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Dose-Related Differences in the Regional Pattern of Cannabinoid Receptor Adaptation and in Vivo Tolerance Development to Δ^9-Tetrahydrocannabinol

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Abstract

Chronic treatment with Δ^9-tetrahydrocannabinol (THC) produces tolerance to cannabinoid-mediated behaviors and region-specific adaptation of brain cannabinoid receptors. However, the relationship between receptor adaptation and tolerance is not well understood, and the dose-response relationship of THC-induced cannabinoid receptor adaptation is unknown. This study assessed cannabinoid receptor function in the brain and cannabinoid-mediated behaviors after chronic treatment with different dosing regimens of THC. Mice were treated twice per day for 6.5 days with the following: vehicle, 10 mg/kg THC, or escalating doses of 10 to 20 to 30 or 10 to 30 to 60 mg/kg THC. Tolerance to cannabinoid-mediated locomotor inhibition, ring immobility, antinociception, and hypothermia was produced by both ramping THC-dose paradigms. Administration of 10 mg/kg THC produced less tolerance development, the magnitude of which depended upon the particular behavior. Decreases in cannabinoid-mediated G-protein activation, which varied with treatment dose and region, were observed in autoradiographic and membrane guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS)-binding assays in brains from THC-treated mice. Agonist-stimulated [^{35}S]GTPγS binding was reduced in the hippocampus, cingulate cortex, periaqueductal gray, and cerebellum after all treatments. Decreased agonist-stimulated [^{35}S]GTPγS binding in the caudate-putamen, nucleus accumbens, and preoptic area occurred only after administration of 10 to 30 to 60 mg/kg THC, and no change was found in the globus pallidus or entopeduncular nucleus after any treatment. Changes in the CB_1 receptor B_{max} values also varied by region, with hippocampus and cerebellum showing reductions after all treatments and striatum/globus pallidus showing effects only at higher dosing regimens. These results reveal that tolerance and CB_1 receptor adaptation exhibit similar dose-dependent development, and they are consistent with previous studies demonstrating less cannabinoid receptor adaptation in striatal circuits.

Cannabinoids are used for their psychoactive effects and for therapeutic treatment of nausea/emesis and cachexia. Previous studies also suggest that cannabinoids may have clinical potential for the treatment of pain and degenerative disorders (Piomelli et al., 2000; van der Stelt and Di Marzo, 2003). Acute administration of cannabinoids produces antinociception, locomotor inhibition, hypothermia, and impairment of short-term memory (Howlett et al., 2002). Δ^9-Tetrahydrocannabinol (THC) and other cannabinoids produce their psychoactive and behavioral effects via activation of CB_1 receptors in the central nervous system (CNS) (Ledent et al., 1999). Recent reports indicate that CB_2 and novel cannabinoid receptors might

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exist in the CNS (Mackie and Stella, 2006), but their role is unclear. CB_1 receptors are widely distributed in the brain where they exhibit a predominantly presynaptic location and modulate neurotransmitter release (Schlicker and Kathmann, 2001). High levels of CB_1 receptors are found in the hippocampus, cerebellum, and basal ganglia (Herkenham et al., 1991), consistent with effects on memory and motor function, respectively. Low to moderate levels of CB_1 receptors are distributed in the hypothalamus and periaqueductal gray (PAG) (Herkenham et al., 1991), consistent with effects on temperature, feeding, and pain. CB_1 receptors are also found in the mesocorticolimbic pathway, which probably contributes to the reinforcing effects of cannabinoids and modulation of reinforcement produced by other psychoactive drugs (van der Stelt and Di Marzo, 2003; Le Foll and Goldberg, 2005). The intracellular effects of CB_1 receptor activation are produced primarily via activation of inhibitory G-proteins, resulting in inhibition of adenylyl cyclase, activation of A-type and inwardly rectifying K^+ channels, and inhibition of N- and P/Q-type Ca^{2+} channels (Howlett et al., 2002).

Preclinical studies have shown that tolerance to cannabinoid-mediated behaviors developed after repeated cannabinoid administration (Carlini, 1968; McMillan et al., 1971). Tolerance to cannabinoid-mediated effects was also found in humans after chronic marijuana use (Jones et al., 1976, 1981). It is of interest that the characteristics of tolerance can vary with regard to time and magnitude in a behavior-specific manner. For example, tolerance to cannabinoid-mediated hypoactivity developed more slowly than tolerance to certain other effects, such as hypothermia (Dewey, 1986; Whitlow et al., 2003). Tolerance to cannabinoid-mediated effects in operant tests also developed more slowly than to hypothermia or analgesia (De Vry et al., 2004). The rate of recovery of tolerance also varied by behavior, with tolerance to cannabinoid-mediated hypomotility disappearing more quickly than for antinociception (Bass and Martin, 2000). Differences in the development of tolerance to cannabinoid-mediated effects have also been reported in humans (Jones et al., 1981; Haney et al., 1999; Hart et al., 2002). Comparison of THC-mediated effects in frequent versus infrequent marijuana users revealed differential tolerance to various subjective measures (e.g., greater tolerance to sedation than “high”) and less tolerance to physiological and psychomotor effects compared with subjective effects (Jones et al., 1976, 1981; Kirk and de Wit, 1999; Hart et al., 2002). Although it is difficult to compare clinical studies, it is clear that greater tolerance developed after administration of higher doses of THC and longer treatment duration (Jones et al., 1976, 1981; Hart et al., 2002). However, the mechanistic basis for these observations is not known.

Repeated cannabinoid administration produces alterations in cannabinoid receptors that include receptor down-regulation and desensitization of receptor-mediated G-protein activation and second messenger effects (reviewed by Sim-Selley, 2003). In fact, down-regulation of CB_1 receptors has recently been found in the brains of human cannabis users (Villares, 2007). Studies have consistently found that cannabinoid receptor adaptation varies by brain region (reviewed by Sim-Selley, 2003). For example, desensitization of receptor-mediated G-protein activity is smaller in magnitude and develops more slowly in the basal ganglia (BG), especially the globus pallidus (GP), entopeduncular nucleus, and substantia nigra, compared with the hippocampus or cerebellum (Breivogel et al., 1999). Treatment paradigms have been developed to examine whether parameters that affect tolerance, such as treatment duration, affect cannabinoid receptor adaptation. Time-course studies have revealed that cannabinoid receptor down-regulation and desensitization were generally greater with increasing treatment duration, although the time course of adaptation varied by brain region (Breivogel et al., 1999). The dose effect of the treatment drug on receptor adaptation is not as well understood. Oviedo et al. (1993) reported that administration of increasing doses of CP55,940 produced greater decreases in CB_1 agonist binding in several forebrain nuclei. Regional differences in the pattern of down-regulation seemed to be present, but they were difficult to assess because analysis was limited to the striatum and adjacent structures. The majority of chronic cannabinoid studies in the literature administer either 1) the threshold dose

to produce tolerance (low dose) or 2) maximal dose to produce greater receptor adaptations (high dose). Therefore, it is unclear whether regional differences in adaptation reflect differential sensitivity to THC (e.g., are dose-dependent) or differential mechanisms of cannabinoid receptor adaptation. Interpretation is further complicated by differences in the cannabinoid drug administered, dose, and treatment duration between studies. This study was designed to directly examine regional differences in adaptation of cannabinoid receptors after administration of varying doses of THC and to assess tolerance produced by these administration paradigms.

