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Functional responses to the cannabinoid agonist WIN 55,212-2 in neonatal rats of both genders: influence of weaning

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Abstract

We have studied behavioural, biochemical and endocrine responses to the cannabinoid agonist WIN 55,212-2 (WIN) in neonatal rats, as well as the effects of weaning on such responses. We used preweanling rats (20 days of age), 25-day-old weaned rats (weaning at Day 22) and 25-day-old nonweaned rats of both sexes. The behavioural effects of WIN were assessed in the nociceptive tail immersion test and in the open field. We also analysed the effect of weaning on corticosterone responses to WIN (radioimmunoassay) as well as on WIN-stimulated [^{35}S] GTPγS binding in periaqueductal grey (PAG) and striatum. The cannabinoid agonist induced a modest increase in pain thresholds, whereas the effect of the drug on open-field activity, particularly on vertical activity, was much more marked. The weaning process appeared to reduce the baseline nociceptive latencies of the female rats. No significant effect of weaning on the behavioural responses to WIN was found. However, the group of weaned females (but not males) showed a significantly reduced WIN-stimulated [^{35}S] GTPγS binding in the striatum. The cannabinoid agonist significantly increased the corticosterone levels of 25-day-old rats with the effect being more marked in weaned than in nonweaned animals. The results suggest that the weaning process might produce some sexually dimorphic developmental changes in CB_1 receptor function.

Keywords: Rat; Weaning; WIN 55,212-2; Nociception; Open field; Corticosterone; Cannabinoid agonist-stimulated [^{35}S] GTPγS binding

1. Introduction

The effects of cannabinoid agonists on adult rodents have been extensively studied and include antinociception, changes in motor activity and anxiety, and activation of the hypothalamus–pituitary–adrenal (HPA) axis (Wenger et al., 1997; Chaperon and Thiébot, 1999; Manzanares et al., 1999a). There is also evidence about the behavioural and neuroendocrine consequences of pre- and perinatal exposure to either cannabis preparations or to its main psychoactive component, Δ^9-tetrahydrocannabinol (THC) in rats (Navarro et al., 1994; Fernández-Ruiz et al., 1999; Ramos et al., 2002). However, there is much less information about the acute effects of cannabinoid agonists in infant rats. The endocannabinoid system appears to play different functional roles at different developmental stages in rodents. The atypical distribution of cannabinoid CB_1 receptors during the perinatal period seems to be related to a specific involvement of the endocannabinoid system in brain development. Thus, during this early developmental period, CB_1-receptor binding is prominent in white-matter areas, including those of the midbrain and brainstem, and CB_1-receptor mRNA is expressed in some forebrain areas which participate in the processes of neuronal and glial-cell proliferation. As for binding in fiber tracts, the importance of this observation is its transient nature (Romero et al., 1997; Fernández-Ruiz et al., 2000; Ramos et al., 2002). In adults rats, the highest values for CB_1 receptor binding are found in striatum, hippocampus and cerebellum (Fernández-Ruiz et al., 2000). The endocannabinoid 2-arachidonoyl glycerol (2-AG) is present in maternal milk (Fride et al., 2001) as well as in pups' brain

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(Berrendero et al., 1999), and the endocannabinoid system appears to play a role in milk suckling, and hence, in neonatal development (Fride et al., 2001; Fride and Shohami, 2002).

The periweanling period, and in particular, the weaning stimulus, appears to be critical for the development of neurochemical systems, such as the opioid system (Kitchen et al., 1995; Terranova and Laviola, 2001). The activation of δ-opioid receptors induced by weaning may be dependent on the loss of certain components of milk which show opioid activity (Goody and Kitchen, 2001), and the manipulation of weaning age induces a number of neurobehavioural changes, including an altered responsivity to δ-opioid agonists (Terranova and Laviola, 2001; Laviola et al., 2003). Thus, both the cannabinoid and the opioid systems appear to be critically involved in developmental changes occurring during the periweanling period. In addition, there is abundant evidence about parallelisms and functional interactions between the two systems in adult animals (Manzanares et al., 1999b; Kirkham and Williams, 2001; Berrendero and Maldonado, 2002; Marín et al., 2003). On the basis of this evidence, we hypothesised that the weaning stimulus might exert an influence on the functional development of cannabinoid CB_1 receptors. In this study, we have investigated a number of functional responses to the cannabinoid receptor agonist WIN in neonatal rats of both genders, as well as the influence of weaning in such responses. We have addressed behavioural and corticosterone responses, as well as WIN-stimulated [^{35}S] GTPγS binding in discrete brain regions. This latter biochemical technique provides quantification of functional receptor–G protein coupling, an index of cannabinoid receptor function. We performed this assay in the striatum, in relation to the motor component of open-field behaviour, and the periaqueductal grey (PAG) as a region related to both emotional responses and nociception.

2. Materials and methods

2.1. Animals and experimental conditions

Experiments were performed on Wistar albino rats of both sexes from the animal house of the Universidad Complutense of Madrid, which is served by Harlan Interfauna Ibérica S.A. (Barcelona, Spain). The animals were maintained at a constant temperature of 21 °C and in a reverse 12-h dark–light cycle (lights on at 2000 h), with free access to food (commercial diet for rodents A04/A03; Panlab, Barcelona, Spain) and water. Male rats were mated with females (one male × two females). On the day of birth (postnatal day 0), litters were sex balanced and culled to 10 ± 1 pups per dam. Only one litter had a fewer number of animals, three males and three females. To investigate the effect of weaning on the diverse behavioural, endocrine and biochemical parameters indicated below, the experiments were performed in preweanling rats (20 days of age), 25-day-old weaned rats (weaning at Day 22: removal of the mother and rehousing of the pups in groups of the same gender) and 25-day-old nonweaned rats. All experimental procedures were carried out between 0930 and 1430 h. On the day of testing, the animals were equilibrated in a quiet laboratory for a 30-min period, before experimental procedures began. Nociceptive and behavioural testing was carried out under the same illumination conditions as those in the animal facilities (red light).

