Prolonged Recovery Rate of CB₁ Receptor Adaptation after Cessation of Long-Term Cannabinoid Administration

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ABSTRACT

Long-term cannabinoid administration produces region-dependent CB₁ receptor desensitization and down-regulation. This study examined the time course for normalization of CB₁ receptors and G-protein activation using [³⁵S]GTPγS binding, respectively, in hippocampus and striatum/globus pallidus (GP). Mice were treated with escalating doses of Δ⁹-tetrahydrocannabinol (Δ⁹-THC) or R(+)-[2,3-dihydro-5-methyl-3-[(morphinyl)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. [³⁵S]SR141716A and WIN55,212-2-stimulated [³⁵S]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. CB₁ receptor binding did not recover to control levels until day 7 or 14 after treatment in striatum/GP and hippocampus, respectively. The mechanism of CB₁ binding site down-regulation was investigated after long-term Δ⁹-THC treatment. Analysis of CB₁ receptor mRNA in hippocampus and striatum/GP showed that transcriptional regulation could not explain prolonged recovery rates from CB₁ receptor down-regulation. In contrast, CB₁ receptor protein, as determined by immunoblot analysis, matched the down-regulation and recovery rates of CB₁ receptor binding sites relatively closely. These data demonstrate that cannabinoid-induced decreases in CB₁ receptor function persist for relatively long time periods after cessation of long-term drug treatment and that CB₁ receptor signaling recovers more quickly in striatum/GP than hippocampus. Moreover, down-regulation of CB₁ receptor binding sites does not seem to result mainly from transcriptional regulation, suggesting that adaptive regulation of CB₁ receptors in brain primarily occurs at the protein level.

The behavioral effects of cannabinoids such as Δ⁹-tetrahydrocannabinol (Δ⁹-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 CB₁ 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). CB₁ receptors primarily activate pertussis toxin-sensitive G-proteins of the Go family 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 Δ⁹-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 CB₁ receptor binding (down-regulation) and CB₁ 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; CB₁, CB₁ receptor immunoreactivity; THC, Δ⁹-tetrahydrocannabinol; [³⁵S]GTPγS, guanosine 5′-O-[3-[³⁵S]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-[(morphinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-[1-naphthalenyl]methanone mesylate.
are believed to contribute to tolerance (Martin et al., 2004). Decreased CB1 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 CB1 receptors in hippocampus and cerebellum exhibiting greater magnitude and more rapid adaptations than those in basal ganglia (Breivogel et al., 1999). Changes in CB1 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 CB1 receptor function might exhibit regional differences. However, the time course of CB1 receptor normalization after cessation of cannabinoid treatment has not been assessed at the cellular level.

The mechanisms by which long-term cannabinoid administration attenuates CB1 receptor function in the brain has also not been resolved. The finding of a close regional and temporal correlation between decreases in CB1 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 CB1 receptors. However, Coutts et al. (2001) reported that although CB1 receptors were internalized by agonist treatment in cultured hippocampal neurons, persistent exposure to agonist did not decrease CB1 receptor protein. Likewise, Cichewicz et al. (2001) reported no decrease in CB1 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 CB1 receptor mRNA during long-term THC treatment did not correspond well with the time course of changes in CB1 receptor binding sites. Thus, it remains to be determined whether down-regulation of CB1 receptor binding sites is mediated by a reduction in CB1 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 Δ9-THC or WIN55,212-2 using a paradigm that produces high levels of tolerance in vivo and CB1 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 CB1 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 CB1 receptors, mediate motor (basal ganglia) and memory (hippocampus) effects of cannabinoids, and differ in the rate of development of CB1 receptor adaptation.

