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Ligand-Induced Regulation and Localization of Cannabinoid CB₁ and Dopamine D₂L Receptor Heterodimers

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d. Abbreviations: A₂A, adenosine ₂A receptor; AM281, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide; BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; CAD, Cath. a differentiated; CB₁, cannabinoid 1 receptor; CE, 1-[7-(2-chlorophenyl)-8-(4-chlorophenyl)-2-methylpyrazolo[1,5-a]-[1,3,5]triazin-4-yl]-3-ethylaminoazetidine-3-carboxylic acid amide benzenesulfonate; CC, Cerulean C-terminus fragment; CFP, cyan fluorescent protein; CN, Cerulean N-terminus fragment; CP55,940, (2)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol; D₂, dopamine D₂ receptor; D₂S and D₂L, short and long form of the D₂ receptor; FRET, fluorescence resonance
energy transfer; MBiFC, multicolor BiFC; PBS, phosphate-buffered saline; VN, Venus N-terminus fragment; YFP, yellow fluorescent protein.

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Abstract

The cannabinoid CB₁ (CB₁) and dopamine D₂ (D₂) receptors are co-expressed in the basal ganglia; an area of the brain involved in such processes as cognition, motor function, and emotional control. Several lines of evidence suggest that CB₁ and D₂ receptors may oligomerize, providing a unique pharmacology in vitro and in vivo. However, limited information exists on the regulation of CB₁ and D₂ receptor dimers. We employed a novel technique, multicolor bimolecular fluorescence complementation (MBiFC) to examine the subcellular localization of CB₁-D₂L heterodimers as well as D₂L-D₂L homodimers in a neuronal cell model, CAD cells. MBiFC was then used to explore the effects of persistent ligand treatment on receptor dimerization at the plasma membrane and intracellularly. Persistent (20 hr) agonist treatment resulted in increased formation of CB₁-D₂L heterodimers relative to the D₂L-D₂L homodimers. The effects of the D₂ agonist, quinpirole were restricted to the intracellular compartment and may reflect increased D₂L receptor expression. In contrast, treatment with the CB₁ receptor agonist, CP55,940 produced increases in both membrane and intracellular CB₁-D₂L heterodimers independent of alterations in CB₁ receptor expression. The effects of CB₁ receptor activation were attenuated by the CB₁ antagonist, AM281 and were both time- and dose-dependent. The effects of CB₁ activation were examined further by combining MBiFC with a constitutively active CB₁ receptor mutant (CB₁T210I). These studies demonstrated that the expression of CB₁T210I increased intracellular CB₁-D₂L heterodimer formation. In summary, agonist-induced modulation of CB₁-D₂L oligomerization may have physiological implications in diseases such as Parkinson’s disease and drug abuse.
Introduction

Increasing evidence suggest that G protein coupled receptors (GPCRs) may function in receptor dimeric or higher-order oligomeric complexes (for review see Milligan, 2008). One set of receptors that has received significant attention relevant to oligomerization is the CB₁ cannabinoid (CB₁) receptor and D₂ dopamine (D₂) receptor (for review see Fuxe, et al., 2008). It is thought that the cannabinoid system negatively modulates dopamine circuits as activation of the CB₁ receptor leads to an attenuation of dopamine signaling (Laviolette and Grace, 2006). The CB₁ receptor is widely expressed in the central nervous system with great abundance in the basal ganglia (Herkenham, et al., 1991). CB₁ receptors are located on striatal GABAergic neurons (Herkenham, et al., 1991) and they are also found on dendrites in both the dorsal striatum and the nucleus accumbens (Pickel, et al., 2006). The D₂ receptor exists as two splice variants, D₂S (short) and D₂L (long). The D₂S is highly expressed on pre-synaptic dopaminergic neurons, whereas the D₂L is found postsynaptically on dopaminergic neurons throughout the striatum (Khan, et al., 1998;Usiello, et al., 2000). These observations reveal that CB₁ and D₂L receptors have overlapping expression patterns in the striatum and also suggest that they are co-localized in neurons in the nucleus accumbens (Pickel, et al., 2006; see references within Kearn, et al., 2005).

It has been reported that CB₁ and D₂ receptors oligomerize providing unique pharmacology in vitro and in vivo (Jarrahian, et al., 2004;Marcellino, et al., 2008;Glass and Felder, 1997;Kearn, et al., 2005). For example, it was demonstrated in primary rat striatal neurons that concurrent activation of Gaᵢ/o-coupled CB₁ and D₂ receptors resulted in stimulation of cAMP accumulation (Glass and Felder, 1997). Subsequent experiments using recombinant CB₁ and D₂L receptors suggested that D₂L receptor activation promoted a switch in CB₁ receptor
coupling from Gαi/o to Gαs (Glass and Felder, 1997). One proposed mechanism for D2 receptor modulation of CB1-G protein coupling may involve receptor oligomerization. This hypothesis was examined by demonstrating a physical interaction between CB1 and D2L receptors using co-immunoprecipitation (Kearn, et al., 2005). The same investigators also revealed that the CB1-D2L receptor complex can be dynamically modulated by receptor agonists. More recent studies have examined CB1-D2L heteromers using FRET techniques (Marcellino, et al., 2008). Using HEK cells transiently transfected with fluorescently tagged CB1 and D2L receptors, a FRET interaction was detected. However, no significant changes in the FRET signal were detected following short term exposure to CB1 or D2L receptor agonists (Marcellino, et al., 2008). The ability of CB1 and D2L receptors to interact is consistent with suggestion of a CB1-D2L heterodimer. Additional behavior and biochemical data support further the physiological relevance of CB1 and D2 receptors heterodimers (see Fuxe et al., 2008). However, limited information exists on the cellular localization and regulation of CB1-D2L receptor heterodimers. Despite the therapeutic potential of drugs targeting these receptors, the effect of persistent receptor activation on the dynamics of receptor oligomerization has not been explored.

The most common techniques currently being used to study the physical association of GPCRs include coimmunoprecipitation and traditional resonance energy transfer (FRET and BRET) techniques (Vidi and Watts, 2009). These techniques are typically limited to the study of a single protein-protein complex. Additionally, coimmunoprecipitation does not allow for detection of an interacting protein complex within a living cell. In order to gain further insight into GPCR dimerization in live cells, we recently established the use of multicolor bimolecular fluorescence complementation (BiFC) (Hu, et al., 2002; Shyu, et al., 2006) as a tool to investigate GPCR homo- and heteromer oligomerization (Vidi, et al., 2008b; Vidi, et al., 2008a). MBiFC
allows for the detection of two separate protein-protein complexes in living cells by visualizing the fluorescence complementation of two distinct spectral variants of GFP (Hu and Kerppola, 2003). Moreover, this technique can be used to measure the relative amounts of homodimer versus heterodimer formation in a cell region-specific fashion (Vidi, et al., 2008b).

