Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 459 (2003) 139–150
www.elsevier.com/locate/ejphar

The effects of Δ^9-tetrahydrocannabinol physical dependence on brain cannabinoid receptors

Christopher S. Breivogel[a,*], Susan M. Scates[b], Irina O. Beletskaya[b], Olivia B. Lowery[a], Mario D. Aceto[b], Billy R. Martin[b]

[a] *Department of Pharmaceutical Sciences, Campbell University School of Pharmacy, P.O. Box 1090, Buies Creek, NC 27506, USA*
[b] *Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, P.O. Box 980613, Richmond, VA 23298, USA*

Received 27 June 2002; received in revised form 25 November 2002; accepted 29 November 2002

Abstract

The effects of chronic Δ^9-tetrahydrocannabinol on cannabinoid receptor levels and receptor–G-protein coupling were investigated. Male Sprague–Dawley rats were infused continuously with low or high dose regimens of Δ^9-tetrahydrocannabinol or vehicle for 4 days. Following treatment, rats were sacrificed for cannabinoid CB_1 receptor binding analysis or challenged with the cannabinoid CB_1 receptor antagonist, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl (SR141716A). The rats receiving Δ^9-tetrahydrocannabinol exhibited antagonist-precipitated withdrawal signs. Each brain region (cerebellum, cortex, hippocampus and basal ganglia) from high-dose rats showed 30–70% decreases in [^{3}H] (−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxyphenyl)cyclohexanol (WIN55212-2) B_{max} values, indicating receptor down-regulation. Most regions showed decreased WIN55212-2-stimulated [^{35}S]guanosine-5′-*O*-3-thiotriphosphate (GTPγS) binding, indicating desensitization of cannabinoid CB_1 receptors. Additional receptor binding assays in cerebellar membranes showed a significantly greater decrease in agonist than in antagonist B_{max} values, indicating a lower fraction of coupled receptors after treatment. Concentration–effect analysis of five agonists revealed that the treatment resulted in greater decreases in the efficacy of low-efficacy agonists.

Keywords: Cannabinoid CB_1 receptor; G-protein; Physical dependence; Desensitization; Down-regulation

1. Introduction

The principal biologically active constituent in marijuana, Δ^9-tetrahydrocannabinol (Gaoni and Mechoulam, 1964), has been shown to produce its effects via brain cannabinoid receptors. The first cannabinoid receptor (designated cannabinoid CB_1 receptor) was cloned only 12 years ago (Matsuda et al., 1990). The only other known cannabinoid receptor was named the cannabinoid CB_2 receptor (Munro et al., 1993), and is localized on cells of the immune system. Both cannabinoid CB_1 and CB_2 receptors couple primarily to inhibitory G-proteins of the G_i and/or G_o subtypes (for review see Childers and Breivogel, 1998). Receptor activation of G-proteins can be measured using agonist-stimulated binding of the non-hydrolyzable GTP analog, [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GT PγS) (Hilf et al., 1989) to membranes.

Via the activation of G-proteins, cannabinoid CB_1 receptors affect adenylyl cyclase (Glass and Felder, 1997; Howlett, 1984), a variety of K^+ (Deadwyler et al., 1993, 1995a; Mackie et al., 1995; Mu et al., 1999) and Ca^{2+} (Mackie and Hille, 1992; Mackie et al., 1995) currents, and the mitogen-activated protein kinase (MAPK) pathway (Bouaboula et al., 1995). Cannabinoid CB_1 receptors have been shown to activate at least 6 subtype of G_i and G_o proteins, supporting the reports that they effect a wide variety of intracellular signaling systems (Prather et al., 2000). Cannabinoid CB_2 receptors also inhibit adenylyl cyclase and activate MAPK and Krox-24 pathways (Bouaboula et al., 1996), but do not appear to affect ion currents (Felder et al., 1995). In vivo, cannabinoid compounds elicit a characteristic spectrum of behaviors in laboratory animals. These include catalepsy, analgesia, decreases in spontaneous activity and body

* Corresponding author. Tel.: +1-910-893-1702; fax: +1-910-893-1697.
E-mail address: breivogel@mailcenter.campbell.edu (C.S. Breivogel).

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(02)02854-6

temperature, (Adams and Martin, 1996) and disruption of memory (Heyser et al., 1993).

It has been proposed that the cannabinoid CB_1 receptor is the mediator of all of the central nervous system (CNS) actions associated with cannabinoid compounds, since: (1) the structure–activity relationship between behavioral potency and receptor affinity are highly correlated (Compton et al., 1993), (2) the neuroanatomical localization of cannabinoid CB_1 receptors corresponds well with the CNS-mediated effects of cannabinoids (Breivogel and Childers, 1998), and (3) most of the CNS-mediated actions of cannabinoids appear to be reversible by the cannabinoid CB_1 receptor-selective antagonist, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide HCl (SR141716A) (Compton et al., 1996). Since this protein appears to be the primary target of cannabinoids such as Δ^9-tetrahydrocannabinol, it is of interest to determine the effects of long-term Δ^9-tetrahydrocannabinol treatment on cannabinoid CB_1 receptors and its activity in order to gain insight into the molecular mechanisms of cannabinoid tolerance and dependence.

Δ^9-Tetrahydrocannabinol has been shown to produce tolerance to its acute effects in both laboratory animals (Abood et al., 1993; Deadwyler et al., 1995b; Fan et al., 1994) and humans (Hollister, 1986). Moreover, the degree of tolerance induced by higher efficacy agonists like levonantradol and (−)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxyphenyl)cyclohexanol (CP55940) appears to be greater than that induced by Δ^9-tetrahydrocannabinol (Dill and Howlett, 1988; Fan et al., 1994). Since the advent of the cannabinoid receptor antagonist SR141716A, Δ^9-tetrahydrocannabinol has also been shown to produce dependence that can be precipitated by administration of the antagonist to Δ^9-tetrahydrocannabinol-tolerant animals (Aceto et al., 1995; Tsou et al., 1995). More recently, evidence of physical dependence on smoked marijuana has been demonstrated in humans (Haney et al., 1999; Kouri et al., 1999). Chronic administration of cannabinoids such as Δ^9-tetrahydrocannabinol has been shown to reduce the number of cannabinoid binding sites in brain (Breivogel et al., 1998a; Fan et al., 1996; Oviedo et al., 1993; Rodríguez de Fonseca et al., 1994; Romero et al., 1997), and cannabinoid activation of G-proteins (Breivogel et al., 1998a; Corchero et al., 1999; Romero et al., 1997; Sim et al., 1996). There is evidence from cell culture models that cannabinoid CB_1 receptors undergo first desensitization, or uncoupling from G-proteins, then internalization and down-regulation in response to chronic exposure to cannabinoid agonists (Garcia et al., 1998). While this appears to be the case in assays performed in slide-mounted brain slices (where the decrease in cannabinoid activity is greater than then decrease in cannabinoid receptor binding), assays in brain membrane homogenate have failed to find evidence for desensitized cannabinoid CB_1 receptors, since the decrease in receptor density is greater than the decrease in receptor activity (Breivogel et al., 1998a; Fan et al., 1996). One goal of these studies is to examine cannabinoid CB_1 receptor binding more thoroughly in brain membranes to quantify the number of uncoupled receptors both before and after treatment with Δ^9-tetrahydrocannabinol.

