ALLOSTERIC INTERACTIONS WITHIN CANNABINOID RECEPTOR 1 (CB₁)
AND DOPAMINE RECEPTOR 2 LONG (D₂L) HETEROMERS

by

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for the degree of Doctor of Philosophy

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ABSTRACT

G protein-coupled receptors (GPCRs) have long been recognized as essential membrane receptors mediating a vast array of functions in eukaryotes. GPCRs have more complex signaling than originally envisioned due to the fact that GPCRs can associate to form homomeric complexes or associate with other GPCRs to form heteromeric complexes. Allosteric communication within complexes influences the range of receptor function. Cannabinoid receptor 1 (CB1) and dopamine receptor 2 long (D2L) are GPCRs that are co-localized in specific neuronal populations in the basal ganglia. These receptors play crucial roles in the coordination of movement. I hypothesized that CB1 and D2L receptors associate in heteromeric complexes and that CB1 and D2L ligands promote bidirectional allosteric interactions within heteromeric complexes. I confirmed that CB1 and D2L receptors form homodimers and that each homodimer was coupled to a Gαi protein. CB1 and D2L receptors formed higher order oligomeric complexes; the minimum functional heteromeric complex was composed of a CB1 and D2L homodimer each coupled to a Gαi protein. Activation of either CB1 or D2L receptors by the agonists, arachidonyl-2-chloroethylamide (ACEA) or quinpirole, respectively, resulted in fast and transient conformational changes among CB1, D2L and Gαi proteins indicative of receptor activation. Treating cells co-expressing CB1 and D2L receptors with both ACEA and quinpirole switched CB1 and D2L receptors coupling and signaling from Gαi to Gαs, enhanced β-arrestin1 recruitment and co-internalization. The high-affinity D2L receptor antagonist, haloperidol, was also able to switch CB1 coupling from Gαi to Gαs but, unlike D2 agonists, haloperidol inhibited β-arrestin1 recruitment to CB1 and inhibited complex internalization. Allosteric interactions within CB1/D2L heteromeric complexes were ligand dose-dependent and bidirectional. CB1/D2L heteromers were detected in the globus pallidus of C57BL/6J mice. Chronic exposure to the cannabinoid CP 55,940 increased CB1/D2L heteromers while the D2 antagonist haloperidol reduced CB1/D2L heteromers in the globus pallidus of C57BL/6J mice indicating that functional heteromers existed in vivo and were affected by chronic drug exposure. The concept of bidirectional allosteric interaction within CB1/D2 heterotetramers has significant implication for the understanding of the complex physiology and pharmacology of CB1 and D2L receptors.
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<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ACEA</td>
<td>Arachidonyl-2-chloroethylamide</td>
</tr>
<tr>
<td>AEA</td>
<td>N-arachidonylethanolamine, or anandamide</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analyses of variance</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BiLC</td>
<td>Bimolecular luminescence complementation</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>BRET_{Eff}</td>
<td>BRET efficiency</td>
</tr>
<tr>
<td>BRET_{Max}</td>
<td>BRET$^2$ signal to reach a maximum saturated value</td>
</tr>
<tr>
<td>BRET_{Min}</td>
<td>BRET minimum</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CB1</td>
<td>Type 1 cannabinoid receptor</td>
</tr>
<tr>
<td>CB1-VC</td>
<td>CB1 fused to the EYFP Venus C-terminal</td>
</tr>
<tr>
<td>CB1-VN</td>
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<tr>
<td>CB2</td>
<td>Type 2 cannabinoid receptor</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CODA-RET</td>
<td>Complemented donor-acceptor resonance energy transfer</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP response element binding protein</td>
</tr>
<tr>
<td>CTx</td>
<td>Cholera toxin</td>
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<tr>
<td>D2L</td>
<td>Type 2 dopamine receptor long isoform</td>
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<td>D2s</td>
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<td>DMED</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DS</td>
<td>Dopamine System</td>
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<td>ECS</td>
<td>Endocannabinoid System</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
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<td>Abbreviation</td>
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<tr>
<td>EYFP</td>
<td>Enhanced yellow fluorescent protein</td>
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<td>FAAH</td>
<td>Fatty acid amide hydrolase</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>Forward primer</td>
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<td>Green fluorescent protein²</td>
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<td>GPCR</td>
<td>G protein coupled receptor</td>
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<td>GRK</td>
<td>G protein receptor kinase</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>L-AP4</td>
<td>L-2-amino-4-phosphonobutyric acid</td>
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<td>L-DOPA</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>mGluR6</td>
<td>Metabotropic glutamate receptor 6</td>
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<tr>
<td>mHtt</td>
<td>Mutant huntingtin protein</td>
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<tr>
<td>MSNs</td>
<td>GABAeric medium spiny neurons</td>
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<tr>
<td>N</td>
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</tr>
<tr>
<td>O.D.</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PBST</td>
<td>Phosphate-buffered saline with 0.1% tween-20</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>pCREB</td>
<td>Phosphorylated CREB</td>
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<td>pERK</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>Sequential resonance energy transfer</td>
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<td>Sulpiride</td>
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<tr>
<td>THC</td>
<td>$\Delta^9$-tetrahydrocannabinol</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid type 1</td>
</tr>
</tbody>
</table>
Acknowledgements

In the name of ALLAH (GOD), the most merciful, the most compassionate

"The more you thank Me, the more I give you." Quran, 14:7

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CHAPTER 1
INTRODUCTION

1.1 Overview

G protein-coupled receptors (GPCRs) are the largest family of transmembrane receptors. GPCRs mediate intracellular signaling via G protein-dependent and G protein-independent signaling pathways. The complexity and diversity of GPCR signaling are much greater than first envisioned because GPCRs can physically interact to form homo- and heteromeric complexes. Allosteric interactions across homo- and heteromeric complexes have profound impacts on GPCR ligand binding, G protein coupling, receptor trafficking and internalization. Cannabinoid receptor 1 (CB₁) and dopamine receptor 2 long (D₂L) are GPCRs that are co-expressed in the basal ganglia and play crucial roles in controlling movement. Antipsychotics, acting as D₂L receptor antagonists, are clinically used to treat psychotic disorders in a variety of clinical settings and to control excessive involuntary movement in Huntington’s disease (HD). Heteromerization between CB₁ and D₂L receptors has been confirmed in heterologous expression systems and striatal neurons. Concurrent activation of both receptors was proposed to alter G protein coupling relative to the effects of independently activating each receptor. The global aim of this thesis was to understand the physical and functional interactions between CB₁ and D₂L receptors within heteromeric complexes. The focus has been placed on elucidating the allosteric interactions within CB₁/D₂L heteromers following the application of CB₁ agonists and D₂ ligands (agonists and antagonists) using a heterologous expression system and cells endogenously expressing both receptors. Specifically, we examined the effects of CB₁ and D₂L ligand co-application on G protein coupling, G protein-dependent downstream signalling, and β-arrestin recruitment. Furthermore, we aimed to understand the stoichiometry of CB₁/D₂L/Gα proteins within heteromeric complexes. Finally, we examined the expression of CB₁/D₂L heteromers in the globus pallidus of C57BL/6J mice following chronic CB₁ and/or D₂L ligand treatment. The studies presented in this thesis will improve understanding of the allosteric interactions within CB₁/D₂L heteromers and the impact of co-administration of cannabinoids on the therapeutic effects of
antipsychotics (D₂ antagonists). This work will guide efforts to improve treatment for patients suffering from movement disorder and psychosis.

1.2 G-protein Coupled Receptors (GPCRs)

GPCRs are the largest family of signal transduction transmembrane receptors, with class A GPCRs being the largest subfamily within the group (Bockaert, 1991; Gether 2000; reviewed in Katritch et al., 2013). These receptors and signal transduction pathways play essential roles in various physiological functions as well as in pathologies. Therefore, GPCRs are considered a highly ‘druggable’ class of receptors and are the targets of a wide range of pharmacological therapies. GPCRs possess seven membrane-spanning regions, coupled to heterotrimeric guanine nucleotide binding proteins (G proteins). G proteins are comprised of a Gα subunit bound to a Gβγ dimer. GPCRs can generate diverse signaling responses based on their coupling to specific Gα subtypes. The three primary subtypes include 1) Gαₛ, which activates adenylyl cyclase (AC) and increases cyclic adenosine monophosphate (cAMP), 2) Gαᵢ, which inhibits AC and decreases cAMP; and 3) Gα₉, which activates the phospholipase C (PLC) signaling pathway resulting in an increase in intracellular calcium (Ca²⁺) (Strathmann and Simon, 1990; Levitzki and Bar-Sinai, 1991; Nurnberg et al., 1995).

The dynamics of GPCR and G protein interaction are still not completely understood. Two models have been proposed to explain the interactions between GPCRs and G proteins and the subsequent activation of G proteins (Limbird, 1983; Gilman 1987; Bockaert, 1991; Brady and Limbird, 2002; reviewed in Oldham and Hamm, 2007; Goricanec et al., 2016; Toyama et al., 2017). The classic model of GPCR-mediated signal transduction was believed to occur through the interaction and activation of different types of Gα protein (Limbird, 1983; Gilman 1987; Bockaert, 1991; Brady and Limbird, 2002). This model implies that the four components of the interacting functional complex including GPCR, Gα, Gβγ, and AC are freely mobile and can interact by random ‘collision coupling’ (Tolkovsky and Levitzki 1978; reviewed in Oldham and Hamm, 2008). In this model, GPCR-mediated signal transduction begins with agonist binding to the orthosteric ligand-binding site at the receptor promoting conformational
changes and the transition of the receptor from the inactive to the active state leading to G protein recruitment in its guanosine diphosphate (GDP)-bound Gαβγ form (Fig. 1.1). Activated G protein coupled-GPCRs trigger guanylyl nucleotide exchange from GDP to guanosine triphosphate (GTP) on the Gα subunit, which leads to rapid dissociation of Gα and Gβγ into active subunits to allow effector activation. Activated Gα binds and activates different second messengers depending on the subtype of coupled Gα protein with the receptor. Finally, signaling is terminated when GTP is hydrolyzed to GDP by intrinsic GTPase activity of the Gα subunit, which promotes dissociation of Gα from AC and reconstitution of Gαβγ heterotrimeric protein (reviewed in Cabrera-Vera, 2003; Oldham and Hamm, 2007).

The second model of GPCR-mediated signal transduction suggests that GPCRs are “pre-assembled” with their cognate heterotrimeric G protein (Braun and Levitzki, 1979; Klein et al., 2000; Philip et al., 2007). The pre-assembly of GPCR and cognate G protein occur early during the biosynthesis of the receptors in the endoplasmic reticulum and the pre- assembled GPCR/G protein complex is trafficked together to the cell membrane (Dupré et al. 2007). The pre-assembly between GPCR and G protein has been confirmed using biochemical approaches such as co-immunoprecipitation (Smith and Limbird, 1981), crystallographic analysis (Rasmussen et al., 2007; Scheerer et al., 2008) and resonance energy transfer (RET)-based approaches (Galés et al., 2005, 2006; Nobels et al., 2005; Ayoub et al., 2007, 2010; Audet et al., 2008; Levoye et al., 2009; Qin et al., 2011). The use of RET, bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) facilitated the study of the interaction between GPCR and G protein in real time in living cells. RET approaches allow for the determination of the proximity and relative conformation between chromophores fused to a GPCR and a G protein (either Gα or Gβγ protein, reviewed in Ayoub et al., 2012). RET approaches have been used to confirm the pre-assembly of various family A GPCR with their cognate Gαi-protein such as α2A-drenergic receptor with Gαi1 (Galés et al., 2006) and Gαo (Nobels et al., 2005), protease-activated receptor 1 and 2 with Gαi1 (Ayoub et al., 2007, 2010), δ-opioid receptor and Gαi1 (Audet et al., 2008), muscarinic M4 receptors and Gαo (Nobels et al., 2005), chemokine CXCR4 and CXCR7 (Levoye et al., 2012).
Figure 1.1: The Life Cycle of a GPCR. GPCRs are translated on ribosomes associated with the endoplasmic reticulum and transported via secretory vesicles to the Golgi apparatus and eventually to the plasma membrane. Signal transduction at GPCRs begins with agonist binding to the receptor, which catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α-subunit of heterotrimeric G proteins (Gαβγ). This allows the activated G protein to act on downstream effectors and produce biological responses. Signaling is then turned off by the hydrolysis of GTP to GDP by the Regulator of G protein Signaling (RGS) proteins. Receptors are internalized following phosphorylation of the intracellular domain of the receptor by G protein kinase (GRK) and then subsequent recruitment of β-arrestin protein (Arr). Internalized receptors are either degraded by the lysosome or recycled back to the cell surface. Figure 1.1 was modified from Wilkie, 2001.
2009) receptors, and muscarinic receptor M3 and Gaq (Qin et al., 2011). RET experiments indicate that binding of an agonist to GPCRs results in rapid conformational changes with rearrangement and/or reorientation of Gα within the pre-assembled GPCR-G protein complex, rather than recruitment of G proteins to GPCR. Such conformational changes result in agonist dose-dependent increase/decrease in RET signal between tagged GPCR and Gα protein (Galés et al., 2005, 2006; Levoye et al., 2009; Levoye et al., 2009; Denis et al., 2012). The agonist-dependent increase in RET signals is followed by the return of RET signals to basal levels indicating the return of GPCR/Gα protein complexes to the inactive conformation rather than dissociation of GPCR from Gα protein (Bunemann et al., 2003; Galés et al., 2006). Moreover, the pre-assembly model suggests that Gα and Gβγ subunit dimers remain associated and pre-assembled to GPCR during activation of GPCRs by agonists (Galés et al., 2006). It has become increasingly clear that GPCRs mediate ligand-dependent cell signaling is far more complex than can be simply explained by the activation of different Gα subtypes since GPCRs are able to couple and activate multiple downstream effector proteins (reviewed in Bosier and Hermans, 2007; Kenakin and Christopoulos, 2013; Ferré et al., 2014, 2015).

After G protein-dependent activation, the primary pathway leading to GPCR desensitization involves the phosphorylation of the intracellular carboxy terminus of the receptor by a G protein receptor kinase (GRK) (Benovic et al., 1985; Lefkowitz, 1993; reviewed in Gurevich et al., 2012; Smith and Rajagopal, 2016). Following receptor phosphorylation, β-arrestin is recruited to the receptor, which blocks the G protein binding site on the receptor thereby desensitizing the GPCRs to the initial stimuli (Ferguson et al., 1996; Lohse et al., 1990; DeGraff et al., 2002; Marion et al., 2006; reviewed in Smith and Rajagopal, 2016). β-arrestin further serves as a scaffold protein, allowing for the formation of clathrin-coated pits followed by endocytosis of the GPCR/β-arrestin complex (Anderson, 1998; Luttrell et al., 2001). The GPCR may then be recycled to the plasma membrane or targeted to lysosomes for degradation (Anderson, 1998; Luttrell et al., 2001; Luttrell and Lefkowitz, 2002).

In addition to the primary roles of β-arrestins in the termination of G protein-dependent signal and receptor internalization, β-arrestins are involved in G protein independent signaling. β-arrestins scaffold and regulate several downstream effectors
(reviewed in Smith and Rajagopal, 2016). In particular \( \beta \)-arrestins scaffold and activate the mitogen-activated proteins (MAPs) including extracellular signal–regulated kinases (ERK1 and ERK2) (Tohgo \textit{et al.}, 2002; Lefkowitz and Shenoy, 2005; Shenoy \textit{et al.}, 2006). Unlike the transient G protein-dependent ERK signaling (peak 2-5 min), \( \beta \)-arrestin-dependent ERK phosphorylation develops slowly (peak 5-10 min) and persists for extended periods (over 30 min) due to the long-lasting association between the receptor and \( \beta \)-arrestin (Ahn \textit{et al.}, 2004; Shenoy \textit{et al.}, 2006; DeWire \textit{et al.}, 2007).

GPCRs do not exist in either active conformations capable of activating G proteins or in inactive conformations unable to activate G proteins. Rather GPCRs can adopt multiple active conformations, and each active conformation favors binding and stimulation of specific effector proteins (Kenakin, 2010; Kenakin and Christopoulos 2013). Biased agonism or functional selectivity is the result of an orthosteric ligand-dependent shift in the conformation of a receptor that favors interaction with specific effector proteins at the expense of other possible effector proteins (Kenakin, 2010; Kenakin and Christopoulos, 2013). For example, different orthosteric cannabinoid agonists that bind the type 1 cannabinoid receptor (CB\(_1\)) can preferentially stabilize different active conformations of the receptor resulting in alteration of the coupling of the receptor to different G proteins (reviewed in Laprairie \textit{et al.}, 2017).

To further increase the complexity of GPCR signaling, the pharmacology of GPCR orthosteric ligands can be modulated by the binding of allosteric modulators. Allosteric modulators are molecules that bind to a site distinct from that of the orthosteric agonist-binding site on a GPCR (conduit) and induce conformational changes within the GPCR that are transmitted from the allosteric binding site to the orthosteric binding site (Fig. 1.2A). Allosteric modulators lack intrinsic efficacy and are unable to activate the receptor in the absence of orthosteric agonist (guest) (Wootten \textit{et al.}, 2013; van der Westhuizen \textit{et al.}, 2015). The binding of the allosteric modulators can either enhance (positive allosteric modulator), or diminish (negative allosteric modulator) the efficacy and potency of orthosteric ligand-dependent signaling through the GPCR (Wootten \textit{et al.}, 2013; van der Westhuizen \textit{et al.}, 2015). G proteins and other effector proteins that physically bind GPCRs also have allosteric modulatory properties that can modify orthosteric ligand binding (reviewed in Leach \textit{et al.}, 2007; Darren \textit{et al.}, 2013; Gentry et
The assembly of homo- and heteromeric complexes influences the conformation of each receptor within the complex (Vilardaga et al., 2008; Maier-Peuschel et al., 2010; Bourque et al., 2017; Devost et al., 2017; Sleno et al., 2017). Taken together, it is now accepted that orthosteric-ligand dependent biased agonism and allosteric modulation due to ligand binding and protein-protein interactions contribute to the diversity of signaling responses. This view is in contrast to early simple models of GPCR function that were based on the classic “one receptor - one G protein -one signaling response” model. The demonstration that many GPCRs physically associate to form homo- and heteromers, and that these interactions have the ability to modulate nearly every aspect of receptor pharmacology and function provides further evidence that GPCR signaling is increasingly more complex than previously assumed.

1.2.1 GPCR Oligomerization

It is now well accepted that class A GPCRs physically associate to form homo and heteromers or higher order oligomeric complexes in heterologous expression systems (reviewed in Rios et al., 2001; Milligan 2004, 2009; Ferré, 2015; Franco et al., 2016; Gaitonde and González-Maeso, 2017). The evidence of GPCR oligomerization emerged during 1970-1980 with the observation of functional interactions among GPCRs. These interactions involve ligand binding to one receptor altering the ligand binding of another receptor. Negative cooperativity among the β2 adrenergic receptors (β2AR) was observed in 1975 in the frog erythrocyte membrane preparation (Limbird et al., 1975). The observed cooperativity effects were proposed to be due to the formation of β2AR homomers (De Lean et al., 1980; Chidiac et al., 1997). Subsequently, the formation of β2AR homomers was confirmed using differential epitope tagging and co-immunoprecipitation (Hébert et al., 1996) and using BRET (Angers et al., 2000). Since these findings, GPCRs oligomerization has been a major subject of research, and increasing evidence suggests that class A GPCRs exist as homo- and heteromers when expressed in a heterologous expression system (reviewed in Milligan 2004, 2009; Ferré, 2015; Franco et al., 2016; Gaitonde and González-Maeso, 2017). Interestingly, more recent evidence has provided evidence for the existence of GPCR heteromers in native tissues and animal models (reviewed in Franco et al., 2016; Gomes et al., 2016).
**Figure 1.2: Allosterism Across GPCR Monomers and Oligomers.** (A) The GPCR monomers can act as the conduit of the allosteric modulator. Small molecule allosteric modulators bind to a region of the receptor that is distant from the orthosteric ligand-binding site. Allosteric modulators can affect the binding and function of orthosteric ligands (guest). In addition, G proteins and other effector proteins that physically bind GPCRs can have allosteric modulatory properties that modify orthosteric ligand binding and receptor functions. (B) GPCR oligomers can have two types of allosteric interactions. GPCR oligomers can be considered as the conduit of the allosteric modulator where the orthosteric ligand of the first GPCR protomer acts as an allosteric modulator to alter the functions of the second orthosteric ligands (guest) bound to the second GPCR protomer (left panel). The second type of allosteric interaction within GPCR oligomers is called ligand-independent allosteric modulation. In this case, one of the GPCR protomer acts as the allosteric modulator, in the absence of ligand, and the second GPCR protomer becomes the conduit that binds the guest ligand (right panel). Figure 1.2 was modified from Kenakin and Miller, 2010.
1.2.2 Functional Consequences of GPCR Oligomerization

Oligomerization of class A GPCRs can affect nearly every aspect of GPCR functions including biosynthesis, trafficking, ligand pharmacology, signal transduction and internalization (Fig. 1.3). Therefore, GPCR homomerization can play important roles in the modulation of GPCR functions and is a vital mechanism to increase the diversity and specificity of GPCR signaling (reviewed in Milligan, 2004, 2009; Terrillon and Bouvier, 2004; Gurevich et al., 2008; Smith and Milligan, 2010; Ferré et al., 2014, 2015; Franco et al., 2016; Gaitonde and González-Maeso, 2017). Several studies have demonstrated that family A GPCR homo- and hetero-oligomerization play a crucial role for proper trafficking of the receptors to the plasma membrane. GPCR oligomers form in the endoplasmic reticulum and appear to be present through all phases of receptor trafficking (Dupré et al., 2006; Herrick-Davis et al., 2006). For example, olfactory receptors reach the cell surface when co-expressed with the α1B adrenergic receptor or the β2 receptor, but not if expressed as single receptors (Hague et al., 2004; Bush et al., 2007; Hall, 2009). Similar observations have been reported for other GPCRs and confirm that homo- and heteromerization of GPCRs is required for the proper maturation and trafficking of GPCRs from the endoplasmic reticulum to the cell membrane (Kobayashi et al., 2009; reviewed in Milligan, 2004, 2009; Terrillon and Bouvier, 2004; Gurevich et al., 2008; Smith and Milligan, 2010; Ferré et al., 2014, 2015). GPCR oligomerization can also affect receptor desensitization and internalization following agonist activation. Most commonly, activation of one receptor in a heteromer will lead to a cross-internalization and a cross-desensitization of the second receptor (Pfeiffer et al. 2002; Hillion et al., 2002; Fiorentini et al., 2008). These observations suggest that GPCR oligomers internalize as intact entities instead of disassociating prior to receptor internalization (reviewed in Prinster et al., 2004; Terrillon and Bouvier, 2004). GPCR heteromerization has also been found to play a role in the recycling of internalized receptors back to the plasma membrane (Pfeiffer et al., 2003; Terrillon et al., 2004; Ellis et al., 2006; Grant et al., 2008). Together, this evidence indicates that oligomerization plays a significant role in the proper trafficking of GPCRs throughout their entire life-cycle starting early during receptor synthesis in the endoplasmic reticulum and being maintained throughout trafficking to the plasma membrane, agonist-induced internalization, and during recycling
Figure 1.3: Functional Consequences of GPCR Oligomerization. (1) GPCR oligomerization plays an important role in receptor maturation and correct trafficking from the endoplasmic reticulum to the plasma membrane. (2) Ligand binding to GPCR oligomers can modulate GPCR oligomer formation. (3) Ligand binding to one GPCR protomer can allosterically modulate the affinity between the ligand and associated protomer within oligomeric complexes. (4) Allosteric interaction within oligomeric complexes might result in enhancing or suppressing downstream signaling or altering G protein coupling. The three Go subtypes include Goi, which inhibits adenylyl cyclase (AC) and decreases cyclic adenosine monophosphate (cAMP), Gos, which activates AC and increases cAMP, and Goq, which activates the phospholipase C (PLC). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). (5) GPCR oligomerization can affect GPCR localization, the rate of internalization and subsequent recycling. +/- indicates an increase or decrease, respectively. Figure 1.3 was modified from Schellekens et al., 2013.
Specific ligand binding has been shown to modulate GPCR oligomer formation. Some studies have suggested that GPCRs either form stable interactions while other studies suggested that the interactions between GPCRs are transient. With the ability to detect oligomerization using RET techniques, several studies reported ligand-induced changes in BRET or FRET signals. These changes in BRET or FRET signals were suggested to represent the formation or disassociation of GPCR oligomers leading to the conclusion that GPCR oligomer formation is dynamic in nature (Angers, 2000; Rocheville, 2000; Cornea et al., 2001; Milligan and Bouvier, 2005; Alvarez-Curto et al., 2010; Elisa et al., 2010; Urizar et al., 2011). However, because BRET and FRET are dependent not only on the number of interacting receptors but also on the relative orientation of the donor and acceptor molecules, it is possible that ligand-induced changes in BRET and FRET are more likely caused by conformational changes than alterations in the number of interacting receptors (Pfleger and Eidne, 2005; Milligan and Bouvier, 2005; Alyarez-Curto, 2010; Ayoub, 2009, 2012). More recent studies using single-molecule total internal reflectance fluorescence microscopy together with SNAP-tag technique reported that GPCR oligomerization is highly dynamic, with the constant formation or disassociation of GPCR oligomers; however, ligand treatment did not modify GPCR oligomerization (Hern et al., 2010; Kasai et al., 2011; Calebiro et al., 2013). In contrast, using post-imaging acquisition spatial intensity distribution analysis of standard laser scanning confocal microscopy images demonstrated that the serotonin 5-HT2C receptors form mainly homodimers, and antagonist treatment decreased the number of homodimers (Ward et al., 2015). Altogether, the effects of acute ligands treatment on GPCR oligomerization are still controversial and might be receptor-dependent.

As mentioned earlier, the first evidence of GPCR oligomerization was the negative cooperativity observed in radioligand binding experiments (Limbird et al., 1975; De lean et al., 1980; Chidiac et al., 1997). Since then, several studies have reported either negative or positive cooperativity of GPCR homo- and heteromers in relation to ligand binding and intrinsic efficacy (Albizu et al., 2006, 2010; reviewed in Ferré et al., 2014). The negative or positive cooperativity effects of ligand binding are a particular type of allostERIC communication between GPCR protomers, within homo or hetero-oligomeric
complexes (Kenakin and Miller, 2010; Kenakin and Christopoulos, 2013). Kenakin and Miller (2010) proposed two models to describe allosteric modulations within GPCR oligomers with respect to ligand binding and efficacy. In the first model, the GPCR oligomers (at least two protomers) are considered as the conduit of the allosteric modulator; the orthosteric ligand of the first GPCR protomer acts as an allosteric modulator to alter the affinity and/or efficacy of the second orthosteric ligand (guest) binding to the second GPCR protomer (Fig. 1.2B). In this model, binding of the allosteric modulator to one of the GPCR protomers leads to either an increase or decrease in the affinity and/or efficacy of the guest ligand, which binds to the second GPCR protomers within homo or hetero-oligomeric complexes (Kenakin and Miller, 2010). An example of this model is the adenosine A2A receptor (A2A)/D2 heterotetramer, where the A2A receptor ligand decreases the affinity and signaling of dopamine at the D2 receptor (Ferré et al., 1992; Azdad, 2009; Bonaventura et al., 2015). Similarly, CB1 ligands can allosterically potentiate the binding and signaling of the δ-opioid receptor agonists (Bushlin et al., 2012; Rozenfeld et al., 2012).

The second model of allosteric modulation within GPCR oligomers with respect to ligand binding and efficacy is known as ligand-independent allosteric modulation in which one of the GPCR protomer acts as the allosteric modulator and the second GPCR protomer becomes the conduit that binds the guest ligand (reviewed in Ferré et al., 2014, 2015). In this model (Fig. 1.2B), the first GPCR protomer acts as the allosteric modulator of the orthosteric ligand binding to the conduit (second GPCR protomer) (reviewed in Ferré et al., 2014, 2015). For instance, the dopamine D2 receptor acts as the allosteric modulator that reduces the binding of SCH-442416 to A2A receptors within A2A/D2 heterotetramers (Orru et al., 2011; Bonaventura et al., 2015). Numerous mathematical models have been developed to analyze the complex ligand binding curves generated from ligand binding to GPCR oligomers. (Casadó et al., 2007, 2009; Rovira et al., 2009; Giraldo, 2013; reviewed in Ferré et al., 2014, 2015).

Allosteric communication between GPCR protomers within GPCR heteromeric complexes might contribute to activation of distinct signaling pathways known as functional selectivity or biased signaling (reviewed in Ferré et al., 2014). Physical interactions between GPCR protomers allosterically induce conformational changes in
each of the individual GPCR protomers (Vilardaga et al., 2008; Hlavackova et al., 2012; Sleno et al., 2017). In some cases, GPCR oligomerization might preferentially stabilize each of the individual GPCR protomers in conformations that favor coupling to specific G proteins (Kenakin and Miller, 2010). Several examples of switches in G protein coupling following GPCR heteromerization have been reported including G protein switching at angiotensin AT_1/CB_1 heteromers (Rozenfield et al., 2011), dopamine D_1/histamine H_3 heteromers (Ferrada et al., 2009), the dopamine D_2/ghrelin GHSR_1a heteromers (Kern et al., 2012, 2015). Other studies have reported that GPCR heteromerization may only potentiate or inhibit receptor signaling through distinct pathways rather than altering G protein coupling (reviewed in Milligan, 2004, 2009; Terrillon and Bouvier, 2004; Gurevich et al., 2008; Smith and Milligan, 2010; Ferré et al., 2014, 2015). Allosteric communication within GPCR heteromeric complexes may result in unique pharmacological properties of GPCR heteromers versus homomers.

