Prolonged Recovery Rate of CB$_1$ Receptor Adaptation after Cessation of Long-Term Cannabinoid Administration

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ABSTRACT

Long-term cannabinoid administration produces region-dependent CB$_1$ receptor desensitization and down-regulation. This study examined the time course for normalization of CB$_1$ receptors and G-protein activation using ^{3}H-labeled N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride (SR141716A) and guanosine 5′-O-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS binding), respectively, in hippocampus and striatum/globus pallidus (GP). Mice were treated with escalating doses of Δ^9-tetrahydrocannabinol (Δ^9-THC) or R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate (WIN55,212-2) for 15 days, and tissue was collected 1, 3, 7, or 14 days after final injection. [^{3}H]SR141716A and WIN55,212-2-stimulated [^{35}S]GTPγS binding were decreased in both regions 1 day after treatment. WIN55,212-2-stimulated G-protein activation in striatum/GP returned to control level at 3 days after cessation of treatment with either drug but did not return to control level in hippocampus until 14 days. CB$_1$ receptor binding did not recover to control levels until day 7 or 14 after treatment in striatum/GP and hippocampus, respectively. The mechanism of CB$_1$ binding site down-regulation was investigated after long-term Δ^9-THC treatment. Analysis of CB$_1$ receptor mRNA in hippocampus and striatum/GP showed that transcriptional regulation could not explain prolonged recovery rates from CB$_1$ receptor down-regulation. In contrast, CB$_1$ receptor protein, as determined by immunoblot analysis, matched the down-regulation and recovery rates of CB$_1$ receptor binding sites relatively closely. These data demonstrate that cannabinoid-induced decreases in CB$_1$ receptor function persist for relatively long time periods after cessation of long-term drug treatment and that CB$_1$ receptor signaling recovers more quickly in striatum/GP than hippocampus. Moreover, down-regulation of CB$_1$ receptor binding sites does not seem to result mainly from transcriptional regulation, suggesting that adaptive regulation of CB$_1$ receptors in brain primarily occurs at the protein level.

The behavioral effects of cannabinoids such as Δ^9-tetrahydrocannabinol (Δ^9-THC), the psychoactive component of marijuana, include motor disturbances and memory impairment (Hollister, 1986; Compton et al., 1993; Heyser et al., 1993). These effects are mediated by CB$_1$ receptors (Ledent et al., 1999; Zimmer et al., 1999; Varvel and Lichtman, 2002), which are widely distributed in the central nervous system, with highest density in basal ganglia, hippocampus, and cerebellum (Herkenham et al., 1991; Tsou et al., 1998). CB$_1$ receptors primarily activate pertussis toxin-sensitive G-proteins of the G$\alpha_{i/o}$ subfamily and produce intracellular effects that include inhibition of adenylyl cyclase (Howlett et al., 1986) and modulation of calcium- and potassium-channel conductance (Mackie et al., 1995; Twitchell et al., 1997).

Long-term administration of cannabinoids, including Δ^9-THC, produces tolerance to cannabinoid-mediated effects in humans and laboratory animals (Carlini, 1968; Jones et al., 1981). Cannabinoid dependence has also been demonstrated experimentally using antagonist-precipitated withdrawal (Aceto et al., 1995; Tsou et al., 1995). At the cellular level, attenuation of both CB$_1$ receptor binding (down-regulation) and CB$_1$ agonist-stimulated G-protein activation (desensitization) occur after long-term cannabinoid administration and

ABBREVIATIONS: BSA, bovine serum albumin; CHO, Chinese hamster ovary; O.D., optical density; GP, globus pallidus; ECL, enhanced chemiluminescence; SSPE, sodium chloride/sodium phosphate/EDTA; TBST, Tris-buffered saline/Tween 20; CB$_1$-ir, CB$_1$ receptor immunoreactivity; THC, Δ^9-tetrahydrocannabinol; [^{35}S]GTPγS, guanosine 5′-O-(3-[^{35}S]thio)triphosphate; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboximide hydrochloride; WIN55,212-2, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo-[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate.

are believed to contribute to tolerance (Martin et al., 2004). Decreased CB$_1$ receptor binding and receptor-mediated G-protein activation occur throughout the brain, although the magnitude of change varies among regions (Sim et al., 1996; Romero et al., 1997; Sim-Selley and Martin, 2002). Moreover, studies have demonstrated the time-dependent nature of the development of down-regulation and desensitization, with CB$_1$ receptors in hippocampus and cerebellum exhibiting greater magnitude and more rapid adaptations than those in basal ganglia (Breivogel et al., 1999). Changes in CB$_1$ receptor mRNA also occur in these regions, but the magnitude and direction of change vary with treatment duration (Zhuang et al., 1998). Studies have also characterized temporal characteristics of the development of tolerance to cannabinoid-mediated memory impairment (Deadwyler et al., 1995), hypomotility (Bass and Martin, 2000; Whitlow et al., 2002), antinociception (Bass and Martin, 2000), and hypothermia (Whitlow et al., 2002) and showed differences in the rate of tolerance development to these effects. Few studies have examined the time course for the disappearance of tolerance to cannabinoid-mediated effects after the cessation of long-term cannabinoid treatment. Bass and Martin (2000) reported that the disappearance of tolerance to cannabinoid-mediated hypomotility preceded that of antinociception, suggesting that recovery of CB$_1$ receptor function might exhibit regional differences. However, the time course of CB$_1$ receptor normalization after cessation of cannabinoid treatment has not been assessed at the cellular level.

The mechanisms by which long-term cannabinoid administration attenuates CB$_1$ receptor function in the brain has also not been resolved. The finding of a close regional and temporal correlation between decreases in CB$_1$ receptor binding sites and decreases in G-protein activation (Breivogel et al., 1999; Sim-Selley and Martin, 2002) suggests that these two adaptive responses share a common mechanism, such as internalization and degradation of CB$_1$ receptors. However, Coutts et al. (2001) reported that although CB$_1$ receptors were internalized by agonist treatment in cultured hippocampal neurons, persistent exposure to agonist did not decrease CB$_1$ receptor protein. Likewise, Cichewicz et al. (2001) reported no decrease in CB$_1$ receptor protein in midbrain or spinal cord of mice administered oral THC for 6.5 days. Moreover, Zhuang et al. (1998) reported that the time course of changes in CB$_1$ receptor mRNA during long-term THC treatment did not correspond well with the time course of changes in CB$_1$ receptor binding sites. Thus, it remains to be determined whether down-regulation of CB$_1$ receptor binding sites is mediated by a reduction in CB$_1$ receptor protein and, if so, to what extent transcriptional regulation plays a role in this adaptive response.

The present study addressed these questions by treating mice with either Δ^9-THC or WIN55,212-2 using a paradigm that produces high levels of tolerance in vivo and CB$_1$ receptor down-regulation and desensitization in situ (Sim-Selley and Martin, 2002) and then collecting brain tissue at different time points after the final drug injection. This paradigm was used to examine the time course of normalization of CB$_1$ receptor function because it was important to maximize receptor adaptation so that an incremental return to baseline function could be quantified. Striatum/globus pallidus (GP) and hippocampus were selected for analysis because these regions contain high levels of CB$_1$ receptors, mediate motor

(basal ganglia) and memory (hippocampus) effects of cannabinoids, and differ in the rate of development of CB$_1$ receptor adaptation.

Materials and Methods

Materials. ICR mice (male, 24–30 g) were purchased from Harlan Laboratories (Indianapolis, IN). [^{35}S]GTPγS (1250 Ci/mmol) and GeneScreen Plus nylon membrane were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]SR141716A (49 Ci/mmol) and Enhanced Chemiluminescence (ECL) and ECL Plus reagent were purchased from GE Healthcare (Piscataway, NJ). Δ^9-THC was provided by the Drug Supply Program of the National Institute on Drug Abuse. WIN55,212-2 and GDP were purchased from Sigma/RBI (Natick, MA). Mouse 18S RNA was obtained from DECAprobe (Ambion, Austin, TX), Prime-It II Random Primer Labeling Kit and UV linker were obtained from Stratagene (La Jolla, CA), and TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). CB$_1$ receptor antisera were generously provided by Dr. Ken Mackie (Department of Anesthesiology, University of Washington, Seattle, WA). Goat anti-rabbit peroxidase and protein molecular weight markers were purchased from Vector Laboratories (Burlingame, CA). All other reagent-grade chemicals were obtained from Sigma Chemical (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA).