In light of the recent evidence for physical dependence on Δ^9-tetrahydrocannabinol or smoked marijuana and the growing interest in the use of marijuana for medical uses, we decided to investigate brain cannabinoid CB_1 receptor molecular alterations associated with physical dependence. The specific objectives of this study were to determine: (1) the relationships between cannabinoid receptor binding and function, and (2) the impact of chronic Δ^9-tetrahydrocannabinol-induced receptor alterations on agonist efficacy and possible implications for function of the endogenous cannabinoid system.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats were obtained from Dominion Laboratories (Dublin, VA). [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) (1250 Ci/mmol), [^{3}H]CP-55940 (120 Ci/mmol) and [^{3}H]WIN55212-2 (50.8–55.0 Ci/mmol) were ordered from New England Nuclear (Boston, MA). Δ^9-Tetrahydrocannabinol, SR141716A and [^{3}H]SR141716A (16.9 Ci/mmol) were provided by the National Institute on Drug Abuse (Rockville, MD). (−)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxyphenyl)cyclohexanol (WIN55212-2) and (*R*)-arachidonic acid propan-1-ol-2-amide (R(+)-methanandamide) were purchased from Research Biochemicals International (Natick, MA). CP55940 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). (−)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxyphenyl)cyclohexanol (HU-210) was a generous gift from Prof. Raphael Mechoulam (Hebrew University, Jerusalem, Israel). Guanosine 5′-diphosphate (GDP) and guanosine-5′-(3-thiotriphosphate) (GTPγS) were purchased from Boehringer Mannheim (New York, NY). Sterile water was acquired commercially (Abbot Labs, N. Chicago, IL). All other reagent grade chemicals and enzymes were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

2.2. Chronic Δ^9-tetrahydrocannabinol infusion and antagonist challenge

Modification of the methodologies previously described (Aceto et al., 1996; Teiger, 1974) was used. Groups of 14 rats were anesthetized with pentobarbital (40 mg/kg, i.p.) and fitted with an i.p. cannula, which passed under the skin from the abdominal area and emerged through the skin at the back of the neck. The cannula then passed through a light-

weight saddle strapped to the back of the rat. Tubing connected the saddle to an infusion pump (Harvard Apparatus, S. Natick, MA) via a rotating flow-through swivel. After cannulation, each rat was placed in its home cage and was allowed to recover for 2–3 days. While recuperating, sterile water was infused continuously at 2.0 ml/day. Food and water were available at all times except during the behavioral observation sessions, and body weight was monitored daily. Animals were randomly assigned to receive either Δ^9-tetrahydrocannabinol or vehicle. Δ^9-Tetrahydrocannabinol was dissolved in 1:1:18 emulphor: ethanol: sterile water (vehicle) daily, and solutions of either Δ^9-tetrahydrocannabinol or vehicle were infused continuously at 0.33 ml/h for 4 days; the starting dose of 12.5 mg/kg/day was doubled each day to a final dose of 100 mg/kg/day. Rats were infused for an additional 25 h with sterile water at 2.0 ml/day, prior to sacrifice for radioligand binding analysis. To express physical dependence, separate groups of rats received the drug treatment followed by an i.p. injection of 10 mg/kg SR141716A or vehicle in place of the decapitation step. This same 4-day infusion (without the 25 h water infusion) and antagonist dose were used to precipitate withdrawal as was done in earlier studies (Aceto et al., 1995, 1996). Prior to SR141716A challenge, the position of each cage was randomly reassigned and the cage number concealed by a laboratory worker so that the observer was blind regarding treatments. The most notable behavioral sign was wet-dog shakes, and was the only sign quantified. There were six or seven animals per group for all experiments. All rats received care according to ''Guide for the Care and Use of Laboratory Animals'', National Research Council, Revised, 1996. These facilities are certified by the American Association for the Accreditation of Laboratory Care, and these studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

2.3. Brain membrane preparation

Cerebellum, frontal cortex, hippocampus and basal ganglia (striatum plus globus pallidus) were dissected from whole brains on ice and frozen at −80 °C until the day of assay. The same brain region from each of 2–6 animals was homogenized separately with a Tissumizer (Tekmar, Cincinnati, OH) in cold membrane buffer (50 mM Tris–HCl pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA) and then centrifuged at 40,000 × *g* for 10 min at 4 °C. Pellets were re-suspended in membrane buffer, and centrifuged again at 40,000 × *g* for 10 min at 4 °C. Pellets from the second centrifugation were homogenized in membrane buffer and preincubated for 10 min at 30 °C in 0.004 U/ml adenosine deaminase (240 U/mg protein, Sigma) to remove endogenous adenosine, and then assayed for protein content before addition to assay tubes in [^{35}S]GTPγS and [^{3}H]WIN55212-2 binding assays.

2.4. Agonist-stimulated [^{35}S]GTPγS binding assays

Assays were conducted at 30 °C for 1 h in membrane buffer that contained 100 mM NaCl including 10–30 μg membrane protein with 0.1% (w/v) bovine serum albumin, 30 μM GDP, 0.10 nM [^{35}S]GTPγS and 0.2–10 nM unlabeled GTPγS in the presence and absence of 10 μM WIN 55212-2 in a final volume of 1 ml. Concentration–effect analyses were performed in the absence of unlabeled GTPγS and the presence of various concentrations of each agonist. Non-specific binding was determined in the absence of agonists and the presence of 30 μM unlabeled GTPγS. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by 3 washes with cold Tris–HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S after overnight extraction of the filters in 4 ml BudgetSolve scintillation fluid (RPI, Mount Prospect, IL).

2.5. [^{3}H]WIN55212-2 binding assays

Assays were conducted at 30 °C for 1 h in membrane buffer including 30–100 μg membrane protein with 0.1% (w/v) bovine serum albumin and 0.05–5 nM [^{3}H]WIN55212-2 in a final volume of 1 ml. Non-specific binding was determined by the inclusion of 1 μM unlabeled SR141716A. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters that had been soaked in 0.1% (w/v) bovine serum albumin. Filters were then washed three times with cold Tris–HCl buffer, pH 7.4, containing 0.05% (w/v) bovine serum albumin. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for ^{3}H after overnight extraction of the filters in 4 ml BudgetSolve scintillation fluid.

2.6. [^{3}H]SR141716A and [^{3}H]CP55940 binding assays

Saturation binding assays were conducted at 30 °C for 1 h in membrane buffer including 20 μg membrane protein with 0.1% (w/v) bovine serum albumin and 0.02–1 nM [^{3}H]SR141716A or [^{3}H]CP55940 in a final volume of 1 ml. Biphasic binding of CP55940 and WIN55212-2 was determined by competition of 0.001–30,000 nM of each compound for the binding of 1 nM [^{3}H]SR141716A under the same conditions used for saturation analyses. Non-specific binding was determined by the inclusion of 5 μM unlabeled SR141716A. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters that had been soaked in 0.5% (w/v) bovine serum albumin. Filters were then washed seven times with cold Tris–HCl buffer, pH 7.4, containing 0.05% (w/v) bovine serum albumin. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for ^{3}H after overnight extraction of the filters in 4 ml Econo Scintisafe 1 (Fisher Scientific) scintillation fluid.

2.7. Data analysis

Behavioral data from the quantified withdrawal sign wet-dog shakes were collated by treatment regimen. Statistical analysis included one-way analysis of variance (ANOVA); post hoc multiple comparisons were made using Newman–Keuls test. Net agonist-stimulated [^{35}S]GTPγS binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values. Specific binding of [^{3}H]WIN55212-2, [^{3}H]SR141716A and [^{3}H]CP55940 was determined by subtracting binding values obtained in the presence (non-specific binding) from those obtained in the absence (total binding) of unlabeled SR141716A. Data analyses of saturation binding curves and agonist concentration–effect curves were conducted by iterative non-linear regression using Prism for Windows (GraphPad Software, San Diego, CA) to obtain K_d, $B_{max,}$ EC_{50}, E_{max} or IC_{50} values. High and low affinity IC_{50} values and the fraction of sites exhibiting each IC_{50} were determined by fitting the data to the equation: $B = 100\% \times (\text{Fraction}1/1 + 10^{[A] - \log\ IC501}) + 100\% \times (1 - \text{Fraction}1/1 + 10^{[A] - \log\ IC502})$, where B is the concentration of [^{3}H]SR141716A bound at agonist concentration [A]. K_i values for WIN55212-2 and CP55940 were calculated according to the equation:

$$K_i = \frac{IC_{50}}{\frac{[L]}{K_d} + 1},$$

where [L] was the concentration of [^{3}H]SR141716A and K_d was that determined by [^{3}H]SR141716A saturation analysis. Differences from control (vehicle) were determined by ANOVA followed by Dunnett's test when there were more than two groups; pairs of groups were compared using Student's *t*-test or Newman–Keuls Multiple Comparison Test. The significance of the correlation was determined by Pearson's test. Data are presented as mean ± S.E.M. obtained from 3–7 animals in experiments performed in duplicate or triplicate. Statistical significance is defined in all cases as $P < 0.05$.

