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Downregulation of Rat Brain Cannabinoid Binding Sites After Chronic Δ^9-Tetrahydrocannabinol Treatment

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RODRÍGUEZ DE FONSECA, F., M. A. GORRITI, J. J. FERNÁNDEZ-RUIZ, T. PALOMO AND J. A. RAMOS. *Downregulation of rat brain cannabinoid binding sites after chronic Δ^9-tetrahydrocannabinol treatment.* PHARMACOL BIOCHEM BEHAV **47**(1) 33–40, 1994.—Specific cannabinoid receptors have been recently described in extrapyramidal and limbic areas and presumably might mediate the effects of marijuana exposure on behavioral processes related to those areas. In this work, we examined whether cannabinoid receptors exhibit downregulation as a consequence of the chronic exposure to Δ^9-tetrahydrocannabinol (THC), which might explain certain tolerance phenomena observed in relation to motor and limbic effects of marijuana. To this end, we first characterized the binding of cannabinoid receptors, by using [^{3}H]CP-55,940 binding assays, in the striatum, limbic forebrain, and ventral mesencephalon of male rats, and, second, we measured the density and affinity of those receptors in these brain areas after 7 days of a daily treatment with THC. Development of a tolerance phenomenon was behaviorally tested by using an open-field technique. Results were as follows. The three areas studies presented specific and saturable binding for the cannabinoid ligand, as revealed by their corresponding association and dissociation curves, displacement by THC, saturation curves, and Scatchard plots. A chronic treatment with THC produced the expected tolerance phenomenon: The decrease caused by an acute dose in spontaneous locomotor (49.4%) and exploratory (59.7%) activities and, mainly, the increase in the time spent by the rat in inactivity (181.7%) were diminished after 7 days of daily treatment (39.4, 40.4, and 31.7%, respectively). This tolerance was accompanied by significant decreases in the density of cannabinoid receptors in the striatum and limbic forebrain, the areas where nerve terminals for nigrostriatal and mesolimbic dopaminergic systems, respectively, which play an important role in those processes, are located. This downregulation phenomenon was also observed in the ventral mesencephalon, the area where cell bodies for both dopaminergic neuronal systems are clustered, but the decrease was smaller and nonsignificant. No modifications were seen in the affinity of these receptors by chronic exposure to THC. In summary, a chronic treatment with THC produced downregulation of cannabinoid receptors in the striatum and limbic forebrain and a nonsignificant trend in the ventral mesencephalon. This observation might explain the tolerance phenomena observed in the effects of THC on motor and limbic behaviors after chronic exposures.

Cannabinoid receptors THC Striatum Limbic forebrain Ventral mesencephalon Tolerance

THE exposure to *Cannabis sativa* derivatives, or to their individual psychoactive constituents, have been reported to produce a variety of neurobiological effects (8), among them cannabinoid-induced analgesia (8,20,22), neuroendocrine effects (11,27), extrapyramidal actions (11,31), effects on learning and cognition (8,12), and others. These effects seem to be originated as a consequence of cannabinoid-induced changes in the activity of neuronal systems involved in those processes, such as dopaminergic (2,5,11,33), GABAergic (31), or serotonergic (11) projections. In particular, the nigrostriatal and mesolimbic dopaminergic pathways, which play an important regulatory role in neurobiological processes such as motor activity and motivational behavior, respectively, seem to be important targets of cannabinoid action [for review, see (11)]. Much information exists showing that administration of Δ^9-tetrahydrocannabinol (THC) or related cannabinoids affected the activity of these neurons at the neurochemical level (3,5, 32,33), as well as their related behaviors (11,12). An interesting aspect derived from these studies is that, although cannabinoids do not produce a phenomenon of tolerance/dependence similar to that observed with opiates, certain tolerance manifestations in relation to motor and limbic effects of marijuana compounds were evident after prolonged treatments [for review, see (8)].

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On the other hand, the synthesis of nonclassical cannabinoid compounds has allowed the study of the molecular mechanisms underlying the pharmacological effects of psychoactive components of *Cannabis sativa* (9,18). One of these, CP-55,940, has been tritiated and used to identify and fully characterize a cannabinoid receptor in rat brain membranes (6). Pharmacological, anatomic, and molecular characteristics of this receptor, including its gene cloning (25), brain distribution (15,16), and analysis of its possible endogenous ligand(s) (10), have been recently studied [for review, see (18)]. Thus, it seems to be associated with a Gi protein and negatively coupled to adenylate cyclase activity (1,6,21). The study of their brain distribution revealed that this class of receptors displays one of the most dense distribution of all receptors in the brain (15,16). Autoradiographic studies confirmed their neuronal location (14) and that they are present in a number of vertebrate species (18), with the greatest density in the basal ganglia, hippocampus, and cerebellum (13,16).