The present study uses MBiFC to examine CB₁-D₂L heterodimers and D₂L-D₂L homodimers in Cath. a differentiated (CAD) cells. CAD cells are a neuronal cell model that express GAP-43, synaptotagmin, and SNAP-25 and upon differentiation, form neurite-like processes (Qi, et al., 1997). The present results provide additional evidence for the existence of CB₁ and D₂L receptor oligomers. We also revealed that persistent agonist (i.e. dopaminergic or cannbinergic) treatment favors the formation of the CB₁-D₂L heterodimer relative to the formation of the D₂L-D₂L homodimer. The D₂ agonist-mediated effects were accompanied by an increase in D₂L receptor expression, whereas, the CB₁ agonist-mediated changes in heterodimer formation appeared to involve primarily CB₁ receptor activation. These results provide further insight into the dynamic nature of CB₁-D₂L oligomerization.
Methods

Materials

Human CB1 and D2L cDNAs were obtained from the Missouri S&T cDNA Resource Center. Growth media (DMEM), quinpirole, and sulpiride were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), and bovine calf serum (BCS) were purchased from Fisher Scientific. Penicillin/streptomycin/amphotericin B antibiotic/antimycotic was purchased from Invitrogen. Forskolin was purchased from Tocris Biosciences (Ellisville, MO). CP55,940 was a generous gift from Pfizer Pharmaceuticals. [3H]cAMP (25 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [3H]Spiperone (91 Ci/mmol) and [3H]-SR141716A (42 Ci/mmol) were obtained from GE Healthcare (Chalfont St. Giles, Buckingham, UK). Specific cellular compartment markers (mCherry-mem, YFP-ER, YFP-Endo and YFP-Golgi) were gifts from Dr. Catherine Berlot (Weis Center for Research, Danville PA).

Expression Vectors

Full-length human CB1 and D2L cDNAs were amplified by polymerase chain reaction (PCR) using oligonucleotides with EcoRI, XbaI or XhoI restriction sites and omitting the stop codons. The PCR products were digested with either EcoRI/XbaI or EcoRI/XhoI and ligated into the corresponding pBiFC vectors. These expression vectors contain non-fluorescent fragments of the N and C termini of the enhanced yellow fluorescent protein (Venus) and the enhanced cyan fluorescent protein (Cerulean). N terminal fragments (VN or CN) include residues 1 to 172, whereas the C terminal fragment of Cerulean (CC) includes residues 155-238. This cloning strategy places the fragment on the C terminus of the receptors. Additionally, the CB1 and D2L receptor PCR products were digested with either EcoRI/XbaI or EcoRI/XhoI and ligated into expression vectors containing the full length Venus or Cerulean proteins resulting in the CB1 and...
D₂L receptors tagged at the C-terminus with either Venus or Cerulean. The CB₁ receptor mutant (CB₁T210I) was generated using the QuikChange kit according to the supplier’s protocol (Stratagene, La Jolla, CA) in pcDNA3-CB₁ and then subcloned into the pBiFC vectors using EcoRI and XbaI restriction enzyme sites. All constructs were verified by DNA sequencing.

**Cell Culture and Transient Transfections**

CAD cells were maintained as described previously (Vortherms and Watts, 2004). For microscopic evaluation of BiFC, CAD cells were grown to approximately 70% confluency in four-well LabTek chambered coverslips (Nunc Rochester, NY). Cells were transfected with 1 µL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. In MBiFC experiments, CB₁-VN (500 ng), D₂L-CC (300 ng), and D₂L-CN (300 ng) were transiently co-transfected with 20 ng of either mCherry-Mem, YFP-Endo, YFP-ER, or YFP-Golgi depending on the experiment. Twenty-four hours post transfection, the growth media and transfection reagent was replaced with 400 µL warm phosphate-buffered saline (PBS) and images were taken using a charge coupled device camera mounted on a TE2000-U inverted fluorescence microscope (Nikon Instruments Inc., Melville, NY) equipped with a 100-W mercury lamp and band-pass filters (Chroma, Rockingham, VT) for Venus (excitation at 500/20 nm; emission at 535/30 nm), Cerulean (excitation at 430/25 nm; emission at 470/30 nm), or mCherry (Texas Red, excitation at 572/23 nm; emission at 625/25 nm). Fluorescent images were acquired using MetaMorph software (Molecular Devices, Sunnyvale, CA). For MBiFC experiments investigating the effects of receptor ligands on receptor dimer population, the cells were transfected as described above and 4 hours post transfection, the appropriate drug treatment was added to the growth medium for an additional 20 hours prior to image acquisition.
Quantitative Image Analysis

Quantification of fluorescent signals was performed as described previously using ImageJ software (http://rsb.info.nih.gov/ij/) (Hu, et al., 2002 and see supplemental Fig. 1). In each experiment, approximately 40-50 individual cells were quantified. Ten microscopic fields at 60x magnification were acquired as stacks of images from the YFP, CFP, and Texas Red channels corresponding to the fluorescent signals from Venus, Cerulean, and mCherry proteins, respectively. Background fluorescence intensity was measured in each channel in an area devoid of cells and subtracted from the fluorescent signals. The signals corrected for background fluorescence were then scaled to a factor equal to that of the inverse of the exposure time for each pixel intensity measurement. The images of the mCherry-Mem membrane marker signal were used to select cells for image analysis and to normalize BiFC signals (Supplemental Fig 1).

Cellular analysis of BiFC signals was performed in two parts. First, the fluorescent signal intensity maximum at the membrane was determined by drawing a perpendicular line through the membrane using the mCherry-Mem image. The maximum signal intensity was determined in all three channels, YFP, CFP, and Texas Red to estimate the BiFC signals at the membrane. The BiFC signal intensity in the intracellular space was determined by outlining the intracellular compartment (excluding the plasma membrane) and determining the average pixel intensity in all three channels, YFP, CFP, and Texas Red to estimate the intracellular BiFC signals. Cells with saturated signals as well as cells with signals that were 1.2 times lower than background were not used for quantification. BiFC experiments assessing bleed-through/overflow of Cerulean or Venus in the opposite channels (i.e. YFP or CFP) revealed minimal crosstalk. Specifically, complemented Cerulean contributed less than 2% of the YFP signal and complemented Venus contributed less than 3% of the CFP signal (data not shown). Venus/Cerulean fluorescence
ratios exhibit a non-Gaussian distribution therefore median values were calculated and averaged between experiments.

