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Chronic cannabinoid administration alters cannabinoid receptor binding in rat brain: a quantitative autoradiographic study

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The active ingredient of marijuana is (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC). Δ^9-THC and other natural and synthetic cannabinoids such as CP-55,940 inhibit spontaneous activity and produce catalepsy in animals in a receptor-mediated fashion. Tolerance develops to the motor effects of Δ^9-THC after repeated administration. To test the hypothesis that tolerance is mediated by changes in cannabinoid receptor binding characteristics, we used quantitative in vitro autoradiography of [^{3}H]CP-55,940 binding to striatal brain sections from rats treated either chronically or acutely with Δ^9-THC, CP-55,940, or the inactive natural cannabinoid cannabidiol. In the chronic condition, rats were given daily i.p. injections of Δ^9-THC (10 mg/kg), cannabidiol (10 mg/kg), or CP-55,940 (1, 3, or 10 mg/kg) for 2 weeks and sacrificed 30 min after the last injection. In the acute condition, animals received a single dose (10 mg/kg) prior to sacrifice. Rats developed tolerance to the inhibitory effects of Δ^9-THC and CP-55,940, assayed in an open field on days 1, 7, and 14. Cannabidiol had no effect on behavior. Densitometry of [^{3}H]CP-55,940 binding to brain sections showed that Δ^9-THC- and CP-55,940-treated animals had homogeneous decreases in binding in all structures measured at the selected striatal level. Cannabidiol had no effect on binding. Analysis of binding parameters showed that alterations in the acute condition were attributed to changes in affinity (K_D), whereas the major changes in the chronic condition were attributed to a lowering of capacity (B_{max}). The effects in the 1, 3, and 10 mg/kg CP-55,940 conditions were dose-dependent and paralleled the behavioral data showing that the animals given the highest dose developed the greatest degree of tolerance. The data suggest that tolerance to cannabinoids results at least in part from agonist-induced receptor down-regulation.

INTRODUCTION

Marijuana (*Cannabis sativa*) is a widely used psychoactive drug, with a history of use dating back thousands of years[1]. In many cultures, marijuana use is heavy and chronic. Tolerance to repeated use has long been suspected, given the fact that experienced users are capable of consuming enormous quantities of the drug with few or no obvious ill effects[12,26,30]. Scores in cognitive tasks, both in human and non-human primate studies, show a paucity of measurable effects associated with chronic use[26,45]. It has been claimed that tolerance to the subjective experience, termed the 'high', does not occur[47], but tolerance to most psychoactive and physiological effects does occur in humans when high doses are administered daily[30].

The principal psychoactive ingredient of marijuana is (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC)[44]. Recently, the receptor to which Δ^9-THC binds to produce its characteristic motor and cognitive effects has been identified, fully characterized, and localized in brain[16,25]. These studies have shown that most well-characterized cannabinoid effects are receptor-mediated, including reductions in spontaneous activity, catalepsy, antinociception, hypothermia, and the marijuana 'high'[25,27,37]. In many animal studies, tolerance to these effects has been reported[11,17,19,34,41,43,54].

The receptor binding assay allows testing of the hypothesis that tolerance to cannabinoids may be receptor-mediated. Chronic cannabinoid administration may lead to compensatory decreases in levels of the cannabinoid receptor (receptor down-regulation), resulting in reduced sensitivity to the drug.

In order to examine this hypothesis, we performed in vitro receptor binding and quantitative autoradiography on slide-mounted brain sections from rats treated

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either acutely or chronically with Δ^9-THC, CP-55,940 (a synthetic cannabinoid compound which has similar effects on behavior as does Δ^9-THC), cannabidiol (a natural cannabinoid which lacks psychoactive properties and which binds with very low affinity to the cannabinoid receptor), or vehicle. The duration of the chronic treatment, once daily for 2 weeks, is consistent with earlier reports of the time-course of tolerance development[17].

MATERIALS AND METHODS

Drug treatments and brain handling

Sixty male Sprague–Dawley rats weighing 230–250 g were separated into 10 treatment groups (6 chronic and 4 acute). In the chronic condition, 6 groups of rats received doses of CP-55,940 (1, 3, or 10 mg/kg), (−)-Δ^9-THC (10 mg/kg), cannabidiol (10 mg/kg), or vehicle injected once daily for 14 days. The acute conditions were CP-55,940 (10 mg/kg), Δ^9-THC (10 mg/kg), cannabidiol (10 mg/kg), or vehicle. In the acute condition, the drugs were administered once, 30 min prior to sacrifice. All drugs were dissolved in vehicle at concentrations allowing delivery of 1 ml/kg. The vehicle used to deliver the drugs i.p. was 5% ethanol, 5% Emulphor, 90% sterile saline[36], except Δ^9-THC which was obtained in ampules at a concentration of 20 mg/ml in ethanol and, therefore, dissolved in an equal volume of Emulphor with no saline. Δ^9-THC and cannabidiol were obtained from the National Institute on Drug Abuse. CP-55,940 was a generous gift from Dr. L. Melvin, Pfizer Inc. Emulphor (EL-620) was from GAF Corp., New York.