Hence, it seems likely that these receptors could be one of the molecular mechanisms underlying in neurobiological effects of cannabinoids. However, the physiological processes in which these receptors might be involved have not been defined yet because the endogenous ligand(s) for this receptor is(are) still unknown and potential antagonists are not available. In this regard, Devane et al. (7) recently presented evidence of an arachidonic acid derivative that, being synthesized in the brain, might bind to cannabinoid receptors.

Presently, there are not any evidence that these receptors can be downregulated after a chronic treatment with cannabinoids. Solely, there is a recent abstract from Herkenham's group (29), who also found downregulation of these receptors after chronic cannabinoid treatment, although the full details of that study have not been published yet. The possibility of downregulation of cannabinoid receptors seems important on the basis of two facts: a) the assumption that exogenous cannabinoids act on neurobiological processes through their binding to specific receptors presumably located on cell bodies and/or axonic terminals of neurons involved in those processes; and b) that chronic exposures to marijuana compounds, mainly THC, have been sometimes associated with tolerance phenomena (8). Downregulation of cannabinoid receptors could be a plausible explanation for these phenomena.

In the present work, we examined whether the behavioral tolerance developed after 7 days of daily treatment with THC was associated with a decrease in the binding of THC to its specific receptors in the brain areas where nigrostriatal and mesolimbic dopaminergic neuronal systems are located. To this end, we specifically analyzed the striatum and limbic forebrain, where nerve terminals for both systems are, respectively, located, and the ventral mesencephalon, where cell bodies for both systems are clustered. In the three areas, we measured the affinity and density of cannabinoid receptors, by using CP-55,940 binding assays, after a chronic exposure to THC at a dose similar to that has been observed to produce neurochemical effects in previous dose-response studies (33). Saturation and dissociation curves, displacement by THC, and Scatchard plots for these areas were also performed for validation of the binding method. Only those in the striatum are drawn as an example.

METHOD

Animals

Male rats of the Wistar strain were housed in a room with a controlled photoperiod (light 1500–0300) and temperature (23 ± 1°C). They had free access to standard food (Panlab, Barcelona, Spain) and water. Rats were used for experimental purposes at adult age (>3 months of life).

Cannabinoid Treatment and Sampling

THC (Sigma Chemical Co., St. Louis, MO), stored in ethanol solution, was used for pharmacological treatments. Immediately before use, the alcohol was evaporated and the residue emulsified in Tween-80 until the appropriate concentration for administration. Two different kinds of treatments were used. In the acute situation, animals received a single IP injection of THC (6.4 mg/kg body weight) or vehicle in a volume of approximately 0.2 ml (0800–0900 h). Thirty minutes later, animals were submitted to a behavioral testing session. In the chronic situation, a different group of animals (this was to discard problems derived from learning of the test) received a daily IP injection of THC (6.4 mg/kg body weight) or vehicle for 7 days (0800–0900 h). Thirty minutes after the last administration, animals were submitted to the behavioral testing session. Immediately later, animals were sacrificed by decapitation. Brains were quickly removed and the striatum, limbic forebrain (this area contains olfactory tubercles, rostral limbic nuclei, nucleus accumbens, septal nuclei, and parts of the anterior amygdaloid nuclei), and ventral mesencephalon were dissected (13). Tissues were immediately frozen at −70°C until assay.

Membrane Preparation

On the day of the analysis, tissues were thawed and homogenized for 20 s with a Polytron at speed 2–3 in 5 ml ice-cold 50 mM Tris-HCl buffer at pH 7.4. The homogenates were centrifuged at 40,000 × *g* for 10 min at 4°C. After one wash, the pellets were resuspended in a volume of the same buffer (variable as a function of the desired protein concentration) and used for the binding assay. An aliquot of membrane fraction was used for determining the protein concentration by using the Lowry method (23). This was approximately 2–3 mg/ml.

