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CB_1 receptor-independent actions of SR141716 on G-protein signaling; co-application with the μ-opioid agonist DAMGO unmasks novel, pertussis toxin-insensitive opioid signaling in MOR-CHO cells[i]

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Running title: CB_1 receptor-independent effects of SR141716 (Rimonabant)

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Abbreviations: [^{35}S]GTPγS, guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate; BSA, bovine serum albumin; CB_1, type 1 cannabinoid receptor; CB_2, type 2 cannabinoid receptor; CHO, Chinese hamster ovary; CNS, central nervous system; DAMGO, Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol; GPCRs, G-protein-coupled receptors; GTP-γ-S-Li_4, guanosine 5′-[γ-thio]triphosphate tetralithium salt; KO, knock-out; MOR, μ-opioid receptor; NaCl, sodium chloride; PTX, pertussis toxin; Win55,212-2, *R*(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)-methyl]pyrrolo[1,2,3-de]-1,4 benzo-xazin-yl]-(1-naphthalenyl) methanone mesylate; SR141716, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride; Tris, tris(hydroxymethyl)aminomethane;wt,wildtype.

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Abstract

The CB_1 cannabinoid receptor antagonist SR141716 has been shown by many investigators to inhibit basal G-protein activity, i.e. to display inverse agonism at high concentrations. However, it is not clear whether this effect is CB_1 receptor-mediated. Using the ligand-stimulated [^{35}S]GTPγS assay, we have found that 10 μM SR141716 slightly, but significantly decreases the basal [^{35}S]GTPγS binding in membranes of the wild-type and CB_1 receptor knock-out mouse cortex, parental Chinese hamster ovary (CHO) cells and CHO cells stably transfected with μ-opioid receptors, MOR-CHO. Accordingly, we conclude that the inverse agonism of SR141716 is CB_1 receptor-independent. While the specific MOR agonist DAMGO saturably and concentration-dependently stimulated [^{35}S]GTPγS binding, SR141716 (10 μM) inhibited the basal activity by 25% and competitively inhibited DAMGO stimulation in the mouse cortex. In MOR-CHO membranes, DAMGO caused a 501 ± 29% stimulation of the basal activity which was inhibited to 456 ± 22% by 10 μM SR141716. The inverse agonism of SR141716 was abolished, DAMGO alone displayed weak, naloxone-insensitive stimulation, whereas the combination of DAMGO + SR141716 (10 μM each) resulted in a 169 ± 22% stimulation of the basal activity (that was completely inhibited by the prototypic opioid antagonist naloxone) due to pertussis toxin (PTX) treatment to uncouple MORs from G_i/G_o proteins. Importantly, SR141716 proved to directly bind to MORs with low affinity (IC_{50} = 5.7 μM). These results suggest the emergence of novel, PTX-insensitive G-protein signaling that is blocked by naloxone when MORs are activated by of the combination DAMGO + SR141716.

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G-protein-coupled receptors (GPCRs), the largest class of cell-surface receptors, are one of the major targets for many current and emerging drugs. In the absence of agonists, many GPCRs exhibit spontaneous activity, which can be selectively blocked by ligands that are referred to as inverse agonists (for a review, see Milligan, 2003).

Cannabinoid CB_1 receptors are the most abundant GPCRs in the brain, with levels ten-fold higher than those of other GPCRs. They are predominantly, but not exclusively, coupled to G_i/G_o GTP-binding proteins (Howlett, 1985; Felder et al., 1998). The CB_1 receptors display a significant level of constitutive activity, either when heterologously expressed in non-neuronal cells or in neurons where the CB_1 receptors are expressed naturally, and hence at lower concentrations (for a review, see Pertwee, 2005). The first highly selective CB_1 receptor antagonist, SR141716 (Rimonabant), has been shown to exert a plethora of pharmacological effects in a number of pathological conditions (Bifulco et al., 2007). These effects are mainly attributed to its antagonistic properties at the CB_1 receptors, although the evidence is increasing that it may also behave as an inverse agonist (for a review, see Pertwee, 2005). The involvement of receptor-mediated G-protein activity in the inverse agonist response is supported by reports that SR141716A inhibits [^{35}S]GTPγS binding in CB_1 receptor-transfected cell lines (MacLennan et al., 1998), neuronal cells and the brain (Breivogel et al., 2001; Sim-Selley et al., 2001; Cinar et al., 2006).

However, recent studies have revealed the existence of CB_1 receptor-independent actions of CB_1 inverse agonists. High concentrations of SR141716 caused inverse agonism in the CB_1 receptor knock-out (CB_1-KO) mouse brain, mediated by neither CB_1 nor the non-CB_1, non-CB_2 putative cannabinoid receptor type (Breivogel et al., 2001). It has been proposed that the inhibitory effect of SR141716 on the basal receptor activity might occur either via a non-receptor-mediated effect or by binding to a site other than the agonist binding site on the CB_1

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receptors, or by binding to GPCRs other than the CB_1 receptors, to which it binds with much lower affinity (Sim-Selley et al., 2001). Although there are data supporting these notions (Savienen et al., 2003; Dennis et al., 2008), the exact mechanism of inverse agonism by SR141716 has not yet been clarified.

The μ-opioid system shares several features with cannabinoids. The pattern of expression of the CB_1 receptors strongly overlaps with that of μ-opioid receptors (MORs) in certain CNS regions (Pickel et al., 2004). Both receptor families are predominantly coupled to $G_{i/o}$-proteins (Howlett, 1985; for a review, see Waldhoer et al., 2004). Several studies have revealed functional interactions of the CB_1 receptors and the MORs at the level of G-proteins in certain regions of the brain (Rios et al., 2006; Canals and Milligan, 2008). It has been reported that the constitutive activity of the CB_1 receptors regulates the function of co-expressed MORs (Canals and Milligan, 2008). Alterations in the constitutive activity of the MORs in response to prolonged opioid exposure have been suggested to contribute to the development of opioid tolerance and/or dependence (Liu and Prather, 2001).

The aim of our study was to assess the inverse agonist effect of SR141716 in systems containing distinct populations of receptors in order to determine whether its effect is CB_1 receptor-dependent, and if not, whether it is non-receptor-mediated, or occurs by binding to GPCRs other than the CB_1 receptor, for example to the closely related MOR. Hence, we have used tissues that a) contain both CB_1 receptors and MORs (wild-type (wt) mouse cerebral cortex); b) lack CB_1 receptors (CB_1-KO mouse cerebral cortex); c) lack both CB_1 receptors and MORs (parental Chinese hamster ovary, (CHO) cells); or d) contain a homogeneous population of over-expressed recombinant MORs (MOR-CHO cells). The ligand-stimulated [^{35}S]GTPγS functional assay, which relies on agonist-promoted GDP/GTP exchange occurring at the G-protein level within the receptor/G-protein complex, was used to explore the inverse agonist effects of

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SR141716 in the above systems. This is a sensitive test of inverse agonism because such ligands selectively block the basal [^{35}S]GTPγS activity assessed in the absence of agonists, thereby representing constitutive receptor activity. Our data show that the inverse agonist effect of SR141716 is CB_1 receptor-independent. Moreover, pertussis toxin (PTX)-insensitive novel opioid signaling was revealed by joint addition of the μ-agonist DAMGO and SR141716 to MOR-CHO membranes, pretreated with PTX.

