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Evidence for a New G Protein-Coupled Cannabinoid Receptor in Mouse Brain

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ABSTRACT

The purpose of these studies was to support the hypothesis that an undiscovered cannabinoid receptor exists in brain. [^{35}S]GTPγS binding was stimulated by anandamide and WIN55212-2 in brain membranes from both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. In contrast, a wide variety of other compounds that are known to activate CB_1 receptors, including CP55940, HU-210, and Δ^9-tetrahydrocannabinol, failed to stimulate [^{35}S]GTPγS binding in $CB_1^{-/-}$ membranes. In $CB_1^{-/-}$ membranes, SR141716A affected both basal and anandamide- or WIN55212-2-induced stimulation of [^{35}S]GTPγS binding only at concentrations greater than 1 μM. In $CB_1^{+/+}$ membranes, SR141716A inhibited only 84% of anandamide and 67% of WIN55212-2 stimulated [^{35}S]GTPγS binding with an affinity appropriate for mediation by CB_1 receptors ($K_B \approx 0.5$ nM). The remaining stimulation seemed to be inhibited with lower potency ($IC_{50} \approx 5$ μM) similar to that seen in $CB_1^{-/-}$ membranes or in the absence of agonist. Further experiments determined that the effects of anandamide and WIN55212-2 were not additive, but that the effect of μ opioid, adenosine A1, and cannabinoid ligands were additive. Finally, assays of different central nervous system (CNS) regions demonstrated significant activity of cannabinoids in $CB_1^{-/-}$ membranes from brain stem, cortex, hippocampus, diencephalon, midbrain, and spinal cord, but not basal ganglia or cerebellum. Moreover, some of these same CNS regions also showed significant binding of [^{3}H]WIN55212-2, but not [^{3}H]CP55940. Thus anandamide and WIN55212-2 seemed to be active in $CB_1^{-/-}$ mouse brain membranes via a common G protein-coupled receptor with a distinct CNS distribution, implying the existence of an unknown cannabinoid receptor subtype in brain.

Although the principal active constituent in marijuana, Δ^9-tetrahydrocannabinol (THC), has been known for more than 35 years (Gaoni and Mechoulam, 1964), the first cannabinoid receptor (CB_1) was cloned only 10 years ago (Matsuda et al., 1990). The only other known cannabinoid receptor is CB_2 (Kaminski et al., 1992; Munro et al., 1993), which is localized primarily on cells of the immune system. Both CB_1 and CB_2 are G protein-coupled receptors that seem to couple to inhibitory G_i and/or G_o proteins (Childers and Breivogel, 1998). CB_1 receptors are known to affect adenylyl cyclase (Howlett, 1984), a variety of potassium (Deadwyler et al., 1995; Mackie et al., 1995; Mu et al., 1999) and calcium (Mackie and Hille, 1992; Mackie et al., 1995) currents, and the mitogen-activated protein kinase pathway (Bouaboula et al., 1995). CB_1 receptors have been shown to activate at least six subtypes of G_i and G_o proteins, supporting the reports that they effect a wide variety of intracellular signaling systems (Prather et al., 2000). CB_2 receptors also inhibit adenylyl cyclase and activate mitogen-activated protein kinase and Krox-24 pathways (Bouaboula et al., 1996), but do not seem to effect ion currents directly (Felder et al., 1995). In vivo, cannabinoids elicit a characteristic spectrum of behaviors in laboratory animals. These include catalepsy, analgesia, and decreases in spontaneous activity and body temperature (Adams and Martin, 1996) and a disruption of memory (Heyser et al., 1993).

Receptor activation of G-proteins can be measured using agonist-stimulated binding of the nonhydrolyzable GTP analog, [^{35}S]guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) to membranes (Breivogel et al., 1997b). Using this technique (Sim et al., 1998) or effects on adenylyl cyclase (Howlett, 1984), it is possible to garner evidence for an unknown receptor before the development of selective, high-affinity radioligands.

There are two brain-derived chemicals that have been widely studied as candidate endogenous ligands for cannabinoid receptors. The first to be discovered was arachidonyl

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ABBREVIATIONS: THC, tetrahydrocannabinol; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); CNS, central nervous system; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; PIA, N^6-(2-phenylisopropyl)adenosine; BSA, bovine serum albumin; ANOVA, analysis of variance.

ethanolamide, or anandamide (Devane et al., 1992). The second was also an arachidonic acid derivative, 2-arachidonyl glycerol (Mechoulam et al., 1995; Sugiura et al., 1995). Neither compound has high affinity or potency at CB_1 or CB_2, yet they seem to conform to many of the criteria for candidate neuromodulator compounds (Di Marzo et al., 1998).

It has been proposed that the CB_1 receptor is the mediator of all of the CNS actions of cannabinoids. Evidence of this relationship is 3-fold: the structure-activity relationship for behavior and receptor affinity is highly correlated (Compton et al., 1993); the neuroanatomical localization of CB_1 receptors corresponds well with the CNS-mediated effects of cannabinoids (Breivogel and Childers, 1998); and most of the CNS-mediated actions of cannabinoids seem to be reversible by the CB_1-selective antagonist SR141716A (Compton et al., 1996). However, a number of recent studies have found support for the concept of additional sites of action for cannabinoids (Jarai et al., 1999). There is evidence that anandamide induces spinal antinociception via a different mechanism than THC or CP55940 (Welch and Eads, 1999; Houser et al., 2000), and that the centrally-mediated in vivo actions of anandamide are not reversed by the CB_1-selective antagonist, SR147161A (Adams et al., 1998). Most recently, a study using transgenic C57BL/6 mice lacking the CB_1 receptor ($CB_1^{-/-}$) (Zimmer et al., 1999) revealed that anandamide still affected spontaneous activity, catalepsy, and analgesia, even though THC was inactive in these mice (Di Marzo et al., 2000). In the same study, anandamide was found to stimulate [^{35}S]GTPγS binding to brain membranes from both $CB_1^{-/-}$ and $CB_1^{+/+}$ mice, but THC was active only in $CB_1^{+/+}$ membranes. In the current study, WIN 55212-2 was also found to stimulate [^{35}S]GTPγS binding in $CB_1^{-/-}$ membranes. The goals of this study were to determine whether additional experiments confirmed or refuted the existence of a novel G protein-coupled cannabinoid receptor in brain. To accomplish this goal, the structure-activity profiles for various cannabinoids were determined, it was established whether anandamide and WIN55212-2 act at the same or separate sites, and it was determined whether there is a correlation between activity and specific binding of [^{3}H] ligands across membranes from various CNS regions and across different ligands.

Materials and Methods

Materials. CB_1-receptor "knock-out" mutants were developed in C57BL/6 mice as described earlier (Zimmer et al., 1999). Mice were maintained on a 14-h/10-h light/dark cycle with free access to food and water. [^{35}S]GTPγS (1250 Ci/mmol) and [^{3}H]WIN55212-2 (55.0 Ci/mmol) were purchased from PerkinElmer Life Science Products (Boston, MA). [^{3}H]CP55940 (180 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). CP55940 and CP55244 were generously provided by Pfizer Inc. (Groton, CT). HU-210 and HU-211 were a generous gift from Prof. Raphael Mechoulam (Hebrew University, Jerusalem, Israel). Cannabinol, cannabidiol, Δ^9-THC, Δ^8-THC, SR144528, SR141716A, and [^{3}H]SR141716A (53.0 Ci/mmol) were provided by the National Institute on Drug Abuse. WIN55212-2, *R*-(+)-methanandamide, and *R*-(−)N^6-(2-phenylisopropyl)adenosine (PIA) were purchased from Research Biochemicals International (Natick, MA). Other compounds were synthesized by Raj Razdan (O-prefix; Organix, Woburn, MA) or John W. Huffman (JWH-prefix; Clemson University, Clemson, SC). GDP and GTPγS were purchased from Roche Molecular Biochemicals (New York, NY). [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol] enkephalin (DAMGO) and all other reagent grade chemicals and enzymes were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Agonist-Stimulated [^{35}S]GTPγS binding assays. Spinal cords were taken whole and brains were taken whole or divided on ice into seven regions designated cerebellum, hippocampus, cortex, basal ganglia (striatum and globus pallidus), brain stem, midbrain, and diencephalon (thalamus and hypothalamus). Each preparation was homogenized with a Tissumizer (Tekmar, Cincinnati, OH) in cold membrane buffer (50 mM Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.7) and centrifuged at 48,000*g* for 10 min at 4°C. Pellets were re-suspended in membrane buffer, then centrifuged again at 48,000*g* for 10 min at 4°C. Pellets from second centrifugation were homogenized in membrane buffer and stored at −80°C. Frozen membranes were thawed and diluted in membrane buffer, homogenized, and preincubated for 10 min at 30°C in 0.004 U/ml adenosine deaminase (240 U/mg protein; Sigma Chemical Co.) to remove endogenous adenosine, then assayed for protein content before addition to assay tubes. Assays were conducted at 30°C for 1 h in membrane buffer including 5 μg of membrane protein with 0.1% (w/v) bovine serum albumin (BSA), 30 μM GDP, and 0.10 nM [^{35}S]GTPγS in a final volume of 0.5 ml. Nonspecific binding was determined in the absence of agonists and the presence of 30 μM unlabeled GTPγS. Reactions were terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filters, followed by three washes with cold Tris-HCl buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for ^{35}S after overnight extraction of the filters in 4 ml of BudgetSolve scintillation fluid (RPI Corp., Mount Prospect, IL).

