Evaluation of the In Vivo Receptor Occupancy for the Behavioral Effects of Cannabinoids Using a Radiolabeled Cannabinoid Receptor Agonist, R-[$^{125/131}$I]AM2233

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ABSTRACT G-protein coupled receptors exist in both high and low agonist affinity conformations, with tracer levels of agonist radioligands preferentially binding to the former. The goal of the present study was to characterize the in vivo binding of the aminoalkyindole-based, CB1 receptor agonist, *R*-[$^{125/131}$I]AM2233 ((2-[$^{125/131}$I]iodo-phenyl)-[1-(1-methyl-piperidin-2-yl-methyl)-1*H*-indol-3-yl]-methanone), and to use this radiotracer to selectively measure the receptor occupancy by the related CB1 receptor agonist, WIN55212-2, to the agonist-preferring affinity state of the receptor. In mouse locomotor assays, both WIN55212-2 and AM2233 (racemic) produced an ~60% reduction in activity at 1 mg/kg, (i.v.) and completely inhibited activity at 3 mg/kg, confirming their agonist nature. In ex vivo autoradiography, preferential uptake of *R*-[^{131}I]AM2233 was apparent in CB1 receptor-rich areas, including globus pallidus, substantia nigra, striatum, cerebellum, and hippocampus. Overall brain uptake of *R*-[^{131}I]AM2233 was 1.3% injected activity/g at 5 min in mice. Coinjection of 3 mg/kg (i.v.) SR141716A, a CB1 receptor antagonist, with *R*-[^{125}I]AM2233 inhibited the radiotracer binding almost to nonspecific levels in the striatum, globus pallidus, and substantia nigra, although residual binding to a non-CB1 receptor remained in the hippocampus. In contrast to the effect of SR141716A, coinjection of 10 mg/kg (i.v.) WIN55212-2, a high dose that produced an immediate and profound immobility and catalepsy in the mice, reduced CB1 receptor-specific binding of *R*-[^{125}I]AM2233 in CB1 receptor-rich areas by only 21–43%. These observations suggest that the behavioral effects of CB1 receptor agonists are manifested with a relatively small fraction of the agonist-preferring affinity state of the receptor occupied. **Synapse 60:93–101, 2006.**

INTRODUCTION

The G-protein coupled CB1 cannabinoid receptor is the receptor responsible for the psychotropic and CNS effects of marijuana and its active constituent δ-9 tetrahydrocannabinol (Pertwee, 1997). Brain contains relatively high densities of CB1 receptors, and there is interest in developing radiotracers capable of imaging these receptors in vivo using positron emission tomography (PET) or single photon emission computed tomography (single photon emission computed tomography (SPECT)) (Gatley et al., 1998; Gifford et al., 2002; Mathews et al., 2000). Questions that could be addressed with such radiotracers include: determining the degree of occupancy of cannabinoid receptors

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necessary to produce therapeutic actions of cannabinoids (Gifford et al., 1998); determining if candidate therapeutic agents possess significant binding to cannabinoid receptors in vivo (Cosenza et al., 2000); determining if cannabinoid receptors are up- or down-regulated as a result of chronic drug use or psychiatric conditions (Berding et al., 2004), and monitoring loss of neuronal cell types possessing cannabinoid receptors (Glass et al., 1993).

The primary classes of chemical compounds that have been found to be active at CB1 cannabinoid receptors are the classical and nonclassical cannabinoids, anandamides, aminoalkylindoles, and pyrazoles (Pertwee, 1997). For the most part, lead compounds for radioligand development have been pyrazoles (Gatley et al., 1998; Gifford et al., 2002; Mathews et al., 2000). These compounds are antagonists or inverse agonists at the CB1 receptor and are typified by SR141716A (Rimonabant), (Fernandez and Allison, 2004; Rinaldi-Carmona et al., 1994). SPECT imaging of primate brain CB1 receptors using the pyrazole [^{123}I]AM281 has been reported by Gatley et al. (1998) and in humans by Berding et al. (2004).

In addition to pyrazoles, the aminoalkylindoles such as WIN55212-2 also have promise as lead compounds for developing in vivo imaging agents for CB1 receptors, as they too are less lipophilic than classical and nonclassical cannabinoids (Thomas et al., 1990). A fluorine-18 labeled aminoalkylindole for CB1 receptors, 3-(4-fluoronapthoyl)-1-(*N*-methylpiperidin-2-ylmethyl)indole, has recently been reported as a candidate PET ligand by Willis et al. (2005). In our own studies, we have reported on development of a radioiodine-labeled aminoalkylindole for CB1 receptors, AM 2233 (2-iodo-phenyl)-[1-(1-methyl-piperidin-2-yl-methyl)-1*H*-indol-3-yl]-methanone) (Deng et al., 2005). This ligand has an affinity for CB1 receptors of 0.2 nM (Deng et al., 2005), ~10 times more potent than that of WIN55212, and in initial studies showed potential as an imaging agent for CB1 receptors in vivo (Gifford et al., 2002).