All the experiments performed in this study are in compliance with the Royal Decree 223/1988 of 14 March (BOE 18) and the Ministerial Order of 13 October 1989 (BOE 18) about protection of experimental animals, as well as with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Drug treatment and behavioural testing

In the first phase of the study, to get an adequate dose for the second experiment, we performed an experiment on 25-day-old weaned animals using 3 and 10 mg/kg of WIN 55,212-2 (WIN; Tocris), which was administered subcutaneously. To study the effect of weaning on antinociceptive and behavioural responses to the cannabinoid agonist (second experiment), we selected the dose of 3 mg/kg. The doses of the compound, the route of administration, and the pretest time (see below) were chosen on the basis of pilot experiments performed in our laboratory and previous results obtained from peripubertal rats (Fox et al., 2001; Romero et al., 2002). In fact, there were no data available in the literature about effects of WIN in rats of the same ages as those used in this study. In all cases, the volume administered was 0.1 ml/20 g. Nociception was assessed using the tail immersion test with water at 50 °C (Kitchen et al., 1984). Nociceptive responses (tail immersion latencies) were measured as the time elapsed prior to removal of the tail from the water surface and a maximum 10-s cutoff was used. Response latencies were measured immediately before the administration of WIN or its corresponding vehicle [ethanol/cremophor/saline (1:1:18); cremophor, Fluka BioChemiKa; baseline latencies], and 60 min after treatment (posttreatment latencies). Antinociception was quantified using the following formula: % MPE (percentage maximum possible effect)=(posttreatment latency − baseline latency/cutoff − baseline latency) × 100. Five minutes after the completion of the tail immersion test, the animals were tested individually in the open field, as previously described (Fernández et al., 1999; Romero et al., 2002). The apparatus consisted of a cylinder (75 cm diameter × 50 cm high) with a floor divided into 19 sections of a similar area by two concentric circles (17 and 45 cm diameter) and a series of radii. The duration of the test was 3 min. The parameters measured were as follows: peripheral ambulation (number of squares adjacent to the wall entered with the four limbs),

internal ambulation (number of squares in the central area entered with at least three limbs) and frequency of rearing (number of times that the animal stood on its rear limbs, vertical activity).

2.3. Corticosterone assay

The serum corticosterone levels were measured in animals corresponding to the experiment on the effect of weaning upon nociceptive and behavioural responses to WIN. Three minutes after completion of the open field, the animals were killed by decapitation. Blood samples were collected from the trunk and centrifuged (3000 rpm for 15 min), and serum was stored at −80 °C. Corticosterone was measured using a solid phase ^{125}I radioimmunoassay (Coat-A Count Rat Corticosterone kit, Diagnostic Products, Los Angeles, CA). The detection limit was 5.7 ng/ml and the intra- and interassay coefficients of variation were less than 10%.

2.4. Basal and WIN-stimulated [^{35}S] GTPγS binding assay

The basal and WIN-stimulated [^{35}S] GTPγS binding was studied in additional naive groups of animals.

2.4.1. Drugs

JWH-133 (6aR,10aR)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran (Dr. J.W. Huffman, Clemson University, USA), WIN 55,212-2 mesylate (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morphonilylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphtalenylmethanone mesylate (Sigma), Rimonabant or SR141716 *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyridazole-3-carboxamide (Sanofi Recherche, Montpellier, France), SR 144528 *N*-[(1S)-endo-1,3,3-trimethyl bicyclo [2.2.1]heptan-2-yl]-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboximide (Sanofi Recherche). Drugs were dissolved at 10 mM in DMSO and further diluted in the appropriate buffer or vehicle prior to its use. DMSO final concentration never exceeded 0.1%.

2.4.2. Procedure

Animals were sacrificed by decapitation and the brains were stored at −20 °C. PAG region and striatum were dissected from 2-mm-thick slices. The tissues were homogenized in 50 volumes of Tris–HCl 50 mM, pH 7.4, using an Ultraturrax (20 s, 20,000 rpm). The samples were centrifuged at 2000 × *g* for 10 min at 4 °C, to eliminate nuclei. The supernatants were then centrifuged at 15,000 × *g* for 15 min. The resulting pellets were resuspended in 50 volumes of the same buffer, keeping (−20 °C) a sample for protein measurements. Finally, the tissues were centrifuged again at 15,000 × *g* for 15 min and the pellet was stored at −20 °C until analysis. In preliminary experiments, it was confirmed that two freeze–thaw cycles did not modify GTPγS binding. The membrane preparations were resuspended in 50 volumes of incubation buffer (Tris–HCl 50 mM, pH 7.4, containing 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EGTA, 0.5mg ml^{-1} BSA). Tubes contained 200 μl membrane preparation (4 mg wet weight tissue/ml), 0.04 nM [^{35}S] GTPγS, 30 μM GDP and 5 μM WIN. Basal activity was determined in the absence of agonist and nonspecific binding in the presence of 10 μM of cold GTPγS. In competition experiments performed on striata from 25-day-old nonweaned rats, increasing concentrations (10^{-10}–10^{-5} M) of the CB_1 receptor selective antagonist Rimonabant (SR 141716A) or the CB_2 receptor selective antagonist SR144528 were added in combination with WIN (5 μM). In other experiments, the effect of increasing concentrations of the CB_2 selective agonist JWH-133 on basal [^{35}S] GTPγS binding was assayed. The samples were incubated for 60 min at 30 °C in a water bath, filtered through GF/C Whatman filters (0.45 μm) using a Brandel harvester, washed three times with ice-cold Tris–HCl 5mM, pH 7.4, containing 0.1 mg ml^{-1} BSA, and the radioactivity counted for 3 min using an LKB scintillation counter. Net stimulation was calculated by the difference of the binding in the presence and in the absence of WIN (basal binding). Data were expressed in fmol/mg prot.

2.5. Statistical analysis

The results from the experiment using WIN at 3 and 10 mg/kg in 25-day-old weaned animals were analysed by two-way ANOVA, with the two factors being sex and pharmacological treatment. The baseline latencies obtained in the tail immersion test and the biochemical data were analysed using two-way ANOVA with the two factors being sex and age condition (20-day-old preweanling rats, 25-day-old nonweaned rats and 25-day-old weaned rats). The % MPE values, the data from the open field and those from the corticosterone determinations, corresponding to the study on weaning (using WIN, 3 mg/kg), were analysed by three-way ANOVA (sex, pharmacological treatment and age condition). The Fisher LSD Method (LSD) was used for post hoc comparisons.

