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Assessment of Anandamide Interaction with the Cannabinoid Brain Receptor: SR 141716A Antagonism Studies in Mice and Autoradiographic Analysis of Receptor Binding in Rat Brain[1]

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ABSTRACT

Anandamide is the newly discovered endogenous cannabinoid ligand that binds to brain cannabinoid receptors and shares most, but not all, of the pharmacological properties of Δ^9-THC. Therefore, this study was undertaken to determine whether its interaction with the CB1 receptor in brain was identical to that of Δ^9-THC. Anandamide depressed spontaneous activity and produced hypothermia, antinociception and immobility in mice after i.v. administration. However, none of these effects was blocked by pretreatment with the selective CB1 antagonist, SR 141716A. However, the metabolically stable analog 2-methyl-2′-fluoroethylanandamide produced reductions in motor activity and antinociception in mice, effects that were blocked by the antagonist. To determine whether anandamide's receptor binding mimicked that of other cannabinoids, an autoradiographic comparison of anandamide, SR 141716A and CP 55,940 competition for [^{3}H]CP55,940 binding was conducted throughout rat brain. The receptor affinities for all three compounds did not change according to brain area. As expected, B_{max} values differed dramatically among differ brain areas. However, the B_{max} values for each brain area were similar regardless of the compound used for displacement. These data suggest that anandamide, SR 141716A and CP 55,940 compete for the same cannabinoid receptor throughout brain despite SR 141716A's failure to block anandamide's pharmacological effects. Although there is no question that anandamide binds to the cannabinoid receptor, failure of SR 141716A to block its pharmacological effects in mice poses a dilemma. The results presented herein raise the possibility that anandamide may not be producing all of its effects by a direct interaction with the CB1 receptor.

The discovery of the CB1 cannabinoid receptor (Devane *et al.,* 1988; Matsuda *et al.,* 1990) resulted in considerable effort to ascertain its relevance to the pharmacological effects produced by cannabinoids. The localization of this receptor throughout the brain was found to be consistent with the pharmacological effects produced by cannabinoids. Autoradiography of cannabinoid receptors from several mammalian species, including human, revealed a conserved and unique pattern of distribution (Herkenham *et al.,* 1990). Binding was most dense in the outflow nuclei of the basal ganglia (the substantia nigra pars reticulata and globus pallidus), the hippocampus and the cerebellum. The high densities of receptors in the forebrain and the cerebellum explain the effects of cannabinoids on cognition and movement. High levels in the hippocampus provide a role for these receptors in cannabinoid impairment of memory. Sparse densities in the brainstem areas controlling cardiovascular and respiratory functions may explain why high doses of marijuana do not suppress respiration. The distribution of cannabinoid receptors in rat brain also was determined with the cannabinoid agonists [^{3}H]-WIN 55,212-2 and [^{3}H]-11-OH-Δ^9-THC-DMH (Jansen *et al.,* 1992; Thomas *et al.,* 1992) and antagonist SR 141716A (Rinaldi-Carmona *et al.,* 1996). Binding distribution was very similar between these agonists and antagonists confirming that these structurally diverse compounds bind to the same receptor.

The pattern of distribution of cannabinoid binding is consistent with that of the CB1 mRNA using *in situ* hybridization (Mailleux and Vanderhaegen, 1992; Matsuda *et al.,* 1990). In the hippocampus, high levels of mRNA for the cannabinoid receptor were found in granule cells of the dentate gyrus and in cells of the pyramidal and molecular layers of the hippocampus. Message for the receptor was also prevalent within the superficial and deep layers of the cerebral cortex and amygdala. In the human brain the distribution of

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ABBREVIATIONS: BSA, bovine serum albumin; CP 55,940, (−)-3-[2-hydroxyl-4-(1,1-dimethylheptyl)phenyl]-4-[3-hydroxyl propyl] cyclohexan-1-ol; HU-243, 11-hydroxyhexahydrocannabinol-3-dimethylheptyl; *nor*-BNI (*nor*-binaltorphimine); PEI, polyethylenimine; PMSF, phenylmethylsulfonyl fluoride; THC, tetrahydrocannabinol; SR141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxyamide

the mRNA encoding for the cannabinoid receptor also has been studied using *in situ* histochemistry and oligonucleotide probes (Mailleux *et al.,* 1992; Westlake and Howlett, 1994).

Despite a wealth of knowledge regarding the CB1 receptor, numerous questions persist, the most noteworthy of which is whether this single receptor is responsible for all of the central actions of cannabinoids. As mentioned above, several structurally diverse cannabinoids, including the aminoalkylindoles (WIN 55,212), the bicyclic compounds (CP 55,940) and dimethylheptyl analogs of THC (11-OH-Δ^9-THC-DMH) appear to exert their effects at the same receptor based on similarities in localization of binding throughout brain and pharmacological profiles. The discovery of the putative endogenous cannabinoid ligand, anandamide (Devane *et al.,* 1992), made the notion of a central cannabinoid system plausible. Anandamide produces a pharmacological profile very similar to that of Δ^9-THC in several behavioral models (Fride and Mechoulam, 1993; Smith *et al.,* 1994; Wiley *et al.,* 1995a) even though its structure deviates dramatically from that of the other cannabinoids. Although anandamide produces many of the same effects as other psychoactive cannabinoids, differences do exist. Comparison between anandamide and Δ^9-THC revealed that anandamide was 4- to 20-fold less potent and had a shorter duration of action than Δ^9-THC (Smith *et al.,* 1994). Anandamide is less efficacious than Δ^9-THC at the N-type calcium channels (Mackie *et al.,* 1993). Anandamide produces antinociception like other cannabinoids, but in contrast to Δ^9-THC, anandamide is not active when administered intracerebroventricularly (Smith *et al.,* 1994). Also, unlike other cannabinoids, anandamide's antinociception is not blocked by the kappa antagonist *nor*-BNI (Smith *et al.,* 1994). The question arises as to whether anandamide is interacting with the same receptor as Δ^9-THC or whether it is acting at a different receptor subtype.

