

Cannabidiol is a negative allosteric modulator of the type 1 cannabinoid receptor.

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Author Contribution Statement

- RB Laprairie performed the research.
- RB Laprairie, AM Bagher and EM Denovan-Wright designed the research study.
- MEM Kelly and AM Bagher contributed essential reagents and tools.
- RB Laprairie analysed the data.
- RB Laprairie, MEM Kelly and EM Denovan-Wright wrote the paper.

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Abbreviations:

2-AG, 2-arachidonyl glycerol;

6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran, O-2050;

ANOVA, analysis of variance;

BRET, bioluminescence resonance energy transfer;

BRET_{Eff}, BRET efficiency

CB₁, type 1 cannabinoid receptor;

CB₂, type 2 cannabinoid receptor;

CBD, cannabidiol;

CRC, concentration-response curve

FAAH, fatty acid amide hydrolase;

GPCR, G protein-coupled receptor

HEK 293A cells, human embryonic kidney 293A cells;

HERG, human ether-a-go-go-related-gene;

NAM, negative allosteric modulator;

PAM, positive allosteric modulator;

PPAR γ , peroxisome proliferator-activated receptor γ ;

SEM, standard error of the mean;

THC, Δ^9 -tetrahydrocannabinol;

TRPV1, transient receptor potential cation channel subfamily V 1;

Abstract

Background and purpose

Cannabidiol has been reported to act as an antagonist of cannabinoid agonists at type 1 cannabinoid receptors (CB₁). We hypothesized that cannabidiol can inhibit cannabinoid agonist activity through negative allosteric modulation of CB₁.

Experimental approach

CB₁ internalization, arrestin2 recruitment, and PLCβ3 and ERK1/2 phosphorylation, were quantified in HEK 293A cells heterologously expressing CB₁ and in the *STHdh*^{Q7/Q7} cell model of striatal neurons endogenously expressing CB₁. Cells were treated with 2-arachidonylglycerol or Δ⁹-tetrahydrocannabinol alone and in combination with different concentrations of cannabidiol.

Key results

Cannabidiol reduced the efficacy and potency of 2-arachidonylglycerol and Δ⁹-tetrahydrocannabinol on PLCβ3- and ERK1/2-dependent signaling in cells heterologously (HEK 293A) or endogenously (*STHdh*^{Q7/Q7}) expressing CB₁. By reducing arrestin2 recruitment to CB₁, cannabidiol treatment prevented CB₁ internalization. The allosteric activity of cannabidiol depended upon polar residues being present at positions 98 and 107 in the extracellular amino-terminus.

Conclusions and implications

Cannabidiol behaved as a non-competitive negative allosteric modulator of CB₁. Allosteric modulation, in conjunction with non-CB₁ effects, may explain the *in vivo* effects of cannabidiol. Allosteric modulators of CB₁ have the potential to treat central nervous system and peripheral disorders while avoiding the adverse effects associated with orthosteric agonism or antagonism of CB₁.

Keywords

2-arachidonylglycerol, Cannabinoid, Cannabidiol, CB1, Allosteric modulator, Tetrahydrocannabinol

Introduction

Allosteric modulation of CB₁

The majority of available drugs that target G protein-coupled receptors (GPCR) act at the receptor's orthosteric site – the site at which the endogenous ligand binds (Christopoulos and Kenakin, 2002). The type 1 cannabinoid receptor (CB₁) is the most abundant GPCR in the central nervous system and is expressed throughout the periphery (reviewed in Ross, 2007; Pertwee, 2008). Orthosteric ligands of CB₁ have been touted as possible treatments for anxiety and depression, epilepsy, neurodegenerative diseases such as Huntington disease and Parkinson disease, and chronic pain (Pertwee, 2008; Piscitelli *et al.*, 2012), and have been tested in the treatment of addiction, obesity, and diabetes (Pertwee, 2008; Piscitelli *et al.*, 2012). Despite their therapeutic potential, orthosteric agonists of CB₁ are limited by their potential psychomimetic effects while orthosteric antagonists of CB₁ are limited by their depressant effects (Ross, 2007).

An allosteric binding site is a distinct domain from the orthosteric site that can bind to small molecules or other proteins in order to modulate receptor activity (Wootten *et al.*, 2013). All class A, B, and C GPCRs investigated to date possess allosteric binding sites (Wootten *et al.*, 2013). Ligands that bind to receptor allosteric sites may be classified as *allosteric agonists* that can activate a receptor independent of other ligands, *allosteric modulators* that alter the potency and efficacy of the orthosteric ligand but cannot activate the receptor alone, and *mixed agonist/modulator ligands*. As therapeutics, allosteric modulators, unlike allosteric agonists and mixed agonist/modulator ligands, are attractive because they lack intrinsic efficacy. Therefore, the effect ceiling of an allosteric modulator is determined by the endogenous or exogenous orthosteric ligand (Wootten *et al.*, 2013). In contrast, exogenous orthosteric ligands may produce adverse effects through supra-physiological overactivation or down-regulation of a receptor (Wootten *et al.*, 2013). Unlike orthosteric ligands, allosteric modulators of CB₁ may not produce these undesirable side effects because their efficacy depends on the presence of orthosteric ligands, such as the two major endocannabinoids anandamide and 2-arachidonylglycerol (2-AG) (Ross, 2007; Wootten *et al.*, 2013).

To date, the best-characterized allosteric modulators of CB₁ are the positive allosteric modulator (PAM) Lipoxin A₄ (Pamplona *et al.*, 2012) and the negative allosteric modulators (NAM) ORG27569 and PSNCBAM-1 (Price *et al.*, 2005; Horswill *et al.*, 2007; Wang *et al.*, 2011; Ahn *et al.*, 2013). ORG27569 and PSNCBAM-1 reduce the efficacy and potency of CB₁ agonists WIN55,212-2 and CP55,940 to stimulate GTPγS³⁵, enhance Gα_{i/o}-dependent signaling and arrestin recruitment, and inhibit CB₁ internalization and cAMP accumulation at submicromolar concentrations (Price *et al.*, 2005; Horswill *et al.*, 2007; Wang *et al.*, 2011; Ahn *et al.*, 2013; Cawston *et al.*, 2013). The well-characterized NAM activities of ORG27569 and PSNCBAM-1 are the archetypes against which novel CB₁ NAMs are compared.

Cannabidiol as a possible negative allosteric modulator of CB₁

Cannabidiol (CBD) is known to modulate the activity of many cellular effectors, including CB₁, the type 2 cannabinoid receptor (CB₂) (Hayakawa *et al.*, 2008), the serotonin 5HT_{1A} receptor (Russo *et al.*, 2005), GPR55 (Ryberg *et al.*, 2007), the μ- and δ-opioid receptors (Kathmann *et al.*, 2006), the transient receptor potential cation channel subfamily V 1 (TRPV1) (Bisogno *et al.*, 2001), the peroxisome proliferator-activated receptor γ (PPARγ) (Campos *et al.*, 2012), and fatty acid amide hydrolase (FAAH) (Bisogno *et al.*, 2001). With regard to cannabinoid receptor-specific effects, several *in vitro* and *in vivo* studies have reported that CBD acts as an antagonist of cannabinoid agonists at CB₁ at doses well below the reported affinity (K_i) for CBD to the orthosteric agonist site of CB₁ (Pertwee *et al.*, 2002; Ryan *et al.*, 2007; Thomas *et al.*, 2007; McPartland *et al.*, 2014). We recently reported that the effects of CBD on intracellular signaling were largely CB₁-independent (Laprairie *et al.*,

2014a). However, CBD inhibited CB₁ internalization *in vitro* at submicromolar concentrations where no other CB₁-dependent effect on signaling was observed (Laprairie *et al.*, 2014a). Given the similarity with ORG27569 and PSNCBAM-1 inhibition of CB₁ internalization, and existing *in vivo* data suggesting CBD can act as a potent antagonist of CB₁ agonists, we hypothesized that CBD has NAM activity at CB₁.

Objective of this study

The objective of this study was to determine whether CBD had NAM activity at CB₁ *in vitro*. The NAM activity of CBD was tested for arrestin, Gα_q (PLCβ3), and Gα_{i/o} (ERK1/2) pathways using 2-AG and Δ⁹-tetrahydrocannabinol (THC) as the orthosteric probes and compared to the competitive antagonist O-2050 (Hudson *et al.*, 2010; Laprairie *et al.*, 2014). While some studies have suggested O-2050 may be a partial agonist of CB₁ (Wiley *et al.*, 2011, 2012), several groups have noted the competitive antagonistic activity of O-2050 at CB₁ (Canals and Milligan, 2008; Higuchi *et al.*, 2010; Ferreira *et al.*, 2012; Anderson *et al.*, 2013). Allosteric effects of CBD were studied using an operational model of allosterism (Keov *et al.*, 2011). Using this operational model, we were able to estimate ligand cooperativity (α), changes in efficacy (β), and orthosteric and allosteric ligand affinity (K_A and K_B) (Keov *et al.*, 2011) and support our hypothesis that CBD displayed NAM activity at CB₁. HEK 293A and *STHdh*^{Q7/Q7} cells were used to test our hypothesis. HEK 293A cells represent a well-characterized heterologous expression system to study CB₁ signaling while *STHdh*^{Q7/Q7} cells model the major output of the indirect motor pathway of the striatum where CB₁ levels are highest relative to other regions of the brain (Tetrell *et al.*, 2000; Laprairie *et al.*, 2013, 2014a), making this cell line ideally suited to studying endocannabinoid signaling in a more physiologically relevant context.

Methods

Drugs

Drug stocks were made up in ethanol (THC) or DMSO [2-AG, CBD, and (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran (O-2050), *N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251)] and diluted to final solvent concentrations of 0.1%. 2-AG, CBD, and O-2050 were purchased from Tocris Bioscience (Bristol, UK). THC was purchased from Sigma-Aldrich (Oakville, ON).

Cell culture

HEK 293A cells were from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained at 37°C, 5% CO₂ in DMEM supplemented with 10% FBS and 10⁴ U mL⁻¹ Pen/Strep.

STHdh^{Q7/Q7} cells are derived from the conditionally immortalized striatal progenitor cells of embryonic day 14 C57BL/6 mice (Coriell Institute, Camden, NJ) (Tetrell *et al.*, 2000). Cells were maintained at 33°C, 5% CO₂ in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 10⁴ U mL⁻¹ Pen/Strep, and 400 μg mL⁻¹ geneticin. Cells were serum-deprived for 24 h prior to experiments to promote differentiation (Tetrell *et al.*, 2000; Laprairie *et al.*, 2013, 2014a,b).

Plasmids and transfection

Human CB₁, CB_{1A}, CB_{1B}, and arrestin2 (β-arrestin1) were cloned and expressed as either green fluorescent protein² (GFP²) or *Renilla* luciferase (Rluc) fusion proteins. CB₁-GFP², and arrestin2-Rluc were generated using the pGFP²-N3 and pRluc-N1 plasmids (PerkinElmer, Waltham, MA) as described previously (Hudson *et al.*, 2010; Laprairie *et al.*, 2014a). The GFP²-Rluc fusion construct, and Rluc plasmids have been previously described (Laprairie *et al.*, 2014a).