1.2.3 Stoichiometry of GPCR/G Protein Complexes Within Homo- and Heterooligomeric Complexes

One question that has not been resolved in the field of GPCR oligomerization is the number of GPCR subunits involved in the formation of oligomeric complexes. For GPCR homomers, at least two GPCRs (homodimers) interact (Banères and Parell, 2003; Herrick-Davis et al., 2005). Strong support for the formation of GPCR homodimers also comes from morphological evidence obtained using atomic force microscopy for rhodopsin receptors in native tissue (Fotiadis et al., 2003; Liang et al., 2003). However, several lines of evidence suggest that higher order homooligomeric complexes can exist (reviewed in Bouvier and Hébert, 2014; Ferré et al., 2014, 2015). The use of protein complementation approaches together with BRET have allowed several investigators to demonstrate that GPCRs could form higher order homooligomeric complexes in systems expressing β_2AR receptors (Rebois et al., 2008), dopamine receptor 2 short (D_2s) (Gua et al., 2008), and A_2A receptor (Vidi et al., 2008). FRET was also used to show that higher order homo-oligomeric structures could be formed following expression of M_2 muscarinic receptors (Pisterzi et al., 2010) and the β_2AR receptors (Fung et al., 2009). However, recent studies suggest that GPCR homodimers are the predominant species
(Herrick-Davis et al. 2013; reviewed in Bouvier and Hébert, 2014; Ferré et al., 2014, 2015). This model is supported by evidence obtained using RET, fluorescence correlation spectroscopy and analysis of single fluorescence-labeled receptor molecules by total internal reflection fluorescence microscopy (Calebiro et al., 2013; Herrick-Davis, et al., 2013; Mazurkiewicz et al., 2015; Ward et al., 2015; Navarro et al., 2016). Crystal structures of the β2AR receptor (Rasmussen et al., 2007), CXCR4 chemokine receptors (Wu et al., 2010), μ and κ opioid receptors (Manglik et al., 2012, Wu et al., 2012), and β1 adrenergic receptor (Haung et al., 2013) have demonstrated the presence of receptor homodimers. Moreover, each GPCR homodimer was reported to couple to one heterotrimeric G protein to form a functional signaling complex (Han et al., 2009; Jastrazebka et al., 2013; Navarro et al., 2016). Asymmetric binding of heterotrimeric G protein to homodimers has been reported, where one heterotrimeric G protein binds to one protomer within the homodimeric complexes (Damian et al., 2006; Han et al., 2009; Zylbergold and Hébert, 2009; Jastrazebka et al., 2013; Pellissier et al., 2011; Jonas et al., 2015; Mishra et al., 2016). Therefore, the minimal composition of the functional unit is a homodimer interacting with one heterotrimeric G protein. Higher order homo-oligomeric complexes are also possible. GPCR heteromers are formed when two or multiple homodimers (each coupled to their cognate G protein) interact (reviewed in Ferré, 2015) to form a heterotetramer (Elisa et al., 2010; Mishra et al., 2014; Guitart et al., 2014; Bonaventura et al., 2015; Cordomí et al., 2015; Navarro et al., 2016). Specifically, Guitart et al. (2014) reported that the dopamine receptor type 1 (D1) and dopamine receptor type 3 (D3) receptors form heterotetramers composed of D1 and D3 homodimers as demonstrated using BRET combined with bimolecular fluorescence complementation (BiFC) and bimolecular fluorescence luminescence complementation (BiLC) assays. The same approach has also been used to uncover the tetrameric structure of A2A and D2 heteromers (Bonaventura et al., 2015). A more recent study, using microscope-based single-particle tracking and molecular modeling, reported that A1 and A2A form mainly heterotetramers composed of two homodimers, while A1 and A2A homomers, homotrimers and homotetramers were scarce (Navarro et al., 2016). Overall, GPCR heterotetramers are formed from at least two homodimers and one homodimer-associated G protein (reviewed in Cordomí et al., 2015).
1.3 The Endocannabinoid System (ECS)

The endocannabinoid system (ECS) is a lipid signaling system comprised of endogenous ligands (endocannabinoids), enzymes for their synthesis and degradation and two well-characterized GPCRs, cannabinoid receptors CB1 and CB2 (reviewed in Howlett et al., 2004; Pacher et al., 2006; Mechoulam and Parker, 2013). Endocannabinoids are lipid mediators derived from arachidonic acid. The primary endocannabinoids are N-arachidonoylethanolamine (Anandamide or AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995; Sugiura et al., 1995). Unlike classical neurotransmitters, the endocannabinoids are not stored in vesicles but are synthesized on demand postsynaptically in response to specific signals, such as increases in intracellular calcium or activation of phospholipase Cβ by Gq/11-coupled metabotropic receptors (Di Marzo et al., 1998; Stella and Piomelli 2001; Piomelli, 2003). The enzyme necessary for the synthesis of AEA are N-acyltransferase (NAT) and N-acylphosphatidylethanolamide-phospholipase D (NAPE-PLD) (Cadas et al., 1996; Di Marzo et al., 1999), while the main enzyme required for the synthesis of 2-AG is diacylglycerol lipase (DAGL) (Stella et al., 1997). Degradation of AEA and 2-AG occurs locally by fatty-acid amide hydrolase (FAAH) and monoaoylglycerol lipase (MGL), respectively (Egertová et al., 1998; Cravatt et al., 1996; McKinney and Cravatt, 2005; Blankman et al., 2007; Ahn et al., 2008).

1.3.1 The Cannabinoid Receptor 1 (CB1)

CB1 is the most abundant GPCR in the central nervous system (CNS) and is expressed at high levels in the basal ganglia, hippocampus, cerebral cortex, amygdala and cerebellum and at lower levels throughout the CNS (Matsuda et al., 1990; Herkenham et al., 1990; Mailleux and Vanderhaeghen, 1992). Accumulating evidence has confirmed that CB1 is also expressed in the periphery in many tissues including the cardiovascular system, reproductive system, intestine, smooth muscle, and eye (Pertwee et al., 1996; Sugiura et al., 1998; Straiker et al., 1999; Stamer et al., 2001; Wang, 2003). The widespread distribution of CB1 allows for its participation in the regulation of a variety of central and peripheral physiological functions, including modulation of neurotransmitter release, energy metabolism, and cardiovascular, respiratory and reproductive function.
(reviewed in Iversen, 2003; Pacher et al., 2006; Vemuri et al., 2008; Smith et al., 2010; Kirilly et al., 2012; Pertwee, 2012; Aizpurua-Olaizola, 2017)

The human CB1 gene (CNR1) is located on chromosome 6 locus q14-q15. Alternative splicing of human CB1 within the coding region (exon 4) results in the formation of the full-length CB1 (472 amino acids), CB1a (411 amino acids) (Shire et al., 1995) and CB1b (439 amino acids) (Ryberg et al., 2005). CB1a is shorter than CB1 by 61 amino acids at its N-terminus, while CB1b is shorter than CB1 by 33 amino acids at the N-terminal tail (Shire et al., 1995; Ryberg et al., 2005). Overlap in the distribution patterns of the mRNAs of the three CB1 protein variants in different regions of the human brain and the periphery has been reported (Shire et al., 1995; Ryberg et al., 2005; Xiao et al., 2008; Gustafsson et al., 2008; Bagher et al., 2013).

In the CNS, CB1 is located presynaptically where it plays a modulatory role in the regulation of noradrenaline, acetylcholine, dopamine, γ-aminobutyric acid (GABA), glutamine, serotonin, and glycine release (Fig. 1.4) (Abood and Martin, 1992; Di Marzo et al., 1998; Wilson and Nicoll, 2001; Howlett et al., 2004; Castillo et al., 2012). Endocannabinoids synthesized postsynaptically diffuse retrogradely to activate presynaptic CB1 receptors, resulting in inhibition of voltage-gated Ca2+ channels (VGCC) and activation of G protein-coupled inwardly rectifying K+ channel (GIRKs) suppressing the release of many different neurotransmitters. Some evidence also suggests the CB1 receptors are also expressed postsynaptically on GABAergic neurons and non-retrograde CB1 signaling has been observed (Hohmann et al., 1999; Ong and Mackie, 1999; Bacci et al., 2004; Nyiri et al., 2005). Repetitive activation of GABAergic interneuron triggers increases in intracellular Ca2+, synthesis of AEA/2-AG, and activation of postsynaptic CB1 receptors that couple to GIRKs. This autocrine activation of postsynaptic CB1 receptors leads to postsynaptic hyperpolarization and reducing excitability (Bacci et al., 2004; Marinelli et al., 2008, 2009; reviewed in Castillo, 2012).

In addition to signaling via Gαi/o, CB1 receptors have been shown to signal through both Gαs and Gαq/11 pathways to increase cAMP levels, and cytosolic [Ca2+], respectively (Demuth and Molleman, 2006; Bosier et al., 2010; Turu and Hunyady, 2010; reviewed in Hudson et al., 2010a; Laprairie et al., 2017). In addition to Gα protein
Figure 1.4: Diagram of CB₁ Retrograde Inhibition of Neurotransmitter Release and Postsynaptic Signaling. An increase in intracellular calcium levels in the postsynaptic terminal activates N-acyltransferase (NAT) or diacylglycerol lipase (DGL), leading to the synthesis of anandamide (AEA) and 2-arachidonyl glycerol (2-AG), respectively, from cellular phospholipids. AEA and 2-AG are released into the synaptic cleft and traverse in a retrograde fashion to activate CB₁ receptors located on the presynaptic terminal. Activation of CB₁ receptors inhibits voltage gated-calcium channel (VGCC) and activates G protein-coupled inwardly rectifying K⁺ (GIRK) channels, in addition to causing other presynaptic changes that hyperpolarize the presynaptic membrane and lowers the probability of Ca⁺² dependent neurotransmitter release. In addition, AEA/ 2-AG activate postsynaptic CB₁ receptors to stimulate GIRK channels, which leads to hyperpolarization and inhibition of neuronal firing. Figure 1.4 was modified from Hosking and Zajicek, 2008 and Castillo et al., 2012.
dependent signaling, CB1 can also signal via Gα protein-independent pathways including β-arrestin 1 and 2. Ligand-dependent coupling of CB1 to β-arrestin may influence the dwell time of receptors at the plasma membrane, and receptor internalization, recycling, and degradation (Jin et al., 1999; Bakshi et al., 2007; van der Lee et al., 2009; Laprairie et al., 2014). Ligand-dependent coupling of CB1 to β-arrestin also affects β-arrestin-dependent ERK phosphorylation and signaling kinetics (reviewed in Laprairie et al., 2017; Ibsen et al., 2017).

Similar to other members of the class A GPCR subfamily, CB1 receptors form both homo-oligomers (Wager-Miller et al., 2002) and hetero-oligomers with other GPCRs. Homomerization of CB1 has been demonstrated by the observation of a high molecular weight band on non-denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using an antibody directed against the C-terminal tail of CB1; the observed high molecular weight bands correspond to the molecular weight of a CB1 homodimer (Wager-Miller et al., 2002). Homomerization of CB1 was further confirmed using BRET (Hudson et al., 2010; Bagher et al., 2013, 2016). Heteromerization of CB1 has been demonstrated with several class A GPCRs such as the D2 dopamine receptor (Glass and Felder, 1997; Kearn et al., 2005), μ-, κ-, and δ-opioid receptors (Rios et al., 2006; Hojo et al., 2008; Ittai et al., 2012), orexin-1 (Ellis et al., 2006; Jäntti et al., 2014), A2a adrenergic receptor (Carriba et al., 2007) β2AR (Hudson et al., 2010b), angiotensin II (Ang II) receptor (Rozenfeld et al., 2011), CB1a and CB1b (Bagher et al., 2013). Heteromerization of CB1 has been reported to affect receptor trafficking, G protein coupling and signaling (Rios et al., 2006; Ellis et al., 2006; Carriba et al., 2007; Hudson et al., 2010; Rozenfeld et al., 2011; Bagher et al., 2013). Therefore, such hetero-oligomeric interactions may play a role in the regional and ligand-specific variability in cannabinoid function.

CB1 orthosteric ligands have been proposed as pharmacotherapeutics for treating neurodegenerative diseases, chronic pain, substance abuse disorders and obesity because CB1 plays important roles in many physiological and pathophysiological processes (Pacher et al., 2006; Vemuri et al., 2008; Pertwee, 2008, 2012; Aizpurua-Olaizola, 2017). CB1 can be activated by plant-derived cannabinoid and synthetic compounds in addition to being activated by endocannabinoids. Cannabinoid agonists are divided into four
structurally distinct groups. The first group contains the ‘classical cannabinoids’ derived from the plant *Cannabis sativa* such as Δ-9-tetrahydrocannabinol (THC) and related synthetic derivatives such as HU-210. The second group contains the non-canonical cannabinoids, which are synthetic derivatives of the classical cannabinoids that lack the dihydropyran ring such as CP 55,940. The third group includes aminoalkylindoles such as WIN 55212-2 and its related compounds. The last group contains the endocannabinoids, which are eicosanoid compounds rather than cannabinoid compounds and includes the endocannabinoids AEA and 2-AG (Bosier *et al.*, 2010). Due to the structural differences of CB₁ agonists, different classes of CB₁ show agonist bias, CB₁ coupling and signaling with various effector proteins including Gα₁₁, Gα₅, and Gαq proteins and β-arrestin 1 and 2 (reviewed in Laprairie *et al.*, 2017; Ibsen *et al.*, 2017). In addition, CB₁ allosteric modulators have been developed and tested. Several studies have reported that CB₁ positive allosteric modulators provide improved safety and drug-pharmacology profiles over orthosteric CB₁ agonists (Ross, 2007; Morales *et al.*, 2016; Laprairie *et al.*, 2017).

1.4 The Dopaminergic System (DS)

Dopamine is a monoamine neurotransmitter that is produced in the dopaminergic neurons (Johnston, 1968; Hadjiconstantinou *et al.*, 1993; Männistö *et al.*, 1992; Sampaio-Maia *et al.*, 2001; Eriksen *et al.*, 2010). Dopamine has various functions in the CNS, including regulation of locomotor activity, reward, learning, memory, and endocrine function. In the periphery, dopamine helps to regulate cardiovascular function, vascular tone, renal function, hormone secretion and gastrointestinal motility (reviewed in Iversen and Iversen, 2007). In the brain, there are four main dopaminergic pathways including mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular pathways. The mesolimbic pathway is involved in motivational behavior. This pathway originates from the ventral tegmental area and innervates the nucleus accumbens and parts of the limbic system. The mesocortical pathway also originates from the ventral tegmental area; however, it innervates regions of the frontal cortex involved in learning and memory. The nigrostriatal pathway originates from the substantia nigra compacta and innervates the striatum, where it participates in the control of movement. Finally, the tuberoinfundibular
pathway originates from the cells of the periventricular and arcuate nuclei of the hypothalamus, reaching the pituitary (Missale et al., 1998; Hall et al., 1994; Wang et al., 2009; Beaulieu and Gainetdinov, 2011).

The physiological and pharmacological actions of dopamine are mediated by five dopamine receptors. The dopamine receptors are subclassified into two groups: the D1-like family (includes D1 and D5) and the D2-like family (includes D2, D3, and D4) receptors (reviewed in Missale et al., 1998; Beaulieu and Gainetdinov, 1995; Vallone et al., 2000). The D1 and D5 receptors, members of the D1-like family, share 80% amino acid sequence similarity and couple with the stimulatory GaS protein. The D2 receptor shares 75% sequence similarity with the D3 receptor and only 53% sequence similarity with the D4 receptor. Receptors in the D2-like family couple with the inhibitory GaI protein (reviewed in Missale et al., 1998; Vallone et al., 2000; Beaulieu and Gainetdinov, 2011).

1.4.1 The Dopamine Receptor 2 (D2)

The dopamine D2 receptor is encoded by the DRD2 gene located on chromosome 11q22-23 (Grandy et al., 1989). Alternative splicing of an 87 bp segment within exon 6, between introns 4 and 5, yield two splice variants including the short D2S receptor isoform and the long D2L receptor isoform (Monsma et al., 1989; Dal Toso et al., 1989; Giros et al., 1989). The D2L receptor is characterized by the inclusion of 29 amino acids in the third intracellular loop, which is absent in D2S receptor (Monsma et al., 1989; Dal Toso et al., 1989; Giros et al., 1989). These variants of D2 receptors have a distinct expression, physiological and signaling properties (Guiramand et al., 1995; Khan et al., 1998; Usiello et al., 2000; Beaulieu et al., 2005; Girault and Greengard, 2004; De Mei et al., 2009; Beaulieu and Gainetdinov, 2011).

D2 receptors are highly expressed in the brain and the periphery. In the CNS, highest levels of D2 receptors are found in the striatum, olfactory tubercle, and nucleus accumbens. D2 receptors are also expressed in the ventral tegmental area, substantia nigra, prefrontal cortex, hypothalamus, amygdala and hippocampus (reviewed in Missale et al., 1998; Vallone et al., 2000; Beaulieu and Gainetdinov, 2011). In the CNS, the D2 receptors control a variety of physiological functions. In the striatum, these receptors
have been implicated in regulating locomotor activity (Khan et al., 1998; Kelly et al., 1998; Schindler and Carmona, 2002). Additionally, the D<sub>2</sub> receptor has also been implicated in reward and motivation (Di Chiara and Bassareo, 2007; Koob and Volkow, 2010; Soares-Cunha et al., 2016), learning and memory (Miller and Marshall, 2005; Hyman et al., 2006), as well as cognitive functions (Sawaguchi and Goldman-Rakic, 1994; Takahashi et al., 2008).

Agonist binding to the D<sub>2</sub> receptor results in G<sub>α</sub><sub>i</sub>-dependent activation leading to inhibition adenylyl cyclase activity, causing an overall decrease in the levels of cAMP. Moreover, the D<sub>2</sub> receptor also increases outward potassium currents, leading to cell hyperpolarization through a mechanism including G<sub>βγ</sub> subunits of the G protein (Missale et al., 1998; Neve et al., 2004). The D<sub>2</sub> receptor also signals via β-arrestins, both β-arrestin1 (Kim et al., 2001) and β-arrestin2 (Masri et al., 2008; Huang et al., 2013) to facilitate receptor internalization and G protein-independent ERK phosphorylation.

Similar to CB<sub>1</sub>, the D<sub>2</sub> receptor can form both homo- and hetero-oligomers. Homomerization of the D<sub>2L</sub> receptor was proposed following the observation of high molecular weight bands on SDS-PAGE using rat and human brain striatal membranes following photoaffinity labeling. D<sub>2L</sub> homodimer, trimer, tetramers, and pentamers were all detected, suggesting that D<sub>2</sub> receptors can form both dimer and higher order homomeric complexes (Zawarynski et al., 1998; Armstrong and Strange, 2001; O'Dowd et al., 2005; George et al., 2014). Homomerization of the D<sub>2L</sub> receptors has also been confirmed using BRET, FRET and co-immunoprecipitation (Lee et al., 2000; Wurch et al., 2001; Gazi et al., 2003; Bagher et al., 2016). Homomerization of the D<sub>2L</sub> receptor results in negative cooperativity, whereby ligand binding at one D<sub>2L</sub> receptor, decreases affinity for further ligand binding to another D<sub>2L</sub> receptor within the oligomeric complex (Armstrong and Strange, 2001; Han et al., 2009). The D<sub>2L</sub> receptor has also been shown to hetero-oligomerize with other class A GPCRs including the D<sub>1</sub> receptor (Lee et al., 2004; Rashid et al., 2007; Hasbi et al., 2009), A<sub>2A</sub> receptor (Ferré et al., 1992; reviewed in Ferré et al., 2014, 2015b; Casadó-Anguera et al., 2016), and ghrelin GHSR<sub>1α</sub> receptor (Kern et al., 2012).

D<sub>2</sub> receptors have been implicated in the etiology of several neurological and neuropsychiatric disorders and drugs acting on these receptors are used to treat several
diseases (reviewed in Noble, 2003; Tost et al., 2010; Rangel-Barajas et al., 2015). Pharmaceutical agents include dopamine agonists, such as pramipexole, ropinirole and retigabine, are used clinically to treat symptoms of Parkinson’s disease (reviewed in Brooks, 2000; Stowe et al., 2008; Tomlinson et al., 2010; Stocchi et al., 2016). Clinically, pharmaceutical agents that block the dopamine receptors are used to treat schizophrenia, bipolar disorder, major depression, Huntington’s disease, and Tourette’s syndrome (Seeman, 2010; Eddy and Rickards, 2011; Frank, 2014). Antipsychotics are classified as “typical” (also known as first-generation) antipsychotics or “atypical” (also known as second-generation) antipsychotics, based on their relative affinity for the different receptors (reviewed in Gerlach, 1991; Kapur and Mamo, 2003; Meltzer, 2013; Murray et al., 2017). Antipsychotic drugs mediate their therapeutic actions by blocking the central mesolimbic and mesocortical dopaminergic pathways. Typical antipsychotics have a high affinity for the D2 receptors. The antagonism of D2 in the nigrostriatal pathway is responsible for the extrapyramidal side effect, akathisia, dystonia, and tardive dyskinesia produced by these drugs. In addition to blocking D2 receptors, these drugs also have various affinities for other receptor types such as 5HT2A- serotonergic, α1-adrenergic, M1,2,3-muscarinic and H1-histaminic receptors. Typical antipsychotic drugs include reserpine, chlorpromazine, thioridazine, and haloperidol. Atypical antipsychotics are as potent in inhibiting serotonin 5HT2A receptors as they are in inhibiting dopamine D2 receptors. Examples of atypical antipsychotics include risperidone, clozapine, olanzapine, quetiapine, sertindole and aripiprazole (reviewed in Gerlach, 1991; Kapur and Mamo D, 2003; Meltzer, 2013; Murray et al., 2017). This group of antipsychotics has a lower risk of extrapyramidal side effects but is associated with a higher incidence of metabolic abnormalities including dyslipidemia, metabolic syndrome and weight gain (reviewed in Tschoner et al., 2007; Ücok and Gaebel, 2008; Leung et al., 2012; Scigliano and Ronchetti, 2013).
1.5 Interactions Between the Endocannabinoid System (ECS) and the Dopaminergic System (DS) in the Basal Ganglia

Dopamine is the key neurotransmitter in the basal ganglia that plays a role in the regulation of movement (reviewed in Smith and Villalba, 2008; Nelson and Kreitzer, 2014). The dorsal striatum receives dopamine from the pars compacta of the substantia nigra through the nigrostriatal dopaminergic pathway (Fig. 1.5). This dopaminergic pathway regulates voluntary movement as part of the basal ganglia motor loop (reviewed in Missale et al., 1998; Beaulieu and Gainetdinov, 1995; Vallone et al., 2000). The globus pallidus also receives dopaminergic projections from the para compacta of the substantia nigra (Mamad et al., 2015; Robison et al., 2015). In the basal ganglia, both dopamine D₁ and D₂ are expressed, whereas D₂L receptor is the predominant dopaminergic receptor in the basal ganglia. Specifically, the D₂L receptor is expressed postsynaptically on dendritic spines of GABAergic medium spiny neurons (MSNs) projecting from the striatum to the external segments of the globus pallidus (indirect pathway), and on the terminals of these neurons in the globus pallidus. The D₂S receptor is expressed presynaptically on dopaminergic terminals, and glutamatergic afferents to the striatum. The D₁ receptor is expressed in the GABAergic MSNs projecting from the striatum to the internal segments of the globus pallidus (direct pathway) (Monsma et al., 1989; Giros et al., 1998; Levey et al., 1993; Khan et al., 1998; Gerfen, 2000; Usiello et al., 2000; Shuen et al., 2008).

Activation of the dopaminergic transition in the basal ganglia is associated with an increase in movement; however blocking dopaminergic receptors in the globus pallidus (Hauber and Lutz, 1999; Mamad et al., 2015) or dopamine depletion is associated by hypokinesia (Lorenc-Koci et al., 1995; Abedi et al., 2013). Alternation in the function of the dopaminergic system (DS) in the basal ganglia has been implicated in the pathophysiology of several basal ganglia disorder including Parkinson’s disease, Huntington’s disease (HD) and schizophrenia (reviewed in Mehler-Wex et al., 2006; Cepeda et al., 2014; García et al., 2016).

Endocannabinoid ligands and CB₁ receptors are highly expressed in the basal ganglia (e.g. striatum, globus pallidus and substantia nigra). Specifically, the CB₁ receptor is located presynaptically on terminals of GABAergic interneurons, and also on the glutamatereic afferents to the striatum but not in dopaminergic terminal (Fig. 1.5).
Figure 1.5: Distribution of CB$_1$ and D$_{2L}$ Receptors in the Basal Ganglia. (A) A simplified diagram of basal ganglia circuits. GABAergic inhibitory pathways are presented in red and glutamatergic excitatory pathways are presented in green. The modulatory dopaminergic nigrostriatal pathway is indicated in blue. GABAergic medium spiny neurons (MSNs) of the indirect movement pathway projecting from the striatum to the external globus pallidus (GPe) are highlighted in red. (B) An Enlarged view of the boxes present in part A. CB$_1$ and D$_{2L}$ receptors are co-localized postsynaptically on the dendritic spine of GABAergic MSNs projecting from the striatum to the GPe (right box), as well as being co-localized presynaptically on the axon terminal of the same neurons in the GPe (left box). In addition, CB$_1$ is expressed presynaptically on terminals of glutaminergic cortical and on GABAergic interneurons. The D$_{2s}$ receptors, but not CB$_1$ receptors, are expressed presynaptically on nigrostriatal dopaminergic neurons and on terminals of glutaminergic cortical neurons. Figure modified from Ferré et al., 2009.
(Herkenham *et al*., 1991; Tsou *et al*., 1998; Köfalvi *et al*., 2005; Pickel *et al*., 2006; Uchigashima *et al*., 2007). In addition, the CB₁ receptor is also located postsynaptically on somatodendritic of GABAergic MSNs of both the direct and indirect pathways (Rodriguez *et al*., 2001; Pickel *et al*., 2004, 2006), and is highly expressed in the terminals of these neurons in the globus pallidus (Herkenham *et al*., 1991; Julian *et al*., 2003; Martín *et al*., 2008). The ECS contributes to the regulation of movement (reviewed in Fernández-Ruiz and Gonzáles 2005; Fernández-Ruiz *et al*., 2009; Kluger *et al*., 2015). Administration of exogenous cannabinoids results in dose-dependent modulation of motor activities where very low doses of cannabinoids produce stimulatory effects, while high doses of cannabinoids cause dose-dependent motor depression and even catalepsy (reviewed in Fernández-Ruiz and Gonzáles, 2005; Fernández-Ruiz *et al*., 2010; García *et al*., 2016; Bloomfield *et al*., 2016). Additionally, cannabinoids were reported to counteract the motor effect of dopamine receptor activation (Aulakh *et al*., 1980; Moss *et al*., 1981; Anderson *et al*., 1996; Giuffrida *et al*., 1999; Andersson *et al*., 2005; Marcelino *et al*., 2008). For example, a single low-dose of the cannabinoid agonist CP 55940, which did not affect locomotor activity when administered alone, reduced quinpirole-induced hyperactivity (Marcellino *et al*., 2008). In contrast, the administration of the CB₁ antagonist SR141716A enhanced the stimulation of motor behavior elicited by administration of D₂ agonist quinpirole, confirming the important role of the CB₁ receptor in the control of movement (Giuffrida *et al*., 1999; reviewed in Fernández-Ruiz and Gonzáles, 2005; Fernández-Ruiz *et al*., 2010; García *et al*., 2016; Bloomfield *et al*., 2016).

Several mechanisms have been proposed to explain the interactions between ECS and DS in the basal ganglia involving the modulation of movement (Fig. 1.6). Classically, the effects of cannabinoids on movement were believed to be mediated indirectly by modulating the release of dopamine in the basal ganglia (reviewed in García *et al*., 2016). CB₁ is expressed presynaptically on the GABAergic interneurons and glutamatergic neurons, located in close proximity to the dopaminergic neurons in the striatum. Activation of CB₁ receptors by cannabinoid agonists acts as a retrograde feedback on presynaptic glutamatergic and GABAergic nerve terminals, modulating dopamine release (reviewed in Fernández-Ruiz and Gonzáles, 2005; Fernández-Ruiz *et al*., 2010; García *et al*., 2016; Bloomfield *et al*., 2016).
Figure 1.6: Different Mechanisms Proposed to Explain the Interactions Between the ECS and DS in the Basal Ganglia. The ECS plays a modulatory role in the control of dopaminergic neurotransmission in the basal ganglia. This influence is indirect and exerted through the actions of endocannabinoids on the presynaptic CB₁ receptor to modulate GABA and glutamate inputs received by dopaminergic neurons. Additionally, there is evidence that certain eicosanoid-related cannabinoids may directly activate the transient receptor potential vanilloid type 1 (TRPV1) receptors, which are expressed in nigrostriatal dopaminergic neurons, allowing a direct regulation of dopamine transmission. In addition, CB₁ and D₂L receptors are co-localized postsynaptically on the dendritic spine of GABAergic MSNs projecting from the striatum to the external globus pallidus as well as the axon terminal of the same neurons. It has been proposed that heteromerization between CB₁ and D₂L receptors provides another mechanism to facilitate direct interactions between the two systems. Through these direct and indirect mechanisms, cannabinoids may interact with the dopaminergic transmission in the basal ganglia and play a role in the control of movement. Figure 1.6 was modified from García et al., 2015.
al., 2010; García et al., 2016; Bloomfield et al., 2016). However, accumulating evidence suggests that other cannabinoid receptor(s) and/or mechanisms might be involved in the interaction between the ECS and DA at the level of the basal ganglia. For example, several researchers have reported that the transient receptor potential cation channel family V member 1 (TRPV1) receptors are expressed in the nigrostriatal dopaminergic neurons (Fig. 1.6) (Mezey et al., 2000) and the activation of this receptor can directly modulate dopamine release in the striatum (Marinelli et al., 2003, 2007; Ferreira et al., 2009). Specifically, the activation of TRPV1 receptors with either capsaicin or with other vanilloid agonists produced hypokinesia in rats (Di Marzo et al., 2001). Similarly, the endocannabinoid AEA produced the same behavioral effects (hypokinesia) accompanied by a reduction in the activity of dopaminergic neurons in the striatum; these effects were partially reversed by co-administration of the vanilloid-like receptor antagonist capsazepine. Thus indicating that these effects might also be mediated through the TRPV1, not only the CB1 receptor following AEA application (de Lago et al., 2004; reviewed in García et al., 2016; Bloomfield et al., 2016).