Aminoalkylindole-based ligands generally act as agonists at the CB1 receptor (Compton et al., 1992; Hosohata et al., 1997). Radiolabeled versions of these compounds could thus potentially be used to reveal information on the affinity states of the receptor in vivo that cannot be obtained using radiolabeled antagonists. This stems from the fact that G-protein coupled receptors are known to exist in both high and low agonist affinity states. Radiolabeled agonists, when used at tracer concentrations, will preferentially bind to the former state whereas radiolabeled antagonists will bind equally to both states of the receptor (Kenakin, 1995). Since the difference in affinity of these two states of the receptor is typically one to two orders of magnitude (De Lean et al., 1980), it can be assumed that most or all of the specific binding following administration of tracer dose of a radiolabled agonist in vivo will be to the agonist-preferring population of receptors. Because of this fact, in the current study we considered it would be of interest to not only further characterize the in vivo binding of [$^{131/125}$I] AM2233, but to use this agonist ligand to address the question of what proportion of the *agonist-preferring high affinity state* of the receptor needs to be occupied in the brain to produce the behavioral effects of cannabinoid drugs. This was achieved by coadministering [^{125}I]AM2233 together with a pharmacological dose of WIN55212-2 and comparing it to the effects of the latter drug on locomotor activity in the mice.

MATERIALS AND METHODS

Materials

WIN55212-2 and WIN55212-3 were purchased from Tocris Cookson (Ballwin, MO). [^{3}H]SR141716A (46 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Sodium [^{131}I] iodide, [^{3}H]choline, and [^{3}H]WIN55212-2 (40 Ci/mmol) were purchased from Dupont NEN (Boston, MA).

Animals

Male Swiss Webser mice (20–25 g) and male Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY).

Chemical syntheses

Details of the synthesis of AM2233 has been published elsewhere (Deng et al., 2005). In brief, 1-(1-methyl-piperidin-2-yl-methyl)-1*H*-indole, prepared by N-alkylation of indole with 2-chloromethyl-1-methyl-piperidine, was reacted with 2-iodobenzoyl chloride to give (2-iodo-phenyl)-[1-(1-methyl-piperidin-2-yl-methyl)-1*H*-indol-3-yl]-methanone (AM2233). Enantiomerically pure *R*- and *S*-AM2233 were prepared by syntheses using R- and S-picolinic acids as starting materials. They were converted to the related tributyltin compounds by reacting with hexamethylditin with Pd(0) catalysis.

Radiochemical syntheses

R-[$^{131/125}$I]AM2233, *S*-[^{131}I]AM2233, and [^{131}I] AM630 (Pertwee et al., 1995) (Fig. 1) were prepared from their tributyltin congeners by radioiododestannylation followed by high-performance liquid chromatography (HPLC) purification. Procedures were similar; 50 μL of the tributyltin precursor (1 mg/mL in ethanol), 10 μL of chloramine-T (freshly prepared, 0.2 mg/mL in water), and 10 μl of 0.5 M HCl were added using microsyringes to 2 mCi of sodium [^{131}I] iodide or sodium [^{125}I] iodide in a septum-sealed reaction vial. After 5 min at room temperature the reaction mixture was injected directly into a HPLC system equipped with a 250 × 6 mm^2 C-18 column (Alltech, Econosil)

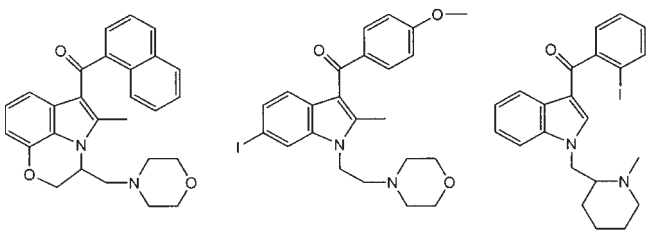

Fig. 1. Structures of the aminoalkylindole cannabinoid receptor ligands AM2233, AM630, and WIN55212-2.

and radiation and UV detectors. The mobile phase consisted of 70% acetonitrile and 30% ammonium formate (2 g/L, pH 6.0). Fractions containing the radiolabeled product were pooled and extracted with ether. The ether was then evaporated to dryness under a stream of nitrogen gas, and the product redissolved in 100 μL ethanol. Specific radioactivity was determined by from the area under the UV peak corresponding to the radioactive peak. The identity of the radioactive product was confirmed in each case by reinjection of an aliquot of product together with a nonradioactive standard and demonstration of identical retention times. Retention times were 18 min for [$^{131/125}$I]*R* and *S*-AM2233 and >30 min for the tributyltin precursor at a flow rate of 2 mL/min. For each product, radiochemical yield (unoptimized) was >60%, radiochemical purity was >95%, and specific radioactivity was >500 Ci/mmol for [^{131}I] compounds and >1500 Ci/mmol for [^{125}I] compounds. This corresponds to a mass of compound injected into the mice of ~1 ng/μCi for [^{131}I]*R*-AM2233 and 0.3 ng/μCi for [^{125}I]*R*-AM223, which in the case of the mice experiments used for autoradiography (25–30 g mice, injected with ~15 μCi [^{125}I]*R*-AM223), equates to an actual dose of *R*-AM2233 of 0.15 μg/kg.

^{131}I labeled radiotracers were used for the majority of the studies because of the lower radiological control concerns compared to ^{125}I. However, a drawback of 131I was that its short half life (8 days) necessitated frequent resynthesis of the radiotracers. The final set of experiments examining SR141716A and WIN55212-2 displacement (Fig. 7) were thus conducted with ^{125}I labeled radiotracers (half life 60 days).

