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The inverse agonist effect of rimonabant on G protein activation is not mediated by the cannabinoid CB1 receptor: Evidence from *postmortem* human brain

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ABSTRACT

Rimonabant (SR141716) was the first potent and selective cannabinoid CB1 receptor antagonist synthesized. Several data support that rimonabant behaves as an inverse agonist. Moreover, there is evidence suggesting that this inverse agonism may be CB1 receptor-independent. The aim of the present study was to elucidate whether the effect of rimonabant over G protein activation in *postmortem* human brain is CB1 dependent or independent.

[^{35}S]GTPγS binding assays and antibody-capture [^{35}S]GTPγS scintillation proximity assays (SPA) were performed in human and mice brain. [^{3}H]SR141716 binding characteristics were also studied.

Rimonabant concentration-dependently decreased basal [^{35}S]GTPγS binding to human cortical membranes. This effect did not change in the presence of either the CB1 receptor agonist WIN 55,212-2, the CB1 receptor neutral antagonist O-2050, or the CB1 allosteric modulator Org 27569. [^{35}S]GTPγS binding assays performed in CB1 knockout mice brains revealed that rimonabant inhibited the [^{35}S]GTPγS binding in the same manner as it did in wild-type mice. The SPA combined with the use of specific antibody-capture of G_α specific subunits showed that rimonabant produces its inverse agonist effect through G_{i3}, G_o and G_z subtypes. This effect was not inhibited by the CB1 receptor antagonist O-2050. Finally, [^{3}H]SR141716 binding assays in human cortical membranes demonstrated that rimonabant recognizes an additional binding site other than the CB1 receptor orthosteric binding site recognized by O-2050.

This study provides new data demonstrating that at least the inverse agonist effect observed with >1 μM concentrations of rimonabant in [^{35}S]GTPγS binding assays is not mediated by the CB1 receptor in human brain.

1. Introduction

The discovery of the endocannabinoid system and its activity as a neuromodulator, has led to the search for new therapeutic strategies through the pharmacological modulation of the different components of this system. Two different cannabinoid receptors have been identified and cloned to date in mammalian tissue: CB1 [1,2] and CB2 [3]. The CB1 receptor is the most abundant G protein-coupled receptor (GPCR) in the brain [4,5] where it plays an important role in the inhibition of neurotransmitter release. This receptor is mostly coupled to $G_{i/o}$ proteins to inhibit adenylyl cyclase, activate mitogen-activated protein kinase (MAPK), inhibit voltage gated Ca^{2+} channels and activate inwardly rectifying K^+ channels [6].

Rimonabant (SR141716) was the first highly potent and selective CB1 receptor antagonist to be developed [7]. Thereafter, it has been widely used in experimental and therapeutic pharmacology. In this way, rimonabant was approved as an

Abbreviations: BSA, bovine serum albumin; cAMP, cyclic 3′,5′-adenosine monophosphate; DPCPX, 1,3-dipropyl-8-cyclopentyl-1,3-dipropylxanthine; DTT, dithiothreitol; EGTA, etylene glycol-bis ((-aminoethyl ether); GDP, guanosine diphosphate; GPCR, G protein-coupled receptor; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; MAPK, mitogen-activated protein kinase; O-2050, (6Ar,10a*R*)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran; Org 27569, (5-chloro-3-ethyl-1*H*-indole-2-carboxylic acid [2-(4-piperidin-1-yl-phenyl)-ethyl]-amide); [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]-thio)triphosphate; SEM, standard error of the mean; SR141716, *N*-piperidin-*O*-5(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-3-carboxamide; Tris–HCl, 2-amino-2-(hydoxymethyl)-1,3-propanediol hydrochloride; WIN 55,212-2, (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone.

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obesity treatment in more than 50 countries worldwide. However, it is well documented that it has inverse agonist properties, that overall have been attributed to the CB1 receptor, since these biochemical or behavioural effects are generally opposite in action to the effects of CB1 agonists. For example, in heterologous expression systems, rimonabant produces a decrease in the [^{35}S]GTPγS basal binding [8], a decrease in MAPK activity [9] and an increase in forskolin stimulated cAMP production [10]. Furthermore, these effects have also been observed in native tissue. Accordingly, rimonabant decreases [^{35}S]GTPγS basal binding in rat [11,12] and mouse brain [13], increases basal cAMP production in rat and human brain membranes [14], and produces an increase in evoked neurotransmitter release [15,16]. Finally, behavioural studies have corroborated that rimonabant also exerts opposite *in vivo* effects to those of cannabinoids, since it produces hyperalgesia [17], an increase in the locomotor activity [18], a decrease in food consumption [19,20], and an improvement in memory [21].

Nevertheless, there is also evidence that the inverse agonist effects of rimonabant are produced in a CB1 receptor-independent manner. Thus, in CB1 knockout mice rimonabant still produces a decrease in the [^{35}S]GTPγS basal binding [13,22].

On the whole, different possible mechanisms have been proposed for the inverse agonist effects of SR141716 [23]: (1) rimonabant may modulate the constitutive activity of CB1 receptors, (2) it may antagonize the responses to the endogenously released endocannabinoids or even other neurotransmitters acting on CB1 receptors, and (3) these inverse agonist effects could be produced by CB1 receptor-independent mechanisms, either through another receptor or by a receptor-independent mechanism.

Research interest in separating "inverse agonist" activity from "neutral antagonist" activity has been increased in recent years as a result of efforts to distinguish therapeutic effects from unwanted side effects for cannabinoid antagonists used as medicinal compounds [24]. This is particularly important for rimonabant since it was withdrawn from the market due to increased rates of depression, anxiety and suicidality related to its use as a treatment for obesity [25]. However, the inverse agonist effect of rimonabant could constitute an advantageous treatment in some pathological states such as dyslipidemia, metabolic syndrome or tobacco addiction [26,27]. In this context, the therapeutic interest of inverse agonism at different receptors has already been suggested [28].

In this study we aimed to evaluate the effect of rimonabant on G protein activation in *postmortem* human brain membranes.

2. Materials and methods

2.1. Postmortem human brain samples

The study was developed in compliance with policies of research and ethical review boards for *postmortem* brain studies. Human brain samples, from subjects who had died by sudden and violent causes (motor-vehicle accidents) were obtained at autopsy in the Basque Institute of Legal Medicine, Bilbao, Spain. All the subjects were determined to be free of neurological or psychiatric disorders based upon both medical histories and *postmortem* tissue examinations. Samples from the prefrontal cortex (Brodmann's area 9) were dissected at the time of autopsy and immediately stored at −70 °C until assay. The *postmortem* delay of the samples used ranged from 8 to 18 h.

2.2. Animals

Animal care was performed in agreement with European Union regulations (O.J. of E.C. L 358/1 18/12/1986). Swiss mice were obtained from the Animal Facility of the University of the Basque Country, Leioa, Spain. CB1 knockout mice were generated as described previously [29]. Animals were killed by cervical dislocation, their brains were removed and stored at −70 °C until assay was performed.

2.3. [^{35}S]GTPγS binding assays

Preparation of plasma membranes (P_2 fraction) and [^{35}S]GTPγS binding assays were performed as previously described [30] with minor modifications. Tissue samples of each subject were homogenized using an Ultra-Turrax T8 Homogenizer (IKA Labortechnik, Satufen, Germany) at maximum speed for 10 s in 30 volumes of homogenization buffer (1 mM EGTA, 3 mM $MgCl_2$, 1 mM DTT, and 50 mM Tris–HCl, pH 7.4) supplemented with 0.25 M sucrose. The crude homogenate was centrifuged for 5 min at 1000 × *g* at 4 °C and the supernatant was recentrifuged for 10 min at 40,000 × *g* (4 °C). The resultant pellet was washed twice with 5 volumes of homogenization buffer and recentrifuged in similar conditions. Aliquots of 0.9 mg protein were stored at −70 °C until assay. Protein content was measured according to the method of Bradford [31] using BSA as standard.

