) spent in social interaction (**A**) and frequency of contacts (**B**) during the 10-min test. Data are expressed as mean ± SEM (n=8/group). *p<0.05, **p<0.01 and ***p<0.001 vs males (Student *t*-test).

The amygdala is a sexually dimorphic brain region critical for the regulation of social, cognitive, and emotional behaviours. Females have a higher amygdala content of endocannabinoid degradation enzymes, fatty acid amid hydrolase (FAAH), and monoacylglycerol lipase than males have, and hence lower amounts of endogenous cannabinoid ligands [83]. Data available so far on sex-dependent differences in CB1 receptor density and function in the amygdala of adult male and female rats are contradictory. In contrast with previous studies that have reported no sex-dependent differences [31, 34] or higher CB1 receptor density in cycling females than in males and OVX females [36], we found that, in the amygdala, CB1 receptor density, but not function, was lower in female than in male rats. In humans, women show lower baseline cerebral CB1 receptor availability in the amygdala than men do, which is related to a high novelty-seeking personality [84]. Notably, a high novelty-seeking trait has been associated with enhanced vulnerability to drug abuse and development of drug addiction in both animals and humans [85-88].

In the present study, finding no sex differences in CB1 receptor density and function in the other brain areas examined differed from earlier studies, which have reported higher CB1 receptor mRNA expression or binding site density in different brain regions of males than in females [80], and a significant modulatory effect of oestradiol [35, 36]. Besides differences in the methodological approaches (homogenate binding vs autoradiographic analysis vs *in situ* hybridization), discrepancies with the above-mentioned studies might also be due to differences in the strains of rats used. In support of this, spontaneous cannabinoid intake in rats has been shown to depend strictly upon the animal strain, and Long Evans and Lister Hooded but not Sprague-Dawley rats are able to acquire and sustain reliable cannabinoid self-administration behaviour [38]. Accordingly, strain differences also exist in the ability of THC to facilitate dopamine efflux from the rat NAc [89].

Interestingly, the present study also demonstrated that up-regulation of CB1 receptors in OVX rats was accompanied by a significant increase in CP55940-stimulated GTPγS binding in the