[^{3}H]Ligand binding. The methods used for radioligand binding were as described previously (Griffin et al., 1998). In initial experiments, binding was initiated by the addition of 25 μg of membrane protein to siliconized tubes containing 10 nM tritiated radioligand (WIN 55212-2, SR141716A, or CP55940), and a sufficient volume of buffer to bring the total volume to 0.5 ml. In subsequent experiments, 100 μg of cerebral cortex or cerebellar membranes and 8 to 85 nM [^{3}H]WIN55212-2 were used to determine dose dependence of specific radioligand binding. The addition of a 2-μM concentration of the corresponding unlabeled ligand was used to assess nonspecific binding. The membranes were then incubated at 30°C for 60 min. The reaction was terminated by addition of ice-cold wash buffer (50 mM Tris HCl and 0.5% BSA, pH 7.4) followed by rapid filtration under vacuum through Whatman GF/C glass-fiber filters using a 12-well sampling manifold. The tubes were washed twice with 2 ml of ice-cold wash buffer and the filters rinsed twice with 4 ml of wash buffer. Filters were placed into 7-ml plastic scintillation vials and 5 ml of BudgetSolve scintillation fluid was added. After shaking for 1 h, bound radioactivity was determined by liquid scintillation spectrophotometry at 45% efficiency for ^{3}H.

Data Analysis. Net agonist-stimulated [^{35}S]GTPγS binding values were calculated by subtracting basal binding values (obtained in the absence of agonist) from agonist-stimulated values (obtained in the presence of agonist). Data analyses (including agonist concentration-effect and competition curves) were conducted by iterative nonlinear regression using Prism for Windows (GraphPad Software, San Diego, CA) to obtain EC_{50}, E_{max}, IC_{50}, and I_{max} values. K_B values were calculated using the equation: $K_B = IC_{50}/[([a]/EC_{50}) + 1]$, where IC_{50} is the concentration of SR141716A that inhibits half of the stimulation by agonist, [a] is the concentration of agonist, and EC_{50} is the concentration of agonists that produces half-maximal stimulation. Percent additivity $= S_{ab}/(S_a + S_b) \times 100\%$, where S_{ab} is stimulation produced by the combination of agonists, S_a is stimulation produced by the first agonist alone (or combination of WIN55212-2 and anandamide), and S_b is stimulation produced by second agonist alone. Significant stimulation by multiple concentrations of each ligand or in each brain region was determined by ANOVA followed by Dunnett's test at the $p < 0.05$ level to compare each concentration of ligand to basal binding. Significant specific binding of single concentrations of radioligand was determined by

one-sample t tests (single concentrations of each ligand) or by ANOVA (for multiple concentrations) followed by Dunnett's test at the $p < 0.05$ level to compare with zero specific binding. All data presented are mean ± S.E. of experiments performed in duplicate or triplicate in membranes from at least two different animals of each genotype, except where noted.

Results

Stimulation of [^{35}S]GTPγS Binding by Various Compounds. Results published previously by this laboratory (Di Marzo et al., 2000) have indicated that, although anandamide seems to be the endogenous ligand for cannabinoid receptors, it still exhibits activity for the stimulation of [^{35}S]GTPγS binding in brain membranes from mice lacking the CB_1 receptor ($CB_1^{-/-}$). Moreover, anandamide activity seemed to be equally potent in both $CB_1^{-/-}$ and $CB_1^{+/+}$ C57BL/6 mice, both for the stimulation of [^{35}S]GTPγS binding and in whole-animal behavioral assays for cannabinoid activity. This activity proved to be insensitive to the CB_1- and CB_2-selective antagonists SR141716A and SR144528, at concentrations sufficient to block activity via each receptor. In contrast, Δ^9-THC was active only in $CB_1^{+/+}$ mice (Di Marzo et al., 2000).

To follow-up the initial experiments, the present study uses agonist-stimulated [^{35}S]GTPγS binding to further characterize the activity and selectivity of this receptor in mouse brain. A total of 24 compounds were assayed for stimulation of [^{35}S]GTPγS binding in both $CB_1^{-/-}$ and $CB_1^{+/+}$ mouse whole-brain membranes. These compounds included an array of commonly used ligands for cannabinoid receptors and a number of analogs of both anandamide and WIN55212-2. This included: anandamide and 2-arachidonyl glycerol, Δ^9-THC, Δ^8-THC, cannabinol, cannabidiol, HU-210, HU-211, CP55940, CP55244, SR141716A, SR144528, the anandamide analogs 1′-methyl-anandamide (O-610), 2-methyl-anandamide (O-680), 2-dimethyl-anandamide (O-687) (Adams et al., 1995b), 2-methylarachidonyl-(2′-fluoroethyl)amide (O-689) (Adams et al., 1995a), WIN55212-2 (and several analogs: JWH-030, JWH-031, JWH-032, JWH-036, JWH-044, JWH-045, and JWH-073) (Wiley et al., 1998). The only compounds that produced statistically significant dose-dependent stimulation of [^{35}S]GTPγS binding in $CB_1^{-/-}$ membranes were anandamide and WIN55212-2 (Fig. 1). All of the commonly used compounds (with the exception of SR144528, the inactive isomer HU-211, and the inactive cannabinol and cannabidiol) produced significant effects on [^{35}S]GTPγS binding in $CB_1^{+/+}$ C57BL/6 whole brain membranes (Table 1 and Fig. 1). The first three anandamide analogs (O-610, O-680, and O-687) differed from anandamide by one or two methyl groups, yet each exhibited greater potency and lower or equal efficacy than anandamide in $CB_1^{+/+}$ (Table 1), and each failed to stimulate [^{35}S]GTPγS binding in $CB_1^{-/-}$ membranes. Although there was no indication of any activity by THC, HU-210, or CP55940, there was a hint of activation by O-610, O-1812 (Fig. 1), and others. However, the degree of stimulation was quite weak compared with anandamide and WIN55212-2, and the results for these ligands failed to achieve statistical significance. Among the analogs of