The incubation buffer for measuring [^{35}S]GTPγS binding to brain membranes contained, in a total volume of 500 μl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM DTT, 50 μM GDP, 50 mM Tris–HCl at pH 7.4 and 0.5 nM [^{35}S]GTPγS. For the binding studies, protein aliquots were thawed and resuspended in resuspension buffer (RB) containing 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, and 50 mM Tris–HCl, at pH 7.4. The incubation was started by addition of the membrane suspension (30 μg of membrane proteins) to the previous mixture and was performed at 30 °C for 120 min with shaking. In order to evaluate the influence of several cannabinoid ligands on [^{35}S]GTPγS binding, different concentrations of rimonabant, O-2050 and/or WIN 55,212-2 were included in the incubation buffer. Incubations were terminated by adding 3 ml of ice-cold incubation buffer followed by rapid filtration through Whatman GF/C filters presoaked in the same buffer. The filters were rinsed twice and transferred to vials containing 5 ml of OptiPhase HiSafe II cocktail (Wallac, UK). The radioactivity trapped was determined by liquid scintillation spectrometry (Packard® 2200CA). Nonspecific binding of the radioligand was defined as the remaining [^{35}S]GTPγS binding in the presence of 10 μM unlabelled GTPγS.

2.4. Antibody-capture [^{35}S]GTPγS scintillation proximity assay (SPA)

Specific activation of different subtypes of Gα proteins was determined using a homogeneous protocol of [^{35}S]GTPγS scintillation proximity assay coupled with the use of specific antibodies essentially as described by DeLapp et al. [32]. [^{35}S]GTPγS binding was performed with the same membrane preparation described above but in 96-well Isoplates (PerkinElmer Life Sciences, Maanstraat, Germany) and in a final volume of 200 μl containing 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM DTT, 50 mM Tris–HCl at pH 7.4, 0.4 nM [^{35}S]GTPγS, 25 μg of protein per well, and different concentrations of GDP depending on the Gα subunit tested (see Table 1). At the end of the 2 h incubation period (30 °C), 20 μl of Igepal 1% were added to each well, and plates were incubated at room temperature (RT) for 30 min with gentle agitation. Specific antibodies for each Gα subunit of interest were then added to each well before an additional 60 min RT incubation period. The antibodies and dilutions employed are described in Table 1. PVT SPA beads coated with secondary anti-rabbit or anti-mouse antibodies (PerkinElmer España, S.L., Tres Cantos, Madrid, Spain) were then added (1 mg of beads per 25 μg protein), and plates were incubated for 3 h at RT with gentle agitation. Finally, plates were centrifuged (5 min at 1000 × *g*), and radioactivity was

Table 1
Antibodies, dilutions and GDP concentrations employed in the [^{35}S]GTPγS scintillation proximity assays.

Target	Description	Commercial firm	Catalog #	[GDP]	Ab. dilution	Beads
$G\alpha_{i1}$	Mouse monoclonal anti-$G\alpha_{i1}$	Santa Cruz	sc-56536	100 μM	1:20	Mouse
$G\alpha_{i2}$	Rabbit Polyclonal anti-$G\alpha_{i2}$	Santa Cruz	sc-7276	50 μM	1:20	Rabbit
$G\alpha_{i3}$	Rabbit polyclonal anti-$G\alpha_{i3}$	ENZO	SA-129	100 μM	1:30	Rabbit
$G\alpha_{o}$	Mouse monoclonal anti-$G\alpha_{o}$	ENZO	SA-280	50 μM	1:75	Mouse
$G\alpha_{S/olf}$	Rabbit Polyclonal anti-$G\alpha_{s/olf}$	Santa Cruz	sc-383	100 μM	1:20	Rabbit
$G\alpha_{q/11}$	Rabbit Polyclonal anti-$G\alpha_{q/11}$	Santa Cruz	sc-392	1 μM	1:20	Rabbit
$G\alpha_{z}$	Rabbit Polyclonal anti-$G\alpha_{z}$	Santa Cruz	sc-388	100 μM	1:20	Rabbit

detected on a MicroBeta TriLux scintillation counter (PerkinElmer España, S.L., Tres Cantos, Madrid, Spain). In order to test their effect on the [^{35}S]GTPγS binding to the different Gα subunit subtypes, 10 μM of WIN 55,212-2, rimonabant and/or O-2050 were used. Nonspecific binding was defined as the remaining [^{35}S]GTPγS binding in the presence of 10 μM unlabelled GTPγS.

2.5. [^{3}H]SR141716 competition assays

Plasma membranes (P_2 fraction) were prepared as follows: brain tissue samples (~200 mg) were homogenized in 5 ml of ice-cold buffer (5 mM Tris–HCl, 250 mM sucrose, pH 7.4) using an Ultra-Turrax T8 at maximum speed for 10 s at 4 °C. The homogenates were centrifuged at 1000 × *g* for 10 min at 4 °C. The pellet (P_1 fraction) was discarded and the supernatants recentrifuged at 40,000 × *g* for 10 min (4 °C). The resulting pellets were resuspended in 2 ml of fresh Tris incubation buffer (50 mM Tris–HCl, pH = 7.5) and recentrifuged under similar conditions. This washing procedure was repeated and the resultant pellets (P_2) were resuspended in an appropriate volume of incubation buffer to a final protein content of approximately 0.5 mg ml^{-1} and homogenized. The protein content was determined by the method of Bradford [31] with BSA as the standard.

Competition binding assays of [^{3}H]SR141716 (5 nM) with unlabelled rimonabant and O-2050 were carried out in order to determine the inhibition constants (K_i) and to compare the maximal inhibition of the [^{3}H]SR141716 specific binding produced by these drugs. To delineate the competition curve, increasing concentrations of the competing drugs were used (10^{-12}–10^{-5} M, 10 concentrations), and the non-specific binding was determined in the presence of 10 μM unlabelled rimonabant. The incubation buffer contained, in a final volume of 500 μl, 50 mM Tris–HCl and 0.5% (w/v) BSA at pH 7.5. The tubes were incubated with shaking for 1 h at 30 °C. Incubations were terminated by dilution of the samples with 3 ml of ice-cold incubation buffer (4 °C) followed by rapid filtration through Whatman GF/C filters presoaked with 0.5% polyethylenimine. The filters were rinsed twice with 3 ml of ice-cold incubation buffer and transferred to vials containing 5 ml of OptiPhase HiSafe II cocktail. The radioactivity trapped was determined by liquid scintillation spectrometry (Packard 2200CA) with a 40–50% counting efficiency and c.p.m. values were transformed to d.p.m. values by an internal calibration program.

2.6. Data analysis and statistical procedures

Pharmacological parameters of the concentration–response curves for the [^{35}S]GTPγS binding, the maximal effect (E_{max}) and the concentration of the drug that determines the half-maximal effect (EC_{50}/IC_{50}), were obtained by non-linear analysis using GraphPad Prism® software. In order to allow better interpretation of the data, the concentration–response curves are displayed as relative stimulations/inhibitions over basal values (% of basal). Basal binding was determined in the absence of the cannabinoid drugs and was defined as 100%.

The statistical comparison of the data sets was performed in GraphPad Prism®, by a co-analysis of the curves, comparing the goodness of fit to a monophasic or biphasic binding model by means of an *F* test (based on the principle of extra sum of squares), described elsewhere [33]. The statistical significance of the improvement was determined with a level of significance $p = 0.05$.

Specific binding data obtained from [^{35}S]GTPγS scintillation proximity assays were transformed to percentage of the basal binding value (binding values observed in the absence of any exogenous drug) obtained for each Gα protein subunit studied. The statistical comparison of the SPA results was carried out by a two-tailed one sample Student's *t*-test with a significance level of $p = 0.05$.

Data obtained from [^{3}H]SR141716 competition assays were also analyzed by non-linear analysis using GraphPad Prism® software, in order to determine the inhibitory constants (K_i) along with the maximal inhibition of the [^{3}H]SR141716 specific binding.

All data are expressed as the mean ± SEM values.

2.7. Drugs, chemicals reagents and other materials

[^{35}S]GTPγS (1250 Ci/mmol) was purchased from PerkinElmer Life Sciences (Maanstraat, Germany) and [^{3}H]SR141716 (44 Ci/mmol) from GE Healthcare Life Sciences (Buckinghamshire, UK). WIN 55,212-2, naloxone and GTPγS were purchased from Sigma–Aldrich (St. Louis, MO, USA); DPCPX, O-2050 and Org 27569 were from Tocris Bioscience (Bristol, UK) and rimonabant was donated by Sanofi-Synthelabo (Longjumeau, France). All other chemical reagents were of analytical quality and were purchased from Merck (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, USA).