Materials and Methods

Materials

[^{35}S]GTPγS (1150–1300 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA). ICR mice (male, 24–30 g) were obtained from Harlan (Indianapolis, IN). CP55,940 and THC were provided by the Drug Supply Program of the National Institute on Drug Abuse. Bovine serum albumin (BSA), guanosine 5′-3-*O*-(thio)triphosphate, GDP, and WIN55,212-2 were purchased from Sigma-Aldrich (St. Louis, MO). Econo-1 scintillation fluid was obtained from Fisher Scientific (Norcross, GA). All other chemicals (reagent grade) were obtained from Sigma-Aldrich or Fisher Scientific.

Drug Treatment

Male ICR mice were housed in an animal care facility maintained at 22 ± 2°C on a 12-h light/dark cycle. Food and water were available ad libitum. All experiments were conducted according to guidelines established by the Institutional Animal Care and Use Committee of Virginia Commonwealth University Medical Center. THC was dissolved in a 1:1:18 solution of ethanol, castor oil 40 mole ethoxylate (Emulphor), and saline. Mice received s.c. injections of THC or vehicle twice daily (7:00 AM and 3:00 PM) for 6.5 days. Mice were divided into four treatment groups as follows: 1) vehicle; 2) 10 mg/kg THC; 3) 10 mg/kg THC on day 1 that was increased every other day to 20 and 30 mg/kg THC, respectively; and 4) 10 mg/kg THC that was increased every other day to 30 and 60 mg/kg THC, respectively. These groups are referred to as “low,” “medium,” and “high” dose, respectively, in graphs. Twenty-four hours after the final injection, separate groups of mice were evaluated in behavioral assays or sacrificed for in vitro assays. Mice were sacrificed by decapitation, and brains for autoradiography were removed and immediately frozen in isopentane at -30°C and stored at -80°C. For membrane homogenate-binding assays, regions of interest [striatum/GP, hippocampus, and cerebellum] were dissected on ice and stored at -80°C until assay.

In Vivo Pharmacological Evaluation

Mice were tested using paradigms previously established to assess tolerance to cannabinoid-mediated hypomotility, hypothermia, immobility, and antinociception (Fan et al., 1994). Antinociception was assessed using tail-flick reaction time to a heat stimulus. Spontaneous activity was assessed by placing mice in standard activity chambers interfaced with a Digiscan Animal Activity Monitor (Omnitech Electronics Inc., Columbus, OH). Rectal temperature was measured using a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH) and thermistor probe. The ring immobility apparatus consisted of an elevated metal ring (5.5-cm diameter, 16-cm height) attached to a wooden stand and was used to assess catalepsy.

On day 7 (24 h after final injection of the dosing regimen), baseline measures of temperature and antinociception were obtained before drug challenge. Mice from vehicle- and THC-treated groups then received a single i.v. injection of THC (10 mg/kg) or vehicle. At 5 min postinjection, mice were placed in individual activity chambers, and activity was measured for 10 min as the number of interruptions of 16 photocell beams per chamber and expressed as a

percentage of inhibition of activity of the vehicle group. Mice were tested in the tail-flick assay at 20 min postinjection. A 10-s maximal latency was used to avoid tail injury. Antinociception was calculated as the percentage of maximal possible effect [%MPE = [(test latency — control latency)/(10 s — control latency)] × 100]. Rectal temperature was measured at 60 min after injection. The change in rectal temperature was calculated by control temperature — test temperature and expressed as Δ°C. At 1.5 h after injection, each mouse was placed on the ring-immobility apparatus for 5 min, and the duration of time (seconds) that the mouse remained motionless (determined by the absence of voluntary movement including snout and whisker movement) was measured. This value was divided by 300 s and multiplied by 100 to calculate the percentage of immobility rating.

Tissue Preparation

Twenty-four hours after the final injection, vehicle- and THC-treated mice were sacrificed by rapid decapitation, and brains were removed and frozen intact in 2-methyl butanol (isopentane) at -30°C for use in autoradiographic studies. Brains from a subset of mice were placed on ice, and the cerebellum, striatum/GP, and hippocampus were dissected and frozen at -80°C. For preparation of the membrane homogenates, tissue was thawed on the day of assay, placed in 20 volumes of cold buffer B (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.4), homogenized with a Polytron homogenizer, and centrifuged at 48,000*g* at 4°C for 10 min. The supernatants were discarded, and the pellets were rehomogenized in buffer B, centrifuged at 48,000*g*, and resuspended in buffer A (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4). Adenosine deaminase (final concentration = 4 mU/ml) was added to the membrane homogenates, which were then preincubated for 10 min at 30°C. Total membrane protein content was measured according to Bradford (1976).

Agonist-Stimulated [^{35}S]GTPγS Autoradiography

Coronal sections (20 μm) were collected at six different brain levels to include the following: 1) striatum and cingulate cortex, 2) globus pallidus and preoptic area (POA), 3) hippocampus, 4) substantia nigra, 5) PAG, and 6) cerebellum. Sections were cut on a cryostat maintained at -20°C, thaw-mounted onto gelatin-subbed slides, and collected in a humidified chamber. Slides were dried under vacuum and stored at 4°C overnight. Slides were stored desiccated at -80°C until use. On the day of assay, slides were brought to room temperature and incubated for 10 min at 25°C in buffer A. Slides were then incubated in buffer A plus 2 mM GDP, 0.5% BSA, and 10 mU/ml adenosine deaminase for 20 min at 25°C. Agonist-stimulated activity was determined by 2-h incubation with agonist (10 μM WIN55,212-2 or 2 μM CP55,940) in buffer A containing 2 mM GDP, 0.5% BSA, 10 mU/ml adenosine deaminase, and 0.04 nM [^{35}S]GTPγS at 25°C. Both WIN55,212-2 and CP55,940 were used in the assay because of recent reports that each can activate different non-CB_1 receptors in the brain (Mackie and Stella, 2006). The rationale is that similar findings with these two agonists would be indicative of CB_1 receptor activity because each seems to produce a somewhat different pharmacological and anatomical profile of non-CB_1 receptor activity. Basal binding was measured in the absence of agonist. After incubation, slides were rinsed twice in Tris buffer (50 mM, pH 7.4) for 2 min at 4°C and once in deionized water for 30 s at 4°C. Slides were dried overnight, placed in cassettes with ^{14}C microscales and Kodak X-Omat MR film and exposed for 24 h. Films were digitized with a Sony XC-77 video camera and analyzed using the NIH Image program for Macintosh computers. Resulting values were expressed as nanocuries of [^{35}S] per gram of tissue and were corrected for [^{35}S] from [^{14}C] standards based on incorporation of [^{35}S] into sections of frozen brain paste. Net agonist-stimulated [^{35}S]GTPγS binding was calculated by subtracting basal binding (obtained in the absence of agonist) from agonist-stimulated binding. Data are reported as mean values ± S.E. of triplicate sections of brains from six mice per treatment group. Statistical comparison between vehicle and THC-treated mice was performed by analysis of variance (ANOVA) followed by post-hoc analysis using the Dunnett's test.

