

Antagonism of Dopamine Receptor 2 Long Affects Cannabinoid Receptor 1 Signaling in a Cell Culture Model of Striatal Medium Spiny Projection Neurons[§]

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ABSTRACT

Activation of dopamine receptor 2 long (D_{2L}) switches the signaling of type 1 cannabinoid receptor (CB₁) from G_{α_i} to G_{α_s}, a process thought to be mediated through CB₁-D_{2L} heteromerization. Given the clinical importance of D₂ antagonists, the goal of this study was to determine if D₂ antagonists could modulate CB₁ signaling. Interactions between CB₁ and D_{2L}, G_{α_i}, G_{α_s}, and β-arrestin1 were studied using bioluminescence resonance energy transfer 2 (BRET²) in *STHdh^{Q7/Q7}* cells. CB₁-dependent extracellular regulated kinase (ERK)1/2, CREB phosphorylation, and CB₁ internalization following cotreatment of CB₁ agonist and D₂ antagonist were quantified. Preassembled CB₁-G_{α_i} complexes were detected by BRET². Arachidonyl-2'-chloroethylamide (ACEA), a selective CB₁ agonist, caused a rapid and transient increase in BRET efficiency (BRET_{Eff}) between G_{α_i}-Rluc and CB₁-green fluorescent protein 2 (GFP²), and a G_{α_i}-dependent increase in ERK phosphorylation. Physical interactions between CB₁ and D_{2L} were observed using

BRET². Cotreatment of *STHdh^{Q7/Q7}* cells with ACEA and haloperidol, a D₂ antagonist, inhibited BRET_{Eff} signals between G_{α_i}-Rluc and CB₁-GFP² and reduced the E_{Max} and pEC₅₀ of ACEA-mediated G_{α_i}-dependent ERK phosphorylation. ACEA and haloperidol cotreatments produced a delayed and sustained increase in BRET_{Eff} between G_{α_s}-Rluc and CB₁-GFP² and increased the E_{Max} and pEC₅₀ of ACEA-induced G_{α_s}-dependent cAMP response element-binding protein phosphorylation. In cells expressing CB₁ and D_{2L} treated with ACEA, binding of haloperidol to D₂ receptors switched CB₁ coupling from G_{α_i} to G_{α_s}. In addition, haloperidol treatment reduced ACEA-induced β-arrestin1 recruitment to CB₁ and CB₁ internalization. D₂ antagonists allosterically modulate cannabinoid-induced CB₁ coupling, signaling, and β-arrestin1 recruitment through binding to CB₁-D_{2L} heteromers. These findings indicate that D₂ antagonism, like D₂ agonists, change agonist-mediated CB₁ coupling and signaling.

Introduction

The type 1 cannabinoid receptor (CB₁) is highly expressed in the central nervous system, where it regulates neuromodulatory processes (Matsuda et al., 1990; Howlett et al., 2004; Bosier et al., 2010). CB₁ is activated by endogenous lipid mediators,

such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and exogenous cannabinoids such as Δ⁹-tetrahydrocannabinol (Mechoulam et al., 1995). CB₁ receptors signal primarily through *Pertussis* toxin (PTx)-sensitive G_{α_{i/o}} proteins (Demuth and Molleman, 2006). In addition, it has been demonstrated that different CB₁ agonists can promote CB₁ signaling through G_{α_s}, G_{α_{q/11}}, and β-arrestin1 (Maneuf and Brochie, 1997; Lauckner et al., 2005; Laprairie et al., 2014).

CB₁ receptors can self-associate to form homomers and can also associate with other class-A G protein-coupled receptors (GPCRs) to form heteromers (Hudson et al., 2010). Specifically, CB₁ is known to heteromerize with the dopamine receptor type 2 long (D_{2L}), the μ-, κ-, and δ-opioid receptors, the orexin-1 receptor, the A_{2A} adenosine receptor (A_{2A}), and β₂ adrenergic receptor (β₂-AR) (Wager-Miller et al., 2002; Kearn et al., 2005; Mackie, 2005; Ellis et al., 2006; Rios et al., 2006; Carriba et al., 2007; Hudson et al., 2010). Heteromerization of CB₁ with the D_{2L} has received significant attention owing to the fact that both receptors are colocalized in the GABA-ergic medium spiny neurons projecting from the striatum to the

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ABBREVIATIONS: ACEA, Arachidonyl-2'-chloroethylamide; AEA, anandamide; ANOVA, analysis of variance; AR, arrestin; BRET, bioluminescence resonance energy transfer; BRET_{Eff}, BRET efficiency; BRET_{Max}, BRET maximum; CB₁, type 1 cannabinoid receptor; CB₁-BP, CB₁ blocking peptide; CREB, cAMP response element-binding protein; CTx, *Cholera* toxin; D₂, type 2 dopamine receptor; D_{2L}, type 2 dopamine receptor long isoform; ERK, extracellular regulated kinase; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HALO, haloperidol; HERG, human ether-a-go-go-related gene; O-2050, (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; PCR, polymerase chain reaction; PTx, *Pertussis* toxin; qRT-PCR, quantitative reverse transcription PCR; Rluc, *Renilla* luciferase.

globus pallidus, as well as on the axon terminals at the globus pallidus (Hermann et al., 2002; Pickel et al., 2006). Medium spiny neurons play important roles in the coordination of movement, emotions, and cognition (Gerfen, 1992; Graybiel, 2005).

Colocalization of CB₁ and D₂L in the basal ganglia may allow for bidirectional functional interaction between the two receptors (reviewed in Fernández-Ruiz et al., 2010). Activation of CB₁ leads to an increase in dopamine release in the nucleus accumbens (Tanda et al., 1997; Solinas et al., 2006). In addition, D₂L activation has been shown to increase endocannabinoid release in the dorsal striatum (Giuffrida et al., 1999; Pan et al., 2008). In vitro functional interactions between CB₁ and D₂L were first observed in striatal neurons by Glass and Felder (1997). Costimulation of both these receptors by their respective agonists in striatal neurons leads to an accumulation of cAMP, whereas stimulation of either receptor alone leads to an inhibition of cAMP (Glass and Felder, 1997). These authors hypothesized that this response was the result of a change in coupling of CB₁ from G_{α_i} to G_{α_s} when the two receptors were coactivated by agonists (Glass and Felder, 1997). Subsequent work demonstrated that D₂ agonists altered CB₁-dependent signaling, CB₁ localization, and receptor expression (Jarrahian et al., 2004; Kearn et al., 2005; Marcellino et al., 2008; Przybyla and Watts, 2010; Khan and Lee, 2014). Functional interactions between CB₁ and D₂L receptors have been attributed to heteromerization between the two receptors, as was demonstrated using coimmunoprecipitation, Förster resonance energy transfer, and bimolecular fluorescence complementation (Kearn et al., 2005; Marcellino et al., 2008; Przybyla and Watts, 2010), suggesting allosteric interactions between CB₁ and D₂L receptors heteromers.

Allosteric ligands modulate orthosteric ligand binding by binding to a distinct allosteric receptor site. In doing so, allosteric modulators can change the potency and efficacy of the orthosteric ligands. In the context of GPCR heteromer, allosteric modulations can be envisioned between the protomers of the heteromer. Each protomer possesses an orthosteric binding pocket (Kenakin and Miller, 2010). Binding of orthosteric ligand to one protomer of the receptor complex may exert allosteric effects on the response of the other protomer to ligand binding. Such allosteric modulation may result in positive or negative cooperativity across the heteromer pair (Kenakin and Miller, 2010; Wootten et al., 2013). A well known example of allosteric interactions between GPCR heteromers is within the D₂-A_{2A} receptor heteromer complex (reviewed in Ferré et al., 2015).

The purpose of the current study was to examine if the high affinity D₂ antagonist haloperidol can allosterically modulate CB₁ pharmacology within the CB₁-D₂L heteromers. D₂ antagonists are widely used as antipsychotics and for the management of movement disorders. We measured the effects of the D₂ antagonist haloperidol on the coupling of CB₁ to G_{α_i}, G_{α_s}, and β-arrestin1 in the presence of the cannabinoid agonist arachidonyl-2'-chloroethylamide (ACEA). ACEA is a stable synthetic analog of the endocannabinoid anandamide (Howlett et al., 2004; Bosier et al., 2010). Bioluminescence resonance energy transfer 2 (BRET²) was used in this study to monitor the coupling between CB₁ to G_{α_i}, G_{α_s}, and β-arrestin1 in *STHdh*^{Q7/Q7} cells, a model of striatal medium spiny projection neurons. These cells endogenously express both CB₁ and D₂L receptors (Trettel et al., 2000; Laprairie et al., 2013, 2014).

Materials and Methods

Drugs. ACEA and the CB₁-selective antagonist O-2050 were purchased from Tocris Bioscience (Bristol, UK). Haloperidol (HALO), sulpiride, *Pertussis* toxin (PTx), and *Cholera* toxin (CTx) were obtained from Sigma-Aldrich (Oakville, ON). Drugs were dissolved in 100% ethanol as 10 mM stocks and the final dimethyl sulfoxide concentration after dilution was 0.1% in assay media. PTx and CTx were dissolved in dH₂O (50 ng/ml) and added directly to the media 24 hours prior to drug treatment. The concentrations of drugs used in this study were based on previous studies (Trincavelli et al., 2010) and work carried out in our laboratory (Laprairie et al., 2014).

Cell Culture and Transfection. In this study *STHdh*^{Q7/Q7} cells were used. *STHdh*^{Q7/Q7} cells endogenously express CB₁, D₂L, D₃, and D₄ receptors (Lee et al., 2007). The *STHdh*^{Q7/Q7} cell line is derived from the conditionally immortalized striatal progenitor cells of embryonic day 14 C57BL/6J male mice (Coriell Institute, Camden, NJ; Trettel et al., 2000). Cells were grown at 33°C, 5% CO₂, in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10,000 IU/ml penicillin-streptomycin, and 400 μg/ml Geneticin (Trettel et al., 2000; Lee et al., 2007; Laprairie et al., 2013).

Generation of Plasmids. Both human CB₁ (GenBank accession number: NM_016083) and D₂L (GenBank accession number: NM_000795) were cloned such that either green fluorescent protein 2 (GFP²) or *Renilla* luciferase (Rluc) were expressed as fusion proteins at the intracellular C-terminus of each receptor using the pGFP²-N3 and pRluc-N1 plasmids (PerkinElmer, Waltham, MA). CB₁-GFP² and CB₁-Rluc constructs were generated as described previously (Hudson et al., 2010). The human D₂L-pcDNA3.1 (+) plasmid was obtained from the Missouri University of Science and Technology cDNA Resource Center (Rolla, MO). D₂L-GFP² and D₂L-Rluc were generated by amplifying the D₂L from D₂L-pcDNA3.1 (+) by polymerase chain reaction (PCR) using the forward primer 5'-CGACAAGCTTATG-GATCCACTGAATCTGTCC-3' and the reverse primer 5'-TGACATG-GATCCCAGCAGTGGAGGATCTTC-3'. The PCR products were digested with *Eco*R1 and *Kpn*1 before being ligated into pGFP²-N3 and pRluc-N1. The CB₁ blocking peptide (CB₁-BP) was amplified from CB₁-Rluc by PCR using the forward primer 5'-GATGGATC-CATGTGTGAAGGCACTGCGCAGCCT-3' and the reverse primer 5'-GGCCTCGAGTCATGAGTCCCCCATGCTGTTATC-3' (Khan and Lee, 2014). The PCR products were cloned into the pcDNA3.1 Zeo (+) using *Bam*H1 and *Xho*I restriction sites. The C-terminus fusion constructs of the β₂ adrenergic receptor (β₂-AR) with GFP², β₂-AR-GFP², and the membrane protein human ether-a-go-go-related gene (HERG), HERG-GFP², were provided by Dr. Terry Herbert and were used as controls (Dupré et al., 2007; Hudson et al., 2010). The following plasmids G_{α_{i1}}-Rluc, G_{α_s}-Rluc, Gβ₁-pcDNA3.1 (+) and Gγ₂-pcDNA3.1 (+) and β-arrestin1-Rluc were provided by Dr. Denis J. Dupré (Dupré et al., 2006) (Dalhousie University, Halifax, CA). For the G_{α_{i1}}-Rluc construct, the Rluc cDNA sequence (GenBank accession number: JQ606807.1) was inserted between nucleotides 273 and 274 of human G_{α_{i1}} (GenBank accession number: NM_001256414), which corresponds to the loop connecting helices A and B of G_{α_i}. The recombinant G_{α_{i1}}-Rluc construct was cloned in pcDNA3.1 (+), as previously described (Ayoub et al., 2007). For the G_{α_s}-Rluc construct, Rluc was inserted between nucleotides 564 and 565 corresponding to the α-helical domain of the human G_{α_s} protein (GenBank accession number: BC108315.1) (Ayoub et al., 2007). For the β-arrestin1-Rluc construct, Rluc was fused to the carboxyl terminus of β-arrestin1 (GenBank accession number: NM_004041.4) (Hamdan et al., 2007). All constructs were sequenced to confirm their full cDNA sequence and reading-frame (Genewiz Inc., South Plainfield, NJ).