Another proposed mechanism that might facilitate the interaction between the ECS and the DS in the basal ganglia is through the formation of CB1 and D2L receptor heteromers in the basal ganglia (Fig. 1.6). CB1 and D2L receptors are co-localized postsynaptically on the dendritic spine of GABAergic MSN projecting from the striatum to the globus pallidus as well as the axon terminal of the same neurons in the external globus pallidus (Fig. 1.5) (Maneuf and Brotchie 1997; Pickel et al., 2006). The fact that CB1 and D2L receptors are co-localized suggests that they could form functional heteromers. The formation of CB1/D2L heteromers would allow for bi-directional interactions between the ECS and DS at the level of GPCR and G protein function (Giuffrida et al., 1999; Meschler and Howlett, 2001; Julian et al., 2003; Martín et al., 2008; Nguyen et al., 2012). Even before heteromerization between these two receptors had been demonstrated in vitro, it was observed that co-stimulation of CB1 and D2 in striatal neurons leads to an accumulation of cAMP, while stimulation of either receptor alone leads to an inhibition of cAMP (Glass and Felder 1997). This response was suggested to be the result of switching CB1 coupling from Ga<sub>i</sub> to Ga<sub>s</sub> proteins following the co-activation of both CB1 and D2 receptors (Kearn et al., 2005). Subsequently, it was
found that co-expression of the D\textsubscript{2} receptor with CB\textsubscript{1} was sufficient to switch CB\textsubscript{1} coupling even in the absence of a D\textsubscript{2} agonist (Jarrahian \textit{et al.}, 2004). Finally, heteromerization between the two receptors was confirmed using co-immunoprecipitation, BRET, FRET, and multicolor BiFC (Kearn \textit{et al.}, 2005; Marcellino \textit{et al.}, 2008; Przybyla and Watts \textit{et al.}, 2010; Khan and Lee, 2014; Bagher \textit{et al.}, 2016). In fact, \textit{in vivo} heteromerization between the two receptors has been recently confirmed in the caudate-putamen of \textit{Macaca fascicularis} brain using \textit{in situ} PLA (Pinna \textit{et al.}, 2014; Bonaventura \textit{et al.}, 2014).

1.5.1 Clinical Relevance: Huntington’s Disease

Alteration in the expression and function of CB\textsubscript{1} and D\textsubscript{2L} receptors has been observed in Huntington’s disease (HD) (Blázquez \textit{et al.}, 2011; reviewed in Laprairie \textit{et al.}, 2015). HD is an inherited dominant negative disorder characterized by movement, psychological and cognitive impairments. Other symptoms include weight loss, metabolic dysfunction, muscle wasting and cardiac abnormalities (Newcombe, 1981; Roos \textit{et al.}, 1993; Foroud \textit{et al.}, 1999; Ross 2010, Roos and Tabrizi 2011; Labbadia and Morimoto, 2013). HD is caused by the expression of a single copy of huntingtin (Htt) with an expanded CAG repeat. Translation of the mutant allele yields the mutant Htt (mHtt) protein containing an expanded polyglutamine region near the amino terminus (Huntington’s Disease Collaborative Research Group, 1993). The N-terminus of mHtt undergoes protein cleavage and accumulates in the nucleus where it forms aggregates (Vonsatte \textit{et al.}, 1985; Luthi-Carter \textit{et al.}, 2002; Atwal \textit{et al.}, 2007; Hogel \textit{et al.}, 2012). mHtt interferes with a variety of cellular processes including excitotoxic stress, mitochondrial dysfunction, an abnormal inflammatory response in the CNS and the transcriptional dysregulation of a subset of genes (reviewed in Zuccato and Cattaneo, 2014; Sharma and Taliyan, 2015). One of the earliest signs of cellular dysfunction in HD brain is a decline in the expression of CB\textsubscript{1} receptors in the basal ganglia (Denovan-Wright and Robertson, 2000; Glass \textit{et al.}, 2000). A significant reduction in CB\textsubscript{1} receptor mRNA and protein were observed in the caudate nucleus, putamen and external segment of globus pallidus of post-mortem human HD brain tissue (Denovan-Wright and Robertson, 2000; Glass \textit{et al.}, 2000; reviewed in Sagredo \textit{et al.}, 2012). Studies using
positron emission tomography and autoradiography demonstrated reduced striatal D2 receptor density even in asymptomatic HD patients (Richfield et al., 1991; Weeks et al., 1996; van Oostrom et al., 2009). These observations indicate that cannabinoid and dopamine signaling is disrupted early in HD progress. Atrophy of the striatum is the hallmark of HD pathogenesis. GABAergic MSNs of the indirect movement pathway that project from the striatum to the globus pallidus are more susceptible to degradation in HD. The loss of GABAergic MSN is responsible for the development of the involuntary movements (chorea) observed in HD (reviewed in Zuccato and Cattaneo, 2014; Sharma and Taliyan, 2015).

Currently, there is no cure for HD. Available therapies aim to reduce the severity of motor symptoms but do not alter disease progression (reviewed in Ross and Tabrizi, 2010; Carroll et al., 2015; Polo et al., 2015; Mason and Barker, 2016; Wyant et al., 2017). Tetrabenazine and deutetrabenazine are the Food and Drug Administration (FDA) approved drugs to control chorea in HD (Hayden et al., 2009; Frank et al., 2014). However, patients who cannot tolerate the side effects of tetrabenazine are prescribed typical or atypical antipsychotic drugs. These drugs are also used in HD patients to control psychosis, delusions, agitation and hallucinations (reviewed in Ross and Tabrizi, 2010; Carroll et al., 2015; Polo et al., 2015; Mason and Barker, 2016; Wyant et al., 2017). Increasing evidence suggests that cannabinoid-based therapies might aid in reducing involuntary movement due to their anti-hyperkinetic properties and may also help in slowing the progression of HD due to their neuroprotective, anti-inflammatory and antioxidant profiles (Blázquez et al., 2011, 2015; Mievis et al., 2011; reviewed in Sagredo et al., 2012; Chiarlone et al., 2014; Naydenov et al., 2014; Laprairie et al., 2016). The effects of co-administration of cannabinoids on dopamine antagonist effects are still unknown. Preclinical studies suggest that co-administration of cannabinoids and D2 antagonist might have different outcomes than administrating either compound alone. Therefore, a better understanding of the allosteric interaction between the CB1 and D2L receptors is directly applicable to the current treatments for HD and the design of therapies for HD.
1.6 Research Objectives

Increasing functional, biochemical and pharmacological evidence suggests that CB₁ and D₂ receptors can form heteromers that have distinct functional properties compared to homomers of either parent receptor. Given that allosteric interactions within hetero-oligomeric complexes result in a unique pharmacology, there is a need to better understand the allosteric interactions within CB₁/D₂L heteromeric and the stoichiometry of CB₁/D₂L/G protein complexes. *In vivo*, CB₁ and D₂L receptors are co-localized in the GABAergic MSNs projecting from the striatum to the globus pallidus, as well as on the axon terminals at the globus pallidus where they play important roles in the coordination of movement. Given the interaction between CB₁ and D₂ receptors, we hypothesized that co-localization of CB₁ and D₂L receptors in the basal ganglia allows for bidirectional allosteric interactions between CB₁ and D₂L ligands within CB₁/D₂L heteromers, which may be physiologically and clinically significant. Therefore, in the present work, I address these issues with three primary research objectives:

1- **Understand the stoichiometry of CB₁/D₂L/G protein complexes.**

2- **Examine the effect of D₂ ligands (agonist and antagonists) on CB₁ pharmacology, and examine the effect of CB₁ agonists on D₂L pharmacology within the CB₁/D₂L heteromers in both heterologous expression system and in cell model endogenously expressing both receptors.**

3- **Examine the effects of chronic cannabinoid and/or antipsychotic treatment on locomotion activity and on CB₁/D₂ heteromer expression in the globus pallidus of C57BL/6J mice.**
CHAPTER 2
MATERIALS AND METHODS

2.1 Generation of DNA Constructs

All cDNA plasmid constructs used in this thesis are listed in Table 2.1. For BRET\textsuperscript{2} assays, the C-terminus of the D\textsubscript{2L} receptor (GenBank accession number: NM_000795) was tagged with green fluorescent protein 2 (GFP\textsuperscript{2}) using the pGFP\textsuperscript{2}-N3 plasmid to generate the D\textsubscript{2L}-GFP\textsuperscript{2} construct. D\textsubscript{2L} was also tagged at the C terminus with Renilla luciferase (Rluc) using the pRluc-N1 plasmid to generate the D\textsubscript{2L}-Rluc construct (PerkinElmer, Waltham, MA). The human D\textsubscript{2L}-pcDNA3.1 (+) plasmid was obtained from the Missouri University of Science and Technology cDNA Resource Center (Rolla, MO). The D\textsubscript{2L}-GFP\textsuperscript{2} and D\textsubscript{2L}-Rluc constructs were generated by amplifying the D\textsubscript{2L} from D\textsubscript{2L}-pcDNA3.1 (+) by PCR using the forward primer (FP) D\textsubscript{2L}-FP and the reverse primer (RP) D\textsubscript{2L}-RP (Tables 2.2 and 2.3). Briefly, to clone the D\textsubscript{2L} receptor into the pGFP\textsuperscript{2}-N3 and pRluc-N1 plasmids the D\textsubscript{2L} receptor was amplified without stop codon from the D\textsubscript{2L} pcDNA3.1 (+) plasmid by PCR utilizing a high-fidelity Pfu DNA polymerase (Thermo Fisher Scientific, ON, Canada) with the FP D\textsubscript{2L}-FP possessing an EcoR1 restriction site and the RP D\textsubscript{2L}-RP possessing a Kpn1 restriction site. PCR reactions contained 1 \(\mu\text{l}\) of 10 pg/\(\mu\text{l}\) D\textsubscript{2L}-pcDNA3.1 (+), 2 mM 10X Pfu buffer with MgSO4, 2 mM each deoxyribonucleoside triphosphate and 1 unit of Pfu DNA polymerase (Thermo Fisher Scientific). Reactions were subjected to an initial denaturation step at 95°C for 3 m, and then 30 cycles of denaturation at 95°C for 30 s, primer annealing at 58°C for 30 s and extension at 72°C for 2 min with a final extension at 72°C for 10 m. The PCR products were fractionated on a 1% agarose gel containing ethidium bromide and visualized with a UV transilluminator and Kodak EDAS 290 docking station. Bands of the expected size were extracted from the agarose gel using the GenElute\textsuperscript{TM} Gel Extraction Kit (Sigma-Aldrich, ON) and digested with FastDigest EcoRI and Kpn1 (Thermo Fisher Scientific) at 37°C for 15 min. The same restriction enzyme digestions were performed on the pGFP2-N3 and pRluc-N1 plasmids (PerkinElmer, Waltham, MA). The FastDigest enzymes were inactivated by heating for 5 min at 80°C. Fragments were ligated into compatibly
Table 2.1: DNA Constructs Used in This Thesis.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Genbank accession number</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB₁-pcDNA</td>
<td>NM_016083</td>
<td>Untagged CB₁ receptor cloned into pcDNA3.1 Zeo (+) (Invitrogen).</td>
<td>Construct was cloned by AMB.</td>
</tr>
<tr>
<td>CB₁-GFP²</td>
<td>NM_016083</td>
<td>CB₁ receptor C-terminally tagged with GFP² cloned into GFP²-N3 (PerkinElmer).</td>
<td>Construct was cloned by Dr. Brian Hudson (Hudson et al., 2010).</td>
</tr>
<tr>
<td>CB₁-Rluc</td>
<td>NM_016083</td>
<td>CB₁ receptor C-terminally tagged with Rluc cloned into Rluc-N1 (PerkinElmer).</td>
<td>Construct was cloned by Dr. Brian Hudson (Hudson et al., 2010).</td>
</tr>
<tr>
<td>CB₁-VC</td>
<td>NM_016083</td>
<td>CB₁ receptor C-terminally tagged with EYFP Venus C-terminal hemiprotein cloned into pBiFC-VC155 (Shyu et al., 2006).</td>
<td>Construct was cloned by AMB.</td>
</tr>
<tr>
<td>CB₁-VN</td>
<td>NM_016083</td>
<td>CB₁ receptor C-terminally tagged with EYFP Venus N-terminal hemiprotein cloned into pBiFC-VN173 (Shyu et al., 2006).</td>
<td>Construct was cloned by AMB.</td>
</tr>
<tr>
<td>CB₁-BP</td>
<td>NM_016083</td>
<td>CB₁ blocking peptide that inhibits the interaction between CB₁ and D₂L cloned into pcDNA3.1 Zeo (+) (Invitrogen).</td>
<td>Construct was cloned by AMB.</td>
</tr>
<tr>
<td>CB₁-Γ₁₁-BP</td>
<td>NM_016083</td>
<td>A blocking peptide that binds to the 3rd intracellular loops of CB₁ (amino acids 316-344) and blocks the interaction between CB₁ and Γ₁₁. The blocking peptide was cloned into pcDNA3.1 Zeo (+). (Invitrogen).</td>
<td>Construct was cloned by AMB.</td>
</tr>
<tr>
<td>D₂L-pcDNA</td>
<td>NM_000795</td>
<td>Untagged D₂L receptor cloned into pcDNA3.1 Zeo (+) (Invitrogen).</td>
<td>Construct was obtained from the Missouri University of Science and Technology cDNA Resource Center (Rolla, MO).</td>
</tr>
</tbody>
</table>
Table 2.1: DNA Constructs Used in This Thesis.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Genbank accession number</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2L-GFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>NM_000795</td>
<td>D&lt;sub&gt;2L&lt;/sub&gt; receptor C-terminally tagged with GFP&lt;sup&gt;2&lt;/sup&gt; cloned into GFP&lt;sup&gt;2&lt;/sup&gt;-N3 (PerkinElmer).</td>
<td>Construct was cloned by AMB.</td>
</tr>
<tr>
<td>D&lt;sub&gt;2L&lt;/sub&gt;-Rluc</td>
<td>NM_000795</td>
<td>D&lt;sub&gt;2L&lt;/sub&gt; receptor C-terminally tagged with Rluc cloned into Rluc-N1 (PerkinElmer).</td>
<td>Construct was cloned by AMB.</td>
</tr>
<tr>
<td>Gα&lt;sub&gt;i1&lt;/sub&gt;-Rluc</td>
<td>001256414</td>
<td>Rluc was inserted between nucleotide 273 and 274 of human Gα&lt;i&gt;i1&lt;/i&gt;. The recombinant Gα&lt;i&gt;i1&lt;/i&gt;-RLuc construct was cloned in pcDNA3.1 (+) (Invitrogen).</td>
<td>Construct was obtained from Dr. Denis Dupré (Ayoub et al., 2007).</td>
</tr>
<tr>
<td>Gα&lt;sub&gt;s&lt;/sub&gt;-Rluc</td>
<td>BC108315.1</td>
<td>Rluc was inserted between nucleotide 564 and 565 corresponding to the α-helical domain of the human Gα&lt;sub&gt;s&lt;/sub&gt;. The recombinant Gα&lt;sub&gt;s&lt;/sub&gt;-RLuc construct was cloned in pcDNA3.1 (+).</td>
<td>Construct was obtained from Dr. Denis Dupré (Ayoub et al., 2007).</td>
</tr>
<tr>
<td>Gβ&lt;sub&gt;1&lt;/sub&gt;pcDNA</td>
<td>NC_000001.11</td>
<td>Untagged Gβ&lt;sub&gt;1&lt;/sub&gt; cloned into pcDNA 3.1 (+) (Invitrogen).</td>
<td>Construct was obtained from Dr. Denis Dupré (Galés, 2005).</td>
</tr>
<tr>
<td>Gγ&lt;sub&gt;2&lt;/sub&gt;-pcDNA3.1</td>
<td>NM_031754</td>
<td>Untagged Gγ&lt;sub&gt;2&lt;/sub&gt; cloned into pcDNA 3.1 (+) (Invitrogen).</td>
<td>Construct was obtained from Dr. Denis Dupré (Galés et al., 2005).</td>
</tr>
<tr>
<td>HERG-GFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>NG_008916.1</td>
<td>HERG sequence was inserted into pGFP&lt;sup&gt;2&lt;/sup&gt;-N3 plasmid (PerkinElmer).</td>
<td>Plasmid was obtained from Dr. Terry Hébert (Dupré et al., 2007).</td>
</tr>
<tr>
<td>mGluR6-GFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>NC_000005.10</td>
<td>mGLuR6 sequence was inserted into the pGFP&lt;sup&gt;2&lt;/sup&gt;-N3 plasmid (PerkinElmer).</td>
<td>Construct was obtained from Dr. Robert Duvoisin.</td>
</tr>
<tr>
<td>β-arrestin1-GFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>NM_004041.4</td>
<td>β-arrestin1 C-terminally tagged with GFP&lt;sup&gt;2&lt;/sup&gt; cloned into pcDNA 3.1 (+) (Invitrogen).</td>
<td>Construct was cloned by AMB.</td>
</tr>
</tbody>
</table>
**Table 2.1: DNA Constructs Used in This Thesis.**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Genbank accession number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-arrestin1-Rluc</td>
<td>NM_004041.4</td>
<td>β-arrestin1 C-terminally tagged with Rluc cloned into pcDNA 3.1 (+) (Invitrogen). Construct was obtained from Dr. Denis Dupré (Ayoub et al., 2007).</td>
</tr>
<tr>
<td>β2AR-GFP²</td>
<td>NM_000024</td>
<td>β2AR C-terminally tagged with GFP² cloned into GFP²-N3 plasmid (PerkinElmer). Construct was cloned by Dr. Brian Hudson (Hudson et al., 2010).</td>
</tr>
<tr>
<td>β2AR-pcDNA</td>
<td>NM_000024</td>
<td>Untagged β2AR cloned into pcDNA3.1 Zeo (+) (Invitrogen). Construct was cloned by Dr. Brian Hudson (Hudson et al., 2010).</td>
</tr>
<tr>
<td>β2AR-VN</td>
<td>NM_000024</td>
<td>β2AR C-terminally tagged with EYFP Venus C-terminal hemiprotein cloned into pBiFC-VC155. Construct was cloned by Dr. Maha Hammad (Hammad and Dupré, 2010).</td>
</tr>
<tr>
<td>β2AR-VC</td>
<td>NM_000024</td>
<td>β2AR C-terminally tagged with EYFP Venus N-terminal hemiprotein cloned into pBiFC-VC155. Construct was cloned by Dr. Maha Hammad (Hammad and Dupré, 2010).</td>
</tr>
</tbody>
</table>
digested pGFP\textsuperscript{2}-N3 and pRluc-N1 plasmids using a T4 DNA ligase overnight at 4°C. The ligation mixture contained 100 ng of each PCR product, 1 μl ligase 10X buffer and 1 unit T4 DNA ligase in 10-μl reaction (Promega Fisher Scientific Ltd., Ottawa, CA). The ligation mix was then transformed into One Shot\textsuperscript{®} TOP10 Chemically Competent \textit{E. coli} (Thermo Fisher Scientific) and plated on agar plates containing either zeocin (25 μg/ml) or kanamycin (30 μg/ml) for selection of D\textsubscript{2L}-GFP\textsubscript{2} and D\textsubscript{2L}-Rluc constructs, respectively. Plates were incubated overnight at 37°C to allow individual colonies to form. Single colonies were isolated and allowed to grow overnight in 2 ml Luria-Bertani (LB) broth containing either zeocin (25 μg/ml) or kanamycin (30 μg/ml). Plasmids were extracted using a GenElute\textsuperscript{™} Plasma Miniprep Kit (Sigma-Aldrich, ON), and clones containing appropriate inserts were identified by restriction digestion of each individual DNA sample with \textit{EcoR1} and \textit{Kpn1} followed by gel electrophoresis. A clone containing appropriate sized insert was subjected to bidirectional sequencing using universal FP and RPs (Genewiz, NJ).

Similarly, the CB\textsubscript{1} receptor (GenBank accession number: NM_016083) was cloned such that either GFP\textsuperscript{2} or Rluc was expressed as fusion proteins on the intracellular C-terminus of each receptor using the pGFP\textsuperscript{2}-N3 and pRluc-N1 plasmids (PerkinElmer, Waltham, MA). Both the CB\textsubscript{1}-GFP\textsuperscript{2} and the CB\textsubscript{1}-Rluc constructs were cloned by Dr. Brian Hudson (Hudson \textit{et al.}, 2010b). To clone CB\textsubscript{1} cDNA into pcDNA3.1 Zeo (+) (Thermo Fisher Scientific), the CB\textsubscript{1} cDNA was amplified from CB\textsubscript{1}-Rluc by PCR using the following primers: CB\textsubscript{1}-FP and CB\textsubscript{1}-RP (Tables 2.2 and 2.3). The PCR products were inserted into the pcDNA3.1 Zeo (+) using \textit{BamH1} and \textit{Xho1} restriction sites to generate CB\textsubscript{1}-pcDNA. After transforming chemically competent \textit{E.coli}, colonies were selected on agar plates with 50 μg/ml carbenicillin. The CB\textsubscript{1} blocking peptide (CB\textsubscript{1}-BP), spanning amino acids 432-456 of the CB\textsubscript{1} sequence that inhibits the interaction between CB\textsubscript{1} and D\textsubscript{2L} receptors, was amplified from CB\textsubscript{1}-Rluc by PCR using the CB\textsubscript{1}-BP-FP and the CB1-BP-RP (Tables 2.2 and 2.3; Khan and Lee, 2014). The PCR products were cloned into the pcDNA3.1 Zeo (+) using \textit{BamH1} and \textit{Xho1} restriction sites. A blocking peptide that binds to the 3\textsuperscript{rd} intracellular loops of CB\textsubscript{1} (amino acids 316-344), and specifically blocks the interaction between CB\textsubscript{1} and Ga\textsubscript{a11} protein (CB\textsubscript{1}-Ga\textsubscript{a11}-BP), was also cloned (Mukhopadhyay and Howlett, 2001). The CB\textsubscript{1}- Ga\textsubscript{a11}-BP was amplified by PCR from
Table 2.2: Primer Sequences Used in RT-PCR and Cloning. Restriction sites are shown in bold.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence (5’ to 3’)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-arrestin-FP</td>
<td>ATATGCTAGCATGGGCGACAAAGGGGACCAG</td>
<td>Designed by AMB</td>
</tr>
<tr>
<td>β-arrestin-RF</td>
<td>ATATAAGCTTCTGTGTTTGAGCTGTGGAGAGCC</td>
<td>Designed by AMB</td>
</tr>
<tr>
<td>CB1-FP</td>
<td>GATGGATCCATGAAGTCGATCTAGAT</td>
<td>Designed by AMB</td>
</tr>
<tr>
<td>CB1-RP</td>
<td>GCCCTCGAGTCAGAGCCCTCGGCAGAGCG</td>
<td>Designed by AMB</td>
</tr>
<tr>
<td>CB1-BP-FP</td>
<td>GATGGATCCATGTGTAAGGCACTCGCGGCCCT</td>
<td>Khan and Lee, 2014</td>
</tr>
<tr>
<td>CB1-BP-RP</td>
<td>GCCCTCGAGTCATGAAGTCCCATGCTGTTATC</td>
<td>Khan and Lee, 2014</td>
</tr>
<tr>
<td>CB1-Gα11-BP-FP</td>
<td>GATGGATCCATGAAGGAGCATCATCAGCAC</td>
<td>Mukhopadhyay and Howlett, 2001</td>
</tr>
<tr>
<td>CB1-Gα11-BP-RP</td>
<td>GCCCTCGAGCTTTGCACTCTAATGTC</td>
<td>Mukhopadhyay and Howlett, 2001</td>
</tr>
<tr>
<td>CB1-VN173-FP</td>
<td>CCGGACGAATTCTATGAAGTCGATCCT</td>
<td>Designed by AMB</td>
</tr>
<tr>
<td>CB1-VN173-RP</td>
<td>ACATGGTACCATGCACAGAGCCCTCGGCAGAGC</td>
<td>Designed by AMB</td>
</tr>
<tr>
<td>CB1-VC155-FP</td>
<td>CCGGACGAATTGTCATGAAGTCGATCCTGTTGAGGCC</td>
<td>Designed by AMB</td>
</tr>
<tr>
<td>CB1-VC155-RP</td>
<td>ACATGGTACCCACAGAGGCCTCGGCAGAC</td>
<td>Designed by AMB</td>
</tr>
<tr>
<td>D2L-FP</td>
<td>CGACAAAACTTATAGTAGATCCACTGAATCTGTCC</td>
<td>Bagher et al., 2016</td>
</tr>
<tr>
<td>D2L-RP</td>
<td>TGACATGGATCCACGGCTGAGGATC</td>
<td>Bagher et al., 2016</td>
</tr>
<tr>
<td>mouse CB1-FP</td>
<td>GGGCAATTTCTCTTGTAGCA</td>
<td>Blázquez et al., 2011</td>
</tr>
<tr>
<td>mouse CB1-RP</td>
<td>GGCTAAGCTGAGAGAAA</td>
<td>Blázquez et al., 2011</td>
</tr>
<tr>
<td>mouse D2L-FP</td>
<td>TTCAGAGCCAACTCTAAGAGACACCA</td>
<td>Coronas et al., 1997</td>
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<tr>
<td>mouse D2L-RP</td>
<td>GCTTTCTGCGGTCTCATCTCATTTA</td>
<td>Coronas et al., 1997</td>
</tr>
<tr>
<td>mouse D2-FP</td>
<td>CTGGAGAGCCAGAACCCTGGAGGAGA</td>
<td>Ikegami et al., 2014</td>
</tr>
<tr>
<td>mouse D2-RP</td>
<td>TAGACGACCAGGCCTAGGAAAC</td>
<td>Ikegami et al., 2014</td>
</tr>
</tbody>
</table>
CB₁-Rluc using the following primers: CB₁-\( \text{G}_{\alpha_{i1}} \)-BP-FP and CB₁-\( \text{G}_{\alpha_{i1}} \)-BP-RP (Table 2.2). The PCR products were cloned into the pcDNA3.1 Zeo (+) using \( \text{BamHI} \) and \( \text{XhoI} \) restriction sites.

The C-terminus fusion constructs of the \( \beta_2 \text{AR} \) with GFP\(^2 \), \( \beta_2 \text{AR-GFP}\(^2 \), and the membrane protein human \text{ether-a-go-go}-related gene (HERG), HERG-GFP\(^2 \), were provided by Dr. Terry Hébert (McGill University, Montreal, CA). These constructs were used as controls as specified (Dupré \textit{et al}., 2007, Hudson \textit{et al}., 2010b). The carboxy-terminus GFP\(^2 \) construct of the human metabotropic glutamate receptor 6 (mGLuR6)-GFP\(^2 \) was obtained from Dr. Robert Duvoisin of the Oregon Health and Science University, Portland, OR, and was generated by the insertion of the mGLuR6 sequence into the pGFP2-N3 plasmid (Hudson \textit{et al}., 2010). Plasmids encoding \( \text{G}_{\alpha_{i1}} \)-Rluc, \( \text{G}_{\alpha_s} \)-Rluc, \( \text{G}_{\beta_1} \)-pcDNA3.1 (+) and \( \text{G}_{\alpha_{i1}} \)-pcDNA3.1 (+) were provided by Dr. Denis Dupré (Dalhousie University, Halifax, CA) (Dupré DJ \textit{et al}., 2006). For the \( \text{G}_{\alpha_{i1}} \)-RLuc construct, the Rluc cDNA sequence (GenBank accession number: JQ606807.1) was inserted between nucleotide 273 and 274 of human \( \text{G}_{\alpha_{i1}} \) (GenBank accession number: NM_001256414), which corresponds to the loop connecting helices A and B of \( \text{G}_{\alpha_i} \). The recombinant \( \text{G}_{\alpha_{i1}} \)-RLuc construct was cloned in pcDNA3.1 (+), as previously described (Ayoub \textit{et al}., 2007). To generate \( \text{G}_{\alpha_s} \)-Rluc construct, Rluc was inserted between nucleotide 564 and 565 corresponding to the \( \alpha \)-helical domain of the human \( \text{G}_{\alpha_s} \) protein (GenBank accession number: BC108315.1) (Ayoub \textit{et al}., 2007). For the \( \beta \)-arrestin1-RLuc construct, Rluc was fused to the carboxyl terminus of \( \beta \)-arrestin1 (GenBank accession number: NM_004041.4) (Hamdan \textit{et al}., 2007). \( \beta \)-arrestin was also tagged at the C-terminus with GFP\(^2 \). The \( \beta \)-arrestin was PCR amplified from \( \beta \)-arrestin-Rluc without its stop codon using the \( \beta \)-arrestin-FP and \( \beta \)-arrestin-RF primers (Tables 2.2 2.3). The PCR products were cloned into \text{Nhel} \) and \text{HindIII} \) sites of pGFP\(^2 \)-N3 to generate \( \beta \)-arrestin-GFP\(^2 \) construct.