Long-Term Drug Administration. All mice were maintained on 14:10 light/dark cycle with ad libitum food and water. All animal procedures were conducted according to established National Institutes of Health guidelines and were approved by the Internal Animal Care and Use Committee of Virginia Commonwealth University. Δ^9-THC and WIN55,212-2 were dissolved in a 1:1:18 solution of ethanol, Emulphor, and saline. Male ICR mice were injected subcutaneously twice daily with Δ^9-THC, WIN55,212-2, or vehicle for 15 days (Sim-Selley and Martin, 2002). Initial doses of drugs were 10 mg/kg Δ^9-THC and 3 mg/kg WIN55,212-2, and the doses were doubled every 3 days to final doses of 160 mg/kg Δ^9-THC and 48 mg/kg WIN55,212-2. Mice were killed by decapitation at 1, 3, 7, and 14 days after the final injection of drug. The striatum/GP and hippocampus were dissected on ice and stored at $-80°C$ until assay.

Agonist-Stimulated [^{35}S]GTPγS Binding. Each tissue sample was placed in 20 volumes of ice-cold membrane buffer (50 mM Tris-HCl, 3 mM MgCl$_2$, and 1 mM EGTA, pH 7.4) and homogenized on the day of assay. The sample was then centrifuged at 48,000g at 4°C for 10 min, resuspended in membrane buffer, and centrifuged at 48,000g. Membranes were resuspended in assay buffer A (50 mM Tris-HCl, 3 mM MgCl$_2$, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4), and protein was determined as described previously (Selley et al., 2004). Concentration-effect curves were generated by incubating the appropriate concentration of membrane (5–10 μg) in assay buffer A with 1 g/l bovine serum albumin (BSA) (assay buffer B) with various concentrations of WIN55,212-2 with 30 mM GDP and 0.08 nM [^{35}S]GTPγS in 1 ml total volume. Basal binding was assessed in the absence of agonist, and nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. The reaction was terminated by filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold (4°C) Tris buffer (50 mM Tris-HCl, pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency after extraction overnight in ScintiSafe Econo 1 scintillation fluid (Fisher Scientific).

[^{3}H]SR141716A Binding. Membranes were prepared as above, diluted with assay buffer A, and incubated under [^{35}S]GTPγS binding assay conditions. Saturation analysis of [^{3}H]SR141716A binding was performed by incubating 5 to 10 μg membrane protein with 0.02 to 5 nM [^{3}H]SR141716A in assay buffer B containing 30 μM GDP (total volume, 1 ml) in the presence and absence of 5 mM concentration of unlabeled SR141716A to determine nonspecific and specific binding, respectively. The assay was incubated for 2 h at 30°C and

terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters that were presoaked in Tris buffer containing 5 g/l BSA followed by five washes with ice-cold Tris buffer containing 5 g/l BSA. Bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency after 1-h shaking of the filters in 4 ml of ScintiSafe Econo 1 scintillation fluid.

Northern Blot. Samples were individually homogenized in 0.75 ml of TRIzol reagent using a Kontes glass tissue grinder. After the addition of 0.2 ml of chloroform, samples were centrifuged at 10,000g for 15 min. The aqueous phase was transferred to separate tubes, and RNA was precipitated with 0.5 ml of isopropyl alcohol. The sample was centrifuged at 10,000g for 15 min, the RNA pellet was washed with 75% ice-cold ethanol, and the sample was centrifuged at 7500g for 5 min. The RNA pellet was resuspended in water and quantified by spectrophotometry. Samples (2.5 μg of RNA) were prepared by adding three volumes of loading buffer (50% formamide, 6% formaldehyde, 20 mM boric acid, 10% glycerol, 0.2 mM EDTA, 0.25% bromphenol blue, and 0.25% xylene cyanol) per sample, denatured at 65°C for 5 min, and chilled on ice. UV visualization of bands was facilitated by 1 μl of a 1 μg/μl ethidium bromide solution added to each sample before loading onto a horizontal agarose gel (0.8% agarose, 20 mM boric acid, 0.2 mM EDTA, and 3% formaldehyde). After electrophoretic separation (100 V, 30 min), gels were submerged in 0.05 N NaOH for 20 min and 20× SSPE for 40 min. RNA was eluted by capillary transfer to a GeneScreen Plus nylon membrane for 16 h and UV-fixed. Membranes were prehybridized at 60°C for 4 h with hybridization buffer (5× SSPE, 10% dextran sulfate, 1% SDS, and 5× Denhardt's solution, with 0.1 mg/ml salmon sperm DNA). For hybridization, the samples were incubated with hybridization buffer (minus salmon sperm DNA) containing radiolabeled probes. Each membrane was hybridized with 1 × 10^7 dpm radiolabeled random prime probe for 16 h with either mouse CB$_1$ receptor cDNA (McLaughlin and Abood, 1993) or mouse 18S RNA used as a loading control. Both random prime probes were produced using a Prime-It II Random Primer Labeling Kit. Membranes were washed twice in 2× SSPE (for 15 min at room temperature) and once in 2× SSPE containing 2% SDS (15 min at 60°C). Washed membranes were exposed to Cyclone phosphor screens and quantified using a Cyclone Storage Phosphor System (PerkinElmer Life and Analytical Sciences, Boston, MA).

Immunoblot. Each sample was individually homogenized in buffer A using a Polytron homogenizer (Kinematica, Basel, Switzerland). Membranes were centrifuged at 50,000g for 10 min at 4°C and resuspended in buffer A. Protein was quantified as described previously (Selley et al., 2004), and samples were diluted as appropriate to obtain equal concentrations of protein. Samples were then dissolved in equal volumes of 2× sample buffer (250 mM Tris-HCL, pH 6.9, 4% SDS, 24% sucrose, 100 mM dithiothreitol, and 0.0025% bromphenol blue) and boiled for 10 min. Samples of equal volume were then loaded onto 10% SDS-polyacrylamide gels, and a protein molecular weight marker was added to each gel. Electrophoresis was conducted at 115 V for 1.5 h. Proteins were transferred electrophoretically to reinforced nitrocellulose (Bio-Rad, Hercules, CA) at 60 V for 1 h. Proteins were visualized using Ponceau S stain [0.5% Ponceau S (w/v) and 1% acetic acid (v/v) in distilled water] for 1 min at room temperature. Blots were blocked overnight at 4°C in TBST (20 mM Tris-HCl, pH 7.4, 0.8% NaCl, and 0.1% Tween 20) with 5% nonfat dry milk. All subsequent incubations were performed on a rocking platform at room temperature. Rinse buffer was discarded and replaced with TBST with 5% nonfat dry milk containing primary antibody at appropriate dilutions and incubated for 1 to 2 h. Two different antisera were used in immunoblot experiments: rabbit anti-rCB$_1$ C terminus (last 15 residues) diluted 1:1000, or rabbit anti-rCB$_1$ N terminus (first 77 residues) diluted 1:1000. After removal of primary antibody, the blot was rinsed for 1 min and then washed five times for 5 min each in TBST. Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was diluted 1:10,000 in TBST with 5% nonfat dry milk and incubated with the blot for 30 min. The blot

was rinsed for 1 min and then five times for 5 min each in TBST. ECL reagent (GE Healthcare) was added for 1 min for visualization of protein. Blots were then exposed to Kodak BioMax MR film (Eastman Kodak, Rochester, NY), the film was scanned, and densitometry was performed using NIH Image software. To facilitate detection of subtle differences in optical density, time course immunoblots were incubated in ECL Plus reagent (GE Healthcare) for 2 min and visualized using the Storm 860 Molecular Imager (GE Healthcare). The scanned images were analyzed with ImageQuant 5.2 software (GE Healthcare) for Windows.