3. Results

3.1. Effects of WIN, 3 and 10 mg/kg, in 25-day-old weaned rats

3.1.1. Tail immersion test

The analysis of the % MPE values showed a significant effect of the treatment [$F(2,54) = 114.7$, $P < .001$], whereas no significant effect of sex or interaction between factors were found. As Fig. 1a shows, WIN induced a modest although significant increase of the % MPE values with the highest dose being more effective (Fig. 1a).

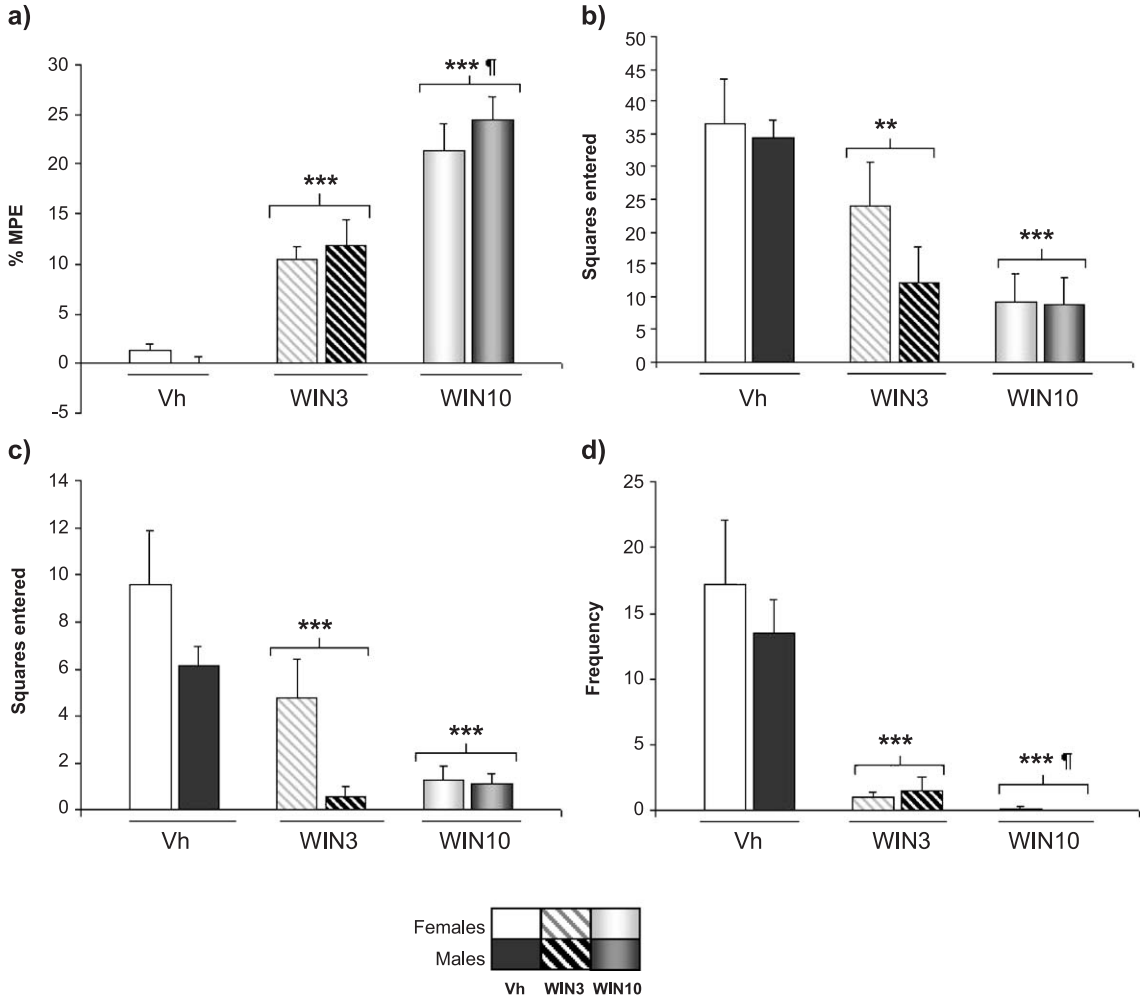

Fig. 1. Effect of WIN 3 and 10 mg/kg on functional responses of 25-day-old weaned female (F) and male (M) rats. Control animals were injected with vehicle (Vh; see text). (a) Antinociceptive responses in the tail immersion test. % MPE (percentage maximum possible effect)=(posttreatment latency – baseline latency/cutoff – baseline latency) × 100. Open-field activity: (b) peripheral ambulation, (c) internal ambulation and (d) rearing frequency (vertical activity). Histograms represent the mean ± S.E.M. of the following number of animals: Vh (F = 10, M = 9), WIN 3 (F = 12, M = 9) and WIN 10 (F = 11, M = 9). LSD test: ** $P < .01$, *** $P < .001$ vs. vehicle-treated animals; ¶ $P < .01$ vs. the animals treated with WIN 3 mg/kg.

3.1.2. Open-field activity

The analysis of open-field data indicated that WIN significantly reduced the three parameters measured (Fig. 1b–d): peripheral ambulation [$F(2,54) = 11.9$, $P < .001$]; internal ambulation [$F(2,54) = 15.8$, $P < .001$] and rearing frequency [$F(2,54) = 72.6$, $P < .001$].

A visual inspection of Fig. 1b and c shows that the effect of WIN on the horizontal ambulation of females was less marked at 3 mg/kg than at 10 mg/kg, whereas in males, the two doses had a similar effect. With respect to vertical activity, WIN induced a drastic reduction of rearing behaviour at the two doses used and in both sexes (Fig. 1d).

3.2. Effects of weaning on functional responses to WIN, 3 mg/kg

Because, in the first experiment, the dose of 10 mg/kg induced an excessive reduction of motor activity, we decided to use the dose of 3 mg/kg to study the effect of weaning on the diverse parameters indicated below. For this part of the study, we obtained additional litters to complete a new group of 25-day-old weaned animals which was tested in parallel with the other two experimental groups, i.e., 20- and 25-day-old nonweaned rats.