An additional 20 rats were used in a replication of a critical feature of the study, namely the dose-dependency aspect. The 4 groups were 1, 3, or 10 mg/kg CP-55,940, and vehicle. Conditions and procedures were identical except that there were only 5 animals in each group, and the study was carried out for 13 days (to conserve drug).

30 min after the last injection for each animal, the rats were sacrificed without anesthesia by rapid decapitation. The brains were removed and briefly frozen in 2-methyl butane at −40°C and then transferred to dry ice. Foil-wrapped brains were stored at −70°C until sectioning. Coronal sections 15 μm-thick were cryostat-cut, thaw-mounted onto gelatin-coated slides, dried briefly at 30°C, and stored with desiccant at −40°C until used.

Behavioral studies

Chronic animals were placed in the center of a circular open field on the first, seventh and fourteenth day, 30 min after dosing (at which time the high-dose acute CP-55,940 animals showed maximal immobility). The open field, measuring 4 ft in diameter, was placed in a moderately lit room distinct from the animal holding area. The open field was divided into 64 equal-area curvilinear boxes. Placement of two forepaws into a box constituted a count. Two observers monitored activity within the open field for 2 min. Acute animals were treated similarly, except they were only tested on the day of injection. On the last day of testing, animals were removed from the open field, taken to another procedure room, and sacrificed. Activity in the open field was analyzed by repeated measures ANOVA with post-hoc analysis by unpaired *t*-test.

Binding assay

[^{3}H](−)-CP-55,940 (specific activity, 79.4 Ci/mmol) was custom radiolabeled at Dupont New England Nuclear and purified[25]. Binding was in cytomailers at 37°C for 2 h in 50 mM Tris-HCl (pH 7.4) containing 5% (w/v) bovine serum albumin (BSA) and 10 nM [^{3}H]CP-55,940. Washing was at 0°C for 4 h in the same buffer containing 1% BSA[25]. Sections were blown dry with room-temperature air.

Binding parameters

Competitive inhibition curves were determined by binding surface analysis using a 1-site competitive model[51,52]. A series of 40 consecutive sections, mounted 2 per slide, was cut from each brain at the striatal level beginning at 1.60 mm rostral to bregma[46]. This level was chosen because structures are relatively invariant across many sections. Binding kinetics were determined by competitively inhibiting 2 concentrations of [^{3}H]CP-55,940 (4 and 20 nM) with 8 different concentrations of unlabeled ligand. For the 4 nM [^{3}H]CP-55,940 concentration, the concentrations of unlabeled CP-55,940 were 1,000, 333.3, 111.1, 37.0, 12.4, 4.1, 1.4, and 0.5 nM. For the 20 nM [^{3}H]CP-55,940 concentration, the concentrations of unlabeled CP-55,940 were 1,500, 500, 166.7, 55.6, 18.5, 6.2, 2.1, and 0.7 nM. Non-specific binding, determined by the addition of 10 μM CP-55,244 (the most potent Pfizer compound), was subtracted from total binding in the absence of displacers to determine total specific binding. Binding was typically 90% specific.

Autoradiography and quantification of binding

Sections were apposed to Hyperfilm-^{3}H (Amersham Corp., Arlington Heights, IL) for 2 weeks. Films were developed (D19, Kodak, Rochester, NY) for 4 min. Image analysis was done on a Macintosh II computer-based densitometry system with solid-state video camera and IMAGE software (Wayne Rasband, NIMH, Bethesda, MD). Anatomical regions appearing on the monitor were traced with mouse cursor control, and light transmittance was determined. For the binding studies, the following structures were analyzed: olfactory tubercle, lateral caudate-putamen, medial caudate-putamen, nucleus accumbens, lateral septum (dorsal and ventral), and the territory comprising the medial septum and vertical and horizontal limbs of the diagonal band[46]. Light transmittance values were determined in 3–5 sections from 5–6 animals for each of the anatomical regions, and converted to fmol/mg wet weight of tissue using a standard curve generated from ^{3}H standards (^{3}H-Microscales, Amersham).

For binding surface studies, light transmittance of each autoradiographic image of the entire section was measured in 2 consecutive sections at each displacer concentration. Light transmittance was converted to fmol/mg wet weight of tissue using the known conversion values of the standards.

In the replication study, slides were scored and broken, and the sections were individually placed in scintillation cocktail and counted for radioactivity (cpm's) the next day in a liquid scintillation counter. Radioactivity was expressed as fmol bound/section.

Analysis of competitive inhibition curves using MLAB

K_D and B_{max} values were determined by binding surface analysis of competitive inhibition curves using a 1-site competitive model[40,51,52] and MLAB software (Civilized Software Inc., Bethesda, MD). MLAB is an iterative curve-fitting program that determines best-fit parameter estimates by minimizing a sum-of-squares value using the Marquardt–Levenberg algorithm. This system has been shown to be a rigorous form of binding kinetics data analysis[51]. The data were fit to the following 1-site equations describing total specific binding (G1S) and binding displacements (GD1)[52]:

$$G1S(L) = (B_{max} \times L)/(L + K_D)$$

$$GD1(L,LL) = G1S(L,LL)/G1S(L)$$

where L = concentration of [^{3}H]CP-55,940 and LL = concentration of CP-55,940. Statistical analysis was done by using the modified F-test[50]:

$$F = [(SS1 - SS2)/(df1 - df2)]/[SS2/df2]$$

where SS1 and SS2 are the sums of squares and df1 and df2 are degrees of freedom. The data of the experimental group were fit to the binding equations

with either the K_D or B_{max} constrained to equal that of the control group. The modified F-test was then used to determine if there was a significant increase in the SS[52].