BRET². Interactions between CB₁ and D₂L, G_{α_i}, G_{α_s}, and β-arrestin1 were assessed via BRET² as described previously (Ramsay et al., 2002; James et al., 2006; Bagheri et al., 2013). For BRET² experiments using G_{α_i}-Rluc or G_{α_s}-Rluc fusion proteins, cells were cotransfected with untagged Gβ₁ and Gγ₂ in pcDNA3.1 (+). For all BRET² experiments, ligands were present throughout the assay and

were not washed out. For BRET² saturation experiments, cells were transfected with fixed amounts of the BRET² donor (Rluc-tagged protein), and increasing amounts of BRET² acceptor (GFP²-tagged protein). BRET efficiency (BRET_{Eff}) values were plotted against the ratio of GFP²/Rluc concentration. The resulting data were fit to a rectangular hyperbola curve using GraphPad version 6.0 (GraphPad Software Inc., San Diego, CA). If the interaction is specific this should result in a hyperbolic increase in BRET signal to reach a maximum value (or “saturate”), BRET maximum (BRET_{Max}), whereas nonspecific interactions will only result in a gradual linear increase in BRET_{Eff}. Changes in BRET_{Max} values reflects the relative orientation, distance, and expression levels of both donor and acceptor molecules (Guan et al., 2009). BRET₅₀ represents the donor/acceptor ratio required to achieve 50% of the BRET_{Max} signal. BRET₅₀ value reflects the affinity of donor and acceptor molecules to each other (Guan et al., 2009). For BRET² kinetic analyses, the BRET² substrate coelenterazine 400a (Biotium, Hayward, CA) was added at time 0 minutes, and light emissions were measured every 25 seconds for 9 minutes. Haloperidol was added at 50 seconds, and vehicle or ACEA was added at 75 seconds following coelenterazine 400a administration.

On-Cell and In-Cell Western Analyses. On-Cell Western analysis (LI-COR Biosciences, Lincoln, NE) was used to assess the cell-surface expression of CB₁ in *STHdh*^{Q7/Q7} cells. In-Cell Western analysis (LI-COR Biosciences, Lincoln, NE) was used to measure total CB₁ expression and to measure phosphorylation of the extracellular regulated kinase 1/2 (pERK1/2; Tyr-205/Tyr-185) and cAMP response element-binding protein (pCREB; Ser-133) using previously described protocol (Laprairie et al., 2014). In cells treated with ACEA and haloperidol, haloperidol was added 25 seconds before the addition of ACEA.

Confocal Microscopy and Immunofluorescence. The colocalization of endogenous CB₁ and D_{2L} in *STHdh*^{Q7/Q7} cells was observed using confocal microscopy as described (Bagher et al., 2013). Cells were incubated with primary monoclonal rabbit N-terminal CB₁ antibody (1:500; Cayman Chemical Company, Ann Arbor, MI) and primary monoclonal mouse N-terminal-D₂ antibody (1:200; Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. The next day, the cells were washed three times with 0.1 M phosphate-buffered saline and incubated with a Cy3-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody and Cy5-conjugated anti-rabbit secondary antibody (1:500; Jackson Immuno Research Laboratories, West Grove, PA) for 1 hour at room temperature. Cover slips were mounted on microscope slides using Calbiochem FluorSave reagent (EMD Millipore, Billerica, MA). Images of cells were acquired with a Nikon Eclipse E800 microscope attached to the D-Eclipse C1 confocal system (Nikon Canada, Mississauga, ON).

Quantitative Reverse-Transcription PCR. Quantitative reverse-transcription PCR (qRT-PCR) was used to quantify the expression of CB₁ and D₂ mRNAs in *STHdh*^{Q7/Q7} cells using a previously described protocol (Laprairie et al., 2013).

Statistical Analyses. Data are presented as the $\pm$ S.E.M. or 95% confidence interval, as indicated. Statistical analysis and curve fitting of the data were performed using GraphPad version 6.0. Concentration-response curves were fit to nonlinear regression model with variable slope (four parameters). The Hill coefficient calculated from the slope of the curve represents the cooperativity of oligomeric allosteric proteins. If the Hill coefficient is larger than 1, it is a positive cooperativity, whereas the Hill coefficient smaller than 1 indicates negative cooperativity. Statistical analyses were conducted by one-way analysis of variance (ANOVA), as indicated. Posthoc analyses were performed using Tukey's honest significance test. The level of significance was set to $P < 0.01$.

Results

CB₁ and D_{2L} Receptors Form Heteromers in *STHdh*^{Q7/Q7} Cells. *STHdh*^{Q7/Q7} cells endogenously express CB₁ and D₂ receptors and other proteins associated with

signaling via these receptors (Trettel et al., 2000; Lee et al., 2007; Laprairie et al., 2013). We confirmed via PCR, qRT-PCR, In-Cell Western, and immunofluorescence that *STHdh*^{Q7/Q7} cells express CB₁ and D₂ receptors (Supplemental Fig. 1). Our immunocytochemistry experiments show colocalization of CB₁ and D₂ in *STHdh*^{Q7/Q7} cells. CB₁ immunofluorescence was not confined to the plasma membrane, but intracellular reactions were also seen, as previously reported (Leterrier et al., 2006; McDonald et al., 2007; Scavone JL et al., 2010). Using PCR primers that span the alternatively spliced exon that distinguished the D₂ long (D_{2L}) and D₂ short (D_{2S}) isoforms (Coronas et al., 1997), we found that *STHdh*^{Q7/Q7} cells only express the D_{2L} isoform (data not shown). The D_{2L} isoform was cloned and used for all BRET² analyses.

BRET² was used to determine whether CB₁ and D_{2L} receptors heteromerize when expressed in *STHdh*^{Q7/Q7} cells. Cells were cotransfected with CB₁-Rluc and D_{2L}-GFP² constructs. Negative control included the human ether-a-go-go-related gene construct HERG-GFP², which is a membrane-localized K⁺ channel that does not interact with GPCRs or G proteins (Hudson et al., 2010). The combination of CB₁-Rluc and D_{2L}-GFP² resulted in greater BRET_{Eff} compared with negative controls obtained from cells expressing CB₁-Rluc and HERG-GFP² (Fig. 1A), indicating that CB₁ and D_{2L} form heteromers when coexpressed in *STHdh*^{Q7/Q7} cells. The interaction between CB₁ and D_{2L} is mediated by the C-terminus of CB₁ and the third intracellular loop of D_{2L} (Khan and Lee, 2014). To disrupt the formation of CB₁ and D_{2L} complexes, a CB₁ blocking peptide (CB₁-BP) that binds to the CB₁ receptor C-terminal region (C417-S431) was cloned (Khan and Lee, 2014). The CB₁-BP inhibits the heteromerization of CB₁ and D_{2L} by competing with CB₁ for binding with D_{2L} (Khan and Lee, 2014). The coexpression of CB₁-Rluc and D_{2L}-GFP² together with the CB₁-BP reduced BRET_{Eff} relative to cells transfected with CB₁-Rluc and D_{2L}-GFP² (Fig. 1A). CB₁-BP did not alter BRET_{Eff} in cells expressing CB₁-Rluc and CB₁-GFP² (Fig. 1A). These differences in BRET² signals did not result from a difference in the expression level of BRET² partners quantified by luminescence and fluorescence measurements (Supplemental Fig. 2). These data indicate that CB₁-BP blocks the formation of CB₁-D_{2L} heteromers, but not CB₁ homomers, suggesting that the protein regions crucial for CB₁ homomerization are different from those involved in CB₁-D_{2L} heteromerization.

A BRET² saturation curve was generated to demonstrate the ability of CB₁ and D_{2L} receptors to form heteromers at constant donor expression levels and increasing acceptor expression levels. For the BRET² saturation curve, cells were cotransfected with a constant amount of CB₁-Rluc with increasing amounts of D_{2L}-GFP² or HERG-GFP² (Fig. 1B). The combination of CB₁-Rluc with D₂-GFP² resulted in a significantly different saturation curve than the control curve, which was generated with the coexpression of CB₁-Rluc with HERG-GFP² (Fig. 1B). The BRET² saturation curve resulted in a BRET_{Max} of 0.28 ± 0.01 and a BRET₅₀ of 0.4 ± 0.03 . Treating cells coexpressing CB₁-Rluc and D_{2L}-GFP² for 30 minutes with 1 μ M ACEA, 10 μ M haloperidol or both resulted in higher BRET_{Max} but not BRET₅₀ compared with the BRET_{Max} observed in vehicle-treated cells. Haloperidol treatment alone did not alter BRET_{Max} or BRET₅₀ compared with vehicle-treated cells (Fig. 1C). The change in BRET_{Max}, but not BRET₅₀, following treatment with cannabinoid alone

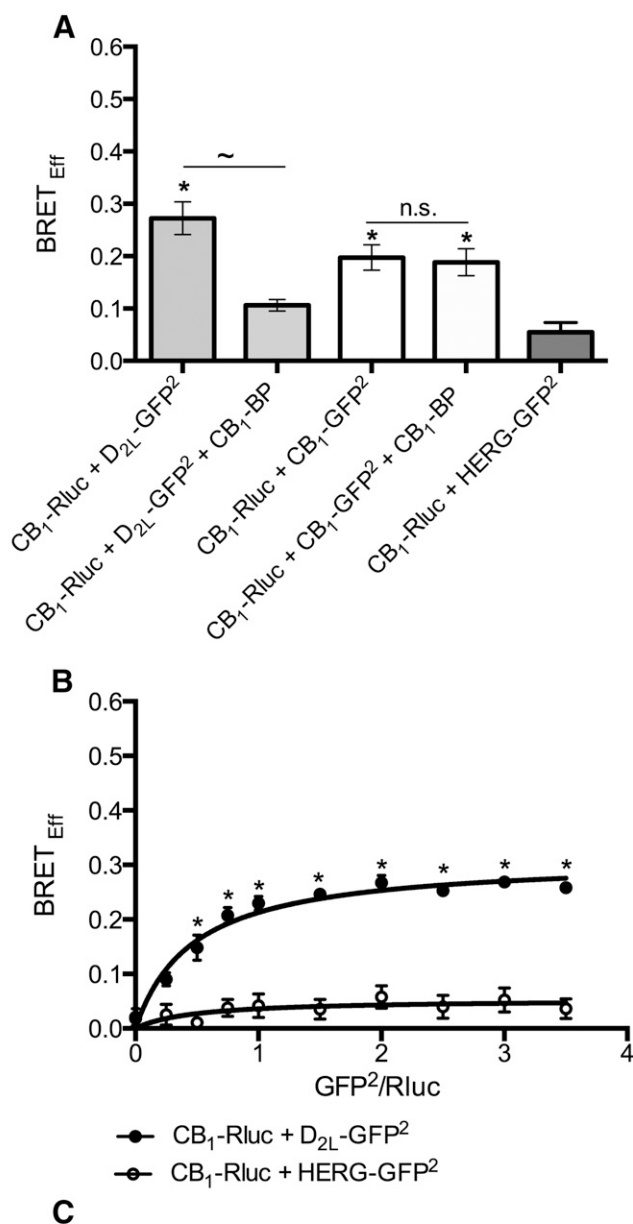


Fig. 1. CB₁ and D_{2L} receptors formed heteromers when expressed in *STHdh*^{Q7/Q7} cells as demonstrated using BRET². (A) BRET_{Eff} was measured in cells expressing CB₁-Rluc and D_{2L}-GFP², or CB₁-Rluc and CB₁-GFP² constructs and the CB₁-BP or pcDNA vector. As a negative control, cells were cotransfected with CB₁-Rluc and HERG-GFP². **P* < 0.01 compared with cells expressing CB₁-Rluc and HERG-GFP². ~*P* < 0.01 compared with cells expressing CB₁-Rluc, D_{2L}-GFP² and pcDNA. *n.s.* > 0.05 compared to cells expressing CB₁-Rluc, CB₁-GFP² and pcDNA. (B) BRET² saturation curves of cells transiently transfected with a constant amount of CB₁-Rluc and an increasing amount of D_{2L}-GFP². **P* < 0.01 compared with cells expressing CB₁-Rluc and HERG-GFP². (C) BRET_{Max} and BRET₅₀ parameters derived from BRET² saturation curves of cells transiently transfected with a constant amount of CB₁-Rluc and an increasing amount of D_{2L}-GFP² and treated for 30 minutes with vehicle and 1 μM ACEA or 10 μM haloperidol, alone or combined. **P* < 0.01 compared with cells treated

or in combination with D₂ antagonist, suggests that ligand binding stabilized the conformation of this heteromer, which enhanced the energy transfer between CB₁ and D_{2L} without increasing the number of receptors involved in heteromerization.