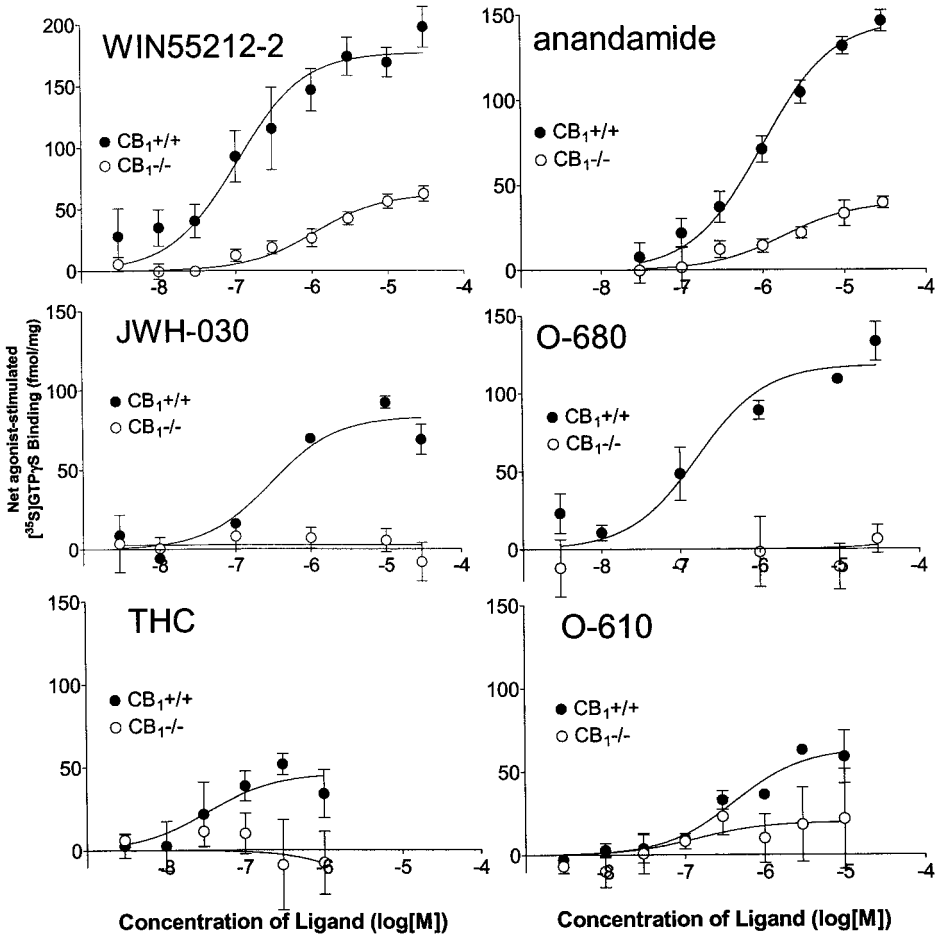

Fig. 1. Concentration-effect curves for stimulation of [^{35}S]GTPγS binding by WIN55212-2, anandamide, O-610, JWH-030, O-680, and THC in $CB_1^{+/+}$ and $CB_1^{-/-}$ C57BL/6 mouse whole brain membranes. O-610 and O-680 are monomethylated analogs of anandamide and JWH-030 is an analog of WIN55212-2. Assays were performed using 5 μg of membranes and included 30 μM GDP, 100 mM NaCl, and 0.1 nM [^{35}S]GTPγS as described under *Materials and Methods*. Net agonist-stimulated [^{35}S]GTPγS binding was determined by subtracting basal binding values obtained in the absence of agonist from values obtained for each concentration of agonist. Curve fits are to a one-site model. Data are mean values ± S.E. from at least three assays performed in duplicate or triplicate. Although stimulation by each ligand was statistically significant in $CB_1^{+/+}$ membranes, only WIN55212-2 and anandamide significantly stimulated [^{35}S]GTPγS binding in $CB_1^{-/-}$ membranes.

WIN55212-2, only those that had previously been reported to compete for binding of [^{3}H]CP55940 to rat brain membranes (K_i values < 10,000 nM) were found to stimulate [^{35}S]GTPγS binding in $CB_1^{+/+}$ C57BL/6 whole brain membranes (Table 1). As with the anandamide analogs, none of the analogs of WIN55212-2 significantly stimulated [^{35}S]GTPγS binding in $CB_1^{-/-}$ membranes. Δ^8-THC (not shown) produced effects in $CB_1^{+/+}$ membranes similar to those of Δ^9-THC (Table 1), but neither produced significant effects in $CB_1^{-/-}$ membranes. The antagonist SR141716A produced significant inhibition of [^{35}S]GTPγS binding that was equivalent in $CB_1^{+/+}$ (IC_{50}, 4.7 ± 0.4 μM; I_{max}, −48 ± 9 fmol/mg) and $CB_1^{-/-}$ membranes (IC_{50}, 5.7 ± 2.1 μM; I_{max}, −50 ± 9 fmol/mg).

In the present study, results for anandamide were similar to those previously published (see Table 2): E_{max} of 41 fmol/mg (30 ± 2% stimulation over basal binding) with an EC_{50} value of 3.6 μM in $CB_1^{-/-}$, whereas the corresponding values in $CB_1^{+/+}$ membranes were 150 fmol/mg (91 ± 4%) and 1.4 μM (Table 2). Similar to anandamide, stimulation of [^{35}S]GTPγS binding by WIN55212-2 exhibited lower efficacy in $CB_1^{-/-}$ (64 fmol/mg; 48 ± 6%) than in $CB_1^{+/+}$ (180 fmol/mg; 110 ± 9%) membranes, but in contrast, WIN55212-2 also exhibited approximately 10-fold lower potency with an EC_{50} value of 1.8 μM in $CB_1^{-/-}$ and 0.17 μM in $CB_1^{+/+}$ (Table 2).

Once the active ligands were found, additional experiments were conducted to optimize assay conditions for [^{35}S]GTPγS binding by anandamide and WIN55212-2 in $CB_1^{-/-}$ membranes and to determine whether this receptor behaves similarly to previously characterized G protein-coupled receptors for the stimulation of [^{35}S]GTPγS binding. Incubation time and concentrations of membrane protein, guanosine diphosphate (GDP), and NaCl were varied (data not shown). All of the results were qualitatively the same as those previously observed using other ligands for other receptor systems (Breivogel et al., 1997b; Selley et al., 1998) or WIN55212-2 in wild-type rat brain membranes (Breivogel et al., 1998). Briefly, increasing incubation times between 5 and 60 min increased the amount of [^{35}S]GTPγS binding obtained in either the absence (basal) or presence of agonist (30 μM anandamide or 10 μM WIN55212-2). There was little change in binding between 45 and 60 min. Increasing the quantity of membrane protein between 3 and 100 μg increased the amount of [^{35}S]GTPγS binding obtained in either the absence (0.47–30 fmol of specific [^{35}S]GTPγS binding) or presence of 30 μM anandamide (0.62–32 fmol of specific [^{35}S]GTPγS binding) or 10 μM WIN55212-2 (0.67–33 fmol of specific [^{35}S]GTPγS binding). However, the increase in binding over basal by agonists (and thus percent stimulation over basal binding) was greatest at the lowest concentration of protein (52% by WIN55212-2) and decreased with increasing protein (9.6% by WIN55212-2). Standard assay conditions in this study included 30 μM GDP and 100 mM NaCl. Lowering either the concentration of GDP or NaCl increased the amount of binding obtained in either the absence (basal) or presence of agonist (30 μM anandamide or 10 μM WIN55212-2). However, because basal binding increased whereas net agonist-stimulated binding remained virtually the same as the concentrations of GDP and NaCl were decreased, percentage stimulation over basal binding decreased dramatically (from 62 to 5.2% by WIN55212-2). Although maximal percentage of stimulation was obtained at increased (100 μM) GDP and 100 mM NaCl, net agonist-stimulated binding was greater, and thus experimental values were more consistent, at standard assay conditions (30 μM GDP). These results indicated that this receptor behaves similarly to previously characterized G protein-coupled receptors with regard to the effects of time, amount of membranes, and concentrations of GDP and NaCl on [^{35}S]GTPγS binding. Moreover, the effects of varying GDP and NaCl were similar between $CB_1^{-/-}$ and $CB_1^{+/+}$ C57BL/6 brain membranes.

