



Cannabigerol Action at Cannabinoid CB₁ and CB₂ Receptors and at CB₁–CB₂ Heteroreceptor Complexes

Gemma Navarro^{1,2}, Katia Varani³, Irene Reyes-Resina^{2,4}, Verónica Sánchez de Medina⁵, Rafael Rivas-Santisteban^{2,4}, Carolina Sánchez-Carnerero Callado⁶, Fabrizio Vincenzi³, Salvatore Casano⁷, Carlos Ferreiro-Vera⁶, Enric I. Canela^{2,4}, Pier Andrea Borea³, Xavier Nadal^{5*†} and Rafael Franco^{2,4*†}

¹ Department of Biochemistry and Physiology, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain, ² Centro de Investigación Biomédica en Red, Enfermedades Neurodegenerativas (CIBERNED), Instituto de Salud Carlos III, Madrid, Spain, ³ Department of Medical Sciences, Institute of Pharmacology, University of Ferrara, Ferrara, Italy, ⁴ Molecular Neurobiology Laboratory, Department of Biochemistry and Molecular Biomedicine, University of Barcelona, Barcelona, Spain, ⁵ Department of R&D – Extraction, PhytoPlant Research S.L., Córdoba, Spain, ⁶ Department of Analytical Chemistry, PhytoPlant Research S.L., Córdoba, Spain, ⁷ Department of Breeding and Cultivation, PhytoPlant Research S.L., Córdoba, Spain

OPEN ACCESS

Edited by:

Fabrizio A. Pamplona,
Entourage Phytolab, Brazil

Reviewed by:

Steven R. Laviolette,
University of Western Ontario, Canada
Attila Köfalvi,
Universidade de Coimbra, Portugal

*Correspondence:

Xavier Nadal
x.nadal@phytoPlant.es
Rafael Franco
rfranco123@gmail.com

† These authors have contributed
equally to this work.

Specialty section:

This article was submitted to
Neuropharmacology,
a section of the journal
Frontiers in Pharmacology

Received: 22 February 2018

Accepted: 25 May 2018

Published: 21 June 2018

Citation:

Navarro G, Varani K, Reyes-Resina I, Sánchez de Medina V, Rivas-Santisteban R, Sánchez-Carnerero Callado C, Vincenzi F, Casano S, Ferreiro-Vera C, Canela EI, Borea PA, Nadal X and Franco R (2018) Cannabigerol Action at Cannabinoid CB₁ and CB₂ Receptors and at CB₁–CB₂ Heteroreceptor Complexes. *Front. Pharmacol.* 9:632. doi: 10.3389/fphar.2018.00632

Cannabigerol (CBG) is one of the major phytocannabinoids present in *Cannabis sativa* L. that is attracting pharmacological interest because it is non-psychoactive and is abundant in some industrial hemp varieties. The aim of this work was to investigate in parallel the binding properties of CBG to cannabinoid CB₁ (CB₁R) and CB₂ (CB₂R) receptors and the effects of the compound on agonist activation of those receptors and of CB₁–CB₂ heteroreceptor complexes. Using [³H]-CP-55940, CBG competed with low micromolar *K_i* values the binding to CB₁R and CB₂R. Homogeneous binding in living cells, which is only technically possible for the CB₂R, provided a 152 nM *K_i* value. Also interesting, CBG competed the binding of [³H]-WIN-55,212-2 to CB₂R but not to CB₁R (*K_i*: 2.7 versus >30 μM). The phytocannabinoid modulated signaling mediated by receptors and receptor heteromers even at low concentrations of 0.1–1 μM. cAMP, pERK, β-arrestin recruitment and label-free assays in HEK-293T cells expressing the receptors and treated with endocannabinoids or selective agonists proved that CBG is a partial agonist of CB₂R. The action on cells expressing heteromers was similar to that obtained in cells expressing the CB₂R. The effect of CBG on CB₁R was measurable but the underlying molecular mechanisms remain uncertain. The results indicate that CBG is indeed effective as regulator of endocannabinoid signaling.

Keywords: cannabinoid receptor, cannabigerol, G-protein-coupled receptor, phytocannabinoid, TR-FRET, partial agonist

Abbreviations: Δ⁸-THC, Δ⁸-tetrahydrocannabinol; Δ⁹-THC, Δ⁹-tetrahydrocannabinol; Δ⁹-THCA, Δ⁹-tetrahydrocannabinolic acid; Δ⁹-THCV, Δ⁹-tetrahydrocannabivarin; 2-AG, 2-arachidonoyl glycerol; AEA, anandamide; CB₁R, cannabinoid receptor 1; CB₂R, cannabinoid receptor 2; CBC, cannabichromene; CBD, cannabidiol; CBDA, cannabidiolic acid; CBDV, cannabidivarin; CBG, cannabigerol; CBGA, cannabigerolic acid; CBN, cannabinol; CNS, central nervous system; DMR, dynamic mass redistribution; HEK, human embryonic kidney; HTRF, homogeneous time-resolved fluorescence; SNAP, protein used as a tag; it contains circa 180 amino acids and may be covalently labeled with different probes; Tb, terbium; TLB, Tag-lite labeling medium.

INTRODUCTION

Cannabinoid compounds bind and activate cannabinoid CB₁ (CB₁R) and CB₂ (CB₂R) receptors, which belong to the superfamily of G-protein-coupled receptors. There are many ways to classify them, but the most used distinguishes between endogenous molecules (endocannabinoids), phytocannabinoids and synthetic cannabinoids. Endocannabinoids and one of the most studied phytocannabinoids, Δ^9 -tetrahydrocannabinol (Δ^9 -THC), are agonists with more or less CB₁R/CB₂R selectivity. Furthermore, synthetic cannabinoids mainly act (as agonists or antagonists) by binding to the orthosteric site of receptors (Mechoulam, 2016). Indeed, there is a limited number of molecules, either synthetic or phytocannabinoids, that behave as allosteric modulators of cannabinoid receptor function.

Anandamide and 2-arachidonoyl glycerol (2-AG) are the two main endocannabinoids, being synthesized from membrane lipids and having an alkyl-amide chemical structure. They are retrograde effectors being produced in the post-synaptic neuron to act in the pre-synaptic neuron where they regulate the release of neurotransmitters (Diana and Marty, 2004).

Phytocannabinoids are phenolic terpenes biosynthesized in nature nearly exclusively in the *Cannabis sativa* L. plant. In the *Cannabis* plant, all cannabinoids are biosynthesized in the acid form, mainly Δ^9 -THCA, CBDA, etc. CBGA is the first molecule formed in the biosynthetic pathway and the substrate of Δ^9 -tetrahydrocannabinol-synthase and CBD-synthase (Fellermeier and Zenk, 1998). The pharmacologic effects of *Cannabis* components, traditionally consumed through inhalation, are attributed to the decarboxylated neutral products of above mentioned acids: Δ^9 -THC, CBD, and CBG.

Synthetic cannabinoids are very different in chemical structure. For instance, they may be indoles like WIN-55,212-2, AM-1241 or JWH-018, or phenolic, phenols lacking the pyrene ring, like CP-55,940 or HU-308. All these compounds have been used in cannabinoid research and have helped to unveil pharmacological aspects of the endocannabinoid system. It should be noted that some of these compounds have recently arrived at the streets sold as *legal highs*, thus raising Public Health concerns (Adams et al., 2017; Weinstein et al., 2017).

The endocannabinoid system is constituted by the endogenous cannabinoids, the enzymes that produce and degrade them, and by the receptors that mediate their actions. Whereas endocannabinoids consist of molecules with aliphatic structure, AEA and 2-AG, the structure of natural cannabinoids, derived from *C. sativa* L., is fairly different [see (Lu and Mackie, 2016) and references therein]. Although it is well established that one of the main active components of the plant and one of the few that are psychoactive, namely Δ^9 -THC, acts via cannabinoid receptors, there is controversy on whether these receptors mediate the action of phytocannabinoids such as CBN, CBD or CBG. As happened the last years for CBD, a new research and revision of the cannabinoid receptor pharmacology must be done with the

rest of phytocannabinoids as CBG. A further phenomenon that may be considered to understand the action of molecules from *C. sativa* L. and its extracts is the fact that cannabinoid receptors may form heteromers, namely CB₁-CB₂ heteroreceptors, which display particular functional properties (Callén et al., 2012). It should be noted that in CNS those heteromers are mainly expressed in pallidal neurons (Lanciego et al., 2011; Sierra et al., 2015) and in activated microglia (Navarro et al., 2018a).

Cannabigerol was isolated, characterized and synthesized by the same researchers than reported the structure of the main psychotropic agent of *Cannabis*, Δ^9 -THC (Gaoni and Mechoulam, 1964). Few years later *in vivo* assays showed that CBG was non-psychoactive (Grunfeld and Ederly, 1969; Mechoulam et al., 1970). The lower concentration and the lack of psychoactivity was probably the cause that CBG was shadowed by Δ^9 -THC. In fact, CBG has attracted less attention than Δ^9 -THC and even than CBD, but nowadays is gaining interest among the scientific community. Some commercial hemp varieties have CBG and CBGA as main cannabinoids and, therefore, CBG is another of the phytocannabinoids to be considered by the unregulated market of hemp oils and derivatives. As recently pointed out, the increased therapeutic potential of *C. sativa* L. components requires a more in deep understanding of the pharmacology of phytocannabinoids other than Δ^9 -THC, namely CBD, CBG, CBN, Δ^9 -THCV, Δ^8 -THC, CBC and CBDV (Turner et al., 2017).