D₂ Antagonism Can Allosterically Inhibit the Association of CB₁ Receptor and Gα_i Protein. Different CB₁ agonists can activate different G proteins including Gα_i and Gα_s proteins (Bosier et al., 2010; Laprairie, et al., 2014). To study coupling of CB₁ to Gα_i and Gα_s proteins, we used BRET² for real-time assessment of receptor-G protein interaction in living *STHdh*^{Q7/Q7} cells transiently transfected with G protein-Rluc and CB₁-GFP².

Our first aim was to investigate the coupling of CB₁ to Gα_i protein in the absence of agonist. *STHdh*^{Q7/Q7} cells were transiently transfected with Gα_i-Rluc and CB₁-GFP². Coexpression of Gα_i-Rluc and CB₁-GFP² resulted in BRET_{Eff} equal to 0.23 ± 0.08, which was higher than cells expressing Gα_i-Rluc and HERG-GFP² (Fig. 2A). We found basal BRET_{Eff} was insensitive to 24-hour PTx treatment (Fig. 2A). Chronic PTx treatment inactivates Gα_i protein. This finding confirms that CB₁ receptors are preassembled with Gα_i prior to the addition of exogenous ligand and does not result from constitutive activation of Gα_i (Ayoub et al., 2007). Next, the influence of CB₁ agonist treatment on CB₁-Gα_i coupling was tested. Treating cells with 1 μM ACEA resulted in an increase in BRET_{Eff} (Fig. 2A). Inactivating Gα_i with PTx suppressed ACEA-induced BRET_{Eff} to the basal level (Fig. 2A). The agonist-induced BRET_{Eff} increase clearly demonstrates a functional coupling of CB₁ and Gα_i protein.

We measured the effect of D₂ antagonism on CB₁ agonist-induced CB₁-Gα_i-dependent BRET_{Eff} in cells cotransfected with Gα_i-Rluc and CB₁-GFP² and untagged D_{2L}-pcDNA. An ACEA concentration response curve was generated. Increasing ACEA concentration resulted in an increase in BRET_{Eff} between Gα_i and CB₁ [EC₅₀ = 0.22 (0.19–0.28)], E_{Max} = 0.45 (0.41–0.48), and Hill coefficient = 1.15 (0.91–1.4) (Fig. 2B). Treating the cells with different concentrations of haloperidol alone did not alter BRET_{Eff} between Gα_i and CB₁ (data not shown). However, pretreating the cells with haloperidol 25 seconds prior the addition of ACEA reduced ACEA-induced BRET_{Eff} signal between Gα_i and CB₁ in a haloperidol concentration-dependent manner (Fig. 2B). Haloperidol produced a concentration-dependent rightward and downward shift in the ACEA concentration response curves. Both the efficacy and the potency of ACEA-dependent Gα_i-CB₁ interaction were diminished by D₂ antagonism. The rightward shift in EC₅₀ for ACEA concentration-response curves was significant at 0.1, 1, and 10 μM haloperidol for ACEA-treated cells (Table 1). The decrease in E_{max} was significant at all concentrations of haloperidol tested (Table 1). The Hill coefficient was significantly less than 1 at 0.1, 1, and 10 μM haloperidol for ACEA concentration-response curves (Table 1). The observed effects of haloperidol on ACEA-dependent Gα_i-CB₁ interaction indicate the presence of negative cooperativity; the Hill coefficient is less than one.

with vehicle. Data are presented as mean ± S.E.M. of four independent experiments. Significance was determined via one-way ANOVA followed by Tukey's posthoc test, *n* = 4.

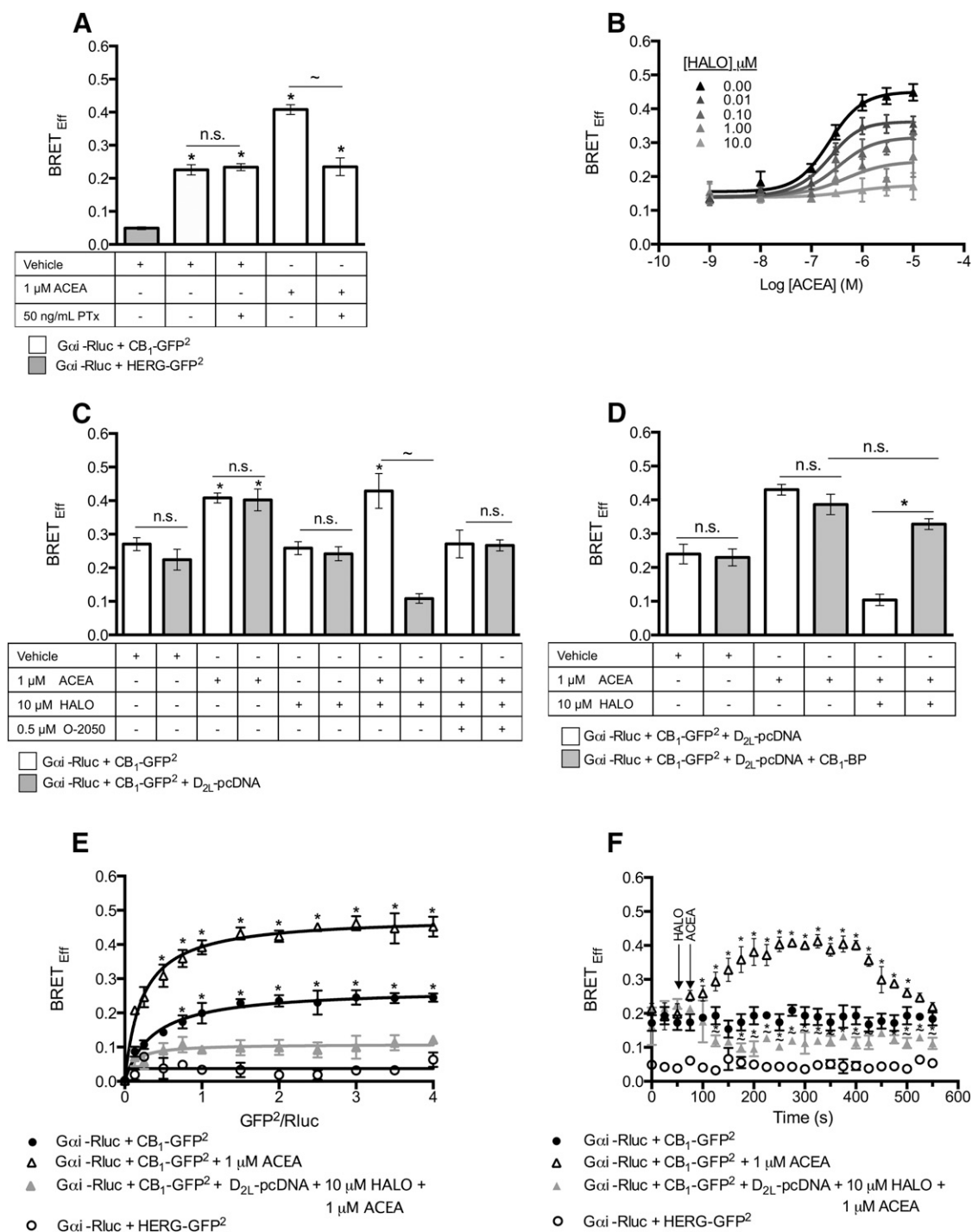


Fig. 2. Haloperidol treatment inhibited interactions between CB_1 and $G_{\alpha i}$ in the presence of ACEA in $STHdh^{Q7/Q7}$ cells. (A) BRET_{eff} was measured at 2 minutes following the addition of vehicle or 1 μ M ACEA $\pm$ 24-hour pretreatment with 50 ng/mL PTx in cells expressing $G_{\alpha i}$ -Rluc and CB_1 -GFP². * P < 0.01 relative to cells expressing $G_{\alpha i}$ -Rluc and HERG-GFP²; ~ P < 0.01 compared with cells expressing $G_{\alpha i}$ -Rluc and CB_1 -GFP² treated with PTx for 24 hours. (B) Concentration-response curves of ACEA-induced BRET_{eff} between $G_{\alpha i}$ -Rluc and CB_1 -GFP² in the absence or presence of haloperidol (HALO) measured at 2 minutes following ACEA application. (C) BRET_{eff} was measured at 2 minutes following the addition of vehicle, 10 μ M HALO, 1 μ M ACEA $\pm$ 10 μ M HALO and pretreated for 30 minutes with 0.5 μ M O-2050 in cells expressing $G_{\alpha i}$ -Rluc and CB_1 -GFP² $\pm$ D_{2L}-pcDNA. n.s. P > 0.05 compared with cells expressing $G_{\alpha i}$ -Rluc and CB_1 -GFP² only; * P < 0.01 relative to cells expressing $G_{\alpha i}$ -Rluc and CB_1 -GFP² and treated with 1 μ M ACEA and 10 μ M HALO; * P < 0.01 compared with cells treated with vehicle. (D) Cells were transfected with $G_{\alpha i}$ -Rluc, CB_1 -GFP², D_{2L}-pcDNA and CB_1 -BP, or pcDNA, and BRET_{eff} was measured at 2 minutes following the addition of vehicle, 10 μ M HALO, 1 μ M ACEA $\pm$ 10 μ M HALO. * P < 0.01 relative to cells expressing $G_{\alpha i}$ -Rluc, CB_1 -GFP² and CB_1 -BP and treated with 1 μ M ACEA and 10 μ M HALO. (E) BRET² saturation curves were generated by cotransfecting constant amounts of $G_{\alpha i}$ -Rluc and increasing amounts of CB_1 -GFP² alone or with D_{2L}-pcDNA or HERG-GFP², and BRET_{eff} was measured following the addition of vehicle, 1 μ M ACEA alone or in combination with 10 μ M HALO. * P < 0.01 compared with cells expressing $G_{\alpha i}$ -Rluc and HERG-GFP². (F) BRET_{eff} was measured over 9 minutes (540 seconds) in cells expressing $G_{\alpha i}$ -Rluc and CB_1 -GFP² alone or together with D_{2L}-pcDNA and treated with vehicle, 1 μ M ACEA $\pm$ 10 μ M HALO. As a negative control, cells were cotransfected with $G_{\alpha i}$ -Rluc and HERG-GFP². * P < 0.01 compared with cells expressing $G_{\alpha i}$ -Rluc and CB_1 -GFP² and treated with vehicle; ~ P < 0.01 compared with cells expressing $G_{\alpha i}$ -Rluc and CB_1 -GFP² and treated with 1 μ M ACEA. Data are presented as mean $\pm$ S.E.M. of four independent experiments, significance was determined via one-way ANOVA followed by Tukey's posthoc test, n = 4.