The human CB₁ receptor was mutagenized at two cysteine residues (Cys-98 and Cys-

107). Mutagenesis was conducted as described previously (Laprairie *et al.* 2013) with the cysteine residues being mutated to alanines (C98A, C107A) or serines (C98S, C107S) using the CB₁-GFP² fusion plasmid and the following forward and reverse primers: CB₁^{C98A}-GFP² forward 5'-AACATCCAGGCTGGGGAGAACT-3', reverse 5'-AGTTCTCCCCAGCCTGGATGTT-3'; and CB₁^{C107A}-GFP² forward 5'-GACATAGAGGCTTTCATGGTC-3', reverse 5'-GACCATGAAAGCCTCTATGTC-3'; CB₁^{C98S}-GFP² forward 5'-AACATCCAGTCTGGGGAGAACT-3', reverse 5'-AGTTCTCCCCAGACTGGATGTT-3'; and CB₁^{C107S}-GFP² forward 5'-GACATAGAGTCTTTCATGGTC-3', reverse 5'-GACCATGAAAGACTCTATGTC-3'. Mutagenesis was confirmed by sequencing (GeneWiz, Camden, NJ).

Cells were grown in 6 well plates and transfected with 200 ng of the Rluc fusion plasmid and 400 ng of the GFP² fusion plasmid according to previously described protocols (Laprairie *et al.*, 2014a) using Lipofectamine 2000® according to the manufacturer's instructions (Invitrogen, Burlington, ON). Transfected cells were maintained for 48 h prior to experimentation.

Bioluminescence resonance energy transfer² (BRET²)

Interactions between CB₁ and arrestin2 were quantified *via* BRET² according to previously described methods (Laprairie *et al.*, 2014a). BRET efficiency (BRET_{Eff}) was determined as previously described (James *et al.*, 2006; Laprairie *et al.*, 2014a) such that Rluc alone was used to calculate BRET_{MIN} and the Rluc-GFP² fusion protein was used to calculate BRET_{MAX}.

On- and In-cellTM western

On-cellTM western analyses were completed as described previously (Laprairie *et al.*, 2014a) using primary antibody directed against N-CB₁ (1:500; Cayman Chemical Company, Ann Arbor, MI, Cat No. 101500). All experiments measuring CB₁ included an N-CB₁ blocking peptide control (1:500; Cayman Chemical Company), which was incubated with N-CB₁ antibody (1:500). Immunofluorescence observed with the N-CB₁ blocking peptide was subtracted from all experimental replicates. In-cellTM western analyses were conducted as described previously (Laprairie *et al.*, 2014a). Primary antibody solutions were: N-CB₁ (1:500), pERK1/2(Tyr205/185) (1:200), ERK1/2 (1:200), pPLCβ3(S537) (1:500), PLCβ3 (1:1000), or β-actin (1:2000) (all from Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody solutions were: IR^{CW700dye} or IR^{CW800dye} (1:500; Rockland Immunochemicals, Gilbertsville, PA). Quantification was completed using the Odyssey Imaging system and software (v. 3.0; Li-Cor, Lincoln, NE).

Data analysis and curve fitting

Data are presented as the mean ± the standard error of the mean (SEM) or mean and 95% confidence interval, as indicated, from at least 4 independent experiments. All data analysis and curve fitting was carried out using GraphPad Prism (v. 5.0). Concentration-response curves (CRC) were fit with the non-linear regression with variable slope (4 parameters), Gaddum/Schild EC₅₀ shift model, or operational model of allosterism (Eq. 1) (Keov *et al.*, 2011) and are shown in each figure according to the best-fit model as determined by R² value (GraphPad Prism v. 5.0). Pharmacological statistics were obtained from non-linear regression models as indicated in figures and tables. Global curve fitting of allosterism data was carried out using the following operational model (Hudson *et al.*, 2014; Keov *et al.*, 2011; Smith *et al.*, 2011):

$$E = \frac{E_{\max} (\tau_A [A] (K_B + \alpha \beta [B]) + \tau_B [B] K_A)^n}{([A] K_B + K_A K_B + [B] K_A + \alpha [A] [B])^n + (\tau_A [A] (K_B + \alpha \beta [B]) + \tau_B [B] K_A)^n} \quad \text{Eq. 1}$$

where E is the measured response, A and B are the orthosteric and allosteric ligand concentrations, respectively, E_{\max} is the maximum system response, α is a measure of the allosteric co-operativity on ligand binding, β is a measure of the allosteric effect on efficacy, K_A and K_B are estimates of the binding of the orthosteric and allosteric ligands, respectively, n represents the Hill slope, and τ_A and τ_B represent the abilities of the orthosteric and allosteric ligands to directly activate the receptor (Smith *et al.*, 2011). To fit experimental data to this equation, E_{\max} and n were constrained to 1.0 and 1.0, respectively, which allowed for estimates of α , β , K_A , K_B , τ_A and τ_B .

Relative receptor activity (RA) was calculated according to equation 2 (Christopoulos and Kenakin, 2002):

$$RA = \frac{(E_{\max} \%)(EC_{50} \text{ Agonist Alone})}{(E_{\max} \text{ Agonist Alone} \%)(EC_{50})} \quad \text{Eq. 2}$$

where $E_{\max} \%$ is the E_{\max} of the concentration-response curve in the presence of a given concentration of CBD, EC_{50} is the EC_{50} (μM) in the presence of a given concentration of CBD; $E_{\max} \text{ Agonist Alone} \%$ is the E_{\max} in the absence of CBD; $EC_{50} \text{ Agonist Alone}$ is the EC_{50} (μM) in the absence of CBD. Statistical analyses were one- or two-way analysis of variance (ANOVA), as indicated, using GraphPad. *Post-hoc* analyses were performed using Dunnett's multiple comparisons, Bonferroni's or Tukey's tests, as indicated. Homogeneity of variance was confirmed using Bartlett's test. The level of significance was set to $P < 0.001$ or < 0.01 , as indicated. To improve the readability of the data, all figures have been laid out such that data from HEK 293A cells appears above data from *STHdh*^{Q7/Q7} cells, and data for O-2050 appears before data for CBD (Fig. 1-3).

Results

CB₁ internalization and kinetic experiments

We had previously observed that CBD reduced CB_1 internalization in *STHdh*^{Q7/Q7} cells (Laprairie *et al.*, 2014a). Here, we sought to determine how CBD affected the kinetics of CB_1 internalization and arrestin2 recruitment in *STHdh*^{Q7/Q7} cells. The fraction of CB_1 at the plasma membrane was dose-dependently decreased by THC (Fig. 1A) and 2-AG in *STHdh*^{Q7/Q7} cells (Fig. 1B). The efficacy and potency of THC- and 2-AG-dependent CB_1 internalization were reduced by increasing concentrations of CBD (Fig. 1A,B). BRET² between arrestin2-Rluc and CB_1 -GFP² was measured every 10 s for 4 min in *STHdh*^{Q7/Q7} cells treated with 1 μM THC (Fig. 1C) or 2-AG (Fig. 1D). Increasing concentrations of CBD decreased the rate of association between arrestin2 and CB_1 over 4 min (Fig. 1E) and decreased maximal BRET_{Eff} observed at 10 min (Fig. 1C-E). The fraction of CB_1 at the plasma membrane was also reduced in *STHdh*^{Q7/Q7} cells treated with 1 μM THC (Fig. 1F) or 2-AG (Fig. 1G) over 60 min. CBD alone increased the fraction of CB_1 at the membrane (Fig. 1F-H). The rates of CB_1 internalization, and the maximum fraction of CB_1 internalized were reduced by increasing concentrations of CBD (Fig. 1F-H). Similarly, Cawston *et al.* (2013) observed that the rate of arrestin recruitment to CB_1 was reduced by the allosteric modulator Org27569. Therefore, CBD delayed interactions between CB_1 and arrestin2 and increased the pool of receptors present at the plasma membrane at sub-micromolar concentrations, which is similar to the actions of the previously described CB_1 allosteric modulator Org27569 (Cawston *et al.*, 2013).

CB₁-arrestin2 BRET² experiments

2-AG and THC enhance the interaction between CB_1 and arrestin2, as indicated by BRET² in *STHdh*^{Q7/Q7} cells (Laprairie *et al.*, 2014a). Here, we used HEK 293A cells as a

heterologous expression system for CB₁ and arrestin2 to determine whether CBD acted as a NAM of CB₁. Treatment of HEK 293A cells with 0.01 – 5.00 μ M THC or 2-AG for 30 min produced a dose-dependent increase in BRET_{Eff} between arrestin2-Rluc and CB₁-GFP² (Fig. 2A-D). The CB₁ antagonist O-2050 (0.01 – 5.00 μ M) produced a dose-dependent rightward shift in the THC and 2-AG CRCs that were best fit using the Gaddum/Schild EC₅₀ non-linear regression model indicative of competitive antagonism (Fig. 2A,B). CBD (0.01 – 5.00 μ M) treatment produced a dose-dependent rightward and downward shift in the THC and 2-AG CRCs that were best fit using the operational model of allosterism (Eq.1, Fig. 2C,D). The rightward shift in EC₅₀ was significant at 1.00 μ M and 0.50 μ M CBD for THC- and 2-AG-treated cells, respectively (Table 1). The decrease in E_{\max} was significant at 0.10 and 0.50 μ M for THC- and 2-AG-treated cells, respectively (Table 1). The Hill coefficient (n) was less than 1 at 0.10 and 0.50 μ M for THC- and 2-AG-treated cells, respectively (Table 1). Relative receptor activity (estimated using Eq. 2) was significantly reduced at 0.01 μ M for THC- and 2-AG-treated cells (Table 1). Schild analyses of these data demonstrated that while O-2050 behaved as a competitive antagonist, inhibition of BRET_{Eff} by CBD was non-linear for THC- and 2-AG-treated HEK 293A cells (Fig. 2E, Table 2). These data demonstrated that CBD behaved as a NAM of THC- and 2-AG-mediated arrestin2 recruitment to CB₁ in the HEK 293A heterologous expression system.

The NAM properties of CBD on CB₁-arrestin2 interactions were confirmed in the *STHdh*^{Q7/Q7} cell culture model of medium spiny projection neurons. As in HEK 293A cells, O-2050 treatment produced a dose-dependent rightward shift in the THC and 2-AG CRCs that were best fit using the Gaddum/Schild EC₅₀ non-linear regression model indicative of competitive antagonism (Fig. 2F,G), and CBD treatment produced a dose-dependent rightward and downward shift in the THC and 2-AG CRCs that were best fit using the operational model of allosterism (Fig. 2H,I) in *STHdh*^{Q7/Q7} cells. The rightward shift in EC₅₀ was significant at 0.50 μ M CBD for THC- and 2-AG-treated cells (Table 1). The decrease in E_{\max} was significant at 1.00 and 5.00 μ M for THC- and 2-AG-treated cells, respectively (Table 1). The Hill coefficient (n) was less than 1 at 5.00 and 0.50 μ M for THC- and 2-AG-treated cells, respectively (Table 1). Relative receptor activity (Eq. 2) was significantly reduced at 0.10 μ M for both THC- and 2-AG-treated cells (Table 1). The Schild regression for these data demonstrated that O-2050 modeled competitive antagonism for THC- and 2-AG-treated *STHdh*^{Q7/Q7} cells (greater slope and R²) (Fig. 2J, Table 2). CBD alone displayed weak partial agonist activity in this assay at concentrations > 2 μ M (Suppl Fig. 1). Taken together these data indicate that CBD behaved as a NAM of THC- and 2-AG-mediated arrestin2 recruitment to CB₁ at concentrations below its reported affinity to CB₁ in a cell culture model endogenously expressing CB₁ (Pertwee, 2008).