### Materials and Methods

**Materials.** ICR mice (male, 24–30 g) were purchased from Harlan Laboratories (Indianapolis, IN). [35S]GTPγS (1250 Ci/mmol) and GeneScreen Plus nylon membrane were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [3H]SR141716A (49 Ci/mmol) and Enhanced Chemiluminescence (ECL) and ECL Plus reagent were purchased from GE Healthcare (Piscataway, NJ). Δ9-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). CB1 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 established National Institutes of Health guidelines and were approved by the Internal Animal Care and Use Committee of Virginia Commonwealth University. Δ9-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 Δ9-THC, WIN55,212-2, or vehicle for 15 days (Sim-Selley and Martin, 2002). Initial doses of drugs were 10 mg/kg Δ9-THC and 3 mg/kg WIN55,212-2, and the doses were doubled every 3 days to final doses of 160 mg/kg Δ9-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 [35S]GTPγS Binding.** Each tissue sample was placed in 20 volumes of ice-cold membrane buffer (50 mM Tris-HCl, 3 mM MgCl2, and 1 mM EGTA, pH 7.4) and homogenized in ScintiSafe Econo 1 scintillation fluid (Fisher Scientific). Membranes were prepared as above, diluted with assay buffer A, and incubated under assay conditions. Saturation analysis of [3H]SR141716A binding was performed by incubating to 5 to 10 μg membrane protein with 0.02 to 5 nM [3H]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 spectrometry 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 isopropanol 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/ml ethidium bromide solution added to each sample before loading onto a horizontal agarose gel (0.8% SDS, and 5% nonfat dry milk) and incubated with the blot for 30 min. 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 [35S]GTPγS binding is defined as agonist-stimulated minus basal [35S]GTPγS binding. Percentage of stimulation is defined as (net-stimulated/basal [35S]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 CB₁ 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. CB₁ receptor binding was assessed using varying concentrations of the CB₁ selective antagonist [3H]SR141617A (rimonabant), and CB₁ receptor-mediated G-protein activation was examined using WIN55,212-2-stimulated [35S]GTPγS binding.

In hippocampus, results showed that WIN55,212-2-stimulated [35S]GTPγS binding was significantly reduced 1 day after cessation of Δ⁹-THC treatment (Fig. 1A), as previously demonstrated using [35S]GTPγS autoradiography (Sim-Selley and Martin, 2002). G-protein activation by WIN55,212-2 in Δ⁹-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 Δ⁹-THC treatment, the E₅₀ value of WIN55,212-2-stimulated [35S]GTPγS binding was 53% of the corresponding vehicle-treated control. Although the EC₅₀ value of WIN55,212-2 was approximately twice that of vehicle-treated mice at 1 day after Δ⁹-THC treatment, this was not a statistically significant difference. The WIN55,212-2 E₅₀ values then increased to 62, 70, and 83% of vehicle control at days 3, 7, and 14 after Δ⁹-THC, respectively. Similar results were obtained in hippocampal membranes from WIN55,212-2-treated mice. The E₅₀ values of WIN55,212-2-stimulated [35S]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 Δ⁹-
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 CB₁ receptor signaling in hippocampus after cessation of long-term cannabinoid agonist administration.

Similar results were obtained in hippocampus with CB₁ receptor binding. The number of [³H]SR141716A binding sites was reduced at 1 day after Δ9-THC treatment (Fig. 1B). Nonlinear regression analysis of [³H]SR141716A saturation binding data confirmed that the $B_{\text{max}}$ value at 1 day after Δ9-THC treatment was 24% of that obtained in vehicle-treated controls, with no difference in $K_D$ value (Table 1). [³H]SR141716A binding in Δ9-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 [³S]GTPγS binding. [³H]SR141716A $B_{\text{max}}$ values in Δ2-THC-treated mice were 37, 43, and 80% of their corresponding vehicle-treated controls at days 3, 7, and 14 after Δ9-THC, respectively (Table 1). It is noteworthy that [³H]SR141716A $K_D$ values did not differ between Δ9-THC- and vehicle-treated mice at any time point after Δ9-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 [³H]SR141716A $B_{\text{max}}$ 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 CB₁ receptor binding sites after cessation of long-term cannabinoid agonist administration, the time course of which closely parallels the recovery of CB₁ receptor-mediated G-protein activation.