Preliminary results using membranes from mice brain or from CHO cells expressing the human CB₂R led to postulate that CBG could be a partial agonist at both CB₁R and CB₂R with K_i values in the 300–500 nM range (Gauson et al., 2007; Pertwee, 2008). The first published data on the binding of CBG to human CB₁R and CB₂R were provided by (Rosenthaler et al., 2014) working with [³H]CP-55,940 as radioligand and with preparations from Sf9 cells co-expressing one receptor and the G α i3 β 1 γ 2 protein. The K_i values obtained in competition assays are 897 and 153 nM for CB₁R and CB₂R, respectively. CBG may modulate the activity of transient receptor potential channels of ankyrin type-1; however, the EC₅₀ values lie in the micromolar range (De Petrocellis et al., 2008). It has been reported that CBG binds to CB₁R (K_i = 381 nM) from mouse brain membranes and CB₂R (K_i = 2.6 μ M) from CHO cells expressing the human receptor; CBG at high concentrations (10 μ M) antagonized [³⁵S]GTP γ S binding in mouse brain membranes treated with AEA or CP-55940 (Cascio et al., 2010). Authors also reported CBG as α_2 -adrenoceptor agonist at nanomolar levels (EC₅₀ = 0.2 nM), and being also able to antagonize [³⁵S]GTP γ S binding upon stimulation of the 5HT_{1A} receptor by 1 μ M 8-OH-DPAT (Cascio et al., 2010). Other findings indicate that CBG can act as (i) agonist/desensitizer of TRPA1 (EC₅₀ = 700 nM), (ii) agonist of TRPV1 (EC₅₀ = 1.3 μ M) (iii) agonist of TRPV2 (EC₅₀ = 1.7 μ M), (iv) antagonist of TRPM8 channels (IC₅₀ = 160 nM) and v) inhibitor of AEA cell uptake (K_i = 11.3 μ M) (De Petrocellis et al., 2011). More recently, the PPAR γ has been reported as target of the phytocannabinoid CBG (K_i = 11.7 μ M) that at high concentrations, in the 10–25 μ M range, may enhance the PPAR γ transcriptional activity (Granja

et al., 2012; Nadal et al., 2017). A recent review substantiates the complexity of the field and highlights that other players, GPR55 for instance, are also targeted by cannabinoids (Solymosi and Kofalvi, 2017).

The aim of this work was to characterize CBG pharmacology on the cannabinoid receptors using binding and measurement of different signal transduction mechanisms in living HEK-293T cells expressing human CB₁R, CB₂R, or CB₁-CB₂ heteroreceptor complexes. The results indicate that, in our experimental conditions, CBG mainly acts on CB₂R and behaves as a partial agonist.

MATERIALS AND METHODS

Reagents

ACEA, JWH133, and AEA were purchased from Tocris Bioscience (Bristol, United Kingdom), CBD and CBG analytical standard solutions were purchased from THCpharm (Frankfurt, DE). Concentrated (10 mM) stock solutions prepared in ethanol (CBG, ACEA, and AEA) or DMSO (JWH133 and CM-157) were stored at -20°C . In each experimental session, aliquots of concentrated solutions of compounds were thawed and conveniently diluted in the appropriate experimental solution. For non-radioactive binding assays, TLB was obtained from Cisbio Bioassays (LABMED; Codolet, France). The Tb derivative of O6-benzylguanine was synthesized by Cisbio Bioassays and is commercialized as SNAP-Lumi4-Tb (SSNPTBC; Cisbio Assays). The plasmid encoding for the SNAP-tagged human CB₂R used for transient transfection was obtained from Cisbio Bioassays (PSNAP-CB2). CB₂R agonist 3-[[4-[2-tert-butyl-1-(tetrahydropyran-4-ylmethyl)benzimidazol-5-yl]sulfonyl-2-pyridyl]oxy]propan-1-amine (CM-157) conjugated to a fluorescent probe was developed in collaboration with Cisbio Bioassays (Martínez-Pinilla et al., 2016).

Cannabinoid Isolation, Purification and Analysis

Cannabidiol was purified from dried leaves and inflorescences of the *Cannabis* variety SARA (CPVO file number: 20150098), CBG from the variety AIDA (CPVO file number: 20160167) following a previously described method (Nadal, 2016) that provides compounds with >95% purity. An Agilent liquid chromatography set-up (Model 1260, Pittsburgh, PA, United States) consisting of a binary pump, a vacuum degasser, a column oven, an autosampler and a diode array detector (DAD) equipped with a 150 mm length \times 2.1 mm internal diameter, 2.7 μm pore size Poroshell 120 EC-C18 column was used for the quality control of the purified cannabinoids. The analysis was performed using water and acetonitrile both containing ammonium formate 50 mM as mobile phases. Flow-rate was 0.2 mL/min and the injection volume was 3 μL . Chromatographic peaks were recorded at 210 nm. All determinations were carried out at 35°C . All samples were analyzed in duplicate. The results of each cannabinoid purity, 96.04% for CBD and 99.9% for CBG, were calculated as weight (%) versus a commercial standard

from THCpharm (CBD batch n° L01258-M-1.0; CBG batch n° L01260-M-1.0).

Radioligand Binding Assays

Cell Culture and Membrane Preparation

For radioligand binding experiments CHO cells, stably transfected with cDNA for human CB₁ or CB₂ cannabinoid receptors, were grown adherently and maintained in Ham's F12 containing 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 $\mu\text{g}/\text{mL}$) and geneticin (G418, 0.4 mg/mL) at 37°C in a humid atmosphere of 5% CO₂. Membranes were prepared from cells washed with PBS and scraped off plates in ice-cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with a Polytron and then centrifuged for 30 min at $40,000 \times g$.

Saturation Binding Experiments

[³H]-CP-55940 saturation binding experiments (specific activity 169 Ci/mmol, Perkin Elmer) were performed incubating different concentrations of the radioligand (0.03 – 10 nM) in binding buffer (50 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 5 mM MgCl₂ for CB₁R or 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM MgCl₂ for CB₂R) using CHO membranes expressing the human versions of CB₁R or CB₂R (10 μg protein/sample) at 30°C . Non-specific binding was determined in the presence of 1 μM WIN-55,212-2. At the end of the incubation period (90 min for CB₁R or 60 min for CB₂R) bound and free radioactivity were separated in a cell harvester (Brandel Instruments) by filtering the assay mixture through Whatman GF/B glass fiber filters. The filter-bound radioactivity was counted in a 2810 TR liquid scintillation counter (Perkin Elmer).

[³H]-WIN-55,212-2 saturation binding experiments (specific activity 48 Ci/mmol, Perkin Elmer) were performed incubating different concentrations of the radioligand (0.5–100 nM for CB₁R or 0.2–40 nM for CB₂R) in binding buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM MgCl₂) with CB₁R- or CB₂R-containing CHO cell membranes (10 μg protein/sample) at 30°C . Non-specific binding was determined in the presence of 1 μM WIN-55,212-2. At the end of the incubation period (60 min) bound and free radioactivity were separated in a cell harvester (Brandel Instruments) by filtering the assay mixture through Whatman GF/B glass fiber filters. The filter-bound radioactivity was counted in a 2810 TR liquid scintillation counter (Perkin Elmer).

Competition Binding Experiments

[³H]-CP-55940 competition binding experiments were performed incubating 0.3 nM of radioligand and different concentrations of the tested compounds with membranes obtained from CHO cells expressing human CB₁ or CB₂ receptors (10 μg protein/sample) for 90 min (CB₁R) or 60 min (CB₂R) at 30°C . Non-specific binding was determined in the presence of 1 μM WIN-55,212-2. Bound and free radioactivity were separated by filtering the assay mixture as above indicated. The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

Competition binding experiments were also performed incubating 3 nM [³H]-WIN-55,212-2 and different concentrations of the tested compounds with membranes obtained from CHO cells transfected with human CB₁ or CB₂ receptors (10 µg protein/sample) for 60 min at 30°C. Non-specific binding was determined in the presence of 1 µM WIN-55,212-2. Bound and free radioactivity were separated by filtering the assay mixture as above indicated. The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer).

Homogeneous Binding Assays in Living Cells

Expression Vector

cDNAs for the human version of cannabinoid CB₂R without their stop codon were obtained by PCR and subcloned to SNAP-containing vector (PSNAP; Cisbio Bioassays) using sense and antisense primers harboring unique restriction sites for HindIII and BamHI generating the SNAP tagged CB₂R (CB₂R-SNAP).

Cell Culture and Transfection

For HTRF assays, HEK-293T cells were used. HEK 293T (HEK-293T) cells were grown in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin/streptomycin, and 5% (v/v) FBS [all supplements were from Invitrogen, (Paisley, Scotland, United Kingdom)]. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and were passaged, with enzyme-free cell dissociation buffer (13151-014, Gibco®, Thermo Fisher, Waltham, MA, United States), when they were 80–90% confluent, i.e., approximately twice a week. Cells were transiently transfected with the PEI (Polyethylenimine, Sigma, St. Louis, MO, United States) method as previously described (Medrano et al., 2017; Navarro et al., 2018b). Experiments were carried out in cells expressing SNAP-tagged CB₂R in the presence or in the absence of CB₁R.

Labeling of Cells Expressing SNAP-Tagged CB₂R

Cell culture medium was removed from the 25-cm² flask and 100 nM SNAP-Lumi4-Tb, previously diluted in 3 mL of TLB 1X, was added to the flask and incubated for 1 h at 37°C under 5% CO₂ atmosphere in a cell incubator. Cells were then washed four times with 2 mL of TLB 1X to remove the excess of SNAP-Lumi4-Tb, detached with enzyme-free cell dissociation buffer, centrifuged 5 min at 1,500 rpm and collected in 1 mL of TLB 1X. Tag-lite-based binding assays were performed 24 h after transfection. Densities in the 2,500–3,000 cells/well range were used to carry out binding assays in white opaque 384-well plates.

Non-radioactive Competition Binding Assays

For competition binding assays, the fluorophore-conjugated CB₂R ligand (labeled CM-157), unconjugated CM-157 and CBG were diluted in TLB 1X. HEK-293T cells transiently expressing Tb-labeled SNAP-CB₂R with or without CB₁R were incubated with 20 nM fluorophore-conjugated CB₂R ligand, in the presence of increasing concentrations (0–10 µM range) of CBG or CM-157. Plates contained 10 µL of labeled cells, and 5 µL of TLB 1X

or 5 µL of CBG or 5 µL CM-157 were added prior to the addition of 5 µL of the fluorescent ligand. Plates were then incubated for at least 2 h at room temperature before signal detection. Detailed description of the HTRF assay is found in Martínez-Pinilla et al. (2016).

Signal was detected using an EnVision microplate reader (PerkinElmer, Waltham, MA, United States) equipped with a FRET optic module allowing donor excitation at 337 nm and signal collection at both 665 and 620 nm. A frequency of 10 flashes/well was selected for the xenon flash lamp excitation. The signal was collected at both 665 and 620 nm using the following time-resolved settings: delay, 150 µs; integration time, 500 µs. HTRF® ratios were obtained by dividing the acceptor (665 nm) by the donor (620 nm) signals and multiplying by 10,000. The 10,000-multiplying factor is used solely for the purpose of easier data handling.