TABLE 1

The effects of haloperidol on BRET² (Gα_i-Rluc + CB₁-GFP²), Gα_i-dependent ERK phosphorylation, BRET² (Gα_s-Rluc + CB₁-GFP²), Gα_s-dependent CREB phosphorylation, and BRET² (β-arrestin1-Rluc + CB₁-GFP²)

Determined using nonlinear regression with variable slope (four parameter) in GraphPad (version 6.0). Data shown are 95% CI, from four independent experiments.

Agonist	HALO (μM)	EC ₅₀ μM (95% CI)	E _{Max} (95% CI)	Hill Coefficient (95% CI)
BRET ² (Gα _i -Rluc + CB ₁ -GFP ²)	0	0.22 (0.19–0.28)	0.45 (0.41–0.48)	1.15 (0.91–1.40)
	ACEA	0.01	0.25 (0.21–0.32)	0.36 (0.33–0.38)*
		0.1	0.38 (0.32–0.43)*	0.33 (0.26–0.39)*
		1	0.68 (0.33–0.92)*	0.23 (0.21–0.25)*
		10	0.98 (0.94–1.41)*	0.17 (0.14–0.20)*
Gα _i -dependent ERK phosphorylation	0	0.27 (0.25–0.29)	0.76 (0.71–0.80)	1.11 (0.89–1.23)
	ACEA	0.01	0.31 (0.26–0.36)	0.69 (0.64–0.75)
		0.1	0.41 (0.37–0.45)*	0.52 (0.48–0.57)*
		1	0.72 (0.67–0.78)*	0.39 (0.37–0.42)*
		10	1.01 (0.82–1.21)*	0.32 (0.29–0.34)*
BRET ² (Gα _s -Rluc + CB ₁ -GFP ²)	0	N.C.	N.C.	N.C.
	ACEA	0.01	0.49 (0.36–0.62)*	0.18 (0.16–0.19)*
		0.1	0.35 (0.29–0.42)*	0.26 (0.24–0.27)*
		1	0.29 (0.24–0.35)*	0.31 (0.28–0.33)*
		10	0.23 (0.18–0.31)*	0.36 (0.32–0.40)*
Gα _s -dependent CREB phosphorylation	0	0.35 (0.31–0.40)	0.26 (0.23–0.28)	1.02 (0.90–1.14)
	ACEA	0.01	0.31 (0.29–0.35)	0.42 (0.40–0.45)*
		0.1	0.29 (0.23–0.48)*	0.56 (0.53–0.59)*
		1	0.23 (0.19–0.27)*	0.65 (0.62–0.68)*
		10	0.21 (0.17–0.25)*	0.70 (0.67–0.74)*
BRET ² (β-arrestin1-Rluc + CB ₁ -GFP ²)	0	0.25 (0.19–0.35)	0.56 (0.52–0.60)	1.21 (0.11–1.23)
	ACEA	0.01	0.27 (0.21–0.37)	0.53 (0.49–0.56)
		0.1	0.33 (0.28–0.51)*	0.48 (0.45–0.52)
		1	0.36 (0.29–0.44)*	0.38 (0.36–0.41)*
		10	0.45 (0.34–0.57)*	0.32 (0.29–0.34)*

CI, confidence interval; N.C. not converged.

**P* < 0.01, compared with ACEA-treated cells; one-way ANOVA with Tukey's posthoc test.

To confirm that the observed allosteric effect of haloperidol was mediated through CB₁-D_{2L} heteromers and not mediated through the direct effect of haloperidol on the CB₁ receptor, cells were cotransfected with Gα_i-Rluc and CB₁-GFP² and treated with 10 μM haloperidol prior to 1 μM-ACEA application. No change in BRET_{eff} between Gα_i-Rluc and CB₁-GFP² was observed (Fig. 2C). These data demonstrate that CB₁-D_{2L} heteromerization was required for a haloperidol effect, as haloperidol had no effect on CB₁-Gα_i interactions in the absence of D_{2L} (Fig. 2C). In addition, the expression of equimolar D_{2L}-pcDNA and CB₁-GFP² in the presence of excess pool of Gα_i-Rluc did not alter CB₁ coupling to Gα_i-Rluc protein in the presence of vehicle or ACEA, compared with cells expressing CB₁-GFP² and Gα_i-Rluc alone (Fig. 2C). The coapplication of the CB₁ orthosteric antagonist O-2050 prior to the application of ACEA and haloperidol, returned BRET_{eff} to basal levels, which confirms that the observed increase in BRET_{eff} between CB₁ and Gα_i was attributable to the binding of ACEA to the orthosteric site of the CB₁ (Fig. 2C). Therefore, expression of D_{2L} receptors did not alter CB₁ coupling to Gα_i, but cotreatment of cells with haloperidol and ACEA resulted in reduced BRET_{eff} signals between Gα_i and CB₁.

Next, we confirmed that the inhibition of BRET_{eff} between Gα_i and CB₁ following haloperidol and ACEA application was mediated through the binding of haloperidol to CB₁-D_{2L} complexes. To confirm this we blocked the heteromerization between CB₁ and D_{2L} receptors by the coexpression of CB₁-BP. Cells cotransfected with Gα_i-Rluc, CB₁-GFP², D_{2L}-pcDNA, and CB₁-BP treated with ACEA and haloperidol had higher BRET_{eff} compared with cells transfected with Gα_i-Rluc, CB₁-GFP², D_{2L}-pcDNA, and no CB₁-BP (Fig. 2D). Thus, haloperidol inhibited ACEA-enhanced CB₁-Gα_i induced BRET² through binding to CB₁-D_{2L} complexes.

BRET² saturation curves were generated between Gα_i-Rluc and CB₁-GFP² in the presence and absence of ACEA to validate the specificity of the interaction between Gα_i and CB₁ (Fig. 2E). Cells were cotransfected with constant amounts of Gα_i-Rluc and increasing amounts of CB₁-GFP² or HERG-GFP² (Fig. 2E). The combination of Gα_i-Rluc and CB₁-GFP² resulted in a BRET_{Max} of 0.26 ± 0.041 and a BRET₅₀ of 0.37 ± 0.05. The BRET_{Max} and BRET₅₀ values were higher compared with cells expressing Gα_i-Rluc and HERG-GFP² (Fig. 2E). Therefore, the interaction between Gα_i and CB₁ was specific and saturable. To test whether ACEA treatment resulted in conformational changes within the preassembled CB₁-Gα_i complexes (observed as changes in BRET_{Max}), rather than the recruitment of more Gα_i to CB₁ (observed as changes in BRET₅₀) (Ayoub et al., 2012), a BRET² saturation curve was created following ACEA (1 μM) treatment (Fig. 2E). The BRET² saturation curve displayed BRET_{Max} of 0.40 ± 0.03 and BRET₅₀ of 0.39 ± 0.04. The BRET_{Max} obtained from treatment with ACEA was significantly higher compared with cells treated with vehicle (BRET_{Max} of 0.26 ± 0.04). No significant change in BRET₅₀ values was observed (Fig. 2E). Therefore, ACEA treatment only induced conformational changes with the CB₁-Gα_i complexes. To test whether haloperidol treatment induces conformational changes with the CB₁-D_{2L}-Gα_i complexes or it promotes the dissociation of CB₁ and Gα_i, a BRET² saturation curve was generated in cells expressing Gα_i-Rluc, CB₁-GFP², and D_{2L}-pcDNA (Fig. 2E). Cotreating the cells with 10 μM haloperidol and 1 μM ACEA significantly reduced BRET_{Max} (0.11 ± 0.04) and BRET₅₀ (0.11 ± 0.07) compared with vehicle-treated cells. Reduction in both BRET_{Max} and BRET₅₀ following haloperidol and ACEA treatment suggested that haloperidol induced dissociation of Gα_i and CB₁ and induced conformational changes between Gα_i-Rluc and CB₁-GFP².

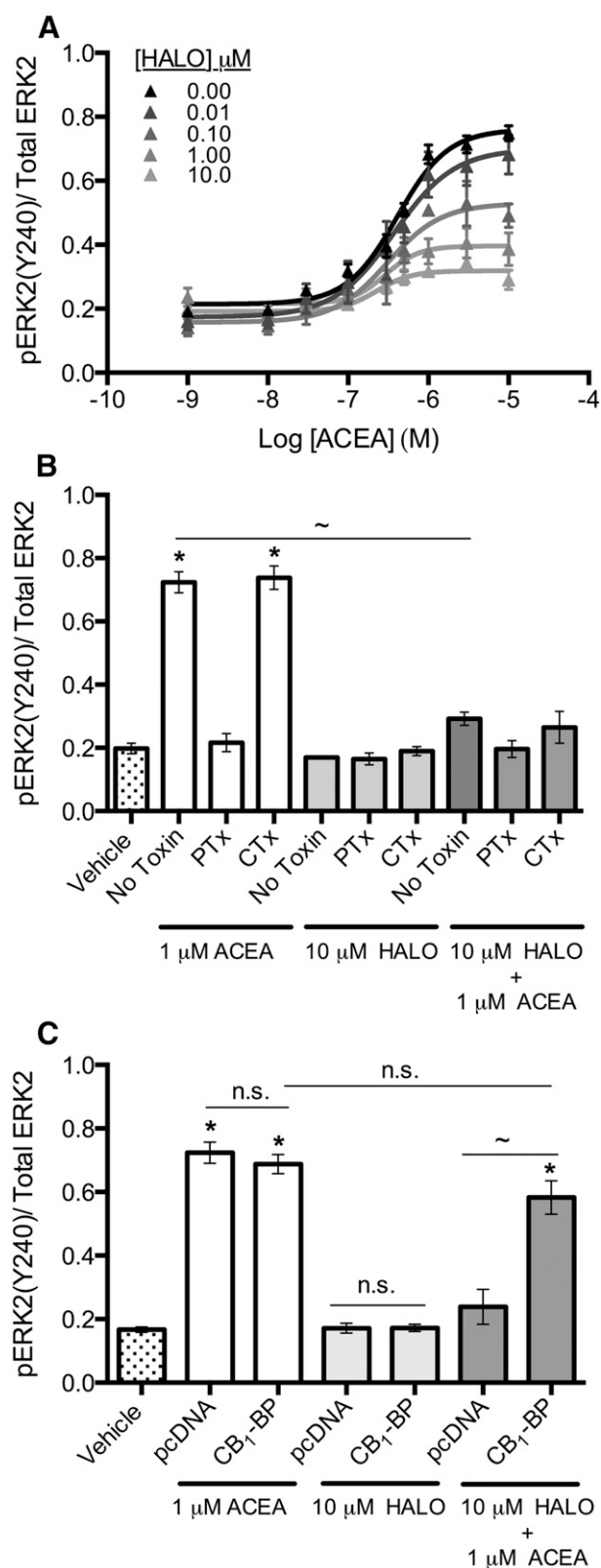


Fig. 3. Haloperidol reduced ACEA-induced ERK phosphorylation. (A) pERK concentration response curve from *STHdh*^{Q7/Q7} cells treated with ACEA alone or in the presence of HALO measured at 5 minutes. (B) *STHdh*^{Q7/Q7} cells were treated with 1 μ M ACEA alone for 5 minutes or in combination with 10 μ M HALO +/- 24-hour pretreatment with 50 ng/ml PTx or CTx. * P < 0.01 compared with vehicle treatment; ~ P < 0.01 compared with cells treated with 1 μ M ACEA. (C) *STHdh*^{Q7/Q7} cells were transfected with the pcDNA or CB₁-BP and treated with 1 μ M ACEA alone for

A kinetic analysis of ACEA-induced BRET² between $G\alpha_i$ and CB₁ was carried out. Cells were cotransfected with $G\alpha_i$ -Rluc and CB₁-GFP² and signals were recorded as repeated measures in vehicle-treated cells for over 9 minutes (540 seconds). Treating cells with 1 μ M ACEA 75 seconds after the addition of coelenterazine 400a resulted in a rapid increase in BRET_{Eff} (Fig. 2F). BRET_{Eff} peaked at ~125 seconds and remained significantly higher for ~400 seconds before declining (Fig. 2F). By ~450 seconds following ACEA application (Fig. 2F), the BRET_{Eff} returned to pre-ACEA levels and remained at this level for 30 minutes (data not shown). However, in cells coexpressing $G\alpha_i$ -Rluc, CB₁-GFP², and D_{2L}-pcDNA, treating the cells with 10 μ M haloperidol (50 seconds following the initiation of the reaction and 25 seconds prior to the application of 1 μ M ACEA) resulted in a rapid reduction in BRET_{Eff} compared with vehicle-treated cells and compared with ACEA-treated cells (Fig. 2F). The reduction in BRET_{Eff} was sustained for the remaining 480 seconds (Fig. 2F). Reduction of BRET_{Eff} below the basal level was observed at 10, 20, and 30 minutes following ACEA application (P < 0.01) (data not shown).