Data Analysis. Data are reported as mean ± S.E. values of at least three independent experiments. All binding experiments were performed in triplicate and were replicated at least three times. Nonspecific binding was subtracted from all binding data. Net stimulated [^{35}S]GTPγS binding is defined as agonist-stimulated minus basal [^{35}S]GTPγS binding. Percentage of stimulation is defined as (net-stimulated/basal [^{35}S]GTPγS binding) × 100%. Nonlinear iterative regression analyses of agonist concentration-effect and saturation binding curves were performed with JMP (SAS Institute, Cary, NC). All blotting experiments were single determinations replicated at least three times. Percentage of control optical density (O.D.) is defined as the optical density value of a THC-treated sample/O.D. value of the adjacent, time-matched vehicle control sample × 100%. Statistical significance of the differences between drug- and vehicle-treated mice in each brain region were determined analysis of variance followed by post hoc analysis with the two-tailed nonpaired Student's t test or Tukey-Kramer test.

Results

Time-Dependent Recovery of CB$_1$ Receptor Function after Long-Term Cannabinoid Agonist Administration. Mice were injected subcutaneously with vehicle or drug as described under *Materials and Methods*, and hippocampus and striatum/GP were collected at various time points after cessation of treatment. CB$_1$ receptor binding was assessed using varying concentrations of the CB$_1$ selective antagonist [^{3}H]SR141617A (rimonabant), and CB$_1$ receptor-mediated G-protein activation was examined using WIN55,212-2-stimulated [^{35}S]GTPγS binding.

In hippocampus, results showed that WIN55,212-2-stimulated [^{35}S]GTPγS binding was significantly reduced 1 day after cessation of Δ^9-THC treatment (Fig. 1A), as previously demonstrated using [^{35}S]GTPγS autoradiography (Sim-Selley and Martin, 2002). G-protein activation by WIN55,212-2 in Δ^9-THC-treated mice slowly returned to levels approaching that of vehicle-treated mice over the 2-week period (Fig. 1A). These results were confirmed by nonlinear regression analysis of the concentration-effect curves (Table 1). At 1 day after Δ^9-THC treatment, the E_{max} value of WIN55,212-2-stimulated [^{35}S]GTPγS binding was 53% of the corresponding vehicle-treated control. Although the EC$_{50}$ value of WIN55,212-2 was approximately twice that of vehicle-treated mice at 1 day after Δ^9-THC treatment, this was not a statistically significant difference. The WIN55,212-2 E_{max} values then increased to 62, 70, and 83% of vehicle control at days 3, 7, and 14 after Δ^9-THC, respectively. Similar results were obtained in hippocampal membranes from WIN55,212-2-treated mice. The E_{max} values of WIN55,212-2-stimulated [^{35}S]GTPγS binding were 61, 65, 70, and 89% of vehicle control on days 1, 3, 7, and 14, respectively (Table 1). Thus, WIN55,212-2 stimulated G-protein activation did not return to control level until 14 days after WIN55,212-2 treatment. In Δ^9-

THC-treated mice, WIN55,212-2 stimulated G-protein activation was still statistically distinguishable from control at day 14. These results demonstrate prolonged attenuation of CB$_1$ receptor signaling in hippocampus after cessation of long-term cannabinoid agonist administration.
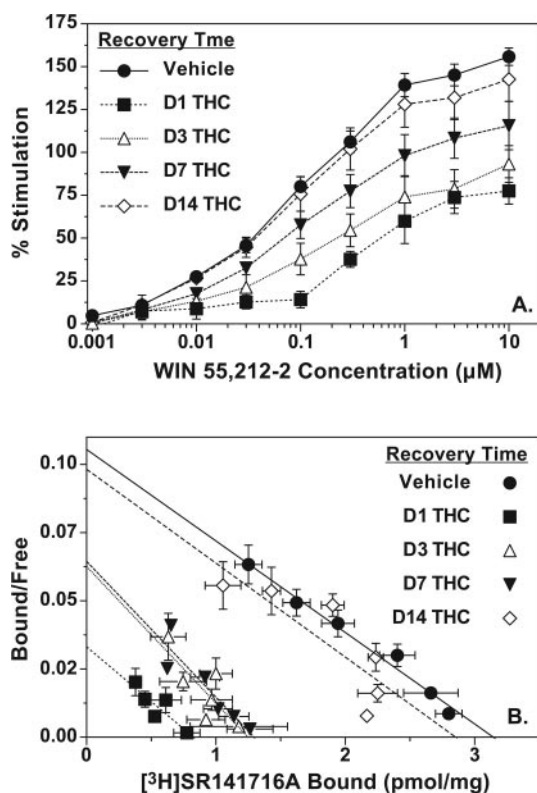
Similar results were obtained in hippocampus with CB$_1$ receptor binding. The number of [^{3}H]SR141716A binding sites was reduced at 1 day after Δ^9-THC treatment (Fig. 1B). Nonlinear regression analysis of [^{3}H]SR141716A saturation binding data confirmed that the B_{max} value at 1 day after Δ^9-THC treatment was 24% of that obtained in vehicle-treated controls, with no difference in K_D value (Table 1). [^{3}H]SR141716A binding in Δ^9-THC-treated mice gradually returned toward vehicle-treated levels over a 2-week period (Fig. 1B), similar to results obtained with WIN55,212-2-stimulated [^{35}S]GTPγS binding. [^{3}H]SR141716A B_{max} values in Δ^9-THC-treated mice were 37, 43, and 80% of their corresponding vehicle-treated controls at days 3, 7, and 14 after Δ^9-THC, respectively (Table 1). It is noteworthy that [^{3}H]SR141716A K_D values did not differ between Δ^9-THC- and vehicle-treated mice at any time point after Δ^9-THC cessation, indicating that there was no residual drug remaining in the membranes. Similar results were obtained in the hippocampi of WIN55,212-2-treated mice, in which [^{3}H]SR141716A B_{max} values were 36, 46, 65, and 84% of their corresponding vehicle-treated controls at 1,3, 7, and 14 days after cessation of WIN55,212-2 treatment, respectively. These results demonstrate a significant loss and relatively slow recovery of hippocampal CB$_1$ receptor binding sites after cessation of long-term cannabinoid agonist administration, the time course of which closely parallels the recovery of CB$_1$ receptor-mediated G-protein activation.

In striatum/GP, the E_{max} value of WIN55,212-2-stimulated [^{35}S]GTPγS binding in Δ^9-THC-treated mice was 64% of vehicle-treated mice 1 day after the last injection of drug (Table 2). Although the EC$_{50}$ value of WIN55,212-2 seemed to be greater in Δ^9-THC- than in vehicle-treated mice, this was not a statistically significant difference. WIN55,212-2-stimulated [^{35}S]GTPγS binding E_{max} values in Δ^9-THC-treated mice were 83 and 119% of vehicle-treated mice on days 3 and 7, respectively, and neither of these values was significantly different from vehicle control. Similar results were obtained

Fig. 1. Time-dependent normalization of CB$_1$ receptor-stimulated G-protein activation and CB$_1$ receptor binding in mouse hippocampus after cessation of long-term THC treatment. Hippocampal membranes obtained from mice 1, 3, 7, or 14 days after cessation of long-term THC treatment were incubated with 30 μM GDP, 0.1 nM [^{35}S]GTPγS, and varying concentrations of WIN55,212-2 (A) or varying concentrations of [^{3}H]SR141716A (B). Data are mean ± S.E.M. of the percentage of stimulation of [^{35}S]GTPγS binding by WIN55,212–2 ($n = 4$–5 mice per group) (A) or Scatchard plots of [^{3}H]SR141716A binding ($n = 3$ mice per group) (B). It should be noted that for visual comparison, data from vehicle-treated samples were averaged from all four time points.