3.2.1. Tail immersion test

The analysis of the baseline latencies revealed a significant effect of the age condition [$F(2,99) = 56.8$, $P < .001$] and post hoc comparisons showed significant differences between the three age groups. As Fig. 2a shows, the preweanling rats showed the lowest baseline latencies. The difference between 25-day-old weaned and nonweaned rats was slight but significant and these latter animals showed the highest latencies. The difference was more clear in females than in males [interaction between factors: $F(2,99) = 2.6$, $P = .08$]. The three-way ANOVA performed on the % MPE

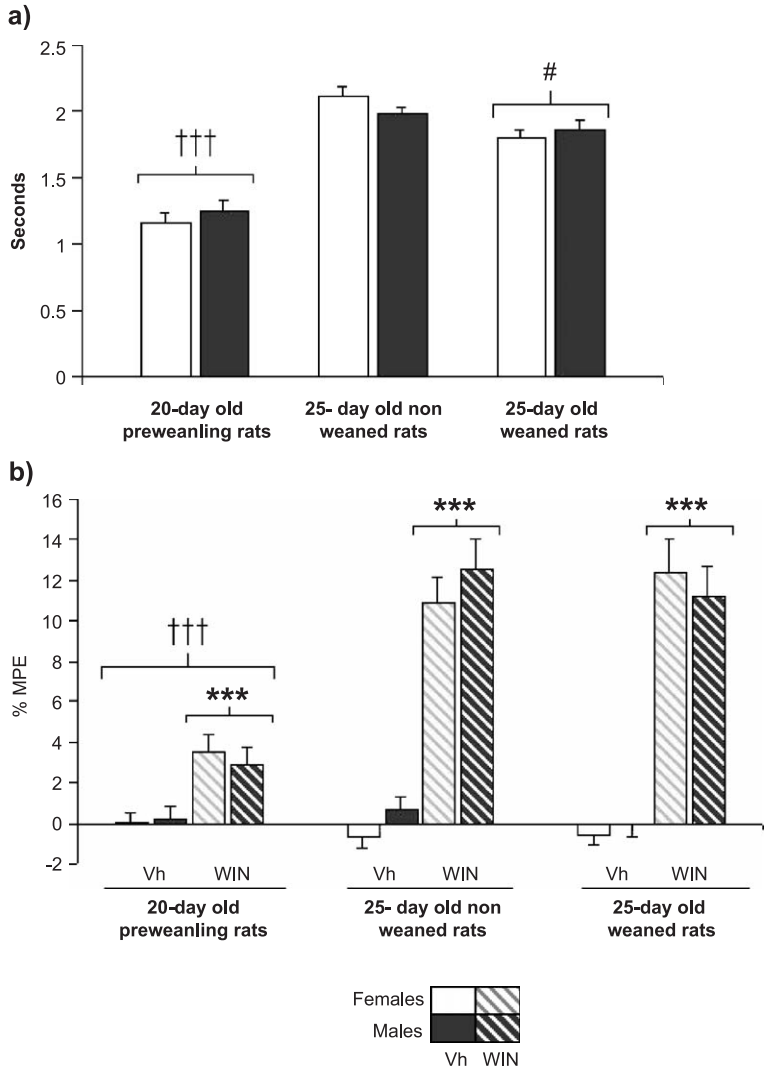

Fig. 2. Effects of weaning on baseline latencies (a) and antinociceptive responses to WIN 3 mg/kg (b) in the tail immersion test. Control animals were injected with vehicle (Vh; see text). F: females, M: males. % MPE (percentage maximum possible effect)=(posttreatment latency – baseline latency/cutoff – baseline latency) × 100. Histograms represent the mean ± S.E.M. of the following number of animals. For baseline latencies: 20-day-old rats (F = 14, M = 15), 25-day-old nonweaned rats (F = 15, M = 16) and 25-day-old weaned rats (F = 22, M = 23). For % MPE: 20-day-old rats, Vh (F = 6, M = 7), WIN (F = 8, M = 8); 25-day-old nonweaned rats, Vh (F = 7, M = 8), WIN (F = 8, M = 8) and 25-day-old weaned rats, Vh (F = 11, M = 12), WIN (F = 11, M = 11). LSD test: $^{†††}P < .001$ vs. 25-day-old weaned and nonweaned rats; $^{\#}P < .05$ vs. 25-day-old nonweaned rats; *** $P < .001$ vs. vehicle-treated animals.

indicated significant effects of treatment [$F(1,93) = 189$, $P < .001$] and age [$F(2,93) = 16.6$, $P < .001$], as well as a significant Treatment × Age interaction [$F(2,93) = 19$, $P < .001$]. As Fig 2b shows, WIN induced a significant increase of the % MPE in the three age groups, and this effect was more marked at 25 days of age (irrespective of weaning) than at 20 days of age.

3.2.2. Open-field activity

The analysis of horizontal activity in the open field (Fig. 3a and b) showed that the pharmacological treatment induced significant decreases in both, (a) peripheral [$F(1,93) = 32.7$, $P < .001$] and (b) internal [$F(1,93) = 53.1$, $P < .001$] ambulation in all the experimental groups, whereas the other main factors and interactions between factors were not significant. The effect of WIN on vertical activity (Fig. 3c) was more marked, and rearing behaviour practically disappeared after drug treatment [$F(1,93) = 182.6$, $P < .001$].

3.2.3. Corticosterone determinations

The three-way ANOVA revealed significant effect of sex [$F(1,73) = 7.6$, $P < .01$], pharmacological treatment [$F(1,73) = 39$, $P < .001$] and age [$F(2,73) = 20.8$, $P < .001$], as well as a significant Treatment × Age interaction [$F(2,73) = 14.1$, $P < .001$]. As Fig. 4 shows, this interaction indicated a different effect of treatment at different ages. Accordingly, we further analysed the effect of WIN within each age condition. The analysis of the data obtained from 20-day-old rats did not render any significant effect. In the