Locomotor activity

Methods have been described elsewhere (Gifford et al., 1998). Briefly, after i.v. injection of AM2233 (racemic), WIN55212-2, or vehicle (0.2 mL 40% cyclodextrin solution) mice were placed in 16″ × 16″ × 16″ plexiglass activity boxes purchased from San Diego Instruments (San Diego, CA). The array of infrared sources and detectors was placed 2″ above the bottom of each box. The number of beam-breaks over a 20 min period was counted for each group of animals, and the data expressed as a percentage of the control for each drug dose.

Synaptosome experiments

Synaptosome experiments were performed as described in detail previously (Gifford et al., 2000). Briefly, the methods were as follows. The hippocampus from a rat was dissected out and homogenized gently in 0.32 M sucrose solution using a glass homogenizer with 3–4 up and down strokes of the pestle. The homogenate was centrifuged at 100 × *g* for 5 min at 4°C and the resulting supernatant removed and centrifuged at 14,000 × *g* for 15 min. The pellet from the second centrifugation was resuspended in 3 mL of Krebs buffer (120 mM NaCl, 3.3 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 11 mM glucose, 0.03 mM EDTA, pH 7.4, saturated with 95% O_2/5% CO_2), containing 15 μCi [^{3}H]choline, and incubated at 35°C for ~20 min to allow [^{3}H]choline uptake into the synaptosomes.

The synaptosomal suspension was subsequently loaded into 10 superfusion chambers that were constructed from Swinnex Millipore filter units. To retain the synaptosomes, glass fiber (GF/B) filters were placed inside the filter units. The chambers were perfused with oxygenated Krebs medium at 35°C and at a superfusion rate of 1.6 mL/min. Physostigmine (1 μM) (to prevent hydrolysis of the released acetylcholine) and 0.3 μM quinuclidinyl benzilate (to prevent autoinhibition of release via presynaptically located muscarinic receptors) were included in the superfusion buffer. After a period of 30 min in calcium-containing Krebs, the superfusion medium was switched to a calcium-free Krebs medium which contained 0.03 mM EGTA (ethylene glycol-bis(-aminoethyl ether)-*N*′-tetraacetic acid). The synaptosomes were perfused with the calcium-free Krebs for 80 min before the addition 1.3 mM $CaCl_2$ to the Krebs medium to evoke [^{3}H]ACh release. Addition of calcium following perfusion with calcium free-buffer produces a pulse of transmitter release from the synaptosomes. Although the mechanism by which calcium addition following perfusion with calcium-free buffer produces transmitter release from synaptosomes is unclear, transmitter release evoked in this way appears to be more readily inhibited by activation of release-regulating presynaptic receptors than that of potassium-evoked release (Bowyer and Weiner, 1987; Gifford et al., 2000).

Stimulation-evoked release was calculated by subtracting the mean level of counts in two 4 min basal fractions collected immediately prior to calcium addition from that in a 4 min fraction collected immediately following calcium addition. Results were calculated as fractional release, representing the ratio of

[^{3}H]ACh-evoked release relative to the total amount of [^{3}H] radioactivity retained on the filters at the end of the experiment. To determine the effects of the drugs on stimulation-evoked release, the fractional release in the drug-exposed chambers was expressed as a percentage of that obtained in the control chambers. Data was analyzed using analysis of variance followed by Dunnett's test for comparing several drug-treated groups to a single control group.

Brain uptake in vivo

Mice were injected via a tail vein with 1 μCi radiotracer dissolved in a volume of 0.2 mL. For most experiments, the vehicle was 40% 2-hydroxypropyl-β-cyclodextrin containing <1% ethanol. The cyclodextrin vehicle appeared to be well-tolerated by the mice and did not appear to produce any obvious behavioral effects. Animals were killed by decapitation and brain structures dissected out. These were weighed and iodine-131 levels determined with a γ-counter. For [^{3}H]WIN 55,212-2, tissues were treated with commercial tissue solubilizer and radioactivity assayed using a liquid scintillation counter. Uptake values were expressed as mean ± standard error percent injected radioactivity per gram of tissue (% IA/g).

Analysis of radioactivity in brain after intravenous injection of R-[^{131}I]AM2233

Brain from a mouse injected i.v. with R-[^{131}I]AM2233 (~50 μCi) 30 min previously was homogenized in 2 mL of acetonitrile/methanol (2:1. v/v) using a Polytron device. After centrifugation (12,000 × *g* for 2 min), the supernatant was analyzed by HPLC using a 250 × 6 mm^2 C-18 column (Alltech Econosil). The mobile phase was 40% acetonitrile and 60% aqueous ammonium acetate (2 g/L). Fractions were collected at 1 min intervals and assayed for radioactivity using a gamma-counter.

Ex vivo autoradiography in rats

R[^{131}I]AM2233 (~50 μCi) dissolved in 40% 2-hydroxypropyl-β-cyclodextrin was administered via a tail vein into rats under light anesthesia (ketamine 100 mg/kg, i.p.). Ketamine anesthesia was used so that the animal remained still and could be easily injected through the tail vein. Animals were killed by decapitation and the brain immediately removed. The brain was cut into two halves, glued to a plastic block, immersed in ice-cold saline, and 500-μm sections cut from the fresh brain tissue using a vibratome. Sections were air-dried on a slide warmer and after drying were apposed to a phosphorimaging plate (Molecular Dynamics, Sunnyvale, CA). The plates were scanned after an exposure time of between 1 to 5 days.