It was previously shown that neither 200 nM SR141716A nor 30 nM SR144528 was able to affect stimulation of [^{35}S]GTPγS binding by anandamide in $CB_1^{-/-}$ membranes (Di Marzo et al., 2000). To further investigate the effects of SR141716A, concentration-effect curves were generated for SR141716A alone and in the presence of anandamide or WIN55212-2 in whole-brain membranes from both $CB_1^{+/+}$ and $CB_1^{-/-}$ mice (Table 2). At concentrations greater than 1 μM, SR141716A decreased basal [^{35}S]GTPγS binding in a concentration-dependant manner in $CB_1^{+/+}$ by 48 ± 9 fmol/mg and $CB_1^{-/-}$ membranes by 50 ± 9 fmol/mg, yielding IC_{50} values of 4.7 ± 0.4 μM and 5.7 ± 2.1 μM, respectively (Fig. 2). When combined with either 10 μM anandamide or 10 μM WIN55212-2, SR141716A concentration-effect curves fit better (F test, $p < 0.05$) to a two-site than to a one-site model in $CB_1^{+/+}$ membranes: there was a high-potency site with IC_{50} values from 2 to 24 nM and a low-potency site with IC_{50} values between 3 and 13 μM (Table 2). The high-potency sites exhibited calculated K_B values of 0.26 nM and 0.40 nM and made up 84% of anandamide-stimulated and 66% of WIN55212-2-stimulated [^{35}S]GTPγS binding sites, respectively (Table 2). In $CB_1^{-/-}$ membranes, concentrations of SR141716A below 1 μM had no effect on 10 μM anandamide or 10 μM WIN55212-2-stimulated [^{35}S]GTPγS binding

TABLE 1

Comparison of CB_1 receptor affinity values from rat brain and concentration-effect parameters for selected agonists in $CB_1^{+/+}$ C57BL/6 mouse brain membranes

K_i values were determined in rat whole brain membranes via displacement of [^{3}H]CP55940. Concentrations-effect parameters for activation of [^{35}S]GTPγS binding to $CB_1^{+/+}$ C57BL/6 mouse brain membranes were determined as described under *Materials and Methods.* The maximal theoretical effect for stimulating [^{35}S]GTPγS binding (E_{max}), and concentrations that produce a half-maximal response (EC_{50}) are given. Values are mean ± S.E. from experiments conducted in triplicate.

Drug	K_i in Rat	E_{max}	EC_{50}
	nM	*% Stimulation*	*μM*
2-AG	470 ± 55[a]	40 ± 8	2.8 ± 0.9
Δ^9-THC	41[b]	40 ± 8	0.081 ± 0.034
Cannabinol	308[c]		
HU-210	0.73[c]	110 ± 10	0.0029 ± 0.0014
CP55940	0.58[c]	120 ± 6	0.0061 ± 0.0015
CP55244	0.11[c]	120 ± 5	0.00012 ± 0.00011
Anandamide	89 ± 10[d]	91 ± 4	1.4 ± 0.3
O-610	137 ± 13[d]	45 ± 9	0.44 ± 0.20
O-680	53 ± 15[d]	92 ± 5	0.35 ± 0.27
O-687	47 ± 3[d]	74 ± 6	0.17 ± 0.17
O-689	5.7 ± 12[d]	94 ± 3	0.0072 ± 0.0065
WIN55212-2	24[b]	110 ± 9	0.17 ± 0.08
JWH-030	87 ± 3[b]	50 ± 1	0.30 ± 0.05
JWH-031	399 ± 109[b]	61 ± 1	0.92 ± 0.10
JWH-032	> 10,000[b]		
JWH-036	309 ± 11[b]	33 ± 6	3.3 ± 2.2
JWH-044	> 10,000[b]		
JWH-045	> 10,000[b]		
JWH-073	8.9 ± 1.8[b]	59 ± 7	0.034 ± 0.018

[a] Mechoulam et al., 1995.
[b] Wiley et al., 1998.
[c] Griffin et al., 1998.
[d] Adams et al., 1995.

(Fig. 2), and seemed to exhibit IC_{50} values of 3.3 μM and 11 μM, respectively. To define the effect of SR141716A on anandamide and WIN55212-2-stimulated [^{35}S]GTPγS binding, in the absence of any unrelated effects of SR141716A alone, binding values obtained in the presence of SR141716A alone were subtracted from those obtained in the presence of each agonist, and the resulting data were analyzed by nonlinear fitting (Fig. 2). These calculations showed stimulation by anandamide and WIN55212-2 that was not completely blocked by SR141716A in $CB_1^{+/+}$ membrane and was not affected at all in $CB_1^{-/-}$ membranes. In $CB_1^{+/+}$ membranes, SR141716A inhibited 78% of anandamide-stimulated [^{35}S]GTPγS binding with a K_B value of 0.42 nM, and 67% of WIN55212-2-stimulated [^{35}S]GTPγS binding with a K_B value of 0.71 nM (Table 2).

Because only anandamide and WIN55212-2, but none of their analogs, were active for the stimulation of [^{35}S]GTPγS binding in $CB_1^{-/-}$ membranes, it was important to determine whether these ligands were working through the same receptor. Experiments were conducted using 10 μM anandamide, 10 μM WIN55212-2, 10 μM DAMGO (Selley et al., 1998), and 1 μM PIA (Breivogel and Childers, 2000), each alone or in combination. Results indicated that combination of any two agonists except anandamide and WIN55212-2 were almost completely additive (between 89 and 104%) and were always higher than for either agonist alone for the stimulation of [^{35}S]GTPγS binding in both wild-type and $CB_1^{-/-}$ membranes (Table 3). Also, combinations of anandamide plus WIN55212-2 with either DAMGO or PIA were also additive compared with anandamide plus WIN55212-2 and either DAMGO or PIA alone (Table 3). The values for combinations of anandamide and WIN55212-2 were higher than for anandamide alone and almost exactly equal to those obtained for WIN55212-2 alone in both $CB_1^{+/+}$ and $CB_1^{-/-}$ membranes.

Although the previous experiments in this study were carried out in membranes prepared from whole mouse brain, a further goal of this study was to determine the brain regional distribution of the activity of the putative new cannabinoid receptor. Concentration-effect curves for the stimulation of [^{35}S]GTPγS binding by both anandamide and WIN55212-2 were performed in eight regions of $CB_1^{-/-}$ mouse CNS, including basal ganglia (caudate-putamen and globus pallidus), brain stem, cerebellum, cerebral cortex, diencephalon, hippocampus, midbrain, and spinal cord. Significant stimulation (ANOVA; $p < 0.05$) was found for both ligands in all regions except basal ganglia and cerebellum, where there was no significant activity of either ligand, and in spinal cord, significant stimulation was observed only with anandamide (Fig. 3A). In $CB_1^{-/-}$ animals, the regions exhibiting the greatest level of activity were cortex, midbrain, and hippocampus, followed by diencephalon and brain stem. Spinal cord contained the lowest level of activity among the regions that showed significant stimulation.

[^{3}H] Ligand Binding. To determine whether the stimulation of [^{35}S]GTPγS binding observed in the $CB_1^{-/-}$ CNS regions could be correlated with specific receptor binding, three radioligands were assayed: [^{3}H]WIN55212-2, [^{3}H]SR141716A, and [^{3}H]CP55940. Initially, it was found that specific [^{3}H]WIN55212-2 binding displayed a linear relationship ($r^2 = 0.963$) and was significantly correlated with protein concentration ($p = 0.019$) in $CB_1^{-/-}$ cerebral cortex membranes between 10 and 100 μg/0.5 ml (Fig. 4). Percent specific binding reached a plateau from 50 to 100 μg at a level of 20%. In subsequent experiments, 25 μg of tissue was used to maximize specific binding while conserving tissue, which was of limited availability.

Radioligands, at concentrations of 10 nM, were then tested in each region prepared from both $CB_1^{+/+}$ and $CB_1^{-/-}$ C57BL/6 mice to determine whether there was significant specific binding. Statistically significant specific binding of each ligand was detected in each region of $CB_1^{+/+}$ membranes (not shown). In contrast, total bound radioligand was very much reduced in $CB_1^{-/-}$ compared with $CB_1^{+/+}$ tissues, although significant specific binding ($p < 0.05$ one-sample t test) of [^{3}H]WIN55212-2 and [^{3}H]SR141716A were observed in some regions (Fig. 5). The areas that demonstrated significant levels of bound [^{3}H]WIN55212-2 were the brain stem, cortex, and hippocampus (Fig. 3B). No statistically-significant binding of [^{3}H]WIN55212-2 was observed in the basal ganglia, cerebellum, diencephalon, midbrain, or spinal cord (not shown). Significant levels of [^{3}H]SR141716A binding ($p < 0.05$ one-sample t test) were observed in the brain stem, cortex, midbrain, and spinal cord, but not in the basal gan-

TABLE 2

Characterization of anandamide and WIN55212-2-stimulated [^{35}S]GTPγS binding and inhibition of agonist-stimulated [^{35}S]GTPγS binding by SR141716A

The first two rows were data obtained using various concentrations of either anandamide or WIN55212-2 alone. The next three rows are data obtained with 10 μM anandamide or 10 μM WIN55212-2 in the presence of various concentrations of SR141716A (see "SR + 10 μM WIN" and "SR + 10 μM anand." in Fig. 2). "High K_B" and "Low IC_{50}" refer to the two sites observed for inhibition of agonist-stimulated [^{35}S]GTPγS binding in $CB_1^{+/+}$ membranes. "High K_B" is the affinity of the high potency site at which SR141716A inhibited agonist stimulation in $CB_1^{+/+}$ membranes and was calculated from the high-affinity IC_{50} value as described under *Materials and Methods*. "Low IC_{50}" is concentration of SR141716A that seem to inhibit half of the remaining stimulation by agonist in $CB_1^{+/+}$ and was the only site observed in $CB_1^{-/-}$ membranes. "% High potency" is the percentage of agonist-stimulated binding inhibited by SR141716A at the high K_B value. The last two rows were obtained by subtracting the effects of SR141716A alone from the data obtained for agonists plus SR141716A for each experiment before nonlinear fitting of the data. "K_B for −SR" is the affinity value exhibited by SR141716A, and "% Blocked −SR" is the percentage of agonist-stimulated binding inhibited by SR141716A (see "SR + 10 μM WIN −SR alone" and "SR + 10 μM anand. − SR alone" in Fig. 2). Data are mean values ± S.E. obtained by nonlinear fitting from at least four experiments.