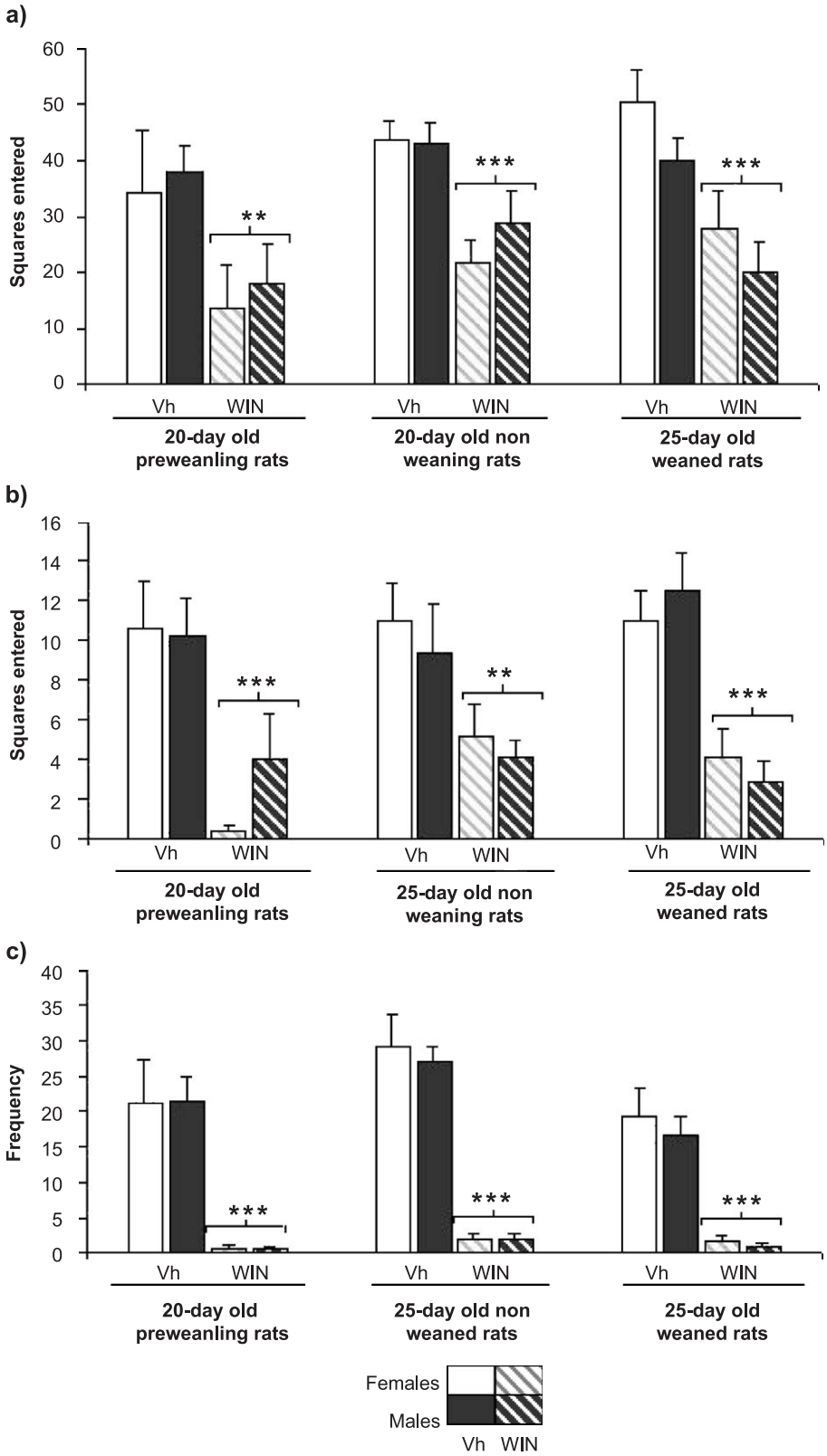

Fig. 3. Effects of weaning on behavioural responses to WIN 3 mg/kg in the open field. Control animals were injected with vehicle (Vh; see text). F: females, M: males. (a) Peripheral ambulation, (b) internal ambulation and (c) rearing frequency. Histograms represent the mean ± S.E.M. of the following number of animals: 20-day-old rats, Vh (F = 6, M = 7), WIN (F = 8, M = 8); 25-day-old nonweaned rats, Vh (F = 7, M = 8), WIN (F = 8, M = 8) and 25-day-old weaned rats, Vh (F = 11, M = 12), WIN (F = 11, M = 11). LSD test: ** $P < .01$, *** $P < .001$ vs. vehicle-treated animals.

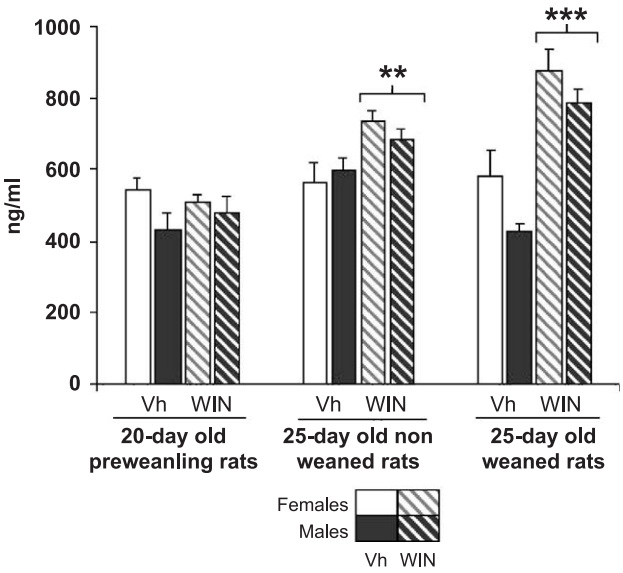

Fig. 4. Effect of weaning on corticosterone responses to WIN. Animals injected with either WIN 3 mg/kg or vehicle were sacrificed by decapitation 3 min after completion of the open field (see text for details). The serum corticosterone levels were measured by radioimmunoassay. Histograms represent the mean ± S.E.M. of the following number of animals: 20-day-old rats, Vh (F = 6, M = 7), WIN (F = 8, M = 8); 25-day-old nonweaned rats, Vh (F = 7, M = 8), WIN (F = 8, M = 8) and 25-day-old weaned rats, Vh (F = 5, M = 7), WIN (F = 6, M = 7). LSD test: ** $P < .01$, *** $P < .001$ vs. vehicle-treated animals. See text for significant overall effect of sex in weaned animals.