3. Results

3.1. Effects of rimonabant on G protein activation in human brain membranes

Initially, the cannabinoid antagonist effect of rimonabant in human prefrontal cortex was demonstrated by [^{35}S]GTPγS binding assays. The [^{35}S]GTPγS assay measures the level of G protein activation following agonist occupation of a GPCR, by determining the binding of the non-hydrolyzable analog [^{35}S]GTPγS to G_α subunits. Thus, the assay measures a functional consequence of receptor occupancy at one of the earliest receptor-mediated events. The assay allows the determination of traditional pharmacological parameters of potency and efficacy, with the advantage that agonist measures are not subjected to amplification or other modulation that may occur when analyzing parameters further downstream of the receptor [34]. Rimonabant (10 μM) significantly right shifted the stimulation curve produced by the cannabinoid agonist WIN 55,212-2 (10^{-12}–10^{-3} M, 10 concentrations). The $-\log EC_{50}$ moved from 5.8 ± 0.1 (EC_{50} = 1.4 μM) to 4.6 ± 0.2 (EC_{50} = 21 μM) and the E_{max} was reduced from 187 ± 2% to 125 ± 3% ($p < 0.001$ for both parameters) in the presence of 10 μM rimonabant (Fig. 1A).

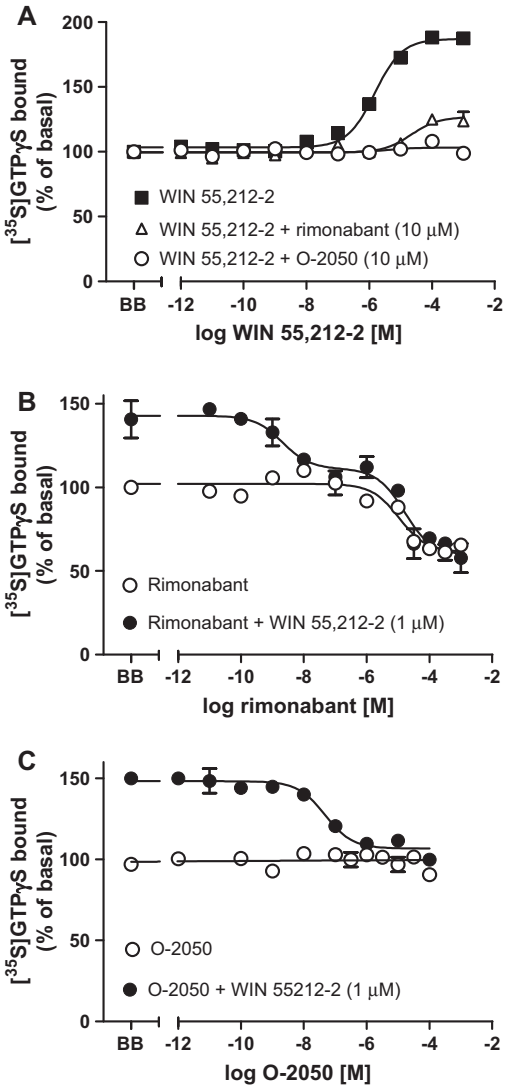

Fig. 1. Concentration–response curves of the stimulation of the [^{35}S]GTPγS specific binding to human brain cortical membranes by: (A) WIN 55,212-2 in the absence and in presence of the cannabinoid antagonists rimonabant (10 μM) or O-2050 (10 μM), (B) rimonabant in the absence and in presence of WIN 55,212-2 (1 μM) and (C) O-2050 in the absence and in presence of WIN 55,212-2 (1 μM). Data shown are mean ± S.E.M. values of three independent experiments performed in brain samples of three different subjects and ran in duplicate.

Rimonabant itself (10^{-12}–10^{-3} M, 12 concentrations) inhibited the basal binding of [^{35}S]GTPγS to human cortical membranes in a concentration dependent manner, with a $-\log IC_{50}$ of 4.7 ± 0.2 (IC_{50} = 20 μM) and a maximal inhibition of 48 ± 2% (Fig. 1B). When the same experiment was performed in the presence of WIN 55,212-2 (1 μM), the curve fitted better to a two site model (Fig. 1B). The increase in the [^{35}S]GTPγS basal binding induced by WIN 55,212-2 (+47%), was antagonized by increasing concentrations of rimonabant with a $-\log IC_{50}$ of 8.7 ± 0.4 (IC_{50} = 2.1 nM). Nevertheless, at rimonabant concentrations higher than 10^{-7}–10^{-6} M, the inhibitory effect of this drug over the [^{35}S]GTPγS basal binding was still observed in the presence of WIN 55,212-2 ($-\log IC_{50}$ = 4.6 ± 0.2; IC_{50} = 28 μM) (Fig. 1B).

Some experiments were performed to validate the O-2050 compound, a neutral antagonist of the CB1 receptor, as a suitable pharmacological tool to further study the effects of rimonabant. In order to confirm its ability to antagonize the effect mediated by cannabinoids, [^{35}S]GTPγS binding assays were performed in human cortical membranes at increasing concentrations of WIN 55,212-2 (10^{-12}–10^{-3} M, 10 concentrations) in the absence or presence of the antagonist. O-2050 (10 μM) completely blocked the effect produced by the cannabinoid agonist WIN 55,212-2 confirming its antagonism of the cannabinoid CB1 receptor (Fig. 1A). In the same way, O-2050 concentration-dependently blocked the increase of the [^{35}S]GTPγS basal binding induced by WIN 55,212-2 (1 μM) ($-\log IC_{50}$ = 7.3 ± 0.2; IC_{50} = 47 nM) (Fig. 1C). On the other hand, when [^{35}S]GTPγS binding assays were carried out at increasing concentrations of O-2050 (10^{-12}–10^{-4} M, 10 concentrations), this drug barely affected the basal binding of [^{35}S]GTPγS (Fig. 1C).

Neither 10 μM nor 100 μM concentrations of O-2050 were able to significantly antagonize the inhibition of the basal binding of [^{35}S]GTPγS to human prefrontal cortex membranes produced by rimonabant (10^{-12}–10^{-3} M, 15 concentrations) (Fig. 2A). The $-\log IC_{50}$ was 4.7 ± 0.1 (IC_{50} = 22 μM) in both cases and the maximal inhibition in the presence of 10 or 100 μM O-2050 was 49 ± 2% and 51 ± 3% respectively.

In order to further investigate the putative involvement of the cannabinoid CB1 receptors in this rimonabant effect, an allosteric modulator of these receptors, Org 27569, was tested. Neither 100 nM nor 1 μM of Org 27569 were able to significantly modify the inhibition of the basal binding of [^{35}S]GTPγS to human prefrontal cortex membranes produced by rimonabant (10^{-8}–10^{-3} M, 12 concentrations) (Fig. 2B). The values obtained were $-\log IC_{50}$ = 4.3 ± 0.1 (IC_{50} = 46 μM) in both cases and a maximal inhibition of 52 ± 2% and 49 ± 3%, in the presence of 100 nM and 1 μM Org 27569, respectively.

In regard to the possible involvement of the opioid and adenosine receptors in this effect of rimonabant [12,22], the same experiments were performed in the presence of the μ, δ and κ opioid receptor antagonist naloxone (10 μM), and the A_1 adenosine receptor antagonist DPCPX (1 μM). None of these drugs significantly affected the concentration–dependent inhibition curve produced by rimonabant ($-\log IC_{50}$ = 4.7 ± 0.2, IC_{50} = 20 μM and $-\log IC_{50}$ 5.0 ± 0.2, IC_{50} = 10 μM; the maximal inhibitions being 44 ± 3% and 46 ± 2%, respectively; Fig. 2C).