[^{35}S]GTPγS Binding in Membrane Homogenates

Membranes (4 – 8 μg of protein) were incubated for2hat 30°C in buffer A containing 0.1 nM [^{35}S]GTPγS and 30 μM GDP, with and without varying concentrations of CP55,940. Basal binding was measured in the absence of agonist, and nonspecific binding was measured with 20 μM guanosine 5′-3-*O*-(thio)triphosphate. The incubation was terminated by filtration through GF/B glass fiber filters and washed three times with 3 ml of ice-cold 50 mM Tris-HCl, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry after overnight extraction of the filters in Econo-1 scintillation fluid.

[^{3}H]SR141716A Binding in Membrane Homogenates

Membranes (5–15 μg) were incubated for 90 min at 30°C in buffer A with 0.5% BSA and varying concentrations of [^{3}H]SR141716A in a total volume of 0.5 ml. Nonspecific binding was assessed in the presence of 5 μM unlabeled SR141716A. Incubations were terminated by vacuum filtration through Whatman GF/B glass fiber filters (Whatman, Clifton, NJ) and washed three times with ice-cold 50 mM Tris-HCl, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for ^{3}H after extraction of the filters in scintillation fluid.

Data Analysis

Separate one-way ANOVAs were used to analyze data for each of the four dependent measures included in the in vivo pharmacological assessment. When an ANOVA was significant ($p < 0.05$), Student-Newman Kuels post-hoc tests ($\alpha = 0.05$) were used to determine differences between group means. All binding assays were performed in duplicate and replicated at least three times; data are reported as specific binding. For [^{35}S]GTPγS binding, basal binding is defined as specific [^{35}S]GTPγS binding in the absence of drug. Net-stimulated [^{35}S]GTPγS binding is defined as [^{35}S]GTPγS binding in the presence of drug minus basal. The percentage of stimulation is expressed as (net-stimulated [^{35}S]GTPγS binding/basal) × 100%. E_{max} and EC_{50} values were calculated from nonlinear regression analysis by iterative fitting of the concentration-effect curves to the Langmuir equation [$E = E_{max}/(EC_{50} + \text{agonist concentration}) \times \text{agonist concentration}$] using Prism 4.0 software (GraphPad Software Inc., San Diego, CA). For [^{3}H]SR141716A binding, B_{max} and K_D values were calculated by iterative fitting of the saturation curves to the Langmuir equation [$B = B_{max}/(K_D + \text{ligand concentration}) \times \text{ligand concentration}$] using Prism 4.0 software (GraphPad Software Inc.).

Results

In Vivo Pharmacological Evaluation

Vehicle- and THC-treated mice received injections with a single challenge of 10 mg/kg THC or vehicle to assess whether tolerance to THC-mediated behaviors developed with chronic treatment (Fig. 1). Administration of vehicle to vehicle-treated (V/V) mice produced no significant effect on any measure. In contrast, administration of THC in vehicle-treated (V/THC) mice produced significant antinociception, ring immobility, suppression of spontaneous activity, and hypothermia. For spontaneous activity, administration of THC to vehicle-treated mice produced 93% inhibition of activity (Fig. 1, top left). Activity in mice, which were repeatedly treated with 10 mg/kg THC (low), was inhibited by 33%, which was intermediate between the vehicle- and ramping THC-treated (medium and high) groups, and did not significantly differ from vehicle-treated mice that received vehicle or THC challenge. The spontaneous activity of mice treated with both ramping paradigms also did not differ from that seen in the V/V mice; however, they exhibited decreased inhibition of spontaneous activity compared to that seen in the vehicle-treated group challenged with THC, suggesting the development of tolerance. For ring immobility (Fig. 1, top right), acute administration of THC

in both vehicle- and 10 mg/kg THC-treated mice produced immobility that differed significantly from vehicle challenge in vehicle-treated mice. Thus, repeated administration of 10 mg/kg THC did not produce tolerance in this test, which was conducted to assess catalepsy. Both ramping treatments produced tolerance, as indicated by significant differences from the group acutely challenged with THC (V/THC) as well as the lack of significant differences compared with vehicle challenge in vehicle-treated mice. Thus, for measures of motor behavior, administration of ramping doses (10–20-30 or 10–30-60 mg/kg) of THC produced tolerance, whereas repeated administration of 10 mg/kg THC did not. A somewhat different result was observed in measures of antinociception and hypothermia. THC challenge in vehicle-treated mice produced 95% MPE in the tail-flick assay (Fig. 1, bottom left). Mice that received chronic treatment with 10 mg/kg THC exhibited 55% MPE after THC challenge, which differed significantly from both vehicle and THC challenge in vehicle-treated mice. Mice treated with the chronic THC-ramping paradigms exhibited reduced antinociception that differed significantly from THC, but not vehicle, challenge in vehicle-treated mice. Rectal temperature produced the same pattern of response. THC challenge in vehicle-treated mice produced a temperature change of -2.8°C. Mice that received chronic administration of 10 mg/kg THC showed a decrease of -1.3°C, which differed from both vehicle and THC challenge in vehicle-treated mice. Mice treated with ramping doses of THC did not exhibit significant hypothermia compared with vehicle challenge in vehicle-treated mice. Thus, 10 mg/kg THC again produced an intermediate response between mice treated with vehicle or ramping doses of THC, but tolerance was observed for THC-mediated hypothermia and antinociception. Treatment with 10 to 20 to 30 or 10 to 30 to 60 mg/kg produced tolerance to both effects, similar to the results in previous tests.

Agonist-Stimulated [^{35}S]GTPγS Autoradiography

Coronal brain sections were collected at six levels to assess basal, WIN55,212-2- (Fig. 2), and CP55,940-stimulated (data not shown) [^{35}S]GTPγS binding in regions of interest from vehicle- and THC-treated mice. The distribution of cannabinoid-stimulated [^{35}S]GTPγS binding corresponded to that described previously; however, CP55,940 produced visibly lower stimulation than WIN55,212-2 in most regions, ranging from <70% (nucleus accumbens, POA, and PAG) to >90% (globus pallidus, substantia nigra, and cerebellum) of WIN55,212-2-stimulated [^{35}S]GTPγS binding in vehicle-treated mice. This observation is consistent with studies in cerebellar membranes showing that CP55,940 is a high-efficacy partial agonist at CB_1 receptors, whereas WIN55,212-2 is a full agonist (Breivogel et al., 1998). Basal [^{35}S]GTPγS binding was also measured to determine whether chronic THC administration had any effect on basal G-protein activity. This is also an important control, because residual THC could increase basal levels of [^{35}S]GTPγS binding in tissue from drug-treated mice. Decreases in basal activity were found only in mice treated with 10 to 20 to 30 mg/kg THC compared with vehicle, and this change was restricted to the caudate-putamen (591.0 ± 43.4 versus 464.7 ± 35.1), nucleus accumbens (593.8 ± 40.0 versus 437.0 ± 38.3), and globus pallidus (552.6 ± 24.5 versus 418.8 ± 18.3). We previously found decreased basal [^{35}S]GTPγS binding in the caudateputamen and globus pallidus of rats treated with THC (10 mg/kg) for 21 days (Sim et al., 1996). No other changes in basal [^{35}S]GTPγS binding were found in any other region.