3. Results

Initial experiments indicated that a 4-day infusion of lower doses of Δ^{9}-tetrahydrocannabinol (2.5, 5.0, 10 and 20 mg/kg/day), though previously shown to produce SR141716A-precipitated withdrawal (Aceto et al., 1996), failed to produce many significant changes in [^{3}H]WIN55212-2 or WIN-55212-2-stimulated [^{35}S]GTPγS binding parameters in any of the four brain regions analyzed (data not shown). Moreover, subsequent experiments using a higher dose regimen outlined in Section 2 (12.5 to 100 mg/kg/day) found evidence of residual Δ^{9}-tetrahydrocannabinol in the brain membranes when animals were sacrificed immediately at the end of the infusion period. This residual Δ^{9}-tetrahydrocannabinol confounded the analysis of the binding data (data not shown). Therefore, all experiments outlined below were conducted in rat brain membranes from rats exposed to a 4-day Δ^{9}-tetrahydrocannabinol continuous infusion regimen as outlined in Section 2, which included doses from 12.5 to 100 mg/kg/day or (equal volumes of vehicle) and a subsequent 25 h washout period prior to sacrifice.

3.1. Behavioral assessment of physical dependence on Δ^{9}-tetrahydrocannabinol

Rats receiving 12.5 to 100 mg/kg/day Δ^{9}-tetrahydrocannabinol or vehicle over 4 days were switched to sterile water for the next 25 h, and then challenged with SR141716A (10 mg/kg, i.p.). Animals were observed for 1 h prior to and 1 h after the SR141716A challenge in order to distinguish antagonist-precipitated from spontaneous withdrawal. The results were similar to those published previously (Aceto et al., 1995, 1996). Δ^{9}-Tetrahydrocannabinol-treated animals showed significantly higher numbers of withdrawal signs than vehicle-treated animals (ANOVA $P < 0.0001$), and only following SR141716A challenge ($P < 0.001$ by Newman–Keuls). Control animals executed an average of 0.4 ± 0.4 wet-dog shakes/h prior to challenge and 3.4 ± 1.2 wet-dog shakes/h post-challenge. Δ^{9}-Tetrahydrocannabinol-treated rats performed an average of 6.6 ± 2.6 wet-dog shakes/h prior to challenge and 40 ± 7.7 wet-dog shakes/h post-challenge.

3.2. Saturation analysis of [^{3}H]WIN55212-2 binding

Each of four brain regions from each rat was analyzed for cannabinoid receptor levels using saturation analysis of [^{3}H]WIN55212-2 binding (Table 1). The number of high-affinity [^{3}H]WIN55212-2 binding sites (B_{max} values) was reduced by Δ^{9}-tetrahydrocannabinol treatment by 55% in frontal cortex, 69% in cerebellum (Fig. 1A), 60% in hippocampus and 46% in basal ganglia membranes ($P < 0.005$)

Table 1
[^{3}H]WIN55212-2 binding saturation analysis in brain membranes from control and Δ^{9}-tetrahydrocannabinol-treated rats

	Vehicle (control)		Δ^{9}-Tetrahydrocannabinol	
	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)
Cerebellum	3.9 ± 0.3	1.3 ± 0.1	1.2 ± 0.1[a]	1.2 ± 0.1
Hippocampus	3.9 ± 0.3	1.8 ± 0.1	1.5 ± 0.1[a]	2.0 ± 0.2
Frontal Cortex	2.8 ± 0.2	1.6 ± 0.2	1.3 ± 0.1[a]	1.6 ± 0.2
Basal Ganglia	2.8 ± 0.3	1.7 ± 0.1	1.5 ± 0.2[a]	1.8 ± 0.3

Membranes from cerebellum, hippocampus, frontal cortex and basal ganglia were analyzed for maximal high-affinity binding by [^{3}H]WIN55212-2. Data are presented as pmol of binding sites per mg of membrane protein (B_{max}) and the dissociation constant (K_d) in nM concentration units. Data are mean values ± S.E.M. from 5–7 animals; each assay was performed in triplicate.
[a] $P < 0.005$ by Student's *t*-test.

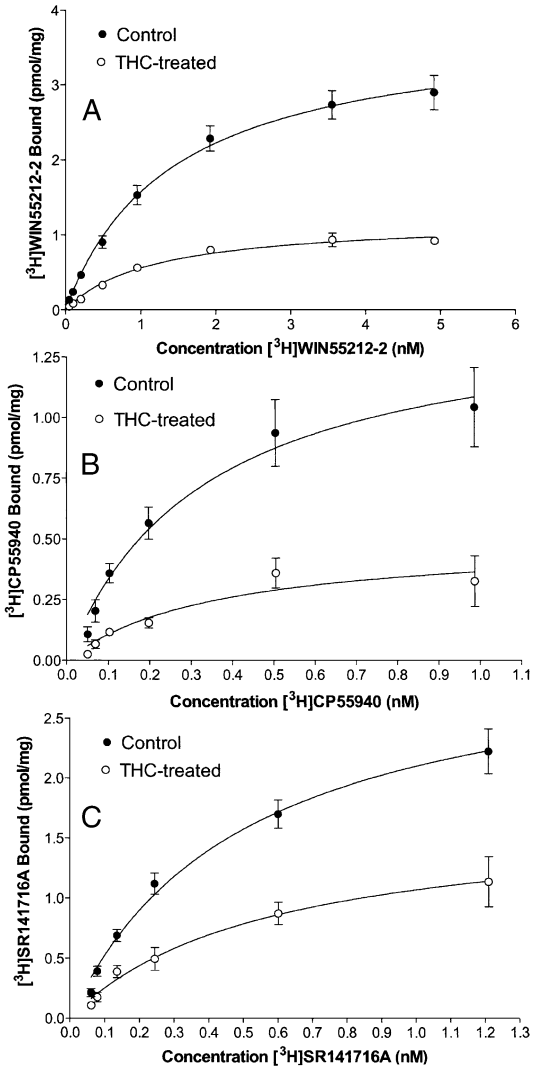

Fig. 1. Saturation analysis of [^{3}H]WIN55212-2 (A), [^{3}H]CP55940 (B) and [^{3}H]SR141716A (C) binding in control and high dose Δ^9-tetrahydrocannabinol-treated rat cerebellar membranes. Assays were performed by incubating membranes with various concentrations of [^{3}H]ligand in the presence and absence of excess (1 or 5 μM) SR141716A. Curve fits are to a one-site model to determine B_{max} and K_d values shown in Table 1 (A) or Table 2 (B and C). Data are means ± S.E.M. from three or four assays performed in triplicate and are expressed as pmol bound/mg membrane protein.

(Table 1). In contrast, there were no significant changes in the K_d values in any brain region ($P>0.05$).

3.3. Saturation analysis of [^{3}H]SR141716A and [^{3}H]CP55940 binding

Since results for each of the four brain regions analyzed by [^{3}H]WIN55212-2 saturation analysis were similar, a single representative brain region, cerebellum, was chosen for more detailed analysis of receptor binding from control and Δ^9-tetrahydrocannabinol-treated rats. High affinity binding by a cannabinoid antagonist, [^{3}H]SR141716A (Fig. 1B), and an additional cannabinoid agonist, [^{3}H]CP55940 (Fig. 1C), were determined by saturation analysis. While there were no effects of Δ^9-tetrahydrocannabinol treatment on K_d values for either ligand, B_{max} values for each ligand were significantly greater in control than in Δ^9-tetrahydrocannabinol-treated membranes (Newman–Keuls test for multiple comparisons $P<0.05$ for each comparison). As determined by [^{3}H]SR141716A, Δ^9-tetrahydrocannabinol treatment decreased the density of binding sites by 47% (from 3.19 to 1.69 pmol/mg) and for [^{3}H]CP55940 the density of binding sites was decreased by 63% (from 1.17 to 0.54 pmol/mg) (Table 2). It is interesting to note that while the B_{max} values for [^{3}H]WIN55212-2 did not match those for the other cannabinoid agonist, [^{3}H]CP55940, in the same groups of animals, the percent change produced by the Δ^9-tetrahydrocannabinol treatment (69% decrease, Table 1) was very similar to the decrease measured by [^{3}H]CP55940 of 63%. In contrast, the decrease measured by the antagonist, [^{3}H]SR147161A, was only 47%.