3.2. Effects of rimonabant on G protein activation in CB1 knockout mice

[^{35}S]GTPγS binding experiments were carried out with brain membranes from Swiss wild-type and CB1R knockout mice. In Swiss control mice, rimonabant (10^{-12}–10^{-3} M, 12 concentrations) inhibited the basal binding of [^{35}S]GTPγS in a similar manner to human cortical membranes (Fig. 3A), producing an inhibition of 63 ± 2% with a $-\log IC_{50}$ of 4.4 ± 0.1 (IC_{50} = 42 μM). When the same experiment was performed in CB1 knockout mice brain membranes, the curve was not different from the one for the wild type mice (Fig. 3A): $-\log IC_{50}$ of 4.4 ± 0.1 (IC_{50} = 36 μM) and an inhibition of 61 ± 3%.

In order to validate that the CB1 receptor was not functional in CB1 knockout mice, [^{35}S]GTPγS specific binding concentration–response curves for WIN 55,212-2 were performed in Swiss and CB1 knockout mice brain membranes. In Swiss control mice WIN 55,212-2 produced a maximal stimulatory effect of 163 ± 2% over basal, with a $-\log EC_{50}$ of 6.5 ± 0.1 (EC_{50} = 0.3 μM). Conversely, in CB1 knockout mice the WIN 55,212-2 stimulation curve was significantly shifted rightward with a maximal effect of 116 ± 2% over basal (Fig. 3B).

3.3. Study of the G protein subtypes involved in WIN 55,212-2 and rimonabant mediated signalling in human brain membranes

[^{35}S]GTPγS scintillation proximity assays coupled to immunoprecipitation with different specific antibodies against several Gα

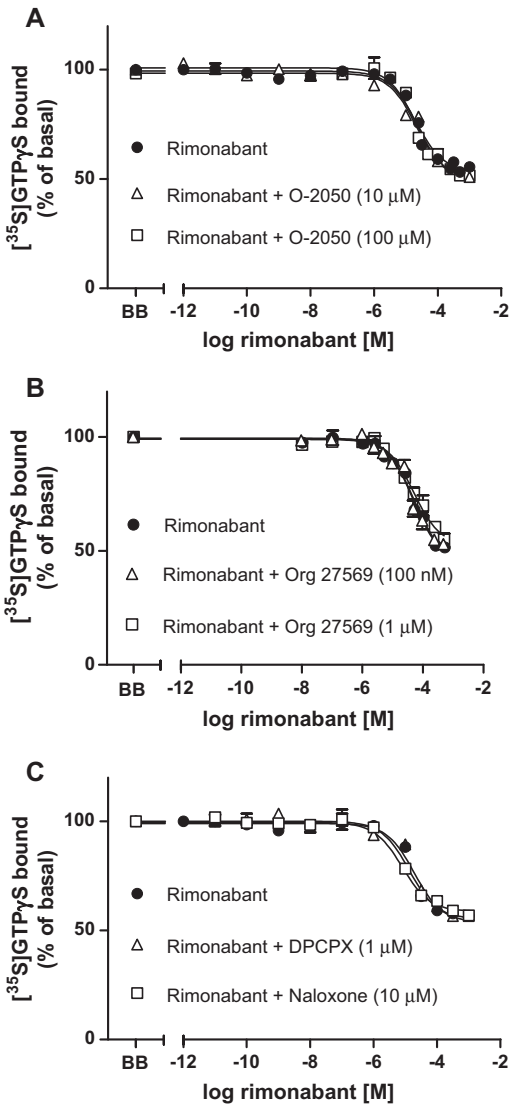

Fig. 2. Effect of increasing concentrations of rimonabant (10^{-12}–10^{-3} M, 15 concentrations) on the [^{35}S]GTPγS specific binding to human brain cortical membranes in the absence and in the presence of: (A) O-2050 (10 and 100 μM), (B) Org 27569 (100 nM and 1 μM) and (C) naloxone (10 μM) or DPCPX (1 μM). Data shown are mean ± S.E.M. values of three independent experiments performed in brain samples of three different subjects and ran in duplicate.

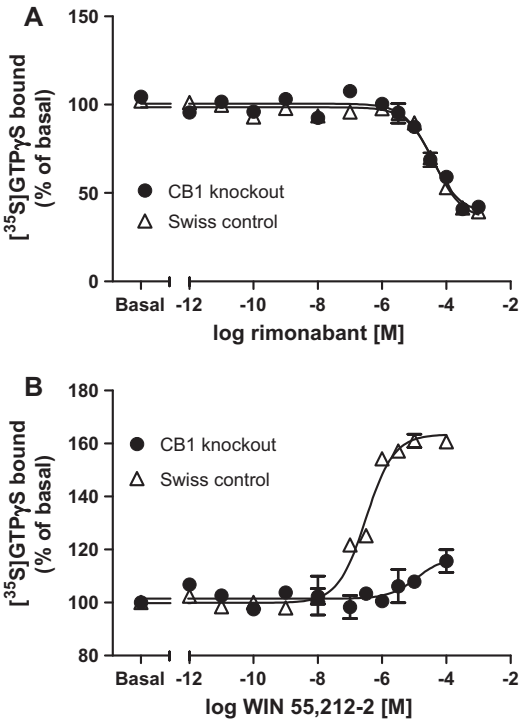

Fig. 3. Concentration-response curves of the stimulation of the [^{35}S]GTPγS specific binding by: (A) rimonabant and (B) WIN 55,212-2 in Swiss control and CB1 knockout mice whole brain membranes. Data shown are mean ± S.E.M. values of three independent experiments.

subunit subtypes showed that 10 μM WIN 55,212-2 induced a statistically significant stimulation of the [^{35}S]GTPγS binding mediated by G_{i1} (137 ± 9%, $p = 0.006$), G_{i2} (133 ± 8%, $p = 0.001$), G_{i3} (196 ± 19%, $p = 0.001$) and G_o (142 ± 10%, $p = 0.001$), but not by $G_{q/11}$ (97 ± 3%, $p = 0.474$), $G_{s/olf}$ (97 ± 4%, $p = 0.412$) or G_z (106 ± 5%, $p = 0.205$) protein subunits (Fig. 4). This WIN 55,212-2 induced stimulation was always antagonized by the co-incubation with 10 μM rimonabant or 10 μM O-2050 (Fig. 4).

When the incubation was carried out in the presence of 10 μM O-2050 alone, neither stimulation nor inhibition of the basal binding values were observed for any of the Gα subtype subunits analyzed (Fig. 4). However, the incubation with 10 μM SR141716 induced a reduction in the basal binding values obtained for some of these Gα subunits.

In order to determine the Gα subunits implicated in the inverse agonist effect of rimonabant, the [^{35}S]GTPγS SPA were carried out in the presence of 10 μM SR141716 alone and in the presence of 10 μM O-2050. In this case, no stimulation over the basal binding was observed with any of the antibodies used. However, a reduction of the [^{35}S]GTPγS basal binding values (considered as 100%) was obtained when specific anti-G_{i3} (70 ± 8%, $p = 0.005$), anti-G_o (83 ± 3%, $p < 0.001$) or anti-G_z (89 ± 2%, $p < 0.001$) antibodies were used, triggering an inhibition of the basal binding values of these subtypes of around 30%, 17% and 11%, respectively (Fig. 4). The presence of 10 μM O-2050 in the incubation with SR141716 was not able to inhibit its inverse agonist effect and statistically significant reductions of the basal binding values were still observed for immunoprecipitations with anti-G_{i3} (83 ± 10%, $p = 0.031$), anti-G_o (87 ± 4%, $p = 0.002$) or anti-G_z (88 ± 4%, $p = 0.008$) antibodies; i.e. inhibitions of 17%, 13% and 12%, respectively (Fig. 4).

Antibody specificity, as previously described in the literature [35–37], was confirmed in our experimental conditions by Western blot (data not shown).

3.4. [^{3}H]SR141716 competition assays

Competition assays of [^{3}H]SR141716 binding (5 nM) by increasing concentrations (10^{-12}–10^{-5} M, 10 concentrations) of unlabelled rimonabant and O-2050 were performed in human brain membranes to further compare both cannabinoid antagonists. These inhibitions showed a pK_i of 8.6 ± 0.1 (K_i = 2.2 nM) and 7.5 ± 0.1 (K_i = 30.0 nM), respectively. However, O-2050 only inhibited by 61 ± 5% the [^{3}H]SR141716 specific binding (Fig. 5). These data indicate that [^{3}H]SR141716 recognizes an additional binding site other than the CB1 receptor recognized by O-2050. On the other hand, naloxone did not significantly displace [^{3}H]SR141716 specific binding, even at high micromolar concentrations (Fig. 5).