RESULTS

Behavioral studies

Within 10 min of the injection on the first day of testing, all animals receiving psychoactive cannabinoids showed characteristic inactivity. When placed into the center of the open maze, animals receiving CP-55,940 or Δ^9-THC exhibited splayed hind limbs and immobility. After varying periods of time, some animals eventually ambulated, typically exhibiting horizontal rotational activity. In contrast, animals receiving cannabidiol or vehicle ambulated in a normal fashion. When tested in the open field on day 7, and clearly by the end of the repeated dosing regimen, most of the CP-55,940- and Δ^9-THC-treated animals exhibited normal levels of activity (Fig. 1). ANOVA revealed a significant effect between treatments on activity counts (Experiment I: $F_{5,56} = 3.327$, $P < 0.02$), a lack of significant effect of repeated treatment, but a significant interaction ($P = 0.001$). When only CP-55,940 doses were compared with vehicle, ANOVA indicated a significant effect in the first experiment ($F_{3,40} = 5.773$, $P = 0.0052$) and the replication ($F_{3,32} = 9.618$, $P = 0.0007$), dose-dependent effects of repeated treatment ($P = 0.001$ and $P = 0.0001$, respectively), as well as significant interactions. In both experiments, the animals receiving the highest dose of CP-55,940 tended to show more rapid return to control levels of activity than did the animals receiving the lowest dose, with the middle-dose animals in-between. Post-hoc analysis showed that there was a significant difference between vehicle and all doses of CP-55,940 and Δ^9-THC on day 1, however, only the effects of 1 mg/kg CP-55,940 remained significantly different from vehicle on both the seventh and the last day of the study (Fig. 1).

Binding levels

Acute condition. The film autoradiographs showed that in all structures evaluated, [^{3}H]CP-55,940 binding density was reduced in the animals treated with a single 10 mg/kg dose of CP-55,940 compared to vehicle-injected controls (Fig. 2; Table I). This reduction ranged from 46% of control, in medial septum/diagonal band region, to 60% of control, in lateral caudate-putamen. The Δ^9-THC-treated animals did not show changes in binding levels. The cannabidiol group showed a trend toward increased binding, ranging from 113% of control in medial caudate-putamen to 119% in lateral caudate-putamen.

Chronic condition. The CP-55,940-treated animals showed a decrease in binding compared to vehicle controls in all structures evaluated (Fig. 3; Table I). These changes followed a dose–response relationship: the high-dose animals had the greatest decrease in binding (ranging from 20% of control, in medial septum/diagonal band region, to 33% of control, in medial caudate-putamen), while the low-dose animals had the smallest decrease in binding (ranging from 50% of control, in medial septum/diagonal band, to 72%, in lateral caudate-putamen). The middle-dose group had an intermediate decrease in binding (ranging from 28%, in medial septum/diagonal band, to 50%, in lateral caudate-putamen). The Δ^9-THC group had a decrease in binding ranging from 32%, in medial septum/diagonal band, to 52%, in lateral caudate-puta-

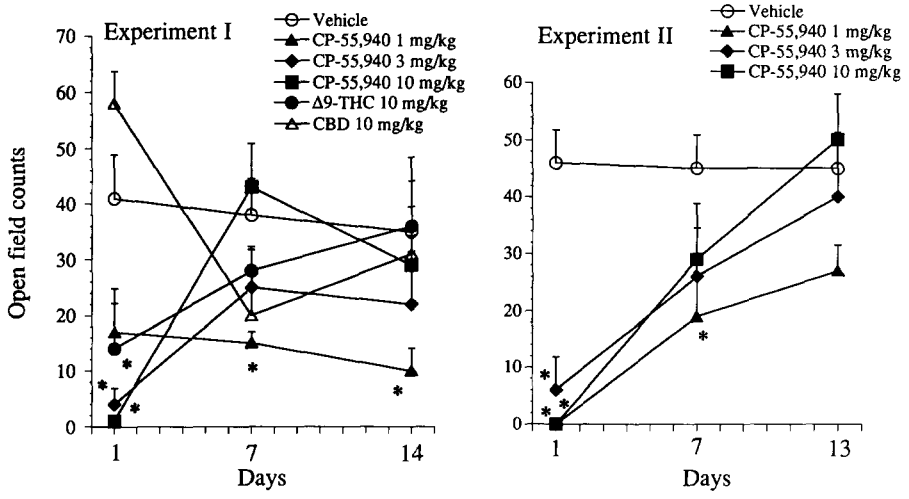

Fig. 1. Activity in the open field during the course of chronic cannabinoid administration (means and S.E.M.; $n = 5$–6 for each group). The dose-dependency relationship of the 3 dose levels of CP-55,940 was replicated in the second experiment, at right. *$P < 0.05$, compared to vehicle.