For SRET\(^2 \) assays combined with BiFC assays, CB₁ receptors were cloned into enhanced YFP (EYFP) Venus vector pBiFC-VN173 (Addgene plasmid # 22010) and pBiFC-VC155 (Addgene plasmid # 22011). The pBiFC-VN173 and pBiFC-VC155 vectors were gifts from Chang-Deng Hu (Shyu \textit{et al}., 2006). The following pairs of primers were used to amplify CB₁ from CB₁-Rluc to be cloned into pBiFC-VN173: CB₁-
<table>
<thead>
<tr>
<th>GenBank accession number</th>
<th>Primer description in table 2.2</th>
<th>Construct was cloned by AMB.</th>
<th>Tag</th>
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<tr>
<td>NM_004041.4 (225-1478)</td>
<td>β-arrestin-2-RF</td>
<td>C-terminal GFP-tag</td>
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</tr>
<tr>
<td>NM_016083</td>
<td>CB-pCDNA</td>
<td>Construct was cloned by AMB.</td>
<td></td>
</tr>
<tr>
<td>NM_016083</td>
<td>CB-BP</td>
<td>Construct was cloned by AMB.</td>
<td></td>
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<tr>
<td>NM_016083</td>
<td>CB-Guo-BP-RP</td>
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<td>CB-VN155-RP</td>
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<td>NM_0000795 (226-1566)</td>
<td>DB1-GFP</td>
<td>C-terminal GFP-tag</td>
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<tr>
<td>NM_016083</td>
<td>DB1-RFP</td>
<td>RLuc tag</td>
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<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Primers, Restriction Sites, and Vectors Used to Clone DNA Constructs.</th>
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</thead>
<tbody>
<tr>
<td>β-arrestin-2-RF</td>
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<td>CB-BP</td>
<td>pGFP-N3, pCMV vectors</td>
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<tr>
<td>CB-pCDNA</td>
<td>NM_016083</td>
<td>CB-BP</td>
<td>pcDNA3.1, zeo (+)</td>
</tr>
<tr>
<td>CB-BP</td>
<td>NM_016083</td>
<td>CB-BP</td>
<td>pcDNA3.1, zeo (+)</td>
</tr>
<tr>
<td>CB-Guo-BP-RP</td>
<td>CB-BP</td>
<td>CB-VN155-RP</td>
<td>pGFP-VC-NC55, zeo (+)</td>
</tr>
<tr>
<td>CB-VN155-RP</td>
<td>CB-BP</td>
<td>CB-VN155-RP</td>
<td>pGFP-VC-NC55, zeo (+)</td>
</tr>
<tr>
<td>DB1-GFP</td>
<td>NM_0000795</td>
<td>DB1-RFP</td>
<td>pGFP-N3, pCMV vectors</td>
</tr>
<tr>
<td>DB1-RFP</td>
<td>NM_0000795</td>
<td>DB1-RFP</td>
<td>RLuc tag</td>
</tr>
</tbody>
</table>

Table 2.3: Primers, Restriction Sites, and Vectors Used to Clone DNA Constructs.
VN173-FP and CB1-VN173-RP. While the following primers pairs were used to amplify CB1 to be cloned into pBiFC-VC155 plasmid: CB1-VC155-FP and CB1-VC155-RP (Tables 2.2 and 2.3). The PCR products were digested with EcoRI and KpnI before being inserted into either pBiFC-VN173 or pBiFC-VC155 to generate CB1-VN and CB1-VC, respectively. All constructs were sequenced to confirm their full cDNA sequence and reading-frame (Genewiz, NJ).

2.2 Material

The CB1 agonist Arachidonyl-2'-chloroethylamide (ACEA) (N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide), and CP 55,940 ((-)cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3 hydroxypropyl)cyclohexanol), and CB1-selective antagonist O-2050 (6aR,10aR)hydroxy-3-(1-Methanesulfonlamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran) were purchased from Tocris Bioscience (Bristol, UK). The D2 agonist quinpirole ((4aR,8aR)-5-propyl-4,4a,5,6,7,8,8a,9-octahydro-1H-pyrazolo[3,4-g]quinolone), and D2-antagonists haloperidol (4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one), sulpiride (N-{[(1-ethylpyrrolidin-2-yl)methyl]-2-methoxy-5-sulfamoylbenzamide), and olanzapine (2-Methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b][1,5] benzodiazepine), β2AR agonist isoprenaline ((RS)-4-[1-hydroxy-2-(isopropylamino)ethyl]benzene-1,2-diol), the mGLuR6 agonist L-AP4 ((2S)-2-amino-4-phosphonobutanoic acid), Pertussis toxin (PTx) and Cholera toxin (CTx) were obtained from Sigma-Aldrich. Drugs were dissolved in 100% ethanol as 10 mM stocks and the final vehicle concentration after dilution was 0.1% (v/v) in assay media. PTx and CTx were dissolved in dH2O (50 ng/mL) and added directly to the media 24 hr prior to drug treatment.

2.3 Cell Culture

The STHdhQ7/Q7 cell line was derived from conditionally immortalized striatal progenitor cells of embryonic day 14 C57BL/6J male mice (Coriell Institute, Camden, NJ) (Trettel et al., 2000; Paoletti et al., 2008). STHdhQ7/Q7 cells endogenously express CB1, D2L, D3 and D4 receptors (Lee et al., 2007). STHdhQ7/Q7 cells were cultured in tissue culture treated flasks (BD) at 33°C, 5% CO2 in Dulbecco’s Modified Eagle’s Medium
(DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 10 µg/ml streptomycin and 400 µg/ml Geneticin® (Thermo Fisher Scientific) (Trettel et al., 2000; Lee et al., 2007; Laprairie et al., 2013). At confluency, cells were subcultured at a 1:10 ratio. All experiments were carried out using cells between passages 3 and 15. STHdhQ7/Q7 cells normally exist in a dividing state. Serum deprivation causes STHdhQ7/Q7 cells to exit the cell cycle, increase neurite outgrowth and increase expression of DARPP-32 and D2L receptors (Trettel et al., 2000; Paoletti et al., 2008). The phenotype of serum-deprived STHdhQ7/Q7 cells resembles that of striatal MSNs (Paoletti et al., 2008; Blázquez et al., 2011). STHdhQ7/Q7 cells were maintained in serum-containing media. To stop cell division and promote neurite outgrowth, media were aspirated from cells, and the cells were rinsed once with 1X phosphate-buffered saline (PBS). Media lacking serum, but otherwise equivalent to STHdh media described above, was then added and cells were allowed to grow for an additional 24 h.

The Human Embryonic Kidney 293A (HEK 293A) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VI, USA). Cells were maintained in high glucose DMEM supplied with 10% (v/v) FBS, 100 U/ml penicillin and 10 µg/ml streptomycin. Cells were cultured at 37°C and 5% CO2. 96-well plate was coated with 0.01% (w/v) poly-D-lysine to provide an adherent substrate for growing cells.

2.4 Transfection

HEK 293A or STHdhQ7/Q7 cells were transfected using Lipofectamine® 2000 reagent (Thermo Fisher Scientific) following the manufacturer’s protocol. For BRET² experiments, cells were plated in 6-well plate (10 cm²/ml) with DMEM and 10% (v/v) FBS for 24-48 h, until cells reached 90% confluence. Each well of the 6-well plate received 400 µg of the required plasmid(s) diluted in 250 µl Opti-MEM® Reduced-Serum Medium (Thermo Fisher Scientific). The total amount of DNA/well was kept constant by using a pcDNA3.1+ empty vector as required. Plasmid DNA was mixed with 250 µl Opti-MEM® Reduced-Serum Medium containing 10 µl of Lipofectamine® 2000 reagent. The solution was then incubated at room temperature for 20 min before being added to a well of the 6-well plate containing fresh DMEM media without serum. Cells
were cultured for 48 h. The same method was used to transfect HEK 293A cells used for SRET² and BiFC assays. For confocal microscopy and Immunofluorescence assays 24-well plate was used, and for In- and On-Cell Western™ analysis 96-well plate was used (Nunc, Rochester, NY) (Table 2.4).

2.5 In-Cell Western™ Analysis

The In-Cell Western™ (ICW) cell-based assay is an immunofluorescences assay that enables the quantification of protein targets in fixed cells in a microplate well. ICW is a very powerful alternative tool to Western blot. ICW allows for quantitative, precise, and rapid detections of target proteins using a 96-or 348-well format (reviewed in Boveia and Schutz-Geschwander, 2015). For ICW the cells are permeabilized, which allow antibodies to reach cell surface and cytoplasmic antigens. ICW functional assays have been used to study the dose and time-dependent pharmacology of GPCR ligands, protein levels and post-transcriptional (phosphorylation) states of signaling proteins (Hudson et al., 2010b; Bagher et al., 2013; Laprairie et al., 2013, 2014, 2016). Levels of protein are normalized to the expression of a housekeeping gene (e.g. β-actin or β-tubulin). The ICW analysis was used to measure phosphorylation of the extracellular kinase 1 and 2 (ERK) and cyclic AMP response element binding protein (CREB). The ICW analysis was used to measure total CB₁ and D₂L immunoreactivity as an estimate of protein levels.

To carry out ICW, cells were plated on either poly-D-lysine-coated 96-well plate (HEK 293A cells) or normal 96-well plate (STHdhQ7/Q7) and cultured for 24-48 hr until confluency was reached. For STHdhQ7/Q7 cells, cell culture media was then replaced with 100 μl of serum-free DMEM and cells were maintained for 24 hr prior to experiments to allow cell differentiation. For HEK 293A, cell culture media was removed and replaced with 100 μl serum-free DMEM. HEK 293A cells were transfected with 200 ng of the required constructs and cells were cultured for 48 hr to allow for protein expression. To carry out ICW, cells were treated as indicated in each figure by the addition of 100 μl of serum-free DMEM containing 2X the desired final concentration of ligand(s) or vehicle. After the indicated agonist exposure time, the media was removed, and cells were fixed for 20 min with 4% (w/v) paraformaldehyde (PFA) in 0.1 M NaPO₄ buffer, pH 7.4. After
Table 2.4: DNA Transfection Protocol for Different Cell Culture Formats Using Lipofectamine® 2000 Reagent.

<table>
<thead>
<tr>
<th>Culture Vessel</th>
<th>Volume of plating medium</th>
<th>Volume of dilution medium</th>
<th>DNA</th>
<th>Lipofectamine® 2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-well</td>
<td>2 mL</td>
<td>2 X 250 μL</td>
<td>4.0 μg</td>
<td>5 μL</td>
</tr>
<tr>
<td>24-well</td>
<td>500 μL</td>
<td>2 X 50 μL</td>
<td>0.8 μg</td>
<td>1 μL</td>
</tr>
<tr>
<td>96-well</td>
<td>100 μL</td>
<td>2 X 50 μL</td>
<td>0.2 μg</td>
<td>0.25 μL</td>
</tr>
</tbody>
</table>
fixation, cells were washed three times with 1X PBS for 5 min each, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 1 hr at room temperature, and then washed three times with 1X PBS while gently shaken. Non-specific antigen binding to cells was blocked using Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE, USA) containing 0.1% (v/v) Tween-20 for 90 min at room temperature while gently shaken. Cells were then incubated overnight at 4°C with either rabbit anti-phospho ERK antibody (Tyr 204; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA, Cat. No: sc-7976) and rabbit anti-total ERK 2 antibody (C-14; Santa Cruz Biotechnology Inc, Cat. No: sc-154) or goat anti-pCREB-1 (Ser 133; Santa Cruz Biotechnology Inc, Cat. No: sc-7978) and rabbit anti-total CREB-1 (C-21; Santa Cruz Biotechnology Inc, Cat. No: sc-186) diluted 1:200 in 20% (v/v) Odyssey Blocking Buffer in 1X PBS.

To measure total CB1 and D2L protein levels following persistent ligand treatment the following primary antibodies were used: Mouse anti-β-Actin antibody (Sigma-Aldrich, ON) and monoclonal rabbit N-terminal CB1 antibody (1:500; Cayman Chemical Company, Ann Arbor, MI, USA) or mouse anti-β-Actin antibody (Sigma-Aldrich, ON) and primary monoclonal rabbit N-terminal-D2 antibody (1:200; Santa Cruz Biotechnology Inc) diluted in 20% (v/v) Odyssey Blocking Buffer in 1X PBS containing 0.1% (v/v) Tween-20. Next day, cells were washed three times with 1X PBS containing 0.1% (v/v) Tween-20 (PBST) for five min each while gently shaken. Cells were incubated for 1 hr with the near infrared (IR) fluorescently tagged secondary antibodies IR800CW-conjugated anti-rabbit IgG secondary antibody (Rockland Immunochemical, Gilbertsville, PA) and Alexa Fluor 680 anti-goat secondary antibody (Thermo Fisher Scientific) or Alexa Fluor 680 anti-mouse secondary antibody (Thermo Fisher Scientific) diluted 1:800 in the 20% (v/v) Odyssey Blocking Buffer in 1X PBS and cells were protected from light and gently shaken. Plates were washed three times with PBST, three times with PBS and once with ddH2O before being allowed to air-dry. Plates were scanned using the Odyssey infrared imaging system (Li-Cor Biotechnology), with intensity settings of 5 for both 700 nm and 800 nm channel and a focus offset of 5 mm.

To obtain relative phosphorylated ERK (pERK) or phosphorylated CREB (pCREB) values, the background fluorescence of channel 700 and 800 obtained from wells receiving only the secondary antibodies was subtracted from the fluorescence of
channel 700 and 800 obtained from the vehicle and drug-treated wells (Fig. 2.1). The ratios of pERK/total ERK (or pCREB/total CREB) was calculated by dividing background-subtracted fluorescence obtained from phosphorylated protein pERK to background-subtracted fluorescence obtained from total protein total ERK for each well. The ratio of the background-subtracted pERK/total ERK signals was then normalized to the ratios obtained from the wells treated with vehicle. To calculate changes in CB1 and D2L protein levels following persistent ligand treatment, the background fluorescence was determined from wells receiving only the secondary antibodies and the background was then subtracted from the total receptor expression fluoresces. The ratio of the background-subtracted total signal/total β-actin fluoresces was then determined for each well.

2.6 On-Cell Western™ Analysis

The On-Cell Western™ (OCW) cell-based assay is used to quantify target protein levels at the cell surface. In OCW, the cell membrane is not permeabilized; therefore, antibody access is restricted to antigens on the cell membrane. The relative signal determined by OCW to ICW signal has been used to study GPCR internalization after ligand treatment. Using antibodies that recognize the extracellular domains of GPCRs (e.g. N-terminal tail of CB1 or D2L), OCW can measure cell surface expression of GPCRs on intact cells following vehicle or ligand treatment (Miller et al., 2004; Hudson et al., 2010b, Laprairie et al., 2015). Following the detection of the cell surface GPCR, cells were permeabilized (using Triton X-1000), and ICW of total GPCR levels were determined. The ratio of GPCR expression on the cell membrane (OCW, non-permeabilized cell) and total GPCR expression (ICW, permeabilized cells) was measured at different times following drug exposure to determine the rate of GPCR internalization.

To measure cell surface expression of CB1 and D2L receptors following vehicle or ligand treatment, OCW analysis was employed using the protocol described previously by Miller et al. (2004). Cells were plated on either poly-D-lysine-coated 96-well plate (HEK 293A cells) or normal 96-well plate (STHdhQ7/Q7 cells) and cultured for 24-48 hr until cell confluency was observed. Following confluency of STHdhQ7/Q7 cells, cell culture media was replaced with 100 μl of serum-free DMEM and cells were maintained
Figure 2.1: In-Cell Western™ Analysis to Measure ERK Phosphorylation. pERK concentration-response curve measured by In-Cell Western™ from HEK 293A cells expressing CB1 receptors treated with increasing concentrations of WIN 55,212-2 for 5 min. (A) ERK phosphorylation was detected using pERK antibody (800 nm, green), while total ERK was detected using total ERK antibody (700 nm, red). Overlaid image (yellow, 800 and 700 nm) indicate pERK and total ERK signals. (B) The concentration-response curve of WIN55,212-2 with pERK signal normalized relative to total ERK. The concentration-response curve was fit to a nonlinear regression with variable slope (four-parameter) model. Figure 2.1 was modified from Bagher et al., 2017 (in press).
for 24 hr prior to experiments. For HEK 293A, culture media was removed and replaced with 100 μl serum-free DMEM and cells were transfected with 200 ng of the required constructs and cells were cultured for 48 hr prior to OCW.

To measure receptor internalization, cells were treated as indicated by the addition of 100 μl of serum-free DMEM containing 2 X the desired final concentration of ligand(s) or vehicle and cells were incubated for 5-60 min at 37°C in a cell culture incubator maintaining a 5% CO2. Cells were fixed with 4% (w/v) PFA for 20 min at room temperature and washed three times with PBS. Cells were blocked using Odyssey Blocking Buffer (Li-Cor Biotechnology) for 90 min at room temperature while gently shaken. Cells were incubated with primary monoclonal rabbit N-terminal CB1 antibody (1:1000; Cayman Chemical Company), and primary monoclonal mouse N-terminal-D2L antibody (1:200; Santa Cruz Biotechnology) diluted in 20% (v/v) Odyssey Blocking Buffer in 1X PBS overnight at 4°C. The following day, cells were washed three times with PBS while gently shaken, before being incubated with an anti-rabbit IR800CW-conjugated secondary antibody (Rockland Immunochemicals) and Alexa Flour 680-conjugated anti-mouse IgG secondary antibody (Invitrogen) diluted 1:800 in 20% (v/v) Odyssey Blocking Buffer in 1X PBS. Finally, cells were washed 5 times with PBS and once with ddH2O while gently shaken. The cell culture plates were scanned using an Odyssey infrared imaging system (Li-Cor Biotechnology) with intensity settings of 5 for both the 700 and 800 nm channels and a focus offset of 3 mm.

After imaging the cell surface expression of the receptors using the Odyssey, total receptor expression was determined. To do this, cells were permeabilized using 0.1% (v/v) Triton X-100 in PBS for 1 hr at room temperature and washed three times with PBST with gentle shaking. Cells were then exposed to primary anti-CB1 and anti-D2L antibodies, secondary antibodies and scanned following the same protocol described for on OWA. To obtain the percent of basal surface expression, the background fluorescence was determined from wells exposed to the secondary antibodies and the background was then subtracted from the surface and total receptor expression signals. The ratio of the background-subtracted surface/total signals was then determined for each well.
2.7 Bioluminescence Resonance Energy Transfer 2 (BRET$^2$)

Bioluminescence Resonance Energy Transfer 2 (BRET$^2$) was used to study protein-protein interactions including the ability of CB$_1$ and D$_{2L}$ receptors to form homo- and heteromers and the physical interaction between CB$_1$ or D$_{2L}$ receptors and G$\alpha_i$, G$\alpha_s$, or $\beta$-arrestin1 using previously described protocol (Ramsay et al., 2002; James et al., 2006; Bagher et al., 2013). In BRET$^2$, Rluc is used as the donor protein, while GFP$^2$ is used as the acceptor protein (Fig. 2.2). BRET$^2$ utilizes a unique Rluc substrate, coelenterazine 400 a, that emits light between 290-400 nm. If the Rluc molecule is in sufficiently close proximity (approximately 50-100 Å) to the GFP$^2$ molecule, then there will be a non-radiative resonance energy transfer to the GFP$^2$, which in turn will lead to its subsequent fluorescent emission at 505-508 nm (Fig. 2.2). The efficiency of energy transfer is dependent upon a number of factors including the relative distance between the donor and acceptor molecules, estimated to be less than 100 Å, and their relative orientation (Pfleger and Eidne, 2005).

To carry out BRET$^2$ experiments, HEK 293A cells or STHdh$^{Q7/Q7}$ cells were plated in 6-well plate and transfected with constructs as indicated in each figure. Forty-eight hours post-transfection, the BRET$^2$ experiment was conducted. Cells were washed twice with cold 1X PBS before being suspended in 90 µl of BRET buffer [1X PBS supplemented with glucose (1 mg/ml), benzamidine (10 mg/ml), leupeptin (5 mg/ml) and a trypsin inhibitor (5 mg/ml)] (James et al., 2006). Cells were dispensed into a white 96-well plate (PerkinElmer). The GFP$^2$ emission was measured using an FLx800 fluorescence plate reader (BioTek Instruments Inc., Winooski, VT) with excitation and emission filters of 485/20 and 510/20 nm respectively. To carry out BRET$^2$, cells were treated with 1 µl of either vehicle or ligand as described in the text and figure legends. Following the addition of 10 µl of 50 µM coelenterazine 400a substrate (Biotium,CA, USA), emissions of Rluc and GFP$^2$ were respectively measured at 405 nm and 510 nm using Luminoskan Ascent plate reader (Thermo Scientific, Waltham, MA), with the integration time set to 10 s and the photomultiplier tube voltage set to 1200 volts. The ratio of 510/405 nm was converted to BRET efficiency (BRET$_{Eff}$) by first determining the 510/405 ratio of each sample, subtracting the minimum 510/405 nm emission
Figure 2.2: Bioluminescence Resonance Energy Transfer 2 (BRET²). (A) GPCRs are tagged at their carboxy-termini with either Rluc or GFP². The left panel illustrates when the tagged GPCRs are not interacting. Following the addition of the Rluc substrate coelenterazine 400a it is oxidized by Rluc, causing Rluc to emit blue light at ~ 405 nm, but no energy is transferred to the acceptor GFP², and therefore no green light is emitted. The right panel illustrates the emission spectra for co-expressed Rluc and GFP² in the presence of coelenterazine 400a when the Rluc and GFP² are not in close proximity. When Rluc and GFP² are not in close proximity resonance energy transfer does not occur; resulting in a peak at 405 nm from Rluc emission. (B) When the tagged GPCRs are interacting, the oxidation of coelenterazine 400a by Rluc emits blue light, which is transferred to the acceptor GFP² when it is in close enough proximity to Rluc. This allows resonance energy transfer to occur, causing GFP² excitation, resulting in the emission of green light at ~ 510 nm. The right panel shows the emission spectra for co-expressed Rluc and GFP² in the presence of coelenterazine 400a when the Rluc and GFP² are sufficiently close to allow for resonance energy transfer to occur. This results in two peaks in the emission spectra; one at ~ 405 nm and one at ~ 510 nm. BRET² signals are measured as the ratio of the 510 nm to the 405 nm peaks.
A) No Protein-Protein Interaction

B) Protein-protein Interaction
obtained from cells expressing only a Rluc-N1 construct, then dividing by the maximum measurable 510/405 nm ratio obtained from cells expressing a GFP\(^2\)-Rluc fusion construct (PerkinElmer).

It is possible that the observed BRET\(^2\) signal may be the result of random collisions of the over-expressed receptors within the cell membrane (Pfleger and Eidne, 2005). BRET\(^2\) saturation assay can distinguish between specific and non-specific interaction (Pfleger and Eidne, 2005). In BRET\(^2\) saturation experiments, cells were transfected with fixed amounts of the BRET\(^2\) donor (Rluc-tagged receptor), together with increasing amounts of BRET acceptor (GFP\(^2\)-tagged receptor). BRET\(_{\text{Eff}}\) values were then plotted against the ratio of GFP\(^2\)/Rluc concentration or plotted against the ratio of GFP\(^2\) fluorescence (obtained by directly exciting GFP\(^2\)) and Rluc emission as described in specific figure legends. The resulting data were fit to a rectangular hyperbola curve using GraphPad version 6.0 (GraphPad Software Inc. San Diego, CA). If the interaction was specific, the curve was hyperbolic indicating a specific and saturable increase in BRET\(^2\) signal to reach a maximum saturated value (BRET\(_{\text{Max}}\)), where all donor molecules are interacting with acceptor molecules. However, non-specific interactions only resulted in a gradual linear increase in BRET\(_{\text{Eff}}\). Changes in BRET\(_{\text{Max}}\) values reflects the relative orientation, distance, and expression levels of both donor and acceptor molecules (Guan et al., 2009). An added benefit to the BRET\(^2\) saturation approach is that the amount of receptor required to achieve 50\% of BRET\(_{\text{Max}}\) signal could be defined as BRET\(_{50}\) values. The BRET\(_{50}\) estimates the affinity of donor and acceptor molecules. In BRET\(^2\) saturation curves that fit a hyperbolic form, B\(_{\text{Max}}\) and K\(_d\) determinations are the BRET\(_{\text{Max}}\) and BRET\(_{50}\) values, respectively (Pfleger and Eidne, 2005; Guan et al., 2009).

The oligomerization state of CB\(_1\) and D\(_{2L}\) homo- and heteromer was assessed by using a modified form of the Veatch and Stryer model (Vrecl et al., 2006; Drinovec et al., 2012). BRET\(^2\) values were fitted to the model curve obtained for simple oligomers with the correction for high-energy transfer efficiencies E (Vrecl et al., 2006; Drinovec et al., 2012):

\[
\frac{\text{BRET}}{\text{BRET}_{\text{Max}}} = 1 - \frac{1}{E + (1 - E) \left(1 + \frac{[A]}{[D]}\right)^N}
\]
Where [D] and [A] are donor and acceptor concentrations and \( N \) is the oligomerization state (\( N=1 \) for dimer, \( N=2 \) for trimer, \( N=3 \) tetramer). The transfer efficiency \( (E) \) was calculated from the emission spectra of donor and acceptor molecules obtained for coelenterazine 400a (Biotium, Hayward, CA, USA) and GFP\(^2\) fluorescence (Vrecl et al., 2006; Drinovec et al., 2012).

BRET\(^2\) experiments were also used to study the interaction between CB\(_1\) or D\(_{2L}\) receptors and Ga\(_1\)-Rluc or Ga\(_5\)-Rluc fusion proteins in the absence or presence of ligands. In these experiments, cells were plated in 6-well plate and transfected with the required constructs (Ga\(_1\)-Rluc or Ga\(_5\)-Rluc together with CB\(_1\)-GFP\(^2\) and/or D\(_{2L}\)-GFP\(^2\)) in addition to un-tagged G\(\beta_1\) and G\(\gamma_2\) in pcDNA3.1 (+). Forty-eight hour later, cells were collected from each well, washed and resuspended in 900 \( \mu l \) BRET buffer. The resuspended cells were dispensed into ten wells of a white 96-well plate (90 \( \mu l / well \)). For BRET\(^2\) kinetic analyses, the BRET\(^2\) substrate coelenterazine 400a (Biotium, Hayward, CA) was added at time 0 min and light emissions were measured every 25 s for 9 min. Haloperidol was added at 50 s, while vehicle or ACEA was added at 75 s following coelenterazine 400a (Biotium, Hayward, CA, USA) administration. Quinpirole and ACEA were co-applied together at 50 s following coelenterazine 400a (Biotium, Hayward, CA, USA) administration. For all BRET\(^2\) experiments, ligands were present throughout the assay and were not washed out.

2.8 Sequential Resonance Energy Transfer (SRET\(^2\)) Combined with Bimolecular Complementation (BiFC)

The Bimolecular Complementation (BiFC) assay can be used to study protein-protein interactions. BiFC relies on the interaction between two non-fluorescent proteins fragments of the enhanced yellow fluorescent protein (EYFP) Venus, resulting in fluorescence EYFP signals that can be qualified (Fig. 2.3) (Hu et al., 2002; Vidi et al., 2010). BiFC was used to confirm that CB\(_1\) receptors could physically associate to form homodimers when expressed in HEK 293A cells. CB\(_1\) cDNA was cloned into expression vectors producing a CB\(_1\) fused to the EYFP Venus N-terminal (VN) using the pBiFC-VN173 plasmid (CB\(_1\)-VN). Similarly, the CB\(_1\) cDNA was cloned to EYFP Venus C-terminal (VC) using pBiFC-VC155 plasmid to produce CB\(_1\) receptor fused to the EYFP
**Figure 2.3: Bimolecular Fluorescence Complementation (BiFC).** (A) GPCRs are tagged at their carboxy-termini with non-fluorescent proteins fragments of the enhanced yellow fluorescent protein (EYFP) Venus, the EYFP Venus N-terminal (VN) or EYFP Venus C-terminal (VC). The left panel illustrates tagged GPCRs that are not interacting. In this case, the two Venus fragments do not come into close proximity and there is no fluorescence. The right panel illustrates the emission spectra for co-expressed Venus-VN and Venus-VC tagged GPCRs when the Venus-VN and Venus-VC tagged are not in close proximity resulting in no detectable signal using an excitation filter of 515 nm and an emission filter of 530 nm. (B) The left panel illustrates tagged GPCRs interactions. As a result of the interaction, the two Venus fragments associate and refold allowing fluorescence to occur. The right panel illustrates the emission spectra for co-expressed Venus-VN and Venus-VC tagged GPCRs when the Venus-VN and Venus-VC are in close proximity allowing the two fragments associate, resulting in a detectable signal using an excitation filter of 515 nm and an emission filter of 530 nm.
Venus C-terminal using (CB1-VC) (Shyu et al., 2006). To conduct BiFC experiments, HEK 293A cells were plated in a 6-well plate and transfected with the required construct (i.e. cells were transfected with either CB1-VN or CB1-VC alone or in combination at 1:1 ratio). Forty-eight hours post-transfection, cells were washed twice with cold 1X PBS before being suspended in 90 µl of BRET buffer. Cells were dispensed into a white 96-well plate (PerkinElmer) and EYFP Venus fluorescence was measured using FLx800 fluorescence plate reader (BioTek Instruments Inc., Winooski, VT) with an excitation at 515 nm and an emission measured at 530 nm.