Cannabinoid Receptor Binding Assay

The measurement of cannabinoid binding sites was performed by using a novel filtration method, based on the procedures described by Bridgen et al. (4) and Houston et al. (17) with slight modifications previously published (3). Two kinds of studies were performed by using this method. In the first, the [^{3}H]CP-55,940 binding was characterized in male rat striatal membranes by studying its kinetics, saturation curve, and binding inhibition by THC (see details in the legend to the corresponding figures). In the second, specific measurements of maximum binding capacity (B_{max}) and affinity (K_d), by using Scatchard analyses, in the different brain areas of males treated with THC or vehicle were done. Assays were always performed in borosilicate tubes silanized with sigmacote (Sigma). The radioactive ligand was [^{3}H]CP-55,940 (104.0 Ci/mmol) purchased from New England Nuclear (Boston, MA). This was used at a range of concentrations of 0.125–2.5 nM (six to seven different concentrations) for the saturation studies and at a fixed concentration of 1 nM for the competition and kinetics experiments. THC, purchased from Sigma, was used as displacer at a concentration of 5 μM. THC solution was prepared the day of the assay by diluting a 10^{-4} M stock solution [THC was dissolved in absolute ethanol and diluted in 5 mg/ml fatty acid-free bovine serum albumin (BSA)]. Both

radioactive ligand and THC were diluted at the above-mentioned concentrations in incubation buffer. This consisted of 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 3 mM $MgCl_2$, and 5 mg/ml BSA. The aliquot of membrane fraction was also diluted in the incubation buffer until a final protein concentration in the incubation volume of 0.2–0.3 mg/ml. The final incubation volume was 0.5 ml for all studies. Incubation was allowed for 60 min at 30°C (different incubation times were used in the association and dissociation studies) and finished by rapid filtration through Whatman GF/C glass fiber filters, presoaked in 1 mg/ml BSA. Filters were washed twice with 5 ml ice-cold Tris-HCl buffer (pH 7.4) containing 1 mg/ml BSA. Radioactivity bound to membranes was determined by liquid scintillation counting. Specific [^{3}H]CP-55,940 binding was calculated as the difference between binding in the presence or absence of THC. Data were analyzed by using a set of computer programs (26): a) Kinetics and saturation curves were analyzed by the iterative procedures KINETIC and EBDA-LIGAND, respectively; and b) competition experiments and Scatchard plots were analyzed by the EBDA program. In all cases, the nonspecific binding was determined by the experimental values and not by the theoretical values estimated by these programs.

Behavioral Testing

Analysis of spontaneous locomotor and exploratory activities and time spent in inactivity was performed by using a modified open-field technique. The method is based on previously published procedures (24,30). The structure consisted of an square (length, 50 × 50 cm; height, 40 cm). The square floor was divided into 25 small squares (length, 10 × 10 cm) using transversal and longitudinal segments. A round hole (diameter, 25 mm) was made in the medium of each small square. The experimental animal was placed in the center and the following activities recorded during a period of 20 min under red light: a) spontaneous locomotor activity, number of sector crossings (a single line-crossing was defined as the rat placing two front paws into an adjacent quadrant); b) spontaneous exploratory activity, the sum of number of rears, number of times in which the animal entries its head into the cleft, and number of times sniffing at the wall, floor, each hole, or fecal boluses; and c) time spent in inactivity. The apparatus was washed with an odoriferous solution after each rat had been tested.

Statistics

Data were assessed by Student' *t*-test.

RESULTS

Validation of the [^{3}H]CP-55,940 Binding Assay in Male Rat Striatal Membranes

Representative association and dissociation experiments of [^{3}H]CP-55,940 binding in male rat striatal membranes are de-