Cyclic AMP Accumulation Assays

CAD cells were grown to 70% confluency in 24-well plates and were transiently transfected as described previously (Vidi et al., 2008a). CAD cells were either transfected with 300 ng/well of the D2L or 500 ng/well CB1 constructs. All drugs were diluted in Earle’s balanced salt solution (EBSS) assay buffer (EBSS containing 2% bovine calf serum, 0.025% ascorbic acid, and 15 mM HEPES, pH 7.4) and added to the cells on ice. Determination of cAMP accumulation was performed by incubating the transfected CAD cells with forskolin (10 µM) in the absence and presence of either CP55,940 (10 µM) or quinpirole (10 µM) for 15 minutes at 37°C. All assays were performed in the presence of the phosphodiesterase inhibitor isobutyl-methylxanthine (IBMX; 500 µM) and terminated with ice-cold 3% trichloroacetic acid. Quantification of cAMP accumulation was determined using a competitive binding assay as previously described (Vortherms and Watts, 2004).

Radioligand Binding Assays

Single point radioligand binding assays were used to estimate CB1 and D2L receptor densities in following drug treatments as described previously (Vidi, et al., 2008a). CAD cells were plated in a 12 well plate and were grown to 70% confluency prior to being transiently transfected with CB1-VN, D2L-CN, and D2L-CC using 2 µL/well of Lipofectamine 2000. Four hours post transfection the appropriate drug treatment was added in triplicate to the growth medium and transfection reagent. The cells were incubated for an additional twenty hours prior to single point radioligand binding assays. Cells were washed three times with 500 µL of receptor binding buffer (50 mM Tris and 4 mM MgCl2, pH 7.4). The cells were lysed with 500
µL ice-cold lysis buffer (1 mM HEPES and 2 mM EDTA, pH 7.4) for 10 min on ice. The cells were removed from each well by trituration and crude cell membranes were collected by centrifugation (15 min at 30,000xg, 4°C). Membrane pellets were resuspended by mechanical homogenization in 1 mL receptor binding buffer. For CB₁ receptor binding, the addition of 0.5% BSA to the receptor binding buffer was employed to decrease non-specific binding. Crude cell membranes (ca. 30 µg in 150 µL) were added in duplicate to the assay tubes to determine both non-specific and total binding. For CB₁ binding, non-specific binding was defined by 10 µM cold SR141716A (essentially identical levels of non-specific binding were obtained using 10 µM AM281, data not shown). All tubes contained a near-saturating amount of [³H]-SR141716A (50 µl, final concentration of ~5.0 nM) in a total volume of 500 µl. Similarly, for D₂ binding, non-specific binding was defined with 5 µM (+)-butaclamol with all reaction conditions containing a near saturating amount of [³H]-spiperone (50 µl, final concentration of ~1.5 nM) in a total volume of 500 µl. The reaction was terminated by filtration onto FB glass fiber plates with ice-cold wash buffer (10 mM Tris and 0.9% NaCl) using a cell harvester (FilterMate, Packard). Radioactivity was determined a Packard TopCount scintillation counter. Specific binding was determined as the difference between the average of the non-specific and total binding conditions. The specific binding amount was normalized to the amount of protein using the Pierce BCA protein assay following the supplier’s protocol. Under the transfection conditions used to explore the effects of drug treatments on BiFC, the following estimated Kᵰ and Bₘₐₓ values were obtained via radioligand saturation binding experiments: [³H]-SR141716A, Kᵰ = 0.74 ± 0.18 nM and Bₘₐₓ = 204 ± 28 fmol/mg; [³H]-spiperone, Kᵰ = 0.051 ± 0.02 nM and Bₘₐₓ = 3550 ± 200 fmol/mg.
Fluorescence Energy Transfer

CAD cells were grown to 70% confluency in 12 well plates prior to transfection. Cells were transiently transfected with three general conditions depending on the receptor dimer species to be studied including: cells only expressing the FRET donor (Cerulean), cells only expressing the FRET acceptor (Venus) and cells expressing both the donor and acceptor. In order to normalize for protein expression in cells only expressing either the donor or acceptor, the total amount of DNA transfected was normalized with the untagged receptor. In each FRET assay, 750 ng/well of the donor (CB1-Cerulean or D2L-Cerulean) and 750 ng/well of the acceptor (CB1-Venus or D2L-Venus) were transiently transfected either alone or in combination 24 hours prior to the experiment. Cells were washed with 500 µL of warm PBS and resuspended in 300 µL of warm PBS. Protein concentration was determined on the cells suspension using the BCA assay method (Pierce, Rockford IL) and normalized to 200 ng/µL with PBS. CAD cells suspensions (40 µg) were transferred into a 96-well black plate (Nunc Rochester, NY) and fluorescence measurements were evaluated on the FUSION plate reader (Packard, Waltham MA). Determination of FRET signals was performed as described previously (Vidi et al., 2008b). Briefly, FRET signals were measured using the sensitized acceptor method. Mock transfected cells were used for background fluorescence. For each sample, Cerulean (C) and Venus (V) was measured using 430/25 nm and 500/20 nm excitation and 470/30 nm and 535/30 nm emission filters. FRET signals (F) were measured using excitation at 430/25 nm and emission at 535/30 nm. Bleed-through coefficients were calculated for the acceptor ($a=F/V$) and for the donor ($d=F/C$) in cells only expressing either Cerulean (donor) or Venus (acceptor) fusion proteins. The FRET signals were corrected (cFRET) for acceptor and donor bleed through using the equation:
\[ \text{cFRET} = F - aV - dC \]

The signals were then normalized to donor (C) and acceptor (Y) intensities as follows:

\[ nFRET = \frac{\text{cFRET}}{\sqrt{C*V}} \]

**Data and Statistical Analysis**

Data and statistical analyses were performed using Prism (GraphPad Software Inc., San Diego, CA). A \( p \) value < 0.05 was considered significant.
Results