Sequential resonance energy transfer 2 (SRET 2) combines both BRET 2 and fluorescence resonance energy transfer (FRET) techniques, which allow identification of heteromers formed by three different proteins (Carriba et al., 2008; Navarro et al., 2013). In SRET 2, the oxidation of Rluc substrate by an Rluc fusion protein triggers acceptor excitation of GFP 2 fusion protein by BRET 2 and subsequent FRET to EYFP fusion protein (Fig. 2.4). SRET 2 combined with BiFC was used to test whether CB 1 and D 2L form heterotetramers according to previously described methods (Carriba et al., 2008; Navarro et al., 2013). In brief, HEK 293A cells were grown in 6-well plates and transiently transfected with different plasmids encoding fusion proteins (D 2L-Rluc, D2L-GFP 2, CB1-NV, and CB1-CV) as indicated for each experiment. Forty-eight hours later, transfected cells were washed twice with cold 1X PBS before being suspended in 360 µl of BRET buffer. The cell suspension was divided into four equal aliquots (90 µl). The first aliquot was used to measure GFP 2. The expression of GFP 2 protein was quantified by determining the fluorescence resulting from direct GFP 2. 90 µl of cell suspension was dispensed into a white 96-well plate and GFP 2 emission was measured using an FLx800 fluorescence plate reader (BioTek Instruments Inc., Winooski, VT) with excitation and mission filters at 485 nm and 510 nm, respectively. The expression of EYFP Venus (CB1-VN and CB1-VC) was qualified by determining the fluorescence resulting from EYFP Venus using a 515 nm excitation filter and a 530 nm emission filter. The second aliquot of cell suspension was used to measure Rluc protein expression. Rluc expression was quantified by determining the luminescence resulting from Rluc. Cells were distributed (90 µL) in a white 96-well plate and the luminescence was determined immediately after addition of 10 µl of 50 µM coelenterazine 400a (Biotium, Hayward, CA, USA) using a
**Figure 2.4:** Sequential Resonance Energy Transfer 2 (SRET^2) Combined with Bimolecular Fluorescence Complementation (BiFC). (A) GPCRs are tagged at their carboxy-termini with Rluc, GFP^{2} or Venus fragments (Venus-VN and Venus-VC). The left panel shows Rluc and GFP^{2} tagged GPCRs interacting. Thus, on the addition of the Rluc substrate, coelenterazine 400a, the oxidation of coelenterazine 400a by Rluc-tagged GPCRs triggers acceptor excitation of GFP^{2} tagged GPCRs by BRET^2. Since Venus-VN and Venus-VC tagged GPCRs interact together, but not with Rluc and GFP^{2} tagged GPCRs, no energy transfer occurs from GFP^{2} tagged GPCRs to Venus tagged GPCRs by FRET. In the right panel, emission spectra for co-expressed Rluc and GFP^{2} in the presence of coelenterazine 400a when the Rluc and GFP^{2} are in close proximity and resonance energy transfer can occur. There is only a peak at 405 nm and 510 nm. (B) The left panel shows Rluc, GFP^{2} or Venus tagged GPCRs interacting. In the left panel, as a result of this, on the addition of coelenterazine 400a, the oxidation of coelenterazine 400a by Rluc emits blue light and triggers the excitation of the acceptor GFP^{2} by BRET^2, which emits green light. Since Venus tagged GPCRs are now in close enough proximity to GFP^{2} tagged GPCRs, resonance energy transfer does occur to the acceptor Venus by FRET. In the right panel, emission spectra for co-expressed Rluc, GFP^{2} and Venus tagged GPCR^{2} in the presence of coelenterazine 400a, when the Rluc, GFP^{2} and Venus tagged GPCR^{2} are sufficiently close to allow resonance energy transfer to occur by BRET^2 and FRET. There will be three peaks at 405 nm resulting from Rluc emission, at 510 nm resulting from GFP^{2} emission and 530 nm resulting from Venus emission. Net SRET^2 signals are measured as the ratio of the 530 nm to the 405 nm peaks.
A) No Dimer-Dimer Interaction

B) Dimer-Dimer Interaction

[Diagram showing molecular interactions and light emission spectra for Rluc, GFP, and VNVC in different conditions.]
Luminoskan Ascent plate reader (Thermo Scientific, Waltham, MA) with detection filter 405 nm. The third aliquot of cell suspension was used to conduct SRET² combined with BiFC experiments. Suspended cells (90 µl) were dispensed into a white 96-well plate (Perkin-Elmer). The SRET² signals were detected immediately following the addition of 10 µl of 50 µM coelenterazine 400a (Biotium, Hayward, CA, USA) using Luminoskan Ascent plate reader (Thermo Scientific, Waltham, MA) with detection filters for 405 nm and wavelength 530 nm. Net SRET² was defined as [(530 nm emission)/(405 nm emission)] – correction factor. The correction factor is the value determined from 530 emission/400 emission for cells expressing only Rluc, GFP², or EYFP (Carriba et al., 2008; Navarro et al., 2013). To confirm the specificity of the interaction, SRET² saturation curves were generated by transfecting cells with a constant amount of protein-Rluc and protein-GFP² and increasing amounts of EYFP Venus constructs (CB₁-NV and CB₁-CV). From these saturation curves, SRET_max and SRET₅₀ values were determined, similar to BRET² assays (Carriba et al., 2008; Navarro et al., 2013).

2.9 Confocal Microscopy and Immunofluorescence

The co-localization of endogenous CB₁ and D₂L in STHdh⁰⁷/⁰⁷ cells was observed using confocal microscopy. STHdh⁰⁷/⁰⁷ cells were plated onto glass coverslips in a 24-well plate. At 50% confluence, cell culture media was then replaced with serum-free DMEM and cells were maintained for 24 hr prior to experiments. Cells were fixed with ice-cold 100% ethanol for 5 min. After washing the cells three times with 1X PBS, non-specific antibody binding was blocked by treating cells with 1% (w/v) bovine serum albumin (BSA) for 60 min at room temperature. Cells were incubated with primary monoclonal rabbit N-terminal CB₁ antibody (1:500; Cayman Chemical Company) and primary monoclonal mouse N-terminal-D₂ antibody (1:200; Santa Cruz Biotechnology) overnight at 4°C. The next day, the cells were washed three times with 1X PBS and incubated with a Cy3-conjugated anti-mouse immunoglobulin G (IgG) secondary antibody and Cy2-conjugated anti-rabbit secondary antibody (1:500, Jackson Immuno Research Laboratories, West Grove, PA) for 1 hr at room temperature, then washed 3 times with 1X PBS and once with H₂O. Finally, coverslips were mounted on microscopic slides (Fisher Scientific) using Fluorsave reagent® (Calbiochem, San Diego, CA).
Images of cells were acquired with a Nikon Eclipse E800 microscope attached to the D-Eclipse C1 confocal system (Nikon Canada Inc., Mississauga, ON). Cy3 was imaged by a 543 nm Helium-Neon laser (JDS Uniphase, Milpitas, CA), while Cy2 was imaged using a 488 nm air-cooled argon laser (Spectra-Physics Lasers Inc., Mountain View, CA). Images were taken using a 100X oil immersion objective.

2.10 RNA Extraction From Cell Culture

$\text{STHdh}^{Q7/Q7}$ cells were cultured in a 24-well plate to approximately 90% confluency. Cells were allowed to differentiate for 24 hr in serum-free DMEM. Trizol® reagent (Thermo Fisher Scientific, ON) was used to extract RNA from $\text{STHdh}^{Q7/Q7}$ cells following the manufacturer’s protocol. Briefly, the culture media was aspirated, and cells were washed once using 1X PBS. Next, 200 $\mu$L of Trizol® was added to each well and samples were mixed by pipetting. Samples were transferred to 1.5 ml microcentrifuge tube, vortexed and incubated on ice for 3 min. Forty $\mu$L of chloroform was then added to each tube, mixed well for 15 sec by shaking and samples were centrifuged at 12,000 x g for 20 min at 4°C. The aqueous phase was removed to a new microcentrifuge tube. To precipitate RNAs, 100 $\mu$L of isopropanol was added, mixed well by inversion and tubes were placed on ice for 15 min before being centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the RNA pellet was washed twice using 200 $\mu$L ice-cold 75% (v/v) ethanol, vortexed and centrifuged at 7,500 x g for 5 min at 4°C. The RNA pellet was allowed to air dry for approximately 10 min before being suspended in 10 $\mu$L ddH$_2$O. The purity and concentration of the collected RNA were determined by measuring the A260/280 ratio of the samples using a spectrophotometer. RNA samples were stored at -80°C.

2.11 Reverse Transcriptase Reaction

Using RNA isolated from $\text{STHdh}^{Q7/Q7}$ cells, first strand cDNA was generated using reverse transcriptase SuperScript® II (Thermo Fisher Scientific, ON) following the protocol supplied by the manufacturer in a 20 $\mu$l reaction volume. Briefly, 2 $\mu$g of total cellular RNA was added to the reverse transcriptase reaction containing 0.5$\mu$M deoxynucleoside triphosphate and 7.5 $\mu$M random primers (mostly hexamers; Invitrogen).
in dH2O to a final volume of 13 μl for +RT reactions, or 14 μl for –RT reactions. The reaction was vortexed, incubated at 65°C for 5 min then chilled on ice for 1 min. The following reagents were then added to the reaction: 20% First-Strand Buffer, 5% RNaseOUT®, 5 mM dithiothreitol, and 200 U SuperScript III® reverse transcriptase (Invitrogen) and the reaction was mixed by pipetting. The reaction was incubated for 1 hr at 50°C, followed by 15 min inactivation at 70°C. The reaction was diluted to a final volume of 40 μL in ddH2O and stored at -20°C.

2.12 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was used to test whether STHdh<sup>Q7/Q7</sup> cells express D<sub>2L</sub> or D<sub>2S</sub> mRNAs. PCR primers that span the alternatively spliced exon that distinguished the D<sub>2L</sub> and D<sub>2S</sub> isoforms were used to detect D<sub>2L</sub> or D<sub>2S</sub> sized variants (Coronas et al., 1997). Amplification using the FP mouse D<sub>2L</sub>-FP and the RP mouse D<sub>2L</sub>-RP (Table 2.2; Coronas et al., 1997) yields two bands of molecular sizes 397 and 310 bp representing D<sub>2L</sub> and D<sub>2S</sub> isoforms of the receptor, respectively. The mouse-CB<sub>1</sub>-FP and the mouse-CB<sub>1</sub>-RP (Table 2.2) were used to amplify CB<sub>1</sub> receptor (Table 2.3) (Blázquez et al., 2011). PCR reactions contained 1 μl cDNA produced from RT reaction, 2 mM of 10X <i>Pfu</i> buffer with MgSO<sub>4</sub> (final concentration of 2 mM), 2 mM each deoxyribonucleoside triphosphate and 1 unit of <i>Pfu</i> DNA polymerase II (Thermo Fisher Scientific, ON) in ddH<sub>2</sub>O to a final volume of 20 μL. These reactions were subjected to an initial denaturation step at 95°C for 3 min, and then 30 cycles of denaturation at 95°C for 30 s, primer annealing at 56 °C for 30 s and extension at 72°C for 1 min with a final extension at 72°C for 10 min. Products were fractionated on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and visualized with a UV transilluminator and Kodak EDAS 290 docking station.

2.13 LightCycler® SYBR Green qRT-PCR

Real-time Quantitative Polymerase Chain Reaction (qRT-PCR) was used to quantify CB<sub>1</sub> and D<sub>2</sub> cDNA expression in STHdh<sup>Q7/Q7</sup> cells using previously described protocol (Laprairie et al., 2013) using the LightCycler® system and software (version 3.0; Roche, Laval, QC). cDNA abundance was measured using SYBR Green (Roche, Laval,
QC), contained in the PCR buffer, which intercalates with double-stranded DNA and fluoresces green at 520 nm. Fluorescence was then quantified by the LightCycler® on a per-sample basis 46 during each round of PCR amplification of cDNA. The following CB1-specific primers were used in qRT-PCR reactions: mouse-CB1-FP and mouse-CB1-RP primers (Table 2.2) (Blázquez et al., 2011), while mouse-D2-FP and mouse-D2-RP were used for D2 (Table 2.2; Ikegami et al., 2014). qRT-PCR reactions were composed of 2 mM MgCl2, 0.5 μM each of FP and RP, 2 μl of LightCycler® FastStart Reaction Mix SYBR Green I [0.3 mM dNTP, 10% SYBR Green I dye, 1.2 U FastStart Taq DNA polymerase], and 1 μl cDNA to a final volume of 20 μl with ddH2O. The PCR program was: 95°C for 10 min, 50 cycles of 95°C 10 s, a primer-specific annealing temperature (Table 2.2) for 5 s, and 72°C for 10 s. Melting curve analysis of PCR products was performed immediately after the PCR program. The melting curve program was 95°C for 10 s, 60°C for 30 s, a ramp to 99°C at 0.20°C/s, and 40°C for 30 s. All qRT-PCR experiments included sample-matched –RT controls, a no-sample ddH2O control, and a standard control containing 1 μl of product-specific cDNA of known concentration in copies/μl. Expression data were quantified by comparing the crossing points (i.e. the cycle number during PCR amplification at which the amount of product measured began to increase at a logarithmic rate) of each sample to a product-specific standard curve generated by plotting the crossing points of known standards against their respective concentrations in copies/μl.

2.14 γ-Aminobutyric Acid (GABA) Assay
To qualify GABA levels in STHdhQ7/Q7 cells culture media, a sandwich enzyme-linked immunosorbent assay (ELISA) was used. ELISA was conducted according to manufacturer’s instructions (Novatein Biosciences, Boston, MA). In the GABA ELISA kit, the 96-well plate was pre-coated with a monoclonal antibody against Mouse GABA. In brief, STHdhQ7/Q7 cells were plated in 96-well plate and cultured until reached 90% confluence. Cell culture medium was then replaced with 100 μl of serum free DMEM and cells were maintained for 24 hr prior to experiments to allow cell differentiation. Twenty-four hours later, 100 μl/well of serum-free DMEM was added to the wells with cells exposed to specific drug treatment. Cells were incubated at 33°C, 5% CO₂ for 30 min or
20 hr; then cell media was collected for analysis of GABA concentration. Next, 50 μl of the collected cell media was added to each sample wells. For controlled defined amounts of GABA (standards) wells, 50 μl of the pre-diluted standards were added to each of the standard wells. GABA standard concentrations ranged from 0.5 μM to 16 μM. 100 μl of the horseradish peroxidase (HRP)-conjugated antibody was added to each well and the plate was mixed well. The plate was incubated for 1 hour at 37°C. Wells were washed five times with 100 μl wash solution for 5 min each to remove all unbound components. The plate was inverted and blotted dry by tapping the plate on absorbent paper towels. Next, 50 μl of Chromogen Solution A and 50 μl Chromogen Solution B were added to each well, sequentially, containing the HRP enzyme substrate tetramethylbenzidine (TMB). The plate was protected from light and incubated for 15 minutes at 37°C to allow the enzyme (HRP) and TMB substrate to react. The enzyme-substrate reaction was terminated by addition of 50 μl of a sulphuric acid stop solution to each well and mixed well. The optical density (O.D.) was measured at 450 nm using SynergyHT fluorescent/luminescent plate reader (BioTek Instruments Inc., Winooski, VT). Background O.D. was collected using a cell-free well and subtracted from each standard and sample reading. For each experiment, a GABA standard curve was created and used to calculate GABA concentration in each sample.

2.15 In situ Proximity ligation Assay (PLA)

In situ proximity ligation assay (PLA) allows for the detection and quantification of protein-protein interactions in intact cells (Fredriksson et al., 2002; Söderberg et al., 2006). In situ PLA involves the use of two secondary antibodies attached to oligonucleotides (PLA probes) that can be joined by ligation only if the antibodies have been brought in close proximity by their respective binding to proteins to form protein-protein complexes. The DNA ligation products that form are then used as a template for in situ PCR amplification for protein detection (Fig. 2.5) (Fredriksson et al., 2002; Söderberg et al., 2006, 2008).

In situ PLA was used to study the interaction between endogenous CB1 and D2L receptors in STHdhQ7/Q7 cells following ligand treatment. CB1/D2L molecular interactions were detected using the Duolink® In Situ Orange Starter Kit Mouse/Rabbit kit (Sigma-
For *in situ* PLA experiments, STHdh\(^{Q7/Q7}\) cells were cultured on glass coverslips (18 mm) on a 24-well plate for 24-48 hr until cells reached 50-60% confluency. Cells were then treated for 18 hr with vehicle or CB\(_1\) and/or D\(_2\) ligands. Eighteen hours later, the cell culture media was removed from each well, and cells were washed three times using 500 µl 1X PBS then fixed using 4% (w/v) PFA for 20 min at room temperature. After that, the cells were washed three times with 1X PBS and coverslips were transferred to a humidity chamber where background fluorescence was blocked using one drop of Duolink In Situ Blocking Solution (Sigma-Aldrich, ON) for 1 hr at 37°C. The blocking buffer was removed and cells were incubated with the primary rabbit N-terminal CB\(_1\) antibody (1:500; Cayman Chemical Company) or the primary monoclonal rabbit N-terminal-D\(_2\) antibody (1:200; Santa Cruz Biotechnology) diluted in Duolink *In Situ* Antibody Diluent (Sigma-Aldrich, ON) overnight at 4°C. The next day, the primary antibodies were removed and the coverslips were transferred to a 24-well plate. Cells were washed four times using 200 µl Duolink *In Situ* Wash Buffer A for 10 min each with gentle shaking. While the cells were being washed, the PLA probes, Duolink\(^{®}\) *In Situ* PLA\(^{®}\) Probe Anti-Rabbit PLUS and the Duolink\(^{®}\) *In Situ* PLA\(^{®}\) Probe Anti-Mouse MINUS, were diluted 1:5 in the in Duolink *In Situ* Antibody Diluent (Sigma-Aldrich, ON) and allowed to incubate for 20 min at room temperature. The coverslips were returned to the humidity chamber, and 30 µl of the diluted probe solution was added to each coverslip. The cells in the humidity chamber were incubated for 60 min at 37°C. Sixty minutes later, the PLA probes were removed, the coverslips were returned to the 24-well plate and cells were washed four times with Duolink *In Situ* Wash Buffer A for 10 min each while gently agitated. During the wash period, the ligation solution was prepared by diluting the 5X ligation stock (Sigma-Aldrich, ON) 1:5 in ddH\(_2\)O. Immediately before applying the ligation solution to the cells, the 1X ligase (1 U/µl; Sigma-Aldrich, ON) was added to the ligation mixture at a 1:40 dilution and the mixture was vortexed. The coverslips were returned to the humidity chamber and 30 µl of the ligation mixture was added to each coverslip. The coverslips were allowed to incubate for 60 min at 37 °C. After removing the ligation mixture, coverslips were placed in the 24-well plate and washed twice using Duolink In Situ Wash Buffer A for 2 min each while gently agitated. In a light protected area, the amplification solution was prepared by
diluting 5X Amplification Orange stock (Sigma-Aldrich, ON) 1:5 in ddH₂O. 1X

**Figure 2.5: In situ Proximity Ligation Assay (PLA).** (A) *In situ* PLA involves the use of two primary antibodies specific for two different GPCRs and two secondary antibodies conjugated to different oligonucleotides (PLA probes). When the two GPCRs are physically separated, the two PLA oligonucleotides cannot hybridize and undergo covalent ligation. As such no PLA signals were detected. (B) When the two GPCRs are in close proximity, the PLA probes will hybridize and ligate together forming a continuous circular DNA structures. The DNA-dependent polymerase will amplify these circular DNA structures through rolling circle amplification. The amplified circular DNA structures can be detected using a fluorescent label. The resulting distinct red spots (PLA signals) are indicative of protein-protein interaction and can be visualized using fluorescence microscopy. Figure 2.5 was modified from Söderberg *et al.*, 2006.
Polymerase (Sigma-Aldrich, ON) was then diluted in the amplification solution at a ratio of 1:80 and vortexed. The Amplification-Polymerase solution was added to the cells and incubated for 100 min at 37°C in the humidity chamber protected from light. Coverslips were placed in the 24-well plate and washed with 1X Wash Buffer B twice for 10 min each followed by a final wash with 0.01X Wash Buffer B for 1 min while gently agitated. Coverslips were allowed to air dry in the dark for 15 min before being mounted on slides using Duolink In Situ Mounting Medium with DAPI (Sigma-Aldrich, ON). Coverslips were edge sealed using clear nail polish and were allowed to air dry in the dark for another 15 min. Images were acquired using Zeiss Axiovert 200M-inverted fluorescence microscopes at 100X objectives and captured with the AxioVision 4.7 Multi Channel Fluorescence software. The following filter sets were used: Amplification Orange (546 nm excitation, 575-640 nm emission) and DAPI (365 nm excitation, 420 nm emission). Slides were stored at -20 in the dark. The same in situ PLA protocol was used to study the interaction between CB1 and D2L receptors in the globus pallidus of brain tissue slides from C57BL/6J mice globus pallidus following chronic ligand treatment.

High-resolution images were analyzed in ImageJ (NIH) to calculate the PLA signals (red spots) using a previously published protocol (Trifilieff et al., 2011). For all experiments, quantifications were performed from at least 9 images from 3 independent experiments per group. A threshold was selected manually to discriminate red PLA dots from background signals. Once selected, this threshold was applied uniformly to all images in the sample set. The built-in macro ‘Analyze Particles’ was then used to count and characterize all objects in an image. Objects larger than 5 μm², such as nuclei, were excluded from the count. The remaining objects were counted as PLA signals. The total number of cells in the field (blue nuclei) was counted manually and included ~ 10-20 cells per image analyzed. Finally, PLA signals (red spots) relative to cell number (nuclei) were calculated (dots/cell).

2.16 Animal Care and Handling

Six-week-old, male, wild-type (C57BL/6J) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were group housed (5 per cage) with ad libitum
access to food, water, and environmental enrichment and maintained on a 12 hr light/dark cycle. Mice were randomly assigned to receive volume-matched, daily intraperitoneal (i.p.) injection of vehicle (10% (v/v) DMSO, 0.1% (v/v) Tween-20 in saline) or 0.01 mg/kg CP 55,940, 0.3 mg/kg haloperidol, or 1.5 mg/kg olanzapine alone or in combination \((n = 10 \text{ per group})\). Mice were weighed daily. All protocols were in accordance with the guidelines detailed by the Canadian Council on Animal Care (CCAC; Ottawa ON: Vol 1, 2nd Ed, 1993; Vol 2, 1984) and approved by the Carleton Animal Care Committee at Dalhousie University.

2.17 Open Field Test

An open field test was performed to assess locomotive activities in mice following drug administration according to previously published protocols (Seibenhener and Wooten 2015). Open field test measurements were performed 24 hr before the first drug injection and on day 1, 7, 14 and 21. The task was performed using an open-filed arena (60 cm width × 60 cm length × 20 cm height). The open-field arena was divided into a 6 × 6 grid of equally sized squares. The central region of the open-field arena was defined as the 4 squares in the middle of the box (i.e. 4 out of 36 squares), while the outer region of the open-field arena was defined as the sum of all the squares, excluding the 4 corner squares and the 4 center squares (i.e. 28 out of 36 squares). At the beginning of the test, each mouse was placed in the same quadrant in the outer section of the arena. The behavior of each mouse was recorded for 2 min using a digital video camera. At the end of each session, the mouse was removed from the open field arena, and the arena was thoroughly cleaned with 70% (v/v) ethanol. The video was scored afterward using The Ethovision® 5.0 software, a video tracking system that automatically records behavioural experiments (Noldus Information Technologie).

2.18 Brain Tissue Preparation

After completion of all drug treatments and behavioral analyses, brains were collected from mice the day after the last drug injection. Mice were deeply anesthetized by an i.p. injection of 100 mg/kg pentobarbital and then perfused intracardially with 1X PBS followed by ice-cold 4% (w/v) PFA solution. Mice brains were then collected and
fixed overnight in 4% (w/v) PFA solution. Next day, the brains were cryoprotected by placing them in 10% (w/v) sucrose (0.1 M PBS, pH 7.4) for several hours until the brain sank to the bottom of a 50 ml Falcon tube. The brains were transferred to 20% (w/v) sucrose for 1 day, then transferred to a 30% (w/v) sucrose solution for several days at 4°C. Brains were flash-frozen on dry ice for 1-2 min and stored at -80°C until use. Sections 20 μm thick were cut using a cryostat and mounted on Superfrost Plus microscopic slides (Fisher Scientific). The mounted brain sections on slides were stored at −20°C until use (Borroto-Escuela et al., 2016).

2.19 Dual-Labeled Quantitative Fluorescence Immunohistochemistry (QF-IHC) Staining of Tissue Sections

Dual-Labeled quantitative fluorescence immunohistochemistry (QF-IHC) staining was used to quantify CB1 and D2 protein levels in the globus pallidus of C57BL/6J mice following chronic ligand treatment. Tissue sections were exposed to IR-labeled antibodies and scanned using infrared-based tissue imaging, which allows for determination of relative protein levels in defined areas (Kearn, 2004; Eaton et al., 2016). PFA-fixed frozen sections mounted on slides were taken out of storage at -80°C, equilibrated to room temperature, then rehydrated in 1X PBS for 10 min. The tissues were blocked using Odyssey Blocking Buffer (Li-Cor Biotechnology) containing 0.1% (v/v) Tween-20 for 90 min at room temperature. The primary monoclonal rabbit N-terminal CB1 antibody (1:500; Cayman Chemical Company) and the primary monoclonal mouse N-terminal-D2 antibody (1:200; Santa Cruz Biotechnology) were diluted in Odyssey Blocking Buffer containing 0.1% (v/v) Tween-20. Tissues were incubated with primary antibodies overnight at 4°C. The next day, slides were washed four times in 1X PBS containing 0.1% (v/v) Tween-20 each for 30 min. The tissues were then incubated for 2 hr with the IR800CW-conjugated anti-rabbit IgG secondary antibody (Rockland Immunochemical) and Alexa Fluor 680 conjugated anti-mouse secondary antibody (Invitrogen) diluted 1:10,000 in 20% (v/v) Odyssey Blocking Buffer in 1X PBS and containing 0.1% (v/v) Tween-20. During antibody exposure sections were protected from light. Slides were washed four times for 30 min in 1X PBS containing 0.1% (v/v) Tween-20 and allowed to air-dry overnight in the dark. Slides were scanned using the Odyssey
infrared imaging system (Li-Cor Biotechnology) with the resolution set at 21 μm, quality set at ‘highest’, focus offset set at 0 mm and the intensity set at 2.0 for both the 700 nm and 800 nm channel. Image quantification of the globus pallidus was carried out using ImageJ (NIH) software.

2.20 Statistical Analyses and Curve Fitting

Data are presented as the Mean ± standard error mean (SEM) 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 non-linear regression model with variable slope (four parameters). Hill coefficients were calculated from the slope of curves and represent the cooperativity of oligomeric allosteric proteins (Edelstein and Le Novère, 2013). If the Hill coefficient is larger than 1, it is a positive cooperativity, whereas the Hill coefficient smaller than 1 indicates negative cooperatively. Statistical analyses were conducted by one-way analysis of variance (ANOVA), as indicated. Post-hoc analyses were performed using Tukey’s honest significance test. The level of significance was set to $P < 0.05$. 

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CHAPTER 3

ANTAGONISM OF DOPAMINE RECEPTOR 2 LONG (D2L) AFFECTS CANNABINOID RECEPTOR 1 (CB1) SIGNALING IN A CELL CULTURE MODEL OF STRIATAL MEDIUM SPINY PROJECTION NEURONS

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This chapter has been previously published in: Amina M. Bagher, Robert B. Laprairie, Melanie E.M. Kelly, and Eileen M. Denovan-Wrigh (2016). Antagonism of dopamine receptor 2 long (D2L) affects cannabinoid receptor 1 (CB1) signaling in a cell culture model of striatal medium spiny projection neurons. Journal of Molecular Pharmacology. 2016 June 89(6): 652-66. The manuscript has been modified to meet formatting requirements. Re-use is permitted with copyright permission (Appendix A).