D₂ Antagonism Reduced the Efficacy and Potency of CB₁-Dependent $G\alpha_i$ -Mediated ERK Phosphorylation.

We had observed a reduction in BRET_{Eff} between $G\alpha_i$ and CB₁ in *STHdh*^{Q7/Q7} cells coexpressing D_{2L} following ACEA and haloperidol treatment, which might suggested that CB₁ receptors are dissociated from $G\alpha_i$ proteins. Thus, we measured whether ACEA-induced and $G\alpha_i$ -mediated ERK phosphorylation was also inhibited by haloperidol treatment. A concentration response curve of ACEA-induced ERK phosphorylation was generated following 5 minutes treatment (EC_{50} = 0.27 (0.25–0.29), E_{Max} 0.76 (0.71–0.80), Hill coefficient = 1.11 (0.89–1.23) (Fig. 3A; Table 1). Haloperidol treatment (0.01–10 μ M) alone did not increase ERK phosphorylation (data not shown). Cotreating the cells with increasing concentrations of haloperidol 25 seconds prior the addition of ACEA produced a concentration-dependent reduction in ACEA E_{Max} and EC_{50} . The reduction in EC_{50} and E_{Max} was significant at 0.1, 1, and 10 μ M haloperidol (Table 1). The Hill coefficient was less than 1 in cells treated with 1 and 10 μ M haloperidol, indicating a negative cooperatively effect (Table 1). To confirm that the CB₁-mediated ERK phosphorylation was mediated through $G\alpha_{i/o}$ protein, *STHdh*^{Q7/Q7} cells were pretreated with PTx for 24 hours, prior to application of ACEA, haloperidol, or both (Fig. 3B). PTx pretreatment inhibited ERK phosphorylation induced by 1 μ M ACEA. However, pretreating the cells with CTx for 24 hours, which suppresses $G\alpha_s$ expression, did not alter ACEA-mediated ERK phosphorylation. These results demonstrated that ACEA treatment induced a PTx-sensitive, $G\alpha_{i/o}$ -mediated increase in ERK phosphorylation. O-2050 pretreatment inhibited ACEA-mediated ERK phosphorylation (data not shown). The coapplication of 10 μ M haloperidol and

5 minutes or in combination with 10 μ M HALO. * P < 0.01 compared with vehicle treatment; ~ P < 0.01 compared with cells transfected with empty pcDNA vector and treated with 1 μ M ACEA and 10 μ M HALO; n.s. P > 0.05 compared to cells transfected with empty pcDNA, or compared to cells transfected with CB₁-BP and treated with 1 μ M ACEA. Data are presented as mean $\pm$ S.E.M. of four independent experiments, one-way ANOVA followed by Tukey's posthoc test, n = 4.

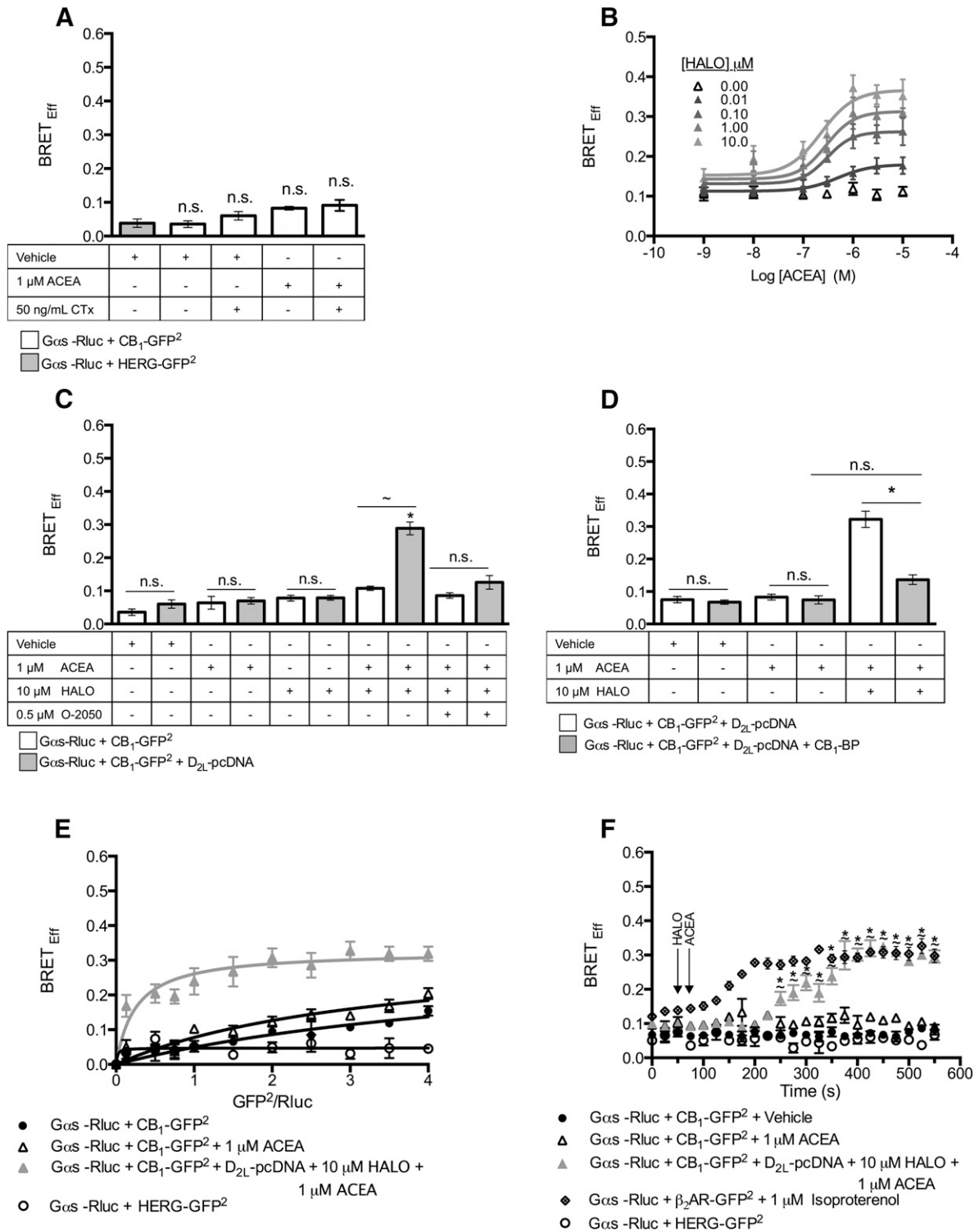


Fig. 4. Cotreatment with ACEA and haloperidol promoted interactions between CB₁ and *Gα_s* in *STHdh*^{Q7/Q7} cells. (A) BRET_{EFF} was measured 5 minutes following the addition of vehicle or 1 μM ACEA or with 500 nM CTx pretreated for 24 hours in cells expressing *Gα_s*-Rluc and CB₁-GFP². n.s., *P* > 0.05 relative to cells expressing *Gα_s*-Rluc and HERG-GFP². (B) Concentration response curves of ACEA-induced BRET_{EFF} between *Gα_s*-Rluc and CB₁-GFP² in the absence or presence of HALO measured at 5 minutes following ACEA application. (C) BRET_{EFF} was measured at 5 minutes following the addition of vehicle, 10 μM HALO or 1 μM ACEA +/- 10 μM HALO and pretreated for 30 minutes with 0.5 mM O-2050 in cells expressing *Gα_s*-Rluc and CB₁-GFP² alone or together with D_{2L}-pcDNA. n.s. *P* > 0.05 compared with cells expressing *Gα_s*-Rluc and CB₁-GFP² only; **P* < 0.01 relative to cells expressing *Gα_s*-Rluc and CB₁-GFP² and treated with 1 μM ACEA and 10 μM HALO; **P* < 0.01 compared with cells treated with vehicle. (D) Cells were transfected with *Gα_s*-Rluc, CB₁-GFP², D_{2L}-pcDNA and CB₁-BP or pcDNA, and BRET_{EFF} was measured at 5 minutes following the addition of vehicle, 10 μM HALO, 1 μM ACEA +/- 10 μM HALO. **P* < 0.01 relative to cells expressing *Gα_s*-Rluc and CB₁-GFP² and CB₁-BP and treated with 1 μM ACEA and 10 μM HALO; n.s. *P* > 0.05 compared to cells expressing *Gα_s*-Rluc, CB₁-GFP², D_{2L}-pcDNA and empty pcDNA. (E) BRET² saturation curves were generated by cotransfected constant amounts of *Gα_s*-Rluc and increasing amounts of CB₁-GFP² alone or with D_{2L}-pcDNA or HERG-GFP², and BRET_{EFF} was measured following the addition of vehicle, 1 μM ACEA +/- 10 μM HALO. **P* < 0.01 compared with cells expressing *Gα_s*-Rluc and HERG-GFP². (F) BRET_{EFF} was measured over 9 minutes in cells expressing *Gα_s*-Rluc and CB₁-GFP² alone or together with D_{2L}-pcDNA and treated with vehicle, 1 μM ACEA alone or together with

ACEA prevented ACEA-induced $G\alpha_{i/o}$ -dependent ERK phosphorylation (Fig. 3B). Transfecting *STHdh*^{Q7/Q7} cells with CB₁-BP did not alter ACEA-induced ERK phosphorylation (Fig. 3C). The expression of CB₁-BP restored ACEA-induced ERK phosphorylation in haloperidol-treated cells (Fig. 3C). Haloperidol inhibited CB₁-dependent and $G\alpha_{i/o}$ -mediated ERK signaling through binding to CB₁-D_{2L} complexes.

Combined D₂ Antagonism and CB₁ Agonism Enhanced BRET_{Eff} Between CB₁ and $G\alpha_s$. We studied next the coupling of CB₁ to $G\alpha_s$ protein in the absence and in the presence of cannabinoid CB₁ agonist. BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP² was similar to that observed in cells coexpressing $G\alpha_s$ -Rluc and HERG-GFP² (Fig. 4A). Twenty-four-hour CTx pretreatment did not affect BRET_{Eff} compared with vehicle-treated cells (Fig. 4A). The higher basal BRET_{Eff} between cells expressing $G\alpha_i$ -Rluc and CB₁-GFP² (Fig. 2A) compared with cells expressing $G\alpha_s$ -Rluc and CB₁-GFP² (Fig. 4A) was not a result of different levels in the expression of $G\alpha_i$ -Rluc, $G\alpha_s$ -Rluc or CB₁-GFP² proteins in the cells because luminescence and fluorescence intensities measured from cells transfected with these constructs were not different (data not shown). In addition, 1 μ M ACEA treatment did not alter BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP² (Fig. 4A). In the absence or presence of CB₁ agonist, no energy transfer was detected between $G\alpha_s$ -Rluc and CB₁-GFP² proteins.