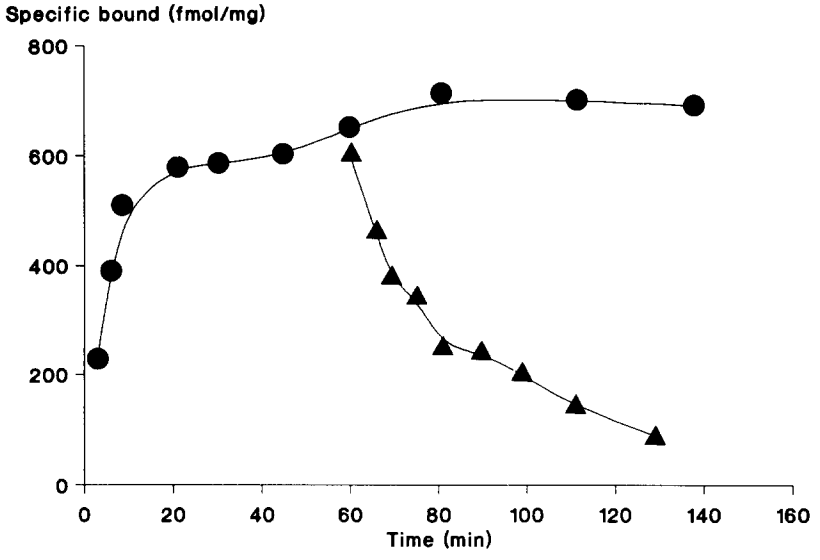


FIG. 1. Association and dissociation kinetics of specific [^{3}H]CP-55,940 binding to membrane preparations from male rat striatum. For the measurement of the association kinetics (-•-), membrane preparations (150 μg protein/tube) were incubated at 30°C in the presence of 1 nM [^{3}H]CP-55,940. The times indicated are those that elapsed between the addition of protein and the start of filtration. Nonspecific binding was defined as the counts in the presence of 5 μM Δ^9-tetrahydrocannabinol (THC). To determine the dissociation rate (-▲-), membrane preparations were incubated at 30°C for 60 min with 1 nM [^{3}H]CP-55,940 to achieve the equilibrium, and the binding was then measured at increasing times following the addition of 500 μl buffer containing THC (5 μM final concentration). The data points are means of triplicate determinations of representative experiments. These experiments were analyzed by the iterative procedure KINETIC (26). Using this algorithm, the associations and dissociations were best fitted to a single binding site model. Assuming a one-site model, the apparent association constant (K_{obs}) was 0.155 ± 0.009 min^{-1} (n = 3) and the dissociation constant (K_{-1}) was 0.029 ± 0.002 min^{-1} (n = 3).

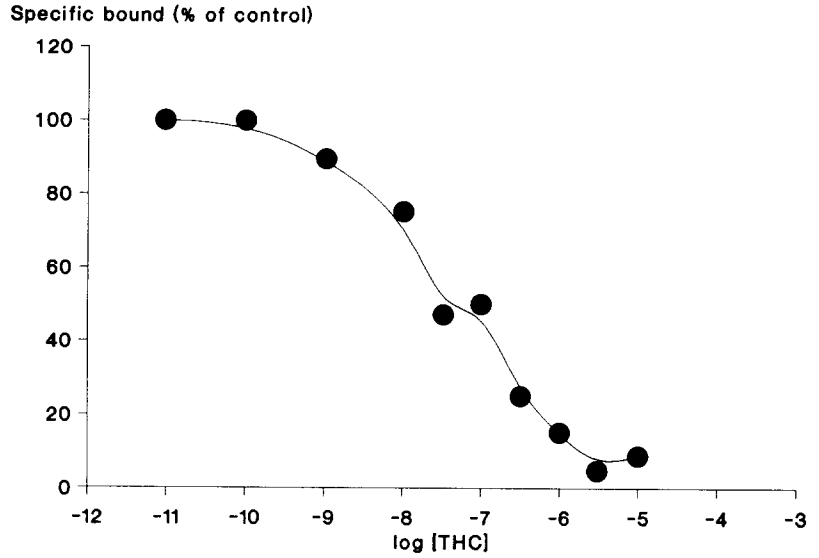


FIG. 2. Competitive inhibition by Δ^9-tetrahydrocannabinol (THC) of specific [^{3}H]CP-55,940 binding to membrane preparations from male rat striatum. Increasing concentrations of THC ranging from 10^{-12} to 5.10^{-5} M with a fixed [^{3}H]CP-55,940 concentration (1 nM) were used. Data points are the means of triplicate determinations from one representative experiment. The ordinate represents binding as a percentage of control (specific binding in the absence of THC). The values of the IC_{50} and K_i from four to five independent experiments, each performed in triplicate, are 40.24 ± 10.70 nM and 16.33 ± 4.92 nM, respectively.