Additionally, B_{max} values for [^{3}H]SR141716A binding were significantly greater than those for [^{3}H]CP55940 in either control (3.19 versus 1.47 pmol/mg) or Δ^9-tetrahydrocannabinol-treated (1.69 versus 0.54 pmol/mg) membranes (Fig. 1 and Table 2), respectively. This analysis yielded values for the percentage of binding sites that were coupled to G-proteins (percent of total antagonist-binding sites that bound agonist with high affinity) of 46% in control and 32% in Δ^9-tetrahydrocannabinol-treated membranes (Table 2).

Because of the relatively low concentrations of [^{3}H]ligand that can practically be added to an assay, saturation binding experiments only measured the amount of high-affinity antagonist and agonist binding. Presumably antagonist binding should all be high affinity, or in other words, high affinity antagonist binding should measure all receptors present. In order to confirm whether the difference in the changes in receptor density measured by the agonist versus the antagonist were attributable to changes in the proportion of coupled receptors (which bind agonist with high affinity) to uncoupled receptors (which bind agonist with low affinity), further experiments were conducted to determine the relative amounts of high and low-affinity agonist binding.

3.4. Biphasic analysis of agonist binding

At the same time that the cerebellar membranes were assayed in the previous section, they were also assayed for high and low-affinity binding of the unlabeled agonists, CP55940 and WIN55212-2, by competition for [^{3}H]SR141716A binding. Results (shown in Table 3) indicated that each agonist competed for antagonist binding with two affinities based on the large range of concentrations that exhibited displacement ($n_H<1$). The differences in the amount of [^{3}H]SR141716A binding between control and treated membranes in the absence of agonist ("Top" in Table 3) reflected the differences in levels of cannabinoid binding sites determined in the

Table 2
[^{3}H]SR141716A and [^{3}H]CP55940 saturation binding in cerebellar membranes

Treatment parameter/ligand	Vehicle (control)		Δ^9-Tetrahydrocannabinol		
	B_{max} (pmol/mg)	K_d (nM)	B_{max} (pmol/mg)	K_d (nM)	%Control B_{max}
[^{3}H]SR141716A	3.19 ± 0.39	0.51 ± 0.08	1.69 ± 0.32[b]	0.56 ± 0.05	53
[^{3}H]CP55940	1.47 ± 0.26[d]	0.34 ± 0.05	0.54 ± 0.29 [a,c]	0.38 ± 0.14	37
% CB_1 coupled	46		32		

Membranes from the cerebella of control and Δ^9-tetrahydrocannabinol-treated rats were analyzed for saturation of [^{3}H]SR141716A and [^{3}H]CP55940 binding. Data are presented as pmol of binding sites per mg of membrane protein (B_{max}) and the dissociation constant (K_d) in nM concentration units. Data for %Control B_{max} were obtained by dividing the B_{max} value for the Δ^9-tetrahydrocannabinol-treated group by the B_{max} value for the control group for each ligand. Data for %CB_1 coupled refer to the percentage of total cannabinoid CB_1 receptors that are able to bind agonist with high affinity, implying that they are coupled to G-proteins, and were calculated by dividing the B_{max} value for the agonist (which represents coupled sites) by the B_{max} value for the antagonist (which represents the total number of sites) for each treatment group. Data are mean values ± S.E.M. from three animals; each assay was performed in triplicate. There were no effects of Δ^9-tetrahydrocannabinol treatment on ligand K_d values. [a]$P<0.05$, [b]$P<0.01$ versus control B_{max} using the same ligand; [c]$P<0.05$, [d]$P<0.01$ versus [^{3}H]SR141716A B_{max} in the same treatment group. All B_{max} comparisons were made by Newman–Keuls multiple comparison test.

previous sections. Differences in the amount of binding agreed well with the above data, since binding in the Δ^9-tetrahydrocannabinol-treatment group was 55% of control in the CP55940 experiments and 42% of control in the WIN55212-2 experiments ([^{3}H]SR141716A B_{max} values in the Δ^9-tetrahydrocannabinol-treated groups were 53% of control values, see Table 2).

Non-linear fitting of the displacement data indicated the presence of multiple binding sites for each agonist, since in four out of six experiments for each ligand the data fit significantly better to a two-site than a one-site model (F test, $P<0.05$), and, moreover, results of non-linear fitting of data pooled for each ligand and each treatment condition were all significantly better fit to a two-site than a one-site model (F test, $P<0.01$ for each condition). K_i values cited here were calculated from each log IC_{50} value (Table 3) as described in Section 2. CP55940 exhibited K_i values of 0.094 nM and 57 nM in membranes from control and 0.160 nM and 120 nM in membranes from Δ^9-tetrahydrocannabinol-treated animals. WIN55212-2 exhibited K_i values of 24 nM and 22 μM in membranes from control and 22 nM and 5.1 μM in membranes from Δ^9-tetrahydrocannabinol-treated animals. Each ligand exhibited different fractions of high versus low affinity binding; CP55940 displaced 47% of antagonist binding with high affinity in control membranes and 28% of antagonist binding with high affinity in membranes from Δ^9-tetrahydrocannabinol-treated animals, whereas WIN55212-2 displaced 79% of antagonist binding with high affinity in control membranes and 72% of antagonist binding with high affinity in membranes from Δ^9-tetrahydrocannabinol-treated animals. There were no statistically significant differences between control and Δ^9-tetrahydrocannabinol-treated animals when comparing across the same parameter in these experiments (see Table 3), except for differences in the amount of [^{3}H]SR141716A bound in the absence of agonist in control versus Δ^9-tetrahydrocannabinol-treated groups. However, there were noticeable trends towards a decrease in the percentage of sites exhibiting high affinity for agonists between control and Δ^9-tetrahydrocannabinol-treated groups ($P=0.22$ for CP55940 and $P=0.06$ for WIN55212-2).

Table 3
Biphasic analysis of WIN55212-2 and CP55940 displacement of [^{3}H]SR141716A binding in cerebellar membranes

	CP55940		WIN55212-2	
	Vehicle	Δ^9-THC	Vehicle	Δ^9-THC
Top (pmol/mg)	1.75 ± 0.25	0.95 ± 0.17	2.12 ± 0.18	1.10 ± 0.12
Log high-affinity IC_{50}	− 9.5 ± 0.9	− 9.3 ± 0.8	− 7.1 ± 0.1	− 7.2 ± 0.1
Log low-affinity IC_{50}	− 6.7 ± 0.7	− 6.4 ± 0.5	− 4.2 ± 0.1	− 4.8 ± 0.2
% High affinity	47 ± 15	28 ± 29	79 ± 3	72 ± 4

Cerebellar membranes from control and Δ^9-tetrahydrocannabinol-treated animals that were assayed in Table 2 were also assayed for displacement of [^{3}H]SR141716A binding by the cannabinoid agonists, CP55940 and WIN55212-2. Data are presented as the amount of [^{3}H]SR141716A bound in the absence of agonist, the log of the high and low-affinity IC_{50} values obtained for each agonist, and the percentage of total [^{3}H]SR141716A binding sites exhibiting high affinity (% High affinity) for each agonist (the remaining sites exhibited low affinity for each agonist). Data are mean values ± S.E.M. from assays performed in membranes from three animals; each assay was performed in triplicate. Δ^9-THC = Δ^9-tetrahydrocannabinol.

