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Chronic Δ^9-Tetrahydrocannabinol During Adolescence Provokes Sex-Dependent Changes in the Emotional Profile in Adult Rats: Behavioral and Biochemical Correlates

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Few and often contradictory reports exist on the long-term neurobiological consequences of cannabinoid consumption in adolescents. The endocannabinoid system plays an important role during the different stages of brain development as cannabinoids influence the release and action of different neurotransmitters and promote neurogenesis. This study tested whether long-lasting interference by cannabinoids with the developing endogenous cannabinoid system during adolescence caused persistent behavioral alterations in adult rats. Adolescent female and male rats were treated with increasing doses of Δ^9-tetrahydrocannabinol (THC) for 11 days (postnatal day (PND) 35–45) and left undisturbed until adulthood (PND 75) when behavioral and biochemical assays were carried out. CB1 receptor level and CB1/G-protein coupling were significantly reduced by THC exposure in the amygdala (Amyg), ventral tegmental area (VTA) and nucleus accumbens (NAc) of female rats, whereas male rats had significant alterations only in the amygdala and hippocampal formation. Neither female nor male rats showed any changes in anxiety responses (elevated plus maze and open-field tests) but female rats presented significant 'behavioral despair' (forced swim test) paralleled by anhedonia (sucrose preference). In contrast, male rats showed no behavioral despair but did present anhedonia. This different behavioral picture was supported by biochemical parameters of depression, namely CREB alteration. Only female rats had low CREB activity in the hippocampal formation and prefrontal cortex and high activity in the NAc paralleled by increases in dynorphin expression. These results suggest that heavy cannabis consumption in adolescence may induce subtle alterations in the emotional circuit in female rats, ending in depressive-like behavior, whereas male rats show altered sensitivity to rewarding stimuli.
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INTRODUCTION

Marijuana and hashish, psychoactive products of the hemp plant, are the illicit drugs most frequently used by human adolescents (Gruber and Pope, 2002). This is facilitated by the fact that users generally perceive these drugs as relatively harmless. Recent data estimate that cannabis use starts in the period from 12 to 18 years of age (SAMHSA, 2004), though even earlier use (9–10 years) is now anecdotally reported.

Despite the constantly spreading use of cannabis, there is little information about its neurobiological long-term consequences in juveniles. Adolescence is a critical phase for cerebral development, characterized by strong neuronal plasticity, with sprouting and pruning of synapses, myelinization of nerve fibers, changes in neurotransmitter concentrations and their receptor levels in brain areas essential for behavioral and cognitive functions (Rice and Barone, 2000). Moreover, the endocannabinoid system plays an important role during early development of the brain as cannabinoids influence the release and action of different neurotransmitters (Viveros *et al*, 2005). Therefore, early exposure to cannabinoids might lead to subtle but lasting neurobiological changes.

Exposure to cannabis during this critical period may even be a risk factor for neuropsychiatric disorders such as schizophrenia, depression, and other mood pathologies (Arseneault *et al*, 2002; Fergusson *et al*, 2003) and early

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regular use of cannabis may well increase the risk of starting to use other illicit drugs (Lynskey *et al*, 2003; Agrawal *et al*, 2004).

Animal data in the literature are still scarce and conflicting, depending on the period of exposure, the dose, and the cannabinoid agonist used. Chronic administration of CP-55,940 in rats during adolescence (from postnatal day (PND) 35–45) resulted in marked behavioral effects in adulthood such as a decrease in the level of emotionality/anxiety in the open field and in the elevated plus maze (EPM) (Biscaia *et al*, 2003). These effects seem sex related and their nature depends on the specific behavioral test involved, with more marked effects in female rats than male rats. Schneider and Koch (2003, 2005) reported that chronic treatment of male rats with the synthetic cannabinoid receptor (CB1) agonist WIN 55,212-2 during puberty (from PND 40–65) had several effects in adulthood that can be considered as the endophenotypes of schizophrenia, such as persistent disturbances in object recognition memory, reduction in progressive ratio instrumental performance, reduction in prepulse inhibition of the acoustic startle reflex, and increased anxiety-related behavior. In adult rats WIN-55,212 had no such effects (Schneider and Koch, 2003).

These findings indicate that adolescence is a vulnerable period for brain development, during which cannabis use can lead to persistent and more severe disturbances than comparable use in adulthood.

The current study was therefore designed to test whether adolescent exposure to THC in rats of both sexes induced long-lasting neurobiological changes leading to altered behavior in adulthood. Adolescence in rats is considered a period during which animals of both sexes and most breeding stocks would be expected to exhibit adolescent-typical neurobehavioral characteristics, such as increases in risk taking and social interaction with peers (Spear, 2000). This age ranges from approximately PND 28–42 (Spear, 2000).

Male and female rats were treated with increasing doses of THC in about mid- or late adolescence (PND 35–45; Andersen, 2003; Ellgren *et al*, 2007), and the effects were tested in different behavioral tests in the adult animals to check for alterations in motor activity, anxiety, and depressive behavior. Autoradiographic binding studies were carried out on CB1 receptor level and efficiency and specific neuronal markers associated with the observed behavior were assayed to define the neurochemical correlates of the altered responses.

MATERIALS AND METHODS

Drugs

Δ^9-Tetrahydrocannabinol (THC), a generous gift from GW Pharmaceutical (Salisbury, UK), was dissolved in ethanol, cremophor, and saline (1 : 1 : 18). The 1 : 1 : 18 vehicle (ethanol:cremophor:saline) has a long history of use in many laboratories for the solubilization of cannabinoids. Ethanol concentration in the THC solution and in the vehicle is 5%, resulting in an ethanol dose of approximately 0.25 g/kg, that in our hands does not induce any significant behavior (data not shown). This was also confirmed by Varlinskaya and Spear (2007).

Animals and Treatment

Male and female Sprague–Dawley rats (PND 28) were obtained from Charles River, Calco, Italy. They were housed in groups of five in standard conditions of temperature and humidity under a 12-h light/12-h dark cycle with *ad libitum* access to food and water. Rats were allowed to acclimate in their new environment for 1 week before the start of the treatment. The treatment began at PND 35 and lasted until PND 45. During this period, rats received increasing i.p. doses of THC twice a day (2.5 mg/kg PND 35–37; 5 mg/kg PND 38–41; 10 mg/kg PND 42–45; see Figure 1) or its vehicle. In all, 62 female and 62 male rats were used.

Experiments were carried out in strict accordance with the guidelines released by the Italian Ministry of Health (DL 116/92) and (DL 111/94-B), and the European Community directives regulating animal research (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering.