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Materials and Methods

Guanosine-5'-*O*-(3-[^{35}S]thio)triphosphate [^{35}S]GTPγS (37–42 TBq/mmol) was purchased from the Isotope Institute Ltd. (Budapest, Hungary). [^{3}H]Tyr-Gly-(NMe)PHe-Gly-ol ([^{3}H]DAMGO) (36 Ci/mmol) was synthetized in the Isotope Laboratory of the Biological Research Center (Szeged, Hungary). *R*(+)-[2,3-Dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl]-(1-naphthalenyl) methanone mesylate (Win55,212-2), (5a)-4,5-epoxy-3,14-dihydro-17-(2-propenyl)morphinan-6-one hydrochloride (naloxone hydrochloride), and (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran (O-2050) were from Tocris (Ellisville, MO, USA). Tris(hydroxymethyl)aminomethane (Tris, free base), sodium chloride (NaCl), ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), guanosine 5′-diphosphate sodium salt (GDP), guanosine 5′-[γ-thio]triphosphate tetralithium salt (GTP-γ-S-Li_4), magnesium chloride hexahydrate ($MgCl_2x6H_2O$), and bovine serum albumin (BSA - essentially fatty acid-free) were from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent was from Bio-Rad Laboratories (Hercules, CA, USA). Unlabeled DAMGO was from Bachem AG (Bubendorf, Switzerland). SR141716 was dissolved in ethanol; Win55,212-2 and O-2050 were dissolved in DMSO as 10 mM stock solutions and stored at -20 °C.

Cell culture and treatment

CHO cells stably transfected with the MORs (MOR-CHO) were cultured as previously described (Szücs et al., 2004). Briefly, the cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose with L-glutamine (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Nova-Tech Inc., Grand Island, NE, USA), 1% penicillin/streptomycin (GIBCO,

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Carlsbad, CA, USA) and 3.6% geneticin (GIBCO, Carlsbad, CA, USA). Cells were grown at 37 ^{0}C in a humidified atmosphere of 10% CO_2, 90% air. One set of cells were treated with 100 ng/ml PTX (List Biological Labs., Inc., Campbell, CA, USA) for the last 24 h in culture. At the end of PTX exposure, the cells were washed twice with ice-cold phosphate-buffered saline (PBS). The cells were harvested with PBS containing 1 mM EDTA. Cell suspension was spun at 2,500 rpm for 5 min, after which preparation of the cell membranes commenced.

Cell membrane preparation

Freshly collected cell pellets were homogenized with a Wheaton teflon-glass homogenizer in 10 vols (v/w) of ice-cold homogenization buffer, pH 7.4, composed of 25 mM HEPES, 1 mM EDTA, 0.5 mg/l aprotinin, 1 mM benzamidine, 100 mg/l bacitracin, 3.2 mg/l leupeptin, 3.2 mg/l soybean trypsin inhibitor and 10% sucrose as reported earlier (Szücs et al., 2004). Homogenates were spun at 1,000 x g for 10 min at 4 ^{o}C, and the supernatant was collected. Pellets were suspended in half of the original volumes of the homogenization buffer and centrifuged as above. Combined supernatants from the two low-speed centrifugations were spun at 20,000 x g for 30 min. The cell pellets were taken up in appropriate volumes of homogenization buffer. Aliquots were stored at -80 ^{o}C until use. Protein content was determined with the Bradford assay (Bradford, 1976), with BSA as a standard.

Cortex membrane preparations

Male mice (CD1) were handled in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv., Section 32). Mutant mice with the CB_1 receptor gene deleted (CB_1-KO) were generated as previously described (Ledent et al., 1999). The animals were housed in a

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temperature- and light-controlled room. Lighting was ensured in a 12-h cycle, and food and water were available *ad libitum.* The animals were decapitated, their brains were removed and the cortex was dissected on ice. The tissues were washed with ice-cold buffer and their weights were measured. They were homogenized in 30 volumes (v/w) of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with 5 strokes in a teflon-pestle Braun homogenizer at 1500 U/min. Homogenates were centrifuged at 20,000 x g for 25 min, and the resulting pellets were suspended in buffer and spun again. The pellets were taken up in the original volume of buffer and incubated for 30 min at 37 ^{0}C, followed by centrifugation at 20,000 x g for 25 min. The supernatants were carefully discarded, and the final pellets were taken up in 5 volumes (v/w) of 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose. Appropriate membrane aliquots were stored at –80 ^{0}C for several weeks. The protein content of the membrane preparation was determined by the method of Bradford (Bradford, 1976), BSA being used as a standard.

Ligand-stimulated [^{35}S]GTPγS binding

The assay was performed as described earlier (Cinar et al., 2008), except that in preliminary experiments the concentration of GDP was optimized at 3 and 30 µM for MOR-CHO cell and mouse cortex membranes, respectively. The highest concentrations of the solvents (0.1% ethanol or DMSO) tested in preliminary experiments had no effect on the basal activity in the assay. Briefly, membrane fractions (10 µg of protein) were incubated with 0.05 nM [^{35}S]GTPγS and appropriate concentrations of ligands in TEM buffer (50 mM Tris-HCl, 1 mM EGTA and 3 mM $MgCl_2$, pH 7.4) containing 100 mM NaCl, 0.1% (w/v) BSA, and an appropriate concentration of GDP, in a total volume of 1 ml, for 60 min at 30 °C. Nonspecific binding was determined with 10 µM GTPγS and subtracted to yield specific binding values. Bound and free [^{35}S]GTPγS were

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separated by vacuum filtration through Whatman GF/F filters with a Brandel Cell Harvester (Gaithersburg, MD, USA). Filters were washed with 3 × 5 ml of ice-cold buffer, and the radioactivity of the dried filters was detected in a toluene-based scintillation cocktail in a Wallac 1409 scintillation counter (Wallac, Turku, Finland).

Receptor binding assays

Heterologous displacement assays were performed with a constant concentration (1 nM) of [^{3}H]DAMGO (spec. activity 36 Ci/mmol), 11 concentrations (10^{-10}-10^{-5} M) of unlabeled Win55,212-2 or SR141716 and the membrane suspension (10 μg protein) in 50 mM Tris-HCl pH 7.4 buffer containing 0.1% (w/v) BSA in a final volume of 1 ml. Nonspecific binding was defined as the radioactivity bound in the presence of 10 μM unlabeled naloxone, and was subtracted from the total binding to obtain the specific binding. The tubes were incubated at 25 °C for 1 h. The reaction was stopped by vacuum filtration through Whatman GF/C glass fiber filters (Whatman, Maidstone, England), using a Brandel M24-R Cell Harvester (Brandel, Gaithersburg, MD, USA). Filters were rapidly washed with 3 x 5 ml of ice-cold 50 mM Tris-HCl pH 7.4 buffer, air-dried and counted in a toluene-based scintillation cocktail in a Wallac 1409 scintillation counter (Wallac, Turku, Finland). All assays were performed in duplicate and repeated at least three times.

Data analysis

Data were fitted with the GraphPad Prism 4.0 software (GraphPad Prism Software Inc., San Diego, CA, USA), using nonlinear regression and sigmoidal curve fitting to obtain potency (ED_{50}: the ligand concentration that elicits the half-maximal effect) and efficacy (E_{max}: maximal effect) values. Basal activities were measured in the absence of receptor ligands and defined as

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0% in each experiment unless otherwise indicated. All data are expressed as percentages of the basal [^{35}S]GTPγS binding and are the means ± S.E.M. of the result of at least three independent experiments performed in triplicate. IC_{50} (the concentration of ligand required to achieve 50% inhibition) values were obtained from the radioligand displacement curves. All receptor binding data are expressed as percentages inhibition of specific binding and are the means ± S.E.M. of the result of at least three independent experiments performed in duplicate. Statistical analysis was performed with GraphPad Prism, using ANOVA or Student`s t-test analysis. Significance was accepted at $p < 0.05$ the level.