Agonist	SR141716A	Parameter	$CB_1^{+/+}$		$CB_1^{-/-}$	
			Anandamide	WIN55212-2	Anandamide	WIN55212-2
	μM					
0.1–30		EC_{50} (μM)	1.4 ± 0.3	0.17 ± 0.08	3.6 ± 2.0	1.8 ± 0.7
0.1–30		E_{max} (fmol/mg)	150 ± 7	180 ± 11	41 ± 3	64 ± 7
10	0.1 –30,000	High K_B (nM)	0.26 ± 0.06	0.40 ± 0.09	none	none
10	0.1 –30,000	Low IC_{50} (μM)	3.0 ± 1.1	13 ± 5	3.3 ± 2.1	11 ± 2
10	0.1 –30,000	% High-potency	84 ± 6%	66 ± 3%		
10	0.1 –30,000	K_B for −SR (nM)	0.42 ± 0.18	0.71 ± 0.21		
10	0.1 –30,000	% Blocked −SR	78 ± 1%	67 ± 5%		

glia, cerebellum, diencephalon, or hippocampus (Fig. 3B). No significant binding of [^{3}H]CP55940 was observed ($p < 0.05$ one-sample t test) in any of the brain regions tested (not shown).

To further characterize the specific [^{3}H]WIN55212-2 binding observed in some regions of $CB_1^{-/-}$ mouse brain membranes, it was important to determine whether the binding was concentration-dependent using higher concentrations of [^{3}H]WIN55212-2 used in the single-concentration analysis described above (Fig. 5). Because analysis of the binding by high concentrations of these lipophilic radioligands was technically difficult, and use of such large amounts of radioligand is impractical, only two regions were selected. Cortex was chosen because it displayed some of the highest levels of both WIN55212-2-stimulated [^{35}S]GTPγS binding and specific [^{3}H]WIN55212-2 binding among the $CB_1^{-/-}$ brain regions, and cerebellum was chosen as a negative control, because it exhibited a lack of activity in either assay. Results indicated significant, concentration-dependent binding of [^{3}H]WIN-55212-2 to $CB_1^{-/-}$ cortex membranes (ANOVA; $p = 0.003$) at 38 and 86 nM ($p < 0.01$ by Dunnett's test versus control (no specific binding). In contrast, no significant specific binding was detected at any concentration of [^{3}H]WIN55212-2 in $CB_1^{-/-}$ cerebellar membranes (ANOVA; $p = 0.06$) (Fig. 5).

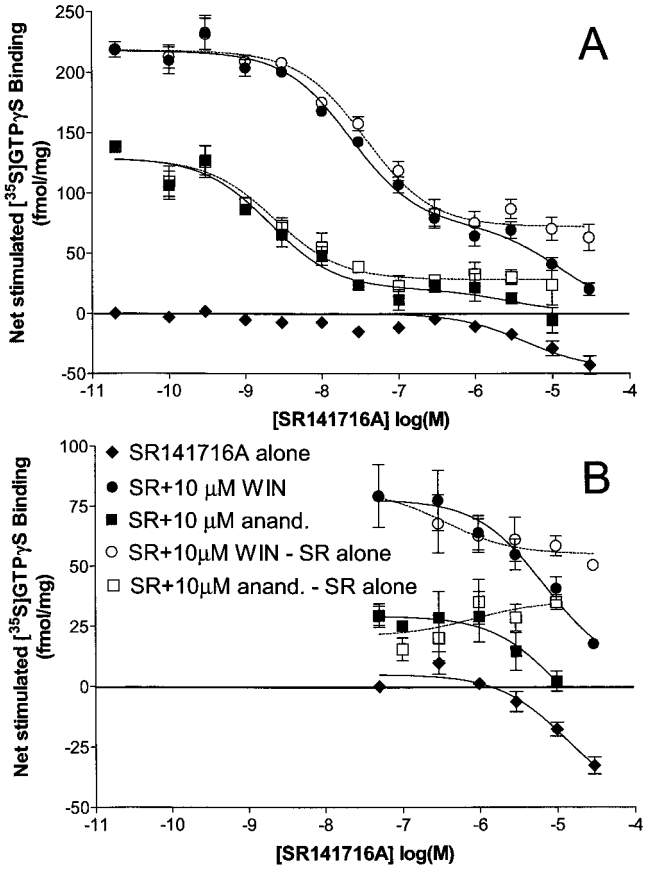

Fig. 2. Comparison of the effects of SR141716A on basal and WIN55212-2 (SR + 10 μM WIN) or anandamide (SR + 10 μM anand.) stimulated [^{35}S]GTPγS binding in $CB_1^{+/+}$ (A) and $CB_1^{-/-}$ brain membranes (B). Net agonist-stimulated [^{35}S]GTPγS binding was determined by subtracting basal binding values obtained in the absence of agonist from values obtained at each concentration of agonist. "SR + 10 μM WIN − SR alone" and "SR + 10 μM anand. − SR alone" curves were obtained by subtracting binding values obtained in the presence of SR141716A alone from values obtained using SR141716A + 10 μM WIN55212-2 or SR141716A + 10 μM anandamide. In other words, the last two curves are the difference between the effect of SR141716A alone and the effects of SR141716A on stimulation of [^{35}S]GTPγS binding by each of these agonists, to account for the effect of SR141716A on basal [^{35}S]GTPγS binding. These manipulations were performed before nonlinear curve fitting of each of the resulting data sets. Curve fits are to one- or two-site models as described under *Results* and in Table 2. Data are means ± S.E. from three or four assays performed in triplicate.

Discussion

A previous study found that anandamide, but not THC, produced pharmacological effects in $CB_1^{-/-}$ mice (Di Marzo et al., 2000). In the present study, none of the classes of compounds represented by THC, CP55940, HU-210 or SR141716A were active, but anandamide and WIN55212-2 activated G proteins in brain membranes from $CB_1^{-/-}$ mice. In contrast, none of the analogs of anandamide or WIN55212-2 were active. However, in an attempt to find ligands selective for the unknown receptor, analogs of WIN55212-2 were chosen that exhibited behavioral activity in mice but failed to displace [^{3}H]CP55940 binding in previous studies (see Table 1). In the present study, these analogs did not activate G proteins in $CB_1^{+/+}$ or $CB_1^{-/-}$ mouse membranes. Among the analogs active in $CB_1^{+/+}$ membranes, all but JWH-073 exhibited lower potency than WIN55212-2 (see Table 1) and contained significant departures from the chemical structure of WIN55212-2. Moreover, each exhibited at least 33% lower efficacy than WIN55212-2, which represents stimulation by WIN55212-2 contributed by the putative new receptor.