Ex vivo autoradiography in mice

R[^{125}I]AM2233 (~15 μCi, corresponding to <4 ng in mass assuming full specific activity), dissolved in 30% 2-hydroxypropyl-β-cyclodextrin was administered via a tail vein to awake mice, either alone or together with WIN55212-2 or SR141716A. Mice were killed by decapitation after 30 min and the brain removed. The 30 min time point was selected on the basis of the duration of action of the locomotor effects of WIN 55,212-2. At the lower doses of WIN55212-2 (1 mg/kg) the mice were starting to show initial signs of recovery at this time point. A sample of plasma and of the cerebellum was taken for counting in the γ-counter and the remainder of the brain placed in ice-cold saline and 300 μm sections cut from the fresh tissue using a vibratome. The sections were dried and placed on a phosphorimaging screen, and the plate scanned after 3 days.

To determine the effects of WIN55212-2 and SR141716A on regional brain uptake of *R*-[^{125}I] AM2233, regions-of-interest (ROIs) were drawn around the lateral striatum, globus pallidus, hippocampus, and substantia nigra in the ex vivo autoradiographic sections taken from each of the mice. ROIs were also drawn for a reference region in each section. The reference region was taken as the dorsal cortex for the striatal sections, the anterior thalamus for the globus pallidus sections, and the mesencephalic nucleus area for the hippocampus and substantia nigra sections. The reference regions were selected on the basis of exhibiting both low levels of radioactivity in the autoradiographic sections and having a known relative paucity of CB1 receptors from immunocytochemical and autoradiographic studies published in the literature. A reference region was taken for each section, rather than just using a single reference region for the whole animal, to normalize for potential variations in sensitivity between different areas of the phosphor screen used to image the sections. However, subsequent analysis of the data indicated little (<3%) difference in radioactivity values between these three reference regions. Receptor-specific uptake was calculated as that in the receptor-rich regions divided by that in the receptor-poor reference regions.

RESULTS

Inhibition of mice locomotor activity

AM2233 reduced the activity level of mice (Fig. 2). In addition, the animals exhibited marked catalepsy and brief bouts of rapid uncoordinated locomotion, typical of that observed with other cannabinoid agonists. AM2233 was approximately equipotent with WIN55212-2.

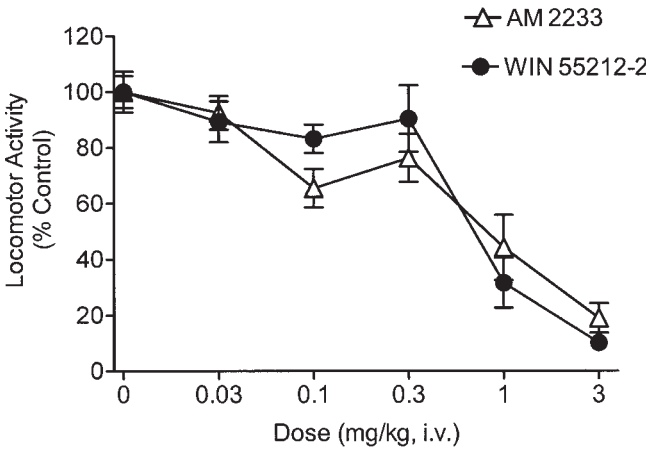

Fig. 2. Inhibition of mouse horizontal locomotor activity by AM2233 (racemic) and by WIN55212-2. Values are the mean ± SEM of 8 replicates.

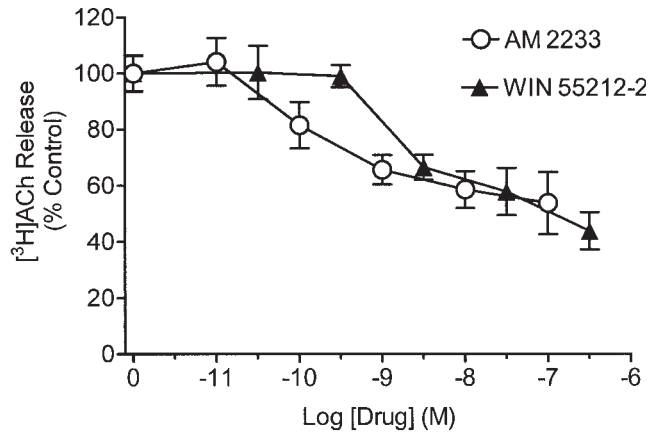

Fig. 3. Inhibition of calcium-evoked [^{3}H]acetylcholine release by AM2233 (racemic) and WIN55212-2 in superfused hippocampal synaptosomes. Data points are means ± SEM of 9–17 replicates.

Inhibition of synaptosomal [^{3}H]acetylcholine release

AM2233 and WIN 55,212-2 were compared in their potency and efficacy to inhibit [^{3}H]acetylcholine release from superfused hippocampal synaptosomes, an effect mediated via presynaptic CB1 receptors (Fig. 3). WIN55212-2 produced a 60% inhibition of the [^{3}H]ACh release evoked by the calcium addition, similar to that we have reported previously (Gifford et al., 2000). AM2233 (racemic) produced an equivalent overall degree of inhibition of [^{3}H]ACh release as WIN55212-2 and had a slightly greater potency, consistent with its greater affinity for the CB1 receptor in receptor binding studies.