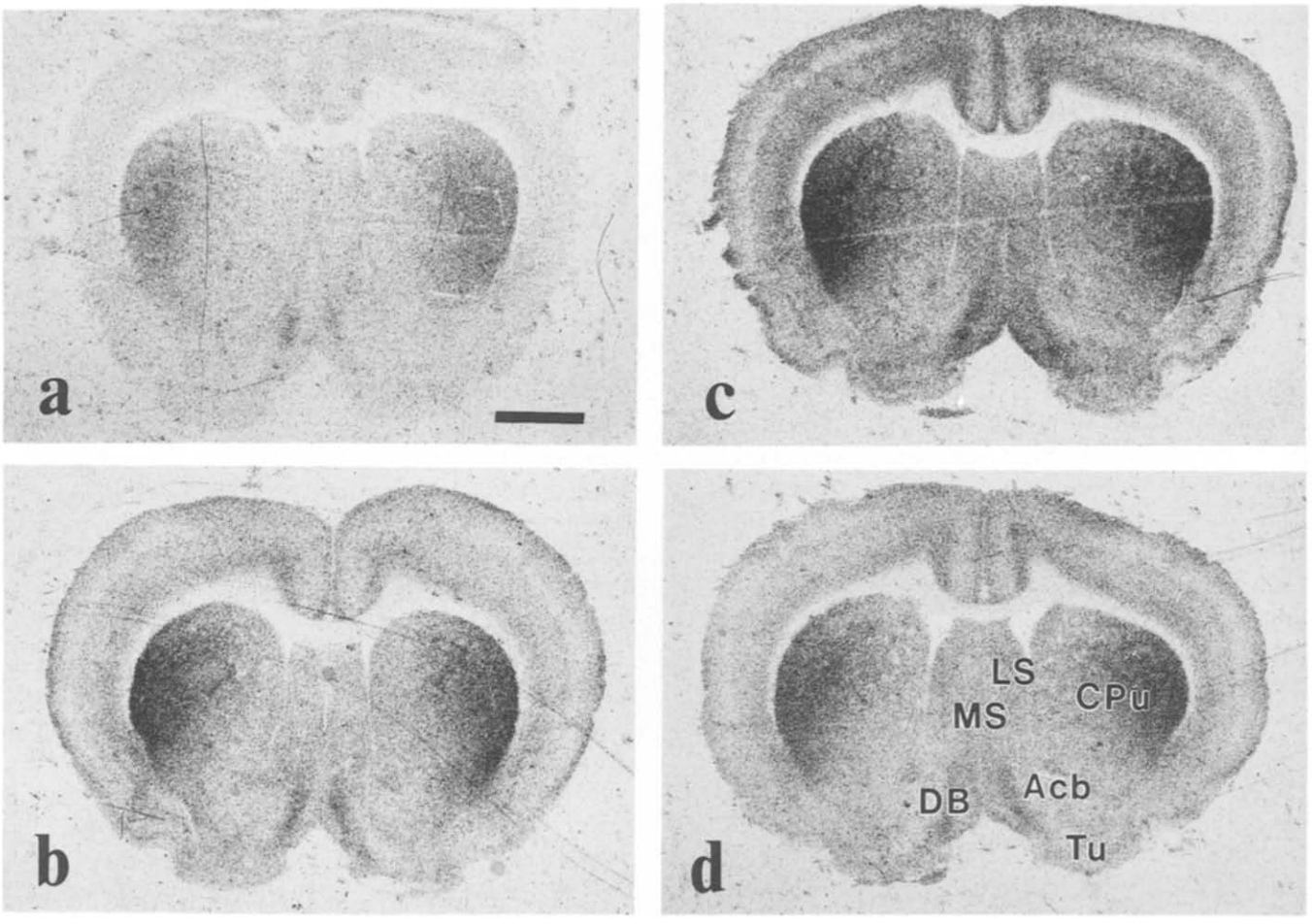

Fig. 2. Acute effects of cannabinoids, seen in autoradiographs of brain sections at the level of the striatum from rats receiving single injections (10 mg/kg i.p.) of CP-55,940 (a), Δ^9-THC (b), cannabidiol (c), or vehicle (d). Bar = 2 mm. Acb, accumbens; CPu, caudate-putamen; DB, diagonal band; LS, lateral septum; MS, medial septum; Tu, olfactory tubercle.

TABLE I

Striatal distribution of [^{3}H]CP 55,940 *binding*

The anatomical distribution of [^{3}H]CP 55,940 binding in different treatment groups. The units are fmol/mg weg weight of tissue. Values are the mean and S.D. The values for each of the drug treatment groups were compared the control groups using one-way ANOVA followed by Student–Newman–Keul's test.