3.5. Net WIN55212-2-stimulated [^{35}S]GTPγS binding saturation analyses

The same four brain regions that were analyzed by [^{3}H]WIN55212-2 saturation analysis, were also analyzed for cannabinoid receptor activity by WIN55212-2-stimulated [^{35}S]GTPγS binding saturation analysis (Fig. 2 and Tables 2 and 3). Saturation analysis of [^{35}S]GTPγS binding induced by agonist measures both apparent K_d and apparent B_{max} values of receptor-activated G-proteins for [^{35}S]GTPγS, indicating the degree to which G-proteins are activated to bind GTP and the number of G-proteins being activated under the current assay conditions, respectively. Treatment with Δ^9-tetrahydrocannabinol increased the net WIN55212-2-stimu-

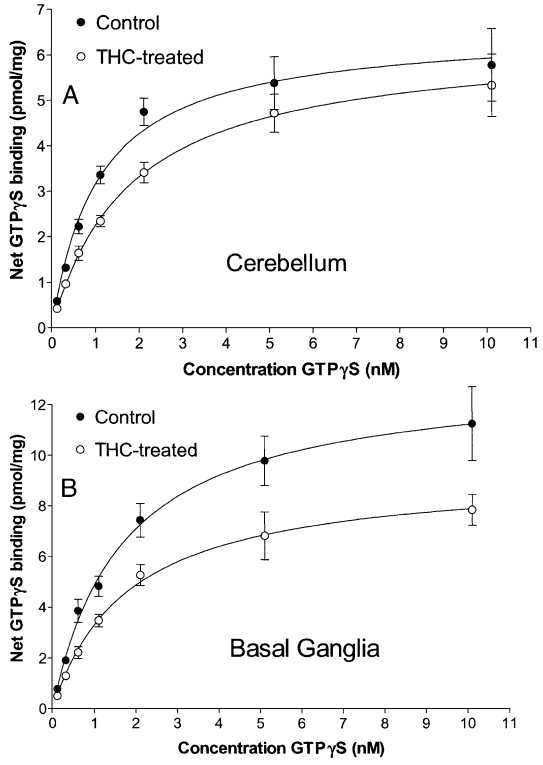

Fig. 2. Net WIN55212-2-stimulated [^{35}S]GTPγS binding in control and high dose Δ^9-tetrahydrocannabinol rat cerebellar (A) and basal ganglia (B) membranes. Assays were performed by incubating membranes with 0.1 nM [^{35}S]GTPγS and various concentrations of unlabeled GTPγS in the presence and absence of 10 μM WIN55212-2. Net agonist-stimulated [^{35}S]GTPγS binding was determined by subtracting values obtained in the presence from those obtained in the absence of WIN55212-2 at each respective concentration of unlabeled GTPγS. Curve fits are to a one-site model to determine apparent B_{max} and apparent K_d values shown in Table 4. Data are means ± S.E.M. from four animals in each group performed in duplicate and are expressed as pmol bound/mg membrane protein.

lated [^{35}S]GTPγS binding apparent K_d value by 60% ($P<0.05$) in hippocampus and decreased the apparent B_{max} value by 25% ($P<0.05$) in hippocampus (Table 4).

As indicated above there were few significant changes in apparent B_{max} or K_d values of agonist-stimulated [^{35}S]GTPγS binding; yet there were significant decreases in [^{3}H]WIN55212-2 binding B_{max} values when comparing control Δ^9-tetrahydrocannabinol-treated samples. However, using a factor that takes the apparent B_{max} and apparent K_d values of WIN55212-2-stimulated [^{35}S]GTPγS binding into account simultaneously, B_{max}/K_d, differences between vehicle and Δ^9-tetrahydrocannabinol treatment groups were revealed (Table 4). The B_{max}/K_d value was decreased in frontal cortex by 30% ($P<0.005$), in cerebellum ($P<0.005$, Fig. 2) and basal ganglia ($P<0.05$) by 40%, and in hippocampus by 50% ($P<0.005$, Table 4).

3.6. Net agonist-stimulated [^{35}S]GTPγS binding concentration effect curves

The previous experiments investigated the effects of a chronic Δ^9-tetrahydrocannabinol treatment on cannabinoid receptors and receptor activity using the full agonist WIN55212-2. In this section, cerebellar membranes were used to determine the effects of this treatment on the concentration–effect parameters of cannabinoid agonists of various efficacies. Using five cannabinoid agonists that represent a wide range of efficacy, agonist concentration–effect curves were generated and analyzed to obtain E_{max} and EC_{50} values (Fig. 3), however, concentrations of CP55940 and HU-210 were too high to accurately determine EC_{50} values for these two ligands, thus these values are omitted from Table 5. Among those agonists for which EC_{50} values were determined (WIN55212-2, methanandamide and Δ^9-tetrahydrocannabinol), there were no differences between EC_{50} values in control and Δ^9-tetrahydrocannabinol-treated tissues; in contrast, the E_{max} values determined for all five agonists were decreased by Δ^9-tetrahydrocannabinol treatment (Table 5). In both control and Δ^9-tetrahydrocannabinol-treated membranes, the rank order of efficacy for the agonists was WIN55212-2>methanandamide ≥ CP55940 ≥ HU-210>Δ^9-tetrahydrocannabinol. However, the efficacy of each ligand relative to WIN55212-2 was lower following chronic Δ^9-tetrahydrocannabinol treatment, and was reduced to the greatest extent for the ligand of lowest

Table 4
Net WIN55212-2-stimulated [^{35}S]GTPγS binding saturation analysis

		Cerebellum	Hippocampus	Frontal cortex	Basal ganglia
Vehicle	B_{max} (pmol/mg)	6.8 ± 0.9	6.1 ± 0.4	8.9 ± 1.2	13.4 ± 1.9
Vehicle	K_d (nM)	1.2 ± 0.2	1.3 ± 0.1	1.9 ± 0.3	1.8 ± 0.3
Δ^9-THC	B_{max} (pmol/mg)	6.5 ± 0.9	4.5 ± 0.3[a]	8.4 ± 0.9	9.6 ± 0.6
Δ^9-THC	K_d (nM)	1.6 ± 0.2	2.1 ± 0.3[a]	2.5 ± 0.4	2.1 ± 0.3
Vehicle	B_{max}/K_d	6.1 ± 0.5	4.5 ± 0.3	4.8 ± 0.3	8.1 ± 0.7
Δ^9-THC	B_{max}/K_d	3.6 ± 0.2[b]	2.3 ± 0.2[b]	3.5 ± 0.2[b]	5.1 ± 0.6[a]

Membranes from cerebellum, hippocampus, frontal cortex and basal ganglia were analyzed for net WIN55212-2-stimulated [^{35}S]GTPγS binding by saturation analysis. Data are presented as pmol of binding sites per mg of membrane protein ([apparent] B_{max}) and the dissociation constant ([apparent] K_d) in nM concentration units. B_{max}/K_d values were determined by dividing the apparent B_{max} value by the apparent K_d value for each sample to yield mean ± S.E.M. Data are mean values ± S.E.M. from 5–7 animals; each assay was performed in triplicate. [a]$P<0.05$, [b]$P<0.005$ by Student's t-test versus control (vehicle) value.

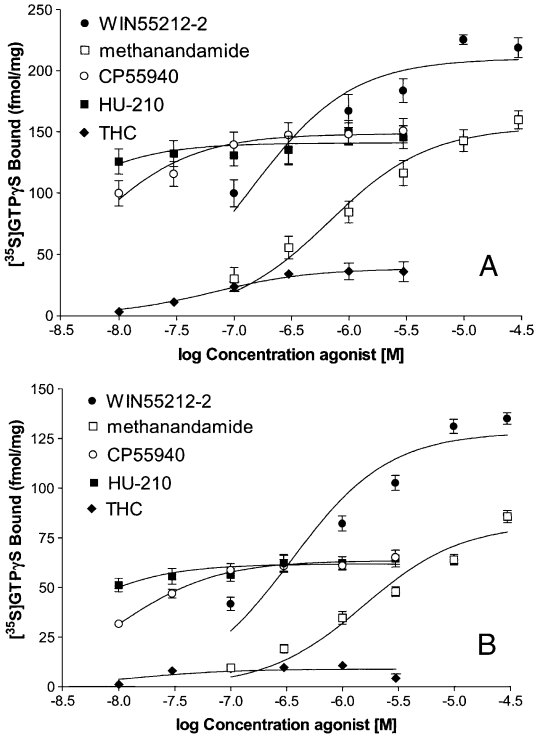

Fig. 3. Cannabinoid stimulation of [^{35}S]GTPγS binding in cerebellar membranes from control (A) and Δ^9-tetrahydrocannabinol-treated rats (B). Net agonist-stimulated [^{35}S]GTPγS binding is shown as fmol of [^{35}S]GTPγS bound/mg membrane protein. Curve fits are to one-site models. Data are mean ± S.E.M. from three animals from each treatment group performed in triplicate.

efficacy (Δ^9-tetrahydrocannabinol) than for the ligands of higher intermediate efficacy (Table 5). In other words, while the E_{max} value for WIN55212-2 in control tissues was decreased by 39%, the E_{max} values for methanandamide, CP55940 and HU-210 were decreased by 46%, 57% and 56%, respectively, and the value for Δ^9-tetrahydrocannabinol was decreased by 73%. Comparison of the E_{max} value of each agonist in control tissue with the %decrease in efficacy following Δ^9-tetrahydrocannabinol treatment yielded a significant inverse correlation (Pearson, $r = 0.958$, $P = 0.010$).