Food Intake and Body Weight

Food intake (FI) and body weight (BW) were monitored throughout the experimental period according to a previously described method (Biscaia *et al*, 2003). Since each cage housed five animals, chow amounts consumed per animal per day were averaged by dividing the amount per cage by 5. For FI the increment (Δ) in grams per animal at each time point was calculated as the difference between FI at a given day (day *t*) and FI at day 28, ie Δg per animal = FI *t*-FI 28. The same criterion was followed to calculate the increment in BW: Δg per animal = BW *t*-BW 28. The corresponding values (Δg per animal) were employed for the statistical analysis. In order to evaluate

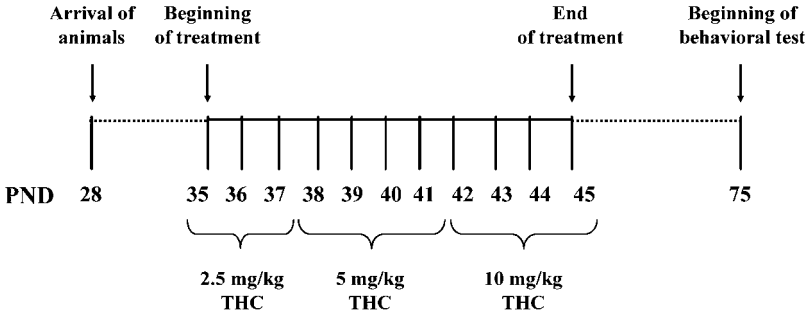

Figure 1 Scheme of treatment.

the short- and long-term effects of the pharmacological treatment, we analyzed the data corresponding to the period of treatment (PND 35–45) as well as at a later stage (PND 70–88).

Behavioral Tests

Behavioral testing started at 75 days of age and was completed within 10 days. On the day of testing, the animals were habituated in a quiet laboratory for a 30-min period, before experimental procedure began. We allowed an interval of 3 days among each test for recovery of animals from the previous testing condition. Animals were randomized in two groups. In the first group, used to provide complementary data about diverse aspects of spontaneous behavior and anxiety-related responses, the order of testing was as follows: locomotor activity, open-field and EPM. In the second group, used to provide complementary data about depressive-like behaviors, the order was as follows: sucrose preference and forced swim test (FST).

The experimenter was not aware of the drug treatment of the animals during behavioral testing.

Spontaneous Locomotor Activity

Spontaneous locomotor activity was evaluated in an activity cage (43 cm × 43 cm × 32 cm) (Ugo Basile, Varese, Italy), placed in a sound-attenuating room. The cage was fitted with two parallel horizontal and vertical infrared beams located 2 and 6 cm from the floor. Cumulative horizontal and vertical movement counts were recorded for 20 min. Testing cage was wiped clean with 0.1% acetic acid and dried after each trial.

Elevated Plus Maze

The elevated plus-maze consisted of two opposite open arms (50 cm × 10 cm) and two enclosed arms (50 cm × 10 cm × 40 cm) that extended from a common central platform (10 cm × 10 cm) based on a design validated by Lister (1987). The apparatus was constructed from gray iron, elevated to a height of 50 cm above floor level and placed in the center of a small quiet room under dim light (about 30 lux). Testing was conducted during the early light phase (0930–1330 hours) of the light cycle. Rats were placed individually onto the center of the apparatus facing an open arm, and the time spent on and entries onto each arm were measured for 5 min. Behavioral parameters comprised both conventional spatiotemporal and ethological measures (Rodgers *et al*, 2003, 2005). Conventional measures were the frequency of open arm entries (arm entry = all four paws into an arm) and the time spent in the same arms. Ethological measures comprised frequency scores for head dips (HD: exploratory movement of head/shoulders over

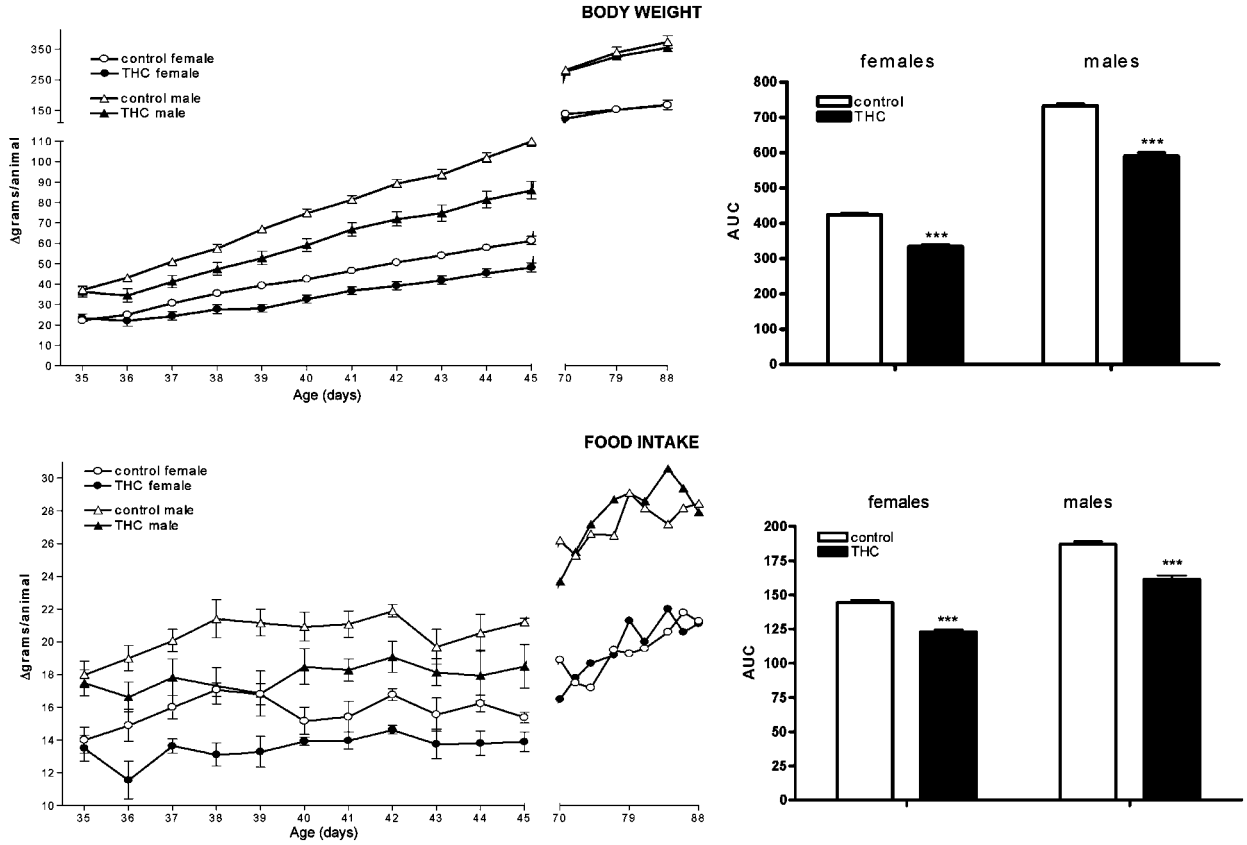

Figure 2 Effect of chronic Δ^9-tetrahydrocannabinol (THC) treatment on growth and food intake (FI) of female and male rats during (postnatal day (PND) 35–45) and after treatment (PND 70–88). On the left, it is presented the increment (Δ) in body weight (BW) (g; upper panel) or FI (g; lower panel) per animal at each time point (day) considering day 28 as the point of reference (see 'Materials and Method'). Each point represents the mean ± SEM of at least 20 animals. On the right, the area under the curve restricted to the period of treatment (PND 35–45) is presented. ***$p < 0.001$ *vs* controls (Bonferroni test).