Densitometric analysis of regions throughout the brain was conducted to determine whether each THC treatment altered cannabinoid receptor-mediated G-protein activity. Similar results were obtained using WIN55,212-2 (Table 1) or CP55,940 (data not shown) as the agonist in [^{35}S]GTPγS autoradiography. Based on this finding, as well as the anatomical distribution of agonist-stimulated [^{35}S]GTPγS binding analyzed densitometrically, it seems that most or all of the activity measured represents activation of CB_1 receptors. Nevertheless, it is possible that these agonists activate non-CB_1 cannabinoid receptors and that agonist-stimulated activity also includes a portion of non-CB_1 receptor-mediated activity. Administration of 10 mg/kg THC

twice daily for 6.5 days reduced net WIN55,212-2- and CP55,940-stimulated activity in a subset of regions: cingulate cortex, hippocampus, PAG, substantia nigra, and cerebellum. Net WIN55,212-2-and CP55,940-stimulated [^{35}S]GTPγS binding in the remaining regions did not significantly differ between brains from mice that received 10 mg/kg THC versus vehicle. Analysis of brains from mice that received 10 to 20 to 30 mg/kg THC revealed the same distribution of reduced activity, with decreased net WIN55,212-2-stimulated [^{35}S]GTPγS binding in the cingulate cortex, hippocampus, substantia nigra, PAG, and cerebellum. Although greater decreases in cannabinoid-stimulated activity were seen in many regions after administration of 10 to 20 to 30 mg/kg THC compared with 10 mg/kg THC, the only statistically significant difference was for net CP55,940-stimulated [^{35}S]GTPγS binding in the cerebellum. Administration of 10 to 30 to 60 mg/kg THC produced the regionally most widespread and greatest magnitude of reductions in cannabinoid-mediated G-protein activity. WIN55,212-2- and CP55,940-stimulated [^{35}S]GTPγS binding was decreased in all regions of the 10 to 30 to 60 mg/kg THC-treated brains examined, with the exception of the globus pallidus and entopeduncular nucleus. The level of WIN55,212-2-stimulated [^{35}S]GTPγS binding in mice treated with 10 to 30 to 60 mg/kg THC differed from that found in both the 10 and 10 to 20 to 30 mg/kg THC treatment groups in the caudateputamen, nucleus accumbens, and POA, and from the 10 mg/kg THC group in the PAG and cerebellum. Reductions in cannabinoid-mediated activity are best illustrated by calculating each value as a percentage of the WIN55,212-2-stimulated [^{35}S]GTPγS binding in vehicle-control brains (Fig. 3).

Agonist-Stimulated [^{35}S]GTPγS and [^{3}H]SR141716A Binding in Membranes

To determine the effect of the three THC treatments on agonist concentration-effect curves for G-protein activation, CP55,940-stimulated [^{35}S]GTPγS binding was performed in isolated membranes prepared from three regions of interest: hippocampus, striatum/GP (includes caudate-putamen, nucleus accumbens, and globus pallidus), and cerebellum. CP55,940 was chosen because greater apparent desensitization of CB_1 receptor-mediated G-protein and effector activity was previously shown using this high-efficacy partial agonist in membrane assays relative to results obtained with the full agonist WIN55,212-2 (Selley et al., 2004). These brain regions were chosen because of the high-expression levels of CB_1 receptors, the reported role in mediating cannabinoid effects on memory, motivation, and locomotor activity and coordination, and for comparison with our previous work on chronic THC-mediated CB_1 receptor adaptation measured in isolated membranes (Breivogel et al., 1999; Selley et al., 2004; Sim-Selley et al., 2006). Although CP55,940 can activate non-CB_1 cannabinoid receptors, its activity in these regions is probably CB_1 receptor-mediated because Breivogel et al. (2001) showed that CP55,940 did not activate [^{35}S]GTPγS binding in these regions in brains from CB_1 receptor null mice.

Results in the hippocampus showed that administration of 10 mg/kg THC appeared to decrease CP55,940-stimulated G-protein activity relative to vehicle-treated mice and that higher dose-ramping regimens appeared to produce only slight further decreases in activity (Fig. 4A). A nonlinear regression analysis of the CP55,940 concentration-effect curves confirmed these observations (Table 2). Treatment with 10 mg/kg THC decreased the E_{max} value of CP55,940 to 75% of the corresponding value obtained in vehicle-treated mice, and the 10 to 20 to 30 and 10 to 30 to 60 mg/kg THC-dosing regimens decreased the CP55,940 E_{max} to 72 and 61%, respectively, of vehicle-treated mice. Although all three chronic THC-treatment regimens appeared to increase the EC_{50} value of CP55,940, only the 10 to 30 to 60 mg/kg THC regimen significantly increased the CP55,940 EC_{50} value (by ~3.4-fold). Basal [^{35}S]GTPγS binding did not differ between the vehicle-treated and any of the THC-treated groups of mice.

In membranes prepared from striatum/GP, 10 mg/kg THC did not appear to affect CP55,940-stimulated [^{35}S]GTPγS binding, whereas the ramping dose THC-treatment regimens appeared

to attenuate stimulation by CP55,940 (Fig. 4B). Nonlinear regression analysis confirmed that there was no difference in CP55,940 E_{max} values between vehicle and 10 mg/kg THC-treated mice (Table 2). In contrast, the ramping dose THC-treatment regimens decreased the E_{max} value of CP55,940-stimulated [^{35}S]GTPγS binding to 75 and 61%, respectively, of that obtained in vehicle-treated mice. The CP55,940 EC_{50} values were not different between vehicle-treated mice and any of the THC-treated groups of mice. It is interesting to note that basal [^{35}S]GTPγS binding was significantly lower in the 10 mg/kg and 10 to 30 to 60 mg/kg dose THC-treated mice than in vehicle-treated mice; however, this apparent difference was not significant between vehicle- and 10 to 20 to 30 mg/kg dose THC-treated mice. These results demonstrate a fundamentally different pattern of adaptation of basal and CP55,940-stimulated G-protein activity in membranes prepared from the striatum/GP than from the hippocampus.

In cerebellar membranes, CP55,940-stimulated [^{35}S]GTPγS binding appeared to be modestly decreased in all three groups of THC-treated mice relative to vehicle-treated mice (data not shown). However, nonlinear regression analysis showed a significant decrease in the CP55,940 E_{max} value only in mice treated with the highest ramping dose of THC, which had an E_{max} value that was 76% of that obtained in vehicle-treated mice. Although CP55,940 EC_{50} values appeared to be greater in 10 mg/kg and 10 to 30 to 60 mg/kg THC-treated than vehicle-treated mice, the EC_{50} value obtained in vehicle-treated mice was not significantly different from the corresponding values in any of the THC-treated groups of mice. Basal [^{35}S]GTPγS binding was not different from vehicle-treated in any of the THC-treated groups of mice. Thus, unlike results obtained in membranes from the hippocampus and striatum/GP, only the highest dose of THC-treatment regimen significantly attenuated CP55,940-stimulated G-protein activity in cerebellar membranes.