4. Discussion

Rimonabant (SR141716) was the first potent and selective antagonist of the brain cannabinoid CB1 receptor to be synthesized [7,38]. In the same way, it was the first therapeutically relevant cannabinoid antagonist, able to suppress the reinforcing/reward-

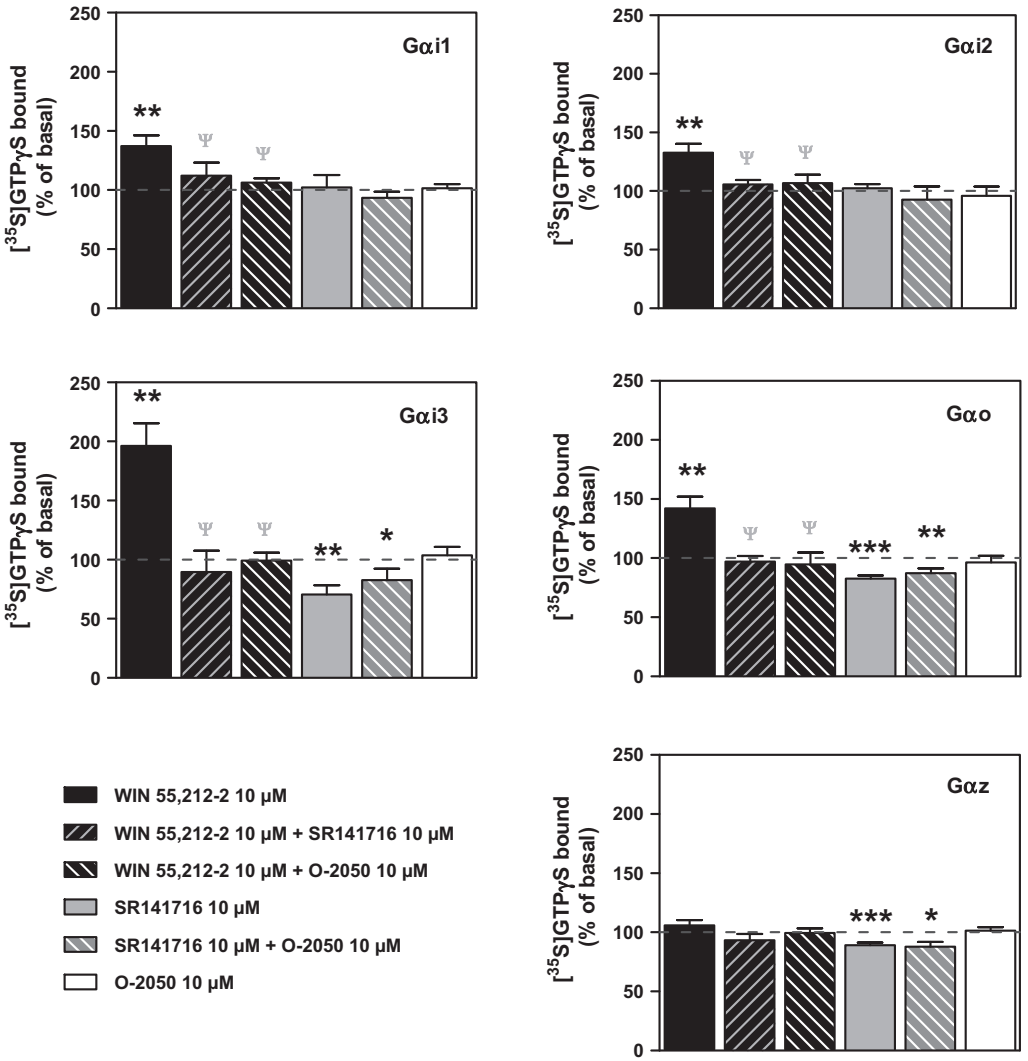

Fig. 4. [35S]GTPγS scintillation proximity assays coupled to immunoprecipitation with different Gα subunits specific antibodies in human brain cortical membranes. Data are shown as percentage of [35S]GTPγS basal binding values obtained for each specific subunit. Bars represent mean ± SEM of four to six different experiments carried out in triplicate. Asterisks highlight those values of stimulation or inhibition of basal binding which are statistically different from basal binding values (Student's *t* test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). WIN 55,212-2 stimulation was always antagonized by the co-incubation with 10 μM rimonabant or 10 μM O-2050 (Student's *t* test between WIN 55,212-2 and WIN 55,212-2 + SR141716 or WIN 55,212-2 + O-2050; $^{\Psi}p < 0.05$; $^{\Psi\Psi}p < 0.01$).

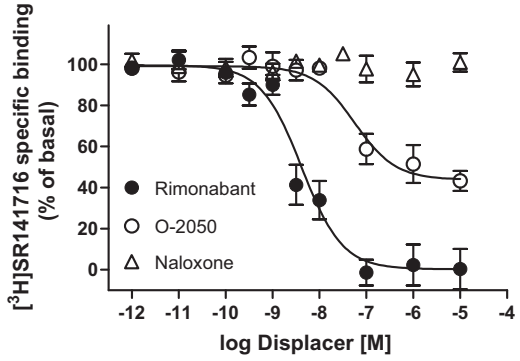

Fig. 5. Inhibition curves of [3H]SR141716 (5 nM) specific binding by increasing concentrations of rimonabant, O-2050 or naloxone (10^{-12}–10^{-5} M, 10 concentrations) in human brain cortical membranes. The non-specific binding was determined in the presence of 10 μM rimonabant. Data shown are mean ± S.E.M. values of three independent experiments performed in brain samples of three different subjects and ran in triplicate.

ing properties of food and different drugs of abuse, and therefore, it is potentially effective for the treatment of drug addiction and obesity-related disorders [39]. The metabolic effects of rimonabant appear to involve the modulation of metabolism through antagonism at the CB1 receptors [40]. However, apart from behaving as an antagonist at the CB1 receptors, it has been suggested that rimonabant could also have inverse agonist properties [8,10–14,18–23]. Moreover, there is evidence suggesting that this inverse agonism is not mediated by the CB1 receptor [23]. In this context, the present study tried to elucidate whether this inverse agonism mechanism is CB1 dependent or independent in the brain. Here, we presented new evidence in *postmortem* human brain, demonstrating that the inverse agonist effect of rimonabant on G proteins observed with >1 μM concentrations in [35S]GTPγS binding assays is not mediated by the CB1 receptor.

Firstly, we demonstrated by [35S]GTPγS binding assays that in human brain cortical homogenates rimonabant antagonizes the effect produced by the CB1 agonist WIN 55,212-2 on the G proteins. This was indeed verified by two different experimental approaches. First, 10 μM rimonabant produced an important rightward shift of the concentration-dependent stimulation

produced by WIN 55,212-2. Second, the stimulation produced by 10 μM WIN 55,212-2 was antagonized by increasing concentrations of rimonabant. Thus, we confirmed that rimonabant acts as a CB1 receptor antagonist in human brain as previously suggested [41,42].

Interestingly, when rimonabant was tested alone in [^{35}S]GTPγS binding experiments, a concentration-dependant inhibition of 48% of the basal binding was observed (Figs. 1B and 2). This effect may be considered as an inverse agonist effect of rimonabant over the G protein's activation. This reduction in the [^{35}S]GTPγS binding by rimonabant has previously been reported in other tissues, either in heterologous expression systems [8], cultured rat brain cells [16] or rodent brain [11–13,22]. Moreover, it has also been reported that in human brain, rimonabant also behaved as an inverse agonist on cAMP production [14]. The slight reduction of potency of rimonabant and WIN 55,212-2 in the [^{35}S]GTPγS binding assays as compared with previously reported values in cells or in other mammalian tissues could represent differences in the receptor:G-protein ratio in humans, differences in the methodological conditions, or could be intrinsic to study conditions in *postmortem* human brain.