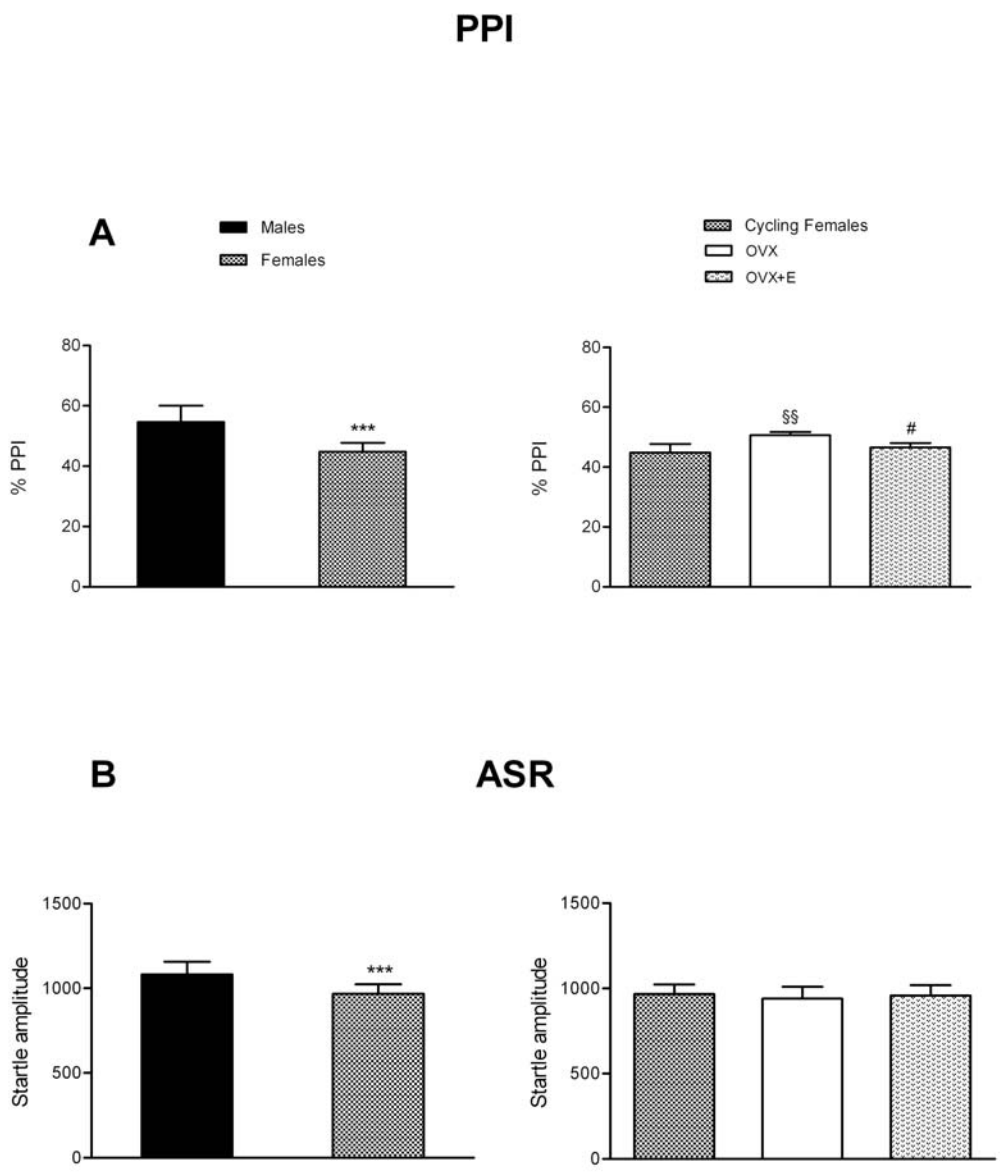


**Fig. (5).** Effect of sex (*left*) and ovarian hormones (*right*) on percentage prepulse inhibition (%PPI) in rats (**A**) and Acoustic Startle Reflex (ASR) (**B**). Data are expressed as the average response over the 3 prepulse intensities (n=11-15). ***p<0.001 vs males (Student *t*-test); §§p<0.01 vs cycling females and #p<0.05 vs OVX (one-way ANOVA followed by Newman-Keuls test).

Cg3, suggesting that, at least in this brain area, endocannabinoid signalling may be more efficient in the absence of ovarian hormones. This hypothesis finds support in the study of Mize and Alper [90], who have reported that acute oestradiol replacement in OVX rats decreases CB1 receptor/G-protein coupling in the prefrontal cortex at 2 hours after administration. Our finding of reduced function of CB1 receptors 2 days after oestradiol administration indicates that this hormone exerts a long-lasting effect on cannabinoid receptor signalling in the Cg3. On the other hand, up-regulation of CB1 receptors did not apparently translate into greater CB1 receptor-mediated G-protein activation in the Cg1 and amygdala of OVX females. It is difficult to explain why, in these two brain regions, the increase in CB1 receptor binding in OVX rats was not coupled with an increase in the efficiency of coupling, although such a limited correlation between alterations in the density and function of CB1 receptors has already been reported in the prefrontal cortex and amygdala of male Lister Hooded rats after cannabinoid self-administration [24]. It could be possible that in Cg3 and amygdala, changes in CB1 receptor density seen 3-4 weeks after ovariectomy are not sufficient to elicit a functional deficit on CB1 receptor coupling, or that such a deficit cannot be detected due to methodological limitations. In fact, the possibility cannot be excluded that the GTPγS assay we used, which measures the total populations of G-proteins (Gαi1, Gαi2 … etc, Gαo) activated by the CB1 receptors, may not be sensitive enough to detect alteration of G-protein subunits.

### Behavioural Findings

We detected sex-dependent differences in the basal level of spontaneous motor activity as well as in social behaviour and sensorimotor gating of Lister Hooded rats. Gonadal hormones seemed to be responsible for most sex differences we observed in such behaviours, but their contribution varied greatly with the behaviour in question.