To determine the relationship between the varying dosage of chronic THC treatment and CB_1 receptor levels, saturation analysis of [^{3}H]SR141716A was conducted in membranes prepared from the brain regions listed above. The results of nonlinear regression analysis are shown in Table 3. In the hippocampus, the [^{3}H]SR141716A B_{max} value was decreased in mice treated with 10 mg/kg THC to 59% of that obtained in vehicle-treated mice. CB_1 receptor down-regulation was maximum in hippocampal membranes from mice treated with the 10 to 20 to 30 mg/kg dose THC regimen and did not progress further with the 10 to 30 to 60 mg/kg treatment; [^{3}H]SR141716A B_{max} values were 38 and 43% of vehicle-treated mice, respectively. None of the THC treatments produced any significant differences in [^{3}H] SR141716A K_D values compared with vehicle-treated mice, indicating a lack of residual THC in the tissue. In striatum/GP, only the highest dose THC-treatment regimen significantly down-regulated CB_1 receptors, such that the [^{3}H]SR141716A B_{max} value was 61% of that obtained in vehicle-treated mice. Although the 10 to 20 to 30 mg/kg THC treatment appeared to decrease the mean [^{3}H]SR141716A B_{max} value to 71% of vehicle-treated mice, this was not a statistically significant difference. As in hippocampus, [^{3}H]SR141716A K_D values in striatum/GP membranes were not different between vehicle-treated mice and any of the THC-treated groups of mice. In cerebellum, chronic THC treatment decreased [^{3}H]SR141716A B_{max} values in 10 mg/kg, 10 to 20 to 30 mg/kg, and 10 to 30 to 60 mg/kg THC-treated mice to 73, 54, and 46% of that obtained in vehicle-treated mice. Again, there were no significant differences in [^{3}H]SR141716A K_D values between any of the groups of THC-treated and vehicle-treated mice. Thus, the dose relationship of chronic THC treatment and CB_1 receptor down-regulation differed among the three brain regions, such that the striatum/GP exhibited less adaptation at lower doses of THC compared with the hippocampus or cerebellum.

Discussion

These results demonstrate differential THC tolerance in vivo and region-dependent adaptation of CB_1 receptors and cannabinoid-mediated G-protein activation as a function of chronic THC

dose. A novel finding is that differences in the dose-effect function for both tolerance and cannabinoid receptor adaptation can be delineated among behaviors and brain regions. For example, development of tolerance to cannabinoid-mediated catalepsy occurred only after ramping doses of THC. In contrast, tolerance to antinociception occurred after 10 mg/kg and ramping doses of THC, and it was associated with desensitization in the PAG. Locomotor inhibition was intermediate, with 10 mg/kg THC producing an effect that differed from neither vehicle nor ramping THC treatments. The finding that 10 mg/kg THC treatment produced less tolerance to cannabinoid-induced motor effects than ramping paradigms is consistent with the finding that cannabinoid receptor adaptation for most basal ganglia nuclei was observed only after treatment with the highest doses of THC or not at all. These results indicate that regional differences in the dose-effect relationship of chronic THC treatment and cannabinoid receptor adaptation have consequences for tolerance to in vivo effects of THC.

It is intriguing that THC challenge in the high ramping-dose paradigm produced motor stimulation. Because stimulation is characteristically a low-dose cannabinoid effect (Sanudo-Pena et al., 2000), stimulation in this context might actually represent tolerance, because tolerance implies that higher doses are required to produce an effect that is usually produced by lower doses. Consistent with this hypothesis, we have previously observed stimulation induced by higher doses of THC after repeated dosing with THC or anandamide analogs in a similar tolerance/cross-tolerance paradigm (Wiley et al., 2005). On the other hand, stimulation has also been reported after administration of SR141617A, but it is not believed to be mediated via interaction with the CB_1 receptor (Bass et al., 2002). Because THC is not entirely CB_1-selective, it is also possible that high-dose THC might have produced adaptation in the activity produced by an as-yet-unknown mechanism that does not seem to be involved in the other pharmacological measures.

Our group and others have shown that cannabinoid receptors in regions with high-receptor density exhibit less adaptation (reviewed by Sim-Selley, 2003). That finding was generally true in the present study but did not apply in all regions, suggesting that factors other than receptor density influence CB_1 receptor adaptation. The basal ganglia output nuclei contain the highest density of CB_1 receptors in the brain (Herkenham et al., 1991) and exhibited the least magnitude of change. In contrast, desensitization was found in the hippocampus and cerebellum, areas that also contain high levels of CB_1 receptors (Herkenham et al., 1991), after all treatments. It is also possible that regional differences in adaptation to THC are influenced by differences in the endogenous cannabinoid tone. A recent study revealed that endocannabinoid levels vary among brain regions (Richardson et al., 2007).

We have previously hypothesized that colocalization with certain signaling proteins, such as G-protein subunits or regulatory proteins, might underlie the apparent resistance of cannabinoid receptors in the striatum/BG to adaptation (Sim-Selley, 2003). It is of interest that basal [^{35}S]GTPγS binding was reduced in the striatum and globus pallidus of brain sections and membranes. This finding, coupled with the result that most striatum/BG nuclei did not exhibit desensitization or were altered only by the highest dose of THC, supports this hypothesis. Regional differences in CB_1 receptor down-regulation could be due to G-protein receptor-associated sorting protein (GASP), which promotes CB_1 receptor down-regulation by augmenting trafficking of the receptor to lysosomes for degradation (Tappe-Theodor et al., 2007). We have shown that CB_1 receptor down-regulation in the hippocampus and striatum is associated with loss of CB_1 protein (Sim-Selley et al., 2006), in agreement with a role for GASP in CB_1 receptor adaptation in the brain. However, the CNS distribution of GASP has not yet been described. In a similar manner, differences in G-protein-coupled receptor kinase (GRK) or β-arrestin distribution could underlie regional differences in CB_1 receptor adaptation. CB_1 receptor desensitization occurred via a GRK3 and β-arrestin2-mediated mechanism in a heterologous expression model (Jin et al., 1999). There is evidence that GRK2, -5, and -6 are

expressed in the striatum/BG, whereas GRK3 expression is low (Arriza et al., 1992; Erdtmann-Vourliotis et al., 2001). Both β-arrestin-1 and -2 are expressed in the striatum/BG, with higher levels of β-arrestin-1 reported in this region (Attramadal et al., 1992; Gurevich et al., 2002). Thus, GRK/β-arrestin-mediated regulation of CB_1 receptors might differ between striatum/BG and other regions.