TABLE 1

Time course of recovery of CB$_1$ receptor function in hippocampus after cessation of long-term cannabinoid administration

Membranes were prepared from hippocampi derived from mice repeatedly treated with vehicle, Δ^9-THC (THC), or WIN55,212-2. Samples were assayed for WIN55,212-2-stimulated [^{35}S]GTPγS binding or [^{3}H]SR141716A binding, and the data were analyzed by nonlinear regression to obtain the curve-fitted E_{max} and EC$_{50}$ or B_{max} and K_D values. Data are reported as mean values ± S.E.M. from three to five individual mice.

Treatment Group	WIN55,212-2-Stimulated [^{35}S]GTPγS Binding		[^{3}H]SR141716A Binding	
	E_{max}	EC$_{50}$	B_{max}	K_D
	% Stim	*nM*	*pmol/mg*	*nM*
Day 1				
Vehicle	155 ± 12	204 ± 80	3.18 ± 0.38	0.16 ± 0.02
THC	82 ± 10**	427 ± 179	0.73 ± 0.09**	0.14 ± 0.06
WIN	94 ± 10**	288 ± 103	1.10 ± 0.04**	0.07 ± 0.01
Day 3				
Vehicle	141 ± 13	80 ± 19	3.21 ± 0.09	0.18 ± 0.05
THC	87 ± 9**	169 ± 52	1.19 ± 0.25**	0.10 ± 0.02
WIN	91 ± 9**	140 ± 30	1.50 ± 0.17**	0.21 ± 0.13
Day 7				
Vehicle	141 ± 6.0	68 ± 8	2.96 ± 0.15	0.20 ± 0.03
THC	109 ± 11*	89 ± 10	1.26 ± 0.16**	0.12 ± 0.04
WIN	98 ± 7**	69 ± 4	1.89 ± 0.17**	0.13 ± 0.02
Day 14				
Vehicle	165 ± 8	96 ± 37	3.25 ± 0.30	0.15 ± 0.01
THC	137 ± 12*	78 ± 11	2.56 ± 0.08*	0.12 ± 0.01
WIN	147 ± 6	60 ± 5	2.69 ± 0.10*	0.09 ± 0.02

* $P < 0.05$, ** $P < 0.01$; different from corresponding value in vehicle-treated mice.

with long-term administration of WIN55,212-2, which decreased the E_{max} value of WIN55,212-2-stimulated [^{35}S]GTPγS binding to 74% of vehicle control at 1 day after cessation of WIN55,212-2 treatment, with no effect on the EC_{50} value. The E_{max} value of WIN55,212-2-stimulated [^{35}S]GTPγS binding recovered to 95% of vehicle on day 3 and therefore was not determined on days 7 or 14 after WIN55,212-2 treatment. Thus, CB_1 receptor-mediated G-protein activation in striatum/GP was slightly less attenuated by long-term cannabinoid agonist treatment and recovered more rapidly than in hippocampus.

Saturation analysis of [^{3}H]SR141716A binding was then performed in striatum/GP membranes prepared from long-term Δ^9-THC- and vehicle-treated mice at various time points after cessation of Δ^9-THC treatment. The effect of long-term WIN55,212-2 treatment was not examined in these experiments because the time course of recovery of CB_1-stimulated G-protein activation in this brain region was identical with that obtained with long-term Δ^9-THC administration. Results showed that the B_{max} value of [^{3}H]SR141716A binding in Δ^9-THC-treated mice was 51% of the corresponding vehicle-treated control value 1 day after cessation of Δ^9-THC treatment (Table 2). As observed in hippocampus, there was no effect of Δ^9-THC treatment on the [^{3}H]SR141716A K_D value. It is interesting that the number of [^{3}H]SR141716A binding sites remained reduced in Δ^9-THC-treated mice (52% of vehicle control) at 3 days after Δ^9-THC treatment and then recovered to 86 and 114% of control levels at days 7 and 14, respectively, which were not different from vehicle control. These results indicate that the time course of recovery of CB_1 receptor binding sites in striatum/GP was slightly slower than that of CB_1 receptor-stimulated G-protein activation. The results from Δ^9-THC-treated mice are summarized in Fig. 2, in which the E_{max} values of WIN55,212-2-stimulated [^{35}S]GTPγS binding and the B_{max} values of [^{3}H]SR141716A binding are plotted as a percentage of their corresponding control values in vehicle-treated mice at each time point after cessation of long-term

Δ^9-THC administration. This presentation of the data clearly illustrates the slow rate of recovery of CB_1 receptor binding sites from Δ^9-THC-treated mice to control levels in both hippocampus and striatum/GP and regional differences in the rate of recovery of CB_1-stimulated [^{35}S]GTPγS binding between these two regions.

Potential Mechanisms of Long-Lasting Attenuation of CB_1 Receptor Binding Sites. To test the hypothesis that transcriptional down-regulation of CB_1 receptors was responsible for the long-lasting decrease in CB_1 receptor binding sites, Northern blot analysis of CB_1 mRNA was performed in hippocampal extracts from mice treated as described above. Results in hippocampus showed that CB_1 receptor mRNA in Δ^9-THC-treated mice was significantly increased to 148% of vehicle-treated mice, with no difference in the 18S rRNA internal control (Fig. 3A). Hippocampal CB_1 receptor mRNA returned to control levels within 3 days after cessation of Δ^9-THC administration and remained at control levels thereafter. In contrast, results in striatum/GP showed that CB_1 receptor mRNA decreased to 57% of vehicle control 1 day after cessation of Δ^9-THC treatment (Fig. 3B). Striatal CB_1 receptor mRNA returned to control level by day 3 but then was elevated to 170% of control level at day 7 after Δ^9-THC, which was significantly greater than control levels. These results suggest that transcriptional down-regulation of CB_1 receptors does not explain the long-lasting decrease in CB_1 receptor binding sites observed after cessation of long-term Δ^9-THC administration. In hippocampus, CB_1 mRNA was increased greater than control level at 1 day after the last dose of Δ^9-THC and returned to control level within 3 days, whereas CB_1 receptor binding site levels were significantly reduced in Δ^9-THC-treated compared with vehicle-treated control mice during this time period. Although CB_1 receptor mRNA was decreased in striatum/GP 1 day after cessation of Δ^9-THC treatment, it returned to control level by 3 days and increased greater than control level at 7 days after Δ^9-THC. These results do not correspond with CB_1 receptor binding sites in this region, which remained significantly

TABLE 2
Time course of recovery of CB_1 receptor function in striatum/GP after cessation of long-term cannabinoid administration

Membranes were prepared from dissected striata/GP derived from mice repeatedly treated with vehicle, Δ^9-THC (THC), or WIN55,212-2. Samples were assayed for WIN55,212-2-stimulated [^{35}S]GTPγS binding or [^{3}H]SR141716A binding, and the data were analyzed by nonlinear regression to obtain the curve-fitted E_{max} and EC_{50} or B_{max} and K_D values. Data are reported as mean values ± S.E.M. from three to seven individual mice.

Treatment Group	WIN55,212-2-Stimulated [^{35}S]GTPγS Binding		[^{3}H]SR141716A Binding	
	E_{max}	EC_{50}	B_{max}	K_D
	% Stim	*nM*	*pmol/mg*	*nM*
Day 1				
Vehicle	109 ± 8.9	115 ± 17	2.60 ± 0.18	0.17 ± 0.04
THC	69 ± 6.9**	161 ± 28	1.30 ± 0.18**	0.37 ± 0.16
WIN	82 ± 8.2*	181 ± 40	N.D.	N.D.
Day 3				
Vehicle	91 ± 7.0	86 ± 15	3.04 ± 0.45	0.14 ± 0.01
THC	74 ± 5.2	105 ± 11	1.69 ± 0.44**	0.13 ± 0.04
WIN	76 ± 6.6	175 ± 64	N.D.	N.D.
Day 7				
Vehicle	107 ± 9.3	94 ± 11	2.81 ± 0.40	0.08 ± 0.02
THC	126 ± 1.1	176 ± 18	2.27 ± 0.06	0.13 ± 0.02
WIN	N.D.	N.D.	N.D.	N.D.
Day 14				
Vehicle	N.D.	N.D.	2.36 ± 0.21	0.09 ± 0.01
THC	N.D.	N.D.	2.65 ± 0.14	0.14 ± 0.04
WIN	N.D.	N.D.	N.D.	N.D.