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RESULTS

Effects of SR141716 on cannabinoid receptors in wt and CB_1–KO mouse cortical membranes

The potency and efficacy of prototypic cannabinoid receptor ligands on G-protein signaling were measured in ligand-stimulated [^{35}S]GTPγS binding assays. The CB_1 receptor, CB_2 receptor agonist Win55,212-2 significantly stimulated [^{35}S]GTPγS incorporation with a potency of 505 ± 138 nM and efficacy of 230 ± 9% in the wt mouse cortical membranes (Fig. 1A). It was noteworthy that, although low concentrations of Win55,212-2 did not exert significant effects in the CB_1-KO mouse cortex, 10 μM of the agonist stimulated [^{35}S]GTPγS binding by 38 ± 5% (Fig. 1C).

O-2050 has been described as a CB_1 receptor neutral antagonist (for a review, see Pertwee, 2005). In accordance with this, O-2050 *per se* did not exhibit any effect on the basal [^{35}S]GTPγS binding: no inverse agonist feature of this ligand at the concentrations tested was observed in either the wt or the CB_1-KO mouse cortical membranes (Fig. 1B, D). In contrast, the stimulation induced by Win55,212-2 (10 μM) was concentration-dependently antagonized by O-2050 in the wt cortex (Fig.1B). The Win55,212-2 stimulation was not antagonized by O-2050 in CB_1-KO membranes, and thus it may not be mediated via the CB_1 receptors (Fig. 1D).

SR141716 dose-dependently inhibited the basal activity, achieving statistically significant inhibition at <1 μM in both the wt and the CB_1-KO membranes (Fig. 1). However, the SR141716-induced inverse agonistic effects were not reversed by O-2050 in either the wt (Fig. 1B) or the CB_1-KO (Fig. 1D) membranes, although the effect of an inverse agonist should be blocked by its respective neutral antagonist. SR141716 fully antagonized the effect of Win55,212-2 stimulation in the wt cortex, and inhibited the basal and the Win55,212-2-stimulated (most likely non-CB_1-receptor–mediated) effects to the same extent in the CB_1-KO

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membranes (Fig. 1A, C). These results suggest that SR141716 displays a CB_1 receptor-independent inverse agonist feature in the mouse cortex.

Effects of SR141716 on MORs in wt and CB_1–KO mouse cortical membranes

We next checked the hypothesis that the inverse agonist effect of SR141716 may be manifested at GPCRs other than the CB_1 receptors, e.g. the closely related MORs. The highly specific MOR agonist DAMGO saturably and concentration-dependently stimulated [^{35}S]GTPγS binding with a potency of ~270 nM (log EC50 = -6.5 ± 0.17) and the efficacy of 80 ± 4% (Fig. 2A). In combination with 10 μM SR141716 (which completely blocked the Win55,212-2 stimulation of the CB_1 receptor), the basal activity was inhibited by about 25% and the DAMGO dose-response curve was shifted to the right. In order to reflect the net effect of SR141716 on the MOR signaling, we expressed the data by defining the [^{35}S]GTPγS binding in the presence of 10 μM SR141716 *per se* as 0% (Fig. 2C). Combination of 10 μM SR141716 with various concentrations of DAMGO significantly ($p < 0.05$) changed the potency of DAMGO, resulting in a log EC_{50} value of -5.8 ± 0.07. The efficacy of DAMGO was not changed by the presence of 10 μM SR141716 (Fig. 2C). Overall, therefore, this indicates that SR141716 acts competitively on MORs in mouse cortex. It should be noted that deletion of the CB_1 receptors did not influence the stimulation of [^{35}S]GTPγS by DAMGO in the absence and presence of SR141716 (Fig. 2B), further supporting the notion that the inhibitory effect of SR141716 seems to be CB_1 receptor-independent.

Effects of SR141716 on MORs in MOR-CHO cell membranes

The mouse brain contains a heterogeneous mixture of receptors, where receptor-receptor interactions (cross-talk, hetero-oligomerization, etc.) might occur. Accordingly, it was of interest

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to examine the mechanism of action of SR141716 by using a cell line that contains a homogeneous population of MORs at high density. With saturating concentrations of the ligands either alone or in appropriate concentrations, it was found that 10 μM DAMGO resulted in a 501 ± 29% stimulation, which was reduced to 86 ± 7% by the prototypic opioid antagonist naloxone (10 μM), indicating that the effect was mediated via the MORs in the MOR-CHO cell membranes (Fig. 3A). SR141716 (10 μM) slightly, but significantly ($p < 0.05$) reduced the basal [^{35}S]GTPγS activity (Fig. 3A). Moreover, SR141716 slightly inhibited the effect of DAMGO, resulting in 456 ± 22% of the basal [^{35}S]GTPγS binding; however, this level was not significantly different from that for DAMGO alone. The combination of SR141716 and naloxone displayed the same inhibition of the DAMGO effect as that of naloxone itself (Fig. 3A).

Previous reports have demonstrated that PTX-sensitive G-proteins participate in the SR141716-induced inhibition of G-protein activity (Glass and Northup, 1999; Sim-Selley et al., 2001; Savinainen et al., 2003). We therefore, pretreated the cells with PTX to uncouple the receptors from the $G_{i/o}$-proteins. SR141716 did not have any significant effect on the basal G-protein signaling in the PTX-treated MOR-CHO (Fig. 3B). Likewise, DAMGO exhibited only a small, naloxone-insensitive effect (~30%) on the [^{35}S]GTPγS binding in the PTX-treated MOR-CHO cell membranes, as expected, since the MORs are predominantly, but not exclusively, coupled to $G_{i/o}$-proteins (Childers, 1991; Szücs et al., 2004; Chakrabarti et al., 2005). The combination of DAMGO and SR141716 (10 μM each) led to a significant ($p < 0.05$) 169 ± 22% stimulation of the G-protein signaling when the MORs were uncoupled from $G_{i/o}$-proteins by PTX (Fig. 3B). This novel signaling was totally blocked by naloxone, indicating that it occurs via the MORs (Fig. 3B).

We examined the concentration-dependence of the above effects of SR141716 with a view to a better understanding of the underlying mechanism (Fig. 4). SR141716 dose-

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dependently, saturably and significantly ($p < 0.05$) reduced the basal [^{35}S]GTPγS activity, with a potency of 6 ± 0.4 µM, achieving a maximal inhibition of about 25% at 100 µM in the MOR-CHO membranes. PTX treatment completely eliminated the inverse agonist effect of SR141716 (Fig. 4A).

In order to determine the net effect of SR141716 on MOR signaling, we expressed the data measured in the joint presence of varying concentrations of SR141716 and a fixed concentration of DAMGO by defining the binding in the presence of 10 µM DAMGO *per se* as 0%. All other data were expressed as percentage stimulation over the normalized basal activity (Fig. 4B). SR141716 displayed a slight tendency in a concentration-dependent manner to inhibit the effect of 10 µM DAMGO in MOR-CHO membranes, but this did not reach the level of statistical significance (Fig. 4B). PTX treatment resulted in a major effect on the intrinsic efficacy of SR141716; SR141716 in the presence of 10 µM DAMGO induced concentration-dependent, significant PTX-insensitive G-protein activation, with a potency of about 3 µM, which reached 118 ± 10% over the 'normalized basal activity' (Fig. 4B).