Treatment group	*n*	*Olfactory Tubercle*	*Nucleus accumbens*	*Caudate-plutamen medial*	*Caudate-putamen lateral*	*Lateral septum, dorsal and ventral*	*Medial septum, horizontal and vertical diagonal band*
Acute							
CP 55,940 (10 mg/kg)	6	31.5± 4.5 *	46.5± 5.6 *	76.0± 9.0 *	109.5±12.6 *	58.0± 8.6 *	58.6±11.4 *
10 mg/kg (−)Δ^9-THC	5	51.4±12.7	88.3±14.6	127.1±11.1	189.2± 8.3	104.1±16.0	104.9±24.5
10 mg/kg cannabidiol	6	73.6±10.6	113.0±16.1	151.3±16.0	217.8±16.0	134.3±11.8	148.1±19.7
Vehicle	6	63.8± 6.7	96.4±10.1	133.6±15.6	182.8±21.7	114.1±12.4	126.2±13.1
Chronic							
1 mg/kg CP 55,940	6	40.6± 4.9 *	59.6± 6.7 *	96.2± 7.2 *	143.8± 8.4 *	69.5± 3.9 *	65.9± 7.1 *
3 mg/kg CP 55,940	6	27.4± 9.2 *	36.2± 6.4 *	62.8± 5.7 *	99.6±10.4 *	45.4± 1.8 *	37.7± 6.4 *
10 mg/kg CP55,940	6	20.4± 9.8 *	29.6± 8.9 *	47.5±12.1 *	64.6±15.3 *	30.8± 5.6 *	26.0± 6.0 *
10 mg/kg (−)Δ^9-THC	5	31.9± 3.8 *	44.4± 9.2 *	66.3± 9.5 *	103.7±21.4 *	56.7±10.1 *	41.9± 7.3 *
10 mg/kg cannabidiol	4	70.3± 5.7	101.4±12.4	133.6±15.7	197.7±26.2	124.5±12.6	119.0±22.2
Vehicle	6	65.1±11.3	106.2±15.0	142.1±22.1	200.7±24.4	124.9±13.7	132.8±17.1

* $P < 0.01$.

men. These decreases are comparable to those in the middle-dose CP-55,940 treatment group. The cannabidiol-treated group had unchanged binding levels.

Binding surface analysis

Acute condition. According to binding surface analysis, CP-55,940 lowered the affinity of binding, whereas cannabidiol raised the affinity. The CP-55,940-treated group had an increase in K_D of 207% (Fig. 4, top; Table II). The cannabidiol-treated group had a K_D decrease to 75% of control, and the Δ^9-THC-treated group had no change in K_D. There were no significant B_{max} changes in any of the acute groups.

Chronic condition. Affinity changes were minor, and capacity changes were large and highly significant (Table II). The K_D decreased to 79% of control in the