Functional Assays

Cell Culture and Transient Transfection

HEK-293T cells were grown in DMEM medium (Gibco, Paisley, Scotland, United Kingdom) supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, MEM Non-Essential Amino Acids Solution (1/100) and 5% (v/v) heat inactivated Foetal Bovine Serum (FBS) (Invitrogen, Paisley, Scotland, United Kingdom). Cells were maintained in a humid atmosphere of 5% CO₂ at 37°C. Cells were transiently transfected with the PEI (Polyethylenimine, Sigma, St. Louis, MO, United States) method as previously described (Medrano et al., 2017; Navarro et al., 2018b) and used for functional assays 48 h later (unless otherwise stated).

cAMP Determination

Signaling experiments have been performed as previously described (Navarro et al., 2010, 2016, 2018b; Hinz et al., 2018). Two hours before initiating the experiment, HEK-293T cell-culture medium was replaced by serum-starved DMEM medium. Then, cells were detached, resuspended in growing medium containing 50 µM zardaverine and placed in 384-well microplates (2,500 cells/well). Cells were pretreated (15 min) with CBG -or vehicle- and stimulated with agonists (15 min) before adding 0.5 µM forskolin or vehicle. Readings were performed after 15 min incubation at 25°C. HTRF energy transfer measures were performed using the Lance Ultra cAMP kit (PerkinElmer, Waltham, MA, United States). Fluorescence at 665 nm was analyzed in a PHERAstar Flagship microplate reader equipped with an HTRF optical module (BMG Lab Technologies, Offenburg, Germany).

ERK Phosphorylation Assays

To determine ERK1/2 phosphorylation, 50,000 HEK-293T cells/well were plated in transparent Deltalab 96-well microplates and kept at the incubator for 24 h. 2 to 4 h before the experiment, the medium was substituted by serum-starved DMEM medium. Then, cells were pre-treated at 25°C for 10 min with vehicle or CBG in serum-starved DMEM medium and stimulated for an additional 7 min with the specific agonists. Cells were then washed twice with cold PBS before addition of lysis

buffer (20 min treatment). 10 μ L of each supernatant were placed in white ProxiPlate 384-well microplates and ERK 1/2 phosphorylation was determined using AlphaScreen® SureFire® kit (Perkin Elmer) following the instructions of the supplier and using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, United States).

Dynamic Mass Redistribution Assays (DMR)

Cell mass redistribution induced upon receptor activation was detected by illuminating the underside of a biosensor with polychromatic light and measuring the changes in the wavelength of the reflected monochromatic light. The magnitude of this wavelength shift (in picometers) is directly proportional to the amount of DMR. HEK-293T cells were seeded in 384-well sensor microplates to obtain 70–80% confluent monolayers constituted by approximately 10,000 cells per well. Previous to the assay, cells were washed twice with assay buffer (HBSS with 20 mM HEPES, pH 7.15) and incubated for 2 h with assay-buffer containing 0.1% DMSO (24°C, 30 μ L/well). Hereafter, the sensor plate was scanned and a baseline optical signature was recorded for 10 min before adding 10 μ L of CBG for 30 min followed by the addition of 10 μ L of specific agonists; all test compounds were dissolved in assay buffer. The cell signaling signature was determined using an EnSpire® Multimode Plate Reader (PerkinElmer, Waltham, MA, United States) by a label-free technology. Then, DMR responses were monitored for at least 5,000 s. Results were analyzed using EnSpire Workstation Software v 4.10.

β -Arrestin 2 Recruitment

Arrestin recruitment was determined as previously described (Medrano et al., 2017; Navarro et al., 2018b). Briefly, BRET experiments were performed in HEK-293T cells 48 h after transfection with the cDNA corresponding to the CB₂R-YFP or CB₁R-YFP and 1 μ g cDNA corresponding to β -arrestin 2-Rluc. Cells (20 μ g protein) were distributed in 96-well microplates (Corning 3600, white plates with white bottom) and were incubated with CBG for 15 min and stimulated with the agonist for 10 min prior the addition of 5 μ M coelenterazine H (Molecular Probes, Eugene, OR, United States). After 1 min of adding coelenterazine H, BRET between β -arrestin 2-Rluc and receptor-YFP was determined and quantified. The readings were collected using a Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany) that allows the integration of the signals detected in the short-wavelength filter at 485 nm and the long-wavelength filter at 530 nm. To quantify protein-Rluc expression luminescence readings were also performed 10 min of adding 5 μ M coelenterazine H.

Data Handling and Statistical Analysis

Affinity values (K_i) were calculated from the IC₅₀ obtained in competition radioligand binding assays according to the Cheng and Prusoff equation: $K_i = IC_{50}/(1 + [C]/K_D)$, where $[C]$ is the free concentration of the radioligand and K_D its dissociation constant (Cheng, 2001).

Data from homogeneous binding assays were analyzed using Prism 6 (GraphPad Software, Inc., San Diego, CA, United States).

K_i values were determined according to the Cheng and Prusoff equation with $K_D = 21$ nM for CM-157 (Cheng, 2001). Signal-to-background (S/B ratio) calculations were performed by dividing the mean of the maximum value (μ_{max}) by that of the minimum value (μ_{min}) obtained from the sigmoid fits.

The data are shown as the mean \pm SEM. Statistical analysis was performed with SPSS 18.0 software. The test of Kolmogorov–Smirnov with the correction of Lilliefors was used to evaluate normal distribution and the test of Levene to evaluate the homogeneity of variance. Significance was analyzed by one-way ANOVA, followed by Bonferroni's multiple comparison *post hoc* test. Significant differences were considered when $p < 0.05$.

RESULTS

Saturation and Competition Radioligand-Based Assays in Membranes Expressing CB₁R or CB₂R

The effect of CBG on radioligand binding to CB₁R or CB₂R was first tested using the classical radioligand-binding assay in membranes isolated from CHO cells expressing human CB₁R or CB₂R and incubated with radioligands: [³H]-CP-55940 or [³H]-WIN-55,212-2. Data obtained from binding isotherms using increasing [³H]-CP-55940 or [³H]-WIN-55,212-2 concentrations lead to a monophasic saturation curve. Saturation curves, receptor density (B_{max} values) and affinity (K_D values) are shown in **Figures 1A–D**. The affinity of the two radioligands was in the nanomolar range for both CB₁R and CB₂R. K_D for [³H]-CP-55940 to CB₁R and CB₂R was similar with values around 0.3 nM. K_D values for WIN-55,212-2 were 9.4 and 3.2 nM for CB₁R and CB₂R, respectively (**Figures 1C,D**). Overall the results agree with previously reported data (McPartland et al., 2007; Merighi et al., 2010).

Competition binding assays of WIN-55,212-2 showed similar K_i values using the two radioligands to CB₁R and CB₂R and agreed with the K_D values for [³H]-WIN-55,212-2 binding (**Table 1** and **Figures 1E,F**). **Table 1** reports the affinity values of CBG. K_i values of CBG obtained using [³H]-CP-55940 as radioligand were in the low micromolar range in both CB₁R and CB₂R. The affinity value of CBG obtained using [³H]-WIN-55,212-2 for CB₂R was 2.7 μ M, about twofold higher than that obtained using [³H]-CP-55940. Using [³H]-WIN-55,212-2 in competition binding experiments on CB₁R, CBG was not able to displace the radioligand (**Figures 2A,B**). In summary, CBG displayed K_i values in the low micromolar range when competing for the binding to the CB₂R. Surprisingly, significant competition in the binding to the CB₁R was only observed when using [³H]-CP-55940 as radioligand.

CBG Binds to the Orthosteric Site of Cannabinoid CB₂R at Nanomolar Concentrations

Competition experiments were performed using 20 nM of a fluorophore-conjugated selective CB₂R agonist (CM-157) and

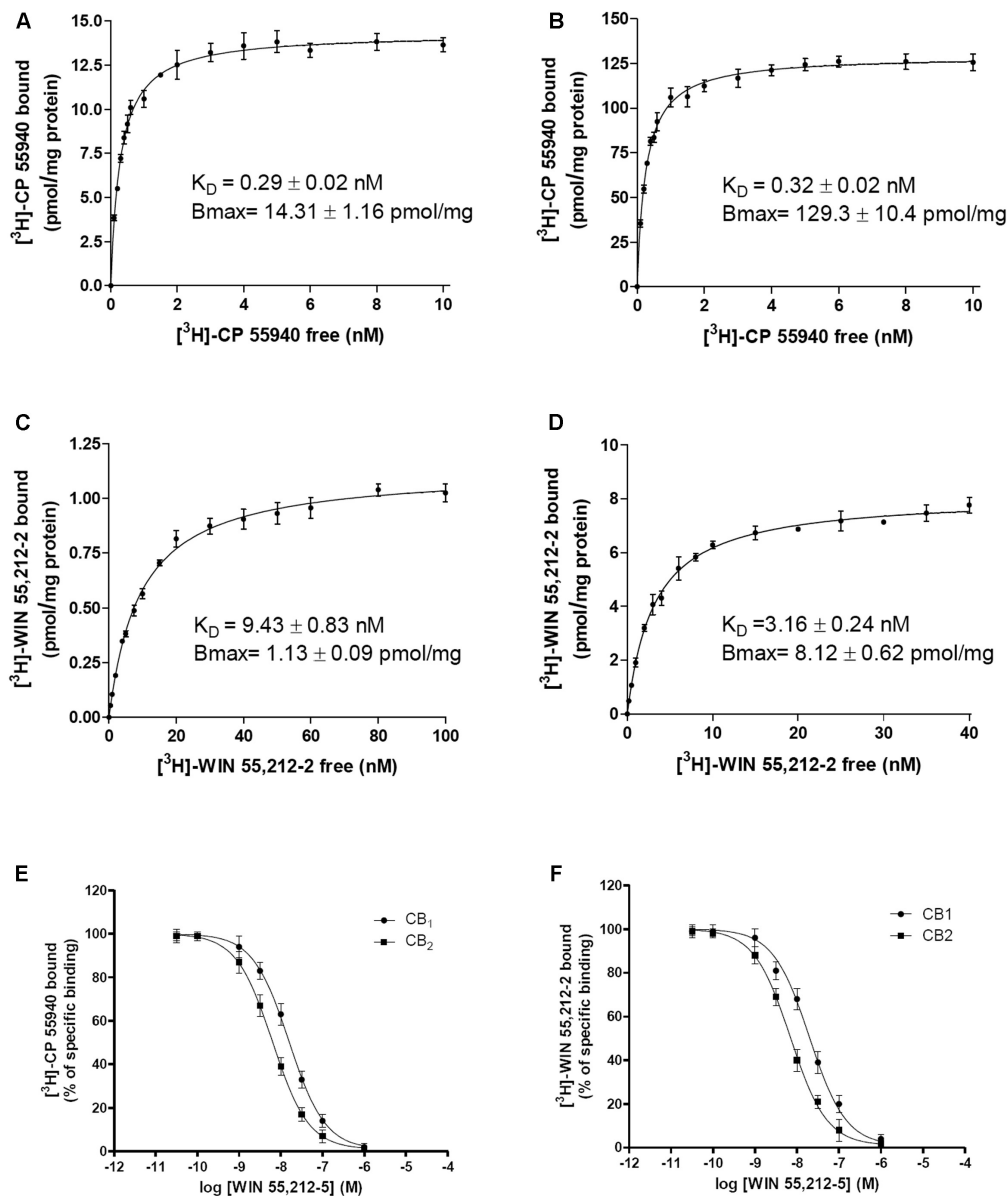


FIGURE 1 | Radioligand binding assays to CB₁R and CB₂R. (A–D) Saturation curves of either $[^3\text{H}]\text{-CP-55940}$ or $[^3\text{H}]\text{-WIN-55,212-2}$ binding on membranes from CHO cells stably expressing human CB₁R (A,C) or CB₂R (B,D). (E,F) Competition curves for WIN-55,212-2 in radioligand-based assays using either $[^3\text{H}]\text{-CP-55940}$ (E) or $[^3\text{H}]\text{-WIN-55,212-2}$ (F) binding on membranes from CHO cells stably expressing human CB₁R or CB₂R. Data are expressed as the mean \pm SEM of five independent experiments performed in duplicate. K_D (obtained from saturation isotherms) are shown in **Table 1**.