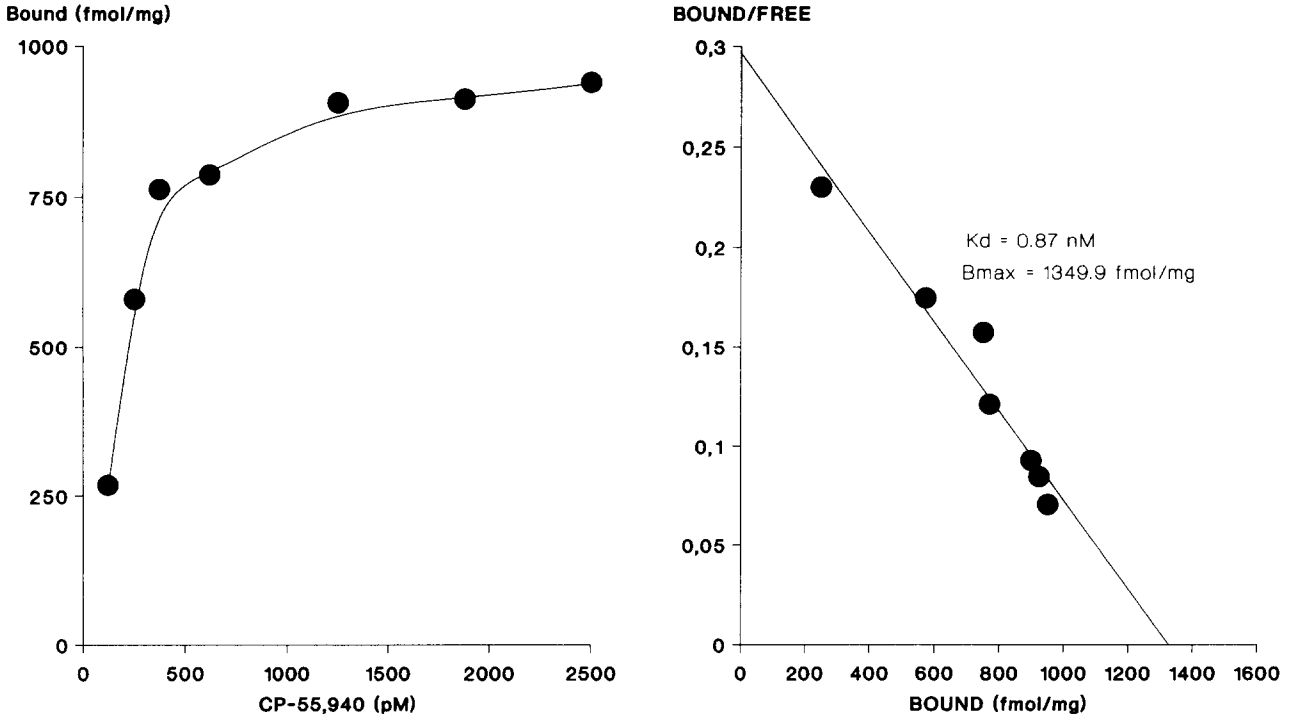


FIG. 3. Representative saturation curve of specific [^{3}H]CP-55,940 binding to membrane preparations from male rat striatum. The data points are the means of duplicate determinations [two tubes for total binding and two tubes for nonspecific counts measured in the presence of 5 μM Δ^9-tetrahydrocannabinol (THC)]. Scatchard transformation is shown beside. Hill transformation displays an n_H close to one (0.987 ± 0.010).

picted in Fig. 1. Association to striatal receptors was rapid. Equilibrium was reached within 30 min at 30°C. Specific binding was stable for at least 2 h, indicating that neither binding sites nor ligand were degraded during the incubation time. Thus, for the competition studies and equilibrium binding that follow the incubation was of 60 min at 30°C. Kinetic analysis yielded a K_{obs} of 0.155 ± 0.009 min^{-1}. The dissociation of [^{3}H]CP-55,940-receptor complex was examined by addition of 5 μM THC after the equilibrium binding was attained (60 min). The half-time displacement value ($T_{1/2}$) was 23.9 min and the dissociation was complete (>90%) after 60 min of THC addition. The dissociation rate constant (K_{-1}) was 0.029 ± 0.002 min^{-1}. The association and dissociation kinetics adjusted to a single binding site model.