25-day-old nonweaned animals, a significant effect of the pharmacological treatment was found [$F(1,27) = 11.6$, $P < .01$], whereas in 25-day-old weaned animals, both main factors, sex [$F(1,21) = 6.2$, $P < .05$] and treatment [$F(1,21) = 43.2$, $P < .001$], were significant. The corticosterone responses to WIN were more marked in weaned than in nonweaned rats. Besides, in weaned animals, the corticosterone levels were significantly higher in females than in males (Fig. 4).

3.3. Basal and WIN-stimulated [^{35}S] GTPγS binding assay

The results obtained in the basal [^{35}S] GTPγS binding assay did not indicate significant differences between the different age-condition groups, in any of the regions analysed: PAG ($n = 4–5$): 20-day-old preweanling rats: females (F) = 29.6 ± 6.5, males (M) = 40.7 ± 6.4; 25-day-old nonweaned rats: F = 38.1 ± 2.8, M = 39.6 ± 3.7 and 25-day-old weaned rats: F = 27.1 ± 1.7, M = 32.4 ± 3.0. Striatum ($n = 4–5$): 20-day-old preweanling rats: F = 35.0 ± 2.0, M = 35.2 ± 2.2; 25-day-old nonweaned rats: F = 42.7 ± 2.1, M = 38.5 ± 6.2 and 25-day-old weaned rats: F = 34.4 ± 1.8, M = 36.3 ± 1.5. With respect to WIN-stimulated [^{35}S] GTPγS binding (Fig. 5a and b), the following results were found: in PAG, an overall effect of sex was found with males showing the highest value [$F(1,21) = 4.7$, $P < .05$] (a); with respect to striatum (b), the statistical analysis rendered a significant effect of sex [$F(1,23) = 7.5$, $P = .01$] as well as a significant Sex × Age Condition interaction [$F(2,23) = 4$, $P < .05$]. According to this interaction, 25-day-old weaned females showed a significant reduction in stimulated binding when compared to both preweanling and 25-day-old nonweaned females, whereas no differences were found between the male experimental groups. Besides, a sex difference was found within the weaned animals with females showing the lowest value.

As stated in Materials and methods, we performed an additional assay in striata to characterize the receptors. Competition curves showed that WIN-stimulated [^{35}S] GTPγS binding was concentration-dependently displaced by the CB_1 receptor selective antagonist Rimonabant, whereas it was not modified by the CB_2 receptor selective antagonist SR144528. Besides, the CB_2 receptor selective agonist JWH-133 did not alter basal [^{35}S] GTPγS binding (data not shown).

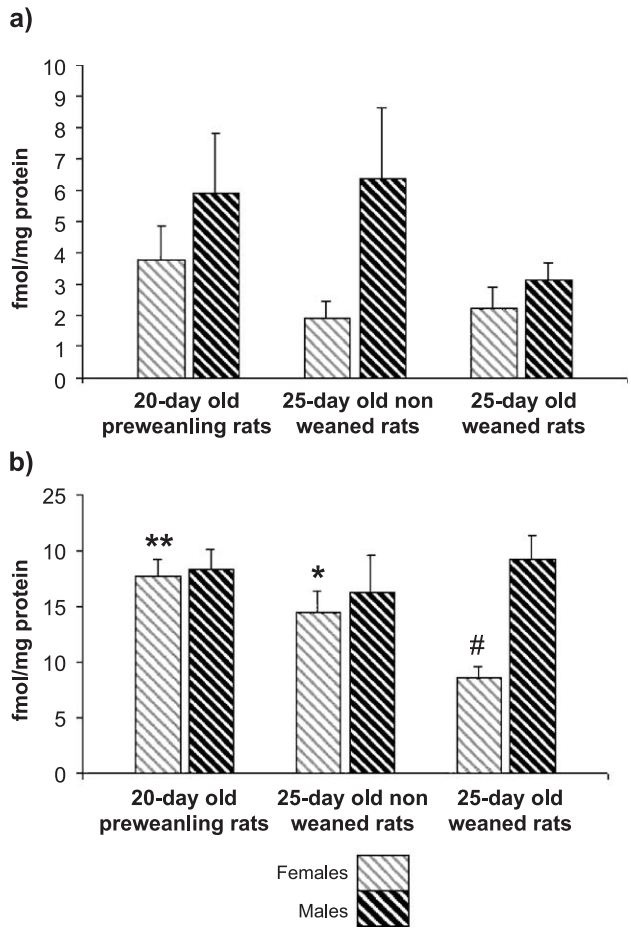

Fig. 5. Effect of weaning on WIN-stimulated [^{35}S] GTPγS binding. The binding assay was performed on samples obtained from naive groups of animals. The data represent net WIN-stimulated [^{35}S] GTPγS binding (difference of binding in the presence and absence of the drug). Two brain regions were analysed: (a) PAG and (b) striatum. Histograms represent the mean ± S.E.M. of four to five animals per group. LSD test: * $P < .05$, ** $P < .01$ vs. 25-day-old weaned females; $^{\#}P < .001$ vs. the corresponding male group.

4. Discussion

The present results indicate that the cannabinoid agonist WIN at the doses employed in this study (3 and 10 mg/kg) induced significant reductions in the motor activity (peripheral ambulation and rearing) of 20- and 25-day-old rats. These data contrast with previous results obtained from developing mice which showed that the cannabinoid agonists anandamide and THC only produced a significant decrease in locomotor activity in 45-day-old mice and in the adults, but not at earlier ages (Fride and Mechoulam, 1996a,b). The discrepancies with respect to the present results might be due to the different type of cannabinoid agonist and/or the different species used. Thus, two additional studies have shown that the potent cannabinoid agonists HU-210 (Martín-Calderon et al., 1998) and CP 55,940 (Mcgregor et al., 1996) produced inhibition of motor activity in neonatal rats. In particular, CP 55,940 inhibited motor activity at postnatal day 12 (Mcgregor et al., 1996) and HU-210 produced immobility at postnatal day 15 (Martín-Calderon et al., 1998). WIN induced a particularly marked effect on vertical activity. In fact, rearing behaviour practically disappeared after the pharmacological treatment. We have previously found a similar result in both peripubertal (Romero et al., 2002) and adult (Marín et al., 2003) rats, indicating that vertical activity is particularly sensitive to the effects of cannabinoid agonists from early postnatal ages. The present results should be taken into account in relation to the potential therapeutic utility of cannabinoids in children (Williamson and Evans, 2000). In contrast with its marked effect on motor activity, the analgesic effect of WIN was very slight (approximately 25% MPE at the highest dose used). These data indicate that the cannabinoid receptors mediating the analgesic effects of cannabinoid agonists are not fully developed at 20–25 days of age. The degree of functional development of different subpopulations of cannabinoid receptors may be related to their respective regional distribution (Romero et al., 1997).