That analogs of anandamide that differed structurally by one or two methyl groups did not activate G proteins in $CB_1^{-/-}$ was somewhat unexpected. These compounds were more potent and exhibited equal or lower efficacy than anandamide in $CB_1^{+/+}$ membranes. One interpretation is that O-610 and O-687 activated only CB_1 receptors and did so with efficacy similar to that of anandamide and that the additional efficacy observed for anandamide came from the activation of the unknown receptor, for which anandamide exhibited lower potency as well (yielding a higher EC_{50} value). In contrast, O-680 exhibited similar efficacy but higher potency than anandamide in $CB_1^{+/+}$ membranes. The lack of activity of O-680 in $CB_1^{-/-}$ membranes may indicate higher intrinsic efficacy than anandamide at CB_1. That each of these modifications to the structure of anandamide yielded compounds that were more potent at CB_1 and were no longer substrates for fatty acid amide hydrolase (Deutsch and Chin, 1993) argues that these alterations are significant and produce changes in three-dimensional structure. Perhaps the putative new receptor's binding pocket is more similar to that of fatty acid amide hydrolase than CB_1. Alternatively, the lower potency of anandamide in $CB_1^{+/+}$ membranes and the activity of anandamide in $CB_1^{-/-}$ membranes might be attributed to degradation of anandamide. This seems unlikely, because pretreatment of the membranes with phenylmethylsulfonyl fluoride in the previous study (Di Marzo et al., 2000) had no effect on anandamide efficacy or potency.

Experiments designed to optimize the [^{35}S]GTPγS binding assay for anandamide and WIN55212-2 in $CB_1^{-/-}$ membranes found that optimal conditions were virtually the same as those for activation in $CB_1^{+/+}$ membranes (Breivogel et al., 1997b) or for other receptor systems in brain (Sim et al.,

1995; Selley et al., 1998) or cell (Breivogel et al., 1997a) membranes. These observations support the concept that the activities of anandamide and WIN55212-2 were due to activation of a G protein-coupled receptor.

In a previous study (Di Marzo et al., 2000), neither SR141716A nor SR144528 affected activation of G proteins by anandamide in $CB_1^{-/-}$ membranes at concentrations at least 50-fold greater than the K_D values of the antagonists at CB_1 and CB_2, respectively. In $CB_1^{+/+}$ membranes in the present study, SR141716A inhibited CB_1 receptor-mediated activity with high potency, and remaining agonist stimulation was inhibited at the same concentrations that produced inhibition of basal [^{35}S]GTPγS binding. If the effect of SR141716A and the agonists at the low potency sites were mediated in a competitive manner, the concentrations of SR141716A necessary to produce inhibition in the presence of agonist would be higher than in the absence of agonist. Moreover, when the effect of SR141716A on basal binding was subtracted from the effect on agonist-stimulated binding, the inhibition of agonist-stimulated [^{35}S]GTPγS binding at the higher concentrations of SR141716A disappeared. Thus, it seems that the low-potency inhibition of [^{35}S]GTPγS binding by SR141716A in both $CB_1^{+/+}$ and $CB_1^{-/-}$ membranes was caused not by blockade of the site of anandamide and WIN55212-2 activity but by additive effects at different sites. These data demonstrate that the "inverse agonist" activity of SR14716A was mediated by neither CB_1 nor the putative new cannabinoid receptor.

Another finding was that the amount of activity of the unknown receptor seemed to be equivalent in $CB_1^{+/+}$ and $CB_1^{-/-}$ membranes. Stimulation produced by anandamide and WIN55212-2 in $CB_1^{-/-}$ membranes, 41 fmol/mg and 64 fmol/mg, respectively, was virtually the same as that refractory to SR141716A in $CB_1^{+/+}$ membranes, 33 and 60 fmol/mg, respectively (calculated from Table 2). This indicated that deletion of the CB_1 gene did not effect the expression or activity of the putative new receptor, an important consideration because the genes for these two receptors may be related. Additivity experiments implied that anandamide and WIN55212-2 acted at the same site in $CB_1^{-/-}$ membranes, and that anandamide is a partial agonist at this site relative to WIN55212-2. Moreover, it seemed that neither μ nor A1 activity was altered by deletion of CB_1.

These data show that anandamide and WIN55212-2 activity was localized to discreet CNS areas of $CB_1^{-/-}$ mice, which coincided to some degree with areas exhibiting specific binding of [^{3}H]WIN55212-2. Both were found in cortex, hippocampus, and brain stem; furthermore, specific binding exhibited linear dependence on protein concentration and [^{3}H]WIN55212-2 concentration when examined in cortex. Two regions, diencephalon and midbrain, exhibited significant stimulation of [^{35}S]GTPγS binding without specific binding of [^{3}H]WIN55212-2. Basal ganglia, cerebellum and spinal cord failed to exhibit significant activity or binding of WIN55212-2, which was confirmed when cerebellum was subsequently assayed using multiple concentrations of [^{3}H]WIN55212-2. In contrast, [^{3}H]CP55940 failed to exhibit specific binding in any CNS region of $CB_1^{-/-}$ mice, in agreement with the lack of activity of CP55940 for [^{35}S]GTPγS binding. [^{3}H]SR141716A exhibited significant binding in some CNS regions of $CB_1^{-/-}$ mouse membranes, but unlike [^{3}H]WIN55212-2, these regions did not correlate with those exhibiting significant stimulation of [^{35}S]GTPγS binding by anandamide and WIN55212-2. Furthermore, SR141716A decreased basal [^{35}S]GTPγS binding in both $CB_1^{+/+}$ and $CB_1^{-/-}$ whole brain membranes. Thus, it seems that the inhibition of [^{35}S]GTPγS binding by SR141716A at higher concentrations is unrelated to the site activated by anandamide and WIN55212-2 in $CB_1^{-/-}$ membranes, but may be the same sites for which [^{3}H]SR141716A showed significant binding in $CB_1^{-/-}$ membranes.

Interestingly, cerebellum and basal ganglia, regions in which anandamide and WIN55212-2 failed to significantly stimulate [^{35}S]GTPγS binding in $CB_1^{-/-}$ mouse brain membranes, are regions that contain high levels of cannabinoid-induced [^{35}S]GTPγS binding (Breivogel et al., 1997b), radioligand binding (Breivogel et al., 1997b), and CB_1 mRNA in situ hybridization (Mailleux and Vanderhaeghen, 1992) in wild-type (nontransgenic) rats. As a direct comparison, WIN55212-2 significantly stimulated [^{35}S]GTPγS binding in C57BL/6 $CB_1^{+/+}$ mouse membranes from cerebellum by 492 ± 44 fmol/mg, basal ganglia by 489 ± 23 fmol/mg, and

TABLE 3
Additivity of the effects of agonists for cannabinoid, μ-opioid, and A1 adenosine receptors in wild-type and $CB_1^{-/-}$ brain membranes

AGONIST				$CB_1^{+/+}$		$CB_1^{-/-}$	
W	A	D	P	Net fmol/mg	% Additivity	Net fmol/mg	% Additivity
+				168 ± 7		53 ± 2	
	+			106 ± 3		22 ± 1	
		+		70 ± 5		74 ± 6	
			+	180 ± 13		159 ± 8	
+	+			170 ± 6	62 ± 1	56 ± 5	75 ± 5
+		+		240 ± 3	101 ± 3	122 ± 4	97 ± 5
+			+	311 ± 11	90 ± 2	200 ± 7	94 ± 1
	+	+		175 ± 7	99 ± 3	84 ± 2	89 ± 5
	+		+	261 ± 11	91 ± 1	188 ± 7	104 ± 2
		+	+	235 ± 16	94 ± 3	217 ± 11	93 ± 1
+	+	+		231 ± 8	96 ± 1	125 ± 3	97 ± 4
+	+		+	315 ± 10	90 ± 2	210 ± 6	97 ± 1

Stimulation of [^{35}S]GTPγS binding by maximally effective concentrations of cannabinoid, μ-opioid, and A1 adenosine agonists to whole-brain membranes from $CB_1^{+/+}$ and $CB_1^{-/-}$ mice. WIN55212-2 (W), anandamide (A), and DAMGO (D) were each added at 10 μM and PIA (P) at 1 μM either alone or in combination to determine the level of additivity among the agonists. Values shown are net-agonist stimulated femtomoles of [^{35}S]GTPγS bound per milligram of membrane protein and percentage of additivity determined as described under *Materials and Methods*. % Additivity values near 100% additivity indicates pairs of ligands that probably act at different targets, whereas values substantially less than 100% imply that the pair of ligands are acting via the same G protein-coupled receptor. Values are mean ± S.E. from experiments conducted in triplicate in whole-brain membranes from three animals of each genotype.

hippocampus by 347 ±19 fmol/mg (D. E. Selley, W. K. Rorrer, C. S. Breivogel, B. R. Martin, and L. J. Sim-Selley, unpublished observations). Thus, the putative new receptor is not localized in all of the same CNS areas as CB_1. That it is missing in basal ganglia implies that this receptor is not involved in cannabinoid influences on locomotion mediated by this brain region. It may still mediate effects on memory via hippocampal receptors (Breivogel and Childers, 1998) or the pyramidal control of locomotion or other cortical functions. Moreover, the presence of these receptors in spinal cord may support previous conclusions that differential binding of cannabinoid ligands in wild-type rat spinal cord versus forebrain regions indicated the presence of multiple receptor subtypes (G. Griffin and B. R. Martin, submitted), and that spinal antinociceptive effects of different cannabinoid ligands are mediated by different receptors (Welch and Eads, 1999; Houser et al., 2000).