Competition studies using increasing concentrations of THC with a fixed amount of [^{3}H]CP-55,940 were also performed in male rat striatal membranes. A representative displacing curve is depicted in Fig. 2. Addition of THC resulted in a substantial decrease in [^{3}H]CP-55,940 binding. IC_{50} and K_i values, obtained from EBDA analysis, were 40.24 ± 10.70 nM and 16.33 ± 4.92 nM, respectively.

Experiments using different concentrations of [^{3}H]CP-55,940 ranging from 0.125 to 2.5 nM revealed evidence of saturable specific binding in male rat striatal membranes. A representative saturation curve and the Scatchard plot obtained therefrom are depicted in Fig. 3. Mean values in this brain area of controls for B_{max} and K_d were 1379.2 ± 144.5 and 0.94 ± 0.16, respectively. Data were also analyzed by the Hill transformation. We observed an n_H close to one (0.987 ± 0.010), which suggests that a single class of binding site is being labeled by the ligand under these assay conditions. A similar validation using association and dissociation experiments, saturation curves, Scatchard plots, and competition studies were also performed in the other brain areas (data not shown). The mean values in controls for B_{max} and K_d were 929.6 ± 72.9 and 0.80 ± 0.08, respectively, in the limbic forebrain, and 487.1 ± 57.6 and 0.84 ± 0.22, respectively, in the ventral mesencephalon.

Cannabinoid Receptor Parameters After Chronic THC Treatment

An acute injection of THC decreased the spontaneous locomotor activity (49.4%) and exploratory behavior (59.7%) (Table 1). These effects also appeared after 7 days of a daily treatment with THC, although the magnitude of changes was significantly smaller (locomotor activity, 39.4%; exploratory activity, 40.4%) (Table 1). Moreover, acute administration of THC increased the time spent in inactivity (181.1%), but this effect disappeared after chronic treatment, although a certain trend to increase could be observed (31.7%) (Table 1). These observations support the existence of a certain tolerance phenomenon. This tolerance was accompanied by significant decreases in the density of cannabinoid receptors in the striatum and limbic forebrain (Fig. 4), the areas where nerve terminals for nigrostriatal and mesolimbic dopaminergic systems, respectively, are located. This downregulation phenomenon was also observed in the ventral mesencephalon (Fig. 4), the area where cell bodies for both dopaminergic neuronal systems are clustered, but the decrease was smaller and nonsignificant. No modifications were seen in the affinity of these receptors by chronic exposure to THC (Fig. 4).

DISCUSSION

The three areas studied presented specific and saturable binding for [^{3}H]CP-55,940, as revealed by the corresponding saturation and dissociation curves, displacement by THC, and Scatchard plots. Specific binding corresponded to a single receptor site as revealed by Hill analysis. [^{3}H]CP-55,940 binding sites characterized in our filtration assay displayed K_d and B_{max} values in the different brain areas that were consistent with those reported in the literature (4,17). These reports present K_d values higher than those described by Devane et al. (6), who used a centrifugation procedure. The binding kinetics accounted for a single binding site as previously reported (6). The binding was saturable and can be displaced by THC with high affinity. However, we found K_i and IC_{50} values for THC displacement lower than those reported by Bridgen et al. (4) and higher than those reported by Devane et al. (6). This discrepancy may due to slight methodological differences, which might be relevant in the management of compounds of extremely hydrophobic characteristics as THC. In this respect, we used fatty acid-free BSA as solubilizer, which has been found to keep the cannabinoid in solution without appreciably affecting binding parameters (6,16).

