Biased Agonism of Three Different Cannabinoid Receptor Agonists in Mouse Brain Cortex

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Cannabinoid receptors are able to couple to different families of G proteins when activated by an agonist drug. It has been suggested that different intracellular responses may be activated depending on the ligand. The goal of the present study was to characterize the pattern of G protein subunit stimulation triggered by three different cannabinoid ligands, Δ⁹-THC, WIN55212-2, and ACEA in mouse brain cortex. Stimulation of the [³⁵S]GTPγS binding coupled to specific immunoprecipitation with antibodies against different subtypes of G proteins (Gαi1, Gαq2, Gαq3, Gαo, Gαz, Gαs, Gαq/11, and Gα12/13) in the presence of Δ⁹-THC, WIN55212-2 and ACEA (submaximal concentration 10 μM) was determined by scintillation proximity assay (SPA) technique in mouse cortex of wild type, CB₁ knock-out, CB₂ knock-out and CB₁/CB₂ double knock-out mice. Results show that, in mouse brain cortex, cannabinoid agonists are able to significantly stimulate not only the classical inhibitory Gαi/o subunits but also other G subunits like Gαz, Gαq/11, and Gα12/13. Moreover, the specific pattern of G protein subunit activation is different depending on the ligand. In conclusion, our results demonstrate that, in mice brain native tissue, different exogenous cannabinoid ligands are able to selectively activate different inhibitory and non-inhibitory Gα protein subtypes, through the activation of CB₁ and/or CB₂ receptors. Results of the present study may help to understand the specific molecular pathways involved in the pharmacological effects of cannabinoid-derived drugs.

Keywords: CB₁ receptor, CB₂ receptor, functional selectivity, scintillation proximity assay (SPA), G proteins, brain

INTRODUCTION

During the last decade a wide number of studies have focused on the potential involvement of the endocannabinoid system in a variety of psychiatric and neurological disorders. The putative psychoactive ingredient of Cannabis sativa (marijuana plant), Δ⁹-tetrahydrocannabinol (Δ⁹-THC), as well as the endogenous cannabinoids anandamide (arachidonoyl ethanolamide) and
2-arachidonoylglycerol (2-AG) act primarily through cannabinoid CB₁ and CB₂ receptors. These cannabinoid receptors are GPCRs mostly coupled to Gα/o proteins (Howlett et al., 2002). The CB₁ receptor is mainly distributed in the CNS, particularly in cortex, basal ganglia, hippocampus, and cerebellum (Mackie, 2005; De Jesus et al., 2006) and generally acts presynaptically inhibiting the release of neurotransmitters. CB₂ receptors are expressed at much lower levels in the CNS compared with CB₁ receptors (reviewed in Atwood and Mackie, 2010). As Gα/o coupled GPCRs, CB₁ and CB₂ receptors inhibit adenylyl cyclase, but moreover, both receptors are able to activate MAPK, inhibit voltage gated Ca²⁺ channels and activate inwardly rectifying K⁺ channels (Childers et al., 1993).

The activation of CB₁ receptor in the brain leads to the modulation of neuronal excitability, which may be in part responsible of the psychoactive effects of exogenous cannabinoids. In this context, a considerable amount of studies have been performed in order to elucidate the effects of cannabinoids (natural or synthetics) in the development of mental alterations, such as addiction, cognitive deficits, anxiety or psychosis. Importantly, different or opposite behavioral effects have been observed after the administration of Δ⁹-THC or synthetic cannabinoid ligands (Fattore et al., 2003; Panagis et al., 2014; Rubino and Parolaro, 2016). It has been demonstrated that for most G protein-coupled receptors, distinct agonists can differentially regulate several signaling pathways through the same receptor by a selective activation of different intracellular effectors. This is a mechanism known as functional selectivity or biased agonism. In this way, cannabinoid receptors have been demonstrated to be capable of coupling to different families of G proteins and/or to beta-arrestin when activated by an agonist drug suggesting that different intracellular responses may be activated depending on the ligand (Glass and Northup, 1999; Bosier et al., 2010). For instance, for the CB₁ receptor has been reported that, whereas 2-AG and WIN55,212 have little preference for inhibition of cAMP and phosphorylation of ERK1/2, anandamide and CP55940 were biased toward cAMP inhibition (Khajehali et al., 2015). Moreover, in a recent study Dhopeshwarkar and Mackie (2016) demonstrated that CB₂ receptor ligands display strong and varied functional selectivity at canonical (inhibition of adenylyl cyclase) and non-canonical (arrestin recruitment) pathways. Moreover, the intracellular signaling activated by a receptor depends on the cellular system where it is expressed, which may vary across different neuronal environments. In this context, it has been demonstrated that opioid and cannabinoid receptors function through the same pool of G proteins when they are co-transfected, whereas in cells endogenously expressing these receptors signaling occurs through distinct pools of G proteins (Shapira et al., 2000). Thus, this fact should be taken into consideration when interpreting results acquired in artificially transfected cells vs. native biological systems.