That WIN55212-2 has previously been shown to be the most efficacious cannabinoid agonist (Sim et al., 1996; Breivogel et al., 1998; Breivogel and Childers, 2000), may be explained by the existence of this second G protein-coupled target for WIN55212-2. It was shown previously that WIN55212-2 was as efficacious as levonantradol in rat cerebellum but was 20 to 30% more efficacious in hippocampus and hypothalamus membranes (Breivogel and Childers, 2000). This agrees with the data in the present study that showed no activity of WIN55212-2 in $CB_1^{-/-}$ cerebellum, but highly significant activity in hippocampus and diencephalon (which included hypothalamus). That anandamide is not more efficacious than other cannabinoid ligands despite its activity at a second target is explained by its lower intrinsic efficacy at both CB_1 (Breivogel and Childers, 2000) and this new receptor.

These data strongly support the existence of a previously uncharacterized, G protein-coupled cannabinoid receptor in brain, and cast light into its kinetic and ligand recognition

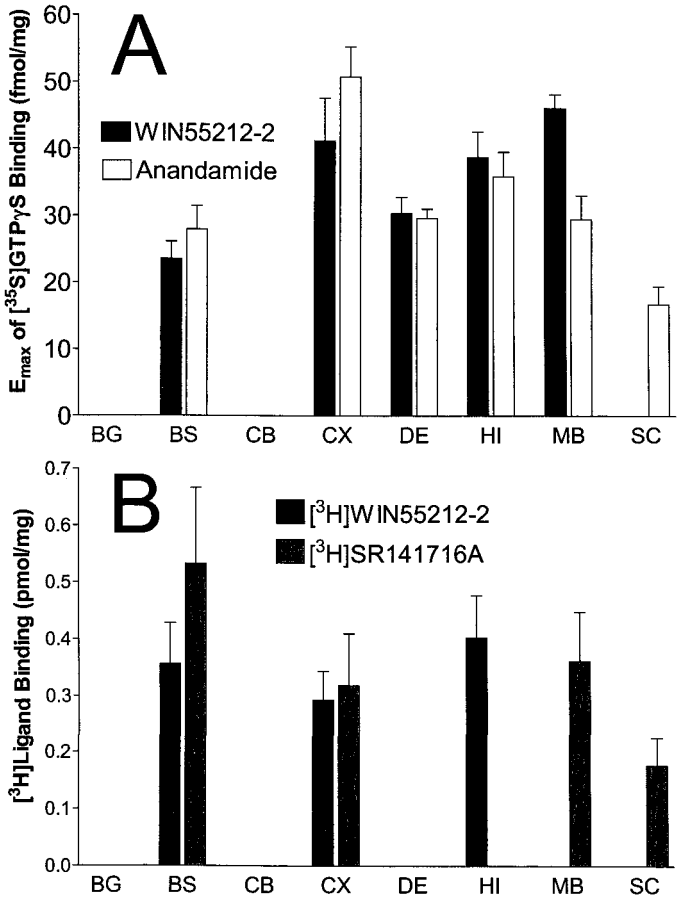

Fig. 3. E_{max} values for stimulation of [^{35}S]GTPγS binding by anandamide and WIN55212-2 (A) and specific binding of 10 nM [^{3}H]WIN55212-2 and 10 nM [^{3}H]SR141716A (B) to membranes from various regions of $CB_1^{-/-}$ mouse CNS. Assays shown in A were performed by incubating membranes with various concentrations of each agonist and 0.1 nM [^{35}S]GTPγS as described under *Materials and Methods*. E_{max} values were determined by nonlinear fitting to a one-site model. For assays in B, specific binding was calculated as total minus nonspecific binding, where nonspecific binding was determined in the presence of a 10-μM concentration of the respective unlabeled ligand. Values are mean ± S.E. from at least three assays performed in triplicate. All data shown were significantly different from basal binding at $p < 0.05$ by repeated measures ANOVA (A) or no specific binding at the $p < 0.05$ level by one-sample *t*-tests (B). BG, basal ganglia; BS, brain stem; CB, cerebellum; CX, cortex; DE, diencephalon; HI, hippocampus; MB, midbrain; SC, spinal cord.

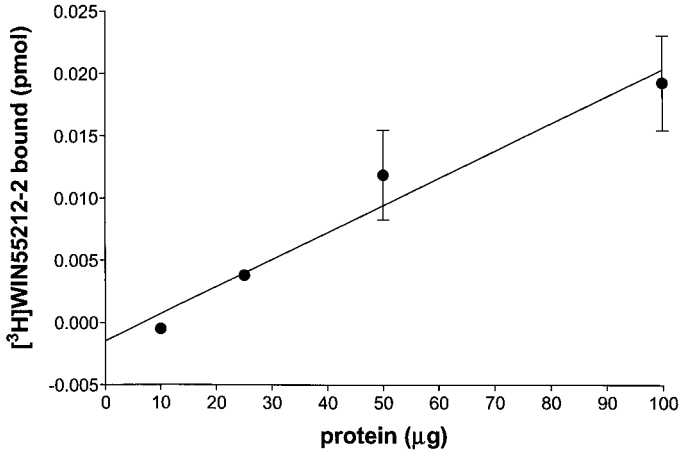

Fig. 4. Relationship between protein concentration and specific binding obtained with 10 nM [^{3}H]WIN55212-2 in membranes prepared from $CB_1^{-/-}$ C57BL/6 mouse cortex. Specific binding was calculated as total minus nonspecific binding at each concentration of protein, where nonspecific binding was determined in the presence of 10 μM WIN55212-2. Data are presented as means ± S.E. determined from two experiments performed in triplicate.

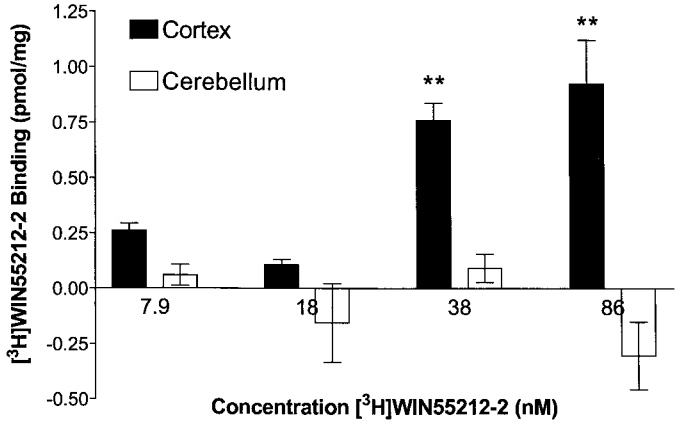

Fig. 5. Specific binding of various concentrations of [^{3}H]WIN55212-2 to membranes from $CB_1^{-/-}$ mouse cortex and cerebellum. Specific binding was calculated as total minus nonspecific binding, where nonspecific binding was determined in the presence of 10 μM unlabeled WIN55212-2. Data are presented as mean ± S.E. determined from three experiments performed in triplicate. $^{**}p < 0.01$ by Dunnett's test versus control (no specific binding).

properties, as well as its CNS distribution. It seems to be related to CB_1 and CB_2, because two chemically unrelated compounds that produce cannabinoid pharmacological effects activate this receptor. It is interesting to note that compounds derived from cannabis, from which "cannabinoid" nomenclature is derived, do not activate this receptor. This receptor may provide a new target for therapeutics, because it seems to have very different structural requirements from CB_1 and may have different physiological effects. However, although these data provide strong evidence for a novel receptor, proof must involve cloning and expression of a novel gene.