a homogeneous non-radioactive method performed in living cells expressing SNAP-CB₂R (details in Martínez-Pinilla et al., 2016; **Figure 2C**). Unfortunately, the equivalent fluorophore-conjugated selective CB₁R ligand is not available to perform HTRF assays in SNAP-CB₁R-expressing living cells. Competition assays were performed in HEK-293T cells expressing Lumi4-Tb-labeled CB₂R fused to the SNAP protein and incubated with a fixed amount of the fluorophore-conjugated agonist and different CBG concentrations. As observed in **Figure 2**, both the unlabelled selective agonist (CM-157) and CBG decreased the binding to SNAP-CB₂R in monophasic fashion and with

K_i values in the nanomolar range (16 nM for CM-157 and of 152 nM for CBG; **Figures 2D,E**). The K_i obtained for CM-157 matches with previously reported dissociation constant K_D values (Martínez-Pinilla et al., 2016). These results indicate that CBG can significantly bind to the orthosteric site of cannabinoid CB₂R at nanomolar concentrations.

Similar experiments were carried out in HEK-293T cells expressing SNAP-CB₂R fusion protein and a similar amount of CB₁R, i.e., in cells that express CB₂R in a CB₁-CB₂ receptor heteromer context. In the presence of cannabinoid CB₁R the K_i for CM-157 was 19 nM (**Figure 2F**) and K_i for CBG was reduced

TABLE 1 | Affinity values of CB compounds obtained from radioligand binding assays.

	$[^3\text{H}]\text{-CP-55940}$ competition binding experiments		$[^3\text{H}]\text{-WIN-55,212-2}$ competition binding experiments	
	CB ₁ – K _D (nM)	CB ₂ – K _D (nM)	CB ₁ – K _D (nM)	CB ₂ – K _D (nM)
	0.29 ± 0.02	0.32 ± 0.02	9.43 ± 0.83	3.16 ± 0.24
	CB ₁ – K _i (nM)	CB ₂ – K _i (nM)	CB ₁ – K _i (nM)	CB ₂ – K _i (nM)
WIN-55,212-2	8.08 ± 0.65	3.22 ± 0.31	9.86 ± 0.84	3.48 ± 0.27
CBG	1,045 ± 74	1,225 ± 85	>30,000	2,656 ± 130
CBD	1,690 ± 110	1,714 ± 70	>30,000	4,019 ± 342

K_D values were obtained from saturation isotherms and K_i from data in competition assays using the indicated radiolabelled compounds ($[^3\text{H}]\text{-CP-55940}$ or $[^3\text{H}]\text{-WIN-55,212-2}$).

(56 nM, **Figure 2G**). These results indicate that in cells expressing both cannabinoid receptors, CB₁ and CB₂, CBG shows higher affinity for cannabinoid CB₂R.

CBG Effects on Cannabinoid Receptor-Agonist-Induced Effects

Previous reports Gauson et al. (2007), Cascio et al. (2010) suggest that CBG may be a partial agonist of cannabinoid receptors. To investigate this possibility, HEK-293T cells expressing CB₁R or CB₂R were treated with increasing concentrations of CBG (1 nM to 10 μM) and cAMP, MAPK, β-arrestin recruitment and dynamic mass cell redistribution (DMR) assays were developed. Interestingly, it was observed that in cells expressing CB₁R (**Figure 3**, blue curves), CBG induced a small decrease in forskolin induced cAMP levels and a small increase in β-arrestin recruitment (**Figures 3A,C**), while having no significant action on MAPK phosphorylation assay (**Figure 3B**). Consequently, CBG in label-free assays induced a slight effect in the DMR signal (**Figure 3D**) that is consistent with a G protein-dependent action on cAMP levels; label-free signal is based on optical detection of DMR following receptor activation and mainly reflects G-protein-coupling (Kebig et al., 2009; Schröder et al., 2009; Hamamoto et al., 2015). On the other hand, in HEK-293T cells expressing CB₂R (**Figure 3**, red curves), the action on forskolin-induced cAMP levels and on the DMR signal was small and similar to that exerted in CB₁R-expressing cells (**Figure 3A**). On the contrary, the activation of the MAP kinase pathway was notable (**Figure 3B**). Also noteworthy was the CBG-induced β-arrestin recruitment (**Figure 3C**). Taken together these data suggest that CBG is a poor agonist of CB₁R, whereas it acts as a partial agonist in some of the signaling pathways analyzed in cells expressing CB₂R.

To further examine the CBG effect over CB₁R, HEK-293T cells expressing CB₁R were treated with the endocannabinoid agonist, AEA, or with ACEA in the presence or in the absence of 100 nM or 1 μM CBG. In forskolin-induced cAMP assays we found that 100 nM or 1 μM CBG pretreatment induced a significant decrease in both, AEA and ACEA induced effects (**Figure 4A**). In contrast, CBG (100 nM or 1 μM) was unable to modify the agonist-induced MAPK phosphorylation and β-arrestin recruitment (**Figures 4B,C**). In label-free DMR assays the results were similar to those obtained in cAMP

determination assays, i.e., CBG reduced the effect of the agonists (**Figure 4D**).

Cannabigerol (100 nM or 1 μM) was also tested in HEK-293T cells expressing CB₂R and using AEA and a receptor selective agonist, JWH133. Pretreatment with CBG reduced the effects of AEA and JWH133 in experiments of forskolin-induced cAMP levels, ERK1/2 phosphorylation and in label-free DMR read-outs (**Figure 4**). In contrast, CBG did not affect the recruitment of β-arrestin induced by agonists (**Figure 4G**). This last result may be due to the low sensitivity of the assay as β-arrestin recruitment BRET signal was virtually negligible. Energy transfer techniques completely depend on the correct orientation of the fusion proteins and the reduced signal may be due to poor recruitment of β-arrestin and/or to a high distance between BRET donor/acceptor in the putative β-arrestin-Rluc/CB₂R-YFP complex. Thus, CBG in cells activated by endocannabinoids or by selective agonists behaves as a partial agonist of the CB₂R.

CBG Effect in HEK-293T Cells Expressing CB₁R and CB₂R

Experiments were finally performed in cells co-expressing the two cannabinoid receptors, which are able to form heteromeric complexes. A CB₁-CB₂ receptor heteromer print consists of a negative cross-talk observed in Akt phosphorylation and neurite outgrowth; i.e., activation of one receptor reduces the signaling originated upon partner receptor activation (Callén et al., 2012). To characterize the CBG effect, experiments were performed in HEK-293T cells expressing the two cannabinoid receptors. Dose-effect curves were provided for cAMP level and ERK1/2 phosphorylation determination, and for label-free DMR signal and β-arrestin recruitment. Interestingly, the effect on cAMP level determination and DMR assays was additive (**Figure 5**), i.e., the presence of CBG blunted the negative cross-talk in these signaling pathways. However, the negative cross-talk was still evident in both ERK1/2 phosphorylation and β-arrestin recruitment experiments (**Figures 5B,C**).

Finally, the effect of 100 nM CBG (100 nM) on AEA, ACEA and/or JWH133 actions was investigated in cells co-expressing CB₁R and CB₂R. CBG pretreatment led to significant effects, always reducing the effect of the agonists, in cAMP-related assays (**Figure 5E**). However, the effect in the other assay types was