To our knowledge, no study has compared G protein signaling by different cannabinoid drugs in native brain tissue. Thus, in the current study, we performed [³⁵S]GTPγS scintillation proximity assay (SPAs) coupled with the use of specific antibodies against different Gα protein subunits to evaluate the functional selectivity of different cannabinoid ligands by activating CB₁ and/or CB₂ cannabinoid receptors in mouse brain cortex.

**MATERIALS AND METHODS**

**Animal Procedures**

Adult C57BL/6J (WT), CB₁ knock-out (CB₁⁻/⁻) (Marsicano et al., 2002), CB₂ knock-out (CB₂⁻/⁻) (Buckley et al., 2000), and CB₁/CB₂ double knock-out (CB₁⁻/⁻/CB₂⁻/⁻) mice were used in this study. Animals (males, aged 7–8 weeks-old) were housed (6–8 animals per cage) in standard cages under controlled conditions of temperature (23 ± 1°C) and photoperiod (light/dark cycle 14 h: 10 h) and free access to standard rodent chow and water.

**Animal Welfare and Ethical Statements**

All experimental procedures using mice were performed in accordance with the European Directive for the Protection of Vertebrate Animals used for experimental and Other Scientific Purposes (European Union Directive #86/606/EEC) and approved by the Ethics Committees for Animal Welfare of the University of the Basque Country (UPV/EHU) permit number CEBA1882011 and by the Institutional Review Board (INIA), permit number CEEA2012/021.

**Rationale for Choice of Cannabinoid Ligands**

In the present study, we decided to investigate the effects of three different cannabinoid ligands. Δ⁹-THC was chosen for being the main psychoactive component of marijuana plant and the putative responsible of the development of mental disorders in humans. WIN55212-2, a synthetic cannabinoid structurally different from Δ⁹-THC, is a potent, non-selective CB₁/CB₂ receptor agonist that is frequently used in the studies that try to elucidate the effects of Cannabis in the brain. Finally, we wanted to study a ligand structurally similar to endogenous cannabinoids, such as the synthetic anandamide analog arachidonyl-2-chloroethylamide (ACEA). O-2050 was chosen as a neutral cannabinoid antagonist. O-2050 has been proved to be a neutral CB₁ receptor antagonist in several studies (Canals and Milligan, 2008; Hudson et al., 2010; Brents et al., 2011; Wiley et al., 2011), with quite similar affinity for CB₁ and CB₂ receptors. Although, there is some data in the literature suggesting its activity as inverse or even partial agonist at CB₁ receptors in various tissues (Makwana et al., 2010; Wiley et al., 2011) in a previous work of our group, we showed that O-2050 has no effect over [³⁵S]GTPγS binding and behaves as an antagonist blocking WIN55212-2-mediated activation (Erdozain et al., 2012) (Figure 1).

**Rationale for Choice of G Protein α Subunit Subtypes**

In the present study, we decided to investigate the ability of cannabinoid receptors to activate different G proteins subtype. We chose at least one G protein subtype representative of each
main G protein family and mainly focusing in the inhibitory G proteins for being the cannabinoid canonical pathway (Figure 2).

Mouse Brain Cortex Membrane Homogenates Preparation

After sacrifice by cervical dislocation, the brains were rapidly removed, cortices dissected and fresh frozen, and stored immediately at −80°C until use. Preparation of membrane enriched fraction (P2 fraction) was performed as previously described (Gonzalez-Maeso et al., 2000) with minor modifications. Mouse brain cortex samples (approximately 200 mg) from seven mice each time were thawed at 4°C and homogenized with a glass/tetlon grinder (IKA labortechnik, Satufen, Germany) (10 strokes at maximum speed) in 30 volumes of homogenization buffer (50 mM Tris-HCl (Invitrogen, Barcelona, Spain), 1 mM EGTA (Sigma-Aldrich, St. Louis, MO, USA), 3 mM MgCl₂ (Sigma-Aldrich, St. Louis, MO, USA) and 1 mM DTT (Invitrogen, Barcelona, Spain); pH 7.4; supplemented with 250 mM sucrose (Panreac Quimica S.A.U, Barcelona, Spain). The homogenates were centrifuged at 1,100 × g for 10 min at 4°C (Sorvall RC-5C centrifuge, SM-24 rotor; FisherScientific, Madrid, Spain). The pellets (P1 fraction) were discarded and the supernatants were then recentrifuged at 40,000 × g for 10 min (4°C). The resultant pellets were resuspended in 20 volumes of fresh cold centrifugation buffer (50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl₂ and 1 mM DTT; pH 7.4) with a glass stick and recentrifuged at 40,000 × g for 10 min (4°C). The obtained pellets were then resuspended in five volumes of centrifugation buffer. Protein content was determined by the method of Bradford with BSA (Sigma-Aldrich®, St. Louis, MO, USA) as standard. Linear regression analysis and extrapolation of the data were carried out with GraphPad Prism 5th software (GraphPad Software, Inc., San Diego, CA, USA). Finally, aliquots of 0.5, 1, and 2 mg were then centrifuged at 21,000 × g (Eppendorf 5810R centrifuge; Eppendorf, Madrid, Spain) during 15 min at 4°C. The supernatant layer was carefully discarded and the pellets stored at −80°C until assay.