Functional cAMP accumulation assays were performed in order to verify the signaling properties of the BiFC tagged CB₁ and D₂L receptors (Fig 1). Because CB₁ and D₂L receptors couple to inhibitory G proteins (i.e. G\(_{\alpha i/o}\)), agonist-induced inhibition of forskolin-stimulated cAMP accumulation was used to evaluate receptor function. The BiFC tagged D₂L receptor, D₂L-CN, and D₂L-CC were functional following stimulation with the D₂ agonist, quinpirole (10µM) revealing approximately 60% inhibition of forskolin-stimulated cAMP accumulation (Fig. 1A). Additional experiments confirming the functionality of the BiFC-tagged CB₁ receptors, CB₁-VN and CB₁-CC were performed. Both constructs were functional following stimulation with the CB₁ receptor agonist, CP55,940 (10 µM) yielding more than 35% inhibition of forskolin-stimulated cAMP accumulation (Fig 1B). A modest, but insignificant degree (ca. 10%) of inhibition was also observed in vector-transfected CAD cells. Receptor signaling in cells co-expressing the wild type or BiFC-tagged receptors was also examined (Fig. 1A and 1B). The D₂ agonist, quinpirole robustly inhibited forskolin-stimulated cAMP accumulation in cells co-expressing CB₁ and D₂L receptors (CB₁ + D₂L or CB₁-VN + D₂L-CC) as well as cells co-transfected with D₂L-CN and D₂L-CC. Similar experiments revealed that CP55,940, inhibited forskolin-stimulated cAMP accumulation in cells co-expressing CB₁ and D₂L receptors (CB₁ + D₂L and CB₁-VN + D₂L-CC). These data suggest that the addition of a C-terminal tag (-VN, -CN or -CC) and fluorescence complementation (see below) does not adversely affect agonist-mediated inhibition of cAMP accumulation.

MBiFC is novel technique that allows for the simultaneous study of two receptor dimer species within living cells (Fig. 2A) (Vidi and Watts, 2009). Initial single color BiFC experiments used the fusion receptors to confirm interactions between CB₁ and D₂L receptors.
Co-expression of either combination of BiFC constructs (CB1-VN + D2L-CC or D2L-VN + CB1-CC) in CAD cells produced a robust Venus signal (Supplemental Fig. 2A). Additional BiFC studies compared the CB1-D2L fluorescent signal with CB1 or D2L receptors in combination with the M4 muscarinic receptor BiFC constructs (i.e. CB1-VN + M4-CC or D2L-CC + M4-VN). The CB1-D2L heterodimer displayed an enhanced fluorescent signal when compared to the M4 containing heterodimers (Supplemental Fig. 2B). The formation of CB1-D2L heterodimers supports previous studies demonstrating interactions between CB1 and D2L receptors (Marcellino, et al., 2008; Kearn, et al., 2005).

One goal of the present study was to assess the dynamic nature of the CB1-D2L heterodimer in response to persistent drug treatment. This required the establishment of MBiFC as previously described for A2A adenosine and D2L dopamine receptors (Vidi et al., 2008a). Using this approach, CAD cells were transiently transfected with CB1-VN, D2L-CC, and D2L-CN to simultaneously visualize CB1-D2L and D2L-D2L receptor dimers using fluorescence microscopy (Fig. 2A). The presence of a Venus signal is indicative of the CB1-D2L heterodimer, whereas, a Cerulean signal corresponds to the D2L-D2L homodimer (Fig. 2B). CAD cells transfected with CB1-VN, D2L-CC, and D2L-CN expressed both Venus and Cerulean signals consistent with the coexistence of CB1-D2L heterodimers and D2L-D2L homodimers (Fig. 2B). Fluorescent signals corresponding to the receptor dimers showed a similar pattern of distribution and were found at the plasma membrane as well as intracellularly. For comparison to the BiFC signals, the localization patterns of CB1-Venus and D2L-Cerulean were evaluated following co-expression (Fig. 2C). The CB1-Venus signal showed significant intracellular localization, whereas, the D2L-Cerulean displayed localization at both the plasma membrane and intracellular compartments. Moderate overlap between the CB1 and D2L signals was also observed. For additional
comparison, the individual expression patterns of CB1-Venus and D2L-Venus were examined (Fig. 2D). When expressed alone, the CB1-Venus signal was primarily localized intracellularly demonstrated by the lack of overlap with the membrane marker (merge panel). Conversely, the D2L-Venus expression was found primarily at the membrane and extensive overlap with the membrane marker was displayed (Fig. 2D).

We also attempted to perform MBiFC experiments to simultaneously examine D2L-CB1 and CB1-CB1 dimers. Unfortunately, the fluorescent signal of the CB1-CB1 dimer under MBiFC conditions was too low to reliably measure, restricting our MBiFC experiments to CB1-D2L and D2L-D2L receptor dimers. The lack of a CB1-CB1 dimer BiFC signal may reflect one of the disadvantages of BiFC. Specifically, the intensity of the fluorescence complementation signal is considerably weaker (2.5 to 5.5 fold) than the signal from the corresponding full-length fluorescent protein under similar transfection conditions (Vidi and Watts, 2009).

One advantage of BiFC is the ability to investigate the localization of the receptor dimers using epifluorescence. With the use of fluorescently-tagged intracellular makers, the patterns of intracellular expression of the CB1-D2L and D2L-D2L receptor dimers were investigated using fluorescent microscopy (Fig. 3). CAD cells were transiently transfected with BiFC constructs that reconstitute Cerulean to either express the CB1-D2L heterodimer (CB1-CN + D2L-CC) or the D2L-D2L homodimer (D2L-CN + D2L-CC). Additionally, these cells were transfected with the indicated YFP-tagged intracellular marker proteins (YFP-Endo, YFP-ER, or YFP-Golgi) (Fig. 3). The endosome marker (YFP-Endo) is a fusion protein with RhoB, a known endosomal protein fused to YFP. The ER marker (YFP-ER) consists of YFP fused to the ER targeting sequence of calreticulin and the KEDL ER retrieval sequence. The golgi marker (YFP-Golgi) is a YFP fusion protein with residues 1-81 of the β1,4-galactosyltransferase protein. Overall, both
receptor dimers, D2L-D2L and CB1-D2L displayed moderate to extensive overlap with endosome and ER structures (Fig. 3A and 3B). However, CB1-D2L and D2L-D2L receptor dimers demonstrated minimal to no overlap with the golgi apparatus. These expression patterns are consistent with receptor dimer assembly at the ER (Herrick-Davis, et al., 1997) and proper trafficking into endosomes (Leterrier, et al., 2004). However, the additional possibility that receptors dimerize at the plasma membrane cannot be excluded in the absence of additional studies.