4. Discussion

The aim of the present study was to determine what changes in the brain cannabinoid receptor system accompanied Δ^9-tetrahydrocannabinol-induced physical dependence and whether cannabinoid activation of G-proteins accounted for differences in desensitization to various cannabinoid ligands (Dill and Howlett, 1988; Fan et al., 1994). It was also of interest to determine whether these changes persisted for a period of time sufficient for significant removal of Δ^9-tetrahydrocannabinol from the brain following Δ^9-tetrahydrocannabinol infusion. It has previously been reported by Aceto et al. (1995) that either a medium (2.5–20 mg/kg/day) or high (12.5–100 mg/kg/day) dose regimen of Δ^9-tetrahydrocannabinol produced equivalent antagonist-precipitated withdrawal signs immediately at the end of the infusion. In this study, the low dose regimen (data not shown) produced few significant changes in the brain cannabinoid receptor system. Results from the first high dose regimen indicated possible interference from residual Δ^9-tetrahydrocannabinol in the assay membranes, evidenced by an increase in the [^{3}H]WIN55212-2 binding K_d value in frontal cortex membranes (data not shown). This prompted treatment of additional groups with the high dose regimen and inclusion of a 25-h "wash out" period. The effect of this 25-h delay on the signs of Δ^9-tetrahydrocannabinol physical dependence/withdrawal signs was also of interest. These groups were assayed for in vitro radioligand binding and for cannabinoid behavioral dependence. The objectives for this second treatment group were: (1) to characterize the magnitude of behavioral dependence syndrome 25 h later than it was done in the original studies that characterized Δ^9-tetrahydrocannabinol dependence (Aceto et al., 1995, 1996), (2) to confirm the changes in the cannabinoid system observed in the previous high dose

Table 5
Binding parameters for [^{35}S]GTPγS concentration–effect analysis of several agonists in brain membranes from control and Δ^9-tetrahydrocannabinol-treated rats

Agonist	Vehicle (control)			Δ^9-Tetrahydrocannabinol		
	E_{max} (fmol/mg)	EC_{50} (nM)	%WIN E_{max}	E_{max} (fmol/mg)	EC_{50} (nM)	%WIN E_{max}
WIN55212-2	212 ± 8	18 ± 5	100	128 ± 4[c]	37 ± 4	100
Methanandamide	154 ± 7	80 ± 23	73 ± 3	83 ± 4[c]	169 ± 33	64 ± 2
CP55940	149 ± 13	N.D.	70 ± 4	64 ± 5[c]	N.D.	49 ± 1[a]
HU-210	141 ± 13	N.D.	67 ± 4	62 ± 5[b]	N.D.	48 ± 2[a]
THC	40 ± 8	74 ± 3	19 ± 4	11 ± 1[a]	24 ± 4	9 ± 1[a]

Cerebellar membranes control and Δ^9-tetrahydrocannabinol-treated rats were analyzed for agonist concentration–effect for the stimulation of [^{35}S]GTPγS binding. Data are presented as maximum fmoles of [^{35}S]GTPγS bound per mg of membrane protein (E_{max}) and the concentration of agonist required to produce half of the maximal effect (EC_{50}) in nM concentration units. The column labeled %WIN E_{max} provides the efficacy of each ligand as a percent of the E_{max} of WIN55212-2 for that treatment and shows how the efficacy of each ligand relative to the full agonist changes as a result of the chronic Δ^9-tetrahydrocannabinol treatment. Data are mean values ± S.E.M. from three animals from each group; each assay was performed in triplicate. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.005$ versus respective control by Student's t-test. N.D., not determined.

group under conditions with no indication of interference from residual treatment drug, (3) to extend the findings from the previous studies to include receptor binding data from multiple cannabinoid ligands and, (4) to determine whether Δ^9-tetrahydrocannabinol-induced desensitization of cannabinoid receptors was different for various cannabinoid ligands which display a range of efficacies.

In the set of animals evaluated for Δ^9-tetrahydrocannabinol physical dependence at 25 h after cessation of drug infusion, results were nearly identical to those obtained immediately after cessation of drug infusion reported previously (Aceto et al., 1995). There were approximately 40 wet-dog shakes/h post-SR141716A challenge in the group exposed to Δ^9-tetrahydrocannabinol, and much lower numbers of signs under the other conditions. As also seen previously, there was no sign of spontaneous withdrawal as there was no significant difference between the number of wet-dog shakes in control and Δ^9-tetrahydrocannabinol-treated animals prior to antagonist challenge. These results confirm the previous findings and extend them to demonstrate that cannabinoid physical dependence persists for at least 25 h virtually unchanged following the end of the infusion. Due to the large doses received and the slow elimination rate of Δ^9-tetrahydrocannabinol, it is possible that there are still significant quantities of Δ^9-tetrahydrocannabinol in the brains of these animals after 25 h of wash out. However, Δ^9-tetrahydrocannabinol levels are certainly lower after 25 h, supporting a hypothesis that the dependence syndrome is the result of alterations in the brain cannabinoid receptor system and not merely antagonism of the effects of residual Δ^9-tetrahydrocannabinol.

This study demonstrated that significant alterations in the brain cannabinoid receptor system accompany the behavioral withdrawal syndrome produced by antagonist injection after continuous exposure to Δ^9-tetrahydrocannabinol. The Δ^9-tetrahydrocannabinol infusion decreased binding of [^{3}H]WIN55212-2 by 45–70% in every region, and the binding of [^{3}H]SR141716A by 47% and of [^{3}H]CP55940 by 63% in cerebellum. In contrast, no significant changes in [^{3}H]WIN55212-2 binding were detected following the low dose regimen. In addition, there were decreases in cannabinoid receptor activation of G-proteins in each region, determined either by net agonist-stimulated [^{35}S]GTPγS saturation or concentration–effect analysis using a number of cannabinoid agonists. Results from the high dose regimen in this study were similar to results from a previous study in which [^{3}H]SR141716A binding and WIN55212-2-stimulated [^{35}S]GTPγS binding were examined in the same brain regions following a Δ^9-tetrahydrocannabinol treatment consisting of daily 10 mg/kg i.p. injections for up to 21 days (Breivogel et al., 1998a). The treatment in the present study was of a much shorter duration, but the cumulative doses of Δ^9-tetrahydrocannabinol were similar (187.5 mg/kg in the high dose regimen in the present study and 210 mg/kg in the previous study, while in the low dose regimen of the present study delivered only 37.5 mg/kg). This implies that the cumulative dose may be a more significant determinant in receptor down-regulation than the dose timing, or at least it is possible to achieve the similar levels of desensitization and down-regulation by giving higher doses over a much shorter time period.

An issue not resolved in the previous study was whether significant levels of desensitized of cannabinoid receptors were produced (whether there were cannabinoid receptors present that could bind antagonist, but were unable to couple to G-proteins), since the decreases in [^{35}S]GTPγS binding were smaller than decreases in receptor binding values determined in membranes. In mu opioid receptor expressing SK-N-SH cells and delta opioid receptor expressing NG108-15 cells treated with opioid ligands, it was clear that receptor desensitization occurred prior to and to a greater degree than down-regulation (Breivogel et al., 1997). It is now a widely accepted model that G-protein-coupled receptor uncoupling/desensitization is a prerequisite for receptor internalization and down-regulation in response to chronic agonist exposure.

In an effort to find evidence for this mechanism in cannabinoid receptors in brain, it was noted that decreases in WIN55212-2-stimulated [^{35}S]GTPγS binding were smaller than decreases in cannabinoid antagonist or agonist receptor binding following chronic Δ^9-tetrahydrocannabinol. This differential between [^{35}S]GTPγS and [^{3}H]receptor ligand binding implies at least small degrees of cannabinoid receptor reserve for the activation of G-proteins in each region. In contrast, comparison of [^{3}H]SR141716A and [^{3}H]CP55940 B_{max} values in cerebellum indicated that the percent of receptors that bound agonist with high affinity, and by implication were able to couple G-proteins (determined by agonist B_{max} divided by antagonist B_{max}), was significantly reduced by Δ^9-tetrahydrocannabinol treatment from 46% to 32%. Determination of the fraction of coupled cannabinoid receptors by biphasic analysis of the displacement of [^{3}H]SR141716A binding suggested that Δ^9-tetrahydrocannabinol treatment decreased the percentage of sites that bound agonist (CP55940 and WIN55212-2) with high affinity, but these changes did not achieve statistical significance due to the variability of the values determined from biphasic fitting of the data.