Antibody-Capture [³⁵S]GTPγS Scintillation Proximity Assay (SPA)

Specific activation of different subtypes of Gα proteins was determined using a homogeneous protocol of [³⁵S]GTPγS SPA coupled with the use of specific antibodies essentially as previously described (Erdozain et al., 2012). [³⁵S]GTPγS binding was performed in 96-well Isoplates (PerkinElmer Life Sciences, Maanstraat, Germany) and in a final volume of 200 ml containing 1 mM EGTA, 3 mM MgCl₂, 100 mM NaCl, 0.2 mM DTT, 50 mM Tris-HCl at pH 7.4, 0.4 nM [³⁵S]GTPγS, 15 µg of protein per well, and different concentrations of GDP depending on the Gα subunit subtype tested. At the end of the 2 h incubation period (30°C), 20 µl of lgepal 1% + SDS 0.1% were added to each well, and plates were incubated at 22°C for 30 min with gentle agitation. Specific antibody for the Gα subunit of interest was then added to each well before an additional 90 min RT incubation period (the antibodies and dilutions employed are described in Table 1). Polyvinyldene fluoride (PVT) SPA beads coated with protein A (PerkinElmer S.L., Tres Cantos, Madrid, Spain) were then added (0.75 mg of beads per well), and plates were incubated for 3 h at RT with gentle agitation. Finally, plates were centrifuged (5 min at 1000 × g), and bound radioactivity was detected on a MicroBeta TriLux scintillation counter (PerkinElmer S.L., Tres Cantos, Madrid, Spain). In order to test their effect on the [³⁵S]GTPγS binding to the different Gα subunit subtypes, a single submaximal concentration of the drugs (10 µM) Δ⁹-THC, WIN55212-2, ACEA and/or O-2050, was used. This submaximal concentration was chosen as previously reported (Erdozain et al., 2012) in our previous experimental assays in which we established the

![Chemical structure of the different cannabinoid ligands used](image-url)
standard conditions for this assays. This concentration is the one which give us binding values around the Emx for any drug and subunit subtype combination studied (Supplementary Figure S1). Non-specific binding was defined as the remaining \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding in the presence of 10 \text{µM} unlabelled \text{GTP} \gamma \text{S}.

**Western Blot**

For Western blot experiments, membrane enriched fraction (P2 fraction) pellets from mouse brain tissue (cortex) were resuspended in TBS, reaching a concentration of 4 \text{mg} protein/ml. Commercial Laemmli 2x (95%) and \(\beta\)-mercaptoethanol (5%) (Sigma-Aldrich\textsuperscript{a}, St. Louis, MO, USA) were added to each sample, reaching a final protein concentration of 2 \text{mg/ml}. Finally, all the samples were heated at 95\(^\circ\)C for 5 min in a Thermoblock (Biometra, Goettingen, Germany) and kept at -20\(^\circ\)C until assay. Electrophoresis was carried out in SDS polyacrylamide gels, composed of 5% stacking (0.5 \text{M} Tris-HCl, pH 6.8, 10% SDS) and 12% resolving (1.5 \text{M} Tris-HCl, pH 8.8, 10% SDS) gels,
using a miniprotein system (Bio-Rad Laboratories). Equal protein loading in the gel was verified by simultaneous immunodetection of β-actin (mouse monoclonal antibody anti-β-actin, Sigma Biosciences, St. Louis, MO, USA) with the different Gα subunit subtypes. Proteins were then transferred to nitrocellulose membranes (1 h, 0.3 A) using an electrophoretic transfer system (Bio-Rad Laboratories). The non-specific binding sites in the membranes were blocked for 1 h at RT in blocking solution (3% non-fat dry milk, pH = 7.4 in PBS). Membranes were incubated overnight at 4°C in incubation buffer (3% non-fat dry milk + 0.1% Tween-20 in PBS) containing the appropriate dilution of the specific primary anti-Gα subunit antibody. Antibody specificity, as previously described in the literature (Gettys et al., 1994; Valdizan et al., 2010), was confirmed in our experimental conditions by Western blot (data not shown). Membranes were washed with PBS and incubated for 1 h at RT and constant agitation with the fluorescent conjugated secondary antibodies (Alexa Fluor® 680 and/or IRDye 800 conjugated antibodies) suitable diluted in incubation buffer. Finally, membranes were re-washed with PBS and immunoreactivity was detected and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and Odyssey Software. Broad-Range pre-stained SDS-PAGE molecular weight standard (Bio-Rad Laboratories, Hercules, CA, USA) was used.

Data Analysis and Statistical Procedures

Data were analyzed with GraphPad Prism™ 5.01 software. In order to allow better interpretation of the data, specific binding obtained from [35S]GTPyS SPAs were transformed to percentage of basal binding (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. Immunodensity data obtained from Western blotting assays were transformed to percentage of the control, being the control the mean of immunodensities obtained for WT mice. The statistical comparison of the Western blot results was carried out by a one-way ANOVA test, followed by Dunnet’s post hoc test for multiple comparisons, with a significance level of p < 0.05. All data are expressed as mean ± SEM values.