Contribution Statement
The manuscript used as the basis for this chapter was written with guidance from Dr. Eileen Denovan-Wright. Data were collected and analyzed by myself. Critical reagents were provided by Drs. Eileen Denovan-Wright and Melanie Kelly.
3.1 Abstract

Activation of dopamine receptor 2 long (D_{2L}) switches the signaling of type 1 cannabinoid receptor (CB_{1}) from G_{α_i} to G_{α_s}, a process which is thought to be mediated through CB_{1}/D_{2L} heteromerization. Given the clinical importance of D_{2} antagonists, the goal of this study was to determine if D_{2} antagonists could modulate CB_{1} signaling. Interactions between CB_{1} and D_{2L}, G_{α_i}, G_{α_s}, and β-arrestin1, were studied using BRET^{2} in STHdh^{Q7/Q7} cells. CB_{1}-dependent ERK1/2, CREB phosphorylation and CB_{1} internalization following co-treatment of CB_{1} agonist and D_{2} antagonist were quantified. Pre-assembled CB_{1}-G_{α_i} complexes were detected by BRET^{2}. Arachidonyl-2'-chloroethylamide (ACEA), a selective CB_{1} agonist, caused a rapid and transient increase in BRET_{Eff} between G_{α_i}-Rluc and CB_{1}-GFP^{2}, and a G_{α_i}-dependent increase in ERK phosphorylation. Physical interactions between CB_{1} and D_{2L} were observed using BRET^{2}. Co-treatment of STHdh^{Q7/Q7} cells with ACEA and haloperidol, a D_{2} antagonist, inhibited BRET_{Eff} signals between G_{α_i}-Rluc and CB_{1}-GFP^{2} and reduced the $E_{\text{Max}}$ and pEC_{50} of ACEA-mediated G_{α_i}-dependent ERK phosphorylation. ACEA and haloperidol co-treatments produced a delayed and sustained increase in BRET_{Eff} between G_{α_s}-Rluc and CB_{1}-GFP^{2} and increased the $E_{\text{Max}}$ and pEC_{50} of ACEA-induced G_{α_s}-dependent CREB phosphorylation. In cells expressing CB_{1} and D_{2L} treated with ACEA, binding of haloperidol to D_{2} receptors switched CB_{1} coupling from G_{α_i} to G_{α_s}. In addition, haloperidol treatment reduced ACEA-induced β-arrestin1 recruitment to CB_{1} and CB_{1} internalization. D_{2} antagonists allosterically modulate cannabinoid-induced CB_{1} coupling, signaling and β-arrestin1 recruitment through binding to CB_{1}/D_{2L} heteromers. These findings indicate that D_{2} antagonism, like D_{2} agonists, change agonist-mediated CB_{1} coupling and signaling.

3.2 Introduction

The type 1 cannabinoid receptor (CB_{1}) 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). The CB_{1} is activated by endogenous lipid mediators, such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and exogenous cannabinoids such as $\Delta^{9}$-tetrahydrocannabinol (THC) (Mechoulam et al., 1995). CB_{1} receptors signal
primarily through *Pertussis* toxin (PTx)-sensitive G\(_{\alpha_{i/o}}\) proteins (Demuth and Molleman, 2006). In addition, it has been demonstrated that different CB\(_1\) agonists can promote CB\(_1\) signaling through G\(_{\alpha_s}\), G\(_{\alpha_{q/11}}\), and \(\beta\)-arrestin1 (Maneuf and Brotchie, 1997; Lauckner *et al*., 2005; Laprairie *et al*., 2014).

CB\(_1\) receptors can self-associate to form homomers and can also associate with other class-A GPCRs to form heteromers (Hudson *et al*., 2010). Specifically, CB\(_1\) is known to heteromerize with the dopamine receptor type 2 long (D\(_{2L}\)), the \(\delta\)-, \(\kappa\)- and \(\delta\)-opioid receptors, the orexin-1 receptor, the A\(_{2A}\) receptor, and \(\beta_2\)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*., 2010b). Heteromerization of CB\(_1\) with the D\(_{2L}\) has received significant attention due to the fact that both receptors are co-localized in the GABAergic medium spiny neurons projecting from the striatum to the 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).

Co-localization of CB\(_1\) and D\(_{2L}\) in the basal ganglia may allow for bidirectional functional interaction between the two receptors (reviewed in Fernández-Ruiz *et al*., 2010). Activation of CB\(_1\) leads to an increase in dopamine release in the nucleus accumbens (Tanda *et al*., 1997; Solinas *et al*., 2006). In addition, D\(_{2L}\) 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 CB\(_1\) and D\(_{2L}\) were first observed in striatal neurons by Glass and Felder (1997). Co-stimulation of both these receptors by their respective agonists in striatal neurons leads to an accumulation of cAMP, while 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 the coupling of CB\(_1\) from G\(_{\alpha_i}\) to G\(_{\alpha_s}\) when the two receptors were co-activated by agonists (Glass and Felder, 1997). Subsequent work demonstrated that D\(_2\) agonists altered CB\(_1\)-dependent signaling, CB\(_1\) 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 CB\(_1\) and D\(_{2L}\) receptors have been attributed to heteromerization between the two receptors as demonstrated using co-
immunoprecipitation, fluorescence resonance energy transfer (FRET), and bimolecular fluorescence complementation (BiFC) (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, 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 cooperatively across the heteromer pair (Kenakin, 2010; Wootten et al., 2013). A well-known example of allosteric interactions between GPCR heteromers is within the D2/A2A receptor heteromer complex (reviewed in Ferré, 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 Gαi, Gαs, and β-арrestин 1 in the presence of the cannabinoid agonist arachidonyl-2'-chloroethylamide (ACEA). ACEA is a stable synthetic analogue 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 Gαi, Gαs, and β-арrestин 1 in STHdhQ7/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).

3.3 Results

3.3.1 CB1 and D2L Receptors Form Heteromers in STHdhQ7/Q7 Cells

STHdhQ7/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 CB_1 and D_2 receptors (Supplementary Fig. 3.1). Our immunocytochemistry experiments show co-localization of CB_1 and D_2 in STHdh^{Q7/Q7} cells. CB_1 immunofluorescence was not confined to the plasma membrane, but those intracellular reactions were also seen, as previously reported (Leterrier et al., 2006; McDonald et al., 2007; Scavone et al., 2010). Using PCR primers that span the alternatively spliced exon that distinguished the D_2 long (D_2L) and D_2 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 BRET^2 analyses.

BRET^2 was used to determine whether CB_1 and D_2L receptors heteromerize when expressed in STHdh^{Q7/Q7} cells. Cells were co-transfected with CB_1-Rluc and D_2L-GFP^2 constructs. Negative control included the human ether-a-go-go-related gene (HERG), HERG-GFP^2, which is a membrane-localized K^+ channel that does not interact with GPCRs or G-proteins (Hudson et al., 2010b). The combination of CB_1-Rluc and D_2L-GFP^2 resulted in greater BRET_{Eff} compared to negative controls obtained from cells expressing CB_1-Rluc and HERG-GFP^2 (Fig. 3.1A), indicating that CB_1 and D_2L form heteromers when co-expressed in STHdh^{Q7/Q7} cells. The interaction between CB_1 and D_2L is mediated by the C-terminus of CB_1 and the third intracellular loop of D_2L (Khan and Lee, 2014). To disrupt the formation of CB_1 and D_2L complexes, a CB_1 blocking peptide (CB_1-BP) that binds to the CB_1 receptor C-terminal region (C417-S431) was cloned (Khan and Lee, 2014). The CB_1-BP inhibits the heteromerization of CB_1 and D_2L by competing with CB_1 for binding with D_2L (Khan and Lee, 2014). The co-expression of CB_1-Rluc and D_2L-GFP^2 together with the CB_1-BP reduced BRET_{Eff} relative to cells transfected with CB_1-Rluc and D_2L-GFP^2 (Fig. 3.1A). CB_1-BP did not alter BRET_{Eff} in cells expressing CB_1-Rluc and CB_1-GFP^2 (Fig. 3.1A). These differences in BRET^2 signals were not due to the difference in the expression level of BRET^2 partners quantified by luminescence and fluorescence measurements (Supplementary Fig. 3.2). These data indicate that CB_1-BP blocks the formation of CB_1/D_2L heteromers, but not CB_1 homomers, suggesting that the protein regions crucial for CB_1 homomerization are different than those involved in CB_1/D_2L heteromerization.
Figure 3.1: CB₁ and D₂L Receptors Formed Heteromers When Expressed in STHdh<sup>Q7/Q7</sup> Cells Demonstrated Using BRET<sup>2</sup>. (A) BRET<sub>Eff</sub> was measured in cells expressing CB₁-Rluc and D₂L-GFP<sup>2</sup>, or CB₁-GFP<sup>2</sup> constructs and the CB₁-BP or pcDNA vector. As a negative control, cells were co-transfected with CB₁-Rluc and HERG-GFP<sup>2</sup>. * P < 0.01 compared to cells expressing CB₁-Rluc and HERG-GFP<sup>2</sup>, ~ P < 0.01 compared to cells expressing CB₁-Rluc, D₂L-GFP<sup>2</sup>, and pcDNA. (B) BRET<sup>2</sup> saturation curves of cells transiently transfected with a constant amount of CB₁-Rluc and an increasing amount of D₂L-GFP<sup>2</sup>. * P < 0.01 compared to cells expressing CB₁-Rluc and HERG-GFP<sup>2</sup>. (C) BRET<sub>Max</sub> and BRET<sub>50</sub> parameters derived from BRET<sup>2</sup> saturation curves of cells transiently transfected with a constant amount of CB₁-Rluc and an increasing amount of D₂L-GFP<sup>2</sup> and treated for 30 min with the vehicle, 1 μM ACEA, 10 μM haloperidol (HALO) alone or treated with ACEA. * P < 0.01 compared to cells treated with vehicle. Data are presented as mean ± SEM of 4 independent experiments. Significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
A

B

C

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<th>Ligand</th>
<th>BRET$_{Max}$</th>
<th>BRET$_{50}$</th>
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<td>Vehicle</td>
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<td>0.41 ± 0.03</td>
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<tr>
<td>1 µM ACEA</td>
<td>0.35 ± 0.01*</td>
<td>0.43 ± 0.03</td>
</tr>
<tr>
<td>10 µM HALO</td>
<td>0.25 ± 0.01</td>
<td>0.41 ± 0.02</td>
</tr>
<tr>
<td>1 µM ACEA + 10 µM HALO</td>
<td>0.33 ± 0.01*</td>
<td>0.37 ± 0.05</td>
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</table>
A BRET² saturation curve was generated to demonstrate the ability of CB₁ and D₂L receptors to form heteromers at constant donor expression levels and increasing acceptor expression levels. For the BRET² saturation curve, cells were co-transfected with a constant amount of CB₁-Rluc with increasing amounts of D₂L-GFP² or HERG-GFP² (Fig. 3.1B). The combination of CB₁-Rluc with D₂-GFP² resulted in a significantly different saturation curve than the control curve, which was generated with the co-expression of CB₁-Rluc with HERG-GFP² (Fig. 3.1B). The BRET² saturation curve resulted in a BRET\textsubscript{Max} of 0.28 ± 0.01 and a BRET\textsubscript{50} of 0.41 ± 0.03. Treating cells co-expressing CB₁-Rluc and D₂L-GFP² for 30 min with 1 μM ACEA +/- 10 μM haloperidol resulted in higher BRET\textsubscript{Max}, but not BRET\textsubscript{50}, compared to the BRET\textsubscript{Max} observed in vehicle-treated cells. Haloperidol treatment alone did not alter BRET\textsubscript{Max} or BRET\textsubscript{50} compared to vehicle-treated cells (Fig. 3.1C). The change in BRET\textsubscript{Max}, but not BRET\textsubscript{50}, following treatment with cannabinoid alone or in combination with the D₂ antagonist, suggests that ligand binding stabilized the conformation of this heteromer, which enhanced the energy transfer between CB₁ and D₂L without increasing the number of receptors involved in heteromerization.

3.3.2 D₂ Antagonism Can Allosterically Inhibit The Association of CB₁ Receptor and Ga\textsubscript{i} Protein

Different CB₁ agonists can activate different G proteins including Ga\textsubscript{i} and Ga\textsubscript{s} proteins (Bosier \textit{et al}., 2010; Laprairie, \textit{et al}., 2014). To study coupling of CB₁ to Ga\textsubscript{i} and Ga\textsubscript{s} proteins, we used BRET² for real-time assessment of receptor-G protein interaction in living STHdh\textsuperscript{Q7/Q7} cells transiently transfected with G-protein-Rluc and CB₁-GFP². Our first aim was to investigate the coupling of CB₁ to Ga\textsubscript{i} protein in the absence of agonist. STHdh\textsuperscript{Q7/Q7} cells were transiently transfected with Ga\textsubscript{i}-Rluc and CB₁-GFP². Co-expression of Ga\textsubscript{i}-Rluc and CB₁-GFP² resulted in BRET\textsubscript{Eff} equal to 0.23 ± 0.08, which was higher than cells expressing Ga\textsubscript{i}-Rluc and HERG-GFP² (Fig. 3.2A). We found basal BRET\textsubscript{Eff} was insensitive to 24 h PTx treatment (Fig. 3.2A). Chronic PTx treatment inactivates Ga\textsubscript{i} protein. This finding confirms that CB₁ receptors are pre-assembled with Ga\textsubscript{i} prior to the addition of exogenous ligand and does not result from constitutive activation of Ga\textsubscript{i} (Ayoub \textit{et al}., 2007). Next, the influence of CB₁ agonist treatment on CB₁-Ga\textsubscript{i} coupling
was tested. Treating cells with 1 μM ACEA resulted in an increase in BRET<sub>Eff</sub> (Fig. 3.2A). Inactivating Ga<sub>i</sub> with PTx suppressed ACEA-induced BRET<sub>Eff</sub> to the basal level (Fig 2A). The agonist-induced BRET<sub>Eff</sub> increase clearly demonstrates a functional coupling of CB<sub>1</sub> and Ga<sub>i</sub> protein.

We measured the effect of D<sub>2</sub> antagonism on CB<sub>1</sub> agonist-induced CB<sub>1</sub>- and Ga<sub>i</sub>-dependent BRET<sub>Eff</sub> in cells co-transfected with Ga<sub>i</sub>-Rluc and CB<sub>1</sub>-GFP<sup>2</sup> and un-tagged D<sub>2L</sub>-pcDNA. An ACEA concentration-response curve was generated. Increasing ACEA concentration resulted in an increase in BRET<sub>Eff</sub> between Ga<sub>i</sub> and CB<sub>1</sub> (EC<sub>50</sub> = 0.22 (0.19 - 0.28), E<sub>Max</sub> = 0.45 (0.41-0.48), Hill coefficient= 1.15 (0.91-1.4) (Fig. 3.2B). Treating the cells with different concentrations of haloperidol alone did not alter BRET<sub>Eff</sub> between Ga<sub>i</sub> and CB<sub>1</sub> (data not shown). However, pre-treating the cells with haloperidol 25 s prior the addition of ACEA reduced ACEA-induced BRET<sub>Eff</sub> signal between Ga<sub>i</sub> and CB<sub>1</sub> in a haloperidol concentration-dependent manner (Fig. 3.2B). Haloperidol produced a concentration-dependent rightward and downward shift in the ACEA concentration-response curves. Both the efficacy and the potency of ACEA dependent Ga<sub>i</sub>-CB<sub>1</sub> interaction were diminished by D<sub>2</sub> antagonism. The rightward shift in EC<sub>50</sub> for ACEA concentration-response curves was significant at 0.1, 1 and 10 μM haloperidol for ACEA-treated cells (Table 3.1). The decrease in E<sub>Max</sub> was significant at all concentrations of haloperidol tested (Table 3.1). The Hill coefficient was significantly less than 1 at 0.1, 1 and 10 μM haloperidol for ACEA-concentration-response curves (Table 3.1). The observed effects of haloperidol on ACEA-dependent Ga<sub>i</sub>-CB<sub>1</sub> interaction indicate the presence of negative cooperatively; the Hill coefficient is less than one.

To confirm that the observed allosteric effect of haloperidol was mediated through CB<sub>1</sub>/D<sub>2L</sub> heteromers and not mediated through the direct effect of haloperidol on the CB<sub>1</sub> receptor, cells were co-transfected with Ga<sub>i</sub>-Rluc and CB<sub>1</sub>-GFP<sup>2</sup> and treated with 10 μM haloperidol prior to 1 μM ACEA application. No change in BRET<sub>Eff</sub> between Ga<sub>i</sub>-Rluc and CB<sub>1</sub>-GFP<sup>2</sup> was observed (Fig. 3.2C). These data demonstrate that CB<sub>1</sub>/D<sub>2L</sub> heteromerization was required for effect of haloperidol, as haloperidol had no effect on CB<sub>1</sub>-Ga<sub>i</sub> interactions in the absence of D<sub>2L</sub> (Fig. 3.2C). In addition, the expression of equimolar D<sub>2L</sub>-pcDNA and CB<sub>1</sub>-GFP<sup>2</sup> in the presence of excess pool of Ga<sub>i</sub>-Rluc did not alter CB<sub>1</sub> coupling to Ga<sub>i</sub>-Rluc protein in the presence of vehicle or ACEA, compared to
**Figure 3.2: Haloperidol Treatment Inhibited Interactions Between CB₁ and Gαᵢ in The Presence of ACEA in STTdh<sup>Q7/Q7</sup> Cells.** (A) BRET<sub>eff</sub> was measured at 2 min following the addition of vehicle or 1 µM ACEA +/- 24 h pre-treatment with 50 ng/ml PTx in cells expressing G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup>. * P < 0.01 relative to cells expressing G<sub>αᵢ</sub>-Rluc and HERG-GFP<sup>2</sup>; ~ P < 0.01 compared to cells expressing G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup> treated with PTx for 24 hr. (B) Concentration-response curves of ACEA- induced BRET<sub>eff</sub> between G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup> in the absence or presence of HALO measured at 2 min following ACEA application. (C) BRET<sub>eff</sub> was measured at 2 min following the addition of vehicle, 10 µM HALO, 1 µM ACEA +/- 10 µM HALO and pre-treated for 30 min with 0.5 µM O-2050 in cells expressing G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup> +/- D<sub>2L</sub>-pcDNA. n.s. P > 0.05 compared with cells expressing G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup> only; ~ P < 0.01 relative to cells expressing G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup> treated with 1 µM ACEA and 10 µM HALO; * P < 0.01 compared to cells treated with vehicle. (D) Cells were transfected with G<sub>αᵢ</sub>-Rluc, CB₁-GFP<sup>2</sup>, D<sub>2</sub>-pcDNA, and CB₁-BP or pcDNA, and BRET<sub>eff</sub> was measured at 2 min following the addition of vehicle, 10 µM HALO, 1 µM ACEA +/- 10 µM HALO. * P < 0.01 relative to cells expressing G<sub>αᵢ</sub>-Rluc, CB₁-GFP<sup>2</sup> and CB₁-BP and treated with 1 µM ACEA and 10 µM HALO. (E) BRET<sup>2</sup> saturation curves were generated by co-transfecting constant amounts of G<sub>αᵢ</sub>-Rluc and increasing amounts of CB₁-GFP<sup>2</sup> alone or with D<sub>2L</sub>-pcDNA or HERG GFP<sup>2</sup>, and BRET<sub>eff</sub> was measured following the addition of vehicle, 1 µM ACEA alone or in combination with 10 µM HALO. * P < 0.01 compared with cells expressing G<sub>αᵢ</sub>-Rluc and HERG-GFP<sup>2</sup>. (F) BRET<sub>eff</sub> was measured over 9 min (540 s) in cells expressing G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup> alone or together with D<sub>2L</sub>-pcDNA and treated with vehicle, 1 µM ACEA +/- 10 µM HALO. As a negative control, cells were co-transfected with G<sub>αᵢ</sub>-Rluc and HERG-GFP<sup>2</sup>. * P < 0.01 compared to cells expressing G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup> and treated with vehicle; ~ P < 0.01 compared to cells expressing G<sub>αᵢ</sub>-Rluc and CB₁-GFP<sup>2</sup> and treated with 1 µM ACEA. Data are presented as mean ± SEM of 4 independent experiments; significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
Table 3.1: The Effects of Haloperidol on BRET^2 (Gα_i-Rluc + CB1-GFP^2), Gα_i-Dependent ERK Phosphorylation, BRET^2 (Gα_s-Rluc + CB1-GFP^2), Gα_s-Dependent CREB Phosphorylation and BRET^2 (β-arrestin1-Rluc + CB1-GFP^2). Determined using nonlinear regression with variable slope (four parameters) in GraphPad (version 6.0). Data are presented as the mean and 95% confidence interval (CI) from four independent experiments. N.C. not converged. *P < 0.01, compared with ACEA-treated cells; one-way ANOVA with Tukey's Post-hoc test.
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<th>E&lt;sub&gt;Max&lt;/sub&gt; (95% CI)</th>
<th>Hill coefficient (95% CI)</th>
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<td>BRET&lt;sup&gt;2&lt;/sup&gt; (G&lt;sub&gt;α&lt;/sub&gt;&lt;sub&gt;i&lt;/sub&gt;- Rluc + CB&lt;sub&gt;1&lt;/sub&gt;- GFP&lt;sup&gt;2&lt;/sup&gt;)</td>
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<td>ACEA</td>
<td>0.22 (0.19-0.28)</td>
<td>0.45 (0.41-0.48)</td>
<td>1.15 (0.91-1.40)</td>
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<td>0.90 (0.86-0.94)</td>
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<tr>
<td>10</td>
<td>0.98 (0.94-1.41)*</td>
<td>N.C.</td>
<td>N.C.</td>
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| G<sub>α</sub><sub>i</sub>-dependent ERK phosphorylation |
|---------|---------------------------|--------------------------|---------------------------|
| ACEA    | 0.27 (0.25-0.29) | 0.76 (0.71-0.80) | 1.11 (0.89-1.23) |
|         | 0.31 (0.26-0.36) | 0.69 (0.64-0.75) | 0.93 (0.84-1.03) |
|         | 0.41 (0.37-0.45)* | 0.52 (0.48-0.57)* | 0.84 (0.78-0.89) |
| 1       | 0.72 (0.67-0.78)* | 0.39 (0.37-0.42)* | 0.79 (0.69-0.79)* |
| 10      | 1.01 (0.82-1.21)* | 0.32 (0.29-0.34)* | 0.74 (0.67-0.81)* |

| BRET<sup>2</sup> (G<sub>α</sub><sub>s</sub>-Rluc + CB<sub>1</sub>- GFP<sup>2</sup>) |
|---------|---------------------------|--------------------------|---------------------------|
| ACEA    | 0.49 (0.36-0.62)* | 0.18 (0.16-0.19)* | 1.12 (0.92-1.32)* |
| 0.01    | 0.35 (0.29-0.42)* | 0.26 (0.24-0.27)* | 1.28 (1.21-1.36)* |
| 0.1     | 0.29 (0.24-0.35)* | 0.31 (0.28-0.33)* | 1.43 (1.36-1.51)* |
| 10      | 0.23 (0.18-0.31)* | 0.36 (0.32-0.40)* | 1.53 (1.43-1.63)* |

| G<sub>α</sub><sub>s</sub>-dependent CREB phosphorylation |
|---------|---------------------------|--------------------------|---------------------------|
| ACEA    | 0.35 (0.31-0.40) | 0.26 (0.23-0.28) | 1.02 (0.90-1.14) |
| 0.01    | 0.31 (0.29-0.35) | 0.42 (0.40-0.45)* | 1.11 (0.98-1.31) |
| 0.1     | 0.29 (0.23-0.48)* | 0.56 (0.53-0.59)* | 1.26 (1.10-1.42)* |
| 10      | 0.23 (0.19-0.27)* | 0.65 (0.62-0.68)* | 1.56 (1.17-1.86)* |

| BRET<sup>2</sup> (β-arrestin1-Rluc + CB<sub>1</sub>- GFP<sup>2</sup>) |
|---------|---------------------------|--------------------------|---------------------------|
| ACEA    | 0.25 (0.19-0.35) | 0.56 (0.52-0.60) | 1.21 (0.11-1.23) |
| 0.01    | 0.27 (0.21-0.37) | 0.53 (0.49-0.56) | 1.12 (0.98-1.10) |
| 0.1     | 0.33 (0.28-0.51)* | 0.48 (0.45-0.52) | 1.01 (0.95-1.21) |
| 10      | 0.36 (0.29-0.44)* | 0.38 (0.36-0.41)* | 1.12 (0.90-1.12) |
| 10      | 0.45 (0.34-0.57)* | 0.32 (0.29-0.34)* | 1.04 (0.85-1.11) |
cells expressing CB1-GFP² and Gαi-Rluc alone (Fig. 3.2C). The co-application of the CB1 orthosteric antagonist, O-2050, prior to the application of ACEA and haloperidol, returned BRET\textsubscript{Eff} to basal levels, which confirms that the observed increase in BRET\textsubscript{Eff} between CB1 and Gαi is due to the binding of ACEA to the orthosteric site of the CB1 (Fig. 3.2C). Therefore, expression of D\textsubscript{2L} receptors did not alter CB1 coupling to Gαi, but co-treatment of cells with haloperidol and ACEA resulted in reduced BRET\textsubscript{Eff} signals between Gαi and CB1.

Next, we confirmed that the inhibition of BRET\textsubscript{Eff} between Gαi and CB1 following haloperidol and ACEA application was mediated through the binding of haloperidol to CB1/D\textsubscript{2L} complexes. To confirm this we blocked the heteromerization between CB1 and D\textsubscript{2L} receptors by the co-expression of CB1-BP. Cells co-transfected with Gαi-Rluc, CB1-GFP², D\textsubscript{2L}-pcDNA and CB1-BP treated with ACEA and haloperidol had higher BRET\textsubscript{Eff} compared to cells transfected with Gαi-Rluc, CB1-GFP², D\textsubscript{2L}-pcDNA, and no CB1-BP (Fig. 3.2D). Thus, haloperidol inhibited ACEA-enhanced CB1-Gαi induced BRET² through binding to CB1/D\textsubscript{2L} complexes.

BRET² saturation curves were generated between Gαi-Rluc and CB1-GFP² in the presence and absence of ACEA to validate the specificity of the interaction between Gαi and CB1 (Fig. 3.2E). Cells were co-transfected with constant amounts of Gαi-Rluc and increasing amounts of CB1-GFP² or HERG-GFP² (Fig. 3.2E). The combination of Gαi-Rluc and CB1-GFP² resulted in a BRET\textsubscript{Max} of 0.26 ± 0.04 and a BRET\textsubscript{50} of 0.37 ± 0.05. The BRET\textsubscript{Max} and BRET\textsubscript{50} values were higher compared to cells expressing Gαi-Rluc and HERG-GFP² (Fig. 3.2E). Therefore, the interaction between Gαi and CB1 was specific and saturable. To test whether ACEA treatment resulted in conformational changes within the pre-assembled CB1-Gαi complexes (observed as changes in BRET\textsubscript{Max}), rather than the recruitment of more Gαi to CB1 (observed as changes in BRET\textsubscript{50}) (Ayoub \textit{et al.}, 2012), a BRET² saturation curve was created following ACEA (1 μM) treatment (Fig. 3.2E). The BRET² saturation curve displayed BRET\textsubscript{Max} of 0.40 ± 0.03 and BRET\textsubscript{50} of 0.39 ± 0.04. The BRET\textsubscript{Max} obtained from treatment with ACEA was significantly higher compared to cells treated with vehicle (BRET\textsubscript{Max} of 0.26 ± 0.04). No significant change in BRET\textsubscript{50} values was observed (Fig. 3.2E). Therefore, ACEA treatment only induced conformational changes with the CB1-Gαi complexes. To test whether haloperidol treatment induces conformational changes with the CB1/D\textsubscript{2L}/Gαi complexes or it promotes the
dissociation of CB₁ and Gαᵢ, a BRET² saturation curve was generated in cells expressing Gαᵢ-Rluc, CB₁-GFP², and D2L-pcDNA (Fig. 3.2E). Co-treating the cells with 10 μM haloperidol and 1 μM ACEA significantly reduced BRETI_{Max} (0.11 ± 0.04) and BRETI_{50} (0.11± 0.07) compared to vehicle-treated cells. Reduction in both BRETI_{Max} and BRETI_{50} following haloperidol and ACEA treatment suggested that haloperidol induced dissociation of Gαᵢ and CB₁ and induced conformational changes between Gαᵢ-Rluc and CB₁-GFP².

A kinetic analysis of ACEA-induced BRET² between Gαᵢ and CB₁ was carried out. Cells were co-transfected with Gαᵢ-Rluc and CB₁-GFP² and signals were recorded as repeated measures in vehicle-treated cells for over 9 min (540 s). Treating cells with 1 μM ACEA 75 s after the addition of coelenterazine 400a resulted in a rapid increase in BRET_{Eff} (Fig. 3.2F). BRET_{Eff} peaked at ~125 s and remained significantly higher for ~400 s before declining (Fig. 3.2F). By ~450 s following ACEA application (Fig. 3.2F), the BRET_{Eff} returned to pre-ACEA levels and remained at this level for 30 min (data not shown). However, in cells co-expressing Gαᵢ-Rluc, CB₁-GFP² and D2L-pcDNA, treating the cells with 10 μM haloperidol added [50 s following the initiation of the reaction and 25 s prior to the application of 1 μM ACEA] resulted in a rapid reduction in BRET_{Eff} compared to vehicle-treated cells and compared to ACEA-treated cells (Fig. 3.2F). The reduction in BRET_{Eff} was sustained for the remaining 480 s (Fig. 3.2F). Reduction of BRET_{Eff} below the basal level was observed at 10, 20 and 30 min following ACEA application (P < 0.01) (data not shown).