Since we have observed an inhibition in BRET² signals between $G\alpha_i$ -Rluc and CB₁-GFP² proteins and inhibition of CB₁-dependent and $G\alpha_{i/o}$ -mediated ERK signaling following ACEA and haloperidol coapplication, we tested if the coapplication of both ligands promoted CB₁ coupling to $G\alpha_s$ protein. An ACEA concentration-response curve was generated to determine the concentration-dependent increase in $G\alpha_s$ -Rluc and CB₁-GFP² association in the presence of D_{2L}-pcDNA and increasing concentrations of haloperidol (0.01–10 μ M) added 25 seconds prior the application of ACEA (Fig. 4B). Increasing ACEA concentration in the presence of increasing concentrations of haloperidol (0.01–10 μ M) resulted in an increase in BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP² in a concentration-dependent manner, shifting the ACEA concentration response curves to the left and upward. The reduction in EC₅₀ and the increase in E_{max} was significant at all haloperidol concentrations tested (0.01–10.0 μ M) (Table 1). Similarly, the Hill coefficient was significantly more than 1 at all haloperidol concentrations tested (Table 1), suggesting that haloperidol exerts a positive cooperativity effect on CB₁ to $G\alpha_s$ interaction.

Previous studies have shown that coexpression of D_{2L} and CB₁ in human embryonic kidney (HEK) 293 cells is sufficient to change the signaling of CB₁ from $G\alpha_i$ to $G\alpha_s$ (Jarrahian et al., 2004). In our study, we found that the coexpression of equimolar of D_{2L}-pcDNA and CB₁-GFP² in the presence of $G\alpha_s$ -Rluc did not change BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP² in the presence of vehicle or ACEA (Fig. 4C). The application of haloperidol alone did not alter BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP² in the absence or presence of D_{2L} (Fig. 4C). Haloperidol promoted BRET² signals between $G\alpha_s$ -Rluc

and CB₁-GFP² only in ACEA-treated cells in the presence of D_{2L}, suggesting that the observed effect of haloperidol and ACEA is mediated through CB₁-D_{2L} heteromers. However, inhibiting the heteromerization between CB₁ and D_{2L} by the expression of CB₁-BP together with $G\alpha_s$ -Rluc, CB₁-GFP², and D_{2L}-pcDNA blocked haloperidol-induced BRET² signals between $G\alpha_s$ -Rluc and CB₁-GFP² in the presence of ACEA (Fig. 4D). Cotreatment with ACEA and haloperidol, therefore, promoted BRET² signals between $G\alpha_s$ -Rluc and CB₁-GFP² through binding to D_{2L} receptors in CB₁-D_{2L} complexes.

BRET² saturation curves were generated to determine the specificity of the interaction between CB₁ and $G\alpha_s$ in the presence and absence of ACEA and/or haloperidol. In cells expressing constant amounts of $G\alpha_s$ -Rluc, increasing the concentration of transfected CB₁-GFP² resulted in a gradual linear increase in BRET_{Eff} in vehicle- or ACEA-treated cells, indicating that the interaction between $G\alpha_s$ -Rluc and CB₁-GFP² was nonspecific (Fig. 4E). However, treating cells expressing $G\alpha_s$ -Rluc, CB₁-GFP², and D_{2L}-pcDNA with 10 μ M haloperidol prior to 1 μ M ACEA application resulted in hyperbolic increase in BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP², with BRET_{Max} of 0.3264 ± 0.01 and BRET_{Min} of 0.25 ± 0.01 (Fig. 4E). The interaction between $G\alpha_s$ and CB₁ was specific and saturable in cells cotreated with ACEA and haloperidol.

Ligand-induced BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP² was recorded for 9 minutes. No BRET_{Eff} signals were observed following the application of 1 μ M ACEA over the 9-minute (540-second) observation period (Fig. 4F). Interestingly, treating cells with 10 μ M haloperidol 50 seconds after coelenterazine addition and 25 seconds prior to 1 μ M ACEA application resulted in a delayed increase in BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP² (225 seconds after the application of ACEA) (Fig. 4F). The signal peaked at 5 minutes (300 second) following the addition of ACEA (375 seconds after coelenterazine addition) (Fig. 4F). BRET_{Eff} signal was still observed at 5, 10 and 20, but not at 30 minutes following ACEA (data not shown). As a positive control, we used β_2 -AR, which has been demonstrated to preassemble with $G\alpha_s$ (Lachance et al., 1999). We measured BRET_{Eff} between $G\alpha_s$ -Rluc and β_2 -AR-GFP² before and following the application of the β_2 -AR agonist isoproterenol (1 μ M). High BRET_{Eff} was observed between $G\alpha_s$ -Rluc and β_2 -AR-GFP² in the absence of exogenous ligand. Isoproterenol led to rapid and sustained elevation in BRET_{Eff} (Fig. 4F). Therefore, the delayed BRET_{Eff} between $G\alpha_s$ and CB₁ following ACEA and haloperidol application could be the result of recruitment of $G\alpha_s$ to CB₁ and its activation instead of the activation of preassembled GPCR-G protein complexes.

Combined D₂ Antagonism and CB₁ Agonism Induced CREB Phosphorylation. We observed that haloperidol treatment increased BRET_{Eff} between $G\alpha_s$ -Rluc and CB₁-GFP² in the presence of ACEA (Fig. 4). To confirm that haloperidol treatment induced functional coupling of $G\alpha_s$ to CB₁ following ACEA treatment, we measured CTx-sensitive, $G\alpha_s$ -dependent CREB phosphorylation (Fig. 5). A concentration

10 μ M HALO. As a negative control, cells were cotransfected with $G\alpha_s$ -Rluc and HERG-GFP². Cells cotransfected with $G\alpha_s$ -Rluc and β_2 -GFP² were used as a positive control. * $P < 0.01$ compared with cells expressing $G\alpha_s$ -Rluc and CB₁-GFP² and treated with vehicle; ~ $P < 0.01$ compared with cells expressing $G\alpha_i$ -Rluc and CB₁-GFP² and treated with 1 μ M ACEA. Data are presented as mean $\pm$ S.E.M. of four independent experiments; significance was determined via one-way ANOVA followed by Tukey's posthoc test, $n = 4$.

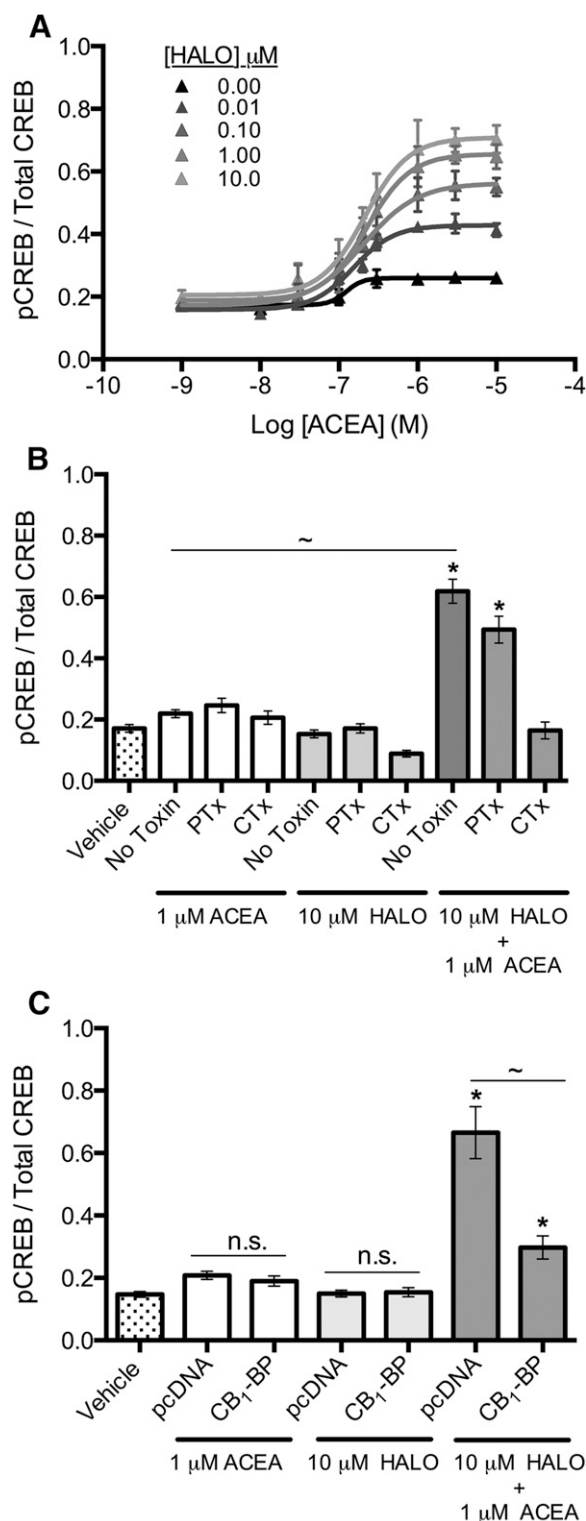


Fig. 5. Cotreatment with haloperidol and ACEA induced CREB phosphorylation. (A) pCREB concentration response curve from *STHdh*^{Q7/Q7} cells treated with ACEA $\pm$ HALO measured at 30 minutes. (B) *STHdh*^{Q7/Q7} cells were treated with 1 μ M ACEA $\pm$ 10 μ M HALO for 30 minutes with or without 24-hour pretreatment with 50 ng/ml PTx or CTx. * P < 0.01 compared with vehicle treatment; ~ P < 0.01 compared with cells treated with 1 μ M ACEA. (C) *STHdh*^{Q7/Q7} cells were transfected with pcDNA or the CB₁-BP and treated with 1 μ M ACEA $\pm$ 10 μ M HALO for 30 minutes. * P < 0.01 compared with vehicle treatment; ~ P < 0.01 compared with cells transfected with empty pcDNA and treated with 1 μ M ACEA and 10 μ M HALO. CREB phosphorylation was quantified via In-Cell Western data are presented as mean $\pm$ S.E.M.

response curve of ACEA-induced CREB phosphorylation was generated following 30 minutes treatment (Fig. 5A). Treating *STHdh*^{Q7/Q7} cells with different concentrations of ACEA did not change CREB phosphorylation compared with vehicle-treated cells (Fig. 5, A and B). Similarly, haloperidol (0.01–10 μ M) treatment did not increase CREB phosphorylation (data not shown). Pretreating the cells with haloperidol 25 seconds prior to the application of ACEA significantly increased CREB phosphorylation. The coapplication of increasing concentrations of haloperidol reduced the EC₅₀, and increased $E_{\max}$ for ACEA-induced CREB phosphorylation (Table 1). The Hill coefficient values were more than 1, suggesting a positive cooperatively effects on CB₁-dependent CREB phosphorylation (Fig. 5A). To confirm that the observed CREB phosphorylation following the application of haloperidol and ACEA was G_{α_s} -dependent, cells were pretreated with CTx for 24 hours. Pretreating the cells with CTx blocked G_{α_s} -dependent CREB phosphorylation in cells cotreated with 1 μ M ACEA and 10 μ M haloperidol (Fig. 5B). CB₁-BP reduced ACEA CREB phosphorylation in haloperidol-treated cells (Fig. 5C). Therefore, cotreatment with haloperidol and ACEA induced G_{α_s} -mediated CREB phosphorylation through binding of haloperidol to CB₁-D_{2L} complexes.

To determine whether the observed effects of haloperidol on CB₁ signaling was specific to haloperidol or common to other high affinity D₂ antagonists, we tested the influence of the high affinity D₂ antagonist sulpiride on the coupling of CB₁ to G proteins and downstream signaling. A reduction in ACEA-enhanced BRET² between G_{α_i} and CB₁ (Supplemental Fig. 3A) and G_{α_i} -dependent ERK phosphorylation (Supplemental Fig. 3B) was observed when *STHdh*^{Q7/Q7} cells were treated with 10 μ M sulpiride and 1 μ M ACEA. In addition, an increase in BRET² signaling between G_{α_s} and CB₁ (Supplemental Fig. 3C) and G_{α_s} -dependent CREB signaling (Supplemental Fig. 3D) was detected in *STHdh*^{Q7/Q7} cells were treated with 10 μ M sulpiride and 1 μ M ACEA. Our findings demonstrated that high affinity orthosteric D₂ antagonists switch CB₁ coupling and signaling from G_{α_i} to G_{α_s} in response to CB₁ agonist when both CB₁ and D_{2L} receptors are expressed.