The development of SR 141716A, a selective CB1 antagonist (Rinaldi-Carmona *et al.,* 1994), has proven to be a valuable tool for identifying receptor-mediated cannabinoid action. SR 141716A has been shown to be effective in blocking the actions of cannabinoids in several mouse behavioral assays (Compton *et al.,* 1996; Rinaldi-Carmona *et al.,* 1994), rat drug discrimination (Wiley *et al.,* 1995b), rat memory tasks (Lichtman and Martin, 1996), mouse vas deferens (Rinaldi-Carmona *et al.,* 1994), adenylyl cyclase (Rinaldi-Carmona *et al.,* 1994), long-term potentiation (Collins *et al.,* 1995), stimulated-arachidonic acid release (Shivachar *et al.,* 1996), and cardiovascular function (Varga *et al.,* 1995). SR 141716A blocks the actions of anandamide in mouse vas deferens (Rinaldi-Carmona *et al.,* 1994), cardiovascular system (Varga *et al.,* 1995), turning behavior after intrastriatal injections (Souilhac *et al.,* 1995), adenylyl cyclase (Felder *et al.,* 1995) and long-term potentiation (Terranova *et al.,* 1995). However, SR 141716A has not been evaluated for blockade of anandamide's pharmacological effects in the mouse tetrad model, a model highly correlated with CB1 receptor affinity (Compton *et al.,* 1993).

The purpose of our investigation was to determine the extent to which the cannabinoid antagonist would block the pharmacological effects of anandamide and whether differences exist between the cannabinoid receptor population that binds CP 55,940, SR 141716A and anandamide. The pharmacological effects of anandamide were assessed in the mouse model of spontaneous activity, antinociception, body temperature and immobility, because most of the most complete characterization of anandamide and its analogs has been done in this model. Autoradiography in rat brain was used to make a direct comparison of receptor affinities of anandamide, CP 55,940 and SR 141716A in different brain regions as well as determine their maximal binding capacities throughout brain. The most complete characterization of cannabinoid receptor localization has used rat brain. Because anandamide does not have high affinity for the cannabinoid receptor, in comparison to other synthetic high-affinity THC analogs, [^{3}H]-anandamide was not used directly to label receptors. Performing autoradiography with compounds possessing weak affinity to a receptor introduces many technical problems, the most serious of which is receptor dissociation. Therefore, the strategy was to compare the abilities of unlabeled anandamide, SR 141716A and CP 55,950 to compete with [^{3}H]CP 55,940 binding. If these agents are binding to the CB1 receptor in brain, then the binding localization should be the same in different brain regions for all three compounds. Differences in binding densities between regions for the three compounds might suggest that a receptor subtype for the central cannabinoid receptor exists. However, these differences can only be detected if [^{3}H]CP 55,940 is labeling more than one receptor. An additional objective was to determine cannabinoid receptor affinities for anandamide, SR 141716A and CP 55,940 and compare the affinities for each compound between brain regions. Differences in affinities may indicate that a particular drug is binding to a brain region in a different manner than to cannabinoid receptors in other regions.

Materials and Methods

Animals. Male Sprague-Dawley rats (150–200 g) from Harlan Laboratories (Dublin, VA) were maintained on a 14:10 hr light/dark schedule and freely received food and water. Male ICR mice weighing 18 to 25 g were used in all *in vivo* experiments. The mice were also maintained on a 14:10 hr light:dark cycle with free access to food and water.

Chemicals. [^{3}H]CP 55,940 was purchased from Du Pont NEN (Wilmington, DE). CP 55,940 and SR 141716A were a gift from Pfizer, Inc. (Groton, CT), and anandamide and 2-methyl-2′-fluoroethylanandamide were kindly provided by Dr. Raj K. Razdan of Organix, Inc. (Woburn, MA). These compounds were prepared as 1 mg/ml stock solutions in absolute ethanol and stored at −20°C for receptor binding. PMSF was dissolved in absolute ethanol as a 20 mg/ml stock solution. Δ^9-THC was obtained from the National Institute on Drug Abuse. For *in vivo* experiments, all drugs were dissolved in 1:1:18 (emulphor-ethanol-saline) to prepare micellular suspensions of drugs suitable for *in vivo* administration (Olsen *et al.,* 1973). Emulphor (EL-620, a polyoxyethylated vegetable oil, GAF Corporation, Linden, NJ) is currently available as Alkmulphor. Drug injections were administered i.v. (tail vein) at a volume of 0.1 ml/10 g of body weight.

Pharmacological evaluation *in vivo*. Mice were acclimated to the evaluation room overnight without interruption of food or water. Mice were pretreated with either vehicle or SR 141716A (i.v.) 10 min before a second i.v. injection of either vehicle or the test drug (anandamide or 2-methyl-2′-fluoroethylanandamide). After the second i.v. injection, one group of mice was evaluated for tail-flick latency (antinociception) response at 5 min and spontaneous (locomotor) activity at 5 to 15 min, although another group was assessed for core (rectal) temperature at 5 min and ring-immobility (catalepsy) at 5 to 10 min, as described elsewhere (Adams *et al.,* 1995; Smith *et al.,* 1994). Inhibition of spontaneous activity was accomplished by plac-

ing mice into individual activity cages (6.5 × 11 in), and recording interruptions of the photocell beams (16 beams per chamber) for a 10-min period using a Digiscan Animal Activity Monitor (Omnitech Electronics Inc., Columbus, OH). Activity in the chamber was expressed as the total number of beam interruptions. Antinociception was assessed using the tail-flick procedure (Dewey *et al.,* 1970). The heat lamp of the tail-flick apparatus was maintained at an intensity sufficient to produce control latencies of 2 to 3 sec. Control values for each animal were determined prior to drug administration. Mice were then reevaluated after drug administration and latency (sec) to tail-flick response was recorded. A 10-sec maximum was imposed to prevent tissue damage. The degree of antinociception was expressed as the % MPE (maximum possible effect) which was calculated as:

$$\%\ \text{MPE} = \left[\frac{(\text{test latency} - \text{control latency})}{(10\ \text{sec} - \text{control latency})}\right] \times 100$$

Hypothermia was assessed by first measuring baseline core temperatures before drug treatment with a telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH) and a rectal thermistor probe inserted to 25 mm. Rectal temperatures were also measured after drug administration. The temperature difference (°C) before and after drug administration was calculated for each animal. Catalepsy was determined by a ring-immobility procedure (Pertwee, 1972). Mice were placed on a ring (5.5 cm in diameter) that was attached to a stand at a height of 16 cm. The amount of time (sec) that the mouse spent motionless during a 5-min test session was recorded. The criterion for immobility was the absence of all voluntary movements (excluding respiration, but including whisker movement). The immobility index was calculated as:

$$\%\ \text{immobility} = \left[\frac{\text{time immobile (sec)}}{\text{length of session (sec)}}\right] \times 100$$

Mice that fell or actively jumped from the ring were allowed five such escapes. After the fifth escape, the test for that animal was terminated and immobility was calculated as a percentage of time that it remained on the ring before being discontinued. Data from mice failing to remain on the ring at least 2.5 min were not included.