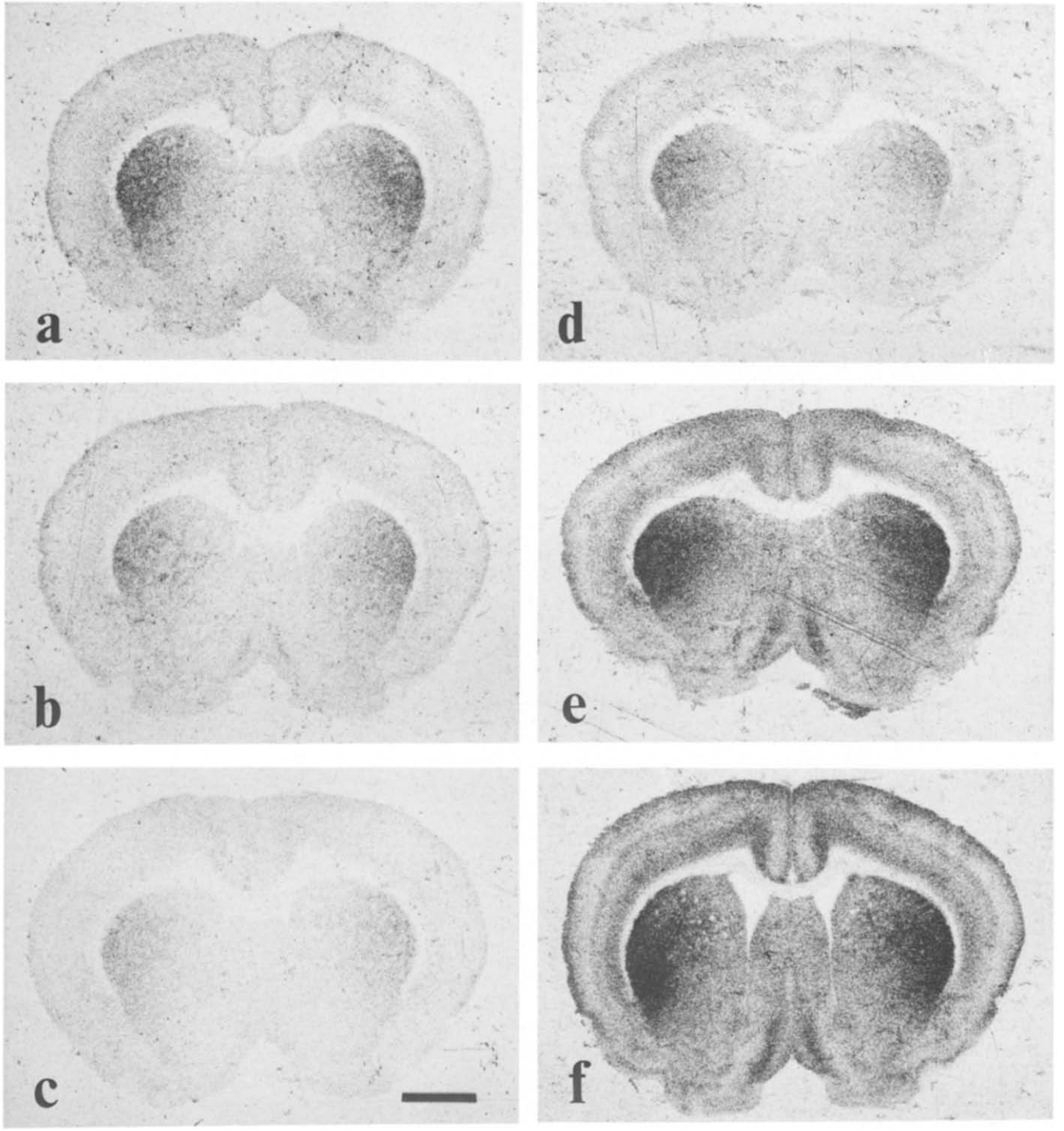

Fig. 3. Chronic effects of cannabinoids seen by autoradiography. Shown are sections from rats receiving repeated injections (for 14 days) of 1 mg/kg CP-55,940 (a), 3 mg/kg CP-55,940 (b), 10 mg/kg CP-55,940 (c), 10 mg/kg Δ^9-THC (d), 10 mg/kg cannabidiol (e), and vehicle (f). Bar = 2 mm.

TABLE II

Kinetic basis of altered cannabinoid receptor binding

K_d and B_{max} values ± S.E.M. are expressed in nM and fmol/mg tissue, respectively. Data and statistical analysis are described in the text.

Group	*n*	K_d	B_{max}
Acute condition			
10 mg/kg CP 55,940	6	46.08 ± 2.01 **	335.97 ± 12.76
10 mg/kg (−)Δ^9-THC	5	20.15 ± 1.7	252.00 ± 12.88
10 mg/kg cannabidiol	6	16.57 ± 0.97 *	292.38 ± 9.59
Vehicle	6	22.23 ± 1.07	307.43 ± 9.27
Chronic condition			
1 mg/kg CP 55,940	6	22.86 ± 1.38	212.68 ± 8.31 **
3 mg/kg CP 55,940	6	22.91 ± 1.96	124.66 ± 6.71 **
10 mg/kg CP 55,940	6	16.79 ± 2.39 *	77.51 ± 6.02 **
10 mg/kg (−)Δ^9-THC	5	28.97 ± 2.16 *	151.27 ± 7.86 **
10 mg/kg cannabidiol	4	20.12 ± 1.42	256.27 ± 10.66
Vehicle	6	21.12 ± 1.32	285.83 ± 11.13

* $P < 0.05$,
** $P < 0.0001$, compared to control.

high dose CP-55,940-treated group (Fig. 4, bottom) and increased to 137% of control in the Δ^9-THC-treated group. There were no other significant changes in K_D. The B_{max} decreased in the CP-55,940- and Δ^9-THC-treated animals but not in the cannabidiol-treated animals. The magnitudes of the B_{max} decreases in the CP-55,940-treated animals were dose-dependent: 27% of control in the high-dose, 44% in the middle-dose, and 74% in the low-dose groups. The B_{max} reduction to 53% of control in the Δ^9-THC group was comparable in size to the middle-dose CP-55,940 group.

Experiment II: Replication of the dose-dependency effect. The dose–response relationships for both open field activity and binding parameters were replicated in the second chronic experiment. The high-, middle-, and low-dose groups had B_{max} values that were 22%, 44%, and 82% of control, respectively (data not shown). All differences were significant ($P < 0.005$).

DISCUSSION

Δ^9-THC and other natural and synthetic cannabinoids including CP-55,940 inhibit activity in mice[33]. The doses of various cannabinoids required to inhibit spontaneous activity and to produce catalepsy[33] correlate highly with their affinity in the in vitro section binding assay[25], indicating that the behavioral inhibition is receptor-mediated.

Tolerance to the motor effects of Δ^9-THC occurs with repeated administrations[4,5,15,22,23,43]. Other behavioral and physiological effects of psychoactive cannabinoids also develop tolerance, such as hypothermia, hypotension, immunosupression, weight loss, and alterations in schedule-controlled behavior[2,3,17]. There is little evidence that chronic administration alters disposition or metabolism of cannabinoids in the brain and periphery[20,38], suggesting that tolerance is pharmacodynamic in nature rather than a consequence of reduced bioavailability of active cannabinoids. The current study replicates the finding of tolerance to the motor effects of Δ^9-THC in rats, and extends this finding to CP-55,940, a synthetic cannabinoid, and suggests a receptor-mediated basis for the tolerance.

In the present study, the initial behavioral consequences of injections of Δ^9-THC and CP-55,940 were quite profound. Most animals were immobile in the open field 10 min following the first injection. Tolerance to the daily injections developed at widely varying rates. Some animals remained mobile in the home cages following the injection on day 3. By the end of 2 weeks of daily injections, all animals showed normal activity after the injections, with the exception of some animals in the low-dose CP-55,940 group who were still hypoactive (Fig. 1).

The dose of Δ^9-THC given (10 mg/kg) is one that is commonly used to show clear inhibitory effects acutely[33,48]. The same dose of cannabidiol, which has a very low affinity at the receptor[25], served as a control for the physicochemical and other non-specific actions of cannabinoids. Predictably, cannabidiol had no effect on behavior. The 3 doses of CP-55,940 used (1, 3, and 10 mg/kg) were actually quite high, given its 28-fold greater affinity for binding[25] and 5–15-fold greater intrinsic efficacy for inhibiting movement than Δ^9-THC[28,33]. As expected, all 3 doses produced immobility on day 1.