Agonist-stimulated [^{35}S]GTPγS binding was examined in brain sections and homogenates. Although results were similar in the striatum/GP and hippocampus, some differences were seen in the cerebellum. The E_{max} value of cannabinoid-stimulated [^{35}S]GTPγS binding decreased in the cerebellum only after treatment with the highest THC regimen when assessed in membranes, whereas desensitization was found after all THC treatments using autoradiography. This finding is similar to previous reports in which greater desensitization was revealed autoradiographically (Breivogel et al., 1999), and it suggests that cytoarchitectural integrity is particularly important in this region. CB_1 receptor levels were also measured in membrane homogenates. [^{3}H]SR141716A binding was reduced in the cerebellum and hippocampus after all treatments, demonstrating CB_1 receptor down-regulation. In contrast, down-regulation was detected in the striatum/GP only after high-dose treatment, consistent with [^{35}S]GTPγS-binding/autoradiographic results. This result differs somewhat from a previous study that reported decreased [^{3}H]CP55,940 binding in the caudate-putamen and adjacent regions after administration of varying doses of CP55,940 (Oviedo et al., 1993). Because THC (present study) and CP55,940 (Oviedo et al., 1993) differ in potency and intrinsic efficacy, it is difficult to directly compare these findings. An important result of the present study was that the K_D of [^{3}H]SR141716A did not change in any region, indicating that residual drug was not present in the tissue. The lack of increased basal [^{35}S]GTPγS binding further confirms this conclusion.

Although it is simplistic to equate a certain behavior with a particular CNS region, specific anatomical circuits clearly mediate distinct behaviors. For example, cannabinoid-mediated antinociception involves the PAG (Lichtman et al., 1996), whereas hypothermia involves the POA (Rawls et al., 2002). Motor behavior involves CB_1 receptors in the basal ganglia and cerebellum. For most behaviors examined, the regional profile of CB_1 receptor adaptation seemed to correlate with tolerance. However, adaptation of downstream effectors probably contributes to tolerance and might vary by region. For example, cannabinoid-mediated inhibition of adenylyl cyclase is reduced in the mouse cerebellum after administration of high doses of THC (Selley et al., 2004). The regional distribution and dose dependence of this effect have not yet been determined. Nonetheless, chronic THC treatment in rats increased cAMP levels in the striatum, cerebellum, and cortex and elevated activity of protein kinase A, a downstream target of adenylyl cyclase, in the cerebellum and cortex (Rubino et al., 2000). Moreover, administration of a protein kinase A inhibitor acutely reversed tolerance to THC-mediated antinociception, catalepsy, and hypoactivity (Bass and Martin, 2000). Other kinases might also be candidates for differential regional regulation of CB_1 receptors. Manipulation of Ras/extracellular-regulated kinase signaling modulates tolerance in a region-specific manner. Inhibition of extracellular-regulated kinase prevented the development of tolerance to THC-mediated hypolocomotion, as well as CB_1 receptor adaptation in the caudate-putamen and cerebellum, whereas CB_1 receptors in the hippocampus and prefrontal cortex were unaffected (Rubino et al., 2005).

The greatest magnitude of desensitization was found in the hippocampus. This result is reminiscent of time-course effects in which the hippocampus rapidly adapted after THC administration (Breivogel et al., 1999). It is perhaps not surprising that receptors in this region exhibited greater plasticity based on their role in learning/memory. The striatum/GP exhibited a fundamentally different response in which adaptation occurred only after treatment with the 10 to 30 to 60 THC paradigm, consistent with the slow time course for adaptation (Breivogel

et al., 1999). In a similar manner, CB_1 receptor adaptation in the nucleus accumbens, included in gross striatal dissections, occurred only after administration of the highest dose of THC. Cannabinoids have psychoactive properties and seem to modulate the reinforcing effects of other drugs. It is interesting to note that some users have less tolerance to the high rather than sedative effects of THC (Kirk and de Wit, 1999), and cannabinoid-mediated effects in operant tests exhibited less tolerance than to hypothermia/antinociception (De Vry et al., 2004). It is always a challenge to make direct comparisons between rodent and human studies because of pharmacokinetic and pharmacodynamic differences between species. Moreover, human consumption of marijuana varies from regular weekend exposure to long-term multiple daily exposures. However, it is evident that tolerance does not develop uniformly to all pharmacological effects in mice and humans. Therefore, a wide-dosing range was chosen for the present study in an effort to mimic human exposure. An s.c. dose of 10 mg/kg was chosen because it produces no overt behavioral effects despite inducing significant tolerance to some THC effects. Therefore, this regimen might be highly relevant to human exposure. On the other hand, the high ramping dose was chosen to induce a high level of tolerance in the shortest period of time.

The results of this study revealed a regional and dose-responsive specificity in the adaptation of cannabinoid receptors that is reflected in vivo as differential tolerance to cannabinoid-mediated behaviors. Moreover, the results of this study are quite consistent with reports on behavioral tolerance to cannabinoids in humans. In light of the recent finding that CB_1 receptor down-regulation occurs in the human brain after chronic cannabis use (Villares, 2007), these results indicate that similar mechanisms of CB_1 receptor adaptation exist in humans and animal models. Moreover, these findings suggest that administering lower doses of THC might mitigate tolerance to certain cannabinoid effects.

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ABBREVIATIONS

THC, Δ^9-tetrahydrocannabinol
CNS, central nervous system
PAG, periaqueductal gray
CP55,940, (-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol
BSA, bovine serum albumin
[^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)tri-phosphate
WIN55,212-2,, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate
GP, globus pallidus
MPE, maximal possible effect
POA, preoptic area
SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamidehydrochloride
ANOVA, analysis of variance
BG, basal ganglia
V/V, vehicle-treated
V/THC, THC in vehicle-treated

GASP, G-protein receptor-associated sorting protein
GRK, G-protein-coupled receptor kinase

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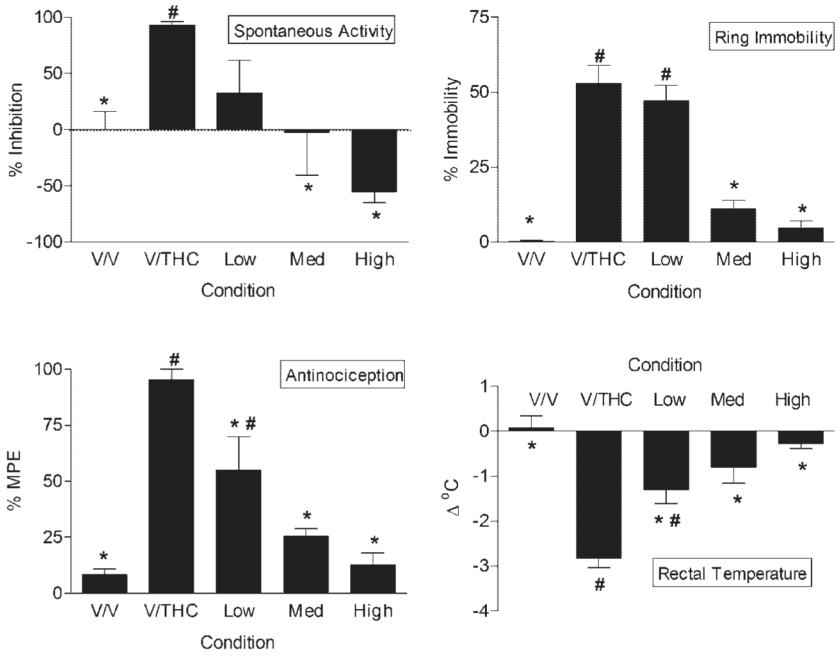

Fig. 1.