Visualization of CB1 receptors by ex vivo autoradiography in rats

After intravenous administration of *R*-[^{131}I]AM-2233, brain sections (Fig. 4) revealed a strong regional selectivity in the distribution of brain radioactivity, similar to that observed using in vitro autoradiography (Deng et al., 2005). In a rat injected with the less active enantiomer, *S*-[^{131}I]AM2233, only very weak selective binding was observed (data not shown).

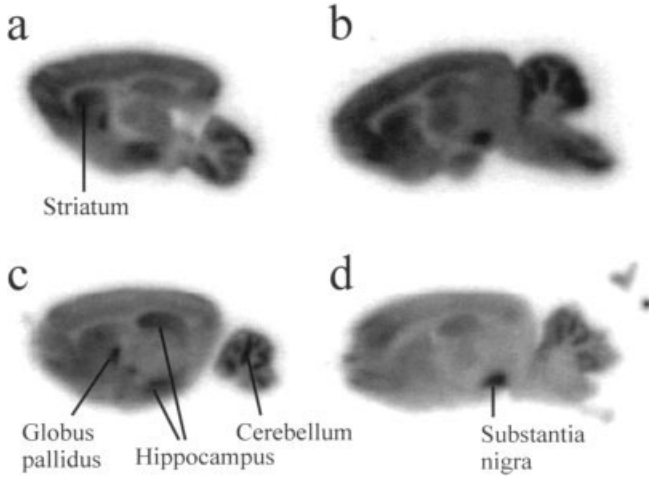

Fig. 4. Ex vivo autoradiography in two rats following intravenous administration of *R*-[^{131}I]-AM2233. The first rat (sections **a** and **b**) was sacrificed at 15 min following radiotracer administration and the second rat (sections **c** and **d**) at 30 min following radiotracer administration. Sections were cut saggitally at the level of the globus pallidus and striatum (**a** and **c**) and at the level of the substantia nigra (**b** and **d**).

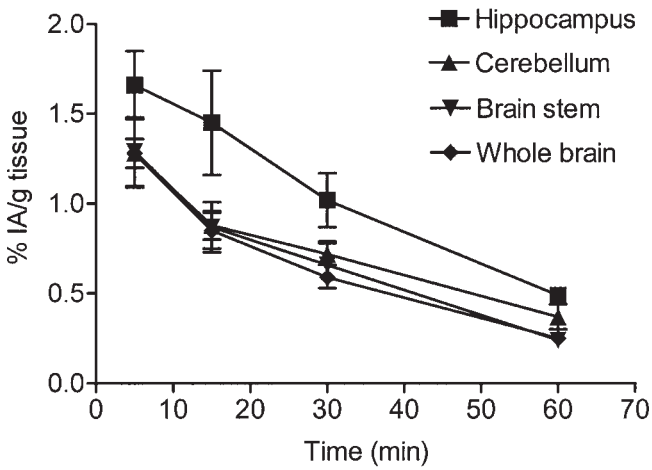

Fig. 5. Time-course of binding in regions of the mouse brain after the intravenous administration of *R*-[^{131}I]-AM2233. Data points are means ± SEM of 7–8 mice.

Time-course of brain uptake of *R*-[^{131}I]AM2233 in mice

After intravenous administration of *R*-[^{131}I]AM2233 to mice, the greatest whole brain uptake of 1.3% injected activity per gram tissue (% IA/g) was observed at the first sacrifice time point of 5 min post-injection (Fig. 5). Thereafter whole brain radioactivity declined, reaching about 0.6% IA/g at 30 min. Uptake

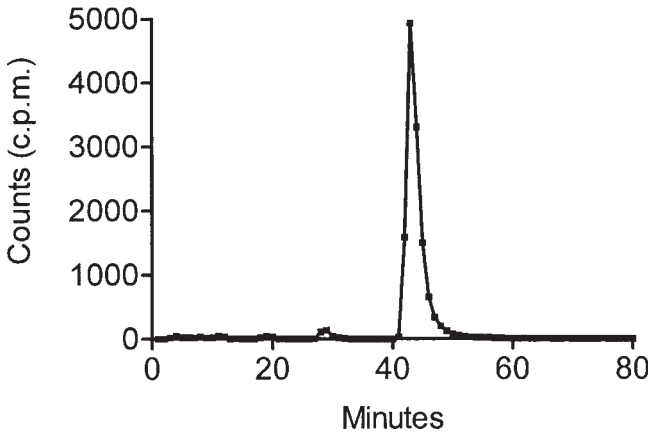

Fig. 6. HPLC analysis of radioiodine in the rat brain after intravenous administration of *R*-[^{131}I]-AM2233.

was significantly higher in the hippocampus, than in the cerebellum, brain stem, and whole brain. Plasma levels of radioactivity were measured in an independent set of experiments using *R*-[^{125}I]AM2233 and were 1.54 ± 0.18% IA/g at 5 min ($n = 10$) and 1.20 ± 0.13% IA/g at 30 min ($n = 10$).