The results demonstrating MBiFC in neuronal cells were further validated by examining dimerization of these receptors using FRET, which has been used previously to investigate interactions of CB1 and D2L receptors (Marcellino, et al., 2008). CAD cells were transiently transfected with either CB1-Venus + CB1-Cerulean, CB1-Venus + D2L-Cerulean, or D2L-Venus + D2L-Cerulean (Fig. 4). A significant FRET signal was detected with all three receptor pairs as compared to the mix control sample in which suspensions of cells only expressing the donor or acceptor was mixed in the FRET sample plate. These results provide further confirmation of our BiFC studies, supporting the hypothesis that CB1 and D2 form both homo- and heteromeric receptor oligomers in a neuronal-like cell model.

Using MBiFC and FRET techniques, we have provided evidence that CB1 and D2 receptors participate in receptor dimer complexes. We next sought to investigate the effects of persistent ligand treatment on the formation of CB1 and D2L heterodimer and D2L homodimers using MBiFC as a tool to monitor changes in relative receptor dimer population. CAD cells were transiently transfected with CB1-VN, D2L-CC, and D2L-CN and the presence of the CB1-D2L heterodimer (Venus) and D2L-D2L homodimer (Cerulean) was simultaneously measured. The fluorescent intensity ratio of Venus to Cerulean in both the plasma membrane and intracellular
compartments were determined following drug treatment. Under the conditions used, an increase in the Venus to Cerulean ratio would be indicative of an increase in the formation of the CB$_1$-D$_{2L}$ receptor dimer relative to the D$_{2L}$-D$_{2L}$ receptor dimer when compared to vehicle-treated cells.

Our previous work with D$_2$ and A$_{2A}$ receptor ligands suggested that a 20 hr drug treatment provided a robust BiFC signal in which drug-induced changes in A$_{2A}$-D$_{2L}$, D$_{2L}$-D$_{2L}$, and A$_{2A}$-A$_{2A}$ dimers could be observed (Vidi, et al., 2008a). In the present study we completed MBiFC time-course experiments with the CB$_1$ receptor ligand CP55,940 to verify that a similar treatment duration produced robust responses in the absence of a ceiling effect. The results of the time course study revealed that CP55,940 treatments shorter than 10 hrs (i.e. 5 hrs) had very low fluorescent signals and did not allow us to quantify an adequate number of cells for analysis (data not shown). However, robust YFP and CFP signals were evident after 10 hrs and the drug effects were time-dependent showing the greatest response at 30 hrs (Fig. 5). The time course study also suggested that the 20 hr time point is on the dynamic portion of the temporal scale potentially allowing us to observe ratiometric changes in both directions as previously shown (Vidi, et al., 2008a). Examination of the overall YFP and CFP intensities at 20hrs indicated that the CP55,940-induced increase in the YFP/CFP ratio reflected a combined increase in the YFP signal (CB$_1$-D$_{2L}$) and a decrease in the CFP signal (D$_{2L}$-D$_{2L}$) when compared to vehicle-treated cells. Specifically, the membrane showed an 11% increase in YFP and a 27% decrease in CFP intensity. Intracellularly, there was 33% increase in the YFP signal and a 15% decrease in the CFP signal (n=4).

Drug-induced changes in the relative receptor dimer population were measured following treatment (20 hr) with either D$_2$ (Fig. 6A) or CB$_1$ (Fig. 6B) receptor ligands. Persistent
activation of the D2L receptor with quinpirole (10 µM) resulted in a significant increase in the Venus to Cerulean ratio consistent with an increase in CB1-D2L heterodimers relative to D2L-D2L homodimers. However, this effect was only significant in the intracellular compartment. The effect of quinpirole was prevented by co-application of the selective D2 receptor antagonist, sulpiride (1 µM). Treatment with sulpiride alone or in combination with quinpirole resulted in a significant decrease in the Venus to Cerulean ratio in both the membrane and intracellular compartments. Because the observed alterations in receptor dimer population may involve changes in receptor expression, single point radioligand binding experiments were used to estimate relative receptor densities following drug treatment. The results of these studies revealed that persistent treatment with quinpirole (10 µM), sulpiride (1 µM), or quinpirole + sulpiride significantly increased D2L receptor density (118±6%, 149±17%, or 129±9%; n = 5) when compared to vehicle treatment (100%). These ligand-induced increases in D2L receptor expression are consistent with our previous report (Vidi, et al., 2008a) and work from others (Sibley and Neve, 1997). No significant changes in CB1 receptor density were observed upon persistent treatment with either of the D2 receptor ligands alone or the combination (data not shown).

MBiFC experiments were also performed using CB1 ligands. Persistent treatment with the CB1 receptor agonist, CP55,940 (10 µM) led to a significant increase in the Venus to Cerulean ratio in both the plasma membrane and the intracellular regions as compared to vehicle-treated cells (Fig 6B). The addition of the CB1 receptor antagonist, AM281 (10 µM) attenuated the CP55,940-induced increase in the Venus to Cerulean ratio. Dose-response experiments revealed that the average EC50 values for CP55,940 increasing the YFP/CFP ratio were 320 nM and 210 nM for membrane and intracellular signals, respectively (Fig. 7). Subsequent single
point radioligand binding experiments revealed that 20 hr treatment with CP55,940 had no effect on CB1 receptor density (106±12%; n = 5), however, a modest decrease in D2L receptor density (82±3%; n = 5) was observed.