Effects of 10 mg/kg THC (i.v.) and vehicle on percentage of inhibition of spontaneous activity (top left), percentage of time of ring immobility (top right), percentage of maximal possible antinociceptive effect (bottom left), and change in rectal temperature (bottom right) in mice in each of the dosing groups. Mice in the V/V condition received injections with vehicle during the dosing regimen and were tested with vehicle. Mice in all of the other groups were tested with 10 mg/kg THC. Mice in the V/THC condition received injections with vehicle during the dosing regimen, and mice in each of the other groups received injections according to the regimen indicated on the abscissa. Values represent the mean ± S.E.M. of data from four to six mice. *, a significant difference from the V/THC condition. #, a significant difference from the V/V condition.

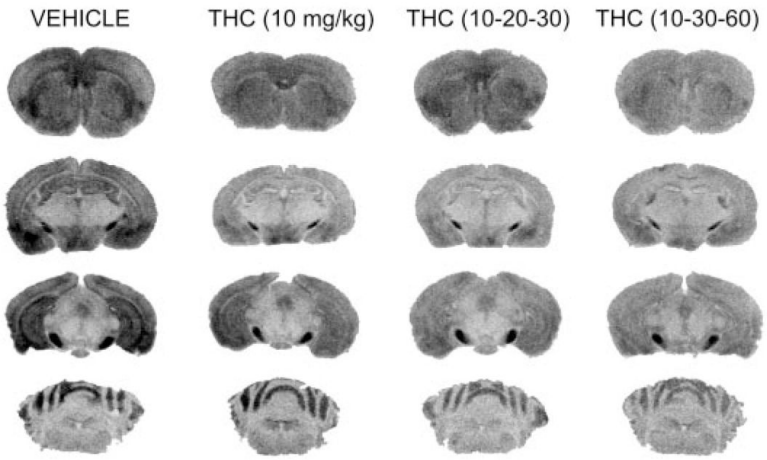

Fig. 2.

Representative autoradiograms showing WIN55,212-2-stimulated [35S]GTPγS binding in the caudate-putamen, nucleus accumbens, and cingulate cortex (row 1), hippocampus and entopeduncular nucleus (row 2), substantia nigra (row 3), and cerebellum (row 4) after chronic treatment with 10, 10 to 20 to 30, or 10 to 30 to 60 mg/kg THC. The regionally distinct pattern of decreases in cannabinoid-mediated G-protein activity is evident in sections shown in row 3, where agonist-stimulated activity was significantly reduced with increasing doses of THC in the hippocampus but unchanged in entopeduncular nucleus.

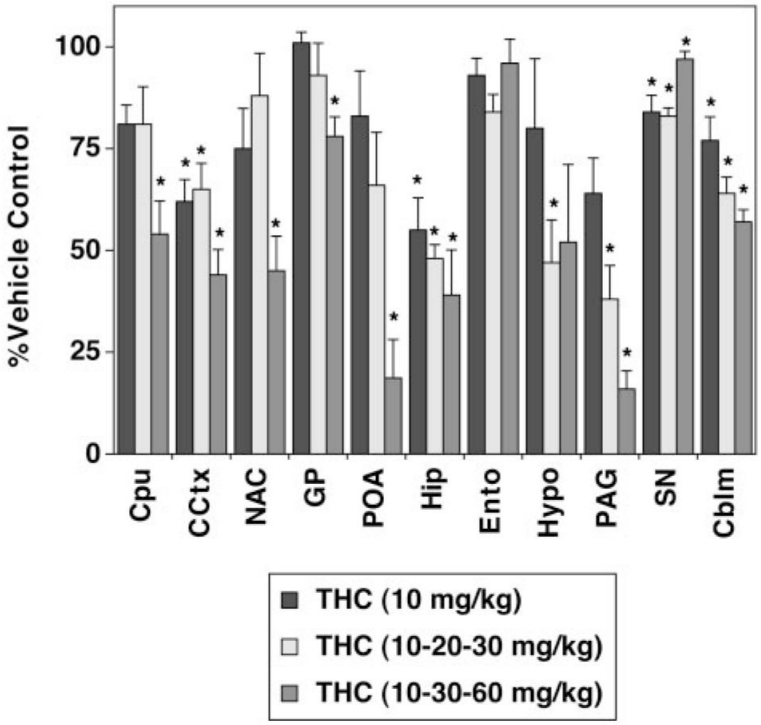

Fig. 3.

Effect of administration of 10, 10 to 20 to 30, or 10 to 30 to 60 mg/kg THC on WIN55,212-2-stimulated [^{35}S]GTPγS binding in brain sections expressed as a percentage of WIN55,212-2-stimulated [^{35}S]-GTPγS binding in brains from vehicle-treated mice.

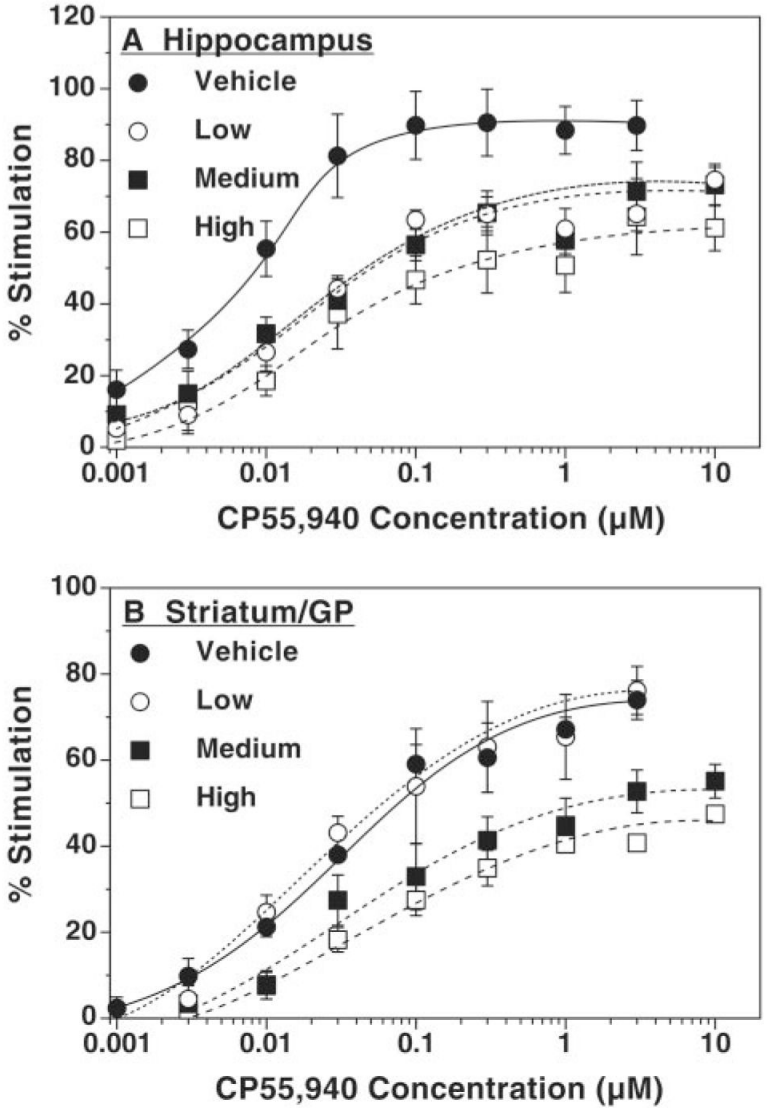

Fig. 4.