Table 1 Effect of Chronic THC Treatment on CB1 Receptor (CB1R) Binding and G-Protein Coupling in Adolescent Animals

	Female				Male			
	CB1R density (fmol/mg tissue)		CB1R/G-protein coupling (% stimulation)		CB1R density (fmol/mg tissue)		CB1R /G-protein coupling (% stimulation)	
Brain area	Vehicle	THC	Vehicle	THC	Vehicle	THC	Vehicle	THC
Prefrontal cortex	66.0 ± 9.4	32.9 ± 3.5**	68.7 ± 6.1	19.1 ± 3.7**	96.9 ± 9.2	62.6 ± 5.2**	51.8 ± 9.3	44.3 ± 4.8
Caudate putamen	132.6 ± 7.1	105.2 ± 5.4**	72.0 ± 12.5	16.0 ± 3.7*	159.6 ± 12.6	126.7 ± 12.2	69.7 ± 7.8	62.8 ± 9.3
Nucleus accumbens	56.9 ± 8.2	42.3 ± 7.2	58.7 ± 15.9	20.0 ± 6.0***	98.5 ± 10.1	69.2 ± 6.1*	65.4 ± 9.2	53.3 ± 7.2
Hypothalamus	40.9 ± 4.0	25.9 ± 3.4*	18.3 ± 4.9	15.7 ± 6.0	84.4 ± 7.2	56.4 ± 3.8**	27.3 ± 7.3	13.7 ± 4.8
Globus pallidus	244.9 ± 22.8	242.8 ± 17.1	170.2 ± 21.1	98.2 ± 13.2*	342.9 ± 6.5	321.9 ± 10.5	143.6 ± 18.2	109.4 ± 17.5
Hippocampus	127.2 ± 6.7	63.8 ± 3.5***	111.3 ± 13.9	34.7 ± 4.1***	177.9 ± 5.6	117.5 ± 8.2***	115.8 ± 30.6	37.1 ± 10.6*
Amygdala	53.8 ± 4.1	28.2 ± 3.2**	91.6 ± 22.2	23.2 ± 8.1*	88.9 ± 5.6	64.1 ± 4.4**	73.3 ± 20.4	10.3 ± 7.7*
Thalamus	50.9 ± 4.7	32.7 ± 3.2***	35.9 ± 6.1	18.8 ± 6.5	78.7 ± 3.6	64.2 ± 5.8*	17.0 ± 4.7	6.0 ± 7.9
Periaqueductal gray	45.9 ± 5.5	25.8 ± 2.1**	57.9 ± 11.7	27.9 ± 8.1	88.1 ± 4.7	70.3 ± 2.7**	20.5 ± 7.8	21.1 ± 2.3
Substantia nigra	265.9 ± 13.1	187.4 ± 12.9**	214.8 ± 18.2	148.1 ± 24.0*	351.0 ± 13.5	314.4 ± 12.7	258.9 ± 22.2	171.4 ± 22.7*
Ventral tegmental area	39.6 ± 3.7	23.4 ± 2.1**	51.2 ± 4.0	21.5 ± 5.7***	96.3 ± 4.8	71.16 ± 4.6**	39.3 ± 6.1	28.0 ± 7.82
Cerebellum	218.4 ± 8.4	140.2 ± 13.1***	98.9 ± 16.1	45.6 ± 13.2*	234.3 ± 6.4	163.4 ± 10.3***	122.2 ± 18.9	65.5 ± 15.4*

Abbreviation: THC, Δ^9-tetrahydrocannabinol.
*$p<0.05$; **$p<0.01$; ***$p<0.001$ *vs* vehicle.

the side of the maze), risk assessment (RA: exploratory posture in which the body is stretched forward and then retracted to the original position without any forward locomotion) and closed-arm returns (CAR: exiting closed arm with forepaws only and doubling back into the same arm).

Open Field

The open-field arena consisted of a Plexiglas cage (60 cm × 60 cm × 60 cm) divided into four squares and a square zone marked in the center of the field (15 cm × 15 cm). A video camera was mounted above the open-field arena, such that the whole arena was visible through a camera. Animals were transported to the experimental room about 1 h before any session started. At the beginning of the test session, each rat was placed in a corner of the open field and allowed to explore the arena for 20 min, and the entire session was videotaped. Number of passages and time spent in the center zone were monitored.

Forced Swim Test

The FST is a 2-day procedure in which rats swim under conditions where escape is not possible (Porsolt *et al*, 1978). On first day, rats are forced to swim for 15 min. Rats initially struggle to escape from the water, but eventually they adopt a posture of immobility characterized by few movements necessary to keep heads above the water. When rats are tested again 24 h later, immobility is increased if animals show depressive-like behavior. On the first day of the FST, rats were placed in a clear 50 cm tall, 20 cm diameter glass cylinder filled to 25 cm with 25°C water. After 15 min of forced swimming, rats were removed from the water, dried with towels and placed under warming lamp until dry. The cylinder was emptied and cleaned between rats. After 24 h, rats were tested again for 5 min under the same conditions. Immobility was defined as the time spent by rats making only movements necessary to keep their head above water, swimming was measured if they were making active swimming movements to the center of the cylinder, climbing if they were making forceful thrashing movements with their forelimbs against the walls of the cylinder. The time rats spent in each of these behaviors was measured.

Sucrose Preference Test

Rats were tested for preference of a 2% sucrose solution, using a two-bottle choice procedure (Monteggia *et al*, 2007) with slight modifications. Subjects were housed singly for the 3 days of test. Rats were given two bottles, one of sucrose (2%) and one of tap water. Every 24 h the amount of sucrose and water consumed was evaluated. To prevent potential location preference of drinking, the position of the bottles was changed every 24 h. The preference for the sucrose solution was calculated as the percentage of sucrose solution ingested relative to the total intake.

Biochemical Assays

Autoradiographic binding studies. A third group of animals was used for this experiment. Adolescent and adult

rats were decapitated and brains were rapidly removed and frozen in liquid nitrogen and stored at −80°C until processing. Adolescent brains were removed 24 h after the last THC injection (PND 46), whereas adult brains were removed between PND 75 and 80. Twenty micron coronal sections were cut on a cryostat and thaw mounted on gelatin-coated slides. Sections were briefly dried at 30°C and stored at −80°C until they were processed for autoradiographic binding studies.