Materials

[35S]GTPyS (4625 × 10^10 Bq/mmol) was purchased from PerkinElmer Life Sciences (Maastrict, Germany). Tetrahydrocannabinol (Δ9-THC) was purchased from THCParm GmbH (Frankfurt, Germany); WIN55212-2 and GTPyS were purchased from Sigma-Aldrich (St. Louis, MO, USA); Arachidonyl-2-chloroethylamide ACEA and O-2050 were from Tocris Bioscience (Bristol, UK). All other chemical reagents were of analytical quality and were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA).

RESULTS

Effects of Δ9-THC, WIN55212-2, and ACEA on G Protein Activation in Mouse Brain Membranes

Cannabinoid receptor ligands were used for the characterization of the functional coupling of cannabinoid receptors to the different G protein α subunit subtypes (Gαq1, Gαq2, Gαq3, Gαq6, Gαo, Gαi1, Gαi2, Gαi3, and Gαi1213) in mouse brain tissue. First, we investigated which Gα subunit subtypes were activated by the natural cannabinoid Δ9-THC (10 μM) in mouse brain cortex membrane homogenates (Figure 3A). As expected, we found that Δ9-THC was able to significantly activate several classical AC inhibitory subunits, as Gαi1 (113 ± 3%), Gαq (110 ± 2%), and Gα12 (115 ± 5%), while exerted no effect on Gα12 or Gαi3. Δ9-THC was also able to activate the Gαi11 subunit (118 ± 5%). However, no changes were observed when we studied AC stimulatory subunit Gαq and the RhoA activator Gα1213 subunit. To further test if these effects of Δ9-THC were cannabinoid-receptor mediated, the same assays were carried out in the presence of a putative neutral antagonist of the CB1 receptor, O-2050. In all cases, the activation of these G protein subunits was blocked when membranes were co-incubated with the cannabinoid antagonist O-2050. Next, we investigated the effects on G protein subunit activation induced by the synthetic cannabinoid agonist WIN55212-2 (Figure 3B). We found that WIN55212-2 significantly increased the binding of [35S]GTPyS to the all the inhibitory subunits Gαi1 (129 ± 6%), Gαi3 (129 ± 5%), Gαq (120 ± 4%) and Gα12 (134 ± 6%), except Gα12. WIN55212-2 was also able to activate the Gαi11 subunit (131 ± 7%), but not the AC stimulatory subunit Gαq. Surprisingly, WIN55212-2 was also able to significantly stimulate the RhoA activator Gα1213 (130 ± 4%). In the same way as previously described for Δ9-THC, the activation of these G protein subunits by WIN55212-2 was always blocked by the co-incubation with the cannabinoid antagonist O-2050, except for the case of Gα12 (106 ± 1%). Finally, we investigated the effect of the synthetic anandamide analog ACEA on G protein subunit activation in mouse brain tissue (Figure 3C). When evaluating the classical AC inhibitory subunits, we found that ACEA stimulated Gαi1 (121 ± 4%), Gαi3 (120 ± 5%), and Gαq (116 ± 4%). However, as occurred with Δ9-THC and WIN55212-2, no stimulation was observed in Gα12, suggesting that none of the cannabinoids evaluated exert their effects through Gα12 signaling. Moreover, ACEA had no effect on Gαq. As previously observed for the other two cannabinoid ligands evaluated, ACEA also activated Gαi11 subunit (122 ± 7%) while had no effect on Gα12. Thus, it seems that none of these cannabinoi ligands are able to activate this AC stimulatory subunit either. No changes were observed when we studied the effects of ACEA on the RhoA activator Gα1213 subunit. Again, the activation of these G protein subunits was blocked when membranes were co-incubated with the cannabinoid antagonist O-2050.
FIGURE 3 | Effects of THC, WIN55212-2, and ACEA on G protein activation in mouse brain membranes. [$^{35}$S]GTPγS scintillation proximity assays coupled to immunoprecipitation with specific antibodies against different Ga subunits (Ga11, Ga12, Ga13, Ga0, Ga2, Ga3, Ga12/11, and Ga12/13) in mouse brain cortical membranes co-incubated with (A) THC (10 µM), (B) WIN55212-2 (10 µM), or (C) ACEA (10 µM) in the presence or absence of the antagonist O-2050 (10 µM). Data are shown as percentage of [$^{35}$S]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 normalized values of stimulation or inhibition of basal binding which are statistically different from 100% (Student’s t-test; *p < 0.05).