The inverse agonism of SR141716 persists in parental CHO cell membranes

We also tested another possible explanation for the observed nonspecific inverse agonist effect of SR141716, i.e. that its effect is non-receptor-mediated (Sim-Selley et al., 2001). Neither DAMGO (10 µM) nor Win55,212-2 (10 µM) had any significant effect on G-protein activation in the parental CHO membranes, indicating that neither the MORs nor the CB receptors are endogenously expressed in this cell line (Fig. 5). It is important that SR141716 (10 µM) still decreased the basal G-protein activity by 20 ± 2% in parental CHO cell membranes (Fig. 5). SR141716 (10 µM) combined with a high concentration of DAMGO, either in the absence or in

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the presence of naloxone, did not significantly change the inhibitory effect of SR141716 *per se* (Fig. 5), further supporting the notion that MORs are not present in these cells. These results confirm the hypothesis that the inverse agonist effect of SR141716 is CB_1 receptor-independent, and possibly even non-receptor-mediated.

SR141716 interacts directly with [^{3}H]DAMGO-binding sites in MOR-CHO cell membranes

The possibility of SR141716 binding with low affinity to GPCR other than the CB_1 receptors has been proposed (Sim-Selley et al., 2001). Using radioligand competition binding assays in MOR-CHO cell membranes, we tested whether SR141716 is able to bind directly to the MORs. Increasing concentrations (10^{-9}-10^{-5} M) of SR141716 and Win55,212-2 were used against 1 nM [^{3}H]DAMGO, and the inhibition of its specific binding was detected. Although the CB_1/CB_2 agonist Win55,212-2 had no effect, SR141716 almost fully displaced [^{3}H]DAMGO, with an IC_{50} of 5.7 μM (Fig. 6). This result suggested that SR141716 may bind directly to the MORs, albeit with low affinity. It should be noted that the inverse agonist effect of SR141716 is also manifested at low (micromolar) concentrations.

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Discussion

The results of the present study indicate that the CB_1 receptor-specific antagonist SR141716 at micromolar concentrations inhibited basal [^{35}S]GTPγS binding, i.e. it behaved as an inverse agonist in the systems tested. This observation agrees with previous results indicating that SR141716 displays inverse agonism with a low potency of about 5 μM (Breivogel et al., 2001; Sim-Selley et al., 2001), while its potency in antagonizing CB_1 receptor-mediated G-protein activation is ~0.5 nM (Sim-Selley et al., 2001).

Further, we have provided evidence that the inverse agonist effect of SR141716 may not be mediated via the CB_1 receptors. This evidence is based on the observation that the extents of SR141716 inhibition of the basal activities were very similar in the wt mouse cortex (containing various kinds of GPCRs including cannabinoid receptors and MORs), MOR-CHO membranes (expressing MORs), and their counterparts lacking CB_1 receptors, i.e. CB_1-KO cortex and parental CHO membranes (also lacking endogenous MORs). Win55,212-2 is a nonselective agonist that binds to CB_1, CB_2 and the non-CB_1, non-CB_2 putative cannabinoid receptors (for a review, see Begg et al., 2005). Since neither the effects of SR141716 nor those of Win55,212-2 were antagonized by the neutral CB_1 antagonist O-2050 in the CB_1-KO mouse cortex (Fig. 1D), it might be postulated that the observed effects could be mediated via the CB_2 or the putative CB receptors. However, these possibilities are unlikely to explain inverse agonism by SR141716. As we have shown, while the inverse agonist effect of SR141716 persisted, Win55,212-2 had no significant effect in the parental CHO cell membranes, indicating that CHO cells do not contain significant levels of endogenous CB_1, CB_2 or the putative CB receptors (Fig. 5).

The inhibitory effect of SR141716 on the G-protein signaling in the parental CHO cell membranes raises the possibilities that SR141716 may act in a non-receptor-mediated fashion (Dennis et al., 2008), e.g. via a direct membrane effect, by changing the membrane fluidity

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(Bloom et al., 1997). However, this is unlikely as SR141716 inhibited the basal [^{35}S]GTPγS binding in a concentration-dependent and saturable manner, which implies receptor-mediated action, as we have demonstrated here (Fig. 4A) and as reported by others (Breivogel et al., 2001; Sim-Selley et al., 2001).

It has also been proposed that SR141716 may act as an inverse agonist at GPCRs other than the CB_1 receptors, binding to these GPCRs with much lower affinity (Sim-Selley et al., 2001). A growing number of orphan GPCRs have been reported to be activated by lipid ligands, such as cannabinoids etc. (Yin et al., 2009). SR141716 behaves as an agonist at the recently discovered GPR55 cannabinoid receptors (Henstridge et al., 2009), and as an antagonist at non-CB_1/non-CB_2 endothelial and CNS cannabinoid receptors (for a rewiew, see Begg et al., 2005). It has been reported that a high concentration of SR141716 exhibits competitive antagonism on the adenosine receptors in the rat brain (Savinainen et al., 2003). Besides the effects of SR141716 on certain GPCRs, it also displays antagonism on TRPV1 (Gibson et al., 2008).

Our data have revealed that SR141716 also influences MOR signaling. SR141716 competitively inhibited, and slightly decreased the DAMGO signaling in the wt and the CB_1-KO mouse cortex (Fig. 2) and MOR-CHO (Figs. 3-4) membranes, respectively. Importantly, SR141716 binds directly to MORs, albeit with a low affinity of 5.7 μM (Fig. 6). SR141716 shares a piperidine ring and aromatic structures with opioid ligands such as loperamide and fentanyl (Kane et al., 2006; di Bosco et al., 2008). Since the piperidine ring is important for binding to the MORs, this may give rise to the direct binding of SR141716 to the MORs at high concentrations.

PTX treatment fully abolished the inhibitory effect of SR141716 on the basal G-protein activity (Fig. 4A). This confirms that the CB_1-independent inverse agonism of SR141716 is mediated via PTX-sensitive G_i/G_o-proteins. It was intriguing that the combination of DAMGO and SR141716 (10 μM each) led to a significant ($p < 0.05$) 169 ± 22% stimulation of the G-

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protein signaling when the MORs were uncoupled from $G_{i/o}$-proteins by PTX in MOR-CHO cells (Fig. 3B). This novel signaling was totally blocked by naloxone, indicating that it occurs via the MORs (Fig. 3B). Similar results were observed when the full agonist DAMGO was replaced by the partial agonist morphine. Combinations of 10 μM morphine and varying concentrations of SR141716 also unmasked a PTX-insensitive novel opioid signaling pathway in PTX-treated MOR-CHO membranes (data not shown). It may be envisaged that the binding of SR141716 and DAMGO to the MORs may induce a conformational change in the receptors, allowing them to interact more readily with PTX-insensitive G-proteins (e.g. G_s, G_z, G_q or G_{12}, etc). The MORs may have higher affinity for the inhibitory G-proteins than for others. Consequently, when these interactions are inactivated by PTX, the stimulatory component may be manifested. In accordance with this observation, it has been reported that the inactivation of G_i/G_o-proteins by PTX unmasks the ability of DAMGO to stimulate adenylyl cyclase activity, which is in contrast with the inhibition observed without PTX treatment in MOR-CHO cells (Szücs et al., 2004). Increasing evidence indicates that a single receptor type may be linked to the formation of multiple, simultaneous intracellular pathways. Chronic morphine treatment caused desensitization of this novel signaling (data not shown). Further studies are required to reveal the G-protein type(s) that participate in this novel MORs-mediated signaling.