It has been shown that rimonabant reduces food intake in obese animals by antagonizing an increased endocannabinoid tone [43]. In this context, some authors suggested that the inverse agonist effect of rimonabant could be due to the antagonism of responses to endogenously released endocannabinoids [16,23]. However, this does not seem to be probable because competitive antagonism of endogenous ligands should require only nanomolar concentrations of rimonabant, while the inhibition of the basal [^{35}S]GTPγS binding occurred at the micromolar range, both in the present study, and in previous ones [8,11,22]. Moreover, the incubation with the cannabinoid receptor antagonist O-2050 did not show an inhibition of the basal binding values under the same experimental conditions (Fig. 1C). Besides, the endogenous ligands present in the tissue would probably have been degraded during incubation.

In the present study, the suspicion that this inverse agonist effect was not mediated by the CB1 receptor rose when 1 μM WIN 55,212-2 did not modify the inhibitory concentration-dependent curve of rimonabant. Similarly, a previous study also observed that the presence of another CB1 receptor agonist, methanandamide at 5 μM, did not significantly alter the effect produced by rimonabant [11].

We considered that the neutral antagonist of the CB1 receptor O-2050 used in the same kind of experiments would provide more precise pharmacological data. Validation experiments were performed beforehand, in order to confirm its reported neutral antagonism over the CB1 receptor [23,44]. As expected, O-2050 completely antagonized the [^{35}S]GTPγS binding stimulation produced by WIN 55,212-2 in human prefrontal cortex (Fig. 1A). When tested alone, O-2050 did not affect the basal binding in a significant manner in [^{35}S]GTPγS binding (Fig. 1C). Moreover, O-2050 did not modulate the basal binding of [^{35}S]GTPγS for any of the studied Gα subunits in our SPA (Fig. 4). These data confirm for the first time that O-2050 behaves *in vitro* as a neutral antagonist of the CB1 receptors in human brain membranes.

The presence of O-2050 at 10 μM or 100 μM was not able to antagonize the inhibition produced by rimonabant over [^{35}S]GTPγS basal binding in [^{35}S]GTPγS binding assays (Fig. 2A), or over the basal binding of the different Gα subunits tested in SPA (Fig. 4), which further supports that this inverse effect is not mediated by the CB1 receptor. These results agree with data obtained in mouse cortical membranes where rimonabant-induced inverse agonist effects on G protein signalling were not reversed by O-2050 [22]. Additional confirmation that rimonabant produced an inverse agonist effect on G protein signalling in a CB1 receptor-independent manner rose from the [^{35}S]GTPγS experiments in CB1 knockout mice brain membranes. These experiments were performed in order to confirm data obtained in human brain. Similarly to previous reports [13,22], rimonabant concentration-dependently inhibited [^{35}S]GTPγS basal binding in CB1 knockout mice brain membranes, in the same manner it did in wild-type brain and with a similar efficacy and potency to that observed in human brain.

It is also possible that the effect of rimonabant could be mediated by its binding to a site on the cannabinoid CB1 receptor distinct from the agonist binding site, or even due to an allosteric modulation of this receptor [11]. In this context, some investigators have found a particular domain in the CB1 receptor which they suggest is necessary for the inverse agonist effects of rimonabant [45,46]. More precisely, in a mutant CB1 receptor where this site was altered, rimonabant no longer exhibited inverse agonism in Ca^{2+} currents, but it could still antagonize the effect of WIN 55,212-2 [45]. This is the reason why we tested the effect of the CB1 receptor allosteric modulator Org 27569. This synthetic compound had been shown to display a number of characteristics commonly associated with allosteric modulators, such as, (a) either enhancement or inhibition of orthosteric CB1 ligand binding depending on the nature of the orthosteric probe, (b) a slowing of the dissociation rate constants for a radioligand from the occupied CB1 receptor, and (c) a non-competitive inhibition of CB1 receptor orthosteric agonist efficacy, for instance, in its mediated G protein signalling [47]. However, the inhibition of the basal G protein signalling produced by rimonabant was not altered in the presence of this allosteric modulator (Fig. 2B), at doses proven to be effective for the allosterism in [^{35}S]GTPγS assays [47].

Several studies have suggested that other GPCRs different from the CB1 receptors might be involved in the inverse agonist effect of rimonabant [11,12,23]. In this regard, in the present study [^{3}H]SR141716 displacement experiments in human cortical membranes have demonstrated that the CB1 receptor neutral antagonist O-2050 inhibited only ~60% of the specific binding displaced by rimonabant (Fig. 5). Certainly, these data reveal that, at least in human brain membranes, rimonabant recognizes an additional binding site other than the CB1 receptor recognized by O-2050. This finding could also explain the biphasic curve produced by rimonabant in the presence of WIN 55,212-2 (Fig. 1B). The first part of the curve could be due to the antagonism of the CB1 receptors by rimonabant. The second part would correspond to the effect of rimonabant over this second binding site.

Different receptors could be responsible for this inhibitory effect on the G proteins. Tonic adenosine A1 receptor-dependent G protein activity has been reported in basal conditions in rat brain [^{35}S]GTPγS binding studies [48,49]. In this regard, it has been observed that rimonabant only inhibited [^{35}S]GTPγS basal binding to rat brain membranes in conditions where tonic adenosine A1 receptor signalling was present [12]. Accordingly, rimonabant no longer produced this effect in the presence of the selective A1 receptor antagonist DPCPX [12]. These findings suggested the hypothesis that the inhibitory effect of rimonabant was due to the blockade of the activation of A1 receptors produced by endogenously released adenosine. Nevertheless, in the present study we have observed no significant effect for DPCPX over the inverse agonist effect produced by rimonabant on the [^{35}S]GTPγS basal binding (Fig. 2C). We therefore suggest that this effect is not mediated by the A1 receptor, at least in our experimental conditions in human prefrontal cortex.

The μ-opioid system has also drawn attention to this issue, due to the similarities between the μ-opioid receptor and CB1 receptor families, and their interactions at the level of G proteins in the brain [50,51]. Furthermore, in the context of alcoholism, the ability of rimonabant to reduce alcohol intake in rodents is significantly potentiated by the concomitant administration of the opioid receptor antagonists naltrexone or naloxone [52–54]. Recently, a

novel pertussis toxin-insensitive opioid signalling has been unmasked by the co-application of DAMGO and rimonabant in cells over-expressing recombinant μ-opioid receptors [22]. However, we have observed that the inverse agonist effect of rimonabant on G protein signalling is not affected in the presence of the μ, δ and κ opioid antagonist naloxone (Fig. 2C). Furthermore, inhibition experiments of [^{3}H]SR141716 with naloxone showed no displacement at all, even at high concentrations of the opioid ligand (Fig. 5). Altogether these results suggest that the inverse agonist effect of rimonabant in human brain is not mediated by opioid receptors. Moreover, the unknown second binding site of [^{3}H]SR141716 is not an opioid receptor.

Since endogenous cannabinoids such as anandamide also activate the vanilloid receptor type 1 [55,56], rimonabant could similarly bind to this receptor. Thus, rimonabant inhibited the effect of anandamide and capsaicin mediated by vanilloid receptor type 1 at concentrations higher than those required for antagonism of the CB1 receptor [57]. Additionally, it has been reported that capsazepine, a selective vanilloid 1 receptor antagonist, completely reversed rimonabant-induced neuroprotective effects [58]. Nevertheless, the vanilloid receptor type 1 is a channel receptor, and since the approach we have used in the present study is the G protein signalling, we rule out the possibility that rimonabant inhibits [^{35}S]GTPγS through this receptor.