3.3.3 D₂ Antagonism Reduced the Efficacy and Potency of CB₁-Dependent Gαᵢ-Mediated ERK Phosphorylation

We had observed a reduction in BRET_{Eff} between Gαᵢ and CB₁ in STHdh^{Q7/Q7} cells co-expressing D₂L following ACEA and haloperidol treatment, which might suggested that CB₁ receptors are dissociated from Gαᵢ proteins. Thus, we measured whether ACEA-induced and Gαᵢ-mediated ERK phosphorylation was also inhibited by haloperidol treatment. A concentration-response curve of ACEA-induced ERK phosphorylation was generated following 5 min 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. 3.3A; Table 3.1). Haloperidol (0.01-10 μM) treatment alone did
Figure 3.3: Haloperidol Reduced ACEA-Induced ERK Phosphorylation. (A) pERK concentration-response curve from STHdhQ7/Q7 cells treated with ACEA alone or in the presence of HALO measured at 5 min. (B) STHdhQ7/Q7 cells were treated with 1 μM ACEA alone for 5 min or in combination with 10 μM HALO +/- 24 h pre-treatment with 50 ng/ml PTx or CTx. * P < 0.01 compared to vehicle treatment; ~ < 0.01 compared to cells treated with 1 μM ACEA. (C) STHdhQ7/Q7 cells were transfected with the pcDNA or CB1-BP and treated with 1 μM ACEA alone for 5 min or in combination with 10 μM HALO. * P < 0.01 compared to vehicle treatment; ~ < 0.01 compared to cells transfected with empty pcDNA vector and treated with 1 μM ACEA and 10 μM HALO. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
not increase ERK phosphorylation (data not shown). Co-treating the cells with increasing concentrations of haloperidol 25 s prior the addition of ACEA, produced a concentration-dependent reduction in ACEA $E_{\text{Max}}$ and $E_{50}$. The reduction in $E_{50}$ and $E_{\text{Max}}$ was significant at 0.1, 1 and 10 μM haloperidol (Table 3.1). The Hill coefficient was less than 1 in cells treated with 1 and 10 μM haloperidol, indicating a negative cooperativity effect (Table 3.1). To confirm that the CB₁-mediated ERK phosphorylation was mediated through Ga$_{i/o}$ protein, STHdh$^{Q7/Q7}$ cells were pre-treated with PTx for 24 hr, prior to ACEA +/-haloperidol application (Fig. 3.3B). PTx pre-treatment inhibited ERK phosphorylation induced by 1 μM ACEA. However, pre-treating the cells with CTx for 24 hr, which suppresses Ga$_s$ expression, did not alter ACEA-mediated ERK phosphorylation. These results demonstrated that ACEA treatment induced a PTx-sensitive, Ga$_{i/o}$-mediated increase in ERK phosphorylation. O-2050 pre-treatment inhibited ACEA-mediated ERK phosphorylation (data not shown). The co-application of 10 μM haloperidol and ACEA prevented ACEA-induced Ga$_{i/o}$-dependent ERK phosphorylation (Fig. 3.3B). Transfecting STHdh$^{Q7/Q7}$ cells with CB₁-BP did not alter ACEA-induced ERK phosphorylation (Fig. 3.3C). The expression of CB₁-BP restored ACEA-induced ERK phosphorylation in haloperidol-treated cells (Fig. 3.3C). Haloperidol inhibited CB₁-dependent and Ga$_{i/o}$-mediated ERK signaling through binding to CB₁/D$_{2L}$ complexes.

**3.3.4 Combined D₂ antagonism and CB₁ agonism enhanced BRET$_{\text{Eff}}$ Between CB₁ and Ga$_s$**

Next, we studied the coupling of CB₁ to Ga$_s$ protein in the absence and in the presence of cannabinoid CB₁ agonist. BRET$_{\text{Eff}}$ between Ga$_s$-Rluc and CB₁-GFP$^2$ was similar to that observed in cells co-expressing Ga$_s$-Rluc and HERG-GFP$^2$ (Fig. 4A). Twenty-four-hour CTx pre-treatment did not affect BRET$_{\text{Eff}}$ compared to vehicle-treated cells (Fig. 3.4A). The higher basal BRET$_{\text{Eff}}$ between cells expressing Ga$_s$-Rluc and CB₁-GFP$^2$ (Fig. 3.2A) compared to cells expressing Ga$_s$-Rluc and CB₁-GFP$^2$ (Fig. 3.4A) was not a result of different levels in the expression of Ga$_s$-Rluc, Ga$_s$-Rluc or CB₁-GFP$^2$ 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 μM ACEA treatment did not alter BRET$_{\text{Eff}}$ between Ga$_s$-Rluc and CB₁-GFP$^2$ (Fig. 3.4A).
Figure 3.4: Co-treatment With ACEA and Haloperidol Promoted Interactions Between CB1 and Ga\(_s\) in STHdh\(^{Q7/Q7}\) Cells. (A) BRET\(_{\text{Eff}}\) was measured 5 min following the addition of vehicle or 1 \(\mu\)M ACEA or with 500 nM CTx pre-treated for 24 h in cells expressing Ga\(_s\)-Rluc and CB1-GFP\(^2\). \(n.s.\) \(P > 0.05\) relative to cells expressing Ga\(_s\)-Rluc and HERG-GFP\(^2\). (B) Concentration-response curves of ACEA-induced BRET\(_{\text{Eff}}\) between Ga\(_s\)-Rluc and CB1-GFP\(^2\) in the absence or presence of HALO measured at 5 min following ACEA application. (C) BRET\(_{\text{Eff}}\) was measured at 5 min following the addition of vehicle, 10 \(\mu\)M HALO or 1 \(\mu\)M ACEA +/- 10 \(\mu\)M HALO and pre-treated for 30 min with 0.5 \(\mu\)M O-2050 in cells expressing Ga\(_s\)-Rluc and CB1-GFP\(^2\) alone or together with D\(_{2L}\)-pcDNA. \(n.s.\) \(P > 0.05\) compared with cells expressing Ga\(_s\)-Rluc and CB1-GFP\(^2\) only; \(\sim P < 0.01\) relative to cells expressing Ga\(_s\)-Rluc and CB1-GFP\(^2\) and treated with 1 \(\mu\)M ACEA and 10 \(\mu\)M HALO; \(P < 0.01\) compared to cells treated with vehicle. (D) Cells were transfected with Ga\(_s\)-Rluc, CB1-GFP\(^2\), and D\(_2\) together with CB1-BP or pcDNA, and BRET\(_{\text{Eff}}\) was measured at 5 min following the addition of vehicle, 10 \(\mu\)M HALO, 1 \(\mu\)M ACEA alone or together with 10 \(\mu\)M HALO. \(P < 0.01\) relative to cells expressing Ga\(_s\)-Rluc, CB1-GFP\(^2\) and CB1-BP and treated with 1 \(\mu\)M ACEA and 10 \(\mu\)M HALO. (E) BRET\(^2\) saturation curves were generated by co-transfected constant amounts of Ga\(_s\)-Rluc and increasing amounts of CB1-GFP\(^2\) alone or with D\(_{2L}\)-pcDNA or HERG GFP\(^2\), and BRET\(_{\text{Eff}}\) was measured following the addition of vehicle, 1 \(\mu\)M ACEA +/- 10 \(\mu\)M HALO. \(P < 0.01\) compared with cells expressing Ga\(_s\)-Rluc and HERG-GFP\(^2\). (F) BRET\(_{\text{Eff}}\) was measured over 9 min in cells expressing Ga\(_s\)-Rluc and CB1-GFP\(^2\) alone or together with D\(_{2L}\)-pcDNA and treated with vehicle, 1 \(\mu\)M ACEA alone or together with 10 \(\mu\)M HALO. As a negative control, cells were co-transfected with Ga\(_s\)-Rluc and HERG-GFP\(^2\). Cells co-transfected with Ga\(_s\)-Rluc and \(\beta_2\)AR-GFP\(^2\) were used as a positive control. \(P < 0.01\) compared to cells expressing Ga\(_s\)-Rluc and CB1-GFP\(^2\) and treated with vehicle; \(\sim P < 0.01\) compared to cells expressing Ga\(_s\)-Rluc and CB1-GFP\(^2\) and treated with 1 \(\mu\)M ACEA. Data are presented as mean ± SEM of 4 independent experiments, significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
In the absence or presence of CB1 agonist, no energy transfer was detected between Gαs-Rluc and CB1-GFP² proteins. Since we have observed an inhibition in BRET² signals between Gαi-Rluc and CB1-GFP² proteins and inhibition of CB1-dependent and Gαi-mediated ERK signaling following ACEA and haloperidol co-application, we tested if the co-application of both ligands promoted CB1 coupling to Gαs protein. An ACEA concentration-response curve was generated to determine the concentration-dependent increase in Gαs-Rluc and CB1-GFP² association in the presence of D2L-pcDNA and increasing concentrations of haloperidol (0.01-10 μM), added 25 s prior the application of ACEA (Fig. 3.4B). Increasing ACEA concentration in the presence of increasing concentrations of haloperidol (0.01-10 μM) resulted in an increase in BRET_Eff between Gαs-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 were significant at all haloperidol concentrations tested (0.01-10.0 μM) (Table 3.1). Similarly, the Hill coefficient was significantly more than 1 at all haloperidol concentrations tested (Table 3.1) suggesting that haloperidol exerts a positive cooperative effect on CB1 to Gαs interaction.

Previous studies have shown that co-expression of D2L and CB1 in HEK 293 cells is sufficient to change the signaling of CB1 from Gαi to Gαs (Jarrahian et al., 2004). In our study, we found that the co-expression of equimolar of D2L-pcDNA and CB1-GFP² in the presence of Gαs-Rluc did not change BRET_Eff between Gαs-Rluc and CB1-GFP² in the presence of vehicle or ACEA (Fig. 3.4C). The application of haloperidol alone did not alter BRET_Eff between Gαs-Rluc and CB1-GFP² in the absence or presence of D2L (Fig. 3.4C). Haloperidol promoted BRET² signals between Gαs-Rluc and CB1-GFP² only in ACEA treated cells in the presence of D2L, suggesting that the observed effect of haloperidol and ACEA is mediated through CB1/D2L heteromers. Furthermore, inhibiting the heteromerization between CB1 and D2L, by the expression of CB1-BP together with Gαs-Rluc, CB1-GFP², and D2L-pcDNA, blocked haloperidol-induced BRET² signals between Gαs-Rluc and CB1-GFP² in the presence of ACEA (Fig. 3.4D). Co-treatment with ACEA and haloperidol, therefore, promoted BRET² signals between Gαs-Rluc and CB1-GFP² through binding to D2L receptors in CB1/D2L complexes.
BRET$^2$ saturation curves were generated to determine the specificity of the interaction between CB$_1$ 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 CB$_1$-GFP$^2$ 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 CB$_1$-GFP$^2$ was non-specific (Fig. 3.4E). However, treating cells expressing G$\alpha_s$-Rluc, CB$_1$-GFP$^2$ and D$_{2L}$-pcDNA with 10 $\mu$M haloperidol prior to 1 $\mu$M ACEA application resulted in a hyperbolic increase in BRET$_{Eff}$ between G$\alpha_s$-Rluc and CB$_1$-GFP$^2$, with BRET$_{Max}$ of $0.33 \pm 0.01$ and BRET$_{Min}$ of $0.25 \pm 0.01$ (Fig. 3.4E). The interaction between G$\alpha_s$ and CB$_1$ was specific and saturable in cells co-treated with ACEA and haloperidol.

Ligand-induced BRET$_{Eff}$ between G$\alpha_s$-Rluc and CB$_1$-GFP$^2$ was recorded for 9 min. No BRET$_{Eff}$ signals were observed following the application of 1 $\mu$M ACEA over the 9 min (540 s) observation period (Fig. 3.4F). Interestingly, treating cells with 10 $\mu$M haloperidol 50 s post-coelenterazine addition and 25 s prior to 1 $\mu$M ACEA application resulted in a delayed increase in BRET$_{Eff}$ between G$\alpha_s$ -Rluc and CB$_1$-GFP$^2$ (225 s after the application of ACEA) (Fig. 3.4F). The signal peaked at 5 min (300 s) following the addition of ACEA (375 s post-coelenterazine addition) (Fig. 3.4F). BRET$_{Eff}$ signal was still observed at 5, 10 and 20, but not at 30 min following ACEA (data not shown). As a positive control, we used $\beta_2$AR, which has been demonstrated to pre-assemble with G$\alpha_s$ (Lachance et al., 1999; Galés et al., 2005). We measured BRET$_{Eff}$ between G$\alpha_s$-Rluc and $\beta_2$AR -GFP$^2$ before and following the application of the $\beta_2$AR agonist isoproterenol (1 $\mu$M). High BRET$_{Eff}$ was observed between G$\alpha_s$-Rluc and $\beta_2$AR-GFP$^2$ in the absence of exogenous ligand. Isoproterenol led to a rapid and sustained elevation in BRET$_{Eff}$ (Fig. 3.4F). Therefore, the delayed BRET$_{Eff}$ between G$\alpha_s$ and CB$_1$ following ACEA and haloperidol application could be due to the recruitment of G$\alpha_s$ to CB$_1$ and its activation instead of the activation of pre-assembled GPCR-G protein complexes.

3.3.5 Combined D$_2$ Antagonism and CB$_1$ Agonism Induced CREB Phosphorylation

We observed that haloperidol treatment increased BRET$_{Eff}$ between G$\alpha_s$-Rluc and CB$_1$-GFP$^2$ in the presence of ACEA (Fig. 3.4). To confirm that haloperidol treatment induced functional coupling of G$\alpha_s$ to CB$_1$ following ACEA treatment, we measured
Figure 3.5: Co-Treatment with Haloperidol and ACEA Induced CREB phosphorylation.

(A) pCREB concentration-response curve from STHdh<sup>Q7/Q7</sup> cells treated with ACEA +/- HALO measured at 30 min. (B) STHdh<sup>Q7/Q7</sup> cells were treated with 1 μM ACEA +/- 10 μM HALO for 30 min with or without 24 h pre-treatment with 50 ng/ml PTx or CTx. *<sup>P</sup> < 0.01 compared to vehicle treatment; ~<sup>P</sup> < 0.01 compared to cells treated with 1 μM ACEA. (C) STHdh<sup>Q7/Q7</sup> cells were transfected with pcDNA or the CB<sub>1</sub>-BP and treated with 1 μM ACEA +/- 10 μM HALO for 30 min. *<sup>P</sup> < 0.01 compared to vehicle treatment; ~ < 0.01 compared to cells transfected with empty pcDNA and treated with 1 μM ACEA and 10 μM HALO. CREB phosphorylation was quantified via In-Cell™ Western Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
CTx-sensitive, G\(\alpha_s\)-dependent CREB phosphorylation (Fig. 3.5). A concentration-response curve of ACEA-induced CREB phosphorylation was generated following 30 min treatment (Fig. 3.5A). Treating STHdh\(^{Q7/Q7}\) cells with different concentrations of ACEA did not change CREB phosphorylation compared to vehicle-treated cells (Fig. 3.5A,B). Similarly, haloperidol (0.01-10 \(\mu\)M) treatment did not increase CREB phosphorylation (data not shown). Pre-treating the cells with haloperidol 25 s prior to the application of ACEA significantly increased CREB phosphorylation. The co-application of increasing concentrations of haloperidol reduced the EC\(_{50}\) and increased \(E_{\text{max}}\) for ACEA-induced CREB phosphorylation (Table 3.1). The Hill coefficient values were greater than 1, suggesting a positive cooperatively effects on CB\(_1\)-dependent CREB phosphorylation (Fig. 3.5A). To confirm that the observed CREB phosphorylation following the application of haloperidol and ACEA was G\(\alpha_s\)-dependent, cells were pre-treated with CTx for 24 hr. Pre-treating the cells with CTx blocked G\(\alpha_s\)-dependent CREB phosphorylation in cells co-treated with 1 \(\mu\)M ACEA and 10 \(\mu\)M haloperidol (Fig. 3.5B). CB\(_1\)-BP reduced ACEA induced CREB phosphorylation in haloperidol-treated cells (Fig. 3.5C). Therefore, co-treatment with haloperidol and ACEA induced G\(\alpha_s\)-mediated CREB phosphorylation through binding of haloperidol to CB\(_1{/}D_{2L}\).

To determine whether the observed effects of haloperidol on CB\(_1\) signaling was specific to haloperidol or common to other high-affinity D\(_2\) antagonists, we tested the influence of the high-affinity D\(_2\) antagonist, sulpiride, on the coupling of CB\(_1\) to G-proteins and downstream signaling. A reduction in ACEA-enhanced BRET\(^2\) between G\(\alpha_i\) and CB\(_1\) (Supplementary Fig. 3.3A) and G\(\alpha_i\) -dependent ERK phosphorylation (Suppl. Fig. 3.3B) was observed when STHdh\(^{Q7/Q7}\) cells were treated with 10 \(\mu\)M sulpiride and 1 \(\mu\)M ACEA. In addition, an increase in BRET\(^2\) signaling between G\(\alpha_s\) and CB\(_1\) (Supplementary Fig. 3.3C) and G\(\alpha_s\)-dependent CREB signaling (Supplementary Fig. 3.3D) was detected in STHdh\(^{Q7/Q7}\) cells were treated with 10 \(\mu\)M sulpiride and 1 \(\mu\)M ACEA. Our findings demonstrated that high affinity orthosteric D\(_2\) antagonists switch CB\(_1\) coupling and signaling from G\(\alpha_i\) to G\(\alpha_s\) in response to CB\(_1\) agonist when both CB\(_1\) and D\(_{2L}\) receptors are expressed.
3.3.6 CB₁ Agonism Resulted in Slow and Sustained β-arrestin1 Recruitment to CB₁ Receptor, Which Was 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 min using BRET². STHdh⁰⁷/⁰⁷ cells endogenously express β-arrestin1 (Laprairie et al., 2014). BRET_{Eff} signals observed from cells expressing β-arrestin1-Rluc and CB₁-GFP² treated with the vehicle was higher than BRET_{Eff} between β-arrestin1-Rluc and HERG-GFP² (Fig. 3.6A). ACEA (1 μM) treatment increased BRET_{Eff} between β-arrestin1-Rluc and CB₁-GFP² starting at 5 min compared to vehicle-treated cells and reached a plateau at 15 min. The signal was sustained for 30 min (Fig. 3.6A). However, treating the cells co-expressing β-arrestin1-Rluc, CB₁-GFP² and D₂L-pcDNA with 10 μM haloperidol 25 s prior to the application of 1 μM ACEA decreased BRET_{Eff} between β-arrestin1-Rluc and CB₁-GFP² over the 30 min (P < 0.01) compared to cells treated with 1 μM ACEA (Fig. 3.6A). BRET_{Eff} signals between β-arrestin1-Rluc and CB₁-GFP² occurred in an ACEA concentration-dependent manner (Fig. 3.6B). The addition of increasing concentrations of (0.1, 1 and 10 μM) haloperidol prior to ACEA application resulted in a lower $E_{Max}$ and EC₅₀ (Table 3.1). 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 D₂L-pcDNA with 10 μM haloperidol or 0.5 μM O-2050 alone did not alter BRET_{Eff} between β-arrestin1-Rluc and CB₁-GFP² compared to vehicle-treated cells (Fig. 3.6C). Our results demonstrated that D₂ antagonism reduced β-arrestin1 recruitment to CB₁ receptors in the presence of CB₁ agonist. CB₁ internalization was measured in STHdh⁰⁷/⁰⁷ cells transfected with CB₁-GFP² and β-arrestin1. CB₁ internalization was measured over 30 min following ligand treatment (Fig. 3.7A,B). Treating STHdh⁰⁷/⁰⁷ cells with 1 μM ACEA resulted in CB₁ internalization starting at 10 min compared to vehicle-treated cells (Fig. 3.7A, B). Treating the cells with 10 uM haloperidol alone or 0.5 μM O-2050 did not alter CB₁ localization compared to vehicle-treated cells (Fig. 3.7B). Pre-treating the cells with 0.5 μM O-2050 before the application of 1 μM ACEA inhibited CB₁ internalization over 30 min (Fig. 3.7A,B). Haloperidol pre-
3.6: **ACEA Treatment Resulted in Slow and Sustained β-arrestin1 Recruitment to CB₁ Receptors, Which Was Inhibited With Haloperidol.** (A) BRET<sub>Eff</sub> was measured over 30-min in cells expressing β-arrestin1-Rluc and CB₁-GFP<sup>2</sup> +/- D<sub>2L</sub>-pcDNA and treated with vehicle, 1 µM ACEA alone or together with 10 +/- HALO. As a control, cells were co-transfected with β-arrestin1-Rluc and HERG-GFP<sup>2</sup>. * P < 0.01 compared to vehicle-treated cells; ~ P < 0.01 compared to cells treated with 1 µM ACEA. (B) Concentration-response curves of ACEA- induced BRET<sub>Eff</sub> between β-arrestin1-Rluc and CB₁-GFP<sup>2</sup> in the absence or presence of increasing concentrations of HALO. (C) BRET<sub>Eff</sub> was measured at 30 min following the addition of vehicle, 1 µM ACEA +/- 10 µM HALO or with 30 min pre-treatment with 0.5 µM O-2050 in cells expressing β-arrestin1-Rluc and CB₁-GFP<sup>2</sup> alone or together with D<sub>2L</sub>-pcDNA. n.s. P > 0.05 compared with cells expressing β-arrestin1-Rluc and CB₁-GFP<sup>2</sup> only; ~ P < 0.01 relative to cells expressing β-arrestin1-Rluc and CB₁-GFP<sup>2</sup> and treated with 1 µM ACEA and 10 µM HALO; * P < 0.01 compared to cells treated with vehicle. Data are presented as mean ± SEM of 4 independent experiments, as determined via one-way ANOVA followed by Tukey's *post-hoc* test.
**A**

- β-arrestin1-Rluc + CB₁-GFP² + Vehicle
- β-arrestin1-Rluc + CB₁-GFP² + 1 µM ACEA
- β-arrestin1-Rluc + CB₁-GFP² + D₂L⁻-pcDNA + 10 µM HALO + 1 µM ACEA
- β-arrestin1-Rluc + HERG-GFP²

**B**

**HALO** [µM]

- 0.00
- 0.01
- 0.10
- 1.00
- 10.0

**C**

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<td>β-arrestin1-Rluc + CB₁-GFP² + D₂L⁻-pcDNA</td>
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Figure 3.7:  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 and CB₁-GFP² + D₂L-pcDNA measured using On-Cell Western™ and In-Cell Western™ in cells treated with vehicle, 1 µM ACEA +/- 10 µM HALO 0.5 µM O-2050. * P < 0.01 compared with vehicle. (B) CB₁ cell surface expression measured at 30 min following ligand treatment. * P < 0.01 compared with vehicle-treated cells. ~ P < 0.01 compared to ACEA-treated cells. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
treatment significantly inhibited ACEA-induced CB₁ internalization compared to ACEA-treated cells. Endogenous CB₁ internalization was also measured in STHdhQ7/Q7 cells (Supplementary Fig. 4). The reduction of BRET² between β-arrestin1-Rluc and CB₁-GFP² following transfection is consistent with the observation that endogenous CB₁ receptor internalization was reduced following haloperidol and ACEA treatment (Supplementary Fig. 3.4).

3.4 Discussion

3.4.1 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/D₂L heteromers. We investigated the influence of D₂ antagonism on CB₁ coupling to Gαᵢ and Gαₛ, as well as β-arrestin1 recruitment and internalization in a model of striatal medium spiny projection neurons (Summarized in Fig. 3.8). In this study, we were able to show that CB₁ and D₂L receptors heteromerize when co-expressed in STHdhQ7/Q7 cells in the absence and presence of exogenous CB₁ ligand. CB₁ agonist treatment stabilized the conformation of the pre-assembled CB₁/D₂L heteromers but did not alter the number of receptors involved in forming CB₁/D₂L complexes. CB₁ was coupled to Gαᵢ in the absence of CB₁ ligand. Agonist-dependent CB₁-activation led to a rapid and transient conformational rearrangement of the pre-assembled CB₁-Gαᵢ complexes, rather than the recruitment of Gαᵢ-proteins to CB₁. Activation was observed as a rapid increase in ERK phosphorylation through the PTx-sensitive Gαᵢ pathway (Galés et al., 2005, 2006; Levoye et al., 2009). Sustained activation of CB₁ was followed by the return of CB₁-Gαᵢ complexes to the inactive conformation rather than dissociation of CB₁ and Gαᵢ protein (Bunemann et al., 2003; Galés et al., 2006). Interactions between Gαᵢ and CB₁ were completely undetectable following CB₁ agonist and D₂ antagonist co-treatment. 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,
Figure 3.8: Kinetic Interaction of CB₁ Receptor and CB₁/D₂ Heteromers With Gaᵢ, Gaₛ, and β-arrestin1. (A, C) BRET² data demonstrated that CB₁ receptor is pre-assembled with Gaᵢ. CB₁ agonist induced fast and transient increases in BRET² indicating conformational changes within the pre-assembled CB₁-Gaᵢ complexes. The deactivation phase of the pre-assembled CB₁-Gaᵢ occurs parallel to the slow and stable recruitment of β-arrestin1. (B, D) CB₁/D₂L pre-assembled complexes are coupled to Gaₛ. CB₁ agonist and D₂ antagonist induced a delayed and sustained recruitment of Gaₛ to CB₁/D₂ complexes. Reduced and sustained recruitment of β-arrestin1 to the CB₁/D₂L/Gaₛ was observed.
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., 2010b). In the presence of ACEA, haloperidol switched the CB1 coupling from Ga to Ga, and induced Ga-dependent CREB phosphorylation. Previous studies have demonstrated that the co-expression and co-activation of both CB1 and D2L receptors are required to switch CB1 signaling from Ga, to Ga, (Glass and Felder, 1997; Kearn et al., 2005). In our study, we found that the co-expression of both receptors and the addition of a CB1 agonist and D2 antagonist was sufficient to induce conformational changes within the pre-assembled CB1/D2L/Ga complexes and favor a higher proportion of CB1 to dissociate from Ga, (Bunemann et al., 2003; Galés et al., 2006).

The delayed interaction between Ga, and CB1 following CB1 agonist and D2 antagonist application cannot be considered as a general feature for Ga, coupling since fast activation of Ga, following ligand binding has been demonstrated for other GPCRs. For example, the β2AR receptor is known to pre-assemble with Ga, (Lachance et al., 1999), which we also observed as a fast increase in BRETeff between Ga, -Rluc and β2AR-GFP2. The delayed interaction between Ga, and CB1 could be due to the recruitment of Ga, to CB1 and its activation instead of the activation of pre-assembled GPCR-G protein complexes (Ayoub et al., 2010). The “pre-assembled model” between GPCRs and G proteins can explain the fast increase in BRETeff signal between Ga,-Rluc and CB1-GFP2 (Janetopoulos et al., 2001; Galés et al., 2006; Ayoub et al., 2007, 2012). However, the interaction between Ga, and CB1 is more compatible with the “free collision model”. This model also proposes that GPCRs can interact 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 Ga, and inducing CREB phosphorylation in cells treated with ACEA, compared to haloperidol. Both haloperidol and sulpiride are D2 antagonists, but the two drugs have different receptor dissociation properties from D2 receptors, which result in different kinetics of D2 blockade. Haloperidol binds with higher affinity to the 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 $E_{\text{Max}}$ for $G\alpha_s$-dependent CREB activation.

### 3.4.2 Haloperidol Reduced $\beta$-arrestin1 Recruitment to CB1

Heteromerization is known to affect $\beta$-arrestin recruitment. $\beta$-arrestin2 (Jin et al., 1999; van der Lee et al., 2009) and $\beta$-arrestin1 facilitate the internalization of CB1 after activation (Laprairie et al., 2014). We demonstrated that D2 antagonism inhibited CB1 agonist-induced recruitment of $\beta$-arrestin1 to CB1/D2L/$G\alpha_s$ complexes and inhibited CB1 receptor internalization in STHdhQ7/Q7 cells in a dose-dependent manner. Therefore, antagonism of one receptor in a GPCR heteromer may allosterically inhibit agonist-induced $\beta$-arrestin1 recruitment of the other receptor. However, a fraction of the response was not antagonized by haloperidol, 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., 2010b). It is unknown at this time how repeated stimulation of CB1 and D2L would affect receptor desensitization.

### 3.4.3 Allosteric Interaction Between CB1 and D2L

Allosteric communications 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 D2L following agonist-dependent co-activation of both receptors, the current work indicates that allosteric interactions are dependent on D2L receptor ligand binding and are not limited to D2L 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 non-linear regression model.
with variable slope (four parameters). Haloperidol treatment was associated with negative cooperativity between Gαi and CB1 because treating the cells with haloperidol decreased both the $E_{\text{Max}}$ and Hill coefficient of the ACEA-mediated Gαi and CB1 interaction and ACEA-mediated pERK concentration–response. However, haloperidol treatment was associated with positive cooperativity between Gαs and CB1 because haloperidol increased the $E_{\text{Max}}$ and Hill coefficient of Gαs and CB1 interaction and ACEA-mediated pCREB response. Whether haloperidol alters cannabinoid agonist affinity to the CB1 receptor is still to be determined.