Tolerance to the motor effects appeared to develop in a dose-dependent manner: the high-dose animals tended to return to control levels of activity more quickly and to a greater extent over the course of the

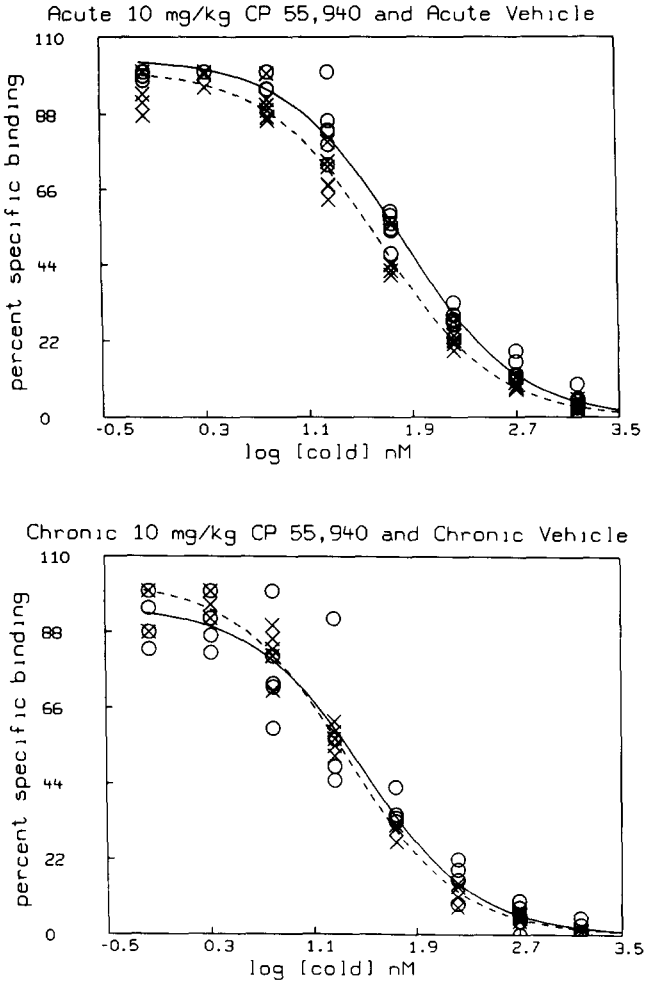

Fig. 4. MLAB-generated displacement curves based on predicted best-fit parameter estimates from the binding surface analysis, superimposed on data collected at the 20 nM [^{3}H]CP-55,940 concentration. Data points are individual determinations from each animal and each concentration of displacer, expressed as percent of specific binding (total minus non-specific) with no displacer. Circles and solid lines represent CP-55,940-treated animals, and ×'s and dashed lines represent vehicle-treated animals. Top is the acute condition and bottom is the chronic 10 mg/kg condition. A decrease in affinity results in a rightward shift of the predicted curve, evident in the acute condition. Based on IC_{50} calculations, there is a 2-fold decrease at top and a 20% decrease at bottom (see Table II for K_D values). Changes in B_{max} (73% reduction in the chronic condition, see Table II) are not apparent because the data are presented as percent of total specific binding for each condition.

14 daily injections than did the low-dose animals. It would seem initially paradoxical that animals receiving the highest doses of cannabinoids would show the greatest and fastest return to normal levels, however, the receptor down-regulation in these animals was so profound that the behavioral correlate may be due to the great loss of functional binding sites. The result has implications for the consequences of chronic high levels of drug use in humans, suggesting diminishing effects with greater levels of consumption.

The behavioral tolerance that developed in each of the drug conditions closely paralleled the binding decrements in these groups. There was no apparent regional selectivity to the decrements, suggesting a lack of involvement of neural circuitry, second messengers, or other intervening variables that might lead to differential effects, however, given the lack of effect of cannabidiol on binding, the reductions do appear to be receptor-mediated. Further support for this conclusion comes from the dose–response relationship observed in the CP-55,940 series.

In the chronic animals, the changes were the result in loss of binding capacity (B_{max}) rather than a change in affinity (K_D). This is an important finding because an alternative explanation for the reduced binding is that 'on-board' drug competes for binding of [^{3}H]CP-55,940 at scarce receptors. Cannabinoids are lipophilic and tend to bind non-specifically to lipids. In addition, they show slow rates of dissociation from the receptor[24]. Competition for binding sites would be reflected as a K_D change. The acute control groups were run to test this possibility. Since both the acutely and chronically treated animals were sacrificed at the same time after the last drug administration (30 min), a similar amount of drug might be pre-bound. The acute Δ^9-THC-treated animals showed no change in binding, ruling out competitive interactions as a possible cause of the binding reduction in the chronic Δ^9-THC group, however, the acute CP-55,940 group did show a 2-fold K_D change (Table II). This reduction in affinity, apparently due to pre-bound competing drug, led us to consider various drug wash-out procedures and mechanistic explanations, given below.