Effects of the Cannabinoid Antagonist O-2050 on G Protein Activation in Mouse Brain Membrane Homogenates

O-2050 was initially synthesized and described as a neutral CB1 receptor antagonist, however, there are some evidences suggesting that is able to act as an inverse agonist or even as a partial agonist (Wiley et al., 2011). For this reason, and in order to validate O-2050 as a useful pharmacological tool to antagonize the effect mediated by cannabinoid receptors, [$^{35}$S]GTPγS SPAs were performed in mouse cortical membranes in the presence of O-2050 (10 µM) alone. Under these experimental conditions, neither stimulation nor inhibition of [$^{35}$S]GTPγS basal binding values were observed for any of the Ga subunit subtypes studied, with the exception of Ga2 (119 ± 1%) (Figure 4).
Effects of Δ⁹-THC, WIN55212-2, and ACEA on G Protein Activation in Cannabinoid Receptors Knockout Mice

To further elucidate the role of each cannabinoid receptor subtype in the agonist-mediated activation of the different Gα subunit subtypes, [35S]GTPγS SPA was performed in brain tissue of CB1−/−, CB2−/−, and CB1−/−/CB2−/− mice. For that purpose, brain membranes were incubated with the different cannabinoid ligands (THC, WIN55212-2, or ACEA) and with the specific antibodies against Gα for which an stimulation with these agonists was observed in WT. Figure 5A shows the stimulation of the different Gα subunits when brain membranes of the four genotypes were incubated with Δ⁹-THC. The significant stimulation of the inhibitory Gα11 subunit observed in the WT mice was completely absent in the CB1−/− and in the CB1/CB2 double ko mice, but was still present in the CB2−/− (108 ± 2\%), which suggests that the Δ⁹-THC-mediated stimulation of Gα11 is induced by the activation of CB1 receptor. On the other hand, opposite results were obtained for Gαi2 and Gαi3, the other two inhibitory subunits that were stimulated by Δ⁹-THC. As previously described, there was a significant Δ⁹-THC-induced stimulation of Gαi2 and Gαi3 subunits in the WT. This stimulation was also observed in the CB1−/− (115 ± 4\% for Gαi2 and 120 ± 4\% for Gαi3) but not in the CB2−/− or the CB1−/−/CB2−/− mice. These data may indicate that, in mouse brain cortical membranes, Δ⁹-THC acts through the CB2 receptor to stimulate these inhibitory Gαi2 and Gαi3 subunits. Finally, the Δ⁹-THC-induced activation of the Gαq/11 subunit observed in the WT mice was not found in the CB1−/− and CB1−/−/CB2−/− mice, while remained unchanged in the CB2−/− (116 ± 2\%). This result indicates that Δ⁹-THC stimulates the Gαq/11 subunit acting mainly through the CB1 receptor.

Figure 5B shows the stimulation of different Gα subunits when brain membranes of the four genotypes were incubated with the synthetic cannabinoid agonist WIN55212-2. The significant stimulation of the inhibitory Gα11 and Gα13 subunits induced by WIN55212-2 in the WT mice was not found in the CB1−/− nor in the CB1−/−/CB2−/− mice, but was still present in the CB2−/− (124 ± 5\% for Gα11 and 123 ± 5\% for Gα13). On the contrary, the inhibitory subunits Gαq and Gαz, which were significantly stimulated in the WT mice, remained stimulated in the CB1−/− (111 ± 2\% for Gαq and 123 ± 7\% for Gαz) but not stimulation was found in the CB2−/− nor in the CB1−/−/CB2−/− mice. These results suggest that the inhibitory signaling of WIN55212-2 in the mouse brain through Gα13 and Gα13 activation seems to be mediated by the CB1 receptor, while the stimulation of Gαq and Gαz would be mediated by the CB2 receptor activation. The significant activation of the Gαq/11 subunit induced by WIN55212-2 in the WT mice was completely absent in the CB1−/− mice, as well as in the CB1/CB2 double ko mice. On the contrary, a significant stimulation of Gαq/11 subunit (120 ± 5\%) was observed in the CB2−/− membranes, suggesting that this stimulation is mediated by the CB1 receptor. Strikingly, the observed stimulation of the RhoA activator subunit Gα12/13 by WIN55212-2 in the WT disappeared in the absence of CB2 receptor (both in CB2−/− and CB1−/−/CB2−/− mice) suggesting an important role of this CB2 receptor in the intracellular signaling via Gα12/13 in the brain.

Finally, the same experiments were performed incubating the brain membranes with the synthetic anandamide analog ACEA (Figure 5C). Surprisingly, the significant stimulation of Gα11 that was observed in the WT mice was still found in all the genotypes evaluated. These results suggest that the inhibitory effect of ACEA mediated by the Gα11 subunit activation may be independent of cannabinoid receptors. On the other hand, the significant stimulation of Gα13 subunit induced by ACEA was not observed in brain membranes of CB1−/− mice but was still significant in CB2−/− membranes (111 ± 3\%). No stimulation was observed in the CB1−/−/CB2−/− mice. Regarding the Gαq subunit, there was a significant stimulation in the absence of CB1 receptors (115 ± 1\%), while this stimulation was not observed in the brain membranes of CB2−/− mice, suggesting the necessary role of this receptor in the activation of Gαq induced by the agonist ACEA. The activation of the Gαq/11 subunit was observed in both CB1−/− (117 ± 2\%) and CB2−/− (118 ± 4\%) but not in the CB1−/−/CB2−/− mice. Thus, as for Δ⁹-THC and WIN55212-2, the activation of Gα13 and Gαq subunits was mediated by their interaction with CB1 and CB2 receptors, respectively. However, the stimulation of [35S]GTPγS binding to Gαq/11 subunit seems to be triggered by the activation of both CB1 and CB2 cannabinoid receptors.
Expression of G Protein Subunits in Knockout Mice for Cannabinoid Receptors