In striatum/GP, the $E_{\text{max}}$ value of WIN55,212-2-stimulated [³S]GTPγS binding in Δ9-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 Δ9-THC- than in vehicle-treated mice, this was not a statistically significant difference. WIN55,212-2-stimulated [³S]GTPγS binding $E_{\text{max}}$ values in Δ9-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

### TABLE 1

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>WIN55,212-2-Stimulated [³S]GTPγS Binding</th>
<th>[³H]SR141716A Binding</th>
</tr>
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<tr>
<td></td>
<td>$E_{\text{max}}$</td>
<td>$EC_{50}$</td>
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<tr>
<td></td>
<td>% Stim</td>
<td>nM</td>
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<tr>
<td>Day 1</td>
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</tr>
<tr>
<td>Vehicle</td>
<td>155 ± 12</td>
<td>204 ± 80</td>
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<tr>
<td>THC</td>
<td>82 ± 10**</td>
<td>427 ± 179</td>
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<tr>
<td>WIN</td>
<td>94 ± 10**</td>
<td>288 ± 103</td>
</tr>
<tr>
<td>Day 3</td>
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<tr>
<td>Vehicle</td>
<td>141 ± 13</td>
<td>80 ± 19</td>
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<tr>
<td>THC</td>
<td>87 ± 9**</td>
<td>169 ± 52</td>
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<tr>
<td>WIN</td>
<td>91 ± 9**</td>
<td>140 ± 30</td>
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<tr>
<td>Day 7</td>
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<tr>
<td>Vehicle</td>
<td>141 ± 6.0</td>
<td>68 ± 8</td>
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<tr>
<td>THC</td>
<td>109 ± 11*</td>
<td>89 ± 10</td>
</tr>
<tr>
<td>WIN</td>
<td>98 ± 7**</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>Day 14</td>
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<tr>
<td>Vehicle</td>
<td>165 ± 8</td>
<td>96 ± 37</td>
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<tr>
<td>THC</td>
<td>132 ± 12*</td>
<td>78 ± 11</td>
</tr>
<tr>
<td>WIN</td>
<td>147 ± 6</td>
<td>60 ± 5</td>
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* $P < 0.05$, ** $P < 0.01$; different from corresponding value in vehicle-treated mice.
with long-term administration of WIN55,212-2, which decreased the $E_{\text{max}}$ value of WIN55,212-2-stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S} \text{ binding to } 74\% \text{ of vehicle control at 1 day after cessation of WIN55,212-2 treatment, with no effect on the } E_{50} \text{ value. The } E_{\text{max}} \text{ value of WIN55,212-2-stimulated } [^{35}\text{S}]\text{GTP} \gamma \text{S binding recovered to } 95\% \text{ of vehicle on day 3 and therefore was not determined on days 7 or 14 after WIN55,212-2 treatment. Thus, CB}_1 \text{ 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]\text{SR141716A binding was then performed in striatum/GP membranes prepared from long-term } \Delta^9\text{-THC- and vehicle-treated mice at various time points after cessation of } \Delta^9\text{-THC treatment. The effect of long-term WIN55,212-2 treatment was not examined in these experiments because the time course of recovery of } \text{CB}_1 \text{ receptor binding sites observed after cessation of } \Delta^9\text{-THC treatment does not explain the long-lasting decrease in } \text{CB}_1 \text{ receptor binding sites, Northern blot analysis of } \text{CB}_1 \text{ mRNA was performed in hippocampal extracts from mice treated as described above. Results in hippocampus showed that } \text{CB}_1 \text{ receptor mRNA in } \Delta^9\text{-THC-treated mice was significantly increased to } 148\% \text{ of vehicle-treated mice, with no difference in the } 18\text{S rRNA internal control (Fig. 3A). Hippocampal } \text{CB}_1 \text{ receptor mRNA returned to control levels within 3 days after cessation of } \Delta^9\text{-THC administration and remained at control levels thereafter.}

### Potential Mechanisms of Long-Lasting Attenuation of CB1 Receptor Binding Sites

To test the hypothesis that transcriptional down-regulation of CB1 receptors was responsible for the long-lasting decrease in CB1 receptor binding sites, Northern blot analysis of CB1 mRNA was performed in hippocampal extracts from mice treated as described above. Results in hippocampus showed that CB1 receptor mRNA in $\Delta^9\text{-THC}$-treated mice was significantly increased to $148\%$ of vehicle-treated mice, with no difference in the $18\text{S rRNA internal control (Fig. 3A).}$