N.D., not determined.
* $P < 0.05$, ** $P < 0.01$; different from corresponding value in vehicle-treated mice.

lower than control levels in Δ^9-THC-treated mice on days 3 through 7 after treatment.

The lack of correlation between CB$_1$ receptor mRNA expression and CB$_1$ receptor binding sites suggested that the long-term Δ^9-THC-induced reduction in functional CB$_1$ receptors might result from either increased degradation or decreased translation of CB$_1$ receptor protein. Therefore, Western immunoblot analysis was performed to test the hypothesis that the long-lasting decrease in CB$_1$ receptor binding sites was due to reduced levels of CB$_1$ receptor protein. Blots were prepared from hippocampal homogenates from mice treated as described above and were probed with an antibody directed against the last 15 amino acids of the CB$_1$ receptor C terminus (Bodor et al., 2005). Initial experiments to confirm antibody specificity detected major immunoreactive bands of approximately 45 and 30 kDa in lysates of mCB$_1$-transfected but not untransfected Chinese hamster ovary K1 (CHO-K1) cell membranes (Fig. 4). Other immunoreactive bands were also evident at approximately 26, 55, 63, 75, and 85 kDa, but the 55- and 85-kDa band seemed to be nonspecific because they also were evident CHO-K1 cell membranes. Similar results were obtained in membranes prepared from cerebellum of wild-type and CB$_1$ receptor knockout C57/Bl6 mice. The major immunoreactive band was 45 kDa, with minor bands at 26, 30, 64, 72, and 80 kDa,

although the 26-, 63-, and 75-kDa bands also were weakly stained in the CB$_1$ receptor knockout mice. No significant labeling was observed when the primary antibody was omitted from the reaction mixture (data not shown). Based on these results, we examined immunoblots in hippocampal membranes prepared from vehicle- and THC-treated ICR mice at 1 day after long-term Δ^9-THC treatment and performed densitometric analysis of the CB$_1$ receptor immunoreactivity (CB$_1$-ir) observed at 45 and 30 kDa. Visual inspec-

Fig. 3. Densitometric analysis of Northern blots of CB$_1$ receptor mRNA in hippocampus and striatum/GP at varying time points after cessation of long-term THC treatment. Blots of total RNA were probed for CB$_1$ receptor mRNA or 18S rRNA. Data are mean $\pm$ S.E.M. of O.D. values in samples from THC-treated mice expressed as a percentage of O.D. values in corresponding vehicle-treated mice at each time point after cessation of treatment ($n = 6$ mice per group).
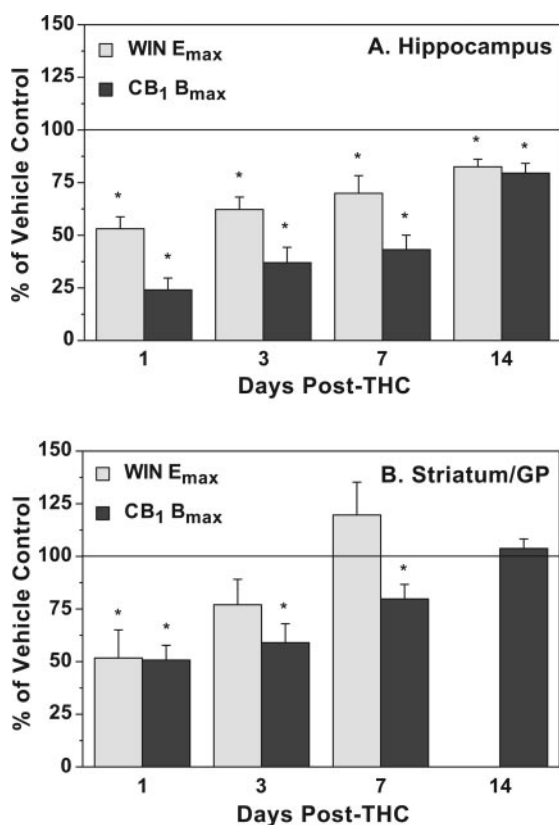
Fig. 2. Time-dependent normalization of CB$_1$ receptor-stimulated G-protein activation and CB$_1$ receptor binding in mouse hippocampus and striatum/GP after cessation of long-term THC treatment: summary graph. Data are mean $\pm$ S.E.M. of E_{max} values derived from concentration-effect curves of WIN55,212-2 (WIN)-stimulated [^{35}S]GTPγS binding or B_{max} values derived from saturation curves of [^{3}H]SR141716A (CB$_1$) binding plotted as a percentage of the corresponding values obtained in vehicle control samples at each time point. E_{max} and B_{max} values were obtained from Tables 1 and 2.
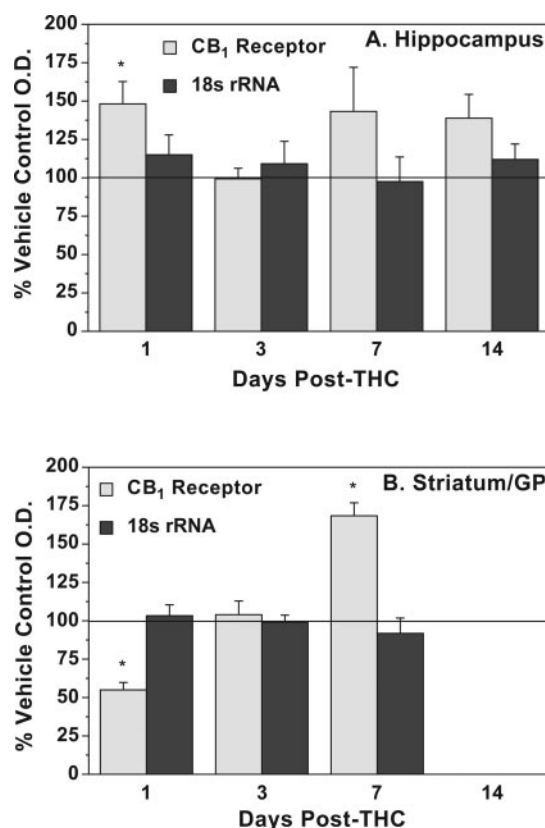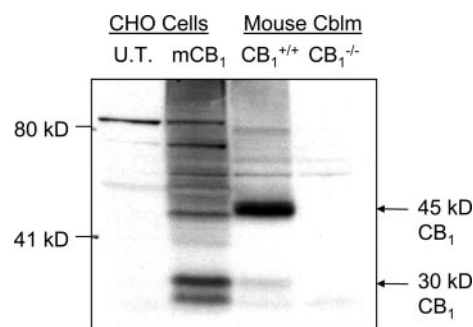
Fig. 4. Immunoblot analysis of CB$_1$ receptor protein in CHO cell and mouse cerebellar membranes. Blots of total protein were probed with an antibody directed against the last 15 residues of the CB$_1$ receptor C terminus. Shown is a representative immunoblot from two independent experiments. U.T., untransfected cells; mCB$_1$, mouse CB$_1$ receptor; Cblm, cerebellum; CB$_1^{-/-}$, CB$_1$ receptor knockout mice on a C57BL6 background; CB$_1^{+/+}$, wild-type litter mate C57BL6 mice.

tion of the immunoblots revealed that long-term Δ^9-THC treatment greatly decreased 45- and 30-kDa CB_1-ir compared with vehicle-treated mice (Fig. 5A). This trend was also observed with higher molecular mass immunoreactive bands, although these bands were faintly labeled. Densitometric analysis of the 45- and 30-kDa bands showed that CB_1-ir in Δ^9-THC-treated mice was 64 and 68%, respectively, of that obtained in vehicle control mice (Fig. 5B). In contrast, α-tubulin-ir was not different between Δ^9-THC- and vehicle-treated mice, suggesting that the decrease in CB_1-ir was not due to neuronal toxicity in vivo or unequal loading or transfer of protein in the immunoblotting procedure. These results are qualitatively similar to those obtained with saturation analysis of [^{3}H]SR141617A binding, although the apparent decrease in CB_1-ir was lesser in magnitude.