In order to determine if a physiological adaptation of knockout mice to the genetic manipulation to inactivate CB1 and/or CB2 receptors may influence our results by the alteration of the expression level of the different Go protein subunits, Western blotting assays were carried out in brain cortex membranes of WT, CB1 knockout (CB1−/−), CB2 knockout (CB2−/−) and double CB1 and CB2 knockout (CB1−/−/CB2−/−/double KO) co-incubated with (A) THC (10 μM) (B) WIN55212-2 (10 μM), or (C) ACEA (10 μM). 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 normalized values of stimulation or inhibition of basal binding which are statistically different from 100% (Student’s t-test; *p < 0.05).

In the case of Ga11 (Figure 6A), no changes were observed for CB1−/− and CB2−/−, but a significant reduction (33 ± 10% from WT) of immunodensity was detected in CB1−/−/CB2−/− mice brain membrane homogenates. No significant differences were found in the expression of Ga0 (Figure 6C) or Ga12/13 (Figure 6F) subunits between the WT, CB1−/−, CB2−/−, and CB1−/−/CB2−/− mice brain membranes. However, an increase in the expression of Ga13 (Figure 6B) in both CB2−/− (139 ± 7%) and CB1−/−/CB2−/− mice (140 ± 7%) was found when compared to the WT and CB1−/− animals. On the contrary,
Figure 6 | Expression of G protein subunits in cannabinoid receptors knockout mice. Immunoreactive signal and representative images obtained by Western blotting with specific antibodies against different Gα subunits: (A) Gαi1, (B) Gαi3, (C) Gαo, (D) Gαz, (E) Gαq/11, and (F) Gα12/13 in mouse brain cortical membranes of WT, CB1 knockout (CB1−/−), CB2 knockout (CB2−/−) and double CB1 and CB2 knockout (CB1−/−/CB2−/−/double KO). Immunoreactivity for β-actin was simultaneously detected on every gel and used as loading control. Normalized values (percentage over controls) of Gα subunits are shown as mean ± SEM of two different experiments carried out in duplicate. Asterisks highlight those values which are statistically different from WT (One-way ANOVA followed by Dunnet’s post hoc test; *p < 0.05).

Goα2 immunodensity was significantly increased in the CB1−/− mice (129 ± 4%) while no changes were found in the rest of the genotypes when comparing to the WT (Figure 6D). Finally, the expression of Goαq/11 (Figure 6E) was significantly increased in brain membranes of CB2−/− mice (137 ± 7%) but not in WT, CB1−/− and CB1−/−/CB2−/− mice.

DISCUSSION

During the last years, a considerable effort has been made to study the effects of cannabinoids in the brain trying to elucidate the mechanisms by which these compounds may facilitate mental disorders, such as addiction, cognitive deficits, anxiety or psychosis. In this context, these studies have been performed with different cannabinoid ligands (natural or synthetics), in cells or in native tissue and/or in different animal species (mouse, rat, human...).

There is wide evidence supporting the idea that for most of GPCRs, distinct drugs are able to regulate different signaling pathways by the selective activation of different intracellular effectors. The pharmacological relevance of this fact is that the biological responses not only depend on targeting a specific GPCR but also on the particular pathway that this receptor activates. Different studies have focused on the evaluation of the functional selectivity of cannabinoid receptors, but most of them have been performed in transfected cells expressing the CB1 receptor (Glass and Northup, 1999; Bosier et al., 2010).

Moreover, much of these studies explore the signaling pathways activated by different agonists by the evaluation of cAMP production or the phosphorylation of intracellular mediators such as ERK or AKT, with no data about the Gα subtype responsible of these downstream effects. These changes on cAMP concentration or ERK/AKT phosphorylation could be the consequence of the activation of different Gα subtypes, Gβγ dimers, etc. On the other hand, and interestingly, opposite behavioral effects have been observed after the administration of Δ9-THC or synthetic cannabinoid ligands (Fattore et al., 2003; Panagis et al., 2014; Rubino and Parolaro, 2016). For example, when evaluating the cannabinoid effects on brain-stimulation reward, Fattore et al. (2003) showed that the potent non-selective CB1/CB2 receptor agonists WIN55,212-2 and CP 55,940, but not Δ8-THC, effectively restored heroin-seeking behavior. In addition, it has been suggested that the signaling of CB1 receptors may differ between humans and rodents (Straiker et al., 2012).

All these frequently contradictory data highlight the relevance of studying, simultaneously, the effects of different cannabinoid ligands in the same tissue and under the same experimental conditions.