CB₁-mediated phosphorylation of PLC β 3

THC and 2-AG treatment both result in a dose-dependent increase in PLC β 3 phosphorylation in HEK 293A cells (Fig. 3A-D) and *STHdh*^{Q7/Q7} cells (Laprairie *et al.*, 2014a; Fig. 3F-I). O-2050 treatment resulted in a dose-dependent rightward shift in the THC and 2-AG CRCs (Fig. 3A,B,F,G), while CBD treatment resulted in a rightward and downward shift in the THC and 2-AG CRCs, in both cell lines (Fig. 3C,D,H,I). O-2050 CRCs were best fit with the Gaddum/Schild EC₅₀ model, while CBD CRCs were best fit with the operational model of allosterism. The rightward shift in EC₅₀ was significant at 0.50 μ M CBD for THC- and 2-AG-treated HEK 293A cells (Table 3) and 0.50 and 1.00 μ M CBD for THC- and 2-AG-treated *STHdh*^{Q7/Q7} cells, respectively (Table 3). The decrease in E_{\max} was significant at 1.00 and 0.50 μ M for HEK 293A and *STHdh*^{Q7/Q7} cells, respectively (Table 3). The Hill coefficient (n) was less than 1 at 0.50 μ M for THC- and 2-AG-treated in both HEK 293A and *STHdh*^{Q7/Q7} cells (Tables 1 and 3). Relative receptor activity was significantly reduced at 0.10 μ M for THC- and 2-AG-treated HEK 293A and *STHdh*^{Q7/Q7} cells (Table 3).

The Schild regression for these data demonstrated that O-2050 modeled competitive antagonism for THC- and 2-AG-treated *STHdh*^{Q7/Q7} cells, while CBD did not (greater slope and R^2) (Fig. 3E,J, Table 2). As with arrestin2 recruitment, CBD alone was a weak partial agonist at concentrations > 2 μ M (Suppl Fig. 1). In the presence of 2-AG or THC, CBD was a NAM of PLC β 3 phosphorylation in HEK 293A cells overexpressing CB₁ and *STHdh*^{Q7/Q7} cells endogenously expressing CB₁.

CB₁-mediated phosphorylation of ERK1/2

2-AG treatment results in the phosphorylation of ERK1/2 in *STHdh*^{Q7/Q7} cells, while THC does not (Laprairie *et al.*, 2014a). 2-AG treatment produced a dose-dependent increase in ERK1/2 phosphorylation in both HEK 293A and *STHdh*^{Q7/Q7} cells (Fig. 4A,B,D,E). O-2050 treatment resulted in a dose-dependent rightward shift in the 2-AG CRCs (Fig. 4A,D), while CBD treatment resulted in a rightward and downward shift in the 2-AG CRCs, in both cell lines (Fig. 4B,E). O-2050 CRCs were best fit with the Gaddum/Schild EC₅₀ model, while CBD CRCs were best fit with the operational model of allosterism. The rightward shift in EC₅₀ was significant at 0.50 and 1.00 μ M CBD for HEK 293A and *STHdh*^{Q7/Q7} cells, respectively (Table 4). The decrease in E_{\max} was significant at 5.00 and 1.00 μ M for HEK 293A and *STHdh*^{Q7/Q7} cells, respectively (Table 4). The Hill coefficient (n) was less than 1 at 0.10 and 0.01 μ M CBD for HEK 293A and *STHdh*^{Q7/Q7} cells, respectively (Table 4). Relative receptor activity was significantly reduced at 0.10 and 0.01 μ M for 2-AG-treated HEK 293A and *STHdh*^{Q7/Q7} cells, respectively (Table 4). The Schild regression for these data demonstrated that O-2050 modeled competitive antagonism in HEK293A (Fig. 3C) and *STHdh*^{Q7/Q7} (Fig. 4F) cells, whereas CBD did not (greater slope and R^2) (Table 2). CBD was a NAM of 2-AG-mediated ERK1/2 phosphorylation in HEK 293A cells overexpressing CB₁ and *STHdh*^{Q7/Q7} cells endogenously expressing CB₁ at lower concentrations than those reported for CB₁ agonist activity (Mechoulam *et al.*, 2007; McPartland *et al.*, 2014) (Suppl Fig. 1). Therefore, CBD behaved as a NAM in these cell lines for arrestin2 recruitment, PLC β 3 and ERK1/2 phosphorylation.

Operational modeling of allosterism

While O-2050 acted as a competitive orthosteric antagonist, CBD acted as a NAM in arrestin2, PLC β 3, and ERK1/2 assays. Global curve fitting of data to the operational model of allosterism was used to assess the NAM activity of CBD. Data were fit to this model by constraining E_{\max} and n (Hill slope) to 1.0 and 1.0, respectively. In this way, the allosteric cooperativity coefficient for ligand binding (α) was found to be less than 1.0 (0.37), with no significant difference between cell lines, orthosteric ligands, or assays (Table 5) indicating that CBD acted as a NAM to reduce the binding of THC and 2-AG. CBD also reduced the efficacy of the orthosteric ligand because β (co-operativity coefficient for ligand efficacy) was consistently less than 1 (0.44). Based on the estimated value of orthosteric ligand affinity (K_A) and the ability of the orthosteric ligand to activate CB₁ (τ_A), 2-AG (241 nM) and THC (97 nM) were able to directly activate CB₁ within a similar concentration range to previously published data (reviewed in Pertwee, 2008). CBD did not display agonist activity, as shown by the estimate of τ_B , but exhibited a greater estimated affinity (304 nM) for CB₁ (K_B) than would be predicted for the orthosteric site (reviewed in Pertwee, 2008). β and $\alpha\beta$ can be used to assess ligand bias (functional selectivity) for allosteric modulators (Keov *et al.*, 2011). No differences in β and $\alpha\beta$ were observed in HEK 293A cells in all assays (Table 5). In *STHdh*^{Q7/Q7} cells, β and $\alpha\beta$ were reduced in PLC β 3 assays compared to arrestin2 recruitment and ERK assays, indicating that CBD was a functionally selective inhibitor of arrestin2 and ERK1/2 pathways (Table 5). Overall, CBD was a NAM of orthosteric ligand binding and efficacy at CB₁.

Negative allosteric modulation of antagonist binding

If CBD reduced the binding of orthosteric agonists to CB₁, as predicted by the operational model of allosterism, then CBD should also reduce the binding of CB₁ inverse agonists and antagonists. In order to test this hypothesis, *STHdh*^{Q7/Q7} cells were treated with the CB₁ inverse agonist AM251 (Pertwee, 2005) and CBD and ERK phosphorylation was measured (Fig. 5A). CBD treatment resulted in a rightward and upward shift in the AM251 CRC (Fig. 5A). CBD CRCs were best fit with the operational model of allosterism. To further test our hypothesis, *STHdh*^{Q7/Q7} cells were treated with 2-AG and 500 nM O-2050, 500 nM CBD, or 500 nM O-2050 and 500 nM CBD (Fig 5B). Treatment of *STHdh*^{Q7/Q7} cells with 2-AG, O-2050 and CBD produced a CRC that was shifted right and down relative to 2-AG alone and left relative to 2-AG and O-2050, indicating that CBD had reduced the competitive antagonistic activity of O-2050 and reduced the efficacy of 2-AG (Fig. 5B). Therefore, CBD was a NAM of orthosteric ligand binding as demonstrated by the reduced potency and efficacy of the CB₁ inverse agonist AM251 and the antagonist O-2050.

Mutagenesis of CB₁

The CB₁ splice variants CB_{1A} and CB_{1B} differ in the first 89 amino acids of the N-terminus relative to CB₁. We compared the allosteric activity of CBD in *STHdh*^{Q7/Q7} cells expressing CB₁, CB_{1A} and CB_{1B} using BRET². BRET_{Eff} did not differ between CB₁-GFP², CB_{1A}-GFP², and CB_{1B}-GFP²-expressing cells treated with 0.01 – 5.00 μM THC or 2-AG ± 0.5 μM O-2050 or 5.00 μM CBD (Suppl Fig. 2A,B). Therefore, the allosteric activity of CB₁ is not contained within amino acids 1 – 89 that differ between CB₁, CB_{1A}, and CB_{1B}, but is associated with the conserved residues common to all three variants (Bagher *et al.*, 2013; Fay and Farrens, 2013).

Fay and Farrens (2013) previously reported that Cys-98 and Cys-107 in the extracellular N-terminus of CB₁ contribute to the allosteric activity of ORG27569 and PSNCBAM-1. They suggested that these residues form a disulfide bridge, which contributed to allosteric modulator activity of ORG27569 and PSNCBAM-1 (Fay and Farrens, 2013). We hypothesized that these residues might similarly influence the allosteric activity of CBD. We wanted to determine whether it was the polarity of Cys-98 and Cys-107 or the formation of a disulfide bridge that contributed to allosteric activity. Each of these residues was individually mutagenized to Ala or Ser in the CB₁-GFP² plasmid (CB₁^{WT}-GFP², CB₁^{C98A}-GFP², CB₁^{C107A}-GFP², CB₁^{C98S}-GFP², CB₁^{C107S}-GFP²) and transfected with arrestin2-Rluc into *STHdh*^{Q7/Q7} cells. Treatment of CB₁^{WT}-, CB₁^{C98A}-, CB₁^{C107A}-, CB₁^{C98S}-, or CB₁^{C107S}-expressing cells with 0.01 – 5.00 μM THC or 2-AG alone resulted in a response that did not differ between CB₁ mutants or between THC and 2-AG treatments (Fig. 6A,B). Further, the competitive antagonistic activity of 0.50 μM O-2050 was not different in CB₁ mutant expressing-cells treated with 0.01 – 5.00 μM THC or 2-AG (Suppl Fig. 2C,D). Together, these data indicated that mutation of Cys-98 or Cys-107 did not alter CB₁ response to orthosteric ligand. Treatment of CB₁^{WT}-expressing cells with 0.01 – 5.00 μM THC or 2-AG and 5.00 μM CBD resulted in a rightward and downward shift in the BRET_{Eff} CRCs (Fig. 6A,B). Similarly, treatment of CB₁^{C98A}- or CB₁^{C107A}-expressing cells with 0.01 – 5.00 μM THC or 2-AG and 5.00 μM CBD resulted in a rightward and downward shift in the BRET_{Eff} CRCs compared to vehicle treatment (Table 6). The magnitude of the rightward and downward shift was less pronounced in CB₁^{C98A}- and CB₁^{C107A}- compared to CB₁^{WT}-, CB₁^{C98S}-, and CB₁^{C107S}-expressing cells treated with CBD (Table 6; Fig. 6A,B). The presence of a polar Ser or Cys at positions 98 or 107 was sufficient to recover the wild-type response to CBD. Therefore, the allosteric activity of CBD at CB₁ depended in part on the presence of polar residues at positions 98 and 107, independent of a disulfide bridge. Additional residues common to CB₁, CB_{1A}, and CB_{1B} may also contribute to the allosteric effect of CBD (Fig. 6C).