In addition to the decreases in the number of cannabinoid receptors, the Δ^9-tetrahydrocannabinol treatment also produced decreases in the number of WIN55212-2-activated G-proteins. Saturation analysis of [^{35}S]GTPγS binding was used since it measures changes in both the number of G-proteins (apparent agonist-induced B_{max} of GTPγS binding) being activated by receptors and of the degree to which the G-proteins are being activated (apparent agonist-induced K_d of GTPγS binding). These terms are labeled "apparent" since the exact values depend on the assay conditions, including the concentration of GDP, which is necessary to observe agonist activity in this assay (Breivogel et al., 1998b). The effects in the low dose group were minimal: the apparent B_{max} value was different in one brain region. The high dose groups

showed more significant effects with changes in apparent B_{max} or apparent K_d in two brain regions.

However, when the factor B_{max}/K_d was examined, highly significant decreases were demonstrated in every brain region of the high dose group. This term takes into account that either a decrease in apparent B_{max} or an increase in apparent K_d value of agonist-stimulated [^{35}S]GTPγS binding measures decreased activation of G-proteins (Selley et al., 1998), since either would lead to decreased binding of a fixed concentration of GTPγS (or GTP) as is used in the agonist concentration–effect curves. As a demonstration of the appropriateness of assessing this factor, the decrease in WIN55212-2 concentration–effect E_{max} value in cerebellum was 40%, indicating that 40% less of the 0.1 nM [^{35}S]GTPγS was bound in these assays. This decrease could have resulted from either a decrease in agonist-induced GTPγS affinity or number of binding sites, which are indicated by the apparent K_d and B_{max}, respectively, and corresponds to the 40% decrease in B_{max}/K_d measured in these tissues (Table 4).

In a previous study of the effects of once daily injections of 10 mg/kg Δ^9-tetrahydrocannabinol, while there were significant decreases in [^{35}S]GTPγS binding in striatum, hippocampus and cortex (as in the present study), there were no significant effects on [^{35}S]GTPγS binding in cerebellum (though there were in the present study) (Breivogel et al., 1998a). In both studies, there were no significant effects of Δ^9-tetrahydrocannabinol in cerebellum in either apparent B_{max} or apparent K_d, and significant differences in the present study were only apparent when B_{max}/K_d values were calculated (this value was not determined in the previous study). However, it appears that even changes in B_{max}/K_d values would have failed to achieve significance in the previous study. The fact that highly significant decreases in G-protein activation in cerebellum were apparent in the present study may be attributed to differences in the administration protocols. Though the total cumulative doses in both studies were similar, in the present study the entire dose of Δ^9-tetrahydrocannabinol (187.5 mg/kg) was administered over only 4 days, while in the previous study, 210 mg/kg was given over 21 days. Moreover, in the present study, rats received increasing doses, so that on the last day they received 100 mg/kg, while in the previous study each rat received 10 mg/kg each day. This difference would result in significantly higher concentrations of Δ^9-tetrahydrocannabinol at cannabinoid CB_1 receptors in the present study, supporting the concept that the concentration of agonist at the receptor is an important factor in determining the level of receptor desensitization.

In the final set of experiments, the concept that low efficacy agonists would be desensitized to a higher degree than higher efficacy agonists by the chronic Δ^9-tetrahydrocannabinol treatment was tested. As hypothesized, each partial agonist exhibited lower efficacy, as a percentage of stimulation by the full agonist WIN55212-2, in the Δ^9-tetrahydrocannabinol-treated animals than in vehicle-treated animals. The magnitude of the decrease showed significant inversely proportionality to agonist efficacy in untreated tissue. It has previously been reported that cannabinoid agonists of higher efficacy than Δ^9-tetrahydrocannabinol, which demonstrates very low comparable efficacy (Breivogel et al., 1998b; Burkey et al., 1997; Sim et al., 1996), can produce greater degrees of tolerance to cannabinoid ligands. This effect has been observed in in vitro experiments N18TG2 cells (Dill and Howlett, 1988) and in whole animal assays (Fan et al., 1994), in which measured responses exhibited a greater magnitude of tolerance following treatment with either levonantradol or CP55940, respectively, than with Δ^9-tetrahydrocannabinol.

The present study has extended the findings of the previous study by Aceto et al. (1995) by demonstrating that the Δ^9-tetrahydrocannabinol dependence syndrome can be precipitated and is essentially unchanged up to 25 h after the end of the high-dose Δ^9-tetrahydrocannabinol infusion. Since spontaneous withdrawal from Δ^9-tetrahydrocannabinol has not been observed in laboratory rodents, it is not known whether this effect of the antagonist is due to interaction with an altered cannabinoid receptor system or merely blocking the actions of the Δ^9-tetrahydrocannabinol still present in the brain. However, these results support the former hypothesis since the syndrome appears to be of the same magnitude after a 25-h washout period. In any case, the effects of SR141716A appear to reveal an alteration in the endogenous cannabinoid receptor system by chronic Δ^9-tetrahydrocannabinol exposure, since these effects are not seen in animals that did not receive Δ^9-tetrahydrocannabinol. Moreover, these results have demonstrated profound decreases in cannabinoid receptor binding and activity that accompany the Δ^9-tetrahydrocannabinol dependence produced by this treatment, and found evidence that cannabinoid receptors in brain tissue become desensitized as well as being down-regulated, as had been indicated from studies in cell culture models of other G-protein coupled receptors. These alterations varied in magnitude across brain regions, similar to a previous report (Breivogel et al., 1998a), and may be the biochemical basis for the observed tolerance (Fan et al., 1994) and dependence (Aceto et al., 1995; Tsou et al., 1995) behaviors observed following chronic Δ^9-tetrahydrocannabinol exposure.

Acknowledgements

This work was supported by NIDA grant DA 03672 (BRM), contract DA 5-8059 (MDA) and training grant DA 07027 (CSB).

References

Abood, M.E., Sauss, C., Fan, F., Tilton, C.L., Martin, B.R., 1993. Development of behavioral tolerance to Δ^9-THC without alteration of cannabi-

noid receptor binding or mRNA levels in whole brain. Pharmacol. Biochem. Behav. 46, 575–579.

Aceto, M.D., Scates, S.M., Lowe, J.A., Martin, B.R., 1995. Cannabinoid precipitated withdrawal by the selective cannabinoid receptor antagonist SR141716A. Eur. J. Pharmacol. 282, R1–R2.

Aceto, M.D., Scates, S.M., Lowe, J.A., Martin, B.R., 1996. Dependence on Δ^9-tetrahydrocannabinol: studies on precipitated and abrupt withdrawal. J. Pharmacol. Exp. Ther. 278, 1290–1295.

Adams, I.B., Martin, B.R., 1996. Cannabis: pharmacology and toxicology in animals and humans. Addiction 91, 1585–1614.

Bouaboula, M., Poinot-Chazel, C., Bourrié, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., Casellas, P., 1995. Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB_1. Biochem. J. 312, 637–641.

Bouaboula, M., Poinot-Chazel, C., Marchand, J., Canat, X., Bourrié, B., Rinaldi-Carmona, M., Calandra, B., Le Fur, G., Casellas, P., 1996. Signaling pathway associated with stimulation of CB_2 peripheral cannabinoid receptor: involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. Eur. J. Biochem. 237, 704–711.

Breivogel, C.S., Childers, S.R., 1998. The functional neuroanatomy of brain cannabinoid receptors. Neurobiol. Dis. 5, 417–431.

Breivogel, C.S., Selley, D.E., Childers, S.R., 1997. Acute and chronic effects of opioids on delta and mu receptor activation of G-proteins in NG108-15 and SK-N-SH cell membranes. J. Neurochem. 68, 1462–1472.

Breivogel, C.S., Childer, S.R., Deadwyler, S.A., Hampson, R.E., Vogt, L.J., Sim-Selley, L.J., 1998a. Chronic Δ^9-tetrahydrocannabinol produces a time-dependent loss of cannabinoid receptors and cannabinoid receptor-activated G-proteins in rat brain. J. Neurochem. 73, 2447–2459.