Tissue preparation. After decapitation, rat brains were quickly removed and frozen in 2-methylbutane (−50°C). The brains were embedded in M-1 embedding matrix and stored at −70°C until sectioning. Brains were mounted onto cryostat chucks with TFM tissue freezing medium. Consecutive coronal brain sections (16 μm) were thaw-mounted onto slides coated with 0.5% gelatin and 0.05% chromium potassium sulfate. Sections were made at the stereotaxic coordinates 1.2 mm from bregma, 0.48 mm from bregma, −5.2 mm from bregma and −12.8 mm from bregma (Paxinos and Watson, 1986). Sections were stored desiccated at −70°C before use in binding assays.

***In situ* cannabinoid binding assays.** Assay conditions for cannabinoid binding have been described previously (Herkenham *et al.,* 1991). Saturation experiments were first performed to determine the K_d of [^{3}H]CP 55,940. Coronal sections containing primarily frontal cortex and caudate-putamen (1.2 mm from bregma) were used for Scatchard analysis. Slides were allowed to return to room temperature and incubated for 2 hr in slide mailers at 37°C in reaction buffer (50 mM Tris-HCl with 5% BSA, pH 7.4). Total binding was determined with seven concentrations of [^{3}H]CP 55,940 (1.1, 2.3, 4.5, 7.5, 15, 23 and 30 nM). Nonspecific binding was determined by incubating [^{3}H]CP 55,940 in the presence of 1 μM CP 55,940. Saturation experiments were also performed with 50 μM PMSF in the incubation buffer. After incubation, slides were washed for 4 hr at 0°C in 50 mM Tris-HCl with 1% BSA (pH 7.4). Sections were scraped from the slides with Whatman GF/C filters. The filters were placed in scintillation vials, and the tissue was solubilized overnight with 1.0 ml of TS-2. Samples were acidified with 10 μl of glacial acetic acid and counted by liquid scintillation spectrometry. Transformation of the data and calculation of K_d values was accomplished using the LIGAND computer software (Munson and Rodbard, 1980) as supplied by Biosoft Inc. (Cambridge, U.K.).

Competition for [^{3}H]CP 55,940 binding. Due to the reported instability of anandamide (Childers *et al.,* 1994; Deutsch and Chin, 1993), optimal binding conditions were first determined in the presence and absence of the enzyme inhibitor PMSF. In the competition experiments, reaction buffers, incubation temperatures and times were identical to the *in situ* binding assay described above. Sections were made from 0.48 mm from bregma, −5.2 from bregma and −12.8 mm from bregma. For CP 55,940, SR 141716A and anandamide, nonspecific binding was determined using 10 μM CP 55,940, and total binding was determined for 10 nM [^{3}H]CP 55,940 (approximately 40% receptor occupation). Eight concentrations of CP 55,940 ranging from 0.1 to 300 nM were assayed; concentrations of anandamide ranged from 0.01 to 10 μM, and concentrations of SR 141716A were 0.001 to 10 μM. Each experiment was conducted in at least triplicate. For anandamide displacement assays, additional experiments were performed either with 50 μM PMSF in the incubation buffer or with sections pretreated for 30 min in a buffer containing 50 μM PMSF before exposure of 50 μM PMSF in the incubation buffer. Anandamide sections were wiped from the slides, solubilized and counted. Optimal conditions for anandamide competition experiments occurred when sections were pretreated for 30 min with PMSF and exposed to PMSF in the incubation buffer.

[^{3}H]CP 55,940 autoradiography. After the wash, slides were rapidly dried with a stream of cool air and stored in a desiccator overnight at 4°C. Sections were apposed to tritium-sensitive film with [^{3}H]-microscales for 3 wk before developing with a D-19 developer. Developed films were analyzed using the NIH Image 1.49 program. Levels of transmittance were converted to dpm/mg protein using a polynominal curve fit of the standards. Brain structures were outlined, and optical density in each area was measured. Curve-fitting of the displacement data and determination of K_i and B_{max} values for anandamide, CP 55,940 and SR 141716A were done using EBDA software. K_i and B_{max} values for anandamide in the substantia nigra and the molecular layer of the cerebellum were determined from autoradiograms apposed to film for 1 wk.

Statistical analysis. Significant differences between K_i values were determined using the ANOVA analysis (Scheffe *post hoc* analysis). To determine if curves were parallel, data from a representative displacement curve from each brain area for anandamide, SR 141716A and CP 55,940 were analyzed using the ALLFIT curve-fitting program. B_{max} values were compared by linear correlations for anandamide and CP 55,940 and SR 141716A and CP 55,940. ANOVA and Scheffe *post hoc* analysis was also used to determine significance in the *in vivo* antagonism studies.

Results

Pharmacological evaluation *in vivo*. The i.v. administration of anandamide produced pharmacological effects in all four mouse pharmacological procedures similar to that described previously (Smith *et al.,* 1994). The ED50's for producing hypomotility in the absence and presence of 3.0 and 30 mg/kg of SR141716A were 11.4, 15.7, and 15.8 mg/kg, respectively (fig. 1). The corresponding ED50's for antinociception were 3.0, 4.2, and 2.9 mg/kg, respectively. The ED50's for producing immobility in the absence and presence of 3.0 and 30 mg/kg of SR141716A were 13.7, 26.9 and 16.0 mg/kg, respectively. Anandamide's effects on rectal temperature were rather modest (maximal effect of 2.4°C decrease at 20 mg/kg) and variable. Despite anandamide's rather weak hypothermic effects, SR141716A still was unable to effectively prevent these effects.