Discussion and Conclusions

Cannabidiol behaves as a negative allosteric modulator of CB₁

In this study, we provide *in vitro* evidence for the non-competitive negative allosteric modulation of CB₁ by CBD. CBD treatment resulted in negative co-operativity ($\alpha < 1$) and reduced orthosteric ligand (THC and 2-AG) efficacy ($\beta < 1$) at concentrations lower than the predicted affinity of CBD for the orthosteric binding site at CB₁ [304 nM (this study) *versus* $> 4 \mu\text{M}$ (reviewed in Pertwee, 2008)]. As a NAM of CB₁ orthosteric ligand-dependent effects, CBD reduced both G protein-dependent signaling and arrestin2 recruitment, which explains both the diminished signaling and diminished BRET observed between CB₁-GFP² and arrestin2-Rluc. In contrast to the NAM activity of CBD, and as shown previously, O-2050 acted as a competitive orthosteric antagonist of CB₁ (Canals and Milligan, 2008; Higuchi *et al.*, 2010; Hudson *et al.*, 2010; Ferreira *et al.*, 2012; Anderson *et al.*, 2013; Laprairie *et al.*, 2014) rather than a partial agonist (Wiley *et al.*, 2011, 2012). To directly test the hypothesis that a disulfide bridge between Cys-98 and Cys-107 regulates the activity of CB₁ allosteric modulators, these residues were mutagenized to either Ala or Ser (Fay and Farrens, 2013). Mutation of these residues to Ala (non-polar) decreased the NAM activity of CBD at CB₁, but not the activity of THC, 2-AG, or O-2050. The NAM activity of CBD depended upon the presence of polar (Ser or Cys) residues at CB₁ positions 98 and 107, rather than a disulfide bridge, because replacement of either Cys residue with Ser did not change CBD NAM activity. These findings suggest that the N-terminal, extracellular residues Cys-98 and Cys-107 either partially regulate the allosteric activity of CBD at CB₁ directly, or the communication between the allosteric and orthosteric sites of CB₁.

Allosteric modulators are probe-dependent, that is, the activity of the allosteric modulator depends on the orthosteric probe being used (reviewed in Christopoulos and Kenakin, 2002). ORG27569 and PSNCBAM-1 both display probe-dependence because they are more potent modulators of CP55,940 binding and CP55,940-mediated CB₁ activation than WIN55,212-2 binding and WIN55,21-2-mediated CB₁ activation (Baillie *et al.*, 2013). 2-AG was chosen as an orthosteric probe in this study because it is the most abundant endocannabinoid in the brain, and therefore 2-AG would be the predominant endogenous orthosteric ligand if exogenous CBD was administered (Sugiura *et al.*, 1999). THC and CBD are the most abundant phytocannabinoids in marijuana and are used together in varying ratios both medicinally and recreationally in marijuana (Thomas *et al.*, 2007). Therefore, THC was selected as an alternative orthosteric probe. In HEK 293A cells, CBD did not display probe-dependence (Table 2). In *STHdh*^{Q7/Q7} cells, CBD was a more potent NAM of CB₁-dependent arrestin2 recruitment when THC was the orthosteric probe compared to 2-AG (Table 2). No probe-dependence was observed for PLC β 3 and ERK1/2 signaling. BRET was used in this study to directly measure the association of CB₁ and arrestin2, which may be a more sensitive method for detecting probe-dependence than In-cellTM western assays that measured PLC β 3 or ERK1/2.

STHdh^{Q7/Q7} cells express several effector proteins that CBD has been shown to modulate, including CB₁, 5HT_{1A}, GPR55, μ -opioid receptors, PPAR γ and FAAH, suggesting that CBD could have acted independently of CB₁ (Tetrell *et al.*, 2000; Lee *et al.*, 2007; Laprairie *et al.*, 2014a). However, the NAM activity of CBD was also observed in HEK 293A cells that heterologously express CB₁, but do not express 5HT_{1A}, GPR55, and μ -opioid receptors demonstrating that these effectors did not alter the actions of CBD (Ryberg *et al.*, 2007). HEK 293A cells do express PPAR γ , but modulation of this nuclear receptor would not affect arrestin and G protein assays used over the duration of these experiments. Importantly, the NAM activity of CBD at CB₁ was dependent on the cannabinoid agonists 2-AG and THC, suggesting that CBD was acting at CB₁. FAAH inhibition would have enhanced, not

diminished, cannabinoid efficacy, which was not observed here. Therefore, the NAM activity of CBD at CB₁ documented in this study adds to the mechanisms of action through which chronic CBD mediates its effects *in vivo*.

No significant signaling bias was observed for CBD in HEK 293A cells because allosteric ligand efficacy (β) and co-operativity ($\alpha\beta$) were not different among arrestin, PLC β 3, and ERK1/2 assays (Table 5). In *STHdh*^{Q7/Q7} cells, we observed that CBD was biased for PLC β 3 signaling compared to ERK signaling and arrestin2 recruitment as indicated by reduced β and $\alpha\beta$ values (Table 5). Previous studies have reported that ORG27569 is also biased against ERK and arrestin signaling (Ahn *et al.*, 2012, 2013; Baillie *et al.*, 2013). The observation that CBD-dependent bias was observed in *STHdh*^{Q7/Q7} cells compared to HEK 293A cells suggests that heterologous expression systems may underrepresent ligand bias (Ahn *et al.*, 2013; Baillie *et al.*, 2013).

Cannabidiol compared to other negative allosteric modulators of CB₁

Based on the functional effects of CBD on PLC β 3, ERK, arrestin2 recruitment and CB₁ internalization, CBD behaved like the well-characterized allosteric modulators ORG27569 and PSNCBAM-1 *in vitro* (Horswill *et al.*, 2007; Cawston *et al.*, 2013). At higher doses (> 2 μ M), CBD was able to enhance PLC β 3 and ERK phosphorylation, and arrestin2 recruitment, as well as limit CB₁ internalization, suggesting that CBD may behave as a weak partial agonist at high concentrations, as observed elsewhere (reviewed in Mechoulam *et al.*, 2007; McPartland *et al.*, 2014). In this study, the primary effect of CBD at CB₁ was negative allosteric modulation at concentrations below 1 μ M. The studies by Price *et al.* (2005) and Baillie *et al.* (2013) demonstrated that ORG27569 and PSNCBAM-1 paradoxically reduce orthosteric ligand efficacy and potency while increasing orthosteric ligand binding affinity and duration. It is thought that, in general, increased ligand binding results in rapid desensitization of receptors (Price *et al.*, 2005; Ahn *et al.*, 2013). In this study, we did not directly test receptor desensitization, or duration of ligand binding. We did, however, estimate ligand co-operativity and found that CBD, unlike ORG27569 and PSNCBAM-1, displayed negative co-operativity for ligand binding ($\alpha < 1$) (Price *et al.*, 2005; Ahn *et al.*, 2013). ORG27569 and PSNCBAM-1 increase the CB₁ receptor pool at the cell surface, and in doing so may potentiate CB₁ signaling (Cawston *et al.*, 2013). *In vivo*, ORG27569 reduces food intake similar to the CB₁ inverse agonist rimonabant (Gamage *et al.*, 2014). However, the *in vivo* actions of ORG27569 are CB₁-independent, suggesting that the *in vitro* pharmacology of ORG27569 does not correlate with *in vivo* observations (Gamage *et al.*, 2014). Like ORG27569, CBD may mediate a subset of its *in vivo* actions through non-CB₁ targets (Campos *et al.*, 2012). For example, the anxiolytic and antidepressant actions of CBD may be 5HT_{1A}-dependent, while the antipsychotic activity of CBD may be TRPV1-dependent (Bisogno *et al.*, 2001; Russo *et al.*, 2005; Ryberg *et al.*, 2007; Campos *et al.*, 2012). Regardless of whether CBD has alternative targets *in vivo*, the work shown here demonstrates that CBD can alter the activity of common endo- and phytocannabinoids at CB₁ and this action is likely to be therapeutically important.

Conclusions

In this *in vitro* study, the NAM activity of the well-known phytocannabinoid, CBD, was characterized for the first time. The data presented here support the hypothesis that CBD binds to a distinct, allosteric site on CB₁ that is functionally distinct from the orthosteric site for 2-AG and THC. Using an operational model of allosteric modulation to fit the data (Keov *et al.*, 2011), we observed that CBD reduced the potency and efficacy of THC and 2-AG at concentrations lower than the predicted affinity of CBD for the orthosteric site of CB₁. Future *in vivo* studies should test whether the NAM activity of CBD explains the ‘antagonist of agonists’ effects reported elsewhere (Thomas *et al.*, 2007). Indeed, the NAM activity of CBD may explain its utility as an anti-psychotic, anti-epileptic and anti-depressant. In conclusion, the identification of CBD as a CB₁ NAM provides new insights into the compound’s medicinal value, and may be useful in the development of novel, CB₁-selective synthetic allosteric modulators or drug combinations.

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All nomenclature conforms to the *British Journal of Pharmacology’s Guide to Receptors and Channels* (Alexander *et al.*, 2009).

Statement of conflicts of interest

The authors declare that they have no conflict of interest.

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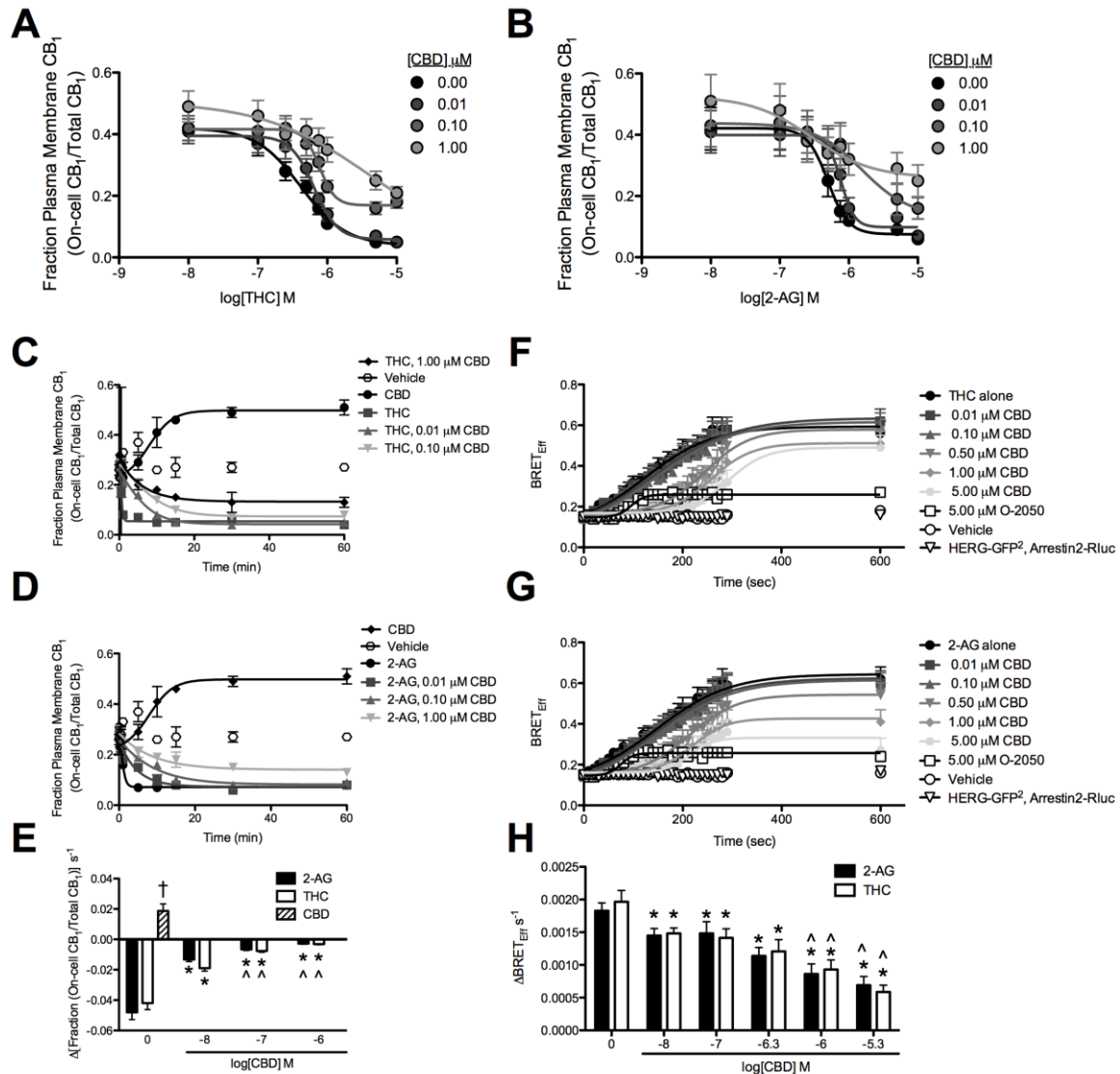