To confirm that decreased CB_1-ir in Δ^9-THC-treated mice was due to a loss in CB_1 receptor protein, the same experiment was repeated using an antibody directed against the first 77 amino acids of the CB_1 receptor N terminus (Twitchell et al., 1997). Control experiments showed that this antibody also recognized apparent 45- and 30-kDa immunoreactive bands in cerebellar membranes from $CB_1^{+/+}$ mice that were not present in $CB_1^{-/-}$ mice (data not shown). In hippocampal membranes from Δ^9-THC-treated ICR mice, CB_1-ir seemed to be greatly decreased compared with vehicle-treated mice (Fig. 6A). Densitometric analysis of the 45- and 30-kDa bands showed that CB_1-ir in Δ^9-THC-treated mice

was 36 and 50%, respectively, of that obtained in vehicle-treated mice, whereas α-tubulin-ir was unchanged (Fig. 6B). These results are similar to those obtained with the CB_1 receptor C-terminal antibody and nearly identical with results obtained with [^{3}H]SR141617A binding analysis.

Similar experiments were performed in membranes prepared from striatum/GP of Δ^9-THC- and vehicle-treated mice using the antibody against the CB_1 receptor N terminus. Visual inspection of immunoblots of striatum/GP showed an apparent decrease in 45-kDa CB_1-ir in Δ^9-THC-treated relative to vehicle control mice, whereas α-tubulin-ir appeared unchanged (Fig. 7A). There also seemed to be minor immunoreactive bands evident at higher and lower molecular mass compared with the 45 kDa CB_1-ir, and the density of some of these bands seemed to be decreased in Δ^9-THC-treated mice. However, these bands were too weakly labeled and variable for accurate quantification. Densitometric analysis of the 45-kDa band confirmed that CB_1-ir in Δ^9-THC-treated mice was 68% of that obtained in vehicle-treated mice, which was significantly different (Fig. 7B). These results are similar to results obtained with saturation analysis of [^{3}H]SR141617A binding and Northern blot analysis of CB_1 receptor mRNA in striatum/GP.

To determine whether down-regulated CB_1 receptor protein in Δ^9-THC-treated mice recovered to vehicle control levels at the same rate as CB_1 receptor binding sites, time course analysis of CB_1 immunoblots were conducted at days 1, 3, and 7 after treatment in both hippocampus and stria-
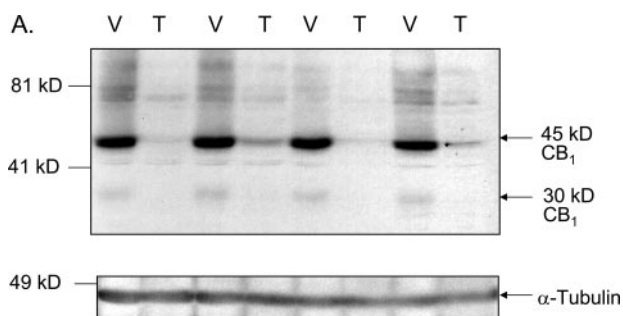

Fig. 5. Immunoblot analysis of CB_1 receptor C terminus in mouse hippocampus 1 day after cessation of long-term THC or vehicle treatment. Blots of total protein were probed with an antibody directed against the last 15 residues of the CB_1 receptor C terminus and then stripped and reprobed with an α-tubulin antibody as a loading control. A, representative blot of hippocampal membrane homogenates prepared from vehicle- or THC-treated mice 1 day after cessation of treatment. B, densitometric analysis of blots of hippocampal membrane homogenates prepared from vehicle- or THC-treated mice 1 day after cessation of treatment. Data are mean ± S.E.M. of O.D. values in samples from THC-treated mice expressed as a percentage of O.D. values in corresponding vehicle-treated mice ($n = 4$ mice per group).
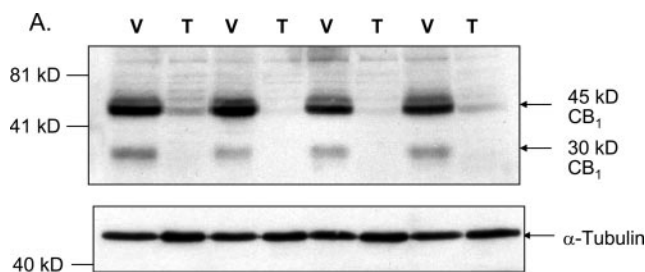
Fig. 6. Immunoblot analysis of CB_1 receptor N terminus in mouse hippocampus 1 day after cessation of long-term THC or vehicle treatment. Blots of total protein were probed with an antibody directed against the first 77 residues of the CB_1 receptor N terminus and then stripped and reprobed with an α-tubulin antibody as a loading control. A, representative blot of hippocampal membrane homogenates prepared from vehicle- or THC-treated mice 1 day after cessation of treatment. B, densitometric analysis of blots of hippocampal membrane homogenates prepared from vehicle- or THC-treated mice 1 day after cessation of treatment. Data are mean ± S.E.M. of O.D. values in samples from THC-treated mice expressed as a percentage of O.D. values in corresponding vehicle-treated mice ($n = 4$ mice per group).

tum/GP. Results in hippocampus showed that CB1-ir in Δ^9-THC-treated mice seemed decreased relative to vehicle control mice at days 1 and 3 after treatment, whereas differences between vehicle and THC-treated mice were less apparent at day 7 after treatment (Fig. 8A). Densitometric analysis of the 45- and 30-kDa CB$_1$-ir confirmed that both immunoreactive bands were significantly decreased in Δ^9-THC-treated mice at days 1 and 3 after treatment (Fig. 8B). Neither 45- nor 30-kDa CB$_1$-ir remained significantly decreased by Δ^9-THC at day 7 after treatment, although there was a trend toward a decrease. Similar results were obtained in striatum/GP, in which CB$_1$-ir seemed to be decreased in Δ^9-THC-treated relative to vehicle-treated mice at days 1 and 3 after treatment, whereas this difference seemed lesser in magnitude at 7 days (Fig. 9A). As in hippocampus, densitometric analysis conformed that 45 kD CB$_1$-ir was significantly decreased at post-treatment days 1 and 3 but not day 7 (Fig. 9B). It is noteworthy that CB$_1$-ir did not increase greater than control levels in Δ^9-THC-treated mice on day 7 after treatment, in contrast to the increase observed with CB$_1$ receptor mRNA. These results are similar to those obtained with [^{3}H]SR141617A binding, although the CB$_1$-ir seemed to recover to control levels somewhat more rapidly in hippocampus than in CB$_1$ binding sites. Nonetheless, overall results of the immunoblotting studies suggest that down-regulation of

CB$_1$ receptor binding sites corresponds with down-regulation of CB$_1$ receptor protein.