[^{3}H]CP-55,940 receptor autoradiographic binding. Slides from THC-pretreated male and female rats were brought to room temperature, then incubated for 2.5 h at 37°C with 10 nM [^{3}H]CP-55,940 (Perkin Eilmer Life Sciences, Milan, Italy) in binding buffer (50 mM Tris-HCl, pH 7.4, 5% BSA). Adjacent cerebral sections were incubated in parallel with 10 μM CP-55,940 to assess nonspecific binding. Sections were washed for 1 h at 4°C in 50 mM Tris-HCl, pH 7.4, 1% BSA buffer and again for 3 h in the same conditions. They were then dipped in 50 mM Tris-HCl Buffer (pH 7.4, 5 min) to remove excess BSA, dipped briefly in distilled water, and dried under a cool air stream. Autoradiograms were generated by exposing the dried sections for 7 days to Hyperfilm ^{3}H (GE Healthcare, Milan, Italy).

CP-55,940-stimulated [^{35}S]GTPγS binding in autoradiography. This was determined as described by Sim *et al* (1996), with slight modifications. Briefly, slides were incubated in assay buffer (50 mM Tris-HCl, 3 mM MgCl2, 0.2 mM EGTA, 100 mM NaCl, 10 mU/adenosine deaminase, 0.1% BSA, pH 7.4) at 25°C for 10 min then in 3 mM GDP in assay buffer at 25°C for 15 min. They were then transferred to assay buffer containing 3 mM GDP and 0.04 nM [^{35}S]GTPγS with (stimulated) or without (basal) 5 μM CP-55,940, and incubated at 25°C for 2 h. Slides were rinsed twice in 50 mM cold Tris buffer and once in deionized water, dried, and exposed to βmax film (GE Healthcare) for 48 h.

Image analysis. The intensity of the autoradiographic films was assessed by measuring the gray levels with an image analysis system consisting of a scanner connected to a PC running Microsoft Windows. The images were analyzed using Image-Pro Plus 5.0 (MediaCybernetics, Silver Spring, USA) as previously described (Rubino *et al*, 2005).

pCREB levels. Cerebral areas (prefrontal cortex, nucleus accumbens (NAc), and hippocampus) were dissected from animals belonging to the first group, 3 days after the last behavioral test. They were obtained within few minutes by regional dissection on ice according to Heffner *et al* (1980), and immediately frozen in liquid nitrogen and stored at –80°C until used. Brain areas were then homogenized and nuclear extracts were prepared as previously described (Rubino *et al*, 2007). The level of activated CREB was measured in the nuclear extracts using transcription factor assay kit (Active Motif, Rixensart, Belgium) based on ELISA method to detect and quantify CREB activation (Rubino *et al*, 2007).

Dynorphin A levels. Single tissue samples from NAc dissected from animals belonging to the second group, 3 days after the last behavioral test, were homogenized by sonication in 0.1 M acetic acid (1 ml/100 mg tissue), heated at 95°C for 10 min, cooled on ice, then centrifuged for 20 min at 17 500*g*. The supernatants were removed and stored at –20°C. Dynorphin A tissue immunoreactivity was measured with a specific radioimmunoassay as previously described (Romualdi *et al*, 1995).

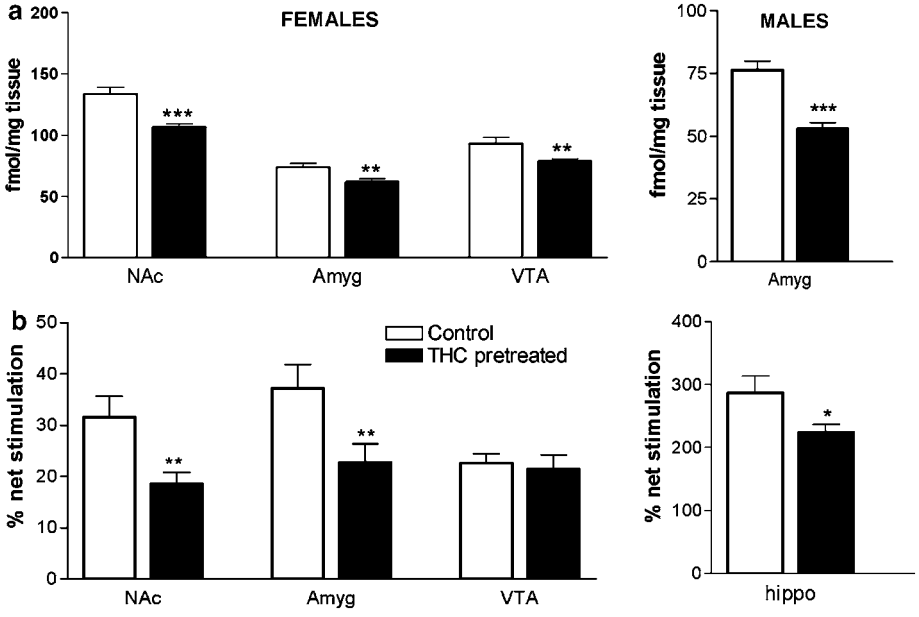

Figure 3 Effect of Δ^9-tetrahydrocannabinol (THC) pretreatment on CB1 receptor binding (a) and net CP-55,940-stimulated [^{35}S]GTPγS binding (b), in adult female and male rats. Gray levels obtained with densitometric analysis in (a) were transformed into fmol/mg tissue using [^{3}H] standards. Bars indicate the mean ± SEM of at least 6 animals. *$p<0.05$; **$p<0.01$; ***$p<0.001$ *vs* controls (Student's *t*-test). Amyg, amygdala; NAc, nucleus accumbens; Hippo, hippocampus; VTA, ventral tegmental area.

Statistical analysis. All the data were expressed as mean ± SEM and analyzed with two-way ANOVA (with the two factors being sex and pharmacological treatment) followed by Bonferroni test or using an unpaired *t*-test where appropriate. All statistical calculations were carried out using Prism, version 4.0 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Body Weight and Food Intake

BWs and FI are presented in Figure 2. THC during adolescence reduced growth in male and female rats, the BW reduction being related to the reduction of FI. This effect seemed restricted to the period of THC exposure, and when the rats reached adulthood neither BW nor FI differed significantly from controls. Two-way ANOVA of the BW, expressed as area under the curve during the period of treatment with THC (Figure 1), showed significant overall effects of both pharmacological treatment ($F_{1,124} = 309.84$; $p < 0.0001$) and sex ($F_{1,124} = 1831.76$; $p < 0.0001$), as well as a significant interaction between these two main factors ($F_{1,124} = 15.84$; $p < 0.0001$). *Post hoc* comparison indicated that THC significantly reduced BW in both male and female rats ($p < 0.001$). The picture was similar for FI, with significant overall effects of both pharmacological treatment ($F_{1,16} = 117.56$; $p < 0.0001$) and sex ($F_{1,16} = 358.27$; $p < 0.0001$) being observed. *Post hoc* comparison indicated that THC also significantly reduced FI in both male and female rats ($p < 0.001$).