Figure 1. CBD reduced the rate and maximal BRET_{Eff} between CB₁ and arrestin2 and CB₁ internalization in THC- and 2-AG-treated *STHdh*^{Q7/Q7} cells. A,B *STHdh*^{Q7/Q7} cells were treated with THC (A) or 2-AG (B) ± CBD for 10 min and the fraction of CB₁ at the plasma membrane was quantified using On- and In-cellTM western analyses. Data were fit to a non-linear regression model with variable slope. **C-E** *STHdh*^{Q7/Q7} cells were transfected with arrestin2-Rluc- and CB₁-GFP²-containing plasmids and BRET² was measured every 10 s for 4 min (240 sec) and again at 10 min (600 sec) after treatment with THC (C) or 2-AG (D) ± O-2050 or CBD. Data were fit to a non-linear regression model with variable slope. **E** The rate of arrestin2 recruitment to CB₁ was measured as the change in BRET_{Eff} s⁻¹ during the first 4 min. **F-H** *STHdh*^{Q7/Q7} cells were treated with THC (F) or 2-AG (G) ± CBD for 60 min and the fraction of CB₁ at the plasma membrane was quantified using On- and In-cellTM western analyses. Data were fit to a non-linear regression model with variable slope. **H** The rate of CB₁ internalization was measured as the change in the Fraction On-cell CB₁/Total CB₁ min⁻¹ prior to plateau. †*P* < 0.01 compared to 2-AG or THC alone, **P* < 0.01 compared to 0 CBD within orthosteric ligand treatment, ^*P* < 0.01 compared to 0.01 μM CBD (log[CBD] M = -8) within orthosteric ligand treatment, as determined *via* two-way ANOVA followed by Bonferroni's *post-hoc* test. *N* = 6.

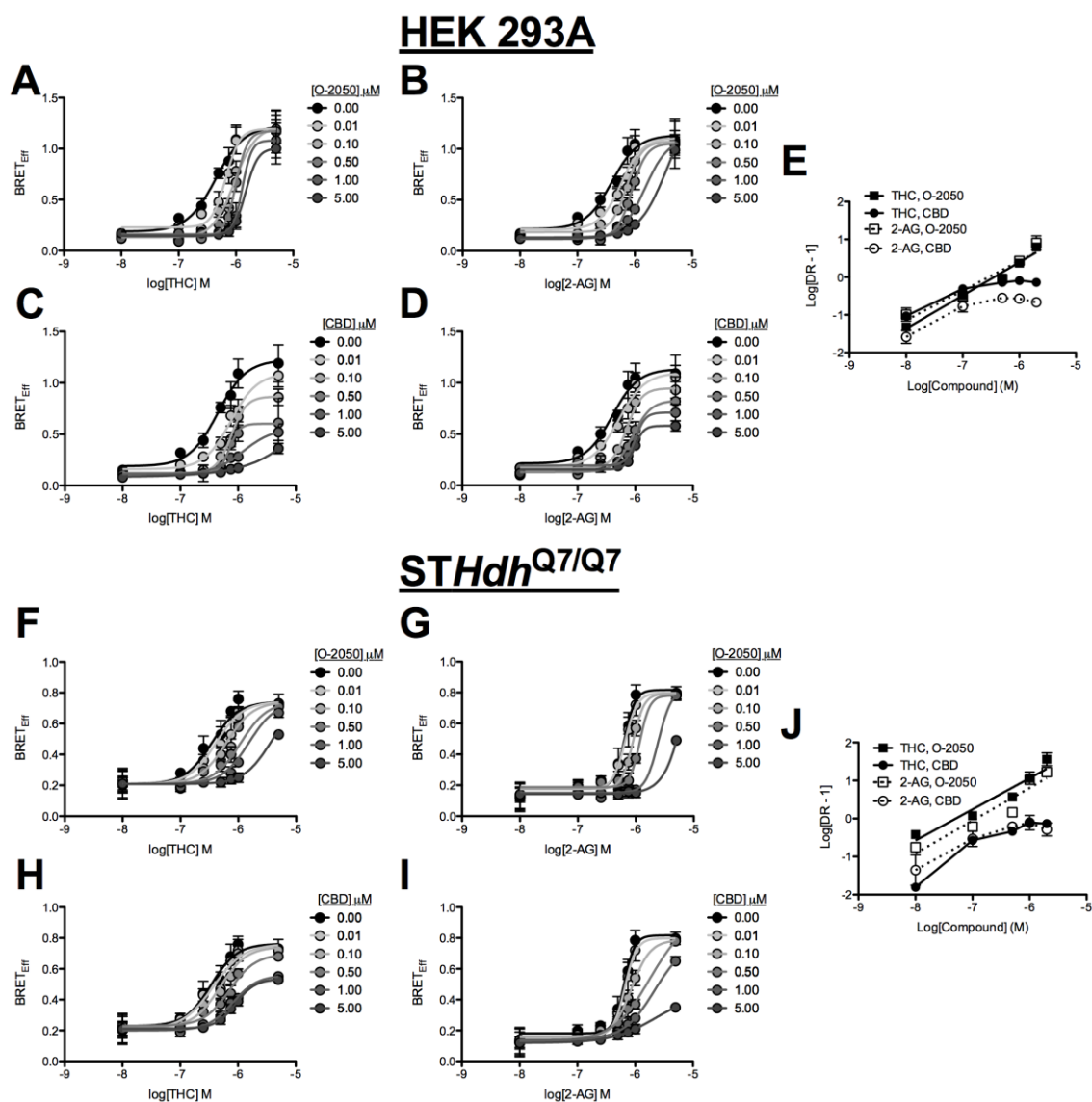


Figure 2. CBD was a NAM of arrestin2 recruitment to CB₁ following THC and 2-AG treatment. HEK 293A (A-E) and STHdh^{Q7/Q7} (F-J) cells were transfected with arrestin2-Rluc- and CB₁-GFP²-containing plasmids and BRET² was measured 30 min after treatment with 2-AG or THC \pm O-2050 or CBD. CRCs were fit using Gaddum/Schild EC₅₀ shift (A,B,F,G) and operational model of allosterism (C,D,H,I) non-linear regression models. E,J) Schild regressions were plotted as the logarithm of 2-AG or THC dose against the logarithm of the dose-response at EC₅₀ - 1. *N* = 6.

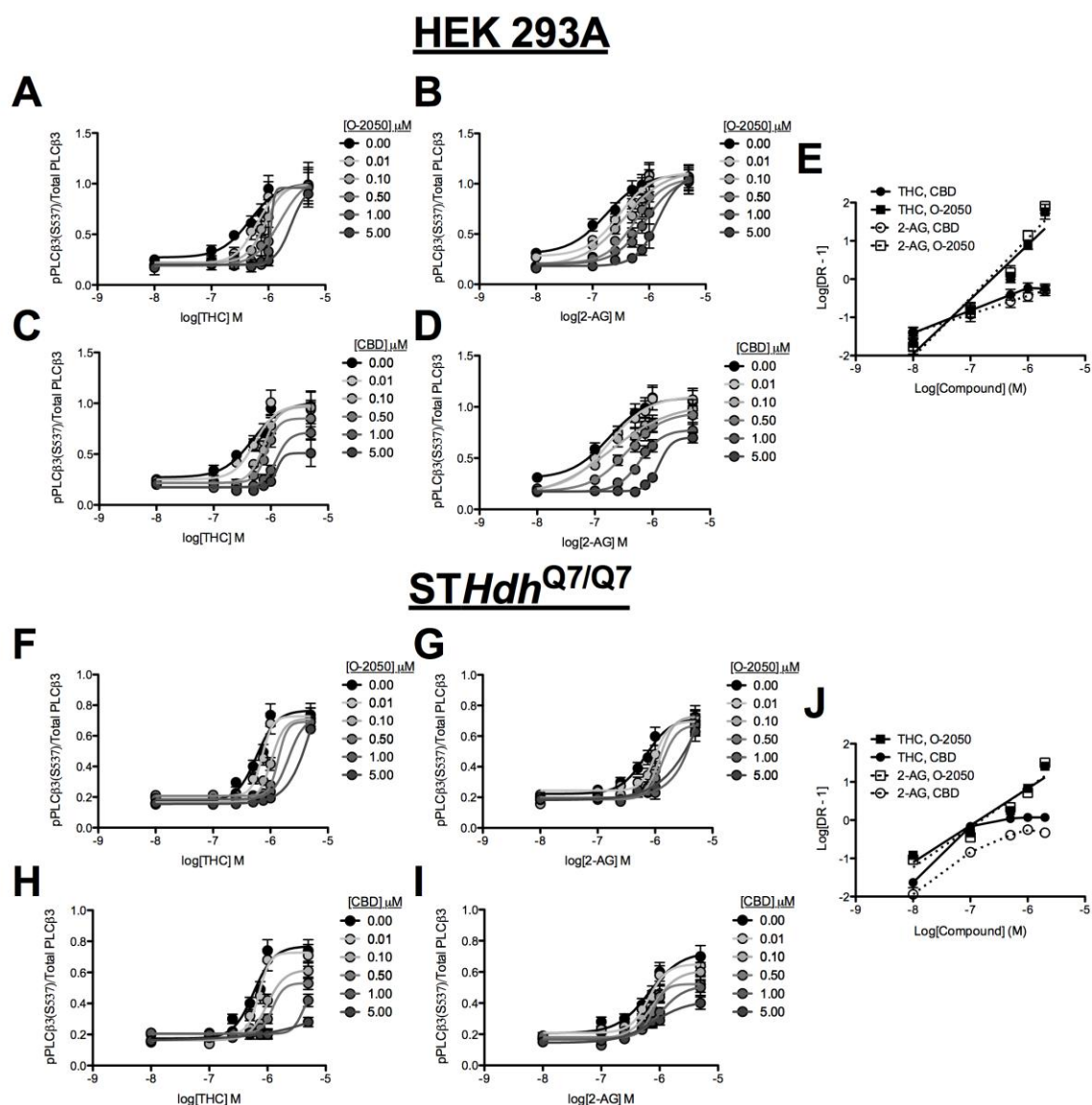


Figure 3. CBD was a NAM of CB₁-dependent PLCβ₃ phosphorylation following THC and 2-AG treatment. HEK 293A cell expressing CB₁-GFP² (A-E) and *STHdh*^{Q7/Q7} cells (F-J) were treated with 2-AG or THC ± O-2050 or CBD and total and phosphorylated PLCβ₃ levels were determined using In-cell™ western. CRCs were fit using Gaddum/Schild EC₅₀ shift (A,B,F,G) and operational model of allosterism (C,D,H,I) non-linear regression models. E,J) Schild regressions were plotted as the logarithm of 2-AG or THC dose against the logarithm of the dose-response at EC₅₀ - 1. *N* = 6.