Breivogel, C.S., Selley, D.E., Childers, S.R., 1998b. Cannabinoid receptor agonist efficacy for stimulating [^{35}S]GTPγS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. J. Biol. Chem. 273, 16865–16873.

Burkey, T.H., Quock, R.M., Consroe, P., Roeske, W.R., Yamamura, H.I., 1997. Δ^9-Tetrahydrocannabinol is a partial agonist of cannabinoid receptors in mouse brain. Eur. J. Pharmacol. 323, R3–R4.

Childers, S.R., Breivogel, C.S., 1998. Cannabis and endogenous cannabinoid systems. Drug Alcohol Depend. 51, 173–187.

Compton, D.R., Rice, K.C., DeCosta, B.R., Razdan, R.K., Melvin, L.S., Johnson, M.R., Martin, B.R., 1993. Cannabinoid structure–activity relationships: correlation of receptor binding and in vivo activities. J. Pharmacol. Exp. Ther. 265, 218–226.

Compton, D.R., Aceto, M.D., Lowe, J., Martin, B.R., 1996. In vivo characterization of a specific cannabinoid receptor antagonist (SR141716A): inhibition of delta 9-tetrahydrocannabinol-induced responses and apparent agonist activity. J. Pharmacol. Exp. Ther. 277, 586–594.

Corchero, J., Romero, J., Berrendero, F., Fernandez-Ruiz, J., Ramos, J.A., Fuentes, J.A., Manzanares, J., 1999. Time-dependent differences of repeated administration with Δ^9-tetrahydrocannabinol in proenkephalin and cannabinoid receptor gene expression and G-protein activation by μ-opioid and CB_1-cannabinoid receptors in the caudate–putamen. Mol. Brain Res. 67, 148–157.

Deadwyler, S.A., Hampson, R.E., Bennett, B.A., Edwards, T.A., Mu, J., Pacheco, M.A., Ward, S.J., Childers, S.R., 1993. Cannabinoids modulate potassium current in cultured hippocampal neurons. Recept. Channels 1, 121–134.

Deadwyler, S.A., Hampson, R.E., Mu, J., Whyte, A., Childers, S.R., 1995a. Cannabinoids modulate voltage-sensitive potassium A-current in hippocampal neurons via a cAMP-dependent process. J. Pharmacol. Exp. Ther. 273, 734–743.

Deadwyler, S.A., Heyser, C.J., Hampson, R.E., 1995b. Complete adaptation to the memory disruptive effects of delta-9-THC following 35 days of exposure. Neurosci. Res. Commun. 17, 9–18.

Dill, J.A., Howlett, A.C., 1988. Regulation of adenylate cyclase by chronic exposure to cannabimimetic drugs. J. Pharmacol. Exp. Ther. 244, 1157–1163.

Fan, F., Compton, D.R., Ward, S., Melvin, L., Martin, B.R., 1994. Development of cross-tolerance between Δ^9-tetrahydrocannabinol, CP55,940 and WIN55,212. J. Pharmacol. Exp. Ther. 271, 1383–1390.

Fan, F., Tao, Q., Abood, M.E., Martin, B.R., 1996. Cannabinoid receptor down-regulation without alteration of the inhibitory effect of CP55,940 on adenylyl cyclase in the cerebellum of CP55,940-tolerant mice. Brain Res. 706, 13–20.

Felder, C.C., Joyce, K.E., Briley, E.M., Mansouri, J., Mackie, K., Blond, O., Lai, Y., Ma, A.L., Mitchell, R.L., 1995. Comparison of the pharmacology and signal transduction of the human cannabinoid CB_1 and CB_2 receptors. Mol. Pharmacol. 48, 443–450.

Gaoni, Y., Mechoulam, R., 1964. Isolation, structure, and partial synthesis of an active constituent of hashish. J. Am. Chem. Soc. 86, 1646–1647.

Garcia, D.E., Brown, S., Hille, B., Mackie, K., 1998. Protein kinase C disrupts cannabinoid actions by phosphorylation of the CB_1 cannabinoid receptor. J. Neurosci. 18, 2834–2841.

Glass, M., Felder, C.C., 1997. Concurrent stimulation of cannabinoid CB_1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a G_s linkage to the CB_1 receptor. J. Neurosci. 17, 5327–5333.

Haney, M., Ward, A.S., Comer, S.D., Foltin, R.W., Fischman, M.W., 1999. Abstinence symptoms following smoked marijuana. Psychopharmacology 141, 395–404.

Heyser, C.J., Hampson, R.E., Deadwyler, S.A., 1993. Effects of delta-9-tetrahydrocannabinol on delayed match to sample performance in rats: alterations in short-term memory associated with changes in task specific firing of hippocampal cells. J. Pharmacol. Exp. Ther. 264, 294–307.

Hilf, G., Gierschik, P., Jakobs, K.H., 1989. Muscarinic acetylcholine receptor-stimulated binding of guanosine 5′-*O*-(3-thiotriphosphate) to guanine–nucleotide-binding proteins in cardiac membranes. Eur. J. Biochem. 186, 725–731.

Hollister, L.E., 1986. Health aspects of cannabis. Pharmacol. Rev. 38, 1–20.

Howlett, A.C., 1984. Inhibition of neuroblastoma adenylyl cyclase by cannabinoid and nantradol compounds. Life Sci. 35, 1803–1810.

Kouri, E.M., Pope, H.G., Lukas, S.E., 1999. Changes in aggressive behavior during withdrawal from long-term marijuana. Psychopharmacology 143, 302–308.

Mackie, K., Hille, B., 1992. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proc. Natl. Acad. Sci. U. S. A. 89, 3825–3829.

Mackie, K., Lai, Y., Westenbroek, R., Mitchell, R., 1995. Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. J. Neurosci. 15, 6552–6561.

Matsuda, L.A., Lolait, S.J., Brownstein, M.J., Young, A.L., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346, 561–564.

Mu, J., Zhuang, S.Y., Kirby, M.T., Hampson, R.E., Deadwyler, S.A., 1999. Cannabinoid receptors differentially modulate potassium A and D currents in hippocampal neurons in culture. J. Pharmacol. Exp. Ther. 291, 893–902.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365, 61–65.

Oviedo, A., Glowa, J., Herkenham, M., 1993. Chronic cannabinoid administration alters cannabinoid receptor binding in rat brain: a quantitative autoradiographic study. Brain Res. 616, 293–302.

Prather, P.L., Martin, N.A., Breivogel, C.S., Childers, S.R., 2000. Activation of cannabinoid receptors in rat brain by WIN55212-2 produces coupling to multiple G protein α-subunits with different potencies. Mol. Pharmacol. 57, 1000–1010.

Rodríguez de Fonseca, F., Gorriti, M.A., Fernandez-Ruiz, J.J., Palomo, T., Ramos, J.A., 1994. Downregulation of rat brain cannabinoid binding sites after chronic Δ^9-tetrahydrocannabinol treatment. Pharmacol. Biochem. Behav. 47, 33–40.

Romero, J., Garcia-Palomero, E., Castro, J.G., Garcia-Gil, L., Ramos, J.A., Fernandez-Ruiz, J.J., 1997. Effects of chronic exposure to Δ^9-tetrahy-

drocannabinol on cannabinoid receptor binding and mRNA levels in several rat brain regions. Mol. Brain Res. 46, 100–108.

Selley, D.E., Liu, Q., Childers, S.R., 1998. Signal transduction correlates of mu opioid agonist intrinsic efficacy: receptor-stimulated [^{35}S]GTPγS binding in mMOR-CHO cells and rat thalamus. J. Pharmacol. Exp. Ther. 285, 496–505.

Sim, L.J., Hampson, R.E., Deadwyler, S.A., Childers, S.R., 1996. Effects of chronic treatment with Δ^9-tetrahydrocannabinol on cannabinoid-stimulated [^{35}S]GTPγS autoradiography in rat brain. J. Neurosci. 16, 8057–8066.

Teiger, D.G., 1974. Induction of physical dependence on morphine, codeine and meperidine in the rat by continuous infusion. J. Pharmacol. Exp. Ther. 190, 408–415.

Tsou, K., Patrick, S.L., Walker, J.M., 1995. Physical withdrawal in rats tolerant to Δ^9-tetrahydrocannabinol precipitated by a cannabinoid receptor antagonist. Eur. J. Pharmacol. 280, R13–R15.