To verify that anandamide and Δ^9-THC did indeed respond

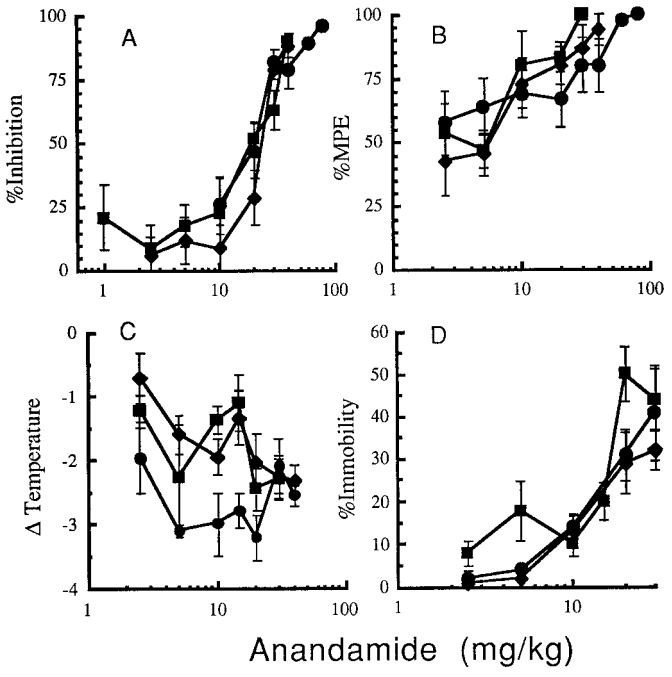

Fig. 1. Failure of SR 141716A to block the effects on anandamide on spontaneous activity (A), tail-flick response (B), rectal temperature (C) and the ring test (D) in mice. The results are presented as means ± S.E. (N = 6 mice per group). Mice were pretreated (i.v.) with either vehicle (■), 3 mg/kg of SR 141716A (◆) or 30 mg/kg of SR 141716A (●) 10 min before the i.v. administration of the indicated doses of anandamide.

differently to pretreatment with SR 141716A, an experiment was conducted in which anandamide and Δ^9-THC were compared in the same experiment. The results in table 1 clearly show that a dose of 3 mg/kg of SR 141716A effectively blocks hypothermia and immobility produced by Δ^9-THC; whereas, this dose of the antagonist was without effects on anandamide-induced hypothermia and immobility.

Failure of SR 141716A to antagonize the effects of anandamide raised the possibility that metabolism of anandamide could be a possible factor. Therefore, studies were carried out to determine whether SR 141716A could block the pharmacological effects of the metabolically stable anandamide analog 2-methyl-2′-fluoroethylanandamide. The results in table 2 demonstrate that pretreatment with SR 141716A at a dose of 3 mg/kg completely reversed the hypoactivity produced by this anandamide analog. The antagonist produced a slight stimulation of motor activity, but this effect was not statistically significant from the vehicle group. However, SR 141716A pretreatment produced a statistically significant reduction in hypoactivity produced by all doses of 2-methyl-2′-fluoroethylanandamide. As for antinociception, SR 141716A also produced a statistically significant reduction in the effect produced by a 10-mg/kg dose of 2-methyl-2′-fluoroethylanandamide. There was not a statistically significant antagonism at the lower doses of 1-methyl-2′-fluoroethylanandamide.

TABLE 1

Pretreatment of SR 141716A before either Δ^9-THC or anandamide in mice[a]

Pretreatment	Treatment	Test Time	Hypothermia	Immobility
Vehicle	Vehicle	30	−0.6 ± 0.3	0 ± 0
Vehicle	Δ^9-THC (3)	30	−4.4 ± 0.4	54 ± 6.3
SR 141716A (3)	Δ^9-THC (3)	30	−0.4 ± 0.4	1.0 ± 0.6
Vehicle	Vehicle	5	−0.3 ± 0.3	0 ± 0
Vehicle	Anandamide (30)	5	−0.4 ± 0.3	9.3 ± 2.9
SR 141716A (3)	Anandamide (30)	5	−1.9 ± 0.3	11 ± 4.4
Vehicle	Anandamide (40)	5	−3.8 ± 0.3	51 ± 6.8
SR 141716A (3)	Anandamide (40)	5	−3.9 ± 0.4	51 ± 5.6

[a] Mice were administered either vehicle or SR 141716A i.v. 10 min before the i.v. administration of the indicated treatment (doses expressed as mg/kg). The animals were tested 30 min after Δ^9-THC treatment and 5 min after anandamide as shown above. The results are presented as mean ± S.E.M. (N = 6–12/group) as changes in body temperature (°C) or percent immobility.

TABLE 2

SR 141716A blockade of hypomotility and antinociception induced by 2-methyl-2′-fluoroethylanandamide

Pretreatment[a] SR 141716A	2-Methyl-2′-Fluoroethyl-anandamide[b]	Spontaneous Activity[c]	Antinociception[d]
0	0		8 ± 2
3	0	−24 ± 10	18 ± 8
0	1	37 ± 12	25 ± 8
3	1	−17 ± 16[e]	39 ± 7
0	3	53 ± 7	38 ± 12
3	3	−30 ± 13[e]	32 ± 7
0	10	81 ± 3	84 ± 8
3	10	−2 ± 14[e]	28 ± 12[e]

[a] Administered i.p. 10 min before i.v. administration of 1-methyl-2′-fluoroethylanandamide. Dose expressed as mg/kg.
[b] Dose expressed as mg/kg.
[c] Data expressed as percent decrease in spontaneous activity of the vehicle/vehicle group and presented as means ± S.E. for 12 mice per group.
[d] %MPE expressed as means ± S.E. for 12 mice per group.
[e] Significantly different from the corresponding vehicle-pretreated group at $P < 0.05$ by Scheffe *post hoc* analysis.

***In vitro* receptor binding.** Cannabinoid receptor affinity (K_d) was determined for CP 55,940 both in the presence and absence of the enzyme inhibitor PMSF. Without PMSF a K_d value of 15.3 ± 1.2 nM (n = 5) was calculated which correlated with the value of 15 ± 3 nM reported in the literature for binding to tissue slices (Herkenham *et al.,* 1990). In the presence of PMSF a K_d value of 12.3 ± 2.1 nM (n = 3) resulted, which is not statistically different from the K_i value obtained without PMSF. Since the presence of PMSF did not influence affinity to the cannabinoid receptor, all K_is were calculated using the K_d value of 15.3 nM.

Because anandamide is susceptible to enzymatic cleavage, conditions were established for determining optimal receptor binding with anandamide. We had previously shown that a concentration of 50 μM of PMSF was effective in preventing metabolism of anandamide in brain homogenates (Adams *et al.,* 1995). Without inclusion of PMSF a K_i of 8,030 ± 1,110 nM (n = 3) resulted when anandamide was incubated with brain slices. PMSF (50 μM) then was added to the buffer during the two-hour incubation period. The K_i decreased to only 2,320 ± 540 nM (n = 6) which suggested that anandamide was probably being degraded. Thus, slices were pretreated for 30 min with 50 μM PMSF in addition to the PMSF exposure during the incubation time which resulted in a K_i of 608 ± 210 nM (n = 3). This K_i value is approximately 10-fold greater than that found in the brain homogenate assay which is consistent with that observed for CP 55,940 (discussed later). Therefore, slices were exposed to PMSF before and during incubation for autoradiography experiments using anandamide, because this treatment appeared to effectively block metabolism.