The physiological relevance of these phenomena is open to question in light of the high concentration of SR141716 needed. After the chronic administration of clinically relevant doses, the concentration of SR141716 in human blood plasma is estimated to be 190 nM (Ken Mackie, personal communication). However, due to its inverse agonist activity under physiological conditions, it was shown that SR141716 induces nausea, emesis and mood depression (Sink et al., 2007). Thus, both *in vitro* and *in vivo* data indicate that the antagonist versus the apparent inverse agonist effects of SR141716 in the brain can be differentiated on the basis of potency (Sim-Selley

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et al., 2001). A variety of human diseases have been ascribed to the constitutive activity of GPCRs (Spiegel, 1996), which may lead to the use of inverse agonists as efficient tools in new therapeutic strategies with which to combat such human disorders.

In summary, we have demonstrated that the inverse agonist effect of the potent CB_1 receptor antagonist SR141716, which is manifested at micromolar concentrations, is CB_1 receptor-independent. Moreover, it may bind directly to the MORs and inhibit their signaling in mouse cortex and MOR-CHO membranes. Novel, PTX-insensitive opioid signaling was unmasked in the joint presence of DAMGO and SR141716 in MOR-CHO membranes pretreated with PTX to uncouple the MORs from the G_i/G_o-proteins. It is concluded that SR141716 exerts a multitude of effects on receptor-mediated G-protein signaling, which should be taken into account during its use.

Acknowledgments

We thank Dr. Alan Gintzler (Brooklyn, NY, USA) for providing MOR-CHO cells, Dr. Ken Mackie (Indianapolis, IN, USA) for the kindly gift of SR141716, Dr. Catherine Ledent (Brussels, Belgium) for providing CB_1 receptor KO mice and Drs. Tamás Freund and István Katona (Budapest, Hungary) for valuable discussions. The technical assistance of Ildikó Németh, and the contribution of Erika Birkás are greatly acknowledged.

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Legends for Figures

Figure 1. CB_1 receptor-independent inverse agonism of SR141716 in mouse cortical membranes. Dose-response curves of SR141716 (X) and Win55,212-2, either alone (●) or in the presence of 10 μM SR141716 (Δ) in membranes from the wt (A) or CB_1-KO (C) mouse cortex. Effects of O-2050 in the absence (■) or in the presence of Win55,212-2 (10 μM, ▲) or SR141716 (10 μM, ○) in membranes from the wt (B) or CB_1-KO (D) mouse cortex. The data are expressed as percentages of the basal activity, binding in the absence of ligands being defined as 0%. Means ± S.E.M., n = 3, all performed in triplicate. The non-visible S.E.M. is within the symbol. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant effects of the ligands on the basal activity, ^{x} indicates significant antagonistic effects of O-2050 on the appropriate ligands, $^\#$ indicates significant inhibition of the Win55,212-2 stimulation by SR141716.

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Figure 2. CB_1 receptor-independent inhibition of μ-opioid signaling by SR141716. Dose-response curves of DAMGO either alone (■) or in the presence of 10 μM SR141716 (▲) in membranes from the wt (A) or CB_1-KO (B) mouse cortex. The data are expressed as percentages of the basal activity, binding in the absence of ligands being defined as 0%. In order to depict the net effect of SR141716 on the μ-opioid signaling, the data are re-plotted and expressed as percentages of the 'normalized basal activity', binding in the presence of 10 μM SR141716 being defined as 0%, in membranes from the wt (C) or CB_1-KO (D) mouse cortex. Means ± S.E.M., n = 3, all performed in triplicate. The non-visible S.E.M. is within the symbol. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant effects of the ligands on the basal activity.

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Figure 3. PTX-insensitive opioid signaling is unmasked by the joint application of DAMGO and SR141716 in PTX-treated MOR-CHO membranes. Each ligand was used at 10 µM, either alone or in combination as shown for the MOR-CHO (A) or PTX-treated MOR-CHO (B) membranes. The data are the means ± S.E.M. of the results of at least three independent experiments, all performed in triplicate and expressed as percentages of the basal activity, binding in the absence of ligands being defined as 0%. Statistical analysis was performed with two-way ANOVA tests followed by Bonferroni post hoc comparisons. * denotes significant effects of the ligands on the basal activity. # denotes significant changes of the DAMGO stimulation by SR141716, x indicates significant antagonism of the agonist effects by naloxone.

Figure 4. Net effects of SR141716 on basal and DAMGO-stimulated G-protein activity in MOR-CHO membranes. A) Dose-response curve of SR141716 in either MOR-CHO (□) or PTX-treated MOR-CHO (x) membranes. B) In another set, we assessed dose-response curves demonstrating the effects of SR141716 on [^{35}S]GTPγS binding stimulated by 10 µM DAMGO in MOR-CHO membranes without (□) or with PTX treatment (x) and expressed as percentages of the 'normalized basal activity', binding in the presence of 10 µM DAMGO being defined as 0%. The data are means ± S.E.M., n = 3, all performed in triplicate. The non-visible S.E.M. is within the symbol. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant effects of SR141716 on the basal activity. # indicates significant changes of the DAMGO stimulation by varying concentrations of SR141716.

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Figure 5. SR141716 inhibits basal G-protein activity in parental CHO membranes. Each ligand was used at 10 µM, either alone or in appropriate combination as shown. The data are means ± S.E.M., n = 3, all performed in triplicate. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant inhibition of the basal activity by SR141716. The presence of DAMGO in the absence or in the presence of naloxone had no significant effect on the inhibitory effect of SR141716.

Figure 6. Competition of the CB_1 receptor inverse agonist SR141716 and the CB_1 agonist Win55,212-2 for the binding sites of $[^3H]$DAMGO. MOR-CHO cell membranes (10 µg) were incubated with the radioligand (1 nM) in the presence of increasing concentrations of SR141716 (○) or Win55,212-2 (X). The nonspecific binding was measured with 10 µM naloxone and subtracted. Specific binding in the absence of competitors, corresponding to 2286 ± 56 fmol x (mg protein)$^{-1}$, was defined as 100%. Data are expressed as percentages of the specific binding. The data are means ± S.E.M., n = 3, all performed in duplicate. Statistical analysis was performed with one-way ANOVA tests combined with Bonferroni post hoc comparisons. * denotes significant inhibition of specific $[^3H]$DAMGO binding by ligands.