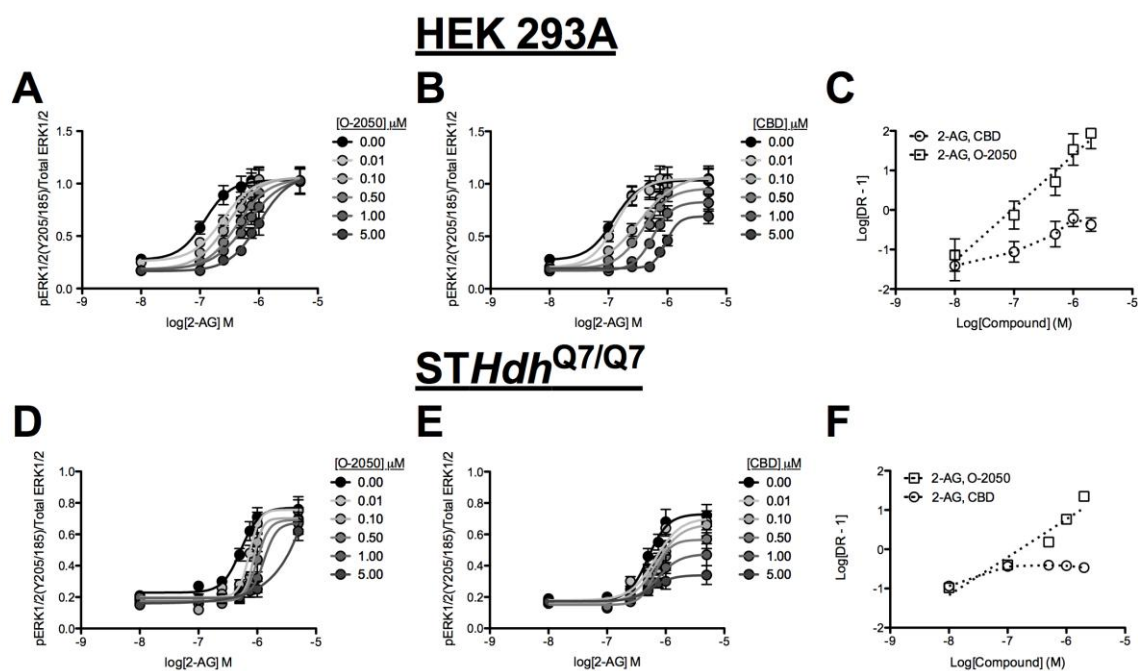


Figure 4. CBD was a NAM of CB₁-dependent ERK1/2 phosphorylation following 2-AG treatment. HEK 293A cell expressing CB₁-GFP² (A-C) and *STHdh*^{Q7/Q7} cells (D-F) were treated with 2-AG ± O-2050 or CBD and total and phosphorylated ERK1/2 levels were determined using In-cellTM western. CRCs were fit using Gaddum/Schild EC₅₀ shift (A,D) and operational model of allosterism (B,E) non-linear regression models. C,F) Schild regressions were plotted as the logarithm of 2-AG or THC dose against the logarithm of the dose-response at EC₅₀ - 1. *N* = 6.

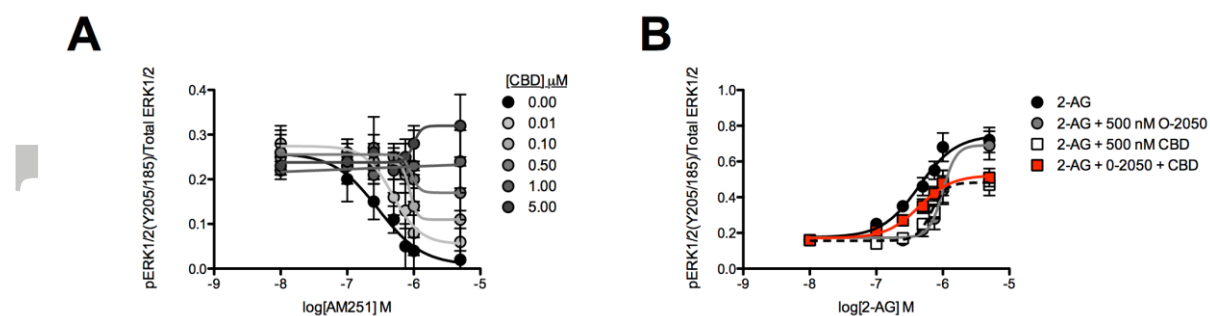


Figure 5. CBD was a NAM of AM251-dependent inverse agonism and O-2050 antagonism. *STHdh*^{Q7/Q7} cells were treated with AM251 \pm CBD (**A**) or 2-AG \pm O-2050, CBD, or O-2050 and CBD (**B**) and total and phosphorylated ERK1/2 levels were determined using In-cell™ western. CRCs were fit using the operational model of allosterism (**A**) or non-linear regression with variable slope (4 parameters) (**B**) models. $N = 6$.

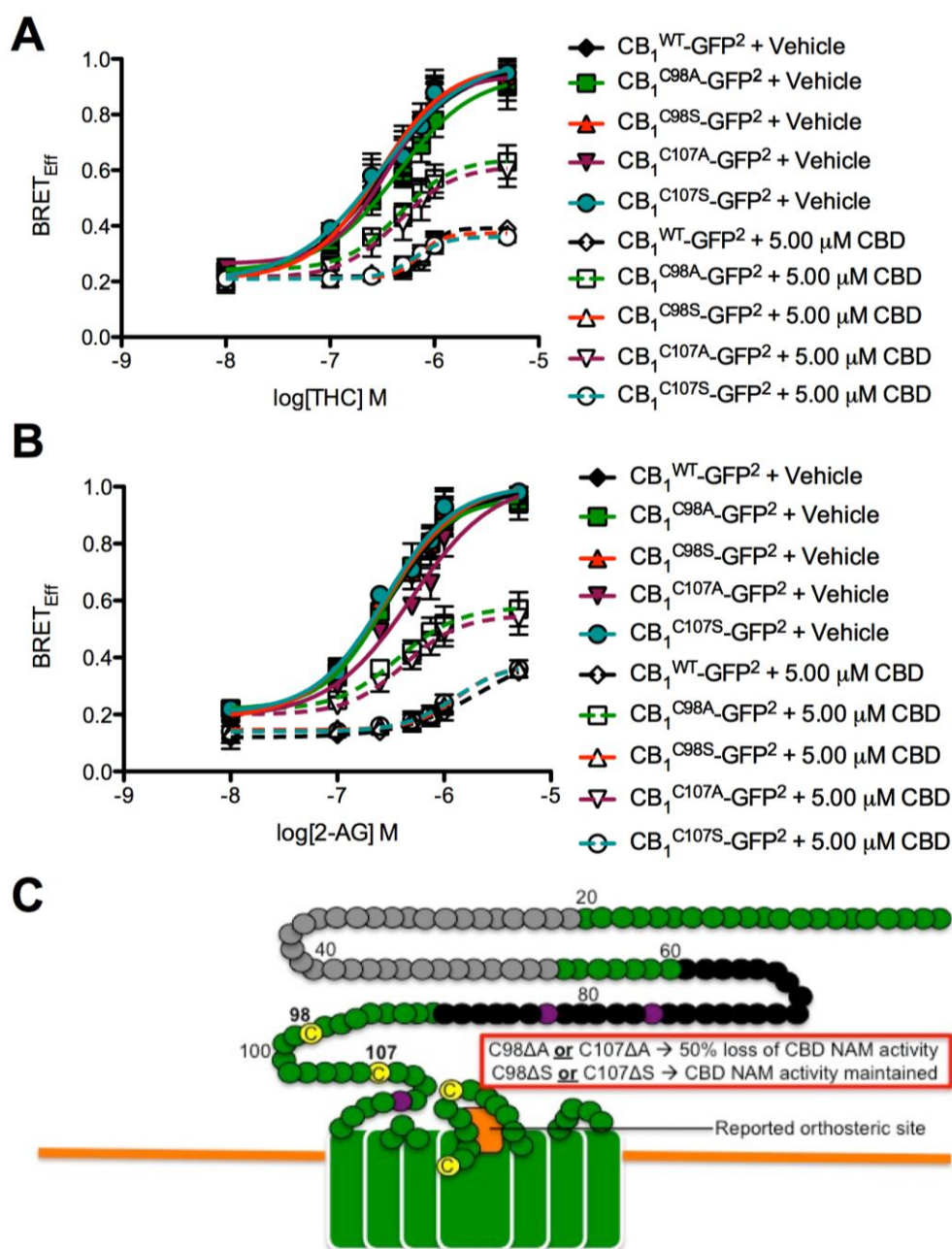


Figure 6. Cys-98 and Cys-107 coordinate the negative allosteric modulatory activity of CBD at CB₁. A,B) *STHdh*^{Q7/Q7} cells were transfected with arrestin2-Rluc- and CB₁^{C98A}-GFP²-, and CB₁^{C98S}-GFP²-, CB₁^{C107A}-GFP²-, and CB₁^{C107S}-GFP²-containing plasmids and BRET² was measured 30 min after treatment with THC (A) or 2-AG (B) ± CBD. CRCs were fit using non-linear regression with variable slope (4 parameter) $N = 4$. C) Schematic of the membrane-proximal region of CB₁ summarizing data presented in this figure (adapted from Fay and Farrens, 2013). Our observations and previous studies suggest that Cys-98 and Cys-107 contribute to CB₁ allosterism, while the orthosteric site is near the second extracellular loop (orange box). In this diagram green represents extracellular surface of CB₁. Black circles represent residues unique to the N-terminus of CB_{1A}. Grey circles represent residues unique to the N-terminus of CB_{1B}. Yellow circles represent Cys. Purple circles represent N-glycosylated residues. Residues mutated in this study are marked in bold. Non-bold numbers indicate amino acid number relative to N-terminus.