References

Adams IB, Compton DR and Martin BR (1998) Assessment of anandamide interaction with the cannabinoid brain receptor: SR 141716A antagonism studies in mice and autoradiographic analysis of receptor binding in rat brain. *J Pharmacol Exp Ther* **284:**1209–1017.

Adams IB and Martin BR (1996) Cannabis: pharmacology and toxicology in animals and humans. *Addiction* **91:**1585–1614.

Adams IB, Ryan W, Singer M, Razdan RK, Compton D and Martin BR (1995a) Pharmacological and behavioral evaluation of alkylated anandamide analogs. *Life Sci* **56:**2041–2048.

Adams IB, Ryan W, Singer M, Thomas BF, Compton D, Razdan RK and Martin BR (1995b) Evaluation of cannabinoid receptor binding and in vivo activities for anandamide analogs. *J Pharmacol Exp Ther* **273:**1172–1181.

Bouaboula M, Poinot-Chazel C, Bourrié B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G and Casellas P (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J* **312:**637–641.

Bouaboula M, Poinot-Chazel C, Marchand J, Canat X, Bourrié B, Rinaldi-Carmona M, Calandra B, Le Fur G and Casellas P (1996) Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor: involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. *Eur J Biochem* **237:**704–711.

Breivogel CS and Childers SR (2000) Cannabinoid signal transduction in rat brain: comparison of cannabinoid agonists in receptor binding, G-protein activation and adenylyl cyclase inhibition. *J Pharmacol Exp Ther* **295:**328–336.

Breivogel CS and Childers SR (1998) The functional neuroanatomy of brain cannabinoid receptors. *Neurobiol Dis* **5:**417–431.

Breivogel CS, Selley DE and Childers SR (1997a) Acute and chronic effects of opioids on delta and mu receptor activation of G-proteins in NG108–15 and SK-N-SH cell membranes. *J Neurochem* **68:**1462–1472.

Breivogel CS, Selley DE and Childers SR (1998) Cannabinoid receptor agonist efficacy for stimulating [^{35}S]GTPγS binding to rat cerebellar membranes correlates with agonist-induced decreases in GDP affinity. *J Biol Chem* **273:**16865–16873.

Breivogel CS, Sim LJ and Childers SR (1997b) Regional differences in cannabinoid receptor/G-protein coupling in rat brain. *J Pharmacol Exp Ther* **282:**1632–1642.

Childers SR and Breivogel CS (1998) Cannabis and endogenous cannabinoid systems. *Drug Alcohol Dep* **51:**173–187.

Compton DR, Aceto MD, Lowe J and Martin BR (1996) *In vivo* characterization of a specific cannabinoid receptor antagonist (SR141716A): inhibition of Δ^9-tetrahydrocannabinol-induced responses and apparent agonist activity. *J Pharmacol Exp Ther* **277:**586–594.

Compton DR, Rice KC, DeCosta BR, Razdan RK, Melvin LS, Johnson MR and Martin BR (1993) Cannabinoid structure-activity relationships: correlation of receptor binding and *in vivo* activities. *J Pharmacol Exp Ther* **265:**218–226.

Deadwyler SA, Hampson RE, Mu J, Whyte A and Childers SR (1995) Cannabinoids modulate voltage-sensitive potassium A-current in hippocampal neurons via a cAMP-dependent process. *J Pharmacol Exp Ther* **273:**734–743.

Deutsch DG and Chin SA (1993) Enzymatic synthesis and degradation of anandamide, a cannabinoid receptor agonist. *Biochem Pharmacol* **46:**791–796.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Wash DC)* **258:**1946–1949.

Di Marzo V, Breivogel CS, Tao Q, Bridgen DT, Razdan RK, Zimmer AM, Zimmer A and Martin BR (2000) Levels, metabolism, and pharmacological activity of anandamide in CB_1 cannabinoid receptor knock-out mice: evidence for non-CB_1, non-CB_2 receptor-mediated actions of anandamide in mouse brain. *J Neurochem* **75:**2434–2444.

Di Marzo V, Melck D, Bisogno T and De Petrocellis L (1998) Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci* **21:**521–528.

Felder CC, Joyce KE, Briley EM, Mansouri J, Mackie K, Blond O, Lai Y, Ma AL and Mitchell RL (1995) Comparison of the pharmacology and signal transduction of the human cannabinoid CB_1 and CB_2 receptors. *Mol Pharmacol* **48:**443–450.

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

Griffin G, Atkinson PJ, Showalter VM, Martin BR and Abood ME (1998) Evaluation of cannabinoid receptor agonists and antagonists using the guanosine-5′-O-(3-[^{35}S]thio)-triphosphate binding assay in rat cerebellar membranes. *J Pharmacol Exp Ther* **285:**553–560.

Heyser CJ, Hampson RE and Deadwyler SA (1993) Effects of delta-9-tetrahydrocannabinol on delayed match to sample performance in rats: alterations in short-term memory associated with changes in task specific firing of hippocampal cells. *J Pharmacol Exp Ther* **264:**294–307.

Houser SJ, Eads M, Embrey JP and Welch SP (2000) Dynorphin B and spinal analgesia: induction of antinociception by the cannabinoids CP55,940, Delta(9)-THC and anandamide. *Brain Res* **857:**337–342.

Howlett AC (1984) Inhibition of neuroblastoma adenylyl cyclase by cannabinoid and nantradol compounds. *Life Sci* **35:**1803–1810.

Jarai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR, Zimmer AM, Bonner TI, Buckley NE, Mezey E, et al. (1999) Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci USA* **96:**14136–14141.

Kaminski NE, Abood ME, Kessler FK, Martin BR and Schatz AR (1992) Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation. *Mol Pharmacol* **42:**736–742.

Mackie K and Hille B (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc Natl Acad Sci USA* **89:**3825–3829.

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

Mailleux P and Vanderhaeghen J-J (1992) Distribution of neuronal cannabinoid receptor in the adult rat brain: a comparative receptor binding radioautography and *in situ* hybridization histochemistry. *Neuroscience* **48:**655–668.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AL and Bonner TI (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature (Lond)* **346:**561–564.

Mechoulam R, Ben-Shabat S, Hanus L, Ligumsky M, Kaminski NE, Schatz AR, Gopher A, Almog S, Martin BR and Compton DR (1995) Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem Pharmacol* **50:**83–90.

Mu J, Zhuang SY, Kirby MT, Hampson RE and Deadwyler SA (1999) Cannabinoid receptors differentially modulate potassium A and D currents in hippocampal neurons in culture. *J Pharmacol Exp Ther* **291:**893–902.

Munro S, Thomas KL and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (Lond)* **365:**61–65.

Prather PL, Martin NA, Breivogel CS and Childers SR (2000) Activation of cannabinoid receptors in rat brain by WIN 55212-2 produces coupling to multiple G protein α-subunits with different potencies. *Mol Pharmacol* **57:**1000–1010.

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

Sim LJ, Hampson RE, Deadwyler SA and Childers SR (1996) Effects of chronic treatment with Δ^9-tetrahydrocannabinol on cannabinoid-stimulated [^{35}S]GTPγS autoradiography in rat brain. *J Neurosci* **16:**8057–8066.

Sim LJ, Liu QX, Childers SR and Selley DE (1998) Endomorphin-stimulated [^{35}S]GTPγS binding in rat brain: Evidence for partial agonist activity at mu-opioid receptors. *J Neurochem* **70:**1567–1576.

Sim LJ, Selley DE and Childers SR (1995) *In vitro* autoradiography of receptor-activated G-proteins in rat brain by agonist-stimulated guanylyl 5′-[γ-[^{35}S]thio]-triphosphate binding. *Proc Natl Acad Sci USA* **92:**7242–7246.

Sugiura T, Kondo S, Sukagawa A, Nakane S, Shinoda A, Itoh K, Yamashita A and Waku K (1995) 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem Biophys Res Commun* **215:**89–97.

Welch SP and Eads M (1999) Synergistic interactions of endogenous opioids and cannabinoid systems. *Brain Res* **848:**183–190.

Wiley JL, Compton DR, Dai D, Lainton JAH, Phillips M, Huffman JW and Martin BR (1998) Structure-activity relationships of indole- and pyrrole-derived cannabinoids. *J Pharmacol Exp Ther* **285:**995–1004.

Zimmer A, Zimmer AM, Hohmann AG, Herkenham M and Bonner TI (1999) Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc Natl Acad Sci USA* **96:**5780–5785.

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