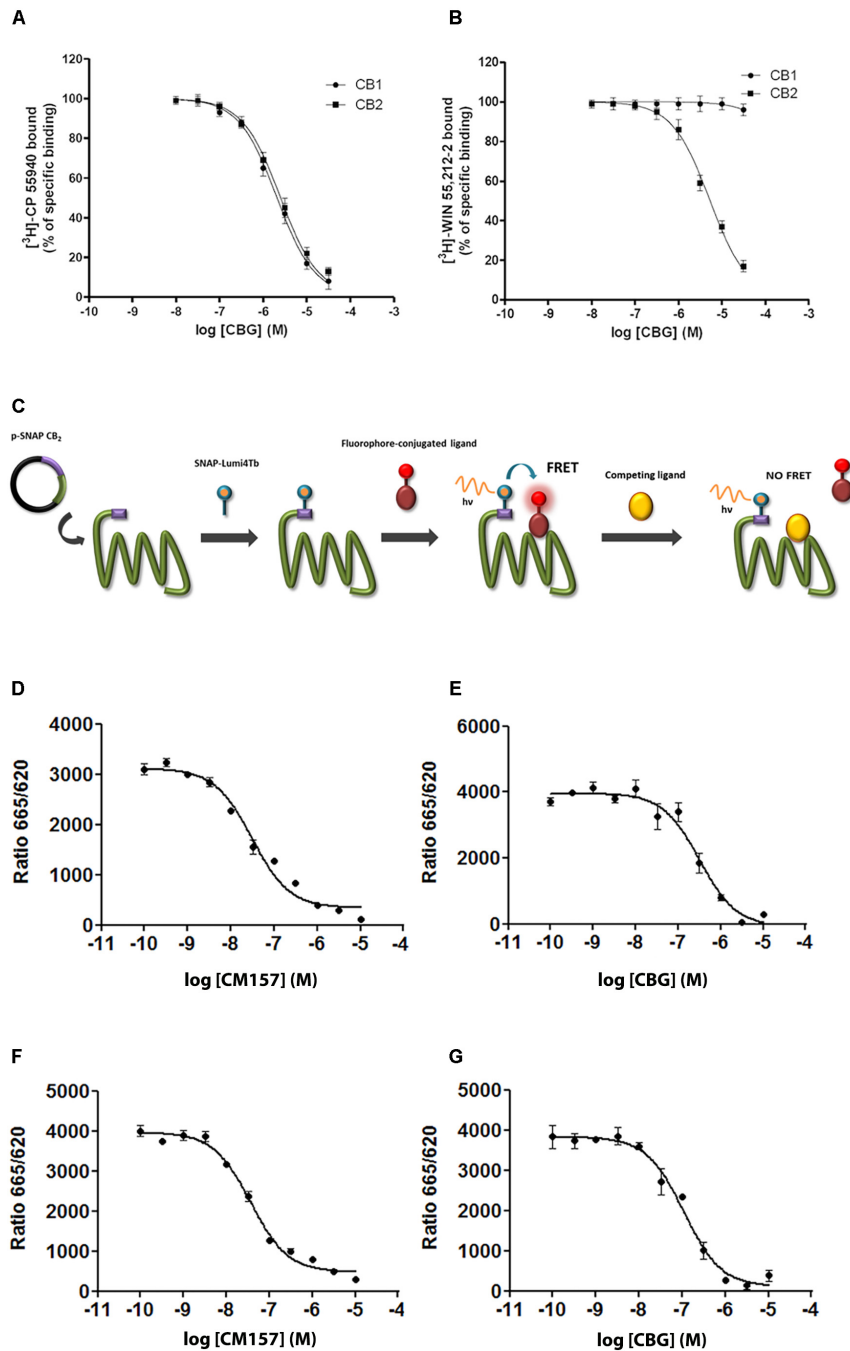
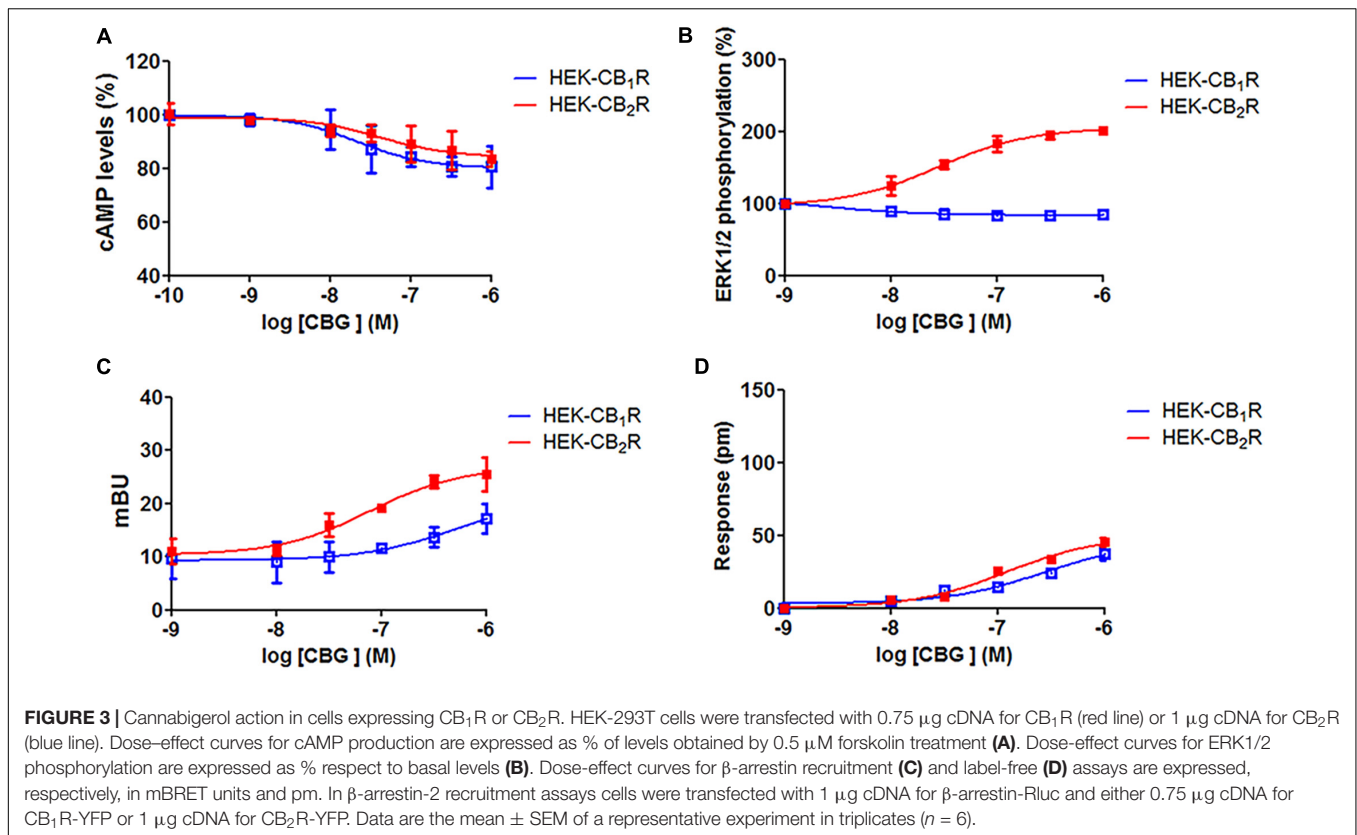


FIGURE 2 | Competition by CBG of agonist binding to CB₁R and/or CB₂R. **(A,B)** Competition curves for CBG in radioligand-based assays using either [³H]-CP-55940 **(A)** or [³H]-WIN-55,212-2 **(B)** binding on membranes from CHO cells stably expressing human CB₁R or CB₂R. **(C)** Scheme of the HTRF-based competitive binding assay. The GPCR of interest with the SNAP-tagged enzyme fused to its N-terminal domain is expressed at the cell surface. SNAP is a commercially available tag consisting of circa 180 amino acids, that can be labeled with fluorophores or other probes in a covalent fashion. The GPCR–SNAP-tagged cells are subsequently labeled with a Tb-containing probe (SNAP-Lumi4-Tb) through a covalent bond between the Tb and the reactive side of the SNAP enzyme. The Tb acts as FRET donor of an acceptor covalently linked to a selective CB₂ receptor ligand. Thus, upon binding of a fluorophore-conjugated ligand (FRET acceptor) on the donor-labeled SNAP-tagged/GPCR fusion protein, an HTRF signal from the sensitized acceptor can be detected since the energy transfer can occur only when the donor and the acceptor are in close proximity. In competition binding assays using CM-157, the unlabelled specific ligand competes for receptor binding site with the fluorophore-conjugated ligand, leading to a decrease in the HTRF signal detected. **(D–G)** HEK-293T were transiently transfected with 1 μg cDNA for SNAP-CB₂R in the absence **(D,E)** or presence of 0.5 μg cDNA for CB₁R **(F,G)**. Competition curves of specific binding of 20 nM fluorophore-conjugated CM-157 using CM-157 (0–10 μM) **(D,F)** or of CBG (0–10 μM) **(E,G)** as competitors are shown. Data represent the mean ± SEM of five experiments in triplicates.

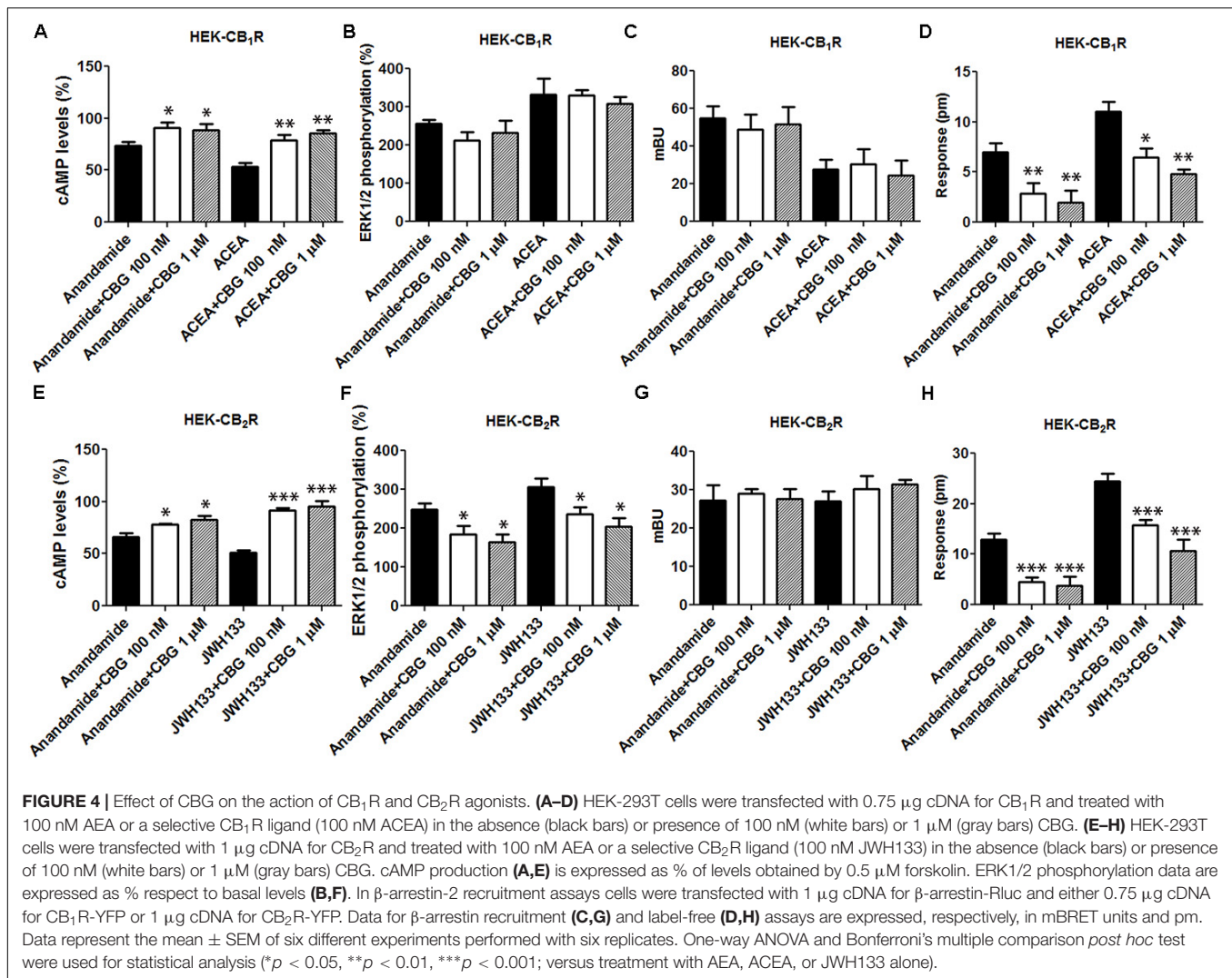


negligible except for the negative modulation of the ACEA effect on ERK1/2 phosphorylation and DMR, and of the AEA effect on DMR read-outs (Figures 5F–H). Therefore, CBG either blunted the cAMP-dependent signaling or did not significantly alter the negative cross-talk when other CB₁/CB₂-mediated signaling read-outs were determined (see Figures 5B,C). It should be noted that cross-talk at the intracellular signaling level, cannot be ruled out to partly explain some of the findings (Bayewitch et al., 1995; Wartmann et al., 1995; McGuinness et al., 2009; Peters and Scott, 2009; Van Der Lee et al., 2009).