The observations described above suggest that persistent activation of the CB1 receptor favors the formation of CB1-D2L heterodimers without alterations in CB1 receptor expression. In order to investigate further the role of persistent activation on receptor dimerization, a constitutively active CB1 receptor mutant was constructed for use in the MBiFC experiments. Threonine 210 of the CB1 receptor was mutated to an isoleucine (CB1T210I) to create a constitutively active receptor (D'Antona, et al., 2006). The presence of an isoleucine at amino acid 210 disrupts the salt bridge in the DRY motif mimicking receptor activation leading to enhanced agonist affinity and increased intracellular localization (D'Antona, et al., 2006). We examined and compared the relative receptor heterodimer (CB1-D2L) and homodimer (D2L-D2L) populations in cells expressing either the wild type (CB1-WT) or the constitutively active CB1 receptor (CB1T210I) using MBiFC (Fig. 8). The Venus (CB1-D2L) to Cerulean (D2L-D2L) ratios at the plasma membrane were similar in cells expressing the wild-type or constitutively active CB1 (Fig. 8A). In contrast, expression of CB1T210I resulted in a significant increase in the intracellular Venus to Cerulean ratio when compared to the wild type CB1 (Fig. 8A). The intracellular to membrane ratio of the Venus signal (i.e. CB1wt-D2L or T210I-D2L dimer) in cells expressing the CB1T210I mutant was also significantly increased (ca. 150%) when compared to cells expressing CB1wt (Fig. 8A and B). The overlapping expression patterns of CB1-D2L and D2L-D2L dimers were markedly reduced in cells co-expressing CB1T210I as indicated by a loss of white signal on the membrane in the merged images. Subsequent localization studies with the CB1T210I-D2L heterodimer revealed significant signal overlap with the endosomes and limited
overlap in the ER consistent with enhanced endocytosis of the CB₁T210I mutant (D'Antona, et al., 2006 and Supplemental Fig. 3).
Discussion

Evidence for the existence and functional significance of CB1 and D2L heterodimers has continued to evolve over the last 10-15 years. However, investigations examining the regulation of these heterodimers and their homodimer counterparts are just beginning as new technological advances for studying protein-protein interactions are developed (Vidi and Watts, 2009). In the present study, we have applied MBiFC as a novel technique to study the dimerization of CB1 and D2L receptors, and reveal for the first time the localization patterns of these receptor heterodimers in a neuronal cell model.

Early studies of CB1 and D2L function were central to the development of the concept of CB1-D2L heterodimer (for review see Glass, et al., 1997). Several studies suggest that the CB1-D2L dimer possesses stimulatory properties toward adenylyl cyclase via the CB1 receptor engaged in the heterodimer (Glass and Felder, 1997;Kearn, et al., 2005;Jarrahian, et al., 2004). However, conflicting conclusions from studies examining the regulation of CB1 and D2L receptor dimerization remain. One potential mechanism for regulating the CB1-D2 dimer is based on observations that the physical association of CB1 and D2L increases in the presence of acute co-activation of both receptors (Kearn, et al., 2005). Activation of either CB1 or D2L receptor individually did not significantly increase the physical association suggesting that co-activation of both receptors is necessary for enhanced receptor dimerization. It was also reported that expression (and not activation) of the D2L receptor was sufficient to induce a switch in CB1-G-protein coupling to a stimulatory pathway, however, measurements of the CB1-D2L receptor dimer were not performed (Jarrahian, et al., 2004). Additionally, another study reported a lack of agonist-mediated increase in the FRET interaction between CB1 and D2 receptors under conditions of both single and concurrent receptor activation (Marcellino, et al., 2008). The lack
of consistency between the reports described above may reflect differences in the choice of receptor ligands, the model systems, technical approaches, or the complex pharmacology of the CB₁-D₂ dimer.

In the present study, we used MBiFC to show that persistent activation of either the CB₁ or D₂L receptor leads to the formation of more CB₁-D₂L heterodimers relative to the D₂L-D₂L homodimers. There are a number of differences between our study of CB₁-D₂L interactions and the previous work described above (e.g. cell type, methods to measure receptors, drug treatment, etc); however, the drug treatment conditions and technology used to assess the receptor dimers are likely to have significant influence. Each of the drug treatments reported here represents an extended drug exposure (i.e. 10-30 hrs). Drugs were added four hours post transfection and were present during the time of ongoing receptor biosynthesis and subsequent oligomerization. Therefore, the dimers observed in our studies are likely to involve mechanisms not reflected in shorter drug treatments or acute studies (Kearn, et al., 2005; Marcellino, et al., 2008). The present study used BiFC technology, which differs from FRET in that the complementation of fluorescent signal is essentially irreversible (Vidi and Watts, 2009). This property of BiFC allows investigators to “capture” and subsequently measure drug-induced changes in receptor dimers over an extended period time in which a sufficient signal can be collected.

Persistent D₂ agonist treatment with quinpirole favored the formation of CB₁-D₂L heterodimers versus D₂L-D₂L homodimers. This effect was accompanied by an increase in D₂L receptor expression and was prevented by the D₂ antagonist, sulpiride. The increase in D₂L receptor expression may suggest a pharmacological chaperone effect on receptor dimer formation where D₂ ligands stabilize the receptor, somehow promoting CB₁-D₂L receptor interactions (Vidi, et al., 2008b). However, treatment with the D₂ antagonist sulpiride also
increased D2L receptor density, but instead favored the formation of D2L-D2L homodimers. These opposing effects of D2 agonists and antagonists on D2L-D2L versus CB1-D2L dimer formation argues against a simple role of increased D2L receptor expression. One explanation for these differential effects may involve ligand-specific changes in receptor dimerization patterns (Vidi, et al., 2008b). In addition to ligands, these dimerization patterns also appear to be influenced by the receptors under investigation. In a previous study of D2L and A2A receptor dimerization, quinpirole increased D2L-D2L homodimers relative to A2A-D2L heterodimers (Vidi, et al., 2008b). The potential scenario gets increasingly complicated when considering a recent BiFC-BRET study providing evidence for a CB1-D2-A2A receptor oligomer (Navarro, et al., 2008). Linking the observations described above and the present results suggests a scenario where striatal neurons expressing D2L, A2A, and CB1 receptors would be subject to a very complicated receptor regulation scheme. For example, persistent D2 agonist treatment would increase overall D2L receptor expression levels and perhaps promote the following pattern of receptor oligomers D2L-CB1 > D2L-D2L > A2A-D2L. The potential physiological and functional significance of these ligand-induced changes in heterodimers are intriguing and await biochemical and behavioral analysis (Marcellino, et al., 2008). In addition to in vivo studies, new molecular tools to study these complex systems are becoming increasingly available as methods to study interactions of higher ordered GPCR oligomers (e.g. trimers and tetramers) such as BiLC-FRET, BiFC-FRET, and BiFC-BRET are developed (Vidi and Watts, 2009).