Motor activity experiments showed that female were spontaneously more active than males, in line with the previous finding of a higher basal activity in female rats [91, 92], at least under the same

experimental conditions, namely, during the dark phase of the cycle [93]. Female rats also display greater locomotor stimulation to psychostimulants and more robust sensitisation with repeated exposure [94]. Many studies have shown a clear relationship between novelty-induced locomotor activity and drug-taking behaviour [95], implying that individual differences in exploratory behaviour can predictably influence drug self-administration, or in broader terms, that a hyperactive behavioural profile may be associated with higher vulnerability to drug addiction. In light of this, and as previously reported for cocaine [48] and amphetamines [41, 42], the finding that adult Lister Hooded female rats exhibit a higher degree of exploration and basal locomotor activity in a novel environment than males might address the question of why they appear more sensitive to the rewarding properties of cannabinoids [20], and self-administer more cannabinoid than males [25]. Ovariectomy typically dampens self-administration of many drugs of abuse [96-98], including cannabinoids [19]. Notably, we found that ovariectomy significantly attenuated basal locomotor activity, whereas acute oestradiol restored behavioural reactivity. Oestradiol increases striatal dopamine release [99, 100], reuptake [101], and turnover [102], thus, it is possible to ascribe, at least in part, the oestradiol enhancing motor effect to its activating effect on the dopaminergic system.

When assessing the role of sex and ovarian hormones on social exploratory behaviour and anxiety by means of the social interaction test, we found that the duration of time spent engaging in social interaction with a novel conspecific (an index negatively associated with social anxiety) was shorter in female than in male rats, suggesting a higher anxiety profile in females. In support of this, female rats also made a significantly higher number of contacts than males when interacting with partners, a behaviour reminiscent of a higher anxiety-like emotional state. This finding disagrees with previous studies using the elevated plus-maze test, where female rats showed a reduced aversion to the open arms compared to male rats [103-105]. This discrepancy might be due to the different type of anxiety measured by the two tests, because measures of anxiety derived from the social interaction test load on an independent factor from those derived in the plus-maze task [106]. Surprisingly, ovariectomy and oestradiol replacement did not have a significant effect on the social interaction test, and they did not affect the length of time spent interacting with the partner or the number of social contacts. In light of the evidence that in rats ovariectomy elicits a reliable increase in anxiety and depression-like behaviours in tests of emotionality, and that oestrogen administration produces anxiolytic and antidepressant-like effects [107, 67], these results were unforeseen to us.

However, because the elevated plus-maze measures changes in innate fear of animals for heights and open spaces, and the social interaction test measures the exploration of an unfamiliar, same-sex partner, it is possible that sex- and hormone-dependent differences in emotional behaviours are task-dependent. Another possibility is that an acute dose of oestrogen may be not sufficient to elicit an anxiolytic effect [108-110], which could occur with subchronic (3-7 days) oestrogen treatment [110-112]. However, a subchronic regimen of oestrogen has also been shown to increase anxiety-like behaviour in certain behavioural tests depending on the animal strains [113], which suggests that, not only the length of oestrogen treatment, but also the species and strain differences may have a role in the effects of gonadal hormones on anxiety-like behaviours.

Finally, we investigated the presence of sex-dependent differences in the PPI and ASR, and on the possible role played by the ovarian hormones in modulating these behavioural parameters. Sensorimotor inhibition is considered to be an early process of stimulus evaluation that acts as a perceptual filter, allowing some stimuli to pass through for further processing while filtering out other less-relevant stimuli [114]. In healthy subjects, presentation of the low-intensity stimulus activates a sensorimotor inhibition mechanism that protects processing from being interrupted by the high-intensity stimulus. PPI of startle involves attenuation of a startle reaction that occurs when a high-intensity startle stimulus is immediately preceded by a low-intensity, non-startling pre-stimulus [59].

The most reliable animal protocol to evaluate sensorimotor gating is the PPI of the ASR. In line with previous studies [115-117], we found that female rats displayed a lower degree of both PPI and ASR, of which only the former seems to depend on ovarian hormonal status. The degree to which the protective process is activated determines the efficiency of information filtering for protecting higher order processing and is measured as the magnitude of PPI. The lower quality of information processing or sustained attention observed in females, that is, less-efficient PPI, might imply poorer attention modulation than in males and ultimately result in ineffective behavioural organisation, thus accounting for their higher vulnerability to cannabinoid self-administration [25]. In support of this hypothesis, a deficit in PPI has been found to constitute a vulnerability marker for alcoholism [53] and nicotine dependence [54]. Deficiency in PPI has been consistently reported among schizophrenic patients. However, a weaker PPI in females does not necessarily imply an enhanced susceptibility to develop schizophrenia-like symptoms after cannabinoid exposure, as also suggested by human studies showing that among cannabis users is the male (and not the female) gender to be associated with an augmented risk for schizophrenia [118]. COMT polymorphism and variation at the AKT1 rs2494732 single nucleotide polymorphism have been shown to influence the risk of psychosis in cannabis users [119-121]. Yet, the moderating effect of gender on these genetic variants has not been investigated so far, leaving unsolved the question whether gender could account for some discrepancies reported in the literature on the link between cannabis use and risk of psychosis [122-124], which strengthens the notion that sex should regularly be taken into consideration in genetic studies, animal research and clinical setting [125].