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Figure 1

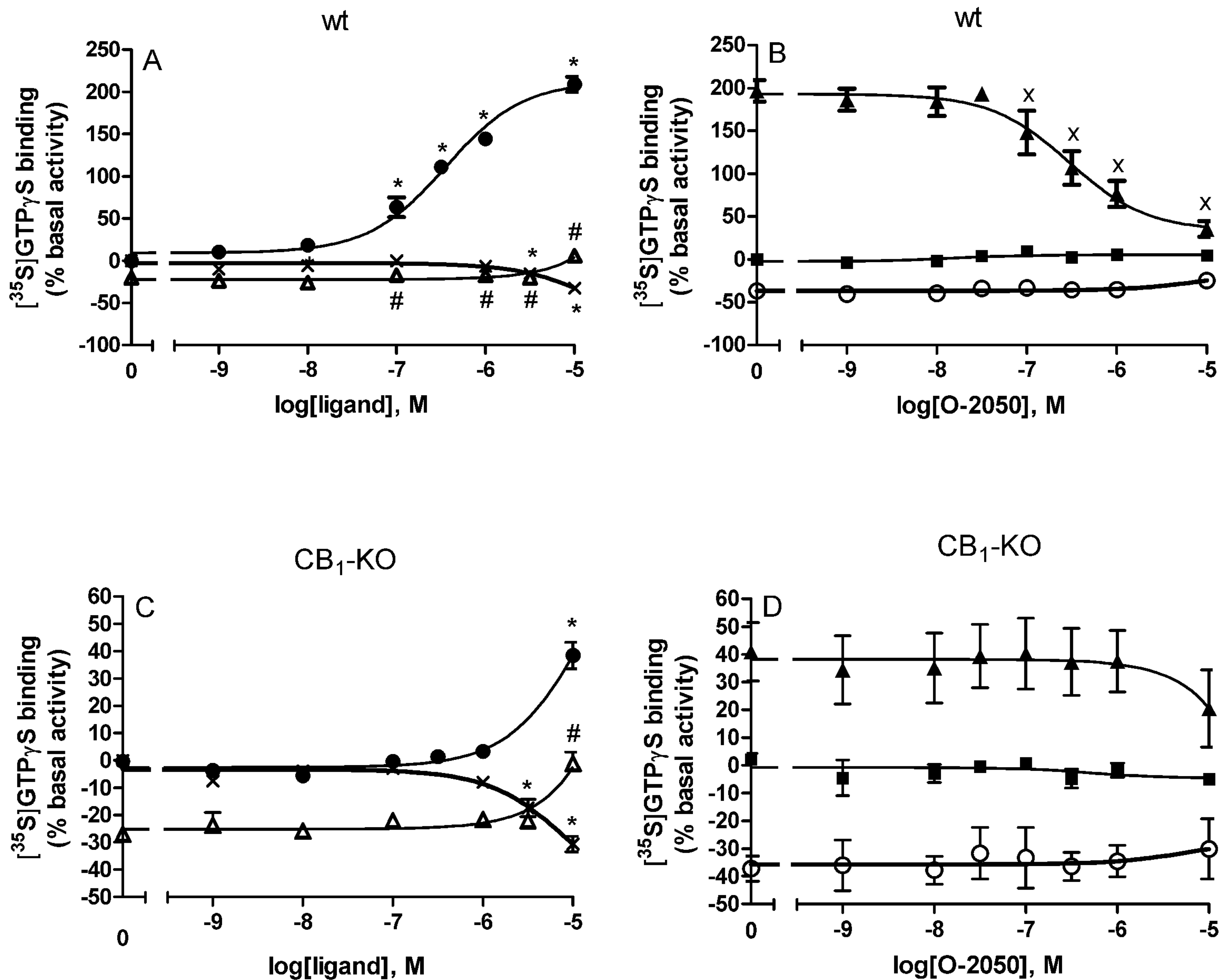

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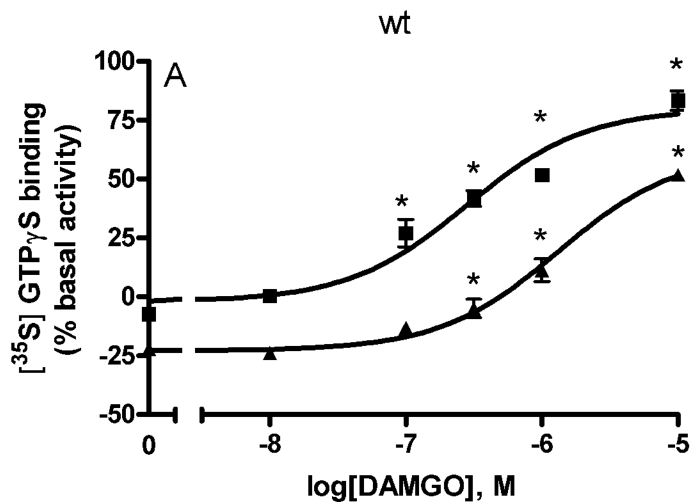
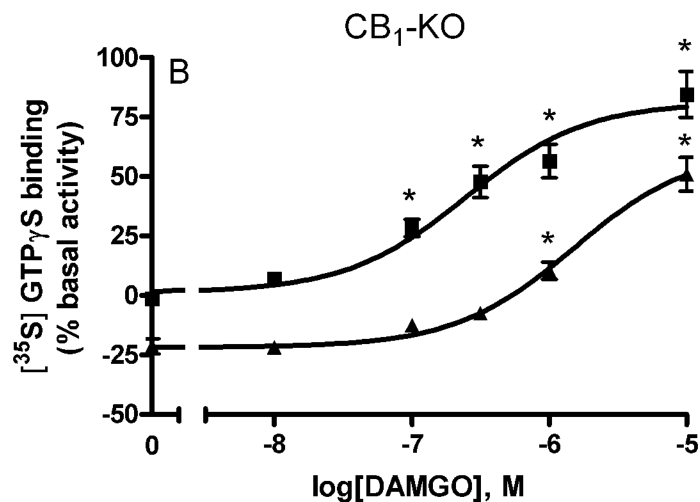
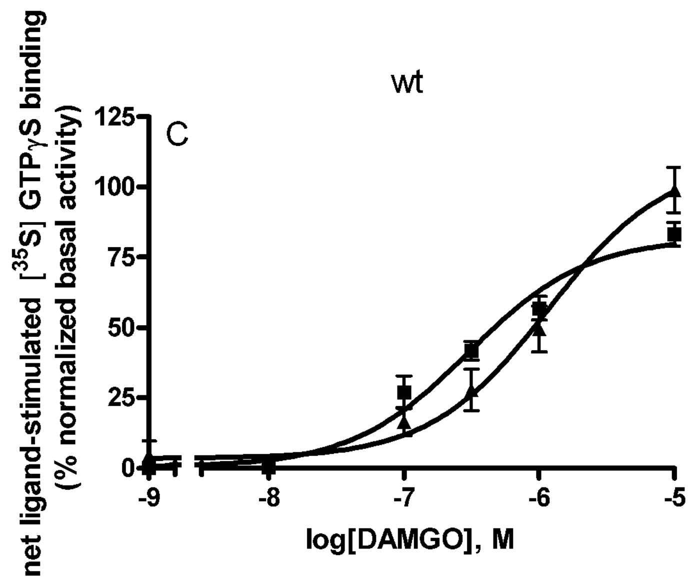
Figure 2