Autoradiographic Binding Studies

On coronal brain sections from adolescent (PND 46) and adult (PND > 75) male and female rats exposed to THC during adolescence, we assessed alterations in CB1 cannabinoid receptor density and coupling to G proteins by autoradiographic binding studies with [^{3}H]CP-55,940 and agonist-stimulated [^{35}S]GTPγS binding, respectively.

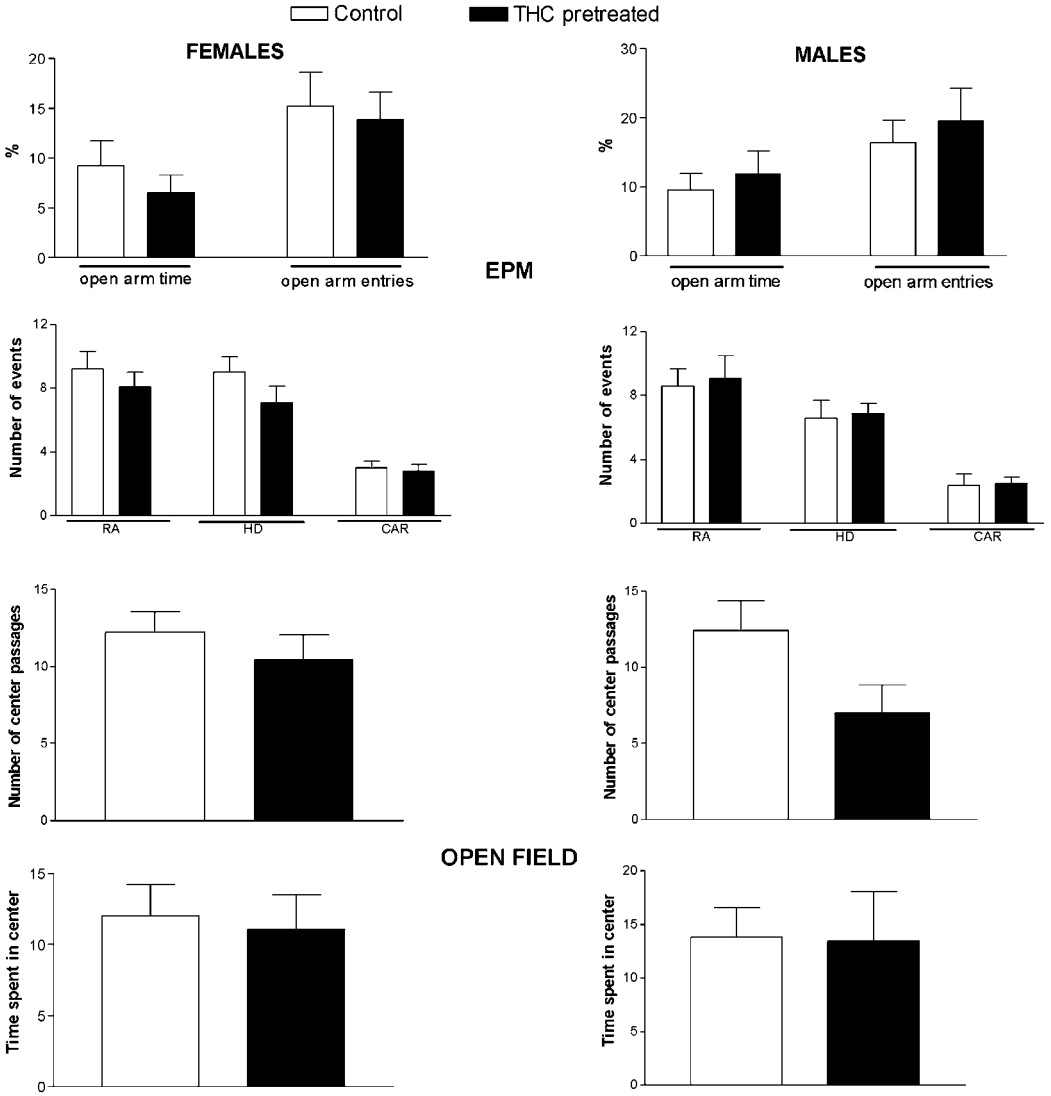

Figure 4 Effect of Δ^9-tetrahydrocannabinol (THC) pretreatment on elevated plus maze (EPM) and open-field activity in adult female and male rats. The following results are shown: percentage of open arm time and entries and number of ethological events (CAR, closed-arm return; HD, head dips; RA, risk assessment) in the EPM; number of entries and time spent in the center of the open field. Mean ± SEM of at least 10 animals.

Table 1 shows the densitometric analysis of [^{3}H]CP-55,940 binding in rat brain coronal sections from adolescent animals 24 h after the last THC injection. Both female and male rats treated with the cannabinoid agonist showed a significant and widespread decrease in receptor binding levels in almost all the brain areas containing CB1 receptors. Quantitative analysis of the net CP55,940-stimulated [^{35}S]GTPγS binding in male and female adolescent rats is illustrated in Table 1, too. Basal [^{35}S]GTPγS binding was not affected in any brain area (data not shown). In female rats, stimulated binding was significantly reduced by THC in almost all the brain areas where there was a significant downregulation of CB1 receptors, consistent with the presence of diffuse desensitization. In male rats, however, the desensitization was less intense, with significant reductions only in the hippocampus ($p<0.05$), amygdala (Amyg) ($p<0.05$), substantia nigra ($p<0.05$) and cerebellum ($p<0.05$) (Table 1).

Autoradiographic binding studies on adult brains (Figure 3) indicated that there were still changes in CB1 receptor functionality but only in certain brain regions. Specifically, adult female rats treated with THC showed significant downregulation in cannabinoid receptors in the NAc (↓20%, $p<0.001$), Amyg (↓17%, $p<0.01$), and ventral tegmental area (VTA) (↓16%, $p<0.01$), but no changes in the other brain regions considered (Figure 3a; data not shown). In parallel with the decrease in CB1 receptor binding, CP-55, 940-stimulated [^{35}S]GTPγS binding was low in the same areas (Figure 3b), the reductions reaching statistical significance in the NAc (↓40%, $p<0.01$) and Amyg (↓40%, $p<0.01$). In contrast, in adult male rats (Figure 3) the effect on CB1 receptors was slighter, with fewer areas involved and alterations only in one parameter. Specifically, CB1 receptor binding decreased in the Amyg (↓30%, $p<0.001$) and CB1 receptor/G-protein coupling was reduced in the hippocampus (↓22%, $p<0.05$).