## CONCLUSIONS

We provided evidence for sex-dependent differences in the density of cannabinoid CB1 receptors in the prefrontal cortex (Cg1 and Cg3) and amygdala, brain areas in which oestradiol seems to be the major factor responsible for the decreased number of CB1 receptors. The endocannabinoid system has been found to control neurotransmitter release from several neuron populations, suggesting a general mechanism for tuning neuronal activity, and thereby regulating emotion and cognition [126, 127]. In female rats, oestradiol engages the endocannabinoid system, potentially through an FAAH-related mechanism, to modulate emotional behaviour [108]. As widely accepted, the amygdala is a critical structure in the modulation of various types of fear and anxiety responses [128, 129], and a neural circuit between the amygdala and the prefrontal cortex is activated in response to novel and emotionally arousing events [115, 130]. These brain regions are also strictly involved in attention filtering and sensorimotor gating as well as in social behaviours. We expected to find sex- and/or hormone-dependent differences also in the VTA, ventral and dorsal striatum, as parts of the functional neuroanatomy subserving motivation, reward-related behaviours and motor activity, where cannabinoid CB1 receptors have been shown to play crucial roles [131, 132]. This was also in light of the evidence that cannabinoids are more potent and in some cases more efficacious in females than in males in altering movement [133-136].

Finally, we demonstrated that male and female rats also differed in their spontaneous motor activity, basal level of social anxiety and PPI of the ASR, with females showing a more vulnerable phenotype to drug addiction, and ovarian hormones playing a significant role in most, but not all, behavioural parameters examined.

The crucial role played by oestradiol in modulating CB1 receptor density and function as well as behaviour of female rats is not

surprising, given the finding that gonadal steroid hormones are able to alter neuronal activity of the brain reward system. An augmented reactivity of the reward system, for example, has been observed in women during the midfollicular phase, that is, when oestrogen is unopposed by progesterone [137]. In women, increased plasma oestradiol is associated with increased vulnerability to the psychostimulant and reinforcing effects of drugs of abuse [138], probably because of its activating effects on the dopaminergic neural circuitry that regulates pleasure and reward [11, 102]. However, oestradiol also affects brain neurotransmission systems other than the dopaminergic one, which are particularly relevant for cognitive activities and affective states. For example, oestradiol acts through nuclear- and membrane-initiated mechanisms to regulate GABA and glutamate signalling, respectively, which are among the most crucial regulators of cognition and mood [139]. Similarly, activation of oestrogen receptors strongly affects serotonin neurotransmission with a resulting modulating effect of anxiety-like state [140].

Future studies are needed to confirm and further explore the precise role of the reported sex differences in CB1 receptor functioning in cannabinoid-induced behavioural responses, and their modulation by sex hormones.

## CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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## ABBREVIATIONS

| | | |
|---|---|---|
| AMY | = | Amygdala |
| ANOVA | = | Analysis of Variance |
| CA1-CA3 | = | CA1-CA2-CA3 fields of Ammon's horn |
| CB1 | = | Cannabinoid type-1 receptor |
| Cg1 | = | Cingulated area 1 |
| Cg3 | = | Cingulated area 3 |
| CP55940 | = | (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol) |
| CPu | = | Caudate-putamen |
| DG | = | Dentate gyrus of hippocampus |
| DSM-IV | = | Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. |
| FAAH | = | Fatty acid amid hydrolase |
| GTP | = | Guanosine-5'-triphosphate |
| GDP | = | Guanosine diphosphate |
| LR | = | Low Responders |
| Nac Core | = | Nucleus accumbens core |
| Nac Shell | = | Nucleus accumbens shell |
| OVX | = | Ovariectomised rats |
| OVX+E | = | Estradiol-primed OVX rats |
| PPI | = | Prepulse inhibition |
| THC | = | Tetrahydrocannabinol |
| VTA | = | Ventral tegmental area |

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