DISCUSSION

The aim of this paper was to comparatively address CBG pharmacology and effects on CB₁ and CB₂ receptors, and on CB₁–CB₂ heteroreceptor complexes. The binding experiments using radiolabelled- and non-radiolabelled-based approaches have provided relevant results. The results on CB₂R are clear an indicate that CBG acts as a competitive partial agonist ligand. There is, however, an interesting observation as the K_i values for competing both [³H]-CP-55940 and [³H]-WIN-55,212-2 are in the low micromolar range (Table 1), whereas displaying a value of 152 nM in HTRF-based assays. As pointed out in previous reports, the conditions of the approach using a fluorescent-conjugated CM-157 allows identification of different states of the receptor. Irrespective of the molecular mechanism, the marked differences in affinity constants suggest different

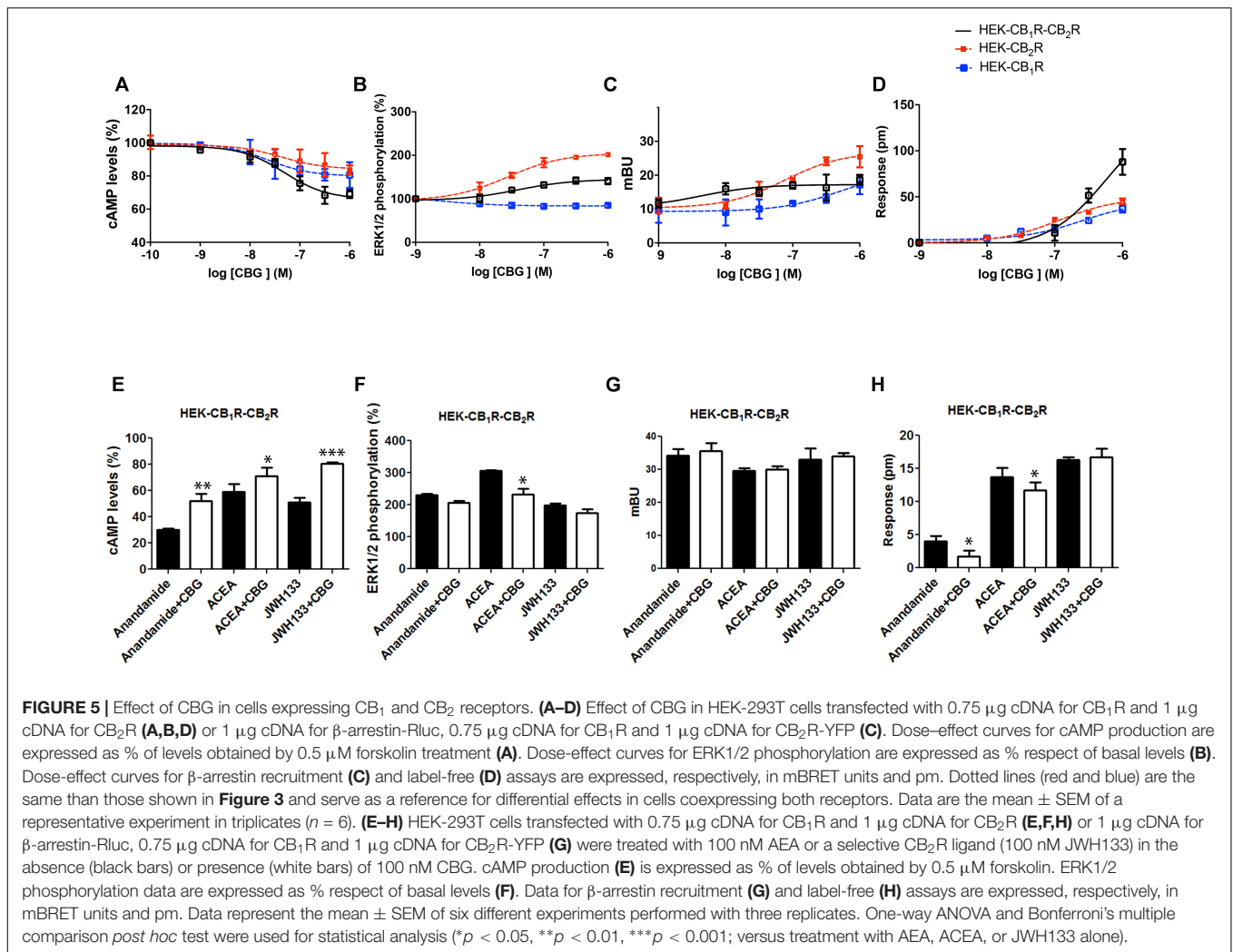
ways to accommodate the ligand within the orthosteric center. To our knowledge this is the first report performed in parallel binding assays using three different ligands that reportedly bind to the orthosteric center of the CB₂R ([³H]-CP-55940, [³H]-WIN-55,212-2 and fluorescence-conjugated-CM-157). In summary, the most reasonable assumption is that CBG binds to the orthosteric center of CB₂R but with marked differences in affinity depending on the assay. It should be noted that differences in affinity may result from the fact that HTRF binding is performed in living cells whereas radioligand binding assays are performed in isolated membranes. The already existing data concerning CBG affinity for CB₁ and CB₂ receptors, all performed using [³H]-CP-55940 also indicate that the affinity may vary depending on the context of the receptor, by *inter alia* the constraints of the membrane, heteromerization or interaction with G-proteins. Comparing our results with similar data using [³H]-CP-55940, the affinity is higher for receptors expressed in HEK-293 cells or in brain membranes (Gauson et al., 2007; Pertwee, 2008; Pollastro et al., 2011) that in receptors expressed in CHO cells (Table 1). In competition assays of radioligand binding to CB₁R or to CB₂R, affinity for CBG is similar to that previously published (Gauson et al., 2007; Pertwee, 2008), except in the case of Sf9 cells (K_i : 897 and 153 nM for, respectively, CB₁R and CB₂R). This piece of data would indicate conformational changes induced by third molecules that affect the binding of the radioligand and/or of CBG. In fact, Sf9 are insect cells that do not express the cognate G_i protein and, therefore, Gαi3β1γ2 was heterologously expressed to perform the



binding assays that led to different affinities for CBG (897 and 153 nM for, respectively, CB₁R and CB₂R) (Rosenthaler et al., 2014).

The results from binding to the CB₁R are not very robust and more difficult to interpret. Unfortunately, there are no ligands available to perform HTRF binding to SNAP-CB₁R-expressing living cells, whereas the data from competition assays using [³H]-CP-55940 or [³H]-WIN-55,212-2 were contradictory. On the one hand, the *K*_i for binding to the CB₁R using [³H]-CP-55940 was in the low micromolar range, as it occurred with data from radioligand binding to the CB₂R. However, CBG was unable to compete [³H]-WIN-55,212-2 binding to the CB₁R. Taking into account that recognition sites for CP-55940 and WIN-55,212-2 are not identical in the CB₁R, one possibility is that CBG binds to the orthosteric center but displaying different equilibrium binding parameters depending on the radioligand. It was early observed that Lys¹⁹² in the CB₁R third transmembrane domain (TM3) was crucial for binding of CP-55940 and AEA but not for WIN-55,212-2 (Bonner et al., 1996; Chin et al., 1998). Later, *in silico* models pointed to an hydrophobic pocket for CP-55940

binding that involved residues in different transmembrane domains (not only in TM3) and in the second extracellular loop (Shim et al., 2003). Those models showed that WIN-55,212-2 not only binds to the hydrophobic pocket described for CP-55940 but to another hydrophobic region involving residues in TM2 and TM3 (Shim and Howlett, 2006). The structure of CBG is more similar to CP-55940 than to WIN-55,212-2, bearing an OH in the A ring that may interact with the TM3 Lys¹⁹² residue. In brief, CBG binds to the orthosteric center of CB₁R as indicated by the fact that CBG affects CP-55940 binding without affecting the binding of [³H]-WIN-55,212-2. In other words, CBG was able to distinguish between two subregions of the CB₁R orthosteric center. We therefore suggest that pharmacological studies concerning the CB₁R should be run in parallel using radiolabelled CP-55940 and WIN-55,212-2. Interestingly CP-55940 and WIN-55,212-2 are able to fix the CB₁R in two different conformations (Georgieva et al., 2008) and, therefore, CBG would affect more the conformation and signaling arising from occupation of the CP-55940 binding site. Other possibilities cannot be ruled out and, in this respect,



we assayed CBD in competition assays and obtained similar results than those obtained using CBG (**Table 1**). Accordingly, CBG could act on CB₁R (but not on CB₂R) as non-competitive (allosteric) modulator, as described for CBD (Laprairie et al., 2015).

When one compound binds to the orthosteric center and affects several signaling pathways with different potency as in the case of CBG in cells expressing CB₁R, the phenomenon is known as functional selectivity or biased agonism. In cells expressing CB₁R, CBG effect is skewed toward the G_i-mediated signaling pathway. This is in agreement with our finding of significant effect in label-free assays; often DMR signals correlate with effect on cAMP levels in the case of receptors coupled to G_i or G_s proteins (Grundmann and Kostenis, 2015a,b; Hamamoto et al., 2015). It is, however, intriguing that CBG was unable to displace the binding of [³H]-WIN-55,212-2 to the CB₁R. Therefore, an action of CBG on a particular state of the receptor, which, in the case of CB₂R may be disclosed by HTRF binding in living cells (Martínez-Pinilla et al., 2016), cannot be ruled out. Taking together all results, an allosteric action of CBG on the CB₁R would not explain why it is able to engage G_i-mediating

signaling. Another possibility, which was suggested for AM630, a previously considered CB₂R antagonist (Bolognini et al., 2012), is that CBG is a protean agonist displaying biased agonism.