Comparison of brain uptake of *R*-[^{131}I]AM2233 with other radiolabeled aminoalkylindole derivatives in mice

Brain uptake of two other radiolabeled aminoalkylindole cannabinoids, [^{131}I]AM630, a CB2 selective aminoalkylindole (Pertwee et al., 1995), and [^{3}H]WIN55212-2, was also evaluated in mice. At 30 min postinjection brain uptake of [^{131}I]AM630 was 0.46 ± 0.04% IA/g ($n = 8$), whereas brain uptake of [^{3}H]WIN55212-2 was 0.07 ± 0.01% IA/g ($n = 5$).

Analysis of radioactivity in brain in mice

At 30 min after i.v. injection, >90% of radioactivity extracted from mouse brain remained in the administered chemical form that eluted at 43 min (Fig. 6), corresponding to that of a [^{131}I]AM2233 standard. Very small peaks (<2% of the AM2233 peak) were seen at 4, 11, 19, and 29 min.

Coadministration of *R*-[^{125}I]AM2233 with WIN55212-2 and SR141716A in mice

Coadministration of a high dose (10 mg/kg) of WIN55212-2 together with *R*-[^{125}I]AM2233 resulted in an immediate and profound catalepsy and immobility in the mice that lasted the entire 30 min until they were sacrificed. Analysis of regional radioactivity levels in autoradiographic sections cut from the mice at the time of sacrifice indicated that receptor-specfic binding was lowered by 21% in the hippocampus, 25% in the striatum, 42% in the globus pallidus, and 43% in the substantia nigra, reaching significance in the two latter areas only (Fig. 7). Coadminstration of 3 mg/kg SR141716A together with *R*-[^{125}I]AM2233, a dose which is sufficient to fully antagonize behavioral effects of WIN55212-2 (Rinaldi-Carmona et al., 1994), produced a much greater inhibition of R-[^{125}I]AM2233 binding, with receptor-specific binding largely eliminated in all regions except for the hippocampus, in which binding was reduced by 69%.

The effects of WIN55212-2 on brain uptake of *R*-[^{125}I]AM2233 was assessed by collecting samples of the cerebellum and blood prior to sectioning of the mice brains for the ex vivo autoradiography analysis. Radioactivity levels of the cerebellum were 0.61 ± 0.06% IA/g ($n = 10$) in the mice given *R*-[^{125}I]AM2233 alone, and 1.02 ± 0.18% IA/g ($n = 8$) in mice given *R*-[^{125}I]AM2233 together with 10 mg/kg WIN 55,212-2. This thus indicates an effect of WIN55212-2 in increasing general brain uptake of *R*-[^{125}I]AM2233 ($P < 0.01$ *t*-test). Total levels of radioactivity in the plasma were similar in the two groups of mice at the time of sacrifice (1.14 ± 0.14% IA/g and 1.23 ± 0.20% IA/g, respectively).

DISCUSSION

Pharmacological effects, distribution of binding sites and, brain uptake of *R*-[^{131}I]AM2233

AM2233 (Deng et al., 2005) is one of a panel of iodine-containing aminoalkylindoles that were examined as potential cannabinoid receptor agonist radioligands, based on earlier studies (D'Ambra et al., 1992). Routine binding screens revealed that AM2233 was the most promising of these compounds, with significantly higher affinity than the commonly used cannabimimetic aminoalkylindole WIN55212-2 (Deng et al., 2005).

In the behavioral experiments in the current study, AM2233 produced the typical CB1 cannabinoid effects of sedation and catalepsy after intravenous injection. These behavioral effects were very similar to that of WIN55212-2 and confirm that AM2233 is able to enter the brain in vivo and acts as an agonist at the CB1 receptor. The agonist nature of AM2233 was also confirmed in the synaptosomal superfusion experiments in which AM2233 was found to inhibit the calcium-stimulated release of [^{3}H]acetylcholine to a similar extent as WIN55212-2, an effect mediated via activation of presynaptic CB1 receptors.

Ex vivo autoradiographic studies of *R*-[^{131}I]AM2233 binding showed a high level of binding throughout substantia nigra and globus pallidus, a high level of binding in regions of hippocampus and cerebellum, medium levels of binding in striatal and cortical areas, and the lowest level of binding in thalamus. The pattern of *R*-[^{131}I]AM2233 binding in the ex vivo experiments in the present study was similar to that observed for in vitro binding experiments with