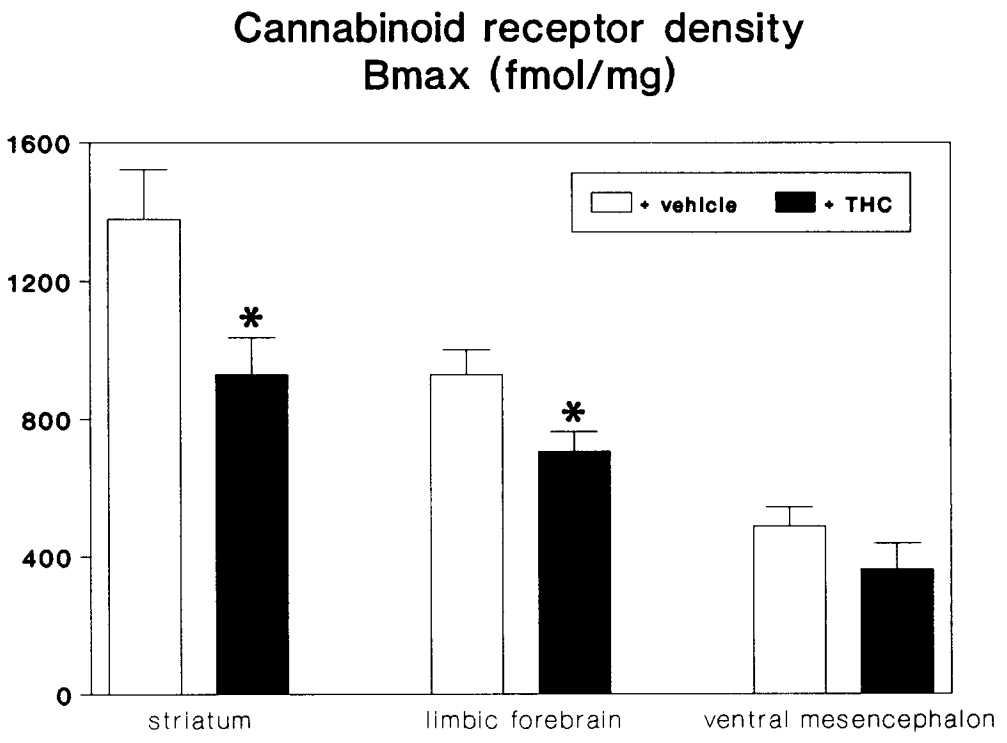
As mentioned in the introductory section, no evidence that brain cannabinoid receptors can be downregulated, as a conse-

TABLE 1

SPONTANEOUS LOCOMOTOR AND EXPLORATORY ACTIVITIES AND TIME SPENT (seconds) IN INACTIVITY IN MALE RATS 30 min AFTER AN ACUTE INJECTION OF THC (6.4 mg/kg BODY WEIGHT), OR VEHICLE, OR AFTER THE LAST INJECTION OF A DAILY CHRONIC TREATMENT WITH THAT THC DOSE

Parameters	Treatment	+ Vehicle	+ THC
Locomotor activity	Acute	172 ± 11	87 ± 18 (49.4%)*
	Chronic	241 ± 26	146 ± 27 (39.4%)†
Exploratory activity	Acute	67 ± 4	27 ± 6 (59.7%)*
	Chronic	94 ± 13	56 ± 11 (40.4%)†
Inactivity	Acute	122 ± 27	343 ± 26 (181.1%)*
	Chronic	186 ± 36	245 ± 37 (31.7%)

These parameters corresponded to a period of 20 min after the beginning of the test. Details in the text. Values are means ± SEM of more than six determinations per group. Percentages of change vs. controls are in parentheses. Statistical differences between THC-injected rats and their corresponding vehicle-treated rats for either the acute or chronic experiment were obtained by Student's t-test (*$p < 0.05$; †$p < 0.005$).