Locomotor Activity

THC had no effects on spontaneous locomotor activity in adult male or female rats (data not shown).

Anxiety States

The results in the EPM and open-field tests are presented in Figure 4. THC and sex had no effects on the percentage of

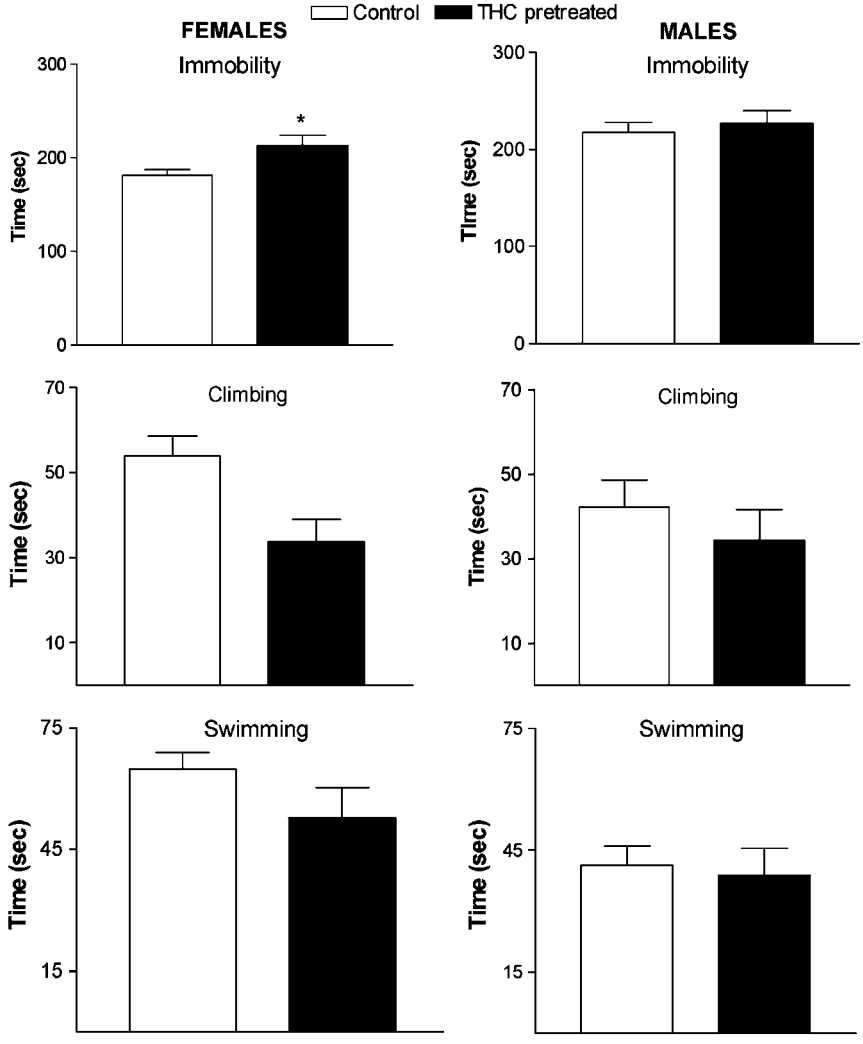

Figure 5 Effect of Δ^9-tetrahydrocannabinol (THC) pretreatment in the forced swim test (FST) in adult female and male rats. The following results are shown: time spent in immobility; time spent climbing; time spent swimming. Mean ± SEM of at least 10 animals. *$p<0.05$ *vs* controls (Bonferroni test).

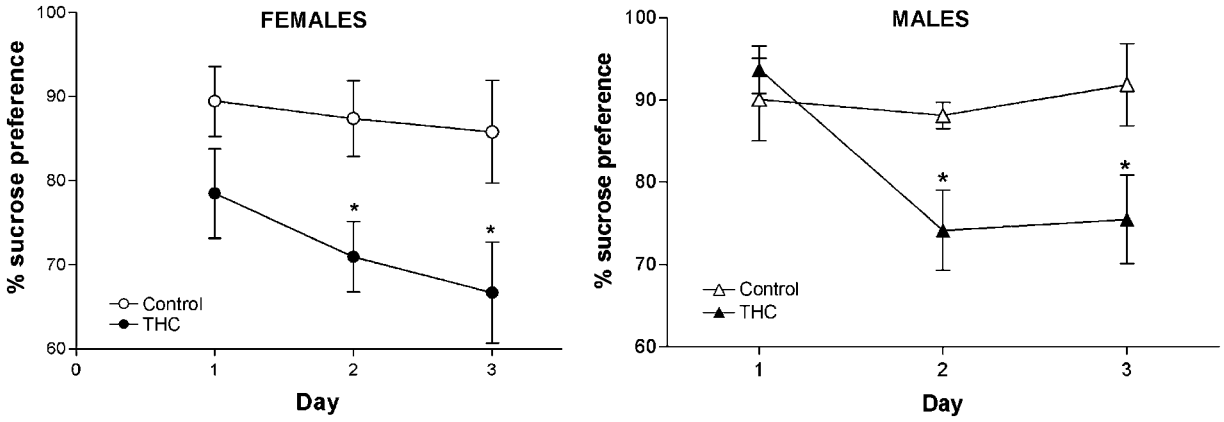

Figure 6 Effect of Δ^9-tetrahydrocannabinol (THC) pretreatment on sucrose preference in adult female and male rats with free choice of water or a sucrose solution (2%). Mean ± SEM of at least six animals. *$p<0.05$.