The ability of quinpirole to alter the formation of receptor oligomers involving D2L receptors may provide insights into the disease states associated with persistent D2 receptor activation, as in the treatment of Parkinson’s disease with L-DOPA and D2 dopamine receptor agonists (Hurley and Jenner, 2006). For example, persistent quinpirole treatment increases A2A-
A2A homodimer formation and A2A signaling (Vidi, et al., 2008a; Vortherms and Watts, 2004). These observations may provide a molecular explanation for the beneficial clinical effects of A2A antagonists in treating L-DOPA-induced dyskinesias (Morelli, et al., 2007; Fuxe, et al., 2008). The current results suggest that persistent treatment with D2 receptor agonist drugs may promote the formation of CB1-D2L heterodimers. The increase in CB1-D2L dimer formation may allow the CB1 receptor to have enhanced antagonistic effects over the D2 receptor signaling (Marcellino, et al., 2008). This scenario would provide for increased CB1 signaling following a dopamine receptor-dependent increase in endocannabinoid release (Giuffrida, et al., 1999; Piomelli, 2003). Additionally, evidence linking the CB1-D2L heterodimer to a stimulatory pathway (Glass and Felder, 1997; Kearn, et al., 2005) may provide a mechanism for CB1 antagonism of D2 signaling at the intracellular level (i.e. cAMP). Further in vivo investigations of CB1 receptor and CB1-D2L heterodimer signaling following persistent D2 receptor activation are warranted, however, studies suggest that the CB1 receptor antagonists/inverse agonists may have beneficial effects in the management of Parkinson’s disease. For example, the CB1 antagonist, CE dose-dependently enhances the anti-Parkinson’s activity of L-DOPA (Cao, et al., 2007). Another study revealed that rimonabant, a CB1 receptor inverse agonist, had beneficial effects in managing L-DOPA induced dyskinesias (van der Stelt M., et al., 2005).

Similar to the D2L receptors, the precise mechanism by which persistent activation of the CB1 receptor favors the formation of CB1-D2L heterodimers relative to D2L-D2L homodimers remains largely unknown. Our observations suggest that the formation of the heterodimer is mediated by receptor activation and not alterations in CB1 receptor expression. It is possible that the activated conformational state of the CB1 receptor possesses enhanced affinity for the D2L receptor and that persistent activation promotes CB1-D2L heterodimerization. This hypothesis is
supported by the report that the CB₁ receptor increases the association with the D₂L receptor in a dose-dependent manner (Kearn, et al., 2005). Further, the present study demonstrated that expression of a constitutively active CB₁ mutant (CB₁T210I) promoted more CB₁-D₂L heterodimerization. Although the identification of a molecular mechanism awaits further study, it is tempting to consider that CB₁-D₂L interactions will represent a new CB₁ receptor signaling pathway that may be subject to functional selectivity (Glass and Northup, 1999; Mukhopadhyay and Howlett, 2005; Urban, et al., 2007).

The physiological significance and functional consequences of CB₁ receptor-induced CB₁-D₂L dimers may have implications in the use of clinical cannabinoids to treat chronic pain as well as chronic marijuana use. Such conditions would involve persistent CB₁ receptor activation providing an impetus to understand the molecular adaptations that occur in the nervous system (Cooper and Haney, 2008). Although we were able to study drug-induced changes of the CB₁-D₂L and D₂L-D₂L receptor dimers, a low BiFC signal between CB₁ receptors prevented us from examining the ratios of CB₁-CB₁ homodimers to CB₁-D₂L heterodimers. In the absence of CB₁-CB₁ studies, the CP55,940-induced increase in the CB₁-D₂L heterodimer may reflect a relative decrease in D₂L-D₂L homodimers and perhaps D₂L function. Consistent with this possibility we observed a modest CP55,940-induced decrease (ca. 15-25%) in D₂L receptor expression and D₂L-D₂L homodimers. These observations may suggest that persistent CB₁ receptor activation and subsequent CB₁-D₂L heterodimer formation could reduce D₂L receptor expression. In partial support of this hypothesis, it has been shown in rats and humans that chronic pre-natal exposure to marijuana decreases the expression of dopamine D₂ receptors in the brain (Walters and Carr, 1986; Wang, et al., 2004).
In the present report, we have visualized simultaneously the localization patterns of CB1-D2L heterodimers and D2L-D2L homodimers in living cells and provided evidence for agonist-regulated effects on receptor dimerization patterns. Recent studies propose that an increasing number of GPCRs may participate in higher order receptor oligomers or “receptor mosaics” and these structures may mediate many signaling events (reviewed in Fuxe, et al., 2008). The present work and other recent studies are consistent with this concept (Carriba, et al., 2008;Navarro, et al., 2008). We anticipate the continued development of new technologies will allow investigators to examine these receptor mosaics in greater detail. Finally, the use of MBiFC provides a new tool to study drug-induced changes in receptor oligomerization and may offer an important asset relevant to the future of drug discovery in the area of receptor heterodimers.
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References


Footnotes

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Personal communications confirmed that the D_{2L} receptor was used in Glass and Felder, 1997 (per Dr. David Sibley, who supplied the CHO-D_{2} cells) as well as in Marcellino et al., 2008 (per Dr. Kjell Fuxe).

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Legends for Figures

Figure 1. Functional characterization of receptor-BiFC fragment fusion proteins by measurement of acute inhibition of forskolin-stimulated cAMP accumulation. CAD cells were transiently transfected as indicated in each panel. Cyclic AMP accumulation was measured following a 15 minute incubation with forskolin (10 µM) in the presence of quinpirole (10 µM) (A.) or CP55,940 (10 µM) (B.) as shown. All data are normalized to the percent of forskolin-stimulated cAMP accumulation under matched transfection conditions. Each bar represents the mean ± S.E.M of three to four independent experiments assayed in duplicate. *, p < 0.05 compared with forskolin-stimulated cAMP accumulation under vehicle conditions (one-sample t test).

Figure 2. CB1-D2L and D2L-D2L dimers detected by MBiFC. A. A schematic representing the MBiFC approach used in these studies. CB1-D2L dimerization reconstitutes the Venus fluorescent protein (yellow) and D2L-D2L dimerization reconstitutes the Cerulean fluorescent protein (cyan). B. Representative images of the fluorescent signals observed in a MBiFC study as described in the schematic in panel A. CAD cells were transiently transfected and imaged as described in the methods section. The merged image (overlapping signal in yellow) represents an overlap of the Venus signal (depicted in red) and the Cerulean signal (depicted in green). Scale bar: 5 µm. C. Representative images of the expression patterns of CB1-Venus and D2L-Cerulean receptors following co-transfection. The merged image (overlapping signal in yellow) represents an overlap of CB1-Venus (depicted in red) and D2L-Cerulean (depicted in green). D. Representative images of the expression patterns of CB1-Venus (top panels) and D2L-Cerulean (bottom panels) following individual transfections in the presence of mCherry-mem. The merge
image (overlapping signal in yellow) represents the overlap of either CB1-Venus or D2L-Venus (green signal) with mCherry-mem (red signal).