Discussion

This study demonstrates a profound and long-lasting decrease in CB$_1$ receptors and CB$_1$-stimulated G-protein activity after cessation of long-term administration of Δ^9-THC or WIN55,212-2. This paradigm has been shown to produce high levels of tolerance to behavioral effects of these drugs and elicit region-dependent decreases in both WIN55,212-2-stimulated G-protein activation and [^{3}H]SR141716A binding measured autoradiographically (Sim-Selley and Martin, 2002). Moreover, this same paradigm produced down-regulation of [^{3}H]SR141716A binding sites and desensitization of CB$_1$ receptor-stimulated G-protein activation and adenylyl cyclase inhibition in cerebellar membranes (Selley et al., 2004). These results agree with previous studies demonstrating regionally variable but widespread desensitization of CB$_1$ receptor-mediated G-protein activation and down-regulation of CB$_1$ receptor binding sites with long-term cannabinoid administration (Sim-Selley, 2003). However, this study is the first to demonstrate that these adaptive changes in CB$_1$ receptor function are paralleled by decreases in immunoreactive CB$_1$ receptor protein and persist for several days after cessation of cannabinoid treatment. The finding that long-
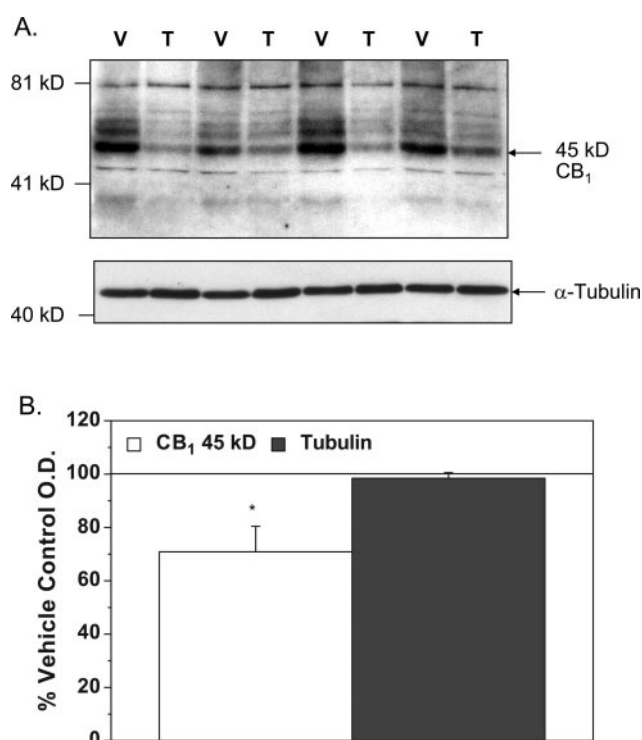

Fig. 7. Immunoblot analysis of CB$_1$ receptor N terminus in mouse striatum/GP 1 day after cessation of long-term THC or vehicle treatment. Blots of total protein were probed with an antibody directed against the first 77 residues of the CB$_1$ receptor N terminus and then stripped and reprobed with an α-tubulin antibody as a loading control. A, representative blot of striatum/GP membrane homogenates prepared from vehicle- or THC-treated mice 1 day after cessation of treatment. B, densitometric analysis of blots of striatum/GP membrane homogenates prepared from vehicle- or THC-treated mice 1 day after cessation of treatment. Data are mean ± S.E.M. of O.D. values in samples from THC-treated mice expressed as a percentage of O.D. values in corresponding vehicle-treated mice (n = 8 mice per group).
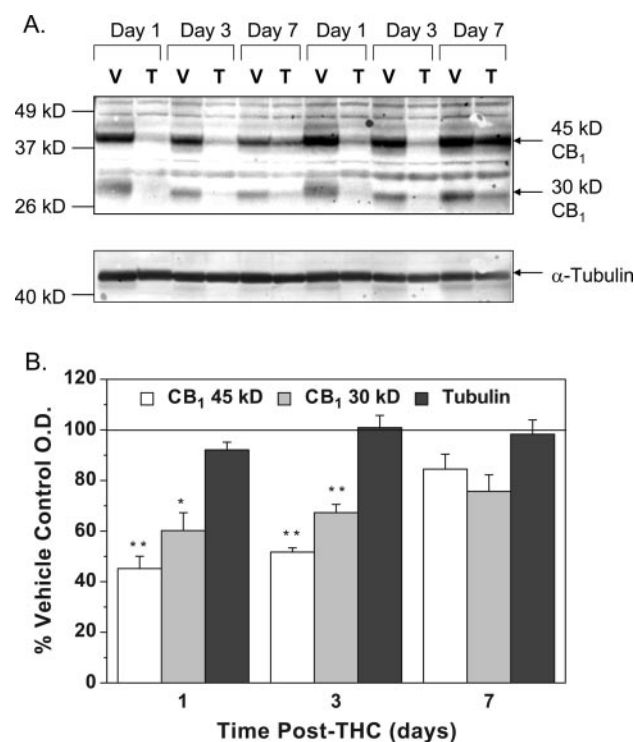
Fig. 8. Immunoblot analysis of CB$_1$ receptor protein in mouse hippocampus at varying time points after cessation of long-term THC treatment. Blots of total protein were probed with an antibody directed against the first 77 residues of the CB$_1$ receptor N terminus and then stripped and reprobed with an α-tubulin antibody as a loading control. A, representative blot of hippocampal membrane homogenates prepared from vehicle- or THC-treated mice 1, 3, 7, or 14 days after cessation of treatment. B, densitometric analysis of blots of hippocampal membrane homogenates prepared from vehicle- or THC-treated mice at the indicated time points after cessation of treatment. Data are mean ± S.E.M. of O.D. values in samples from THC-treated mice expressed as a percentage of O.D. values in corresponding vehicle-treated mice at each time point after cessation of treatment (n = 3 mice per group per time point).

term treatment with Δ^9-THC or WIN55,212-2 produced a similar time course for recovery of CB_1 receptor function suggests that this prolonged adaptive response is a fundamental property of CB_1 receptor regulation. The finding that the K_D values of [^{3}H]SR141716A binding in cannabinoid-treated mice were not different from vehicle at any time point after treatment with either drug indicates a lack of effect of residual drug in the tissue. Furthermore, the lack of decrease in either α-tubulin protein or 18S rRNA at any time point suggests that there was no neuronal loss due to toxicity, as suggested by some previous studies (Landfield et al., 1988; Chan et al., 1998).

Potential Mechanisms of CB_1 Receptor Adaptation. The interpretation that CB_1 receptor protein is down-regulated by long-term Δ^9-THC treatment is based on results of immunoblot analyses. It is interesting to note that CB_1-ir was detected at multiple apparent molecular masses, suggestive of post-translational processing, multiple splice variants, and/or multimerization. The finding that multiple bands of CB_1-ir were identified in $CB_1^{+/+}$ but not $CB_1^{-/-}$ mice indicated that they represent CB_1 receptor protein. The predicted molecular mass of unmodified CB_1 receptor protein is 53 kDa, but the major immunoreactive band was observed at an apparent mass of 45 kDa. This enhanced mobility of unglycosylated CB_1 receptor has been reported previously and is commonly observed for other membrane proteins as a result of their biophysical properties in SDS micelles (Andersson et

Fig. 9. Immunoblot analysis of CB_1 receptor protein in mouse striatum/GP at varying time points after cessation of long-term THC treatment. Blots of total protein were probed with an antibody directed against the first 77 residues of the CB_1 receptor N terminus and then stripped and reprobed with an α-tubulin antibody as a loading control. A, representative blot of striatal/GP membrane homogenates prepared from vehicle- or THC-treated mice 1, 3, 7, or 14 days after cessation of treatment. B, densitometric analysis of blots of striatal/GP membrane homogenates prepared from vehicle- or THC-treated mice at the indicated time points after cessation of treatment. Data are mean ± S.E.M. of O.D. values in samples from THC-treated mice expressed as a percentage of O.D. values in corresponding vehicle-treated mice at each time point after cessation of treatment ($n = 3$ mice per group per time point).

al., 2003). There was another prominent band of 30 kDa in the hippocampus, whereas multiple higher mass forms were apparent in striatum/GP. In both regions, these multiple forms were present in vehicle-treated mice, and most were decreased after long-term Δ^9-THC administration. Thus, it did not seem that CB_1-ir was being transformed from higher to lower mass as a result of limited proteolysis induced by agonist treatment. Moreover, the 30-kDa CB_1-ir in hippocampus was detected by antisera directed against both the C and N termini, indicating that it is unlikely to be a proteolytic product or N-terminal splice variant (Shire et al., 1995; Ryberg et al., 2005).