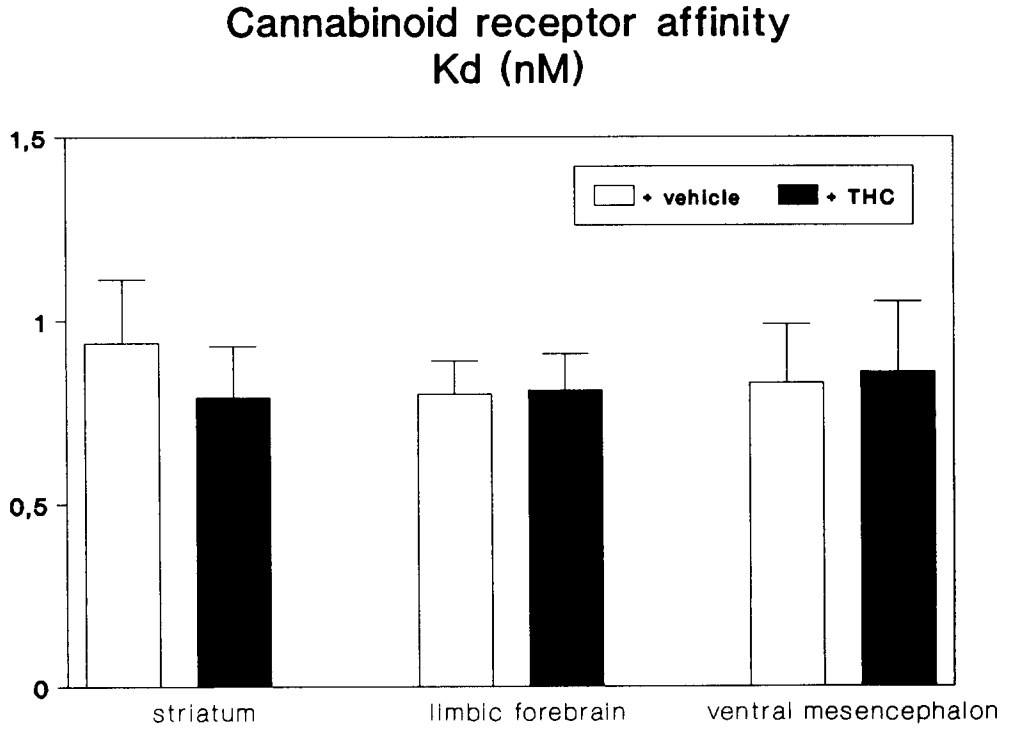


FIG. 4. Maximum binding capacity (B_{max}) and affinity (K_d) of cannabinoid receptors in the striatum, limbic forebrain, and ventral mesencephalon of male rats chronically (7 days) treated with Δ^9-tetrahydrocannabinol (THC) (6.4 mg/kg body weight) or vehicle. Details in the text. Values are means ± SEM of more than six determinations per group. Statistical analysis was assessed by Student's *t*-test (*$p < 0.05$).

quence of the chronic exposure to their natural agonists, has been presented. Solely, there are two recent communications about it: a recent abstract from Herkenham's group (29), but the full details have not been published yet, and a recent report from Howlett's group (35), who found a failure of chronic THC treatment in producing an irreversible alteration of cannabinoid receptors in rat and monkey brains. However, these authors examined cannabinoid receptor density during the period of cannabinoid withdrawal, concretely 2 months after the end of chronic THC exposure in rats, but not during the period of cannabinoid exposure. The existence of a downregulation phenomenon might be expected during the period of chronic cannabinoid treatment, whereas a normalization should be expected 2 months after the end of cannabinoid exposure. In our opinion, this makes difficult to support the view of absence of a downregulation phenomenon, as was properly suggested by these authors (35). In our study, significant reductions in the number of cannabinoid receptors without affecting the affinity were observed in the striatum and limbic forebrain on the seventh day after a daily THC treatment. A similar trend, although nonstatistically significant, was found in the ventral mesencephalon.

These areas displayed a high presence of cannabinoid receptors, as previously reported (15,16), but their main interest resides in the fact that they contain the cell bodies of nigrostriatal and mesolimbic dopaminergic neurons (ventral mesencephalon) or the nerve terminals for both systems (striatum and limbic forebrain, respectively). This does not necessarily imply that cannabinoid receptors must be located on these neurons. They might be present on nondopaminergic neurons but related to dopaminergic nets. Thus, in the case of the striatum cannabinoid receptors seem to be located in GABAergic neurons projecting to substantia nigra (14), which constitute a functional unit with the nigrostriatal dopaminergic pathway.

Anyway, the activity of brain dopaminergic neurons was markedly affected by cannabinoids (see the introductory section) and tolerance phenomena in behavioral processes regulated by these systems have been previously published (8). These have been also observed in the present work because we found a reduction, pronounced in the time spent in inactivity, in the effects caused by chronic THC exposure as compared with the effects observed after an acute treatment. Thus, both spontaneous locomotor activity and exploratory behavior decreased and the time spent in inactivity increased after acute THC, as previously reported (8,28). However, the magnitude of these effects diminished when the THC treatment was repeated daily during 7 days. Even the effect of acute THC increasing the time spent in inactivity disappeared in the chronic situation because there was not any statistical differences between THC- and vehicle-injected animals for chronic experiment. It is likely that this tolerance should be related to the downregulation of cannabinoid receptors observed in the brain areas where reside the neuronal systems involved in the regulation of those behavioral processes.

In summary, chronic treatment with THC produced downregulation of cannabinoid receptors in the striatum and limbic forebrain and a nonsignificant trend in the ventral mesencephalon. This observation might explain the tolerance phenomena observed in the effects of THC on motor and limbic behaviors after chronic exposures.

ACKNOWLEDGEMENT

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