For that reason the goal of the present study was to compare the pattern of G protein subunit stimulation triggered by three structurally different cannabinoids, Δ9-THC, WIN55212-2 and ACEA in mouse brain cortex. To our knowledge, this is the first study evaluating the cannabinoid-induced stimulation of the different Gα subunits in mouse brain tissue.
WIN55212-2, a synthetic cannabinoid structurally different from Δ⁹-THC, is a potent, non-selective CB₁/CB₂ receptor agonist that has been used in many studies of cannabinoid receptor function (Pertwee et al., 2010). The synthetic anandamide analog ACEA is a highly selective agonist for the CB₁ receptor with a low affinity for CB₂ receptors (Hillard et al., 1999).

This study demonstrates that each ligand displays functional selectivity acting as biased agonist for a subset of different G protein subunits. It represents the first characterization of the activation of individual Gα subunits by endogenous cannabinoid receptors in brain cortex. Firstly, we demonstrated that phytocannabinoid Δ⁹-THC differs from the synthetic agonists WIN55212-2 and ACEA in its ability to stimulate Gαᵢ₀ protein subunits in brain cortex.

The Gαᵢ subfamily members Gαᵢ₁, Gαᵢ₂, and Gαᵢ₃ were originally identified by their ability to inhibit AC activity (Plummer et al., 2012; Busnelli et al., 2013; Minetti et al., 2014). Our results show that Δ⁹-THC, WIN55212-2, and ACEA significantly stimulate Gαᵢ₁ subunit. Moreover, data from knockout mice suggest that this effect may be CB₁-mediated in the case of Δ⁹-THC and WIN55212-2. However, the Gαᵢ₁ stimulation is still significant in membranes of all genotypes incubated with ACEA, suggesting that this is a non-CB₁ non-CB₂ dependant effect and supporting putative actions of ACEA over other receptors (Pertwee et al., 2010). Gαᵢ₃ subunit was also stimulated in the presence of WIN55212-2 and ACEA, but not of Δ⁹-THC. This stimulation seems to be mediated by CB₁ receptors as is blocked in the presence of O-2050 and absent in CB₁⁻/⁻ or CB₁⁻/⁻/CB₂⁻/⁻ mice. In the case of Gαᵢ₂, it has been previously described that WIN55212-2 is able to activate this subunit in rat (Prather et al., 2000) and in human brain cortical membranes (Erdozain et al., 2012). However, none of the agonists in the present study stimulated the Gαᵢ₂ subunit. This discrepancy may be due to inter-species and/or regional differences, suggesting that WIN55212-2 may signal through different G protein pools in human and mouse brain cortex.

These three Gαᵢ subunits form the Gαᵢ₁₀ subfamily with the neuronal α₁-subunit Gα₁₅, which corresponds to the most abundant Gα protein in brain (Sternweis and Robishaw, 1984). In our experimental approach, and in accordance with other studies (Glass and Northup, 1999; Presley et al., 2016), Δ⁹-THC, WIN55212-2 and ACEA significantly stimulated Gα₁₅. Results obtained in knockout animals show that the stimulation of Gα₁₅ in mouse cortex is mediated, at least in part, by CB₂ receptors, suggesting a necessary role of this receptor in the cannabinoid-induced activation of Gα₁₅.

The Gα₁ subtype is the most divergent member of the inhibitory subfamily and is distributed primarily in neuronal and neuroendocrine cells (Hinton et al., 1999). While Δ⁹-THC and WIN55212-2, similarly, stimulated Gα₁₂, no stimulation of this subunit was observed when membranes were incubated with ACEA, suggesting that ACEA may not signal through this subunit. Additionally, results obtained with knockout mice suggest that the stimulation of Gα₁₂ by Δ⁹-THC and WIN55212-2 may be induced by a CB₂-mediated mechanism.

Unlike Gα₁₂, the Gα₁₅ family is ubiquitously expressed and couples receptors to AC in a stimulatory fashion (Milligan and Kostenis, 2006). Under the present assay conditions, nor Δ⁹-THC, WIN55212-2 or ACEA were able to activate this stimulatory subunit. Thus, there is no evidence of Gα₁₅ coupling of cannabinoid receptors in the presence of any of these drugs in brain tissue. There are contradictory results about the ability of cannabinoid drugs to activate Gα₁₅ proteins. In this way, there are data from both CHO cell lines (Rinaldi-Carmona et al., 1996; Bonhaus et al., 1998) and HEK cells (Presley et al., 2016) expressing CB₁ receptor, showing the absence of effect as well as a modest but significant coupling of CB₁ to Gα₁₅ triggered by different cannabinoids. It has been proposed that ACEA may elevate cAMP through a non-CB₁ mechanism, since there is an increase in cAMP in both cells transfected and non-transfected with CB₁ and pretreated with pertussis toxin (Presley et al., 2016). It is important to point out to all these studies have been performed in cell lines. Moreover, they use the accumulation of cAMP in the presence of the Gα₁₀ inhibitor pertussis toxin as an indirect evaluation of potential coupling of CB₁ receptors to Gα₁₂. This increase in cAMP production can be mediated by a mechanism different from Gα₁₅ activation, as they did not explore directly the activation of this subunit. Therefore, the possible increase in cAMP induced by other actors different from Gα₁₅ subunits could not be discarded.