With respect to the nociceptive test, our results also show a modest although significant difference between 25-day-old weaned and nonweaned rats, with these latter animals (particularly the females) showing increased baseline latencies. The present results suggest that the weaning process might play a modulatory role in pain sensitivity. It has been shown that intraoral infusions of milk increased pain thresholds of newborn rats. This antinociceptive effect of milk, that appears to be independent of the social contact with the mother, was blocked by the opioid antagonist naltrexone (Blass, 1994). It is likely that peptide fragments with opioid activity (Goody and Kitchen, 2001) and perhaps also endocannabinoids (Fride et al., 2001) contained in maternal milk contribute to such antinociceptive effect.

The present results did not show any significant effect of weaning on the behavioural responses to WIN that we recorded. However, the biochemical data showed a significant decrease in the cannabinoid agonist-stimulated GTPγS binding in the striatum of 25-day-old weaned females with respect to both the other groups of females and the corresponding male group. The pharmacological characterization of the striatal receptors indicated that, as expected, WIN-stimulated [^{35}S] GTPγS binding was occurring through CB_1 receptor stimulation under our conditions. Thus, our results suggest that the weaning process may induce a reduced functional activity of the striatal CB_1 receptors in females. The development of the opioid system, which shows numerous interactions with the cannabinoid system, is also affected by weaning (Kitchen et al., 1995). The loss of dietary casein, which is known to produce peptide fragments that can exert opioid activity, appeared to be a critical factor for this effect (Goody and Kitchen, 2001). The endocannabinoid 2-AG is present in maternal milk and is accompanied by 2-palmitoyl glycerol and 2-linoleoyl glycerol, which enhance its activity (entourage effect; Fride et al., 2001). 2-AG, when administered orally, albeit in high doses, was active in the mouse tetrad (Di Marzo et al., 1998). This finding suggests that the endocannabinoids and the ‘‘entourage’’ compounds that are present in milk might reach, in part at least, the central nervous system (Fride and Shohami, 2002). Previous data also suggest that in certain brain regions, such as the striatum, there may be a relationship between the functional expression of CB_1 receptors and the levels of anandamide (Di Marzo et al., 2000). If weaning (removal of maternal milk) affects the levels of endocannabinoids in the pups, this may account for the reduced functional activity of the striatal CB_1 receptors in our weaned animals. The sexual dimorphism observed in this effect might be related to the previously reported sex differences in the developmental pattern of striatal CB_1 receptors (Rodriguez de Fonseca et al., 1993). The results also indicated another interesting sexual dimorphism in the PAG, with the agonist-stimulated GTP binding being greater in males than in females. It has been shown that there are sex differences in the mesencephalic cannabinoid receptor density of developing rats, with males exhibiting a higher receptor density than females (Rodriguez de Fonseca et al., 1993). As for other sexual dimorphisms found in this study, these gender differences in both cannabinoid receptor density and activation might be dependent on the effects of perinatal androgens during the critical period for the sexual differentiation of the brain (Mclusky and Naftolin, 1981).

There is substantial evidence indicating that cannabinoid receptor agonists induce a CB_1-receptor-mediated activation of the HPA axis in both adult (Wenger et al., 1997; Manzanares et al., 1999a) and 40-day-old (Romero et al., 2002) rats. The present results show that this response was absent at postnatal day 20, at least at a dose of 3 mg/kg of WIN, suggesting that the CB_1 receptors mediating this response are still immature at this age. The cannabinoid agonist induced modest although significant corticosterone responses in 25-day-old nonweaned rats. However, the most marked effect of WIN on adrenocorti-

cal activity was found in the weaned animals. Thus, the weaning process appears to play a role in the functional development of the CB_1 cannabinoid receptors mediating the adrenocortical responses to cannabinoid agonists. The levels of corticosterone were significantly higher in female than in male weaned rats, whereas no sex differences appeared in the other age groups. The gender difference in the HPA axis function has been previously described in both 40-day-old (Romero et al., 2002) and adult (Viveros et al., 1988) rats. Thus, the weaning process may also contribute to the establishment of this sexual dimorphism. The present results contrast with previous data from rats of 10, 20, 30 and 40 days of age, indicating that the cannabinoid agonist HU-210 did not increase plasma corticosterone levels until postnatal day 40 (Martín-Calderon et al., 1998). Our animals were subjected to behavioural testing before sacrifice, and this fact may have facilitated the effect of the cannabinoid agonist. In fact, the stimulatory effects of cannabimimetics on pituitary–adrenal responses appear to be centrally mediated, probably through an increased release of corticotropin-releasing factor, and with the contribution of extrahypothalamic inputs from stress-responsive limbic nuclei (Wenger et al., 1997; Martín-Calderon et al., 1998).

In summary, the present results suggest that, as for the opioid system, the weaning process might affect the functional development of CB_1 cannabinoid receptors, with some of these effects showing sexual dimorphism. The specific component of the weaning process (social/nutritional) involved in this effect and the functional interactions between the cannabinoid and the opioid systems during the periweanling period deserve further investigation.

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References

Berrendero F, Maldonado R. Involvement of the opioid system in the anxiolytic-like effects induced by Delta (9)-tetrahydrocannabinol. Psychopharmacology 2002;163:111–7.

Berrendero F, Sepe N, Ramos JA, Di Marzo V, Fernández-Ruiz JJ. Analysis of cannabinoid receptor binding and mRNA expression and endogenous cannabinoid contents in the developing rat brain during late gestation and early postnatal period. Synapse 1999;33:181–91.

Blass EM. Behavioral and physiological consequences of suckling in rat and human newborns. Acta Paediatr Suppl 1994;397:71–6.

Chaperon F, Thiébot M-H. Behavioral effects of cannabinoid agents in animals. Crit Rev Neurobiol 1999;13:243–81.

Di Marzo V, Sepe N, De Petrocellis L, Berger A, Crozier G, Fride E, et al. Trick or treat from food endocannabinoids? Nature 1998;396:636–7.