CB₁ Agonism Resulted in Slow and Sustained β -Arrestin1 Recruitment to CB₁ Receptor Inhibited by D₂ Antagonism. CB₁ activation is followed by C-terminal tail phosphorylation and β -arrestin1 (Laprairie et al., 2014) or β -arrestin2 (Jin et al., 1999; van der Lee et al., 2009) recruitment to CB₁, leading to receptor internalization. β -arrestin1 recruitment to CB₁ following ligand application was measured over 30 minutes using BRET². *STHdh*^{Q7/Q7} cells endogenously express β -arrestin1 (Laprairie et al., 2014). BRET_{eff} signals observed from cells expressing β -arrestin1-Rluc and CB₁-GFP² treated with vehicle was higher than BRET_{eff} between β -arrestin1-Rluc and HERG-GFP² (Fig. 6A). ACEA (1 μ M) treatment increased BRET_{eff} between β -arrestin1-Rluc and CB₁-GFP² starting at 5 minutes compared with vehicle-treated cells, and reached a plateau at 15 minutes. The signal was sustained for 30 minutes (Fig. 6A). However, treating the cells coexpressing β -arrestin1-Rluc, CB₁-GFP² and D_{2L}-pcDNA with 10 μ M haloperidol 25 seconds prior to the application of 1

of four independent experiments, one-way ANOVA followed by Tukey's posthoc test, n = 4.

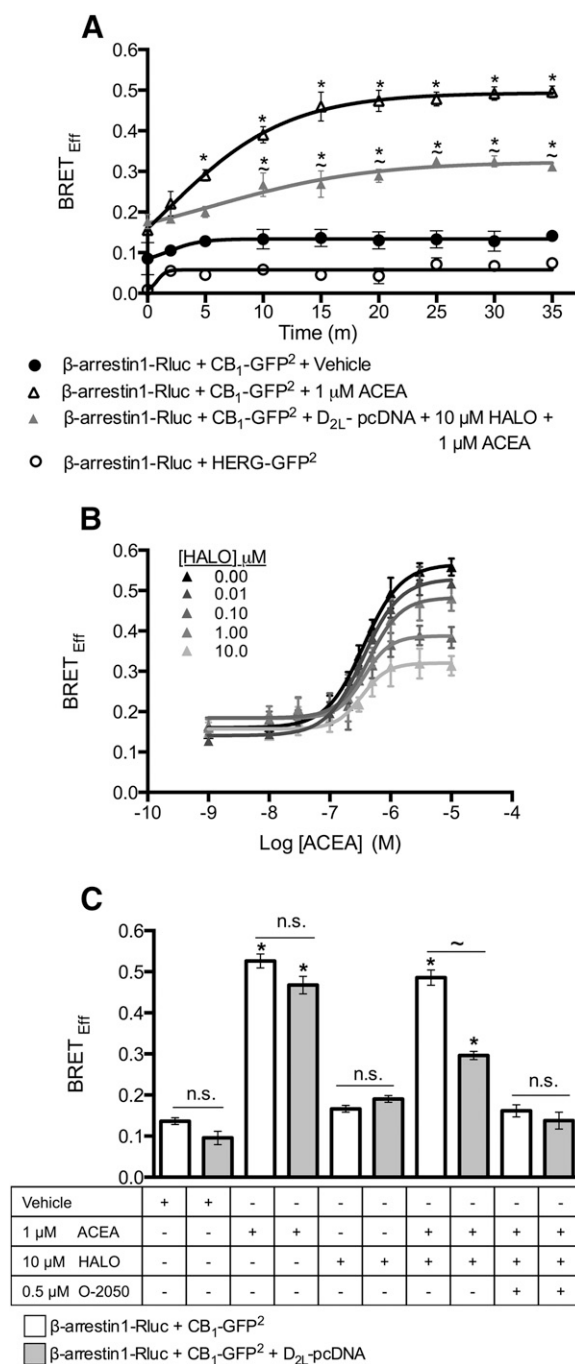


Fig. 6. ACEA treatment resulted in slow and sustained β -arrestin1 recruitment to CB₁ receptors, which was inhibited by haloperidol. (A) BRET_{Eff} was measured over 30-minutes in cells expressing β -arrestin1-Rluc and CB₁-GFP² +/- D_{2L}-pcDNA and treated with vehicle, 1 μ M ACEA alone or together with 10 +/- HALO. As a control, cells were cotransfected with β -arrestin1-Rluc and HERG-GFP². * P < 0.01 compared with vehicle-treated cells; ~ P < 0.01 compared with cells treated with 1 μ M ACEA. (B) Concentration-response curves of ACEA-induced BRET_{Eff} between β -arrestin1-Rluc and CB₁-GFP² in the absence or presence of increasing concentrations of HALO. (C) BRET_{Eff} was measured at 30 minutes following the addition of vehicle, 1 μ M ACEA +/- 10 μ M HALO or with a 30-minute pretreatment with 0.5 μ M O-2050 in cells expressing β -arrestin1-Rluc and CB₁-GFP² alone or together with D_{2L}-pcDNA. n.s., P > 0.05 compared with cells expressing β -arrestin1-Rluc and CB₁-GFP² only; ~ P < 0.01 relative to cells expressing β -arrestin1-Rluc and CB₁-GFP² and treated with 1 μ M ACEA and 10 μ M HALO; * P < 0.01 compared with cells treated with vehicle. Data are presented as mean $\pm$ S.E.M. of four independent experiments, as determined via one-way ANOVA followed by Tukey's posthoc test, n = 4.

μ M ACEA decreased BRET_{Eff} between β -arrestin1-Rluc and CB₁-GFP² over the 30 minutes (P < 0.01) compared with cells treated with 1 μ M ACEA (Fig. 6A). BRET_{Eff} signals between β -arrestin1-Rluc and CB₁-GFP² occurred in an ACEA concentration-dependent manner (Fig. 6B). The addition of increasing concentrations of (0.1, 1, and 10 μ M) haloperidol prior to ACEA application resulted in a lower E_{Max} and EC_{50} (Table 1). The reduction in ACEA-induced β -arrestin1 recruitment to CB₁ mediated by haloperidol is consistent with the interpretation that haloperidol acts as a negative allosteric modulator of CB₁- β -arrestin1 interactions. Treating the cells expressing β -arrestin1-Rluc, CB₁-GFP², and D_{2L}-pcDNA with 10 μ M haloperidol or 0.5 μ M O-2050 alone did not alter BRET_{Eff} between β -arrestin1-Rluc and CB₁-GFP² compared with vehicle-treated cells (Fig. 6C). Our results demonstrated that D₂ antagonism reduced β -arrestin1 recruitment to CB₁ receptors in the presence of CB₁ agonist.

CB₁ internalization was measured in *STHdh*^{Q7/Q7} cells transfected with CB₁-GFP² and β -arrestin1. CB₁ internalization was measured over 30 minutes following ligand treatment (Fig. 7, A and B). Treating *STHdh*^{Q7/Q7} cells with 1 μ M ACEA resulted in CB₁ internalization starting at 10 minutes compared with vehicle-treated cells (Fig. 7, A and B). Treating the cells with 10 μ M haloperidol alone or 0.5 μ M O-2050 did not alter CB₁ localization compared with vehicle-treated cells (Fig. 7B). Pretreating the cells with 0.5 μ M O-2050 before the application of 1 μ M ACEA inhibited CB₁ internalization over 30 minutes (Fig. 7, A and B). Haloperidol pretreatment significantly inhibited ACEA-induced CB₁ internalization compared with ACEA-treated cells. Endogenous CB₁ internalization was also measured in *STHdh*^{Q7/Q7} cells (Supplemental Fig. 4). The reduction of BRET² between β -arrestin1-Rluc and CB₁-GFP² following transfection is consistent with the observation that endogenous CB₁ internalization was reduced following haloperidol and ACEA treatment (Supplemental Fig. 4).

Discussion

Haloperidol Allosterically Alters CB₁-G Protein Coupling and Downstream Cellular Signaling. Given the clinical importance of D₂ antagonists and previous reports that D₂ agonists influence CB₁ signaling, the goal of this study was to determine if D₂ antagonists act as modulators of CB₁ signaling. Specifically, we wanted to investigate how D₂ antagonists and CB₁ agonists influence the activity of CB₁-D_{2L} heteromers. We investigated the influence of D₂ antagonism on CB₁ coupling to G α_i and G α_s , as well as β -arrestin1 recruitment and internalization in a model of striatal medium spiny projection neurons (summarized in Fig. 8). In this study, we were able to show that CB₁ and D_{2L} receptors heteromerize when coexpressed in *STHdh*^{Q7/Q7} cells in the absence and presence of exogenous CB₁ ligand. CB₁ agonist treatment stabilized the conformation of the preassembled CB₁-D_{2L} heteromers but did not alter the number of receptors involved in forming CB₁-D_{2L} complexes. CB₁ was coupled to G α_i in the absence of CB₁ ligand. Agonist-dependent CB₁-activation led to a rapid and transient conformational rearrangement of the preassembled CB₁-G α_i complexes, rather than the recruitment of G α_i -proteins to the CB₁ receptor. Activation was observed as a rapid increase in ERK phosphorylation through the PTx-sensitive G α_i pathway (Galés et al., 2005, 2006; Levoye et al., 2009). Sustained activation of CB₁ receptors

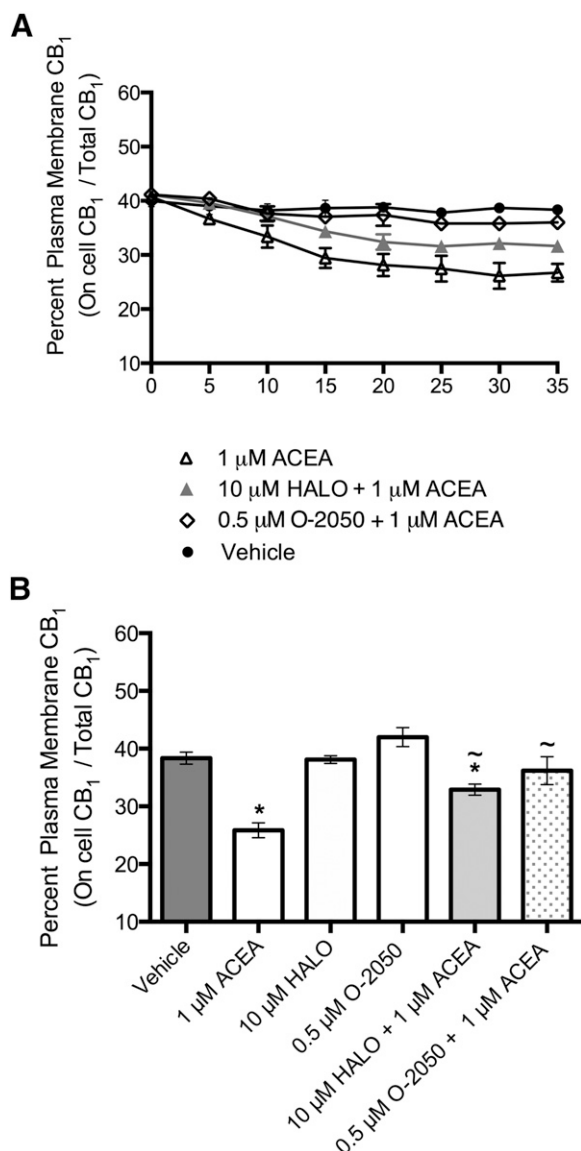


Fig. 7. Haloperidol inhibited CB₁ internalization following ACEA treatment. (A) Time-course analysis of CB₁ cell surface expression and total protein levels over 30 min in cells expressing β -arrestin1-Rluc, CB₁-GFP² and D_{2L}-pcDNA measured using On-Cell Western and In-Cell Western in cells treated with vehicle or 1 μ M ACEA and pretreated with 10 μ M HALO or 0.5 μ M O-2050. * P < 0.01 compared with vehicle. (B) CB₁ cell surface expression measured at 30 minutes following ligand treatment. * P < 0.01 compared with vehicle-treated cells. ~ P < 0.01 compared with ACEA-treated cells. Data are presented as mean $\pm$ S.E.M. of four independent experiments, one-way ANOVA followed by Tukey's posthoc test, n = 4.

was followed by the return of CB₁-G α_i complexes to the inactive conformation rather than dissociation of CB₁ and G α_i protein (Bunemann et al., 2003; Galés et al., 2006). Interactions between G α_i and CB₁ were completely not detected following CB₁ agonist and D₂ antagonist cotreatment. In contrast, the efficacy of ACEA-dependent ERK phosphorylation was reduced by only ~80% in the presence of haloperidol relative to ACEA-treated cells. The residual pERK signal (~20%) was retained in the presence of haloperidol suggesting that a portion of ACEA-dependent ERK signaling occurred through CB₁ monomers, CB₁ homomers, or CB₁-GPCR heteromers, or CB₁-independent mechanisms. (Wager-Miller et al.,

2002; Rios et al., 2006; Carriba et al., 2007; Hudson et al., 2010). In the presence of ACEA, haloperidol switched the CB₁ coupling from G α_i to G α_s and induced G α_s -dependent CREB phosphorylation. Previous studies have demonstrated that the coexpression and coactivation of both CB₁ and D_{2L} receptors is required to switch CB₁ signaling from G α_i to G α_s (Glass and Felder, 1997; Kearn et al., 2005). In our study, we found that the coexpression of both receptors and the addition of a CB₁ agonist and D₂ antagonist was sufficient to induce conformational changes within the preassembled CB₁-D_{2L}-G α_i complexes and favor a higher proportion of CB₁ to dissociate from G α_i (Bunemann et al., 2003; Galés et al., 2006).