A strategy for eliminating the contribution of pre-bound drug is to preincubate the tissue sections in a buffer containing allosteric effectors that will promote rapid dissociation of the drug from the receptor. Addition of ions or guanine nucleotides may accelerate dissociation by switching the receptor to a low-affinity conformational state. We conducted an exhaustive set of experiments with the intention of dissociating pre-bound drug without compromising affinity, coupling, or viability of the receptor. Necessarily long preincubation in buffer alone, predicted by the slow dissociation rate of CP-55,940[24], reduced binding at parallel rates regardless of presence or level of pre-bound drugs, apparently by degrading the receptor. Inclusion of NaCl did not change the dissociation rate. Preincubation with GTP or its non-hydrolysable analogs, strong allosteric inhibitors of [^{3}H]CP-55,940 binding[24], promotes fast dissociation of pre-bound drug; however, its mechanism seems more consistent with alteration of receptor–effector coupling and a profound and irreversible change in subsequent binding affinity (as well as an unpredicted and hitherto unobserved regional

selectivity which will be discussed in a later publication). Because no preincubation attempted served to dissociate drug from the receptor while preserving its high affinity state, capacity, and normal distribution, we abandoned this approach in the present study.

Relying instead on the validity of the binding surface analysis[51,52], we found that the greatest and most consistent change in binding was the reduction in B_{max} occurring only in the chronic condition and only for the psychoactive cannabinoids Δ^9-THC and CP-55,940 (Table II). The time–course, direction, and selectivity of the effect leads us to propose that the major consequence of daily repeated cannabinoid administration is a reduction in the number of receptors. Whether the change occurs at the level of gene transcription is currently under investigation using in situ hybridization histochemistry of oligonucleotide probes to measure cannabinoid receptor mRNA levels[39].

The phenomenon of agonist-induced receptor down-regulation has been described for many CNS receptor systems. Chronic administration of α- and β-adrenergic agonists[53], dopaminergic agonists[13], and benzodiazepine agonists[55] is reported to induce a decrease in the number of binding sites in rat brain. Chronic administration of opiate agonists, however, does not appear to unequivocally down-regulate opiate receptor binding sites in vivo[8,49], and can up-regulate μ receptor subtypes[9]. Chronic nicotine up-regulates nicotinic receptors[35]. In these studies, the effects are rather small (10–40%) and region-specific. Thus, both the lack of consistent direction and small magnitude of regulation observed in other drug receptor systems stands in stark contrast to the massive and homogeneous changes in cannabinoid receptor levels found in the present study. The magnitude of the present effect, like the striking behavioral tolerance, may stem in part from the fact that, unlike other psychoactive agonist drugs, cannabinoids can be administered in very high doses. It is ironic that the magnitude of both tolerance (complete disappearance of the inhibitory motor effect) and receptor down-regulation (78% loss with high dose CP-55,940) is so large, whereas cannabinoid dependence and withdrawal phenomena are minimal[2,26,45]. This supports the claim that tolerance and dependence are independently mediated in the brain[32].

An interaction in vivo between cannabidiol and Δ^9-THC has been proposed because pretreatment with cannabidiol in [^{3}H]Δ^9-THC-treated mice caused increased levels of radioactivity to be recovered from these animals' brains[29]. Both attenuation[6,7,14,31] and enhancement[31,56] of the effects of Δ^9-THC by cannabidiol have been reported in different species and systems. Although we did not specifically look at these interactions in our studies, some of the data from the acute cannabidiol-treated animals suggest a mechanism by which cannabidiol alters Δ^9-THC effects. On the one hand, the decrease in K_D produced by cannabidiol in the acute condition (Table II) could be explained by its action to bind to and make unavailable sites other than the presently defined cannabinoid receptor, effectively elevating the concentration of [^{3}H]CP-55,940 at the receptor. The high lipophilic nature of cannabinoids suggests that the other sites are lipophilic and may act as high-affinity carriers. On the other hand, the much more potent CP-55,940 at 10 mg/kg binds so tightly to the receptor that it does not dissociate quickly and thus effectively reduces the affinity of [^{3}H]CP-55,940. These effects apparently adapt out in the chronic condition. There are generally no differences in metabolism or disposition of cannabinoids in Δ^9-THC-tolerant animals[18,20,38,42]. One possibility not completely ruled out by the present data is that circulating CP-55,940 accumulates over time at the receptor and, due to its long dissociation time (pseudo-irreversible binding), makes the receptor unavailable for [^{3}H]CP-55,940 binding, resulting in the appearance of down-regulation. Studies employing longer survival times after the last injection are in progress to address this possibility.

In conclusion, we have shown that chronic Δ^9-THC and CP-55,940, but not cannabidiol, produce behavioral tolerance and cannabinoid receptor down-regulation. The reduced number of receptors available for binding may be responsible for the behavioral tolerance. Further evidence for this mechanism could come from time-course studies showing parallel changes at the molecular and behavioral levels. Supporting work has already been done in cultured cells. Continued exposure of neuroblastoma (N18TG2) cell lines to Δ^9-THC produces a desensitization of the regulation of adenylate cyclase by Δ^9-THC[21]. The effect is selective to Δ^9-THC (ruling out changes in second messengers), is time- and dose-dependent, and is reversible, and thus appears to be cannabinoid-receptor mediated. We propose by extension that cannabinoid tolerance in vivo results, in part at least[10], from cannabinoid receptor down-regulation.

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