Quantities and patterns of [^{3}H]CP 55,940 binding (figs. 2–5) were in agreement with previously reported data (Herkenham *et al.,* 1991; Jansen *et al.,* 1992; Thomas *et al.,* 1992). High levels of binding were found in the substantia

nigra pars reticulata, molecular layer of the cerebellum, dentate gyrus and CA1 and CA3 regions of Ammon's horn. Moderate levels of cannabinoid receptor binding was observed in all of the cortical areas and throughout most of the brain. Little binding was found in the brainstem or the corpus callosum indicating a lack of receptors in these areas.

A qualitative examination of the autoradiographic film demonstrates the nonspecific binding that results when an excess of unlabeled CP 55,940 is added (fig. 2). Three sections of the rat brain were examined. The stereotaxic coordinates were 0.48 mm, −5.2 mm and −12.8 mm from bregma. At 0.48 mm from bregma measurements were made in the lateral and medial caudate-putamen and the frontal and occipital cortices (fig. 3A). At −5.2 mm from bregma measurements were made from the CA1 and CA3 regions from Ammon's horn, dentate gyrus, entorhinal and occipital cortices and substantia nigra (fig. 4A). At −12.8 mm from bregma measurements were made from the molecular layer of the cerebellum (fig. 5A).

Also apparent from a visual inspection of the developed brain images is the similarity of the displacement patterns for SR 141716A, CP 55,940 and anandamide. Figures 3, 4 and 5 show that binding density, qualitatively, appears not to differ between SR 141716A, CP 55,940 and anandamide at 30, 3 and 3000 nM, respectively. These concentrations represent approximately 50% displacement of [^{3}H]CP 55,940.

To quantitate receptor affinity and number, the competition binding data were analyzed autoradiographically in selected brain areas, and displacement curves were constructed. Figure 6 shows representative displacement curves for anandamide, SR 141716A and CP 55,940 in the lateral caudate-putamen. Because anandamide is a weaker ligand for the cannabinoid receptor, anandamide's displacement

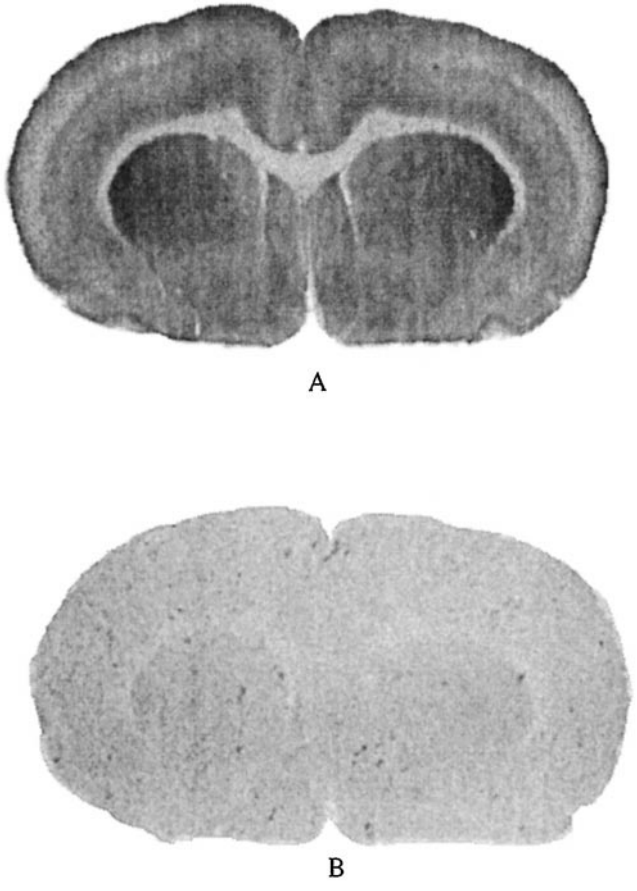

Fig. 2. Autoradiogram of total (A) and nonspecific (B) binding of [^{3}H]CP 55,940 to coronal sections of rat brain.

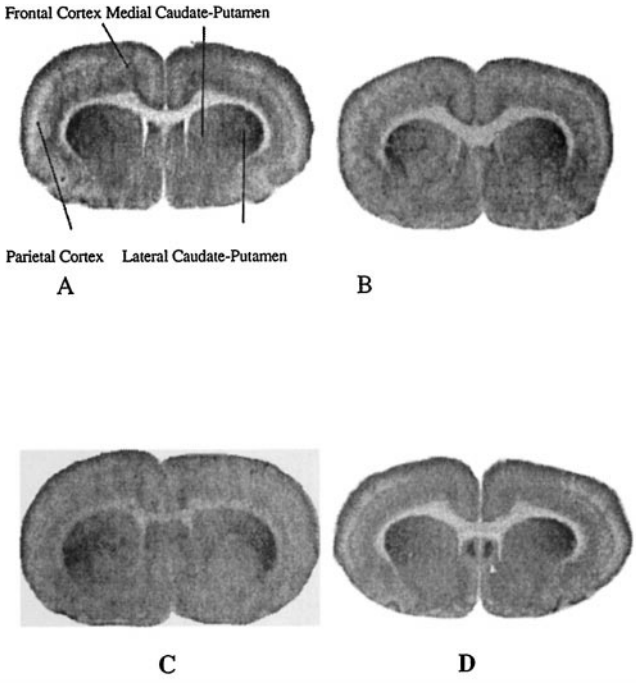

Fig. 3. Autoradiogram of total [^{3}H]CP 55,940 binding to a coronal section of rat brain 0.48 mm from bregma (A); [^{3}H]CP 55,940 displacement at 0.48 mm from bregma by SR 141716A (B, 30 nM), CP 55,940 (C, 3 nM) and anandamide (D, 300 nM).