Data from CB₂R-mediated functional assays were easier to interpret. First of all, the efficacy was lower compared to selective synthetic agonists and endocannabinoids. Also, CBG led to biased agonism as the effect on cAMP levels was small while being quite marked in ERK phosphorylation and β-arrestin recruitment. Therefore, CBG acted as a partial agonist and, as such, it was able to reduce the effects of other cannabinoid agonists. At 1 μM the effect of CBG on receptor activation by other agonists was similar to that exerted by 100 nM (**Figure 4**) thus suggesting that the *effective* affinity in living cells is that obtained in HTRF non-radioactive-based assays.

Due to the complex pharmacology of cannabinoids this research was undertaken to investigate whether CBG could be exerting a differential action on the CB₁–CB₂ receptor heteromers. Previous data have shown that the interplay between the two receptors in an heteromeric context is also complex. Whereas Callén et al. (2012) showed a negative cross-talk in

a heterologous expression system, the allosteric interaction in the CB₁-CB₂ heteroreceptor complex is synergistic in primary cultures of activated microglia activated with LPS and interferon gamma and in primary cultures of microglia from a transgenic model of Alzheimer's disease (Navarro et al., 2018a). Dose-effect experiments here undertaken in the HEK-293T-based heterologous expression system showed that CBG treatment in the absence of any other agonist, led to additive/synergistic effects on cAMP and label-free read-outs. In contrast, in ERK phosphorylation and β -arrestin recruitment, we found the negative cross-talk already described for this heteromer when full agonists are used to activate the receptors (Callén et al., 2012). These results suggest that partial agonism on the CB₂R is regulated by the presence of CB₁R; however, more complex alternative scenarios cannot be ruled out as CBG may act on the orthosteric site of the CB₂R protomer and as protean agonist of the CB₁R protomer. In cells expressing the two receptors, the overall effect of 100 nM CBG on agonist-induced activation is more consistent with acting on CB₂R than on CB₁R. In fact, the results in co-expressing cells, which likely express heteromers, are similar to those encountered in CB₂R-expressing cells. In summary, CBG significantly modulates CB₂R- or CB₁R/CB₂R-mediated endocannabinoid action, while the effects are weak in CB₁R-expressing cells. Our findings demonstrating the action of CBG on the cannabinoid receptors are in complete agreement and may explain the *in vitro* results, reporting the protection of macrophages against oxidative stress (Giacoppo et al., 2017), and the beneficial *in vivo* effects in a model of inflammatory bowel disease (Borrelli et al., 2013). In the first of these two studies CBG-mediated protection is blocked by AM630, a selective CB₂R ligand, whereas the CB₁R antagonist, SR141716A, had no effect on CBG action (Giacoppo et al., 2017). The second study reported that CBG may both reduce the histological and molecular changes of experimental colitis and nitrite release from macrophages after LPS stimulation; again these effects were seemingly mediated by CB₂R (Borrelli et al., 2013). These results can be explained by our findings; CBG acting as a partial agonist

and exerting actions via CB₂R in macrophages (Giacoppo et al., 2017) or “antagonizing” the effects of endogenous or synthetic cannabinoids, as in LPS-stimulated macrophages (Borrelli et al., 2013). In conclusion, the results presented in this study reveal that the non-psychotropic phytocannabinoid, CBG, may exert beneficial actions with therapeutic potential via cannabinoid receptors.

AUTHOR CONTRIBUTIONS

XN and RF had the original idea, designed and coordinated actions in the different participating institutions, and wrote the initial manuscript. GN performed non-radiolabelled-based homogeneous binding assays, participated in the signaling experiments, and significantly contributed to manuscript preparation. IR-R participated in the signaling experiments and in writing methods. RR-S actively participated in data analysis and parameter calculation. EC supervised data analysis, provided pharmacological expertise, and insight into data interpretation. FV performed the radioligand binding experiments. KV and PB performed the radioligand binding data analysis and interpretation. SC selected the *Cannabis* varieties and supervised the production of the vegetal raw material used for the isolation and purification of cannabinoids. VSM performed the isolation and purification of cannabinoids. CS-CC and CF-V performed the analytical quality control to the purified cannabinoids. All co-authors critically revised, contributed to the editing, and approved the manuscript.

FUNDING

This work was partially supported by grants from the Spanish Ministry of Economy and Competitiveness (Ref. No. BFU2015-64405-R and SAF2017-84117-R; they may include FEDER funds) and by a grant 201413-30 from: *Fundació la Marató de TV3*.

REFERENCES

- Adams, A. J., Banister, S. D., Irizarry, L., Trecki, J., Schwartz, M., and Gerona, R. (2017). “Zombie” outbreak caused by the synthetic cannabinoid AMB-FUBINACA in New York. *N. Engl. J. Med.* 376, 235–242. doi: 10.1056/NEJMoa1610300
- Bayewitch, M., Avidor-Reiss, T., Levy, R., Barg, J., Mechoulam, R., and Vogel, Z. (1995). The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling. *FEBS Lett.* 375, 143–147. doi: 10.1016/0014-5793(95)01207-U
- Bolognini, D., Grazia, A., Parolaro, D., and Pertwee, R. G. (2012). AM630 behaves as a protean ligand at the human cannabinoid CB₂ receptor. *Br. J. Pharmacol.* 165, 2561–2574. doi: 10.1111/j.1476-5381.2011.01503.x
- Bonner, I., Song, Z. H., and Bonner, T. I. (1996). A lysine residue of the cannabinoid receptor is critical for receptor recognition by several agonists but not WIN55212-2. *Mol. Pharmacol.* 49, 891–896.
- Borrelli, F., Fasolino, I., Romano, B., Capasso, R., Maiello, F., Coppola, D., et al. (2013). Beneficial effect of the non-psychotropic plant cannabinoid cannabigerol on experimental inflammatory bowel disease. *Biochem. Pharmacol.* 85, 1306–1316. doi: 10.1016/j.bcp.2013.01.017
- Callén, L., Moreno, E., Barroso-Chinea, P., Moreno-Delgado, D., Cortés, A., Mallol, J., et al. (2012). Cannabinoid receptors CB₁ and CB₂ form functional heteromers in brain. *J. Biol. Chem.* 287, 20851–20865. doi: 10.1074/jbc.M111.335273
- Cascio, M. G., Gauson, L. A., Stevenson, L. A., Ross, R. A., and Pertwee, R. G. (2010). Evidence that the plant cannabinoid cannabigerol is a highly potent α 2-adrenoceptor agonist and moderately potent 5HT 1A receptor antagonist. *Br. J. Pharmacol.* 159, 129–141. doi: 10.1111/j.1476-5381.2009.00515.x
- Cheng, H. C. (2001). The power issue: determination of KB or Ki from IC₅₀ - A closer look at the Cheng-Prusoff equation, the Schild plot and related power equations. *J. Pharmacol. Toxicol. Methods* 46, 61–71. doi: 10.1016/S1056-8719(02)00166-1
- Chin, C. N., Lucas-Lenard, J., Abadji, V., and Kendall, D. A. (1998). Ligand binding and modulation of cyclic AMP levels depend on the chemical nature of residue 192 of the human cannabinoid receptor 1. *J. Neurochem.* 70, 366–373.
- De Petrocellis, L., Ligresti, A., Moriello, A. S., Allarà, M., Bisogno, T., Petrosino, S., et al. (2011). Effects of cannabinoids and cannabinoid-enriched *Cannabis* extracts on TRP channels and endocannabinoid metabolic enzymes. *Br. J. Pharmacol.* 163, 1479–1494. doi: 10.1111/j.1476-5381.2010.01166.x
- De Petrocellis, L., Vellani, V., Schiano-Moriello, A., Marini, P., Magherini, P. C., Orlando, P., et al. (2008). Plant-derived cannabinoids modulate the activity of transient receptor potential channels of ankyrin type-1 and melastatin type-8. *J. Pharmacol. Exp. Ther.* 325, 1007–1015. doi: 10.1124/jpet.107.134809