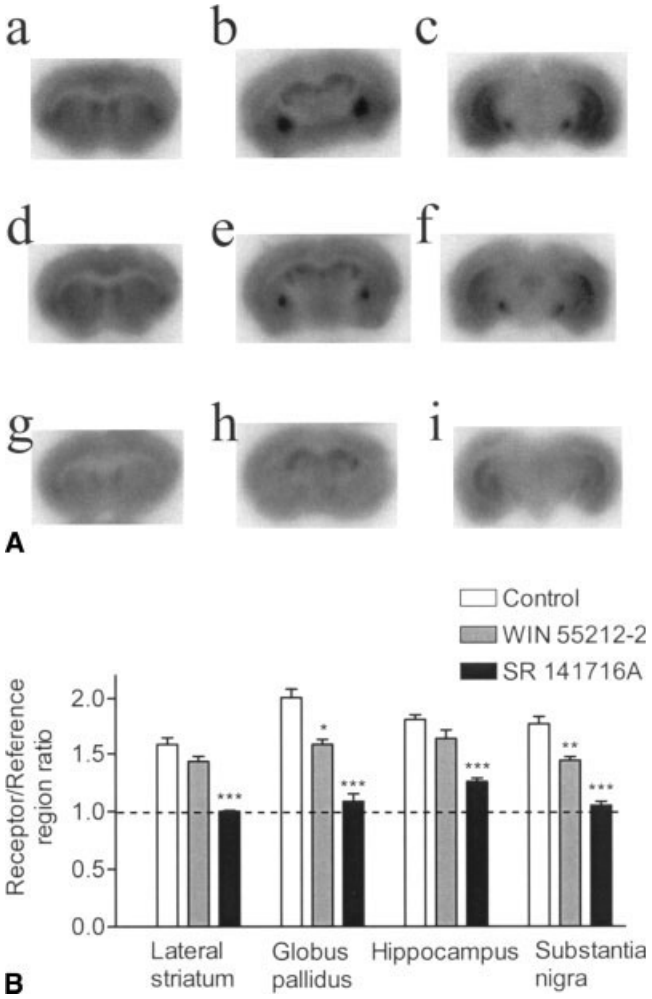

Fig. 7. Effect of coadministration of WIN55212-2 (10 mg/kg, i.v.) or SR141716A (3 mg/kg, i.v.) on regional brain uptake of *R*-[^{125}I]AM2233 in mice. (**A**) Representative ex vivo autoradiograms from a mouse injected with *R*-[^{125}I]AM2233 alone (sections a–c), *R*-[^{125}I]AM2233 together with WIN55212-2 (sections d–f) or *R*-[^{125}I]AM2233 together with SR141716A (sections g–i). Sections shown are at the level of the striatum (a, d, and g), globus pallidus (b, e, and h) and hippocampus/substantia nigra (c, f, and i). (**B**) Quantification of regional brain uptake of *R*-[^{125}I]AM2233 from ex vivo autoradiographic sections. Data are means ± SEM of 10 mice (controls), 8 mice (WIN55212-2), and 6 mice (SR141716A). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control for each brain region (*t*-test with Bonferroni's correction).

R-[^{131}I]AM2233 (Deng et al., 2005), and closely correlated to the reported distribution of CB1 receptors (Gatley et al., 1997; Herkenham et al., 1990; Jansen et al., 1992). In our previous studies, selectivity of *R*-[^{131}I]AM2233 to CB1 receptors was also confirmed by the absence of selective binding in most brain regions in CB1 knockout mice, with the exception of the hippocampus (Deng et al., 2005; Gifford et al., 2002).

Peak brain uptake of *R*-[^{131}I]AM2233 of 1.3% IA/g (or 0.039% (IA/kg)/g for a 0.030 kg mouse) in the present study was similar to the brain uptake of [^{131}I] AM630 and was comparable to the value of 0.033% (IA/kg)/g reported for peak brain uptake of the ^{18}F-labeled aminoalkylindole-derivative, 3-(4-fluoronapthoyl)-1-(*N*-methylpiperidin-2-ylmethyl)indole, in the study by Willis et al. (2005). However, it should be noted that these values are lower than those reported for antagonist pyrazole-based radiotracers (Gatley et al., 1998; Mathews et al., 2000; Mathews et al., 2002) and this fact may limit the potential of aminoalkylindole-based radiotracers for PET and SPECT studies.

Effects of coadministration of SR141716A on radioligand binding

Intravenously administered SR141716A fully inhibited binding of *R*-[^{125}I]AM2233 in most brain regions except in the hippocampus, in which binding was inhibited by only 69%. The residual binding in this region appears to be to an unidentified non-CB1 receptor, and was observed also in experiments in CB1-receptor knockout mice (Gifford et al., 2002). Although this binding-site was clearly available in the hippocampus in the ex vivo binding experiments, it was not observed in in vitro autoradiography experiments (Deng et al., 2005). We were also unable to detect this binding site in homogenate binding experiments using hippocampal tissue from CB1 receptor knockout mice (A.N. Gifford, unpublished observations). Possible explanations for the failure to observe this novel binding site in the in vitro assays are that (1) the binding site is to a soluble rather than membrane-bound protein, (2) a necessary soluble cofactor for the binding site was missing, or (3) the binding site is very sensitive to physiological conditions. Because of the absence of a binding signal in the in vitro assays we were not able to conduct a further pharmacological characterization of this site.

Effects of coadministration of WIN55,212-2 on radioligand binding

In a previous study, we found that intravenouis doses of WIN 55,212-2 that induced rapid immobility and catalepsy in the mice had only a small effect on the in vivo binding of the antagonist radioligand [^{131}I]AM281 (Gifford et al., 1998), whereas SR141716A completely inhibited binding. We proposed on the basis of these observations and parallel experiments in brain slices comparing inhibition of AM281 binding and inhibition of acetylcholine release, that there exists a very large receptor reserve for CB1 receptors in the brain. In other words, the behavioral effects of WIN55212-2 are produced at concentrations that occupy only a small fraction of the available brain CB1 receptors. In the present study, we anticipated that because *R*-[^{131}I]AM2233 is also an agonist, a behaviorally active dose of WIN55212-2 would be competing for the same population of CB1 receptors, namely those in the high agonist affinity state, and thus may have a greater effect on AM2233 binding than AM281 binding. A comparable effect has