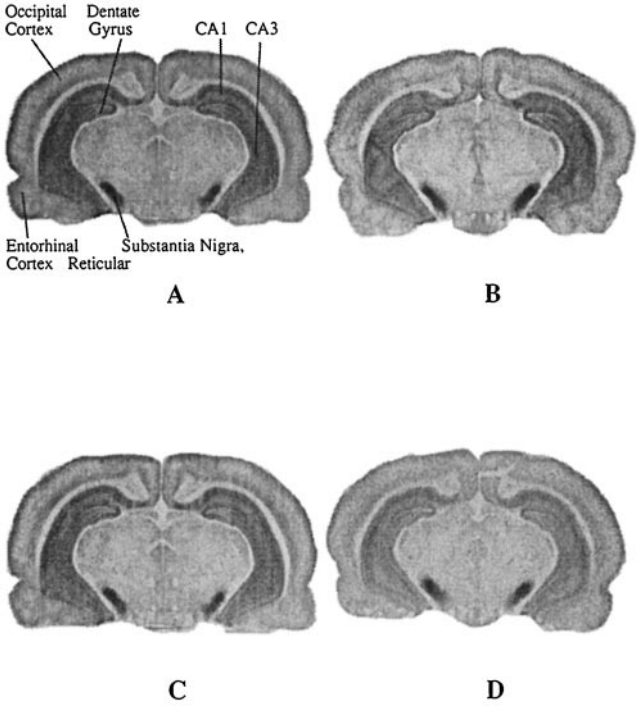

Fig. 4. Autoradiogram of total [^{3}H]CP 55,940 binding to a coronal section of rat brain −5.2 mm from bregma (A); [^{3}H]CP 55,940 displacement at −5.2 mm from bregma by SR 141716A (B, 30 nM), CP 55,940 (C, 3 nM) and anandamide (D, 300 nM).

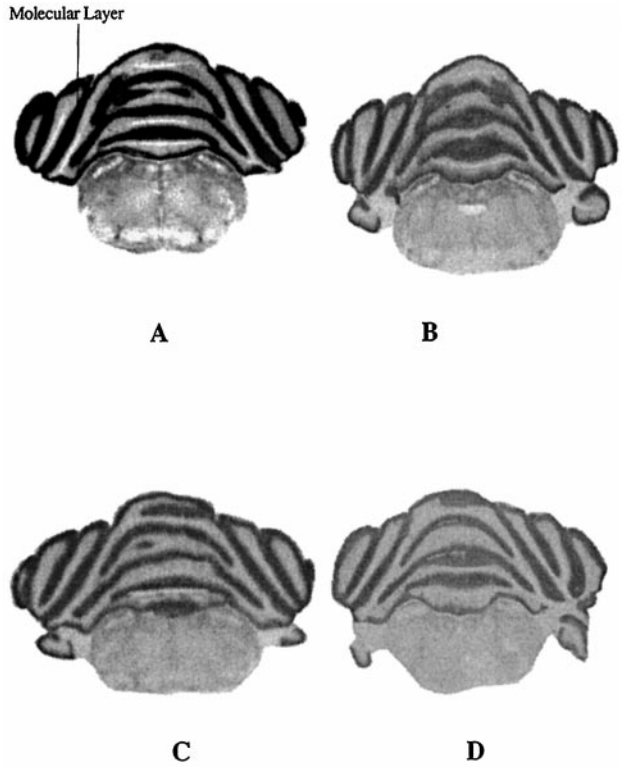

Fig. 5. Autoradiogram of total [^{3}H]CP 55,940 binding to a coronal section of rat brain −12.8 mm from bregma (A); [^{3}H]CP 55,940 displacement at −12.8 mm from bregma by SR 141716A (B, 30 nM), CP 55,940 (C, 3 nM) and anandamide (D, 300 nM).

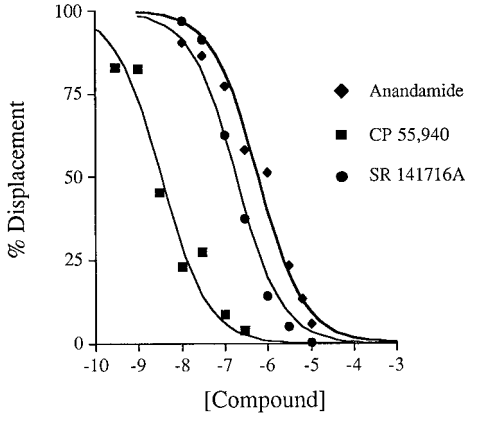

Fig. 6. Anandamide, SR 141716A and CP 55,940 displacement of [^{3}H]CP 55,940 in the lateral caudate-putamen. The curves are representative displacement curves.

curve lies to the right of CP 55,940's curve. The curve for SR 141716A is to the right of CP 55,940's curve and to the left of anandamide's curve because SR 141716A has a higher affinity to the cannabinoid receptor than anandamide and has lower affinity than CP 55,940. Similar curves were generated from the other regions.

The binding affinity for anandamide, SR 141716A and CP 55,940 in each brain area are found table 3. The average K_i value for anandamide in the rat brain homogenate binding assay in the presence of PMSF was 90 nM (Adams *et al.,* 1995), and the average K_i value for anandamide in the present autoradiography experiments was 548 nM. Higher K_i and K_d values are obtained using *in situ* binding than in homogenate membrane binding. These differences are consistent for numerous ligands and are thought to reflect methodological differences between the two types of assays (Herkenham *et al.,* 1991). Average K_i values were 9 nM for CP 55,940 and 47 nM for SR 141716A. Binding affinities were analyzed statistically to determine if differences existed for anandamide's affinity for the CB1 receptor between different brain regions. Statistical analyses were also performed for SR 141716A and CP 55,940. Binding affinities for anandamide, SR 141716A and CP 55,940 were not statistically significant between brain areas. The K_i for the entorhinal cortex for anandamide and CP 55,940 were not included in the statistical analysis since the value was determined from an average of only two experiments. The cannabinoid receptor had the same affinity for anandamide in all regions analyzed. Binding affinities for CP 55,940 and SR 141716A also did not differ between brain regions.

A representative displacement curve was selected from each brain region for each compound. The curves for anandamide, CP 55,940 and SR 141716A were analyzed for parallelism by the program ALLFIT. All displacement curves for anandamide and CP 55,940 from each brain area were parallel. All displacement curves for SR 141716A, except for the entorhinal curve, were parallel.