### Table 2

#### Time course of recovery of CB1 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, $\Delta^9\text{-THC}$ (THC), or WIN55,212-2. Samples were assayed for WIN55,212-2-stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S} \text{ binding and } [^{3}H]\text{SR141716A binding, and the data were analyzed by nonlinear regression to obtain the curve-fitted } E_{\text{max}} \text{ and } E_{50} \text{ or } B_{\text{max}} \text{ and } K_{D} \text{ values. Data are reported as mean values ± S.E.M. from three to seven individual mice.}

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>WIN55,212-2-Stimulated $[^{35}\text{S}]\text{GTP} \gamma \text{S} \text{ Binding}</th>
<th>$[^{3}H]\text{SR141716A Binding}</th>
<th>$E_{\text{max}} \text{ pmol/mg}$</th>
<th>$E_{50} \text{ nM}$</th>
<th>$B_{\text{max}} \text{ pmol/mg}$</th>
<th>$K_{D} \text{ nM}$</th>
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<tr>
<td>Vehicle</td>
<td>109 ± 8.9</td>
<td>115 ± 17</td>
<td>2.60 ± 0.18</td>
<td>0.17 ± 0.04</td>
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<td>THC</td>
<td>69 ± 6.9**</td>
<td>161 ± 28</td>
<td>1.30 ± 0.18**</td>
<td>0.37 ± 0.16</td>
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<tr>
<td>WIN</td>
<td>82 ± 8.2*</td>
<td>181 ± 40</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
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<td>Day 3</td>
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<tr>
<td>Vehicle</td>
<td>91 ± 7.0</td>
<td>86 ± 15</td>
<td>3.04 ± 0.45</td>
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<td>THC</td>
<td>74 ± 5.2</td>
<td>105 ± 11</td>
<td>1.69 ± 0.44**</td>
<td>0.13 ± 0.04</td>
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<tr>
<td>WIN</td>
<td>76 ± 6.6</td>
<td>175 ± 64</td>
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<td>Day 7</td>
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<tr>
<td>Vehicle</td>
<td>107 ± 9.3</td>
<td>94 ± 11</td>
<td>2.81 ± 0.40</td>
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<td>THC</td>
<td>126 ± 1.1</td>
<td>176 ± 18</td>
<td>2.27 ± 0.06</td>
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<tr>
<td>WIN</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>Day 14</td>
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<tr>
<td>Vehicle</td>
<td>N.D.</td>
<td>N.D.</td>
<td>2.36 ± 0.21</td>
<td>0.09 ± 0.01</td>
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<td>THC</td>
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<td>2.65 ± 0.14</td>
<td>0.14 ± 0.04</td>
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N.D., not determined.

* $P < 0.05$, ** $P < 0.01$; different from control value.
lower than control levels in Δ⁹-THC-treated mice on days 3 through 7 after treatment.

The lack of correlation between CB₁ receptor mRNA expression and CB₁ receptor binding sites suggested that the long-term Δ⁹-THC-induced reduction in functional CB₁ receptors might result from either increased degradation or decreased translation of CB₁ receptor protein. Therefore, Western immunoblot analysis was performed to test the hypothesis that the long-lasting decrease in CB₁ receptor binding sites was due to reduced levels of CB₁ 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 CB₁ 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 mCB₁-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 CB₁ 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 CB₁ 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 Δ⁹-THC treatment and performed densitometric analysis of the CB₁ receptor immunoreactivity (CB₁-ir) observed at 45 and 30 kDa. Visual inspec-

Fig. 2. Time-dependent normalization of CB₁ receptor-stimulated G-protein activation and CB₁ receptor binding in mouse hippocampus and striatum/GP after cessation of long-term THC treatment: summary graph. Data are mean ± S.E.M. of Eₘ₉₉₉ values derived from concentration-effect curves of WIN55,212-2 (WIN)-stimulated [³⁵S]GTPγS binding or Bₘ₉₉₉ values derived from saturation curves of [³H]SR141716A (CB₁) binding plotted as a percentage of the corresponding values obtained in vehicle control samples at each time point. Eₘ₉₉₉ and Bₘ₉₉₉ values were obtained from Tables 1 and 2.