been reported with the in vivo binding of dopaminergic radioligands, where receptor-specific binding of ^{3}H and ^{11}C-labeled versions of the dopamine (DA) receptor agonist *N*-propyl-norapomorphine were inhibited by endogenously released DA from amphetamine treatment to a greater extent than that of the DA receptor antagonist, [^{11}C]raclopride (Cumming et al., 2002; Hwang et al., 2005). A greater potency of agonist in displacing agonist radiotracers than antagonist radiotracers is also apparent in in vitro receptor binding studies. Thus, in our own studies we observed that WIN55212-2 showed about a 10-fold greater potency in inhibiting binding of a tracer concentration of *R*-[^{131}I]AM2233 ($K_i = 0.2$ nM) than a tracer concentration of [^{3}H]SR14176A ($K_i = 1.6$ nM; Deng et al., 2005) in membrane homogenates. A similar phenomenon has also been reported in in vitro receptor binding studies comparing agonist displacement of [^{3}H] WIN55212-2 with agonist displacement of [^{3}H]SR1-41716A binding in the presence and absence of guanine nucleotides (Petitet et al., 1997). In the current in vivo study, coadministration of WIN55212-2 (10 mg/kg) with *R*-[^{125}I]AM2233 did appear to reduce binding of this radiotracer by a slightly greater amount than that observed in our in vivo study with [^{131}I]AM281 (~30% at 10 mg/kg WIN55212-2; Gifford et al., 1998) in the globus pallidus and substantia nigra, although not in the striatum and hippocampus. However, two caveats should be borne in mind comparing these studies. Firstly, in the case of the hippocampus a precise quantification of the effect of WIN55212-2 on binding is complicated by the presence of a non-CB1 receptor binding of the radioligand in this region. Secondly, the fact that the 10 mg/kg dose of WIN 55,212-2 increased overall brain uptake of *R*-[^{125}I]AM2233 may have had some influence on the regional specific to nonspecific binding ratios of this tracer. The increase in brain uptake of radioactivity by WIN55212-2 could reflect either displacement of radiotracer binding from plasma proteins, decreased clearance of radiotracer from the plasma, or a combination of both. Additional experiments would be needed to interpret the exact mechanism for this effect.

It is notable that the size of the reduction in CB1-receptor specific of binding of *R*-[^{125}I]AM2233 of ~21–43% observed in the present study by the 10 mg/kg dose of WIN 55,212-2 can be considered relatively small when remembering that this dose of WIN55212-2 was at least 10-fold above that at which a substantial effect on locomotor activity of the animals was apparent. A relatively high dose of WIN 55,212-2 was employed to ensure that an effect on radiotracer binding would be discernable above the expected level of experimental variance. However, if some assumptions are made it is possible to extrapolate the data to lower doses of WIN 55,212-2. Thus, if it is assumed that the in vivo displacement of *R*-[^{125}I]AM2233 by increasing doses of WIN 55,212-2 has the same hill slope as observed in our in vitro competition experiments, namely 0.64 (Deng et al., 2005), and the occupancy of the receptor by *R*-[^{125}I] AM2233 is at tracer levels (see calculations on injected mass in methods) then the receptor occupancy of the agonist-preferring affinity state by WIN 55,212-2 in the substantia nigra and globus pallidus can be determined to be ~14% at 1 mg/kg and 4% at 0.1 mg/kg. These values would be still lower for a steeper value for the binding curve. The 1 and 0.1 mg/kg doses represent doses just above and below the 50% level for the inhibition of locomotor activity by WIN 55,212-2, respectively. It is of note that the dose of WIN 55,212-2 producing inhibition of locomotor activity in this study is comparable to that reported in the literature for other behavioral effects of WIN 55,212-2. For example, values of 0.17 mg/kg i.v., 0.43 mg/kg i.v., and 1.1 mg/kg i.v. were reported by Compton et al. (1992) for the ED50 effect of WIN 55,212-2 in drug-discrimination, tail-flick latency, and ring-immobility, respectively, in mice.

In the case of other brain neurotransmitter receptors, few studies have examined the ability of agonist drugs to displace agonist radiotracer binding in vivo. Almost all studies involve antagonist drugs. This may in part be due to the potential toxicity issues from giving animals high doses of neuroactive agonist-acting drugs. Cannabinoids though have an advantage in this respect in that even high doses, evoking strong behavioral effects, are generally safe to the animals. Some literature is available agonist displacement of radiotracer binding in the opiate system. In this case it has been reported that pretreatment with an analgesic dose of the opiate agonist methadone had no effect in inhibiting binding of the opiate antagonist, [^{11}C]diprenorphine, in the brain of rats (Melichar et al., 2005). However, a study in humans examining the effect of buprenorphine on binding of the opiate agonist, [^{11}C]carfentanil, found that the radiotracer binding was reduced by 41, 80, and 84% at 2, 16, and 32 mg doses of drug (Greenwald et al., 2003). In this case though the competing drug, buprenorphine, is a partial agonist rather than full agonist at the opiate receptor, and may thus have a higher receptor occupancy at behaviorally active doses.

In conclusion, the effect of a pharmacological dose of WIN 55,212-2 in inhibiting binding of *R*-[^{125}I]AM2233 in vivo suggests that percentage of the brain CB1 receptors that need to be occupied to evoke behavioral effects of cannabinoids appears to be relatively small, even when as in the present study just the agonist-preferring state of the receptor is considered.

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