The maximal displacement of [^{3}H]CP 55,940 binding by the three compounds are summarized in table 4. B_{max} values of [^{3}H]CP 55,940 are listed according to areas with low (the

TABLE 3
K_i values for anandamide, CP 55,940 and SR 141716A

Brain Region	Anandamide K_i (nM)	CP-55,940 K_i (nM)	SR 141716A K_i (nM)
Entorhinal cortex	956[a]	10.2[a]	36 ± 4
Parietal cortex	713 ± 7	6.7 ± 2.1	35 ± 14
Occipital cortex	328 ± 86	17.5 ± 7.8	34 ± 7
Frontal cortex	661 ± 145	8.5 ± 0.2	26 ± 10
Medial caudate-putamen	566 ± 93	9.0 ± 2.0	31 ± 8
Lateral caudate-putamen	515 ± 68	5.3 ± 1.3	52 ± 17
Dentate gyrus	400 ± 72	8.5 ± 0.7	36 ± 5[b]
CA1	438 ± 87	6.0 ± 0.9	24 ± 3[b]
CA3	489 ± 176	7.4 ± 1.3	38 ± 5[b]
Substantia nigra	512 ± 169	7.3 ± 2.9	95 ± 29[b]
Molecular layer of the cerebellum	861 ± 32	12.5 ± 4	114 ± 19[b]

Results are presented as means ± S.E. for three experiments unless indicated otherwise.
[a] $N = 2$.
[b] $N = 6$.

TABLE 4
B_{max} values for anandamide, CP 55,940 and SR 141716A

Brain Region	Anandamide B_{max} (pM)	CP-55,940 B_{max} (pM)	SR 141716A B_{max} (pM)
Entorhinal cortex	44 ± 7	28 ± 7	48 ± 14
Parietal cortex	50 ± 2	34 ± 4	66 ± 6
Occipital cortex	59 ± 7	42 ± 7	43 ± 2
Frontal cortex	76 ± 11	56 ± 7	94 ± 6
Medial caudate-putamen	108 ± 7	44 ± 2	107 ± 13
Lateral caudate-putamen	185 ± 33	109 ± 9	189 ± 19
Dentate gyrus	211 ± 13	110 ± 22	149 ± 16[a]
CA1	258 ± 22	151 ± 29	147 ± 15[a]
CA3	771[b]	163 ± 33	150 ± 20[a]
Substantia nigra	209 ± 4	212 ± 34	204 ± 35[a]
Molecular layer of the cerebellum	263 ± 44	250 ± 55	248 ± 22[a]

Results are presented as means ± S.E. for three experiments unless indicated otherwise.
[a] $N = 6$.
[b] $N = 2$.

cortices), moderate (the caudate-putamen) and high values. The molecular layer of the cerebellum had the highest B_{max} for anandamide (with the exception of CA3), CP 55,940 and SR 141716A. For an unknown reason, the B_{max} for anandamide in the CA3 region was extremely high. Because data were available for only two assays; the CA3 region was not used when determining correlations between anandamide and CP 55,940. The entorhinal cortex had the lowest B_{max} for both anandamide and CP 55,940. The lowest value for SR 141716A was in the occipital cortex. To compare densities, linear correlations were made between CP 55,940 and SR 141716A. Correlations also were made between CP 55,940 and anandamide. An excellent correlation ($r = 0.89$) was obtained when the B_{max} values for anandamide and CP 55,940 were compared (fig. 7). A correlation coefficient of 0.92 was obtained when B_{max} values for SR 141716A were compared to those of CP 55,940.

Discussion

The failure of SR 141716A to block the effects of AN in the mouse behavioral assays was quite surprising given the wide range of anandamide effects that it blocks in other systems that include smooth muscle preparations (Rinaldi-Carmona *et al.,* 1994), the cardiovascular system of rats (Varga *et al.,* 1995), turning behavior after intrastriatal injections (Souilhac *et al.,* 1995), adenylyl cyclase (Felder *et al.,* 1995) and long-term potentiation (Terranova *et al.,* 1995). In addition, SR 121716A is very effective in blocking the behavioral effects of Δ^9-THC in mice with AD50s of approximately 0.1 mg/kg (Compton *et al.,* 1996). There is the possibility that methodological issues could account for SR 141716A's ineffectiveness against anandamide. However, the antagonism of Δ^9-THC occurred rapidly after administration of SR 141716A and was of long duration so that the time course of SR141716A would not seem to be an issue. Furthermore, the doses of SR141716A are more than sufficient for producing a complete blockade of THC's effects.

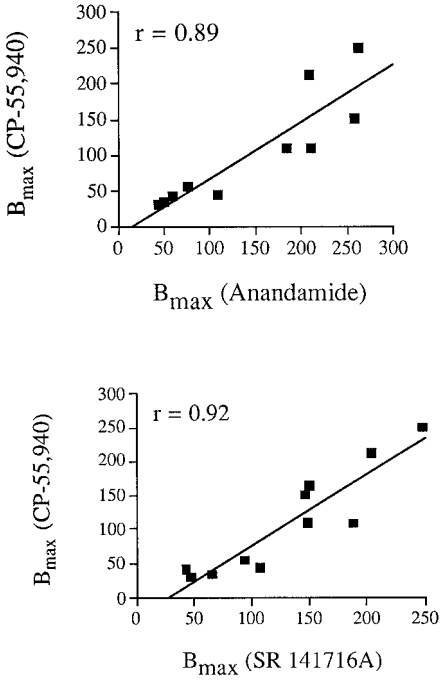

Fig. 7. Relationship between B_{max} values for anandamide, SR 141716A and CP 55,940 in different brain areas.

The question of whether anandamide is capable of interacting with the central cannabinoid receptor is not an issue. Several laboratories have shown that anandamide competes for cannabinoid binding in brain homogenates (Adams *et al.,* 1995; Childers *et al.,* 1994; Devane *et al.,* 1992; Felder *et al.,* 1993) with an affinity that is consistent with its relatively weak pharmacological potency. Although receptor binding studies suggest that CP 55,940, SR 141716A and anandamide are all acting at the same binding site, it is possible that they bind differently to the CB1 receptor in discrete brain areas or receptor subtypes exist that have not yet been discovered. The autoradiographic examination undertaken in the present studies provided a means for systematically examining the interactions of these compounds with cannabinoid receptors throughout the brain. The importance of receptor distribution has been amply demonstrated in several mammalian species (Herkenham *et al.,* 1990, 1991). The densest binding occurs in the basal ganglia (substantia nigra pars reticulata, globus pallidus, entropeduncular nucleus and lateral caudate putamen), and the molecular layer of the cerebellum. Binding in these regions may explain cannabinoid interference with movement. Intermediate levels of binding were found in the CA pyramidal cell layers of the hippocampus, the dentate gyrus and layers I and VI of the cortex. Δ^9-THC disrupts short-term memory in humans (Chait and Pierri, 1992). Cannabinoid effects on memory and cognition are consistent with receptor localization in the hippocampus and cortex. The presence of cannabinoid receptors in regions associated with mediating brain reward (ventromedial striatum and nucleus accumbens) suggests an association with dopamine neurons. Sparse levels were detected in the brainstem, hypothalamus, corpus callosum and the deep cerebellum nuclei. Low levels of receptors in brainstem areas controlling respiratory function is also consistent with the lack of lethality of marijuana. A similar binding profile between CP 55,940 and anandamide would reinforce the premise that these two cannabinoids have similar pharmacological characteristics.