Fig. 3. Densitometric analysis of Northern blots of CB₁ 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 CB₁ receptor mRNA or 18S rRNA. 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 = 6 mice per group).

Fig. 4. Immunoblot analysis of CB₁ 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 CB₁ receptor C terminus. Shown is a representative immunoblot from two independent experiments. U.T., untransfected cells; mCB₁, mouse CB₁ receptor; Chbm, cerebellum; CB₁−/−, CB₁ receptor knockout mice on a C57Bl6 background; CB₁+/−, wild-type litter mate C57Bl6 mice.
tion of the immunoblots revealed that long-term ∆9-THC treatment greatly decreased 45- and 30-kDa CB1-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 CB1-ir in ∆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 ∆9-THC- and vehicle-treated mice, suggesting that the decrease in CB1-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 [3H]SR141617A binding, although the apparent decrease in CB1-ir was lesser in magnitude.

To confirm that decreased CB1-ir in ∆9-THC-treated mice was due to a loss in CB1 receptor protein, the same experiment was repeated using an antibody directed against the first 77 amino acids of the CB1 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 CB1+/− mice that were not present in CB1−/− mice (data not shown). In hippocampal membranes from ∆9-THC-treated ICR mice, CB1-ir seemed to be greatly decreased compared with vehicle-treated mice (Fig. 6A). Densitometric analysis of the 45- and 30-kDa bands showed that CB1-ir in ∆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 CB1 receptor C-terminal antibody and nearly identical with results obtained with [3H]SR141617A binding analysis.

Similar experiments were performed in membranes prepared from striatum/GP of ∆9-THC- and vehicle-treated mice using the antibody against the CB1 receptor N terminus. Visual inspection of immunoblots of striatum/GP showed an apparent decrease in 45-kDa CB1-ir in ∆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 CB1-ir, and the density of some of these bands seemed to be decreased in ∆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 CB1-ir in ∆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 [3H]SR141617A binding and Northern blot analysis of CB1 receptor mRNA in striatum/GP.

To determine whether down-regulated CB1 receptor protein in ∆9-THC-treated mice recovered to vehicle control levels at the same rate as CB1 receptor binding sites, time course analysis of CB1 immunoblots were conducted at days 1, 3, and 7 after treatment in both hippocampus and stria-

Fig. 5. Immunoblot analysis of CB1 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 CB1 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 CB1 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 CB1 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 Δ9-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 CB1-ir confirmed that both immunoreactive bands were significantly decreased in Δ9-THC-treated mice at days 1 and 3 after treatment (Fig. 8B). Neither 45- nor 30-kDa CB1-ir remained significantly decreased by Δ9-THC at day 7 after treatment, although there was a trend toward a decrease. Similar results were obtained in striatum/GP, in which CB1-ir seemed to be decreased in Δ9-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 confirmed that 45 kD CB1-ir was significantly decreased at post-treatment days 1 and 3 but not day 7 (Fig. 9B). It is noteworthy that CB1-ir did not increase greater than control levels in Δ9-THC-treated mice on day 7 after treatment, in contrast to the increase observed with CB1 receptor mRNA. These results are similar to those obtained with [3H]SR141617A binding, although the CB1-ir seemed to recover to control levels somewhat more rapidly in hippocampus than in CB1 binding sites. Nonetheless, overall results of the immunoblotting studies suggest that down-regulation of CB1 receptor binding sites corresponds with down-regulation of CB1 receptor protein.