The Gα₉/₁₁ proteins, widely expressed through the CNS, mediate PLC activation, leading to the activation of downstream calcium signaling pathways including PKC and MAPKs activation (Sanchez-Fernandez et al., 2014). In this study, a significant stimulation of Gα₉/₁₁ was observed in the presence of the three cannabinoids evaluated. It has been previously reported that WIN55212-2 induces the coupling of CB₁ to Gα₉/₁₁ in different cellular types (Lauckner et al., 2005; McIntosh et al., 2007). Our results show that not only WIN55212-2 but also Δ⁹-THC and ACEA can activate Gα₉/₁₁ subunit in mouse brain. Moreover, the activation of this subunit induced by Δ⁹-THC and WIN55212-2 seem to be mediated by the CB₁ receptor, as demonstrate the data obtained with knockout animals. In the case of ACEA, our data suggest that ACEA modulate Gα₉/₁₁ through both CB₁ and CB₂ cannabinoid receptors.

The Gα₁₂/₁₃ proteins regulate important signaling events by the activation of the small GTPase protein RhoA, involved in the regulation of the actin cytoskeleton and cell motility (Kozasa et al., 2011; Yu and Brown, 2015). Under our experimental conditions, a significant stimulation of Gα₁₂/₁₃ subunit was observed when membranes were incubated with WIN55212-2 but not with Δ⁹-THC or ACEA. To our knowledge, this is the first study reporting that WIN55212-2 signals through Gα₁₂/₁₃ in brain cortex. These data are concordant with other studies suggesting that cannabinoids induce the stimulation of this RhoA-activator (Dalton et al., 2013; Roland et al., 2014). Moreover, our results from knockout mice show that the WIN55212-2-induced signaling through Gα₁₂/₁₃ in the brain seems to be mediated, mainly, by the CB₂ receptor.

Although O-2050 had been described as a CB₁ antagonist, it displays a complex pharmacological profile. In this context, its good affinity for CB₂ receptors complicates its use as a tool
to evaluate the unique contribution of CB<sub>1</sub> receptor (Wiley et al., 2011). We observed that, when alone, O-2050 activated the G<sub>α<sub>z</sub></sub> subunit. Therefore, in co-incubations, O-2050 behaved always as an agonist of the effects of Δ<sup>9</sup>-THC, WIN55212-2 and ACEA over all the studied Gα subunit subtypes, except for the G<sub>α<sub>z</sub></sub> subunit. When WIN55212-2 and O-2050 were co-incubated, the stimulation of G<sub>α<sub>z</sub></sub> was lower but still significant. In this way, the blockade exerted by O-2050 pharmacologically confirmed the involvement of cannabinoid receptors in the observed stimulations.

Studies in knockout mice provide very valuable data in basic research but in addition to the absence of the targeted protein, we cannot discard the appearance of putative neurodevelopmental compensatory mechanisms. In this work, we have used CB<sub>1</sub> and/or CB<sub>2</sub> receptor knockout mice to elucidate the role of each receptor in the observed effects of different ligands on the stimulation of Gα subunits. Moreover, Western blotting assays were carried out in order to unmask the role of a possible adaptation of cannabinoid receptors knockout mice affecting the expression level of the different Gα subtypes on the different genotypes. The observed stimulations in knockout mice may not be influenced by putative neurodevelopmental compensatory mechanisms involving G proteins density. In this way, although expression of some Gα subunits in knockout mice is different from the WT, these changes do not explain the absence of stimulation in CB<sub>1</sub> or CB<sub>2</sub> knockout mice. The convergence of our pharmacological and genetic data demonstrate that the results obtained herein with the cannabinoid receptors knockout mice are likely due to the absence of the CB<sub>1</sub> and/or CB<sub>2</sub> receptors and not to non-specific changes due to neurodevelopmental adaptations.

**CONCLUSION**

Our results demonstrate that, in mouse brain native tissue and under our experimental conditions different exogenous cannabinoids are able to selectively activate different inhibitory and non-inhibitory Gα protein subtypes, through the activation of CB<sub>1</sub> and/or CB<sub>2</sub> receptors. However, it is important to be aware of potential limitations. It has been suggested that the signaling of CB<sub>1</sub> receptors is significantly diminished in humans compared to that of rodents, a finding that may have implications for the use of rodent models for studies of CB<sub>1</sub> receptor function related to human disease and therapy (Straiker et al., 2012).

Results of the present study may help to dissect the specific signaling pathways involved in the different pharmacological actions of cannabinoids. Moreover, the knowledge of the specific molecular target responsible of these different physiological effects will help in the design of new biased cannabinoid drugs with more specific therapeutic effect and a reduced range of adverse effects.

**AUTHOR CONTRIBUTIONS**

RD-A, II-L, and AL-C performed the experiments, LC, EA, AG-A, JM, and LU designed the study, JM, LC, RD-A, AG-A, and LU analyzed and interpreted the results, RD-A and LU drafted the manuscript. All the contributors revised critically and gave their approval to the final version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2016.00415/full#supplementary-material

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Frontiers in Pharmacology | www.frontiersin.org 12 November 2016 | Volume 7 | Article 415

Diez-Alarcia et al. Functional Coupling of Cannabinoid Receptors

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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