TABLE 1Effect of CBD on Arrestin-2 recruitment in HEK 293A and *STHdh*^{Q7/Q7} cellsData are mean \pm S.E.M. or with 95% CI of four independent experiments.**HEK 293A**

Agonist	[CBD] (μ M)	EC ₅₀ μ M (95% CI) ^a	E_{\max} (95% CI) ^{a,b}	n (95% CI) ^{a,c}	RA \pm S.E.M. ^d
THC	DMSO	0.44 (0.27 - 0.72)	1.22 (0.99 - 1.46)	1.00 (0.89 - 1.06)	1.00 \pm 0.0
	0.01	0.75 (0.53 - 1.06)	1.09 (0.90 - 1.29)	0.76 (0.65 - 0.89)	0.50 \pm 0.05*
	0.10	0.77 (0.64 - 0.92)	0.87 (0.75 - 0.89)†	0.63 (0.46 - 0.85)†	0.39 \pm 0.04*
	0.50	0.71 (0.49 - 1.03)	0.60 (0.41 - 0.80)†	0.55 (0.43 - 0.69)†	0.29 \pm 0.05*
	1.00	1.29 (0.89 - 1.41)†	0.56 (0.35 - 0.77)†	0.38 (0.26 - 0.41)†	0.15 \pm 0.03*
	5.00	1.41 (1.04 - 1.77)†	0.15 (0.09 - 0.31)†	0.17 (0.08 - 0.24)†	0.04 \pm 0.03*
2-AG	DMSO	0.39 (0.23 - 0.67)	1.13 (0.91 - 1.36)	1.00 (0.86 - 1.13)	1.00 \pm 0.0
	0.01	0.52 (0.36 - 0.75)	1.10 (0.92 - 1.28)	0.81 (0.68 - 1.05)	0.72 \pm 0.04*
	0.10	0.71 (0.59 - 0.86)	0.95 (0.82 - 1.07)	0.78 (0.73 - 0.93)	0.46 \pm 0.07*
	0.50	0.91 (0.69 - 1.08)†	0.83 (0.59 - 1.09)†	0.64 (0.51 - 0.74)†	0.31 \pm 0.02*
	1.00	1.00 (0.87 - 1.16)†	0.71 (0.63 - 0.79)†	0.33 (0.21 - 0.53)†	0.24 \pm 0.04*
	5.00	1.09 (0.87 - 1.18)†	0.58 (0.52 - 0.64)†	0.27 (0.18 - 0.37)†	0.18 \pm 0.02*
<i>STHdh</i>^{Q7/Q7}					
THC	DMSO	0.34 (0.21 - 0.46)	0.76 (0.65 - 0.88)	1.00 (0.93 - 1.31)	1.00 \pm 0.0
	0.01	0.37 (0.18 - 0.56)	0.76 (0.58 - 0.93)	0.87 (0.54 - 1.24)	0.91 \pm 0.3
	0.10	0.49 (0.32 - 0.66)	0.74 (0.63 - 0.86)	0.81 (0.43 - 1.07)	0.68 \pm 0.1*
	0.50	0.72 (0.50 - 0.94)†	0.70 (0.59 - 0.79)	0.80 (0.35 - 1.06)	0.43 \pm 0.1*
	1.00	0.80 (0.56 - 1.05)†	0.54 (0.48 - 0.64)†	0.74 (0.36 - 0.95)	0.31 \pm 0.1*
	5.00	0.91 (0.70 - 1.17)†	0.50 (0.48 - 0.59)†	0.65 (0.30 - 0.84)†	0.26 \pm 0.0*
2-AG	DMSO	0.64 (0.56 - 0.73)	0.82 (0.74 - 0.90)	1.00 (0.71 - 1.37)	1.00 \pm 0.0
	0.01	0.66 (0.52 - 0.84)	0.80 (0.65 - 0.94)	0.89 (0.70 - 1.09)	0.94 \pm 0.2
	0.10	0.86 (0.69 - 1.08)	0.78 (0.68 - 0.89)	0.56 (0.32 - 0.83)	0.72 \pm 0.2*
	0.50	1.80 (1.42 - 2.18)†	0.76 (0.65 - 1.05)	0.29 (0.14 - 0.42)†	0.34 \pm 0.1*
	1.00	2.18 (2.06 - 3.53)†	0.74 (0.68 - 1.04)	0.25 (0.16 - 0.38)†	0.27 \pm 0.1*
	5.00	2.20 (1.95 - 3.55)†	0.44 (0.25 - 0.57)†	0.25 (0.18 - 0.37)†	0.16 \pm 0.0*

^aDetermined using non-linear regression with variable slope (4 parameter) analysis; ^bMaximal agonist effect BRET_{Eff}; ^cHill coefficient; ^dRelative Activity, as determined in Eq. 2.

†Significantly different from the DMSO vehicle as determined by non-overlapping CI.

* $P < 0.01$ compared to DMSO vehicle as determined by one-way ANOVA followed by Dunnett's multiple comparison.

TABLE 2Schild analysis of Arrestin-2, PLC β 3, AND ERK modulation by CBDData are mean \pm S.E.M. or with 95% CI of four independent experiments.**HEK 293A**

Agonist	Slope ^a	R ²	pA ₂ (μ M) \pm S.E.M. ^b	IC ₅₀ (μ M) (95% CI) ^c
BRET² (Arrestin-2-Rluc and CB₁-GFP²)				
THC, O-2050	1.02 \pm 0.11	0.89	0.84 \pm 0.06	0.42 (0.22 - 0.64)
THC, CBD	0.54 \pm 0.06*	0.62	-	0.31 (0.19 - 0.37)
2-AG, O-2050	1.06 \pm 0.06	0.95	0.38 \pm 0.04**	0.57 (0.29 - 0.67)
2-AG, CBD	0.54 \pm 0.07*	0.41	-	0.36 (0.21 - 0.47)
Gα_q-coupled Phosphorylation of PLCβ3				
THC, O-2050	0.99 \pm 0.05	0.90	1.04 \pm 0.13	0.45 (0.35 - 0.58)
THC, CBD	0.59 \pm 0.09*	0.68	-	0.39 (0.29 - 0.51)
2-AG, O-2050	1.03 \pm 0.07	0.96	0.29 \pm 0.03**	0.58 (0.31 - 0.73)
2-AG, CBD	0.48 \pm 0.07*	0.38	-	0.31 (0.17 - 0.46)
G$\alpha_{i/o}$-coupled Phosphorylation of ERK1/2				
2-AG, O-2050	0.93 \pm 0.15	0.88	0.26 \pm 0.03	0.39 (0.09 - 0.46)
2-AG, CBD	0.15 \pm 0.02*	0.62	-	0.26 (0.19 - 0.59)
STHdh^{Q7/Q7}				
BRET² (Arrestin-2-Rluc and CB₁-GFP²)				
THC, O-2050	0.92 \pm 0.09	0.95	0.83 \pm 0.21	0.35 (0.27 - 0.46)
THC, CBD	0.34 \pm 0.10*	0.78	-	0.23 (0.16 - 0.27)
2-AG, O-2050	0.97 \pm 0.10	0.99	0.35 \pm 0.13**	0.52 (0.45 - 0.59)
2-AG, CBD	0.35 \pm 0.13*	0.70	-	0.63 (0.57 - 0.89)††
Phosphorylation of PLCβ3				
THC, O-2050	1.05 \pm 0.17	0.97	0.93 \pm 0.15	0.79 (0.42 - 0.85)
THC, CBD	0.22 \pm 0.08*	0.70	-	0.94 (0.62 - 1.19)
2-AG, O-2050	1.02 \pm 0.05	0.99	0.36 \pm 0.09**	0.83 (0.46 - 1.17)
2-AG, CBD	0.29 \pm 0.05*	0.71	-	0.96 (0.75 - 1.25)
Phosphorylation of ERK1/2				
2-AG, O-2050	1.06 \pm 0.11	0.97	0.36 \pm 0.06	0.87 (0.57 - 0.99)
2-AG, CBD	0.17 \pm 0.08*	0.60	-	0.27 (0.18 - 0.36)†

^{a,b,c}Determined using non-linear regression analysis with a Gaddum/Schild EC₅₀ shift for data presented in Figs 1-3. IC₅₀ determined at 1 μ M agonist. pA₂ was not determined where Schild slope was different from 1.

†Significantly different from the same agonist treatment; ††significantly different from the same modulator treatment; as determined by non-overlapping CI.

* P < 0.01 compared to the same agonist treatment; ** P < 0.01 compared to the same modulator treatment; as determined by one-way ANOVA followed by Dunnett's multiple comparison.



TABLE 3Effect of CBD on PLC β 3 activation in HEK 293A and *STHdh*^{Q7/Q7} cellsData are mean \pm S.E.M. or with 95% CI of four independent experiments.**HEK 293A**

Agonist	[CBD] (μ M)	EC ₅₀ μ M (95% CI) ^a	<i>E</i> _{max} (95% CI) ^{a,b}	<i>n</i> (95% CI) ^{a,c}	RA \pm S.E.M. ^d
THC	DMSO	0.47 (0.27 - 0.69)	1.01 (0.82 - 1.20)	1.00 (0.76 - 1.26)	1.00 \pm 0.0
	0.01	0.58 (0.34 - 0.81)	0.98 (0.80 - 1.17)	0.83 (0.70 - 1.13)	0.79 \pm 0.17
	0.10	0.76 (0.59 - 0.97)	0.97 (0.81 - 1.13)	0.73 (0.67 - 0.93)	0.60 \pm 0.08*
	0.50	0.86 (0.70 - 1.07)†	0.85 (0.70 - 1.00)	0.54 (0.41 - 0.72)†	0.46 \pm 0.05*
	1.00	1.23 (0.85 - 1.80)†	0.71 (0.63 - 0.79)†	0.36 (0.18 - 0.51)†	0.27 \pm 0.03*
	5.00	1.26 (0.82 - 1.58)†	0.51 (0.41 - 0.61)†	0.16 (0.04 - 0.26)†	0.19 \pm 0.02*
2-AG	DMSO	0.48 (0.28 - 0.72)	1.09 (0.90 - 1.29)	1.00 (0.86 - 1.15)	1.00 \pm 0.0
	0.01	0.63 (0.37 - 0.96)	1.11 (0.91 - 1.30)	0.92 (0.81 - 1.02)	0.84 \pm 0.07
	0.10	0.83 (0.58 - 1.03)	1.03 (0.74 - 1.32)	0.84 (0.74 - 1.00)	0.60 \pm 0.07*
	0.50	1.11 (0.95 - 1.35)†	0.95 (0.80 - 1.10)	0.57 (0.46 - 0.79)†	0.41 \pm 0.08*
	1.00	1.62 (1.23 - 1.51)†	0.78 (0.67 - 0.88)†	0.22 (0.07 - 0.36)†	0.23 \pm 0.01*
	5.00	2.48 (1.72 - 3.22)†	0.60 (0.54 - 0.66)†	0.13 (0.04 - 0.24)†	0.12 \pm 0.06*
<i>STHdh</i>^{Q7/Q7}					
THC	DMSO	0.58 (0.42 - 0.79)	0.77 (0.65 - 0.89)	1.00 (0.71 - 1.25)	1.00 \pm 0.0
	0.01	0.72 (0.61 - 0.85)	0.73 (0.63 - 0.82)	0.54 (0.44 - 0.82)	0.77 \pm 0.3
	0.10	0.99 (0.78 - 1.22)	0.62 (0.54 - 0.69)	0.51 (0.42 - 0.78)	0.48 \pm 0.1*
	0.50	1.22 (0.85 - 1.57)†	0.53 (0.48 - 0.58)†	0.55 (0.23 - 0.64)†	0.33 \pm 0.1*
	1.00	4.00 (2.76 - 4.32)†	0.49 (0.37 - 0.52)†	0.51 (0.17 - 0.62)†	0.10 \pm 0.0*
	5.00	>5.00 †	-	< 0.50 †	0.03 \pm 0.0*
2-AG	DMSO	0.66 (0.40 - 0.85)	0.73 (0.59 - 0.87)	1.00 (0.70 - 1.18)	1.00 \pm 0.0
	0.01	0.67 (0.48 - 0.86)	0.65 (0.56 - 0.74)	0.77 (0.55 - 0.89)	0.88 \pm 0.2
	0.10	0.78 (0.58 - 1.01)	0.61 (0.52 - 0.70)	0.57 (0.34 - 0.74)	0.71 \pm 0.2*
	0.50	0.87 (0.63 - 0.92)	0.52 (0.46 - 0.58)†	0.39 (0.15 - 0.58)†	0.60 \pm 0.1*
	1.00	1.04 (0.87 - 1.61)†	0.51 (0.43 - 0.56)†	0.39 (0.12 - 0.50)†	0.45 \pm 0.1*
	5.00	1.78 (1.07 - 2.05)†	0.42 (0.32 - 0.51)†	0.36 (0.09 - 0.49)†	0.21 \pm 0.0*