**Figure 3.** Intracellular localization patterns of the D2L-D2L homomers and CB1-D2L heteromers. CAD cells were transiently transfected with both D2L-CC and D2L-CN (cyan signal in Panel A) or CB1-VN and D2L-CC (cyan signal in Panel B) along with the indicated YFP fluorescent marker proteins (yellow signal). The merged image (overlapping signal in yellow) represents an overlap of the BiFC signal (depicted in red) and the fluorescent marker signal (depicted in green). Images are representative of 3 independent transfections.

**Figure 4.** CB1 and D2L receptor form homo- and heteromeric receptor oligomers as measured by FRET. CAD cells were transiently transfected with 750 ng/well of either CB1-Venus and CB1-Cerulean, D2L-Venus and D2L-Cerulean, or CB1-Venus and D2L-Cerulean. Mix samples represent a mixture of CAD cell suspensions individually expressing the respective fluorescently tagged receptors of interest. Data represent the mean ± S.E.M of three independent experiments assayed in triplicate. **, p < 0.01 compared with mixed samples (one-way ANOVA followed by Dunnett’s post hoc test).

**Figure 5.** Time course examining the effects of persistent CP55,940 treatment on heteromer (D2L-CB1-Venus) and homomer (D2L-D2L-Cerulean) formation. CAD cells were transiently transfected with CB1-VN, D2L-CC, and D2L-CN followed by quantitative image analyses of the Venus/Cerulean ratios for the membrane and intracellular compartments. A. Cells were incubated with 10 µM CP55,940 for 10, 20, or 30 hrs prior to image analysis. Data represents
the average median Venus to Cerulean ratio values normalized to percent vehicle treatment (± S.E.M) in 4 independent experiments. B. Images from 20 hr time point depicting the effects of CP55,940 on Venus and Cerulean signals.

**Figure 6.** The effects of persistent ligand treatment on heteromer (D\textsubscript{2L-CB\textsubscript{1}}-Venus) and homomer (D\textsubscript{2L-D\textsubscript{2L}}-Cerulean) formation. CAD cells were transiently transfected and imaged as described for Figure 5. A. Cells were incubated with 10 µM quinpirole (Quin), 1 µM sulpiride (Sulp), or quinpirole + sulpiride (Quin + Sulp) for 20 hrs. B. Cells were incubated with 10 µM CP55,940 (CP), 10 µM AM281, or CP55,940 + AM281 (CP + AM281) for 20 hrs. Data represent the average median Venus to Cerulean ratio values normalized to percent vehicle treatment (± S.E.M). * p < 0.05 (compared with vehicle, one-sample t test, n = 5-8).

**Figure 7.** Dose-response analysis for CP55,940 modulation of the Venus/Cerulean ratio. CAD cells were transiently transfected and imaged as described for Figure 5. Cells were incubated with increasing concentrations of CP55,940 for 20 hrs. Data represent the average median Venus to Cerulean ratio values normalized to percent vehicle treatment (± S.E.M) in 3 independent experiments.

**Figure 8.** Effect of constitutively active CB\textsubscript{1} receptor (T210I) on relative dimer population at the plasma membrane or intracellular compartment. CAD cells were transiently transfected with either CB\textsubscript{1}wt-VN or CB\textsubscript{1}T210I-VN with D\textsubscript{2L}-CC and D\textsubscript{2L}-CN. A. Left panel: Quantitative image analyses of the Venus/Cerulean ratios were determined for the membrane and intracellular compartments as described in the methods. The Venus to Cerulean ratio induced by CB\textsubscript{1}T210I
was normalized to the Venus to Cerulean ratio measured in cells expressing CB$_1$wt receptor *, p < 0.05 (compared with wild-type, one sample $t$ test). Right panel: Quantitative image analyses of the Intracellular/Membrane ratios were determined for the CB$_1$-D$_{2L}$-Venus signal in cells either expressing CB$_1$wt or CB$_1$T210I. *, p < 0.05 (compared with wild-type, $t$ test). Data for both analyses were generated from the same experiments and represent the average median ± S.E.M from 3 independent experiments. B. Representative images of CAD cells expressing either CB$_1$wt (top panel) or CB$_1$T210I (bottom panel) to reconstitute the BiFC signals, CB$_1$(wt or T210I)-D$_{2L}$-Venus and D$_{2L}$-D$_{2L}$-Cerulean, and the membrane marker (mCherry-mem). The merge panel represents overlap of the three channels and overlapping pixel intensity is presented in white.
Figure 1

A. Quinpirole Inhibition of Cyclic-AMP Accumulation (% Vehicle)

- Vehicle = 100%
- * indicates significant difference from Vehicle

B. CP55,940 Inhibition of Cyclic-AMP Accumulation (% Vehicle)

- * indicates significant difference from Vehicle

Transfection Condition
Figure 2

A.

B. Venus  Cerulean  Merge

C. CB₁-Venus  D₂L-Cerulean  Merge

D. mCherry-Mem  CB₁-Venus  Merge

mCherry-Mem  D₂L-Venus  Merge
Figure 3

A. YFP
   - Endo
   - ER
   - Golgi

B. YFP
   - Endo
   - ER
   - Golgi

D_2L - D_2L

Merge
Figure 4

[Graph showing normalized FRET values for CB₁-CB₁, D₂L-D₂L, and CB₁-D₂L. Bars with error bars represent the FRET values, and a mix of symbols indicates statistical significance.]
Figure 5.

A. Median YFP/CFP Ratio (of Vehicle) vs. CP Treatment Time.

B. Images showing Venus and Cerulean fluorescence under Vehicle and CP treatments.
Figure 6

A.  

![Graph showing D₂ Ligand Treatment (20hrs)]

- **Membrane**
- **Intracellular**

Venus/Cerulean (% Vehicle)

- Quin
- Quin + Sulp
- Sulp

D₂ Ligand Treatment (20hrs)

B.  

![Graph showing CB₁ Ligand Treatment (20hrs)]

- **Membrane**
- **Intracellular**

Venus/Cerulean (% Vehicle)

- CP
- CP + AM281
- AM281

CB₁ Ligand Treatment (20hrs)
Figure 7

![Graph showing median YFP/CFP ratio as a function of log (CP55,940) M. The graph compares membrane and intracellular conditions.](https://example.com)
Figure 8

A.

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