The delayed interaction between G α_s and CB₁ following CB₁ agonist and D₂ antagonist application cannot be considered as a general feature for G α_s coupling since fast activation of G α_s following ligand binding has been demonstrated for other GPCRs. For example, the β_2 -AR receptor is known to preassemble with G α_s (Lachance et al., 1999), which we also observed as a fast increase in BRET_{EFF} between G α_s -Rluc and β_2 -AR-GFP². The delayed interaction between G α_s and CB₁ could result from the recruitment of G α_s to CB₁ and its activation instead of the activation of preassembled GPCR-G protein complexes (Ayoub et al., 2010). The "preassembled model" between GPCRs and G proteins can explain the fast increase in BRET_{EFF} signal between G α_i -Rluc and CB₁-GFP² (Janetopoulos et al., 2001; Galés et al., 2006; Ayoub et al., 2007, 2012). However, the interaction between G α_s and CB₁ is more compatible with the "free collision model." This model also proposes that GPCRs can interact with and activate many G proteins depending on the ligand (reviewed in Oldham and Hamm, 2008). The "free collision model" may explain the ability of CB₁ receptor to activate different G protein pathways observed in previous studies (Laprairie et al., 2014).

Sulpiride is less effective in promoting D₂ coupling to G α_s and inducing CREB phosphorylation in cells treated with ACEA, compared with haloperidol. Both haloperidol and sulpiride are D₂ antagonists, but the two drugs have different receptor dissociation properties from D₂ receptors, which results in different kinetics of D₂ blockade. Haloperidol binds with higher affinity to D₂ receptor and displays slow dissociation from D₂. Conversely, sulpiride displays a lower affinity and a much faster dissociation rate, which would produce rapidly reversible antagonism (Kapur and Seeman, 2001). In addition, highly lipophilic antagonists, such as haloperidol, can accumulate in cell membranes and can reach receptors in membrane folds more easily than hydrophilic D₂ antagonists, such as sulpiride (Rayport and Sulzer, 1995; Sahlholm et al., 2016). Therefore, lipophilic D₂ antagonists with slow dissociation rates, such as haloperidol, have higher E_{Max} for G α_s -dependent CREB activation.

Haloperidol Reduced β -Arrestin1 Recruitment to CB₁. Heteromerization is known to affect β -arrestin recruitment. β -arrestin2 (Jin et al., 1999; van der Lee et al., 2009) and β -arrestin1 facilitate the internalization of CB₁ after activation (Laprairie et al., 2014). We demonstrated that D₂ antagonism inhibited CB₁ agonist-induced recruitment of β -arrestin1 to CB₁-D_{2L}-G α_s complexes and inhibited CB₁ receptor internalization in *STHdh*^{Q7/Q7} cells in a dose-dependent manner. Therefore, antagonism of one receptor in a GPCR heteromer may allosterically inhibit agonist-induced β -arrestin1 recruitment of the other receptor. However, a fraction of the response was not antagonized by haloperidol,

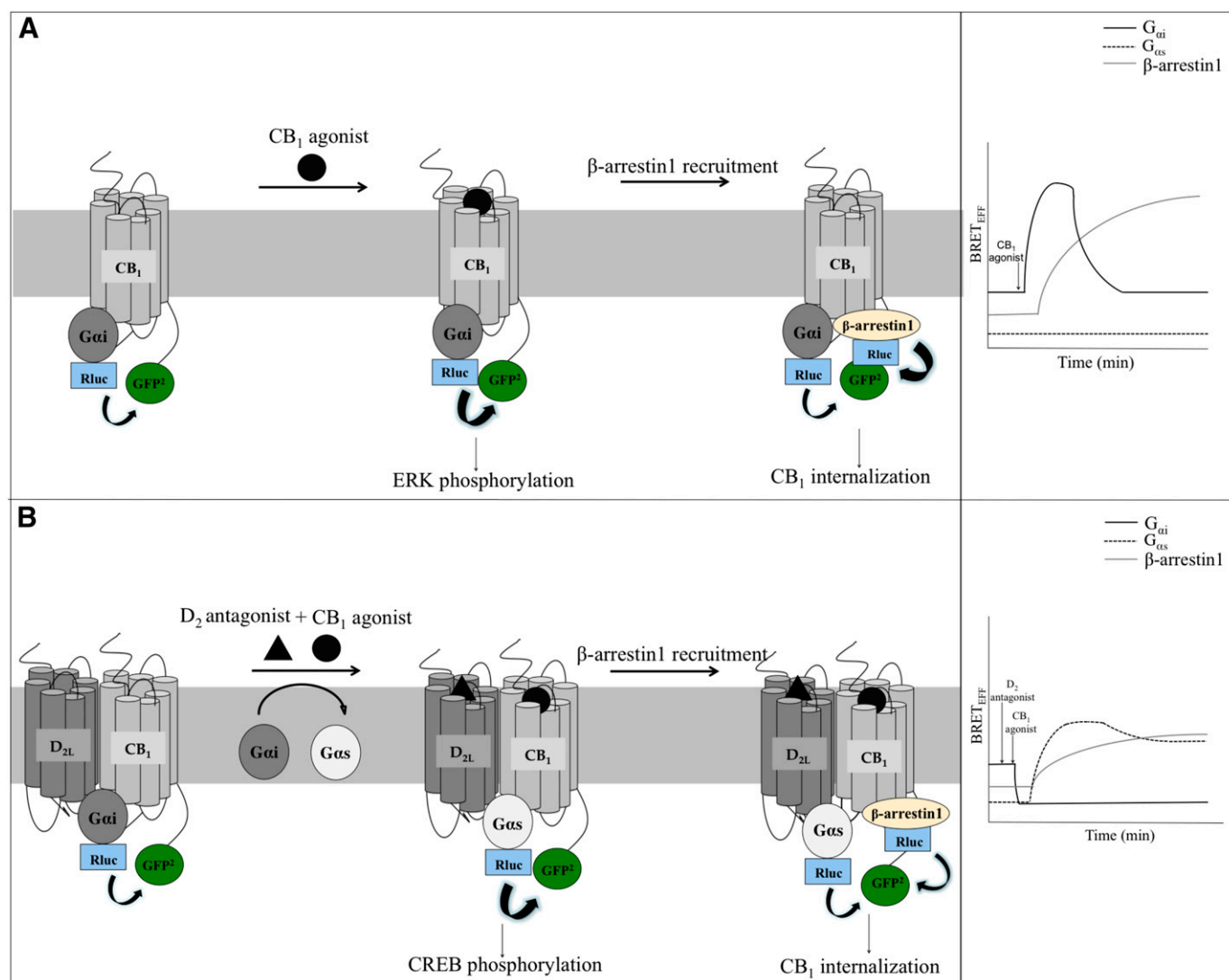


Fig. 8. Kinetic interaction of CB₁ receptor and CB₁-D_{2L} heteromers with G_{α_i}, G_{α_s}, and β-arrestin1. (A) BRET² data demonstrated that CB₁ receptor is preassembled with G_{α_i}. CB₁ agonist-induced fast and transient increases in BRET² indicating conformational changes within the preassembled CB₁-G_{α_i} complexes. The deactivation phase of the preassembled CB₁-G_{α_i} occurs parallel to the slow and stable recruitment of β-arrestin1. (B) CB₁-D_{2L} preassembled complexes are coupled to G_{α_i}. CB₁ agonist and D₂ antagonist induced a delayed and sustained recruitment of G_{α_s} to CB₁-D_{2L} complexes. Reduced and sustained recruitment of β-arrestin1 to the CB₁-D_{2L}-G_{α_s} was observed.

suggesting that some CB₁ functioned as monomers, homomers, or heteromers (Wager-Miller et al., 2002; Rios et al., 2006; Carriba et al., 2007; Hudson et al., 2010). It is unknown at this time how repeated stimulation of CB₁ and D_{2L} would affect receptor desensitization.

Allosteric Interaction Between CB₁ and D_{2L}. Allosteric effects between GPCR heteromers resulting from orthosteric ligand binding have been reported for different GPCRs (reviewed in Ferré et al., 2014). While previous work has highlighted a functional interaction between CB₁ and D_{2L} following agonist-dependent coactivation of both receptors, the current work indicates that allosteric interactions are dependent on D_{2L} receptor ligand binding and are not limited to D_{2L} agonism. Cooperativity effects resulting from allosteric interactions between GPCR protomers have been analyzed using a number of different models (reviewed in Giraldo, 2013). In our study, cooperativity between protomers was assessed using the Hill coefficients obtained from

fitting the data to a nonlinear regression model with variable slope (four parameters). Haloperidol treatment was associated with negative cooperativity between G_{α_i} and CB₁ because treating the cells with haloperidol decreased both the E_{Max} and Hill coefficient of the ACEA-mediated G_{α_i} and CB₁ interaction and ACEA-mediated pERK concentration-response. However, haloperidol treatment was associated with positive cooperativity between G_{α_s} and CB₁ because haloperidol increased the E_{Max} and Hill coefficient of G_{α_s} and CB₁ interaction and ACEA-mediated pCREB response. Whether haloperidol alters cannabinoid agonist affinity to the CB₁ receptor is still to be determined.

In addition to defining pharmacological interactions between CB₁ and D_{2L} receptors, this work may have clinical implications. Many central nervous system diseases, including schizophrenia, Huntington disease, and Parkinson's disease, are treated with drugs that bind D₂ receptors either as antagonists or agonists. Patients who are prescribed such

drugs might also be exposed to cannabinoids in the forms of medically prescribed cannabinoids or illicit agents. The dosing regimen for such cannabinoids might be chronic or intermittent. On the basis of our data, the combined effect of D₂ antagonists and CB₁ agonists are likely to differ from the predicted effect of either drug alone. Typical antipsychotics, including haloperidol, are commonly prescribed to Huntington patients to control chorea and psychosis (Ross and Tabrizi, 2011). In the context of Huntington disease, in which levels of CB₁ and D₂ decline with disease progression (Augood et al., 1997; Denovan-Wright and Robertson, 2000), drug response and response to coadministration of CB₁ agonists and D₂ antagonists may be even more complex than that observed for non-Huntington's patients (Sagredo et al., 2012). A better understanding of the interaction between drugs acting on the dopaminergic and endocannabinoid systems are required for symptom management of Huntington disease and other disorders.

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Authorship Contributions

Participated in research design: Bagher, Laprairie, Kelly, Denovan-Wright.

Conducted experiments: Bagher.

Contributed new reagents or analytic tools: Kelly, Denovan-Wright.

Performed data analysis: Bagher, Denovan-Wright.

Wrote or contributed to the writing of the manuscript: Bagher, Laprairie, Kelly, Denovan-Wright.

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