Discussion

This study demonstrates a profound and long-lasting decrease in CB1 receptors and CB1-stimulated G-protein activity after cessation of long-term administration of Δ9-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 [3H]SR141716A binding measured autoradiographically (Sim-Selley and Martin, 2002). Moreover, this same paradigm produced down-regulation of [3H]SR141716A binding sites and desensitization of CB1 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 CB1 receptor-mediated G-protein activation and down-regulation of CB1 receptor binding sites with long-term cannabinoid administration (Sim-Selley, 2003). However, this study is the first to demonstrate that these adaptive changes in CB1 receptor function are paralleled by decreases in immunoreactive CB1 receptor protein and persist for several days after cessation of cannabinoid treatment. The finding that long-
term treatment with Δ⁹-THC or WIN55,212-2 produced a similar time course for recovery of CB₁ receptor function suggests that this prolonged adaptive response is a fundamental property of CB₁ receptor regulation. The finding that the Kᵦ values of [³H]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 CB₁ Receptor Adaptation.**

The interpretation that CB₁ receptor protein is down-regulated by long-term Δ⁹-THC treatment is based on results of immunoblot analyses. It is interesting to note that CB₁-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 CB₁-ir were identified in CB₁⁻/- but not CB₁⁻/⁻ mice indicated that they represent CB₁ receptor protein. The predicted molecular mass of unmodified CB₁ receptor protein is 53 kDa, but the major immunoreactive band was observed at an apparent mass of 45 kDa. This enhanced mobility of unglycosylated CB₁ 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 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 Δ⁹-THC administration. Thus, it did not seem that CB₁-ir was being transformed from higher to lower mass as a result of limited proteolysis induced by agonist treatment. Moreover, the 30-kDa CB₁-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 CB₁ receptor protein and function demonstrates that these cellular adaptations persist after the cessation of drug treatment. The finding that CB₁ 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 CB₁ receptor regulation. This study examined the possibility that transcriptional regulation could be responsible for this persistent decrease in CB₁ receptors. However, results in hippocampus clearly showed no decrease in CB₁ receptor mRNA; in fact, it was increased greater than control levels 1 day after Δ⁹-THC treatment. In striatum/GP, however, CB₁ receptor mRNA was significantly decreased by Δ⁹-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 CB₁ receptor transcription (Rubino et al., 1994; Zhuang et al., 1998). Although decreased CB₁ receptor mRNA in striatum/GP could contribute to CB₁ receptor down-regulation, the time course of recovery of CB₁ mRNA in this region (3 days) did not match the normalization rate of CB₁ receptor protein or binding (7 days). It is possible that CB₁ receptor protein synthesis could be delayed relative to transcription, but Howlett and colleagues have reported that CB₁ 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 CB₁ receptors in both regions examined.

One alternative mechanism for long-term agonist-induced adaptations of CB₁ 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 CB₁ 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 CB₁ receptors via the clathrin-coated pit pathway in transfected AtT20 cells (Hsieh et al., 1999). In that study, return of CB₁ 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 CB₁-ir using immunoblot analysis (Coutts et al., 2001). Likewise, no change in immunoprecipitated CB₁ receptor protein was observed in N18TG2 neuroblastoma cells after 10-h agonist incubation (McIntosh et al., 1998). Other studies have found that CB₁ 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 CB1 receptor down-regulation depends on cell type.

**Implications of Regional Differences in CB1 Receptor Adaptation.** The properties of CB1 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 CB1 receptors after cessation of long-term ∆9-THC treatment differs between these regions. The most striking observation was that CB1 receptor levels and stimulation of [35S]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 CB1 receptor function in striatum/GP exhibited a slower time course of adaptation compared with hippocampus during the development of ∆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 [3H]SR141716A binding sites, whereas in striatum/GP, CB1 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 CB1 receptor levels. There were also regional differences in the regulation of CB1 receptor mRNA by long-term ∆9-THC treatment. CB1 receptor mRNA was decreased in striatum/GP 1 day after treatment, whereas CB1 mRNA in the hippocampus was not decreased at any time point, as discussed above.

The persistent and regionally variable decrease in CB1 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 ∆9-THC for 6.5 days (Bass and Martin, 2000). Mice remained tolerant to ∆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 CB1 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 ∆9-THC or WIN55,212-2 produced attenuation of CB1 receptor-mediated G-protein activation and down-regulation of CB1 receptors that persisted for several days after the cessation of treatment. CB1 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 CB1 receptor binding sites by long-term cannabinoid treatment seemed to involve decreased CB1 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 CB1 receptor protein or binding. These results suggest that post-transcriptional mechanisms, such as persistent internalization and degradation of CB1 receptors, might underlie CB1 adaptation to long-term agonist exposure.

**References**


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