Because anandamide is degraded in homogenate binding, it was also necessary to determine optimal binding conditions for anandamide. Consistent results were obtained when slices were pretreated with PMSF and exposed to PMSF during the reaction incubation. These results confirm previous reports of anandamide's instability in a biological system. Because slices had to be both pretreated and exposed to PMSF during the entire 2-hr experiment, this finding suggests that levels of the enzyme that degrade anandamide are high in the brain. If anandamide is a neurotransmitter, then mechanisms must exist in the CNS to rapidly remove anandamide and prevent continuous stimulation. As such, one would not expect an endogenous compound to possess great stability. Development of more stable analogs would eliminate the need of exposing brain tissue to enzyme inhibitors. PMSF inhibits a wide variety of enzymes, not just amidases. Therefore, it was necessary to determine if PMSF produced an effect on cannabinoid receptor affinity, as determined from saturation experiments. Unlike the homogenate receptor binding assay, PMSF did not influence cannabinoid receptor affinity for brain slice binding. The K_d determined in the presence of PMSF was not statistically different from the

K_d obtained in the absence of PMSF. It is unknown why PMSF caused a 2-fold shift in the receptor affinity in the homogenate receptor assay, but the shift is probably due to methodological differences between slice and homogenate binding.

Establishing conditions for incubation of anandamide with tissue slices enabled us to compare the abilities of anandamide, CP 55,940 and SR 141716A to bind to the cannabinoid receptor in the following brain areas: lateral and medial caudate-putamen, frontal, occipital, entorhinal and parietal cortices, dentate gyrus, substantia nigra and the molecular layer of the cerebellum. These areas were selected because cannabinoids affect the functioning of these regions as discussed above. Also, they are all large enough so that a sufficient number of consecutive 16 μm slices could be made. Several of the areas, including the molecular layer of the cerebellum and the substantia nigra, have very dense levels of receptors. The dentate gyrus, CA1, CA3 and lateral caudate-putamen also have dense receptor populations. The cortical regions have moderate levels of cannabinoid receptors.

For anandamide, CP 55,940 and SR 141716A, no statistical difference existed between their K_is in different brain regions. Because anandamide is a weak ligand, it is possible that at the lower concentrations in anandamide's displacement curves the percentage of displacement is less accurate because the amount of displacement is overshadowed by the high numbers of receptors. If any of these compounds were binding to a receptor subtype possessing either a higher or lower K_i from the other regions, a statistical difference should result. No such differences were found for CP 55,940, anandamide or SR 141716A. Therefore, these findings support the notion of a common receptor for both CP 55,940 and anandamide.

An additional objective was to analyze representative curves for parallelism from each region for anandamide, SR 141716A and CP 55,940. Representative displacement curves from each brain region for anandamide were analyzed using the statistical program ALLFIT to determine if they were parallel. Differences in parallelism would provide evidence that a compound was interacting with the cannabinoid receptor in a different manner from other regions. All 11 curves for anandamide were parallel, as were curves for CP 55,940. For an unknown reason, the entorhinal cortex curve for SR 141716A was not parallel to the other brain regions analyzed. Thus, these three compounds appear to bind to the CB1 receptor in a similar manner.

Cannabinoid receptor densities were calculated for each brain region for anandamide, SR 141716A and CP 55,940. The purpose of calculating B_{max} values for each region was to determine if one compound might bind more or less in one brain area than in other regions. The relationship between B_{max} values for SR 141716A and CP 55,940 and anandamide and CP 55,940 were compared by linear plots of the respective values, and correlation coefficients were determined. A high correlation was obtained both when comparing the B_{max} values of SR 141716A and anandamide to those of CP 55,940. These correlations indicate that the three compounds are fully capable of maximal binding to the same population of receptors in all of the brain regions.

The finding that SR 141716A is capable of blocking the pharmacological effects of the stable anandamide analog 2-methyl-2′-fluoroethylanandamide underscores the point that the arachidonyl derivatives interact with the cannabinoid receptor. A reasonable conclusion could be that anandamide is rapidly converted to metabolites that are responsible for its pharmacological effects. Regardless of whether the effects are due to anandamide or active metabolites, the pharmacological profile is consistent with the activation of CB1 receptors, and therefore the effects should be antagonized by SR 141716A.

Several conclusions may be drawn from these results. The lack of difference between receptor affinity, receptor distribution and parallelism of the displacement curves suggests that anandamide, SR 141716A and CP 55,940 are binding to the same receptor in the same manner. No evidence of receptor subtypes in the brain was found. However, evidence is mounting that anandamide's actions may not be identical to those of THC. Our earlier studies showed that the relationship between receptor binding (CB1) and pharmacological potency for anandamide analogs (Adams *et al.,* 1995) does not correlate to the same extent as for other cannabinoids (Compton *et al.,* 1993). The greater variation in the correlation with anandamide analogs could be due to either pharmacokinetics factors or to differences in receptor interactions between anandamide and THC. We have also shown recently that the time courses of anandamide's behavioral effects and brain levels do not correspond (Willoughby *et al.,* 1997). Specifically, brain levels of anandamide decrease dramatically despite the persistence of pharmacological effects. Although these latter studies are not definitive, they suggest anandamide is producing its effects in mice in an indirect manner. The ability of SR 141716A to block the effects of the stable anandamide derivative 2-methyl-2′-fluoroethylanandamide (Adams *et al.,* 1995) further suggests that metabolism may play a role in anandamide's effects. However, there has been extensive research demonstrating the relationship between the cannabinoid pharmacological effects in mice and receptor affinity for CB1 receptors so that the effects of either anandamide or its metabolites should be attenuated by the cannabinoid antagonist. Differences in the manner with which anandamide and traditional cannabinoids produce their pharmacological effects can be exploited to gain a better understanding of the endogenous cannabinoid system.

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