The orphan GPR55 has been suggested to be a putative cannabinoid receptor, since it responds to several cannabinoid ligands [59–61]. Interestingly, rimonabant is also a ligand for the GPR55 receptor, although in some studies it behaves as an agonist and in others as an antagonist [61]. So far the effect of rimonabant on the G protein signalling produced by this receptor has not been determined in heterologous expression systems. Thus, the possibility that the observed inhibition in G protein signalling produced by rimonabant is through the GPR55 cannot be discarded. However, GPR55 is coupled to G_q, G_{12} or G_{13} for signal transduction, and the classical [^{35}S]GTPγS binding assay measures mainly the activity of those GPCRs coupled to $G_{i/o}$, since these are generally more abundant than the other families and have higher rates of nucleotide exchange [34]. Moreover, in the [^{35}S]GTPγS SPA we have not observed any effect, neither activation nor inhibition, of rimonabant over the $G_{q/11}$ protein subunit (G_{12} and G_{13} were not tested), which indicates that this effect might not be GPR55-dependent.

Finally, there is some evidence suggesting that other GPCRs could mediate this effect, since several cannabinoid ligands interact with them. The antihyperalgesic effect of WIN 55,212-2 has been suggested to be mediated through interaction with spinal metabotropic glutamate-5 receptors in rats [62]. However, metabotropic glutamate-5 receptors couple preferentially to $G_{q/11}$ isoforms [63] and we have observed that rimonabant does not inhibit the signalling of this subunit. The anxiolytic effects of THC seem to be modulated by serotonergic 5-HT_{1A} receptors [64]. Indeed, 5-HT_{1A} receptors are coupled to $G_{i/o}$ proteins and our SPA showed that rimonabant inhibits the signalling of G_{i3} and G_o subtypes. There is also evidence for novel non-CB1/non-CB2 cannabinoid receptors, localized in the endothelium [65] and the central nervous system [66] that could also mediate the observed effects of rimonabant.

It has also been suggested that rimonabant could inhibit the basal [^{35}S]GTPγS binding by a non-receptor-mediated mechanism. This is unlikely in our study because the effect that we observed is concentration-dependent and saturable. Besides, the inhibitory effect observed in rat brain was only observed at concentrations of GDP that support receptor-mediated effects on [^{35}S]GTPγS binding, such as stimulation by agonists [11].

[^{35}S]GTPγS scintillation proximity assays coupled with immunoprecipitation with different Gα subunit specific antibodies showed that WIN 55,212-2 stimulates the [^{35}S]GTPγS binding to G_{i1}, G_{i2}, G_{i3} and G_o subtypes. On the other hand, rimonabant specifically inhibits the signalling of G_{i3}, G_o and G_z subtypes in human brain, while it has no effect over the [^{35}S]GTPγS binding due to G_{i1}, G_{i2} $G_{q/11}$ or $G_{s/olf}$ subunits. Moreover, this effect of SR141716 reducing the [^{35}S]GTPγS basal binding is not blocked by incubation in the presence of O-2050. All these data could reinforce the hypothesis that the mechanism through which rimonabant exerts its inverse agonist effect is not CB1-dependant.

To conclude, the present study demonstrates in *postmortem* human brain that rimonabant produces a concentration-dependent inverse agonist effect on the G protein signalling, which is CB1 independent.

Conflict of interest statement

The authors have no conflict of interest to declare.

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References

[1] Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 1990;346:561–4.
[2] Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, et al. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science 1992;258:1946–9.
[3] Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. Nature 1993;365:61–5.
[4] Devane WA, Dysarz 3rd FA, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 1988;34:605–13.
[5] Herkenham M, Lynn AB, Johnson MR, Melvin LS, de Costa BR, Rice KC. Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. J Neurosci 1991;11:563–83.
[6] Childers SR, Pacheco MA, Bennett BA, Edwards TA, Hampson RE, Mu J, et al. Cannabinoid receptors: G-protein-mediated signal transduction mechanisms. Biochem Soc Symp 1993;59:27–50.
[7] Rinaldi-Carmona M, Barth F, Heaulme M, Shire D, Calandra B, Congy C, et al. SR 141716A, a potent and selective antagonist of the brain cannabinoid receptor. FEBS Lett 1994;350:240–4.
[8] MacLennan SJ, Reynen PH, Kwan J, Bonhaus DW. Evidence for inverse agonism of SR 141716A at human recombinant cannabinoid CB1 and CB2 receptors. Br J Pharmacol 1998;124:619–22.
[9] Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, et al. A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. J Biol Chem 1997;272:22330–9.
[10] Felder CC, Joyce KE, Briley EM, Glass M, Mackie KP, Fahey KJ, et al. LY320135, a novel cannabinoid CB1 receptor antagonist, unmasks coupling of the CB1 receptor to stimulation of cAMP accumulation. J Pharmacol Exp Ther 1998;284:291–7.
[11] Sim-Selley LJ, Brunk LK, Selley DE. Inhibitory effects of SR 141716A on G-protein activation in rat brain. Eur J Pharmacol 2001;414:135–43.
[12] Savinainen JR, Saario SM, Niemi R, Jarvinen T, Laitinen JT. An optimized approach to study endocannabinoid signaling: evidence against constitutive activity of rat brain adenosine A1 and cannabinoid CB1 receptors. Br J Pharmacol 2003;140:1451–9.
[13] Breivogel CS, Griffin G, Di Marzo V, Martin BR. Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. Mol Pharmacol 2001;60:155–63.
[14] Mato S, Pazos A, Valdizan EM. Cannabinoid receptor antagonism and inverse agonism in response to SR 141716A on cAMP production in human and rat brain. Eur J Pharmacol 2002;443:43–6.