The prolonged change in CB_1 receptor protein and function demonstrates that these cellular adaptations persist after the cessation of drug treatment. The finding that CB_1 receptor binding sites did not fully recover to baseline until 1 to 2 weeks had elapsed suggests that there is a very stable mechanism for CB_1 receptor regulation. This study examined the possibility that transcriptional regulation could be responsible for this persistent decrease in CB_1 receptors. However, results in hippocampus clearly showed no decrease in CB_1 receptor mRNA; in fact, it was increased greater than control levels 1 day after Δ^9-THC treatment. In striatum/GP, however, CB_1 receptor mRNA was significantly decreased by Δ^9-THC at 1 day after treatment. Both of these results are in agreement with previously published findings on brain region-dependent effects of long-term cannabinoid administration on CB_1 receptor transcription (Rubino et al., 1994; Zhuang et al., 1998). Although decreased CB_1 receptor mRNA in striatum/GP could contribute to CB_1 receptor down-regulation, the time course of recovery of CB_1 mRNA in this region (3 days) did not match the normalization rate of CB_1 receptor protein or binding (7 days). It is possible that CB_1 receptor protein synthesis could be delayed relative to transcription, but Howlett and colleagues have reported that CB_1 receptors are synthesized rapidly (<2 h) in neuroblastoma cells (McIntosh et al., 1998). These results suggest that an alternative mechanism contributes to the persistent down-regulation of CB_1 receptors in both regions examined.

One alternative mechanism for long-term agonist-induced adaptations of CB_1 receptors is phosphorylation by G-protein-coupled receptor kinases followed by β-arrestin-mediated desensitization, internalization, and degradation. Evidence for this mechanism has been obtained in oocytes, in which coexpression of G-protein-coupled receptor kinase 3 and β-arrestin-2 enhanced agonist-induced CB_1 receptor desensitization in a manner requiring putative phosphorylation sites in the C-terminal tail (Jin et al., 1999). Also consistent with this mechanism was the finding of agonist-induced internalization of CB_1 receptors via the clathrin-coated pit pathway in transfected AtT20 cells (Hsieh et al., 1999). In that study, return of CB_1 receptors to the cell surface required protein synthesis after long (>90 min) but not short (20 min) incubations, suggesting that receptor degradation was occurring during longer agonist exposures. However, in contrast to studies performed in non-neuronal cells, incubation of cultured hippocampal neurons with agonist for 16 h produced no change in CB_1-ir using immunoblot analysis (Coutts et al., 2001). Likewise, no change in immunoprecipitated CB_1 receptor protein was observed in N18TG2 neuroblastoma cells after 10-h agonist incubation (McIntosh et al., 1998). Other studies have found that CB_1 receptors
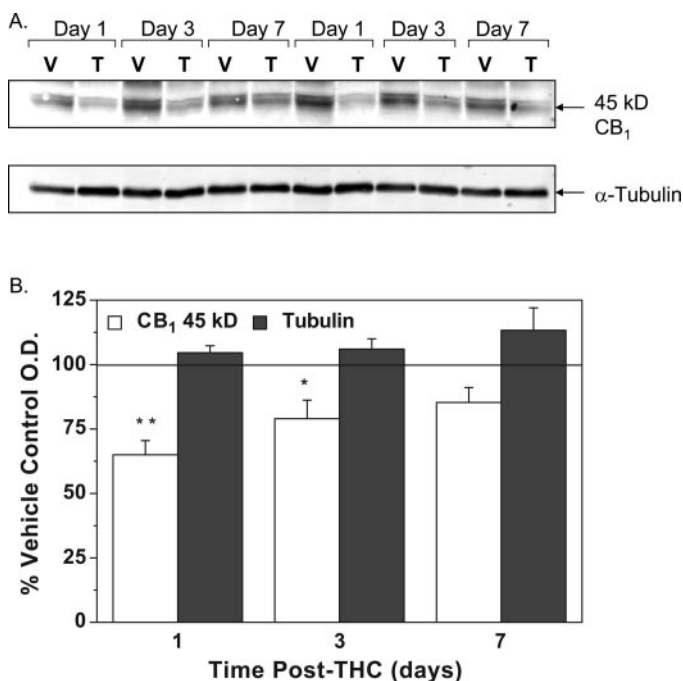
heterologously expressed in human embryonic kidney 293 cells (Shapira et al., 2003) or CHO cells (Rinaldi-Carmona et al., 1998) were or were not down-regulated by agonist treatment, respectively, suggesting that CB$_1$ receptor down-regulation depends on cell type.

Implications of Regional Differences in CB$_1$ Receptor Adaptation. The properties of CB$_1$ receptor adaptation to long-term cannabinoid administration have been shown previously to vary between hippocampus and basal ganglia, which exhibited differences in magnitude and rate of development of desensitization and down-regulation (Breivogel et al., 1999). The results of the present study further demonstrate that normalization of CB$_1$ receptors after cessation of long-term Δ^9-THC treatment differs between these regions. The most striking observation was that CB$_1$ receptor levels and stimulation of [^{35}S]GTPγS binding returned to control levels within 3 to 7 days after treatment in striatum/GP, whereas in hippocampus, these measures remained decreased for 14 days after treatment cessation. These results are interesting, considering previous results showing that CB$_1$ receptor function in striatum/GP exhibited a slower time course of adaptation compared with hippocampus during the development of Δ^9-THC tolerance (Breivogel et al., 1999). Together, these studies indicate a greater tendency for adaptation in hippocampus compared with striatum/GP. Other differences include the finding that in hippocampus, the loss in G-protein activation closely paralleled the decrease in [^{3}H]SR141716A binding sites, whereas in striatum/GP, CB$_1$ receptors remained decreased lower than control levels for a longer duration than G-protein activation. This finding suggests the presence of receptor reserve for G-protein activation in striatum/GP, such that normal levels of receptor-mediated G-protein activation did not require full recovery to normal CB$_1$ receptor levels. There were also regional differences in the regulation of CB$_1$ receptor mRNA by long-term Δ^9-THC treatment. CB$_1$ receptor mRNA was decreased in striatum/GP 1 day after treatment, whereas CB$_1$ mRNA in the hippocampus was not decreased at any time point, as discussed above.

The persistent and regionally variable decrease in CB$_1$ receptor-mediated G-protein activation has implications for recovery from tolerance to the behavioral effects of cannabinoids. These results suggest that tolerance to cannabinoid-mediated memory impairment might persist longer than tolerance to motor effects, but this question has not been addressed directly. The time course for recovery of tolerance to cannabinoid-mediated hypomotility was examined in mice treated with Δ^9-THC for 6.5 days (Bass and Martin, 2000). Mice remained tolerant to Δ^9-THC-mediated hypomotility when tested 4.5 days after treatment cessation, but tolerance had disappeared by 7.5 days after treatment. This time course is consistent with the present finding that CB$_1$ receptors and G-protein activity returned to control levels by 3 to 7 days after treatment.

Summary and Conclusions. In conclusion, long-term administration of Δ^9-THC or WIN55,212-2 produced attenuation of CB$_1$ receptor-mediated G-protein activation and down-regulation of CB$_1$ receptors that persisted for several days after the cessation of treatment. CB$_1$ receptor function returned to vehicle control levels more rapidly in striatum/GP than hippocampus, suggesting that tolerance to the memory-impairing effects of cannabinoids might persist

longer than tolerance to behaviors mediated by striatum/GP. The mechanism underlying down-regulation of CB$_1$ receptor binding sites by long-term cannabinoid treatment seemed to involve decreased CB$_1$ receptor protein in both regions, but evidence for decreased transcription was obtained only in striatum/GP, in which the recovery rate of the transcript was more rapid than that of CB$_1$ receptor protein or binding. These results suggest that post-transcriptional mechanisms, such as persistent internalization and degradation of CB$_1$ receptors, might underlie CB$_1$ adaptation to long-term agonist exposure.

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