Di Marzo V, Breivogel CS, Tao Q, Bridgen DT, Razdan RK, Zimmer AM, et al. Levels, metabolism, and pharmacological activity of anandamide in CB_1 cannabinoid receptor knockout mice: evidence for non-CB_1, non-CB_2 receptor-mediated actions of anandamide in mouse brain. J Neurochem 2000;75:2434–44.

Fernández B, Antelo MT, Kitchen I, Viveros MP. Effects of neonatal naltrindole treatment on antinociceptive and behavioral responses to μ and κ agonists in rats. Pharmacol Biochem Behav 1999;62:145–9.

Fernández-Ruiz J, Berrendero F, Hernández ML, Romero J, Ramos JA. Role of endocannabinoids in brain development. Life Sci 1999;65:725–36.

Fernández-Ruiz J, Berrendero F, Hernández ML, Ramos JA. The endogenous cannabinoid system and brain development. Trends Neurosci 2000;23:14–20.

Fox A, Kesingland A, Gentry C, Mcnair K, Patel S, Urban L, et al. The role of central and peripheral cannabinoid$_1$ receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. Pain 2001;92:91–100.

Fride E, Mechoulam R. Developmental aspects of anandamide: ontogeny of response and prenatal exposure. Psychoneuroendocrinology 1996a;21:157–72.

Fride E, Mechoulam R. Ontogenetic development of the response to anandamide and Δ^9-tetrahydrocannabinol in mice. Dev Brain Res 1996b;95:131–4.

Fride E, Shohami E. The endocannabinoid system: function in survival of the embryo, the newborn and the neuron. NeuroReport 2002;13:1833–41.

Fride E, Ginzburg Y, Breuer A, Bisogno T, Di Marzo V, Mechoulam R. Critical role of the endogenous cannabinoid system in mouse pup suckling and growth. Eur J Pharmacol 2001;419:207–14.

Goody RJ, Kitchen I. Influence of maternal milk on functional activation of delta-opioid receptors in postnatal rats. J Pharmacol Exp Ther 2001;296:744–8.

Kirkham TC, Williams CM. Synergistic effects of opioid and cannabinoid antagonists on food intake. Psychopharmacology 2001;153:267–70.

Kitchen I, Mcdowell J, Winder C, Wilson JM. Low level lead exposure alters morphine antinociception in neonatal rats. Toxicol Lett 1984;22:119–23.

Kitchen I, Leslie FM, Kelly M, Barnes R, Crook TJ, Hill RG, et al. Development of delta-opioid receptor subtypes and the regulatory role of weaning: radioligand binding, autoradiography and in situ hybridation studies. J Pharmacol Exp Ther 1995;275:1597–607.

Laviola G, Macri S, Morley-Fletcher S, Adriani W. Risk-taking behavior in adolescent mice: psychobiological determinants and early epigenetic influence. Neurosci Biobehav Rev 2003;27:19–31.

Manzanares J, Corchero J, Fuentes JA. Opioid and cannabinoid receptor-mediated regulation of the increase in adrenocorticotropin hormone and corticosterone plasma concentrations induced by central administration of Δ^9-tetrahydrocannabinol in rats. Brain Res 1999a;839:173–9.

Manzanares J, Corchero J, Romero J, Fernández-Ruiz JJ, Ramos JA, Fuentes JA. Pharmacological and biochemical interactions between opioids and cannabinoids. Trends Pharmacol Sci 1999b;20:287–94.

Marín S, Marco E, Biscaia M, Fernández B, Rubio M, Guaza C, et al. Involvement of the κ-opioid receptor in the anxiogenic-like effect of CP 55,940 in male rats. Pharmacol Biochem Behav 2003;74:649–56.

Martín-Calderon JL, Muñoz RM, Villanua MA, Del Arco I, Moreno JL, Rodriguez De Fonseca F, et al. Characterization of the acute endocrine actions of (−)-11-hydroxy-Δ^8-tetrahydrocannabinol-dimethylheptyl (HU-210), a potent synthetic cannabinoid in rats. Eur J Pharmacol 1998;344:77–86.

Mcgregor IS, Dastur FN, Mclellan RA, Brown RE. Cannabinoid modulation of rat pup ultrasonic vocalizations. Eur J Pharmacol 1996;313:43–9.

Mclusky NJ, Naftolin F. Sexual differentiation of the central nervous system. Science 1981;211:1294–303.

Navarro M, Rubio P, Rodriguez de Fonseca F. Sex-dimorphic psychomotor activation after perinatal exposure to (−)-Δ^9-tetrahydrocannabinol. An ontogenic study in Wistar rats. Psychopharmacology 1994;116:414–22.

Ramos JA, De Miguel R, Cebeira M, Hernandez M, Fernandez-Ruiz J.

Exposure to cannabinoids in the development of endogenous cannabinoid system. Neurotox Res 2002;4:363–72.

Rodriguez de Fonseca F, Ramos JA, Bonin A, Fernández-Ruiz JJ. Presence of cannabinoid binding sites in the brain from early postnatal ages. NeuroReport 1993;4:135–8.

Romero J, García-Palomero E, Berrendero F, García-Gil L, Hernández ML, Ramos JA, et al. Atypical location of cannabinoid receptors in white matter areas during rat brain development. Synapse 1997;26:317–23.

Romero EM, Fernández B, Sagredo O, Gomez N, Urigüen L, Guaza C, et al. Antinociceptive, behavioural and neuroendocrine effects of CP 55,940 in young rats. Dev Brain Res 2002;136:85–92.

Terranova ML, Laviola G. Delta-opioid modulation of social interactions in juvenile mice weaned at different ages. Physiol Behav 2001;73: 393–400.

Viveros MP, Hernández R, Martinez I, González P. Effects of social isolation and crowding upon adrenocortical reactivity and behavior in the rat. Rev Esp Fisiol 1988;44:315–22.

Wenger T, Jamali KA, Juaneda C, Leonardelli J, Tramu G. Arachidonyl ethanolamide (anandamide) activates the parvocellular part of hypothalamic paraventricular nucleus. Biochem Biophys Res Commun 1997; 237:724–8.

Williamson EM, Evans FJ. Cannabinoids in clinical practice. Drugs 2000; 60:1303–14.