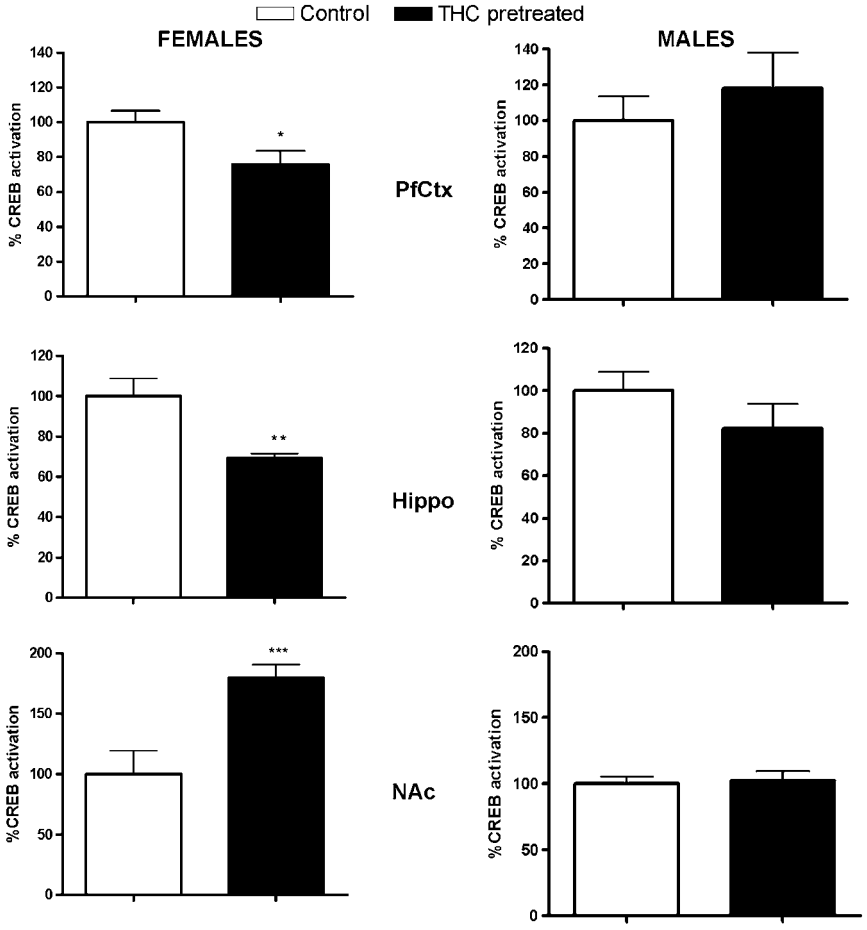

Figure 7 Effect of Δ^9-tetrahydrocannabinol (THC) pretreatment on CREB activation in nuclear extracts of prefrontal cortex (PfCtx), hippocampus (Hippo) and nucleus accumbens (NAc) of adult female and male rats. Mean ± SEM of at least eight animals. *$p<0.05$, **$p<0.01$, ***$p<0.001$ *vs* controls (Student's *t*-test).

time and entries into the open arms of the EPM, and ethological parameters also showed no real changes. In the open-field test neither THC nor sex significantly affected the number of passages and time spent in the center, reinforcing the observation that THC had no effect on anxiety behavior.

Depressive-Like Behavior

The effects of THC in the FST are presented in Figure 5. With respect to immobility, ANOVA showed significant overall effects of both pharmacological treatment ($F_{1,34}=5.25$; $p<0.05$) and sex ($F_{1,34}=6.87$; $p<0.05$), as well as a

significant interaction between these two main factors ($F_{1,34} = 5.20$; $p < 0.05$). *Post hoc* comparison indicated that THC induced significant behavioral changes only in adult female rats, which spent significantly more time immobile (26%; $p < 0.05$). This suggests there was some sort of depressive-like behavior in female rats. Two-way ANOVA did not indicate any significant effect of sex for climbing. However, treatment had a significant overall effect on climbing ($F_{1,34} = 4.24$; $p < 0.05$), with a 40% reduction in climbing activity in THC-treated female rats and less than 20% in THC-treated male rats.

Anhedonia

The sucrose preference test was run to evaluate anhedonia, the main symptom of depression, which reduces the responsiveness to pleasurable stimuli. During the first day of the test, ANOVA showed no significant effect of either pharmacological treatment or sex. However, on the second and third day, two-way ANOVA found significant overall effects of treatment ($F_{1,14} = 14.6$; $p < 0.01$ and $F_{1,14} = 6.98$; $p < 0.05$; Figure 6). Water consumption by treated and control animals of both sexes during the same period remained unchanged (data not shown).

Biochemical Parameters of Depression

Evidence is emerging that CREB activity can either produce or reduce depressive-like behavior in laboratory animals, depending on the brain region involved (see for review Carlezon *et al*, 2005). In adult female and male rats we investigated the activation of CREB in selected cerebral areas involved in depressive-like behaviors, such as the prefrontal cortex, hippocampus and NAc, using a specific ELISA kit. As reported in Figure 7, THC significantly reduced pCREB in the prefrontal cortex (24%, $p < 0.05$), and in the hippocampus (30%, $p < 0.05$) of female rats. However, the levels increased in the NAc (80%, $p < 0.001$) and this was correlated with a significant rise (100%, $p < 0.001$) in dynorphin A (Figure 8), an endogenous ligand of κ-opioid receptors involved in regulating aversive emotions. Male adult rats showed no changes in pCREB levels (Figure 7).

DISCUSSION

In the present study, we set up an experimental protocol of repeated THC injections in adolescent male and female rats, followed by behavioral and biochemical analysis in adulthood, about 35 days after the last THC injection. The use of THC rather than other synthetic cannabinoid compounds (WIN 55,212 or CP-55,940) is particularly important since humans who consume marijuana are exposed to THC. This is a major difference especially at receptor level, since at CB1 receptors THC behaves as a partial agonist, whereas CP-55,940 and WIN 55,212 have higher relative intrinsic activity (Howlett, 2002), very likely triggering different cellular adaptations from THC.

Our findings raise several important behavioral and biochemical points.

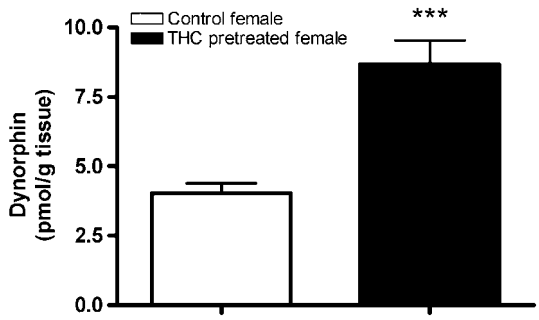

Figure 8 Effect of Δ^9-tetrahydrocannabinol (THC) pretreatment on dynorphin levels in NAc (nucleus accumbens) of adult female rats. Mean ± SEM of at least nine animals. ***$p < 0.001$ *vs* controls (Student's *t*-test).

Body Weight and Food Intake

Surprisingly, THC reduced FI and BW during the treatment period, in contrast to its documented ability to induce FI, while SR141716A (the CB1 antagonist) reduces it (see Matias and Di Marzo, 2007). Our data are in agreement with previous findings that chronic CP-55,940 given during peri-adolescence period (Biscaia *et al*, 2003) reduced FI and BW. The lower FI, already significant after the first day of treatment, may be partly attributable to the decrease in motor activity caused by the acute effect of the drug. In addition, the idea that endocannabinoid control of FI might differ in adolescents and in adults cannot be discarded, since their sensitivity to the pharmacological stimulation of CB1 receptors may differ. This point calls for further investigation.

Finally, binding studies showed a profound downregulation of hypothalamic CB1 receptors, suggesting reduced ability of the cannabinoid system to stimulate orexigenic signals (Matias and Di Marzo, 2007). This hypothesis, however, is weakened by the significant reductions in both BW and FI after the first day of treatment, when downregulation is not yet likely to be present.