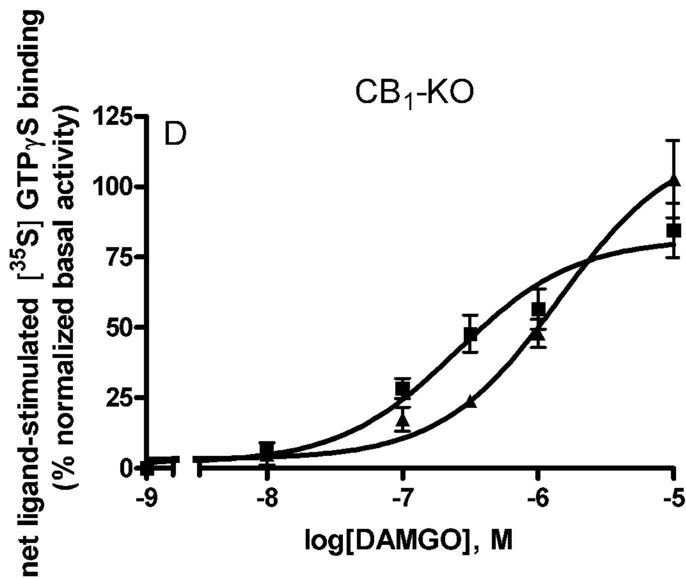


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Figure 3

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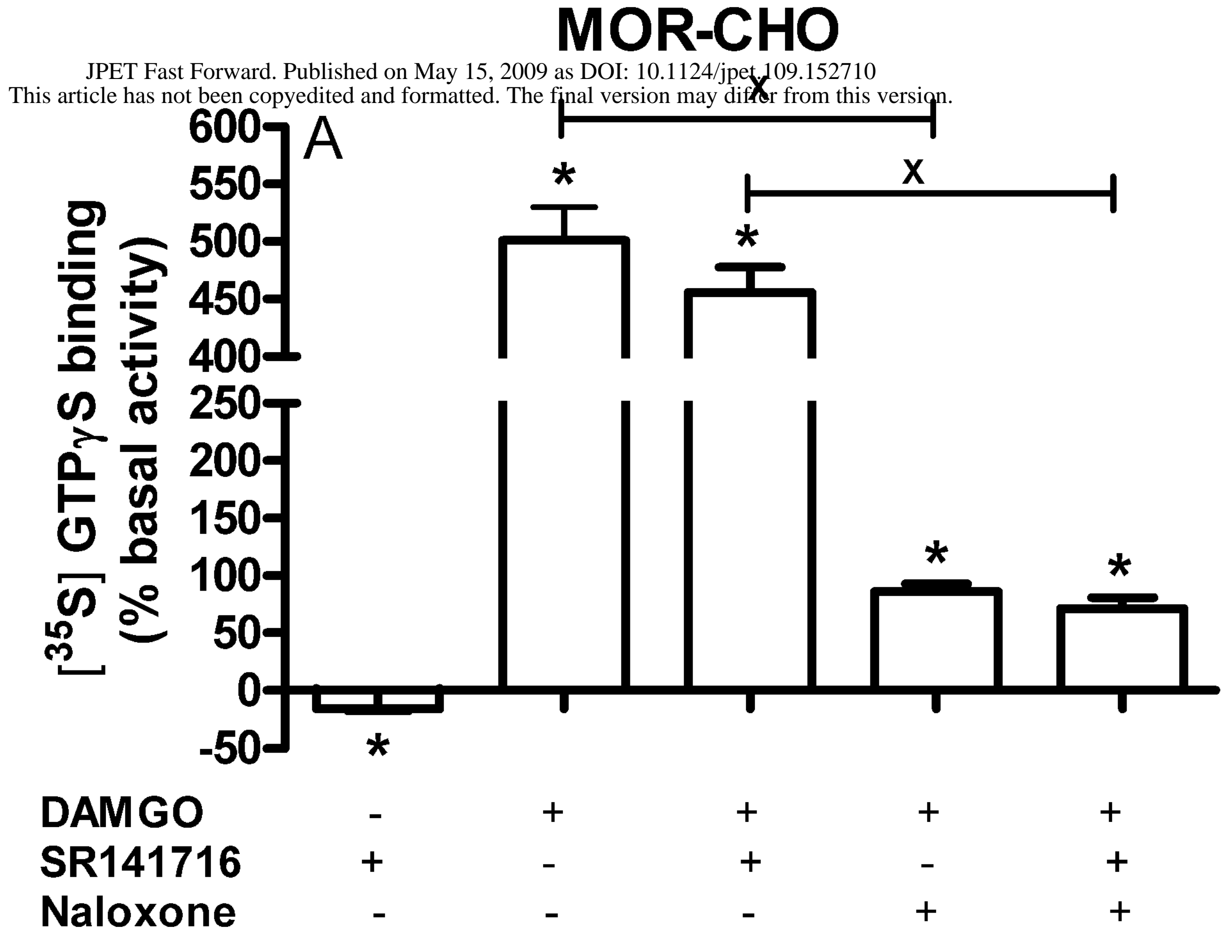

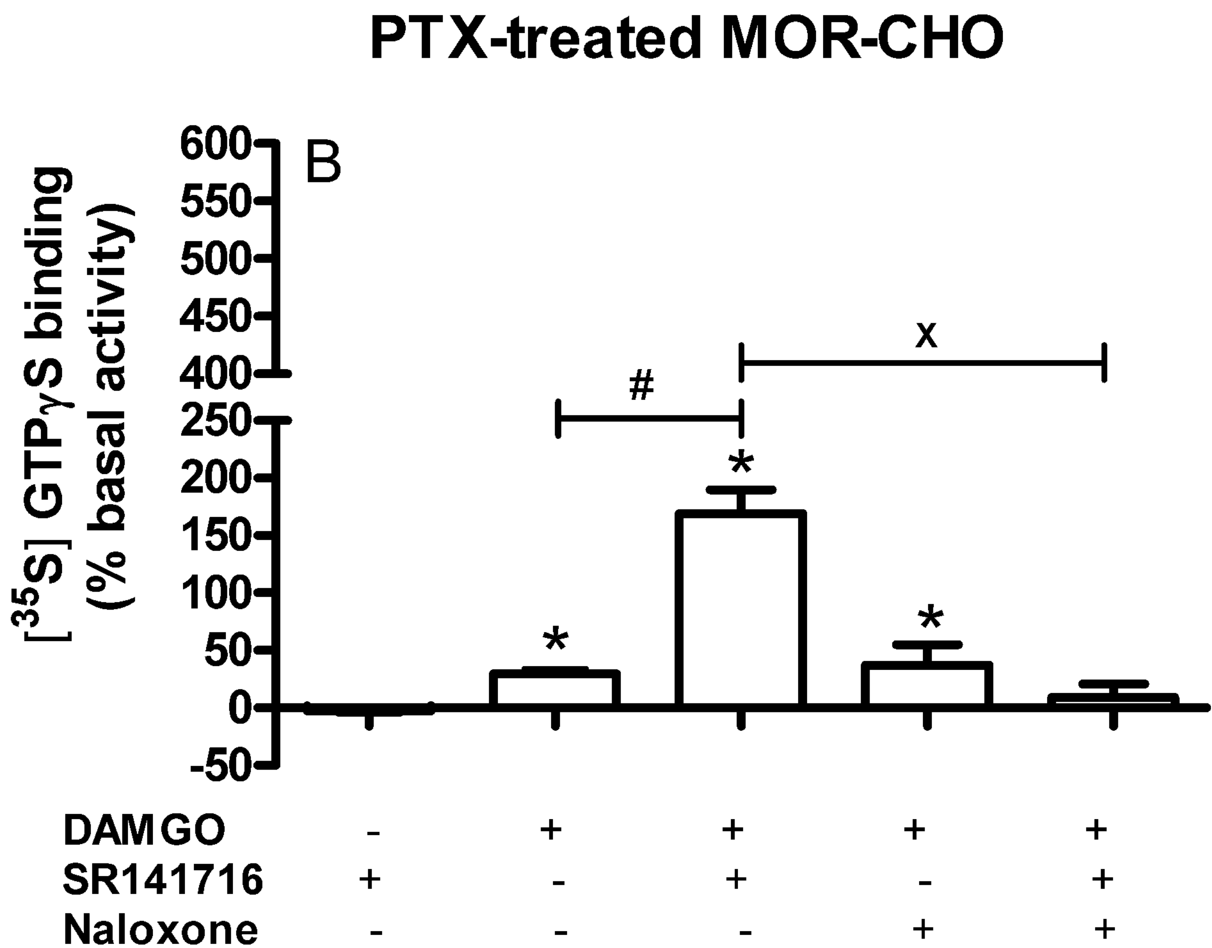

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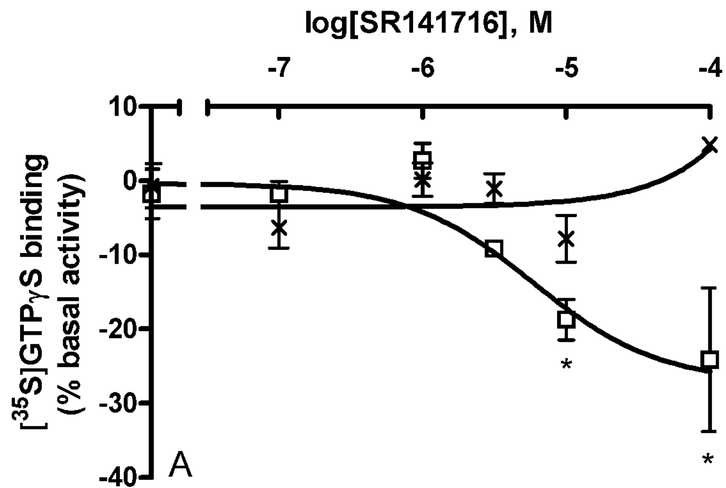
Figure 4