Effects of 10, 10 to 20 to 30, or 10 to 30 to 60 mg/kg THC on CP55,940-stimulated [^{35}S]GTPγS binding in isolated membranes. Membranes prepared from the hippocampus (A) or striatum/GP (B) were incubated with 0.1 nM [^{35}S]GTPγS, 30 mM GDP, and the indicated concentrations of CP55,940 as described under *Materials and Methods*. Data are the mean ± S.E.M. percentage of stimulation values from four to five mice per treatment group.

TABLE 1

Net WIN55,212-2-stimulated [^{35}S]GTPγS binding in brain sections

Brain sections were incubated with 0.04 nM [^{35}S]GTPγS, 10 μM WIN55,212-2, and 2 mM GDP as described under *Materials and Methods*. Data shown are mean net [^{35}S]GTPγS-binding values (nCi/g) ± S.E.M.

Region	Vehicle	10	10–20–30	10–30–60
			mg/kg	
Caudate-putamen	334.6 ± 10.9	303.3 ± 17.7	305.1 ± 34.7	202.9 ± 30.3[a,b,c]
Nucleus accumbens	316.1 ± 14.1	235.6 ± 31.3	277.4 ± 32.7	142.5 ± 27.0[a,b,c]
Cingulate cortex	481.8 ± 44.1	297.0 ± 26[a]	312.3 ± 30.9[a]	211.0 ± 29.7[a]
Globus pallidus	929.5 ± 35.6	934.3 ± 23.9	859.9 ± 73.5	726.7 ± 44.4
Preoptic area	291.6 ± 72.8	242.4 ± 32.4	193.8 ± 38.1	61.4 ± 33.0[a,b,c]
Hippocampus	407.2 ± 66.1	222.3 ± 32.2[d]	196.9 ± 13.8[d]	159.0 ± 45.3[a]
Entopeduncular nucleus	908.2 ± 62.4	840.5 ± 37.8	765.5 ± 38.6	867.4 ± 53.4
Substantia nigra	997.7 ± 12.6	834.5 ± 40.8[a]	825.6 ± 20.3[a]	806.6 ± 44.2[a]
PAG	235.6 ± 21.5	151.4 ± 20.6[d]	90.27 ± 19.5[a]	37.3 ± 10.4[a,b]
Cerebellum	657.7 ± 21.4	504.2 ± 38.4[a]	419.9 ± 26.1[a]	377.5 ± 19.6[a,b]

[a] $P < 0.01$ different from vehicle group by ANOVA with post-hoc Dunnett test.

[b] $P < 0.05$ different from 10 mg/kg THC group by ANOVA with post-hoc Newman-Keuls test.

[c] $P < 0.05$ different from 10 to 30 to 60 mg/kg THC group by ANOVA with post-hoc Newman-Keuls test.

[d] $P < 0.05$ different from vehicle group by ANOVA with post-hoc Dunnett's test.

TABLE 2

E_{max} and EC_{50} values of CP55,940-stimulated [^{35}S]GTPγS binding in membranes from brain regions

Membranes were incubated with 0.1 nM [^{35}S]GTPγ S, 30 μM GDP, and varying concentrations of CP55,940 as described under *Materials and Methods*. Data are the mean E_{max},EC_{50}, and basal [^{35}S]GTPγ S-binding values ± S.E.M. derived from four to six mice per group.

Region	Vehicle	10	10–20-30	10–30-60
			mg/kg THC	
Hippocampus				
E_{max} (% stimulation)	94 ± 5.4	69 ± 4.6[a]	67 ± 4.6[b]	58 ± 7.4[b]
EC_{50} (nM)	7.5 ± 1.3	17 ± 4.2	14 ± 0.9	26 ± 7.1[a]
Basal (fmol/mg)	167 ± 33	158 ± 28	189 ± 35	170 ± 34
Striatum/GP				
E_{max} (% stimulation)	70 ± 3.1	72 ± 7.4	52 ± 4.4[a]	43 ± 1.2[b]
EC_{50} (nM)	32 ± 6.6	24 ± 3.2	49 ± 24	38 ± 6.3
Basal (fmol/mg)	336 ± 58	170 ± 22[a]	250 ± 35	170 ± 14[a]
Cerebellum				
E_{max}(% stimulation)	255 ± 21	210 ± 7.8	202 ± 17	189 ± 16[a]
EC_{50} (nM)	26 ± 9.2	56 ± 15	28 ± 11	43 ± 18
Basal (fmol/mg)	106 ± 20	100 ± 11	88 ± 4.5	91 ± 8.6

[a] $P < 0.05$ different from vehicle control as determined by ANOVA with the Dunnett's post-hoc test.

[b] $P < 0.01$ different from vehicle control as determined by ANOVA with the Dunnett's post-hoc test.

TABLE 3

B_{max} and K_D values of [^{3}H]SR141716AA binding in membranes from brain regions

Membranes were incubated with varying concentrations of [^{3}H]SR141716A as described under *Materials and Methods*. Data are the mean B_{max} and K_D values ± S.E.M. derived from three to five mice per group.

Region	Vehicle	10	10–20-30	10–30-60
			mg/kg THC	
Hippocampus				
B_{max} (pmol/mg)	4.34 ± 0.24	2.52 ± 0.17[a]	1.62 ± 0.04[a]	1.85 ± 0.15[a]
K_D (nM)	0.25 ± 0.02	0.38 ± 0.13	0.20 ± 0.03	0.34 ± 0.06
Striatum/GP				
B_{max} (pmol/mg)	3.41 ± 0.51	2.98 ± 0.22	2.25 ± 0.39	1.86 ± 0.23[a]
K_D (nM)	0.31 ± 0.06	0.27 ± 0.03	0.17 ± 0.04	0.30 ± 0.07
Cerebellum				
B_{max} (pmol/mg)	4.52 ± 0.31	3.26 ± 0.26[b]	2.42 ± 0.34[a]	2.09 ± 0.18[a]
K_D (nM)	0.34 ± 0.05	0.34 ± 0.13	0.28 ± 0.12	0.24 ± 0.05

[a] $P < 0.02$ different from vehicle control as determined by ANOVA with the Dunnett's post-hoc test.

[b] $P < 0.05$ different from vehicle control as determined by ANOVA with the Dunnett's post-hoc test.