CB1 Receptor Density and G-Protein Coupling

When the consequence of chronic THC on CB1 receptor density and G-protein coupling was evaluated at the end of the adolescent treatment, the magnitude of downregulation was similar for male rats and female rats, while there was less intense desensitization in male rats with few areas involved. Thus it appears that chronic THC treatment in adolescence produced the same cellular adaptation in CB1 functionality as widely reported in adults (Parolaro *et al*, 2005), but with different sex-sensitivity for desensitization. Craig *et al* (2007) too reported a significant difference in the magnitude of desensitization between adolescent male and female rats chronically exposed to THC. The lack of studies on the effects of chronic cannabinoid treatment on CB1 receptors in adults of either sex does not allow us to speculate whether a similar sex-dependent desensitization occurs in adult brain too. Future studies will be aimed at clarifying this important point.

From autoradiographic binding studies on adult brains, we found that THC had different effects on CB1 receptor

density and function in male and female rats. In female rats both CB1 density and functionality were significantly reduced in brain areas (VTA, NAc, and Amyg) belonging to a circuit involved in emotional processing and reward. Male rats showed smaller changes, localized in the Amyg for receptor binding, and in the hippocampus for G-protein coupling. Changes in female rats appeared more substantial, each area showing concordant alterations in both CB1 receptor binding and coupling, whereas in male rats the positive areas showed a reduction in either density (Amyg) or coupling (hippocampus). Thus the cannabinoid system might be more vulnerable to pharmacological treatment in adolescent female rats than male rats. These effects were still present 30 days after the last treatment so they can be considered lasting consequences.

To our knowledge this is one of the first studies of CB1 receptor function in adult animals after exposure to THC in adolescence. Only Ellgren *et al* (2007) have previously carried out these experiments, reporting no change in CB1 receptor density and function in male rats exposed to a low-moderate dose of THC (1.5 mg/kg) given once every 3 days during PND 28–49. The difference from our results in male rats might reflect the different treatment paradigm, consisting in a low THC dosing regimen in Ellgren's study and a higher one in ours, and/or the different times when binding studies were carried out.

Behavioral Findings

The behavioral findings and the related biochemical parameters also indicated female rats were more susceptible to adolescent treatment than male rats. Locomotor activity and anxiety response, tested on the EPM and open field, were not significantly affected in either sex, but female rats presented 'behavioral despair', demonstrated by the greater time spent in immobility, that is suggestive of depression. Then too, in the sucrose preference test female rats showed a significant reduction in preference that might be interpreted as anhedonia. In contrast, male rats showed no behavioral despair, but did present anhedonia.

Anhedonia is a core symptom of depression (Anisman and Matheson, 2005), reflecting changes in motivation, hence also changes in the sensitivity of the rewarding dopaminergic system (Nestler and Carlezon, 2006). However, anhedonia occurs in other human neuropsychiatric disorders too, such as post-traumatic stress disorder, drug abuse and schizophrenia (Wolf, 2006; Paterson and Markou, 2007; Kashdan *et al*, 2006). Pistis *et al* (2004) reported that adolescent exposure of male rats to WIN 55,212 altered the response of midbrain dopamine neurons to several drugs of abuse, suggesting that cannabinoid-induced lack or blunting of the dopamine neurons' responses to pharmacological stimuli might reverberate into reduced responses to natural rewarding and motivational stimuli. Pistis' theory provides support for our results on male rats, who displayed anhedonia without depressive-like behavior.

Biochemical Parameters of Depression

The different behavioral picture in male and female rats is supported by the biochemical parameters of depression, in this case changes in CREB. According to previous studies, in the hippocampus and prefrontal cortex CREB appears to be a vital mediator of antidepressant effects since a variety of antidepressant treatments increase CREB activity in these brain regions (Nibuya *et al*, 1996; Thome *et al*, 2000; Sairanen *et al*, 2007; Tiraboschi *et al*, 2004). In addition, significant reductions in CREB protein levels have been found in the prefrontal cortex and hippocampus of suicide subjects (Dwivedi *et al*, 2003).

Conversely, elevated CREB activity in the NAc produces various depressive-like effects in rodents (see for review Carlezon *et al*, 2005). For example, Pliakas *et al* (2001) used herpes simplex virus vectors to raise CREB expression in the NAc or to overexpress a dominant-negative mutant CREB that blocks CREB function. They showed that sustained elevation of CREB activity in the NAc increased immobility behavior in the FST but that reductions had antidepressant-like effects in the same test (Pliakas *et al*, 2001) and the learned helplessness paradigm (Newton *et al*, 2002).

Regulation of depressive-like behavior by changes in CREB activity within the NAc appears to be partly due to dynorphin, an endogenous ligand of κ-opioid receptors that is a target gene of CREB (Newton *et al*, 2002). In our study, female rats showing depressive-like behavior had high CREB activity in the NAc paralleled by a high dynorphin level, and low CREB activity in the hippocampus and prefrontal cortex. Male rats showed no noteworthy alterations in CREB activity in the three brain regions.

It has been suggested that the endocannabinoid system, through the activation of CB1 receptors, is implicated in the control of emotional behavior (see for review Viveros *et al*, 2005). Indeed, endocannabinoids may act as a buffer system in the stress response (Tasker, 2004) since they dampen stress-induced behavioral changes, including the expression of active escape behaviors during acute and repeated restraint stress episodes (Patel *et al*, 2005a, b), immobility time in a tail suspension test (Gobbi *et al*, 2005), swimming time in the FST (Gobbi *et al*, 2005), and decreased sensitivity to natural reward (Rademacher and Hillard, 2007).

In our hands, female rats showed significant reductions in the function of the CB1 receptor in the NAc, Amyg, and VTA, specific cerebral areas belonging to an emotional circuit. If activation of CB1 receptors by endocannabinoids in these brain areas is fundamental for normal emotional behavior and stress responses, as proposed by Laviolette and Grace (2006), then the reduction in cannabinoid receptor function we found might indeed be involved in the altered emotional responses.

CONCLUSIONS

Our results suggest that heavy cannabis consumption in adolescence may, mainly in female rats, induce subtle alterations in the emotional circuit ending in depressive-like behavior, whereas in male rats it can alter the sensitivity to rewarding stimuli. However, we did not investigate whether a similar picture was produced as a long-term consequence after chronic THC treatment in adult rats, although recent papers support the idea of adolescents being specifically vulnerable to enduring adverse effects of cannabinoids (Quinn *et al*, 2007).

Therefore, the potential problems arising in relation to marijuana consumption in adolescence suggest that this developmental phase is a vulnerable period for persistent adverse effects of cannabinoids.

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DISCLOSURE/CONFLICT OF INTEREST

None of the authors report any biomedical financial interests or potential conflicts of interest.

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