[15] Gifford AN, Bruneus M, Gatley SJ, Volkow ND. Cannabinoid receptor-mediated inhibition of acetylcholine release from hippocampal and cortical synaptosomes. Br J Pharmacol 2000;131:645–50.
[16] Breivogel CS, Walker JM, Huang SM, Roy MB, Childers SR. Cannabinoid signaling in rat cerebellar granule cells: G-protein activation, inhibition of glutamate release and endogenous cannabinoids. Neuropharmacology 2004;47:81–91.
[17] Richardson JD, Aanonsen L, Hargreaves KM. SR 141716A, a cannabinoid receptor antagonist, produces hyperalgesia in untreated mice. Eur J Pharmacol 1997;319:R3–4.
[18] Bass CE, Griffin G, Grier M, Mahadevan A, Razdan RK, Martin BR. SR-141716A-induced stimulation of locomotor activity. A structure–activity relationship study. Pharmacol Biochem Behav 2002;74:31–40.
[19] Colombo G, Agabio R, Diaz G, Lobina C, Reali R, Gessa GL. Appetite suppression and weight loss after the cannabinoid antagonist SR 141716. Life Sci 1998;63:PL113–7.
[20] Verty AN, McGregor IS, Mallet PE. Consumption of high carbohydrate, high fat, and normal chow is equally suppressed by a cannabinoid receptor antagonist in non-deprived rats. Neurosci Lett 2004;354:217–20.
[21] Lichtman AH, Hawkins EG, Griffin G, Cravatt BF. Pharmacological activity of fatty acid amides is regulated, but not mediated, by fatty acid amide hydrolase in vivo. J Pharmacol Exp Ther 2002;302:73–9.
[22] Cinar R, Szucs M. CB1 receptor-independent actions of SR 141716 on G-protein signaling: coapplication with the mu-opioid agonist Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol unmasks novel, pertussis toxin-insensitive opioid signaling in mu-opioid receptor-Chinese hamster ovary cells. J Pharmacol Exp Ther 2009;330:567–74.
[23] Pertwee RG. Inverse agonism and neutral antagonism at cannabinoid CB1 receptors. Life Sci 2005;76:1307–24.
[24] Howlett AC, Reggio PH, Childers SR, Hampson RE, Ulloa NM, Deutsch DG. Endocannabinoid tone versus constitutive activity of cannabinoid receptors. Br J Pharmacol 2011;163:1329–43.
[25] Christensen R, Kristensen PK, Bartels EM, Bliddal H, Astrup A. Efficacy and safety of the weight-loss drug rimonabant: a meta-analysis of randomised trials. Lancet 2007;370:1706–13.
[26] Le Foll B, Gorelick DA, Goldberg SR. The future of endocannabinoid-oriented clinical research after CB_1 antagonists. Psychopharmacology 2009;205:171–4.
[27] Giraldo J. How inverse can a neutral antagonist be? Strategic questions after the rimonabant issue. Drug Discov Today 2010;15:411–5.
[28] Greasley PJ, Clapham JC. Inverse agonism or neutral antagonism at G-protein coupled receptors: a medicinal chemistry challenge worth pursuing? Eur J Pharmacol 2006;553:1–9.
[29] Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, et al. Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. Science 1999;283:401–4.
[30] Gonzalez-Maeso J, Rodriguez-Puertas R, Gabilondo AM, Meana JJ. Characterization of receptor-mediated [^{35}S]GTPgammaS binding to cortical membranes from postmortem human brain. Eur J Pharmacol 2000;390:25–36.
[31] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[32] DeLapp NW, McKinzie JH, Sawyer BD, Vandergriff A, Falcone J, McClure D, et al. Determination of [^{35}S]guanosine-5′-O-(3-thio)triphosphate binding mediated by cholinergic muscarinic receptors in membranes from Chinese hamster ovary cells and rat striatum using an anti-G protein scintillation proximity assay. J Pharmacol Exp Ther 1999;289:946–55.
[33] Barturen F, Garcia-Sevilla JA. Long term treatment with desipramine increases the turnover of alpha 2-adrenoceptors in the rat brain. Mol Pharmacol 1992;42:846–55.
[34] Harrison C, Traynor JR. The [^{35}S]GTPgammaS binding assay: approaches and applications in pharmacology. Life Sci 2003;74:489–508.
[35] Gettys TW, Sheriff-Carter K, Moomaw J, Taylor IL, Raymond JR. Characterization and use of crude alpha-subunit preparations for quantitative immunoblotting of G proteins. Anal Biochem 1994;220:82–91.
[36] Cussac D, Newman-Tancredi A, Duqueyroix D, Pasteau V, Millan MJ. Differential activation of Gq/11 and Gi(3) proteins at 5-hydroxytryptamine(2C) receptors revealed by antibody capture assays: influence of receptor reserve and relationship to agonist-directed trafficking. Mol Pharmacol 2002;62:578–89.
[37] Valdizan EM, Castro E, Pazos A. Agonist-dependent modulation of G-protein coupling and transduction of 5-HT1A receptors in rat dorsal raphe nucleus. Int J Neuropsychopharmacol 2010;13:835–43.
[38] Rinaldi-Carmona M, Barth F, Heaulme M, Alonso R, Shire D, Congy C, et al. Biochemical and pharmacological characterisation of SR 141716A, the first potent and selective brain cannabinoid receptor antagonist. Life Sci 1995;56:1941–7.
[39] Carai MA, Colombo G, Gessa GL. Rimonabant: the first therapeutically relevant cannabinoid antagonist. Life Sci 2005;77:2339–50.
[40] Di Marzo V. CB_1 receptor antagonism: biological basis for metabolic effects. Drug Discov Today 2008;13:1026–41.
[41] Steffens M, Szabo B, Klar M, Rominger A, Zentner J, Feuerstein TJ. Modulation of electrically evoked acetylcholine release through cannabinoid CB1 receptors: evidence for an endocannabinoid tone in the human neocortex. Neuroscience 2003;120:455–65.
[42] Vinod KY, Arango V, Xie S, Kassir SA, Mann JJ, Cooper TB, et al. Elevated levels of endocannabinoids and CB1 receptor-mediated G-protein signaling in the prefrontal cortex of alcoholic suicide victims. Biol Psychiatry 2005;57:480–6.
[43] Di Marzo V, Goparaju SK, Wang L, Liu J, Bátkal S, Járal Z, et al. Leptin-regulated endocannabinoids are involved in maintaining food intake. Nature 2001;410:822–5.
[44] Martin B, Stevenson LA, Pertwee RG, Breivogel CS, Williams A, Mahadevan A, et al. Agonists and silent antagonists in a series of cannabinoid sulfonamides. In: Symposium on the cannabinoids, International Cannabinoid Research Society; 2002.p. p.2.
[45] Pan X, Ikeda SR, Lewis DL. SR 141716A acts as an inverse agonist to increase neuronal voltage-dependent Ca^{2+} currents by reversal of tonic CB1 cannabinoid receptor activity. Mol Pharmacol 1998;54:1064–72.
[46] Hurst DP, Lynch DL, Barnett-Norris J, Hyatt SM, Seltzman HH, Zhong M, et al. N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR 141716A) interaction with LYS 3.28(192) is crucial for its inverse agonism at the cannabinoid CB1 receptor. Mol Pharmacol 2002;62:1274–87.
[47] Price MR, Baillie GL, Thomas A, Stevenson LA, Easson M, Goodwin R, et al. Allosteric modulation of the cannabinoid CB1 receptor. Mol Pharmacol 2005;68:1484–95.
[48] Laitinen JT. Selective detection of adenosine A1 receptor-dependent G-protein activity in basal and stimulated conditions of rat brain [^{35}S]guanosine 5′-(gamma-thio)triphosphate autoradiography. Neuroscience 1999;90:1265–79.
[49] Moore RJ, Xiao R, Sim-Selley LJ, Childers SR. Agonist-stimulated [^{35}S]GTPgammaS binding in brain modulation by endogenous adenosine. Neuropharmacology 2000;39:282–9.
[50] Rios C, Gomes I, Devi LA. Mu opioid and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis. Br J Pharmacol 2006;148:387–95.
[51] Canals M, Milligan G. Constitutive activity of the cannabinoid CB1 receptor regulates the function of co-expressed Mu opioid receptors. J Biol Chem 2008;283:11424–3.
[52] Gallate JE, Mallet PE, McGregor IS. Combined low dose treatment with opioid and cannabinoid receptor antagonists synergistically reduces the motivation to consume alcohol in rats. Psychopharmacology (Berl) 2004;173:210–6.
[53] Manzanares J, Ortiz S, Oliva JM, Perez-Rial S, Palomo T. Interactions between cannabinoid and opioid receptor systems in the mediation of ethanol effects. Alcohol Alcohol 2005;40:25–34.
[54] Maccioni P, Colombo G, Carai MA. Blockade of the cannabinoid CB1 receptor and alcohol dependence: preclinical evidence and preliminary clinical data. CNS Neurol Disord Drug Targets 2010;9:55–9.
[55] Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sorgard M, Di Marzo V, et al. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. Nature 1999;400:452–7.
[56] Toth A, Blumberg PM, Boczan J. Anandamide and the vanilloid receptor (TRPV1). Vitam Horm 2009;81:389–419.
[57] De Petrocellis L, Bisogno T, Maccarrone M, Davis JB, Finazzi-Agrò A, Di Marzo V. The activity of anandamide at vanilloid VR1 receptors requires facilitated transport across the cell membrane and is limited by intracellular metabolism. J Biol Chem 2001;276:12856–63.
[58] Pegorini S, Zani A, Braida D, Guerini-Rocco C, Sala M. Vanilloid VR1 receptor is involved in rimonabant-induced neuroprotection. Br J Pharmacol 2006;147:552–9.
[59] Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J, et al. The orphan receptor GPR55 is a novel cannabinoid receptor. Br J Pharmacol 2007;152:1092–101.
[60] Henstridge CM, Balenga NA, Schroder R, Kargl JK, Platzer W, Martini L, et al. GPR55 ligands promote receptor coupling to multiple signalling pathways. Br J Pharmacol 2010;160:604–14.
[61] Sharir H, Abood ME. Pharmacological characterization of GPR55, a putative cannabinoid receptor. Pharmacol Ther 2010;126:301–13.
[62] Hama AT, Urban MO. Antihyperalgesic effect of the cannabinoid agonist WIN55,212-2 is mediated through an interaction with spinal metabotropic glutamate-5 receptors in rats. Neurosci Lett 2004;358:21–4.
[63] Hermans E, Challiss RA. Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. Biochem J 2001;359:465–84.
[64] Braida D, Limonta V, Malabarba L, Zani A, Sala M. 5-HT1A receptors are involved in the anxiolytic effect of Delta9-tetrahydrocannabinol and AM 404, the anandamide transport inhibitor, in Sprague–Dawley rats. Eur J Pharmacol 2007;555:156–63.
[65] Jarai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR, et al. Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. Proc Natl Acad Sci USA 1999;96:14136–41.
[66] Hajos N, Ledent C, Freund TF. Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. Neuroscience 2001;106:1–4.