^aDetermined using non-linear regression with variable slope (4 parameter) analysis; ^bMaximal agonist effect BRET_{Eff}; ^cHill coefficient; ^dRelative Activity, as determined in Eq. 2.

†Significantly different from the DMSO vehicle as determined by non-overlapping CI.

**P* < 0.01 compared to DMSO vehicle as determined by one-way ANOVA followed by Dunnett's multiple comparison.

TABLE 4Effect of CBD on ERK activation in HEK 293A and *STHdh*^{Q7/Q7} cellsData are mean \pm S.E.M. or with 95% CI of four independent experiments.**HEK 293A**

Agonist	[CBD] (μ M)	EC ₅₀ μ M (95% CI) ^a	E _{max} (95% CI) ^{a,b}	n (95% CI) ^{a,c}	RA \pm S.E.M. ^d
2-AG	DMSO	0.12 (0.07 - 0.22)	1.03 (0.89 - 1.17)	1.00 (0.97 - 1.07)	1.00 \pm 0.0
	0.01	0.13 (0.08 - 0.22)	1.05 (0.92 - 1.18)	0.91 (0.82 - 1.03)	0.96 \pm 0.09
	0.10	0.33 (0.19 - 0.47)	1.09 (0.90 - 1.28)	0.63 (0.57 - 0.72)†	0.40 \pm 0.06*
	0.50	0.39 (0.26 - 0.58)†	0.96 (0.82 - 1.10)	0.39 (0.29 - 0.58)†	0.30 \pm 0.05*
	1.00	0.57 (0.45 - 0.72)†	0.83 (0.73 - 0.93)	0.27 (0.17 - 0.39)†	0.17 \pm 0.05*
	5.00	0.95 (0.81 - 1.11)†	0.69 (0.61 - 0.76)†	0.19 (0.11 - 0.30)†	0.09 \pm 0.02*
<i>STHdh</i>^{Q7/Q7}					
2-AG	DMSO	0.50 (0.37 - 0.68)	0.73 (0.63 - 0.83)	1.00 (0.91 - 1.22)	1.00 \pm 0.0
	0.01	0.66 (0.44 - 0.99)	0.70 (0.58 - 0.83)	0.78 (0.57 - 0.83)†	0.74 \pm 0.2*
	0.10	0.69 (0.48 - 0.95)	0.67 (0.56 - 0.77)	0.79 (0.56 - 0.77)†	0.67 \pm 0.1*
	0.50	0.77 (0.52 - 0.87)	0.57 (0.48 - 0.65)	0.73 (0.63 - 0.87)†	0.56 \pm 0.1*
	1.00	0.84 (0.69 - 1.21)†	0.47 (0.37 - 0.57)†	0.70 (0.46 - 0.81)†	0.44 \pm 0.1*
	5.00	1.27 (0.81 - 1.47)†	0.33 (0.26 - 0.41)†	0.57 (0.27 - 0.72)†	0.30 \pm 0.1*

^aDetermined using non-linear regression with variable slope (4 parameter) analysis; ^bMaximal agonist effect BRET_{Eff}; ^cHill coefficient; ^dRelative Activity, as determined in Eq. 2.

†Significantly different from the DMSO vehicle as determined by non-overlapping CI.

**P* < 0.01 compared to DMSO vehicle as determined by one-way ANOVA followed by Dunnett's multiple comparison.

TABLE 5Operational model analysis of CBD at CB₁ in the presence of THC or 2-AGData are mean \pm S.E.M. or with 95% CI of four independent experiments.**HEK 293A**

Agonist Modulator	BRET _{Eff}		pPLC β 3		pERK1/2
	THC	2-AG	THC	2-AG	2-AG
	CBD	CBD	CBD	CBD	CBD
-log α	0.47 \pm 0.06	0.53 \pm 0.07	0.47 \pm 0.12	0.57 \pm 0.11	0.48 \pm 0.13
-log β	0.25 \pm 0.09	0.41 \pm 0.03	0.28 \pm 0.07	0.42 \pm 0.09	0.30 \pm 0.07
log τ_A^a	1.14 \pm 0.26	1.04 \pm 0.19	1.01 \pm 0.20	1.12 \pm 0.18	1.02 \pm 0.12
log τ_B^b	0.21 \pm 0.04	0.13 \pm 0.05	0.25 \pm 0.10	0.15 \pm 0.06	0.06 \pm 0.04
K _A ^a (nM)	128 (56.7 - 159)	262 (197 - 308)	91.9 (82.2 - 103)	255 (176 - 328)	236 (195 - 275)
K _B ^b (nM)	270 (148 - 349)	352 (272 - 409)	268 (197 - 292)	326 (279 - 382)	318 (255 - 369)
$\alpha\beta$	0.19	0.11	0.18	0.10	0.17

STHdh^{Q7/Q7}

Agonist Modulator	BRET _{Eff}		pPLC β 3		pERK1/2
	THC	2-AG	THC	2-AG	2-AG
	CBD	CBD	CBD	CBD	CBD
-log α	0.31 \pm 0.09	0.23 \pm 0.12	0.46 \pm 0.18	0.38 \pm 0.15	0.42 \pm 0.13
-log β	0.25 \pm 0.08	0.33 \pm 0.09	0.60 \pm 0.12*	0.58 \pm 0.09*	0.27 \pm 0.06
log τ_A^a	0.78 \pm 0.21	0.81 \pm 0.17	0.81 \pm 0.17	0.79 \pm 0.12	0.74 \pm 0.18
log τ_B^b	0.31 \pm 0.11	0.21 \pm 0.09	0.29 \pm 0.12	0.18 \pm 0.07	0.19 \pm 0.05
K _A ^a (nM)	95.7 (58.6 - 118)	237 (181 - 294)	72.3 (59.1 - 107)	255 (178 - 318)	198 (137 - 238)
K _B ^b (nM)	278 (148.4 - 335)	333 (291 - 376)	259 (194 - 280)	315 (281 - 362)	329 (241 - 346)
$\alpha\beta$	0.28	0.28	0.09	0.11	0.20

All values estimated using the operational model of allosterism described in Eq. 1. ^alog τ_A and K_A determined for THC or 2-AG; ^blog τ_B and K_B determined for CBD.

* $P < 0.01$ compared to BRET_{Eff} with the same agonist as determined by one-way ANOVA followed by Dunnett's multiple comparison.

TABLE 6Effect of CBD on Arrestin-2 recruitment to mutant CB₁ in *STHdh*^{Q7/Q7} cells

Data are mean with 95% CI of four independent experiments.

Agonist	Receptor	Modulator	EC ₅₀ μ M (95% CI) ^a	E _{max} (95% CI) ^{a,b}
THC	CB ₁ ^{WT}	<i>DMSO</i>	0.34 (0.21 - 0.46)	0.96 (0.75 - 1.01)
		5.00 μ M CBD	0.91 (0.70 - 1.17) [†]	0.30 (0.24 - 0.49) [†]
	CB ₁ ^{C98A}	<i>DMSO</i>	0.35 (0.26 - 0.57)	0.94 (0.78 - 1.11)
		5.00 μ M CBD	0.55 (0.37 - 0.67) [^]	0.64 (0.54 - 0.74) ^{†^}
	CB ₁ ^{C107A}	<i>DMSO</i>	0.36 (0.23 - 0.46)	0.94 (0.82 - 1.07)
		5.00 μ M CBD	0.56 (0.48 - 0.67) ^{†^}	0.61 (0.54 - 0.73) ^{†^}
	CB ₁ ^{C98S}	<i>DMSO</i>	0.30 (0.17 - 0.41)	0.98 (0.83 - 1.12)
		5.00 μ M CBD	0.97 (0.79 - 1.10) [†]	0.37 (0.32 - 0.42) [†]
	CB ₁ ^{C107S}	<i>DMSO</i>	0.31 (0.16 - 0.48)	1.00 (0.82 - 1.18)
		5.00 μ M CBD	0.91 (0.80 - 1.02) [†]	0.36 (0.31 - 0.41) [†]
2-AG	CB ₁ ^{WT}	<i>DMSO</i>	0.64 (0.56 - 0.73)	0.82 (0.74 - 0.90)
		5.00 μ M CBD	2.20 (1.95 - 3.55) [†]	0.44 (0.25 - 0.57) [†]
	CB ₁ ^{C98A}	<i>DMSO</i>	0.62 (0.54 - 0.78)	0.96 (0.84 - 1.09)
		5.00 μ M CBD	1.37 (1.09 - 1.59) ^{†^}	0.67 (0.59 - 0.71) ^{†^}
	CB ₁ ^{C107A}	<i>DMSO</i>	0.59 (0.43 - 0.69)	1.03 (0.89 - 1.18)
		5.00 μ M CBD	1.42 (1.23 - 1.64) ^{†^}	0.66 (0.58 - 0.72) ^{†^}
	CB ₁ ^{C98S}	<i>DMSO</i>	0.68 (0.59 - 0.74)	1.01 (0.90 - 1.12)
		5.00 μ M CBD	2.32 (1.97 - 2.57) [†]	0.37 (0.24 - 0.50) [†]
	CB ₁ ^{C107S}	<i>DMSO</i>	0.67 (0.59 - 0.79)	1.00 (0.90 - 1.11)
		5.00 μ M CBD	2.28 (2.14 - 2.40) [†]	0.38 (0.24 - 0.52) [†]

[†]Significantly different from DMSO vehicle within receptor group as determined by non-overlapping CI.[^]Significantly different from response to 5.00 μ M CBD and DMSO vehicle in CB₁^{WT} vehicle as determined by non-overlapping CI.