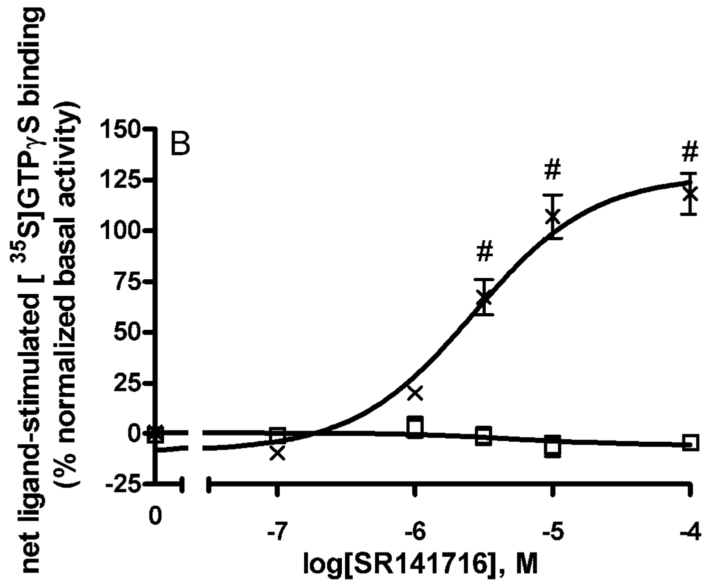


Figure 5

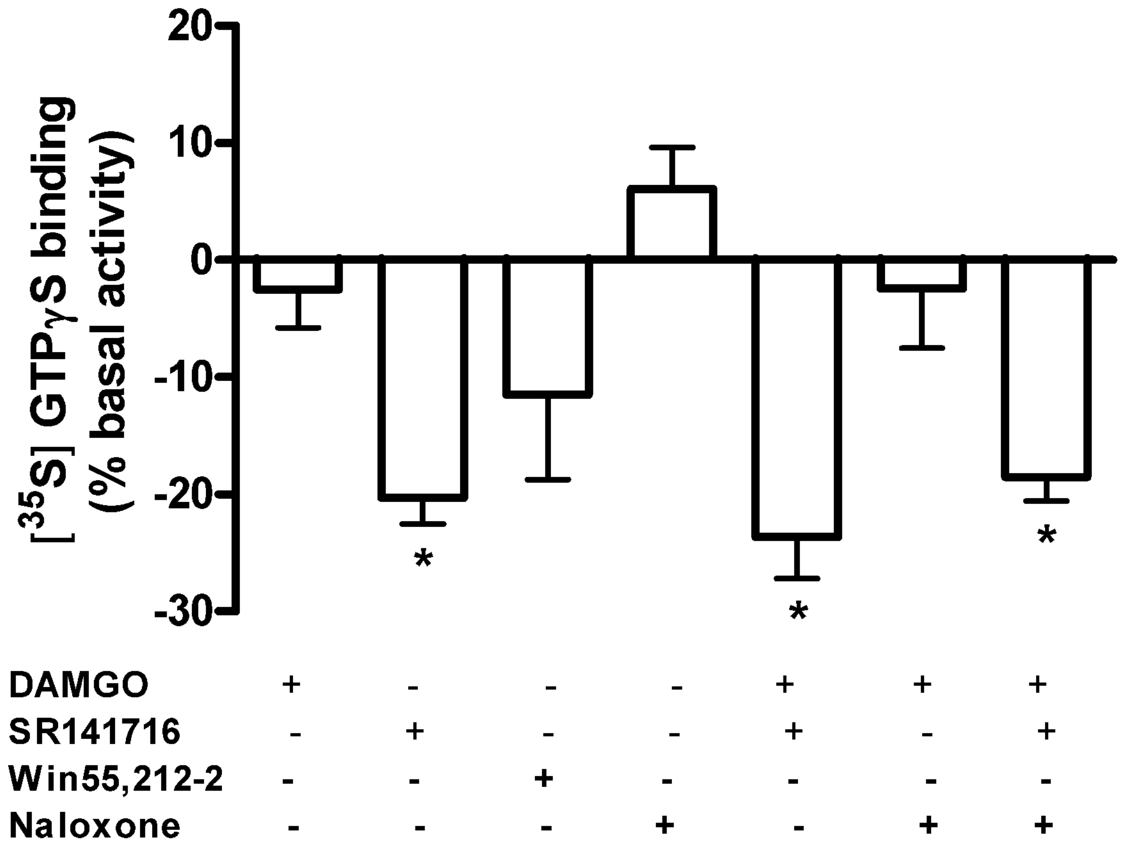


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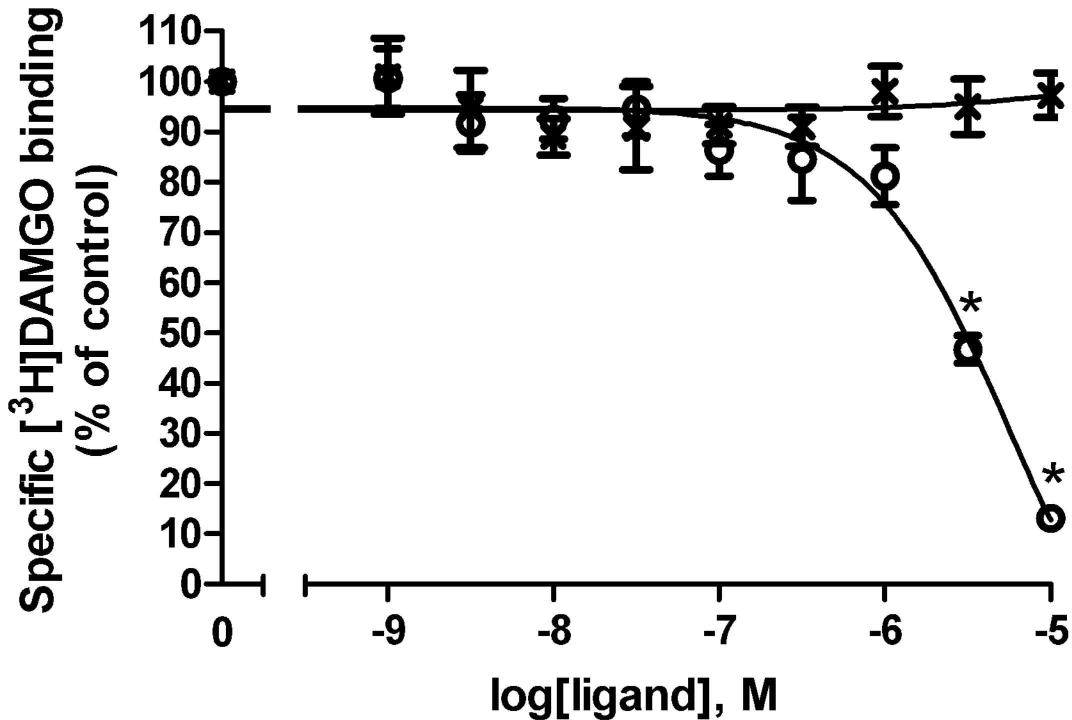
Figure 6



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