- Diana, M. A., and Marty, A. (2004). Endocannabinoid-mediated short-term synaptic plasticity: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE). *Br. J. Pharmacol.* 142, 9–19. doi: 10.1038/sj.bjp.0705726
- Fellermeier, M., and Zenk, M. H. (1998). Prenylation of olivetolate by a hemp transferase yields cannabigerolic acid, the precursor of tetrahydrocannabinol. *FEBS Lett.* 427, 283–285. doi: 10.1016/S0014-5793(98)00450-5
- Gaoni, Y., and Mechoulam, R. (1964). Structure synthesis of cannabigerol new hashish constituent. *Proc. Chem. Soc. Lond.* 82, 2189–2192.
- Gauson, L. A., Stevenson, L. A., Thomas, A., Baillie, G. L., Ross, R. A., and Pertwee, R. G. (2007). “Cannabigerol behaves as a partial agonist at both CB1 and CB2 receptors,” in *Proceedings of the 17th Annual Symposium on the Cannabinoids*, (Burlington, VT: International Cannabinoid Research Society), 206.
- Georgieva, T., Devanathan, S., Stropova, D., Park, C. K., Salamon, Z., Tollin, G., et al. (2008). Unique agonist-bound cannabinoid CB1 receptor conformations indicate agonist specificity in signaling. *Eur. J. Pharmacol.* 581, 19–29. doi: 10.1016/j.ejphar.2007.11.053
- Giacoppo, S., Gugliandolo, A., Trubiani, O., Pollastro, F., Grassi, G., Bramanti, P., et al. (2017). Cannabinoid CB2 receptors are involved in the protection of RAW264.7 macrophages against the oxidative stress: An in vitro study. *Eur. J. Histochem.* 61, 1–13. doi: 10.4081/ejh.2017.2749
- Granja, A. G., Carrillo-Salinas, F., Pagani, A., Gómez-Cañas, M., Negri, R., Navarrete, C., et al. (2012). A cannabigerol quinone alleviates neuroinflammation in a chronic model of multiple sclerosis. *J. Neuroimmune Pharmacol.* 7, 1002–1016. doi: 10.1007/s11481-012-9399-3
- Grundmann, M., and Kostenis, E. (2015a). Holistic methods for the analysis of cNMP effects. *Handb. Exp. Pharmacol.* 238, 339–357. doi: 10.1007/164_2015_42
- Grundmann, M., and Kostenis, E. (2015b). Label-free biosensor assays in GPCR screening. *Methods Mol. Biol.* 1272, 199–213. doi: 10.1007/978-1-4939-2336-6_14
- Grunfeld, Y., and Edery, H. (1969). Psychopharmacological activity of the active constituents of hashish and some related cannabinoids. *Psychopharmacologia* 14, 200–210. doi: 10.1007/BF00404218
- Hamamoto, A., Kobayashi, Y., and Saito, Y. (2015). Identification of amino acids that are selectively involved in Gi/o activation by rat melanin-concentrating hormone receptor 1. *Cell. Signal.* 27, 818–827. doi: 10.1016/j.cellsig.2015.01.008
- Hinz, S., Navarro, G., Borroto-Escuela, D., Seibt, B. F., Ammon, C., de Filippo, E., et al. (2018). Adenosine A2A receptor ligand recognition and signaling is blocked by A2B receptors. *Oncotarget* 9, 13593–13611. doi: 10.18632/oncotarget.24423
- Kebig, A., Kostenis, E., Mohr, K., and Mohr-Andrä, M. (2009). An optical dynamic mass redistribution assay reveals biased signaling of dualistic GPCR activators. *J. Recept. Signal Transduct.* 29, 140–145. doi: 10.1080/10799890903047437
- Lanciego, J. L., Barroso-Chinea, P., Rico, A. J., Conte-Perales, L., Callén, L., Roda, E., et al. (2011). Expression of the mRNA coding the cannabinoid receptor 2 in the pallidal complex of *Macaca fascicularis*. *J. Psychopharmacol.* 25, 97–104. doi: 10.1177/0269881110367732
- Laprairie, R. B., Bagher, A. M., Kelly, M. E. M., and Denovan-Wright, E. M. (2015). Cannabidiol is a negative allosteric modulator of the cannabinoid CB1 receptor. *Br. J. Pharmacol.* 172, 4790–4805. doi: 10.1111/bph.13250
- Lu, H.-C. C., and Mackie, K. (2016). An introduction to the endogenous cannabinoid system. *Biol. Psychiatry* 79, 516–525. doi: 10.1016/j.biopsych.2015.07.028
- Martínez-Pinilla, E., Rabal, O., Reyes-Resina, I., Zamarbide, M., Navarro, G., Sanchez-Arias, J. A., et al. (2016). Two affinity sites of the cannabinoid subtype 2 receptor identified by a novel homogeneous binding assay. *J. Pharmacol. Exp. Ther.* 358, 580–587. doi: 10.1124/jpet.116.234948
- McGuinness, D., Malikzay, A., Visconti, R., Lin, K., Bayne, M., Monsma, F., et al. (2009). Characterizing cannabinoid CB2 receptor ligands using DiscoverX PathHunter™ β -arrestin assay. *J. Biomol. Screen.* 14, 49–58. doi: 10.1177/1087057108327329
- McPartland, J. M., Glass, M., and Pertwee, R. G. (2007). Meta-analysis of cannabinoid ligand binding affinity and receptor distribution: Interspecies differences. *Br. J. Pharmacol.* 152, 583–593. doi: 10.1038/sj.bjp.0707399
- Mechoulam, R. (2016). Cannabis - The Israeli perspective. *J. Basic Clin. Physiol. Pharmacol.* 27, 181–187. doi: 10.1515/jbcpp-2015-0091
- Mechoulam, R., Shani, A., Edery, H., and Grunfeld, Y. (1970). Chemical basis of hashish activity. *Science* 169, 611–612. doi: 10.1126/science.169.3945.611
- Medrano, M., Aguinaga, D., Reyes-Resina, I., Canela, E. I., Mallol, J., Navarro, G., et al. (2017). Orexin A/Hypocretin modulates leptin receptor-mediated signaling by allosteric modulations mediated by the Ghrelin GHS-R1A receptor in hypothalamic neurons. *Mol. Neurobiol.* 55, 4718–4730. doi: 10.1007/s12035-017-0670-8
- Merighi, S., Simioni, C., Gessi, S., Varani, K., and Borea, P. A. (2010). Binding thermodynamics at the human cannabinoid CB1 and CB2 receptors. *Biochem. Pharmacol.* 79, 471–477. doi: 10.1016/j.bcp.2009.09.009
- Nadal, X. (2016). Methods of purifying cannabinoids, compositions and kits thereof. U.S. Patent No 9,765,000. Washington, DC: U.S. Patent and Trademark Office.
- Nadal, X., del Río, C., Casano, S., Palomares, B., Ferreira-Vera, C., Navarrete, C., et al. (2017). Tetrahydrocannabinolic acid is a potent PPAR γ agonist with neuroprotective activity. *Br. J. Pharmacol.* 174, 4263–4276. doi: 10.1111/bph.14019
- Navarro, G., Borroto-Escuela, D., Angelats, E., Etayo, Í., Reyes-Resina, I., Pulido-Salgado, M., et al. (2018a). Receptor-heteromer mediated regulation of endocannabinoid signaling in activated microglia. Role of CB1 and CB2 receptors and relevance for Alzheimer's disease and levodopa-induced dyskinesia. *Brain Behav. Immun.* 67, 139–151. doi: 10.1016/j.bbi.2017.08.015
- Navarro, G., Cordero, A., Brugarolas, M., Moreno, E., Aguinaga, D., Pérez-Benito, L., et al. (2018b). Cross-communication between Gi and Gs in a G-protein-coupled receptor heterotetramer guided by a receptor C-terminal domain. *BMC Biol.* 16:24. doi: 10.1186/s12915-018-0491-x
- Navarro, G., Cordero, A., Zelman-Femiak, M., Brugarolas, M., Moreno, E., Aguinaga, D., et al. (2016). Quaternary structure of a G-protein-coupled receptor heterotetramer in complex with Gi and Gs. *BMC Biol.* 14:26. doi: 10.1186/s12915-016-0247-4
- Navarro, G., Ferré, S., Cordero, A., Moreno, E., Mallol, J., Casadó, V., et al. (2010). Interactions between intracellular domains as key determinants of the quaternary structure and function of receptor heteromers. *J. Biol. Chem.* 285, 27346–27359. doi: 10.1074/jbc.M110.115634
- Pertwee, R. G. (2008). The diverse CB 1 and CB 2 receptor pharmacology of three plant cannabinoids: Δ 9-tetrahydrocannabinol, cannabidiol and Δ 9-tetrahydrocannabivarin. *Br. J. Pharmacol.* 153, 199–215. doi: 10.1038/sj.bjp.0707442
- Peters, M. F., and Scott, C. W. (2009). Evaluating cellular impedance assays for detection of GPCR pleiotropic signaling and functional selectivity. *J. Biomol. Screen.* 14, 246–255. doi: 10.1177/1087057108330115
- Pollastro, F., Tagliatela-Scafati, O., Allarà, M., Muñoz, E., Di Marzo, V., De Petrocellis, L., et al. (2011). Bioactive prenylogous cannabinoid from fiber hemp (*Cannabis sativa*). *J. Nat. Prod.* 74, 2019–2022. doi: 10.1021/np200500p
- Rosenthaler, S., Pöhn, B., Kolmanz, C., Nguyen Huu, C., Krewenka, C., Huber, A., et al. (2014). Differences in receptor binding affinity of several phytocannabinoids do not explain their effects on neural cell cultures. *Neurotoxicol. Teratol.* 46, 49–56. doi: 10.1016/j.ntt.2014.09.003
- Schröder, R., Merten, N., Mathiesen, J. M., Martini, L., Kruljac-Leticic, A., Krop, F., et al. (2009). The C-terminal tail of CRTH2 is a key molecular determinant that constrains Gi α and downstream signaling cascade activation. *J. Biol. Chem.* 284, 1324–1336. doi: 10.1074/jbc.M806867200
- Shim, J. Y., and Howlett, A. C. (2006). WIN55212-2 docking to the CB 1 cannabinoid receptor and multiple pathways for conformational induction. *J. Chem. Inf. Model.* 46, 1286–1300. doi: 10.1021/ci0504824
- Shim, J. Y., Welsh, W. J., and Howlett, A. C. (2003). Homology model of the CB1 cannabinoid receptor: sites critical for nonclassical cannabinoid agonist interaction. *Biopolym. Pept. Sci. Sect.* 71, 169–189. doi: 10.1002/bip.10424
- Sierra, S., Luquin, N., Rico, A. J., Gómez-Bautista, V., Roda, E., Dopeso-Reyes, I. G., et al. (2015). Detection of cannabinoid receptors CB1 and CB2 within basal ganglia output neurons in macaques: changes following experimental parkinsonism. *Brain Struct. Funct.* 220, 2721–2738. doi: 10.1007/s00429-014-0823-8
- Solymosi, K., and Kofalvi, A. (2017). Cannabis: a treasure trove or Pandora's box? *Mini Rev. Med. Chem.* 17, 1223–1291. doi: 10.2174/1389557516666161004162133
- Turner, S. E., Williams, C. M., Iversen, L., and Whalley, B. J. (2017). Molecular pharmacology of phytocannabinoids. *Prog. Chem. Org. Nat. Prod.* 103, 61–101. doi: 10.1007/978-3-319-45541-9_3

- Van Der Lee, M. M. C., Blumenröhr, M., Van Der Doelen, A. A., Wat, J. W. Y., Smits, N., Hanson, B. J., et al. (2009). Pharmacological characterization of receptor redistribution and β -arrestin recruitment assays for the cannabinoid receptor 1. *J. Biomol. Screen.* 14, 811–823. doi: 10.1177/1087057109337937
- Wartmann, M., Campbell, D., Subramanian, A., Burstein, S. H., and Davis, R. J. (1995). The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Lett.* 359, 133–136. doi: 10.1016/0014-5793(95)00027-7
- Weinstein, A. M., Rosca, P., Fattore, L., and London, E. D. (2017). Synthetic cathinone and cannabinoid designer drugs pose a major risk for public health. *Front. Psychiatry* 8:156. doi: 10.3389/fpsy.2017.00156

Conflict of Interest Statement: Authors declare that this research was undertaken in collaboration with PhytoPlant Research S.L. Co-authors working in the Spanish and Italian public institutions do not receive honoraria from the company and do not have any participation in the company (stock shares or similar).

Copyright © 2018 Navarro, Varani, Reyes-Resina, Sánchez de Medina, Rivas-Santisteban, Sánchez-Carnerero Callado, Vincenzi, Casano, Ferreiro-Vera, Canela, Borea, Nadal and Franco. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.