### 3.5 Conclusion

In addition to defining pharmacological interactions between CB1 and D2L receptors, this work may have clinical implications. Many central nervous system diseases, including schizophrenia, Huntington disease (HD) 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. Based on 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 HD where levels of CB1 and D2 decline with disease progression (Augood et al., 1997; Denovan-Wright and Robertson, 2000) drug response and response to co-administration of CB1 agonists and D2 antagonists may be even more complex than that observed for non HD’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 HD and other disorders.
3.6 Supplementary Figures

Supplementary Figure 3.1: STHdhQ7/Q7 Cells Endogenously Co-Express CB1 and D2 Receptors. STHdhQ7/Q7 cells express CB1 and D2 mRNAs as demonstrated by RT-PCR (A) and qRT-PCR (B) using RNA extracted form STHdhQ7/Q7 cells. (C) Total CB1 and D2 protein abundance was determined In-Cell™ Western normalized to β-actin levels. * P < 0.01, as determined via t-test. n =4. (D) CB1 and D2 proteins are co-localized as confirmed by confocal images of a representative STHdhQ7/Q7 cells stained by immunofluorescence for CB1 using a Cys2-conjugated secondary antibody (left panel) and for D2 using a Cy3 conjugated secondary antibody (middle panel); and the merged image (right panel).
Supplementary Figure 3.2: The Expression of The CB₁ blocking peptide (CB₁-BP) Did Not Alter the Expression of Rluc and GFP² Tagged Receptors. Quantification of the Rluc activity and GFP² fluorescence measured in cells expressing of CB₁-Rluc and D₂L-GFP² (1:2 ratios) or CB₁-Rluc and CB₁-GFP² (1:2 ratios) together with CB₁-BP or pcDNA vector. n.s. > 0.05 relative to cells expressing CB₁-Rluc, D₂L-GFP² and pcDNA or CB₁-Rluc, CB₁-GFP² and pcDNA. Data are presented as mean ± SEM of four independent experiments, one-way ANOVA followed by Tukey’s post-hoc test.
Supplementary Figure 3.3: Similarly to Haloperidol, Sulpiride Reduced ACEA-Induced ERK Phosphorylation. BRET$_{Eff}$ was measured in cells expressing $\alpha_i$-Rluc and $\text{CB}_1$-GFP$^2$ +/- with D$_{2L}$-pcDNA (A) or $\alpha_i$-Rluc and $\text{CB}_1$-GFP$^2$ +/- with D$_{2L}$-pcDNA (C) and treated with vehicle, 10 µM sulpiride (SULP), 1 µM ACEA +/- 1 µM SULP and pre-treated for 30 min with 0.5 µM O-2050. n.s. $P > 0.05$ relative to cells expressing $\alpha_i$-Rluc and $\text{CB}_1$-GFP$^2$; $\sim P < 0.01$ relative to cells expressing $\alpha_i$-Rluc and $\text{CB}_1$-GFP$^2$ and treated with 1 µM ACEA and 10 µM SULP; * $P < 0.01$ compared to cells treated with vehicle. STHdh$^{Q7/Q7}$ cells were treated with 1 µM ACEA +/- 10 µM SULP +/- 24 h pretreatment with 50 ng/ml PTx or CTx and ERK phosphorylation was measured following 5 min treatment (B), while CREB phosphorylation was measured following 30 min treatment (D). * $P < 0.01$ compared to vehicle treatment; $\sim P < 0.01$ compared to cells treated with 1 µM ACEA. Data are presented as mean ± SEM of four independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
Supplementary Figure 3.4: Haloperidol Inhibited Endogenous CB₁ Internalization Following ACEA Treatment. (A) Time-course analysis of CB₁ cell surface expression and total protein levels over 30 min measured using On-Cell Western™ and In-Cell Western™ in cells treated with vehicle, 10 μM HALO, 0.5 μM O-2050 1 μM ACEA +/- 10 μM HALO or 0.5 μM O-2050. * P < 0.01 compared with vehicle. (B) CB₁ cell surface expression measured at 30 min following ligand treatment. * P < 0.01 compared with vehicle-treated cells. ~ P < 0.01 compared to ACEA-treated cells. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
A

Percent Plasma Membrane CB₁
(On cell CB₁ / Total CB₁)

Time (min)

△ 1 μM ACEA
△ 10 μM HALO + 1 μM ACEA
◆ 0.5 μM O-2050 + 1 μM ACEA
● Vehicle

B

Percent Plasma Membrane CB₁
(On cell CB₁ / Total CB₁)

Vehicle
1 μM ACEA
10 μM HALO
0.5 μM O-2050
10 μM HALO + 1 μM ACEA
0.5 μM O-2050 + 1 μM ACEA
CHAPTER 4

BIDIRECTIONAL ALLOSTERIC INTERACTIONS BETWEEN CANNABINOID RECEPTOR 1 (CB₁) AND DOPAMINE RECEPTOR 2 L (D₂L) HETEROTETRAMERS

Copyright Statement

This chapter has been previously published in: Amina M. Bagher, Robert B. Laprairie, J. Thomas Toguri, Melanie E.M. Kelly, and Eileen M. Denovan-Wrigh (2016). Bidirectional Allosteric Interactions Between Cannabinoid Receptor 1 (CB₁) and Dopamine Receptor 2 Long (D₂L) Heterotetramers. the European Journal of Pharmacology July 2017 (in press). The manuscript has been modified to meet formatting requirements.

Contribution Statement

The manuscript used as the basis for this chapter was written with guidance from Dr. Eileen Denovan-Wright. Data were collected and analyzed by myself. Critical reagents were provided by Drs. Eileen Denovan-Wright and Melanie Kelly.
4.1 Abstract

Type 1 cannabinoid (CB₁) and dopamine 2 long form (D₂L) receptors can physically interact to form heteromers that display unique pharmacology in vitro compared to homomeric complexes. Co-expression of CB₁ and D₂L and co-application of CB₁ and D₂ agonists increases cAMP levels while administration of either agonist alone decreases cAMP levels. To understand the observed co-agonist response, our first goal of the current study was to define the stoichiometry of CB₁/D₂L/Gα protein complexes. Using bioluminescence resonance energy transfer 2 (BRET²), we confirmed that, CB₁ homodimers, D₂L homodimers, and CB₁/D₂L heteromers are formed. By using sequential energy transfer 2 (SRET²) combined with bimolecular fluorescence complementation (BiFC), we were able to demonstrate that CB₁/D₂L form heterotetramers consisting of CB₁ and D₂L homodimers. We demonstrated that CB₁/D₂L heterotetramers are coupled to at least two Gα proteins. The second aim of the study was to investigate allosteric effects of a D₂L agonist (quinpirole) on CB₁ receptor function and to investigate the effects of a CB₁ agonist [arachidonyl-2-chloroethylamide (ACEA)] on D₂L receptor function within CB₁/D₂L heterotetramers. Treating cells co-expressing CB₁ and D₂L with both ACEA and quinpirole switched CB₁ and D₂L receptor coupling and signaling from Gαi to Gαs proteins, enhanced β-arrestin1 recruitment and receptor co-internalization. The concept of bidirectional allosteric interaction within CB₁/D₂ heterotetramers has important implications for understanding the activity of receptor complexes in native tissues and under pathological conditions.

4.2 Introduction

It is well established that family A G protein-coupled receptors (GPCRs) can physically associate to form both homo- and hetero-oligomeric complexes (reviewed in Milligan, 2013; Bouvier and Hébert, 2014; Ferré et al., 2014, 2015; Gomes et al., 2016; Franco et al., 2016). To date, the evidence suggests that a minimum of two GPCR homodimers interact to form hetero-oligomeric complexes and each GPCR homodimer associates with one G protein within hetero-oligomeric complexes (Han et al., 2009; Jastrazebka et al., 2013; Guitart et al., 2014; Bonaventura et al., 2015; Navarro et al., 2016). GPCR oligomerization allosterically induces conformational changes in each
receptor within the complex (Vilardaga et al., 2008; Maier-Peuscher et al., 2010; Bourque et al., 2017; Sleno et al., 2017). Allosteric interactions within hetero-oligomeric complexes result in unique pharmacology compared to homo-oligomeric complexes. Binding of a ligand to one of the GPCR homodimeric partners can modify the affinity or efficacy of ligands for the other GPCR homodimeric unit. Such allosteric modulation may result in positive or negative cooperativity across the heteromeric pairs and alter signaling bias (Kanakin and Christopoulos, 2013; Wootten et al., 2013).

The type 1 cannabinoid receptor (CB$_1$) and the dopamine receptor 2 long (D$_{2L}$) can physically interact to form CB$_1$ and D$_{2L}$ homomers as well as with each other to form CB$_1$/D$_{2L}$ heteromers (Wager-Miller et al., 2002; Kearn et al., 2005; Guo et al., 2008; Marcellino et al., 2008; Przybyla and Watts 2010; Bagher et al., 2016). Heteromerization between CB$_1$ and D$_{2L}$ is associated with altered function of hetero- compared to homo-oligomeric complexes. Stimulation of either CB$_1$ or D$_{2L}$ leads to an inhibition of cAMP via Pertussis toxin (PTx)-sensitive Ga$_{i/o}$ proteins (Felder et al., 1992; Sibley and Monsma 1992; Demuth and Molleman, 2006). In contrast, co-stimulation of both receptors by their respective agonists leads to an accumulation of cAMP (Glass and Felder, 1997; Kearn et al., 2005; Marcellino et al., 2008; Khan and Lee, 2014; Bagher et al., 2016). Switching in coupling from Ga$_i$ to Ga$_s$ proteins has been proposed to contribute to the observed increase in cAMP following co-activation of both receptors (Glass and Felder, 1997; Kearn et al., 2005). To date, there is no evidence of a physical association between CB$_1$/D$_{2L}$ heteromers and Ga$_s$ proteins following agonist co-treatment. Similar to other GPCRs, CB$_1$ and D$_{2L}$ also signal via β-arrestins. Both β-arrestin1 (Kim et al., 2001; Bakshi et al., 2007; Amar et al., 2008; Laprairie et al., 2014) and β-arrestin2 (Jin et al., 1999; Kim et al., 2001; Masri et al., 2008; van der Lee et al., 2009; Huang et al., 2013) are recruited to agonist-activated CB$_1$ and D$_{2L}$ and facilitate receptor internalization and G protein-independent extracellular signal-regulated kinase (ERK) activation (Laprairie et al., 2014). Whether simultaneous treatment with CB$_1$ and D$_{2L}$ agonists also alters β-arrestin1 recruitment to CB$_1$/D$_{2L}$ receptor complexes and leads to receptor co-internalization has not been studied.

Given that CB$_1$/D$_{2L}$ dimerize and that higher order hetero-oligomers are minimally composed of homodimeric pairs, we hypothesized that CB$_1$ homodimers
selectively dimerize with D2L homodimers and that one Gαi protein couples to the CB1-homodimer while another Gαi protein couples to D2L-homodimer within CB1/D2L heterotetramers. We further hypothesized that CB1/D2L complexes respond differentially to combinations of CB1- and D2-selective agonists compared to either agonist alone.

4.3 Results

4.3.1 CB1 and D2L Form Higher order Heteromers

The first objective of our study was to measure the relative affinities of CB1 and D2L homomers compared to CB1/D2L heteromers. BRET^2 saturation curves of CB1 homomers, D2L homomers and CB1/D2L heteromers were generated. For BRET^2 saturation curves, HEK 293A cells were transfected with a constant amount of one Rluc-tagged receptor and increasing amounts of a second GFP^2-tagged receptors. BRET^50 values obtained from BRET^2 saturation curves are indicative of the affinity of receptors to form complexes when they are co-expressed (Guan et al., 2009). The negative control included human mGLuR6, mGLuR6-GFP^2, a family A GPCR that does not interact with CB1 (Hudson et al., 2010; Bagher et al., 2013). The combination of CB1-Rluc and mGLuR6-GFP^2 resulted in BRET_Eff of 0.08 ± 0.03, which is significantly lower compared to the BRET_Eff observed for CB1 homomers, D2L homomers and CB1/D2L heteromers (Fig. 4.1A). The CB1 homomer saturation curve obtained from cells transfected with CB1-Rluc and CB1-GFP^2 yielded a BRET^50 of 0.31 ± 0.05 (Fig. 4.1A, 1B). The D2L homomer saturation curve obtained from cells expressing D2L-Rluc and D2L-GFP^2 resulted in BRET^50 value of 0.28 ± 0.04 (Fig. 4.1A,B). The CB1-Rluc and D2L-GFP^2 heteromer saturation curve yielded a BRET^50 value of 0.27 ± 0.03 (Fig. 4.1A,B). There were no significant differences in BRET^50 values among CB1 homomers, D2L homomers and CB1/D2L heteromers. These findings demonstrated CB1 and D2L receptors have similar affinities to form both homo and heteromers when expressed in HEK 293A cells.

The oligomerization state of CB1 and D2L homo- and heteromers were assessed by fitting BRET^2 saturation curve values to the mathematical model of Veatch and Stryer model (Eq. 1; Vrecl et al., 2006; Drinovec et al., 2012). In our experiments, the E values
Figure 4.1: CB$_1$ and D$_{2L}$ Receptors Formed Both Homomers and Heteromers When Expressed in HEK 293A Cells Demonstrated Using BRET$^2$. (A) BRET$^2$ saturation curves obtained from cells transiently transfected with CB$_1$-Rluc and CB$_1$-GFP$^2$, D$_{2L}$-Rluc and D$_{2L}$-GFP$^2$ or CB$_1$-Rluc and D$_{2L}$-GFP$^2$. As a negative control, cells were co-transfected with CB$_1$-Rluc and mGLuR6-GFP$^2$. BRET$_{Eff}$ was plotted against the ratio of GFP$^2$ fluorescence and Rluc emission. The data were fit to a rectangular hyperbola. (B) BRET$_{Max}$ and BRET$_{50}$ parameters derived from BRET$^2$ saturation curves. A model curve BRET= BRET$_{Max}$ (1−1/(E +(1−E)(1+[A]/[D])$^N$)) was used, where [D] and [A] are donor and acceptor concentrations, E is energy transfer efficiency and N is oligomerization state (1 = dimer, 2 = trimer, 3=tetramer). Data are presented as mean ± SEM of 4 independent experiments.
A

B

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<th>Constructs</th>
<th>BRET&lt;sub&gt;Max&lt;/sub&gt;</th>
<th>BRET&lt;sub&gt;50&lt;/sub&gt;</th>
<th>N</th>
<th>E</th>
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<tr>
<td>CB₁-Rluc + CB₁-GFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.23 ± 0.02</td>
<td>0.31 ± 0.05</td>
<td>1.0 ± 0.31</td>
<td>0.18</td>
</tr>
<tr>
<td>D₂L-Rluc + D₂L-GFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.26 ± 0.02</td>
<td>0.28 ± 0.04</td>
<td>1.0 ± 0.22</td>
<td>0.20</td>
</tr>
<tr>
<td>CB₁-Rluc + D₂L-GFP&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.32 ± 0.01</td>
<td>0.27 ± 0.03</td>
<td>2.6 ± 0.24</td>
<td>0.21</td>
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were low \((E < 0.2)\) and not significantly different for the CB\(_1\) and D\(_{2L}\) homono- and heteromers BRET\(^2\) saturation curves (Vrecl \textit{et al}., 2006; Drinovec \textit{et al}., 2012). The calculated oligomerization state \((\bar{N})\) suggested that CB\(_1\) \((\bar{N} = 1.00 \pm 0.31)\) and D\(_{2L}\) \((\bar{N} = 1.0 \pm 0.22)\) form mainly homodimers as \(\bar{N}\) is not different from 1 (Fig. 4.1B). In contrast, co-expression of CB\(_1\) and D\(_{2L}\) resulted in a calculated oligomerization state value of \(\bar{N} = 2.61 \pm 0.24\) (Fig. 4.1B), which implied that CB\(_1\)/D\(_{2L}\) heteromers were present as tetramers or higher-order oligomeric complexes.

To directly test the mathematical prediction that CB\(_1\) and D\(_{2L}\) homodimers form a heterotetrameric structure, we utilized SRET\(^2\) combined with BiFC (Fig. 4.2A). In this approach, the oxidation of the Rluc substrate coelenterazine 400a by the donor Rluc-fused protein (D\(_{2L}\)-Rluc) excites the BRET\(^2\) acceptor GFP\(^2\)-fused protein (D\(_{2L}\)-GFP\(^2\)) and emission from GFP\(^2\) then excites the FRET acceptor EYFP Venus. The EYFP Venus acceptor is composed of CB\(_1\) fused to the EYFP Venus N-terminal hemiprotein (CB\(_1\)-VN), and CB\(_1\) fused to the EYFP Venus C-terminal hemiprotein (CB\(_1\)-VC) (Carriba \textit{et al}., 2008; Navarro \textit{et al}., 2013) (Fig. 4.2A).

We first confirmed that EYFP Venus was reconstituted following CB\(_1\) homodimerization using BiFC. An increase in fluorescence was observed when HEK 293A cells were transfected with CB\(_1\)-VN and CB\(_1\)-VC at 1:1 ratio (Supplementary Fig. 4.1A). In cells expressing only CB\(_1\)-VN or CB\(_1\)-VC no fluorescence was detected (Supplementary Fig. 4.1A). The reconstitution of functional EYFP in the presence of CB\(_1\)-VN and CB\(_1\)-VC confirmed that CB\(_1\) forms homodimers when expressed in HEK 293A cells. Using fluorescence microscopy, we observed that the CB\(_1\)-VN and CB\(_1\)-VC were co-localized with CB\(_1\)-GFP\(^2\) (data not shown). The ratio of CB\(_1\)-VN and CB\(_1\)-VC was kept constant at a ratio of 1:1 for all subsequent experiments.

To test the hypothesis that D\(_{2L}\) homodimers associate with CB\(_1\) homodimers to form heterotetramers or higher-order oligomers we generated SRET\(^2\) combined with BiFC saturation curve. We selected the D\(_{2L}\)-Rluc and D\(_{2L}\)-GFP\(^2\) cDNA ratio that produced the BRET\(_{50}\) value calculated from the D\(_{2L}\) homodimer saturation curve (Fig. 4.1B). The ratio used for all subsequent experiments was 1:0.5 ratio for D\(_{2L}\)-Rluc and D\(_{2L}\)-GFP\(^2\). Higher D\(_{2L}\)-Rluc and D\(_{2L}\)-GFP\(^2\) ratios resulted in excessive emission and overlap and obscured EYFP Venus emission (data not shown). Cells were transfected
Figure 4.2: CB1 and D2L Receptors Form Heterotetramers in HEK 293A Cells Demonstrated by SRET² Combined with BiFC. (A) Scheme of SRET² combined with BiFC, D2L was tagged with Rluc (D2L-Rluc) and GFP² (D2L-GFP²), while CB1 was tagged with EYFP Venus N-terminal hemiprotein (CB1-VN) and the EYFP Venus C-terminal hemiprotein (CB1-VC). The oxidation of coelenterazine 400a by Rluc triggers the acceptor GFP² excitation by BRET² and subsequent energy transfer to the FRET acceptor EYFP Venus. Numbers indicate the peak wavelength of the emitted light. (B) SRET² saturation curves were obtained using HEK 293A cells transfected with a constant amount of D2L-Rluc and D2L-GFP² (1:0.5) and increasing amounts of EYFP Venus-tagged CB1 (CB1-VN and CB1-VC at 1:1 ratio). Net SRET² was plotted against the ratio of EYFP fluorescence and Rluc emission. As a negative control, cells were transfected with equivalent amounts of D2L-Rluc + mGluR6-GFP², and increasing amounts of EYFP Venus-tagged CB1 (CB1-VN and CB1-VC at 1:1 ratio). (C) SRET² assays in cells transfected with D2L-Rluc, D2L-GFP², CB1-VN, and CB1-VC or negative controls. * P < 0.01 compared to cells expressing D2L-Rluc + mGluR6-GFP² + CB1-VN + CB1-VC; ~P < 0.01 compared to cells expressing D2L-Rluc + D2L-GFP² + CB1-VN + CB1-VC. # P < 0.01 compared to cells expressing D2L-Rluc, D2L-GFP², β2AR-VN, and β2AR-VC. Data are presented as mean ± SEM of 3 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
with a constant amount of the D2L-Rluc and D2L-GFP2 constructs (1:0.5 ratio) and increasing amounts of the constructs encoding the EYFP Venus protein (CB1-VN + CB1-VC, 1:1 ratio) (Fig. 4.2B). Increasing the concentration of EYFP Venus protein (CB1-VN+ CB1-VC) resulted in a hyperbolic increase in net SRET. From the saturation curve, we calculated that the SRET Max value for hetero-oligomerization was 0.18 ± 0.01 and the SRET50 value was 6.5 ± 0.84. As a negative control, cells were transfected with a constant amount of the D2L-Rluc and mGLuR6-GFP2 (1:0.5 ratio) and increasing concentration of EYFP Venus protein construct (CB1-VN + CB1-VC, 1:1 ratio). Cells expressing the negative controls yielded a weak and non-saturating SRET signal (Fig. 4.2B) demonstrating the lack of specific interaction when mGLuR6 was present. Based on these experiments, we selected the optimal cDNA ratio of 1:0.5:4:4 for D2L-Rluc: D2L-GFP2: CB1-VN: CB1-VC for subsequent SRET determinations. The SRET efficiency was minimal or negligible when we expressed constructs encoding GFP instead of D2L-GFP2 or EYFP instead of CB1-VC + CB1-VN (Fig. 4.2C). As a control for the specificity of the interaction between D2L homodimer and CB1 homodimer, we performed SRET combined with BiFC in cells expressing D2L and β2AR, which do not interact with the D2L. We confirmed, using BiFC, that EYFP Venus can be reconstituted when the β2AR fused to the EYFP Venus N-terminal hemiprotein (β2AR-VN) and β2AR fused to the EYFP Venus C-terminal hemiprotein (β2AR-VC) were co-expressed in HEK 293A cells (Supplementary Fig. 4.1B). Significant fluorescence was observed in HEK 293A cells transfected with β2AR-VN and β2AR-VN, confirming that β2AR-VN and β2AR-VN formed β2AR homodimers in HEK 293A cells (Supplementary Fig. 4.1B) as demonstrated previously (Hammad and Dupré, 2010). Net SRET values were significantly higher between D2L and CB1 compared to D2L and β2AR indicating that the interaction between D2L and CB1 was selective (Fig. 4.2C). Taken together, our results demonstrate a selective interaction between D2L and CB1 homodimers into oligomeric complexes composed of at least two D2L and two CB1 receptors.

The CB1/D2L hetero-oligomer blocking peptide (CB1-BP) binds to the C-terminal tail of CB1 and blocks the interaction between CB1 and D2L, but not the interactions between CB1 homomers (Khan and Lee, 2014; Bagher et al., 2016). Co-expression of CB1-BP with CB1-VN and CB1-VC did not change EYFP fluorescence observed when
CB1-VN and CB1-VC were expressed alone (Supplementary Fig. 4.1A). In contrast, co-expression of CB1-BP with D2L-Rluc + D2L-GFP^2 + CB1-VN and CB1-VC interrupting the energy transfer from D2-GFP^2 to EYFP Venus and resulted in significantly lower SRET\(^2\) value compared to cells transfected only with D2L-Rluc + D2L-GFP^2 + CB1-VN and CB1-VC (Fig. 4.2C). Selective inhibitions of the energy transfer between D2L and CB1 constructs demonstrated that CB1-BP interferes with the formation of CB1/D2L hetero-oligomers without interrupting the formation of CB1 homodimers. Although we acknowledge that higher order structures are possible, these experiments define the minimum complex of D2L and CB1 receptors as being a heterotetramer composed at least one D2L and one CB1 homodimer.

### 4.3.2 CB1/D2L Receptors Form Heterotetramers Consisting of CB1 and D2L Homomers in Complex with at Least Two Ga Proteins

Based on our finding that CB1/D2L minimally form heterotetramers and recent studies that suggested GPCRs form heterotetramers in complex with two Ga proteins (Navarro et al., 2016), we hypothesized that one Ga\(_i\) protein couples to a CB1 homodimer while another Ga\(_i\) protein couples to a D2L-homodimer within CB1/D2L heterotetramers. The interaction between Ga\(_i\) and CB1 was studied using BRET\(^2\). Higher BRET\(_{Eff}\) signals were observed between Ga\(_i\)-Rluc and CB1-GFP^2 compared to BRET\(_{Eff}\) obtained from cells transfected with Ga\(_i\)-Rluc and β2AR (Fig. 4.3A). The β2AR receptor is known to pre-assemble with Ga\(_s\) (Lachance et al., 1999; Galés et al., 2005). Such an increase in BRET\(_{Eff}\) was insensitive to 24 hr PTx treatment. PTx inhibits the activity and dissociation of Ga\(_i\) following ligand-dependent activation or constitutive activity of GPCRs (Ayoub et al., 2007). PTx does not inhibit the physical association of Ga\(_i\) with GPCRs (Ayoub et al., 2010). As PTx did not inhibit the association between Ga\(_i\)-Rluc and CB1-GFP^2, the increase in BRET\(_{Eff}\) was not due to constitutive activation of CB1 receptors (Fig. 4.3A). This data confirmed that CB1 receptors are pre-assembled with Ga\(_i\) protein (Demuth and Molleman, 2006). BRET\(^2\) saturation curve was generated to determine that the interaction between Ga\(_i\)-Rluc and CB1-GFP^2 (data not shown). The CB1 agonist ACEA (1 μM) increased the observed BRET\(_{Eff}\) between Ga\(_i\)-Rluc and CB1-GFP^2 compared to the BRET\(_{Eff}\) observed in vehicle-treated cells (Fig. 4.3A). The increase
Figure 4.3 CB₁/D₂L Heterotetramers are Pre-Coupled to Gαᵢ Proteins. (A) BRET$_{\text{Eff}}$ was measured in cells expressing with CB₁-GFP² and Gαᵢ-Rluc +/- un-tagged D₂L-pcDNA following the addition of vehicle, 1 μM ACEA, 1 μM quinpirole and pre-treated for 24 hr min with 50 ng/ml PTx.; * $P < 0.01$ compared to cells expressing only Gαᵢ-Rluc and HERG- GFP²; – $P < 0.01$ relative to cells expressing only Gαᵢ-Rluc and CB₁-GFP² and treated with 1μM quinpirole; n.s. $P > 0.05$ compared to cells expressing Gαᵢ-Rluc and CB₁-GFP² only. (B) Scheme of BRET². A more efficient energy transfer was observed between Gαᵢ-Rluc and CB₁-GFP² in the presence of un-tagged D₂L following CB₁ agonist treatment compared to D₂L agonist treatment. (C) BRET$_{\text{Eff}}$ was measured in cells expressing Gαᵢ-Rluc and D₂L-GFP² +/- un-tagged CB₁-pcDNA following the addition of vehicle, 1 μM quinpirole, 1 μM ACEA and pre-treated for 24 hr min 50 ng/ml PTx. * $P < 0.01$ compared to cells expressing Gαᵢ-Rluc and β₂-GFP²; – $P < 0.01$ relative to cells expressing Gαᵢ-Rluc and D₂L-GFP² only and treated with 1μM ACEA; n.s. $P > 0.05$ compared to cells expressing only Gαᵢ-Rluc and D₂L-GFP². (D) Scheme of BRET². A more efficient energy transfer was observed between Gαᵢ-Rluc and D₂L-GFP² in the presence of un-tagged CB₁ following D₂L agonist treatment compared to CB₁ agonist treatment. Data are presented as mean ± SEM of 3 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
in BRETEff following ACEA application was rapid and transient; BRETEff peaked at ~125 sec following ACEA application and remained significantly elevated for ~ 400 sec before declining (Supplementary Fig. 4.2A) (Bagher et al., 2016). Therefore, all BRETEff measured between Ga\textsubscript{i}-Rluc and CB\textsubscript{1}-GFP\textsuperscript{2} was performed ~125 sec following ligand application. CB\textsubscript{1} agonism stabilized the conformation of CB\textsubscript{1}/Ga\textsubscript{i} increasing maximal energy transfer in the BRET\textsuperscript{2} assay. The co-expression of un-tagged D\textsubscript{2L}-pcDNA receptors did not alter the interaction between CB\textsubscript{1} and Ga\textsubscript{i} in the presence of vehicle or ACEA (Fig. 4.3A). Regardless of the presence of un-tagged D\textsubscript{2L}-pcDNA, PTx blocked ACEA-dependent increases in BRETEff demonstrating that ACEA was acting on the Ga\textsubscript{i}-coupled CB\textsubscript{1} receptor (Fig. 4.3A). A more efficient energy transfer was observed between Ga\textsubscript{i}-Rluc and CB\textsubscript{1}-GFP\textsuperscript{2} only in the presence of un-tagged D\textsubscript{2L} and the D\textsubscript{2} agonist (quinpirole 1 \textmu M) treatment (Fig. 4.3A,B); this increase in BRETEff was PTx-sensitive. Together these observations indicate that CB\textsubscript{1} was pre-assembled with Ga\textsubscript{i} proteins and that treating cells expressing both CB\textsubscript{1} and D\textsubscript{2L} receptors with either CB\textsubscript{1} or D\textsubscript{2} agonists increased BRETEff signals between Ga\textsubscript{i} protein and CB\textsubscript{1} (Fig. 4.3B).

Next, the interaction between Ga\textsubscript{i}-Rluc and D\textsubscript{2L}-GFP\textsuperscript{2} was studied using BRET\textsuperscript{2} (Fig. 4.3C). Co-expression of Ga\textsubscript{i}-Rluc and D\textsubscript{2L}-GFP\textsuperscript{2} resulted in an increase in BRETEff, which was insensitive to PTx treatment (Fig. 3.3C) indicating that D\textsubscript{2L} was pre-assembled with Ga\textsubscript{i} proteins. Treating the cells with the D\textsubscript{2} agonist quinpirole (1 \textmu M) resulted in a rapid and transient increase in BRETEff, which was indicative of D\textsubscript{2L} receptors activation (Fig. 4.3C; Supplementary Fig. 4.2C) Therefore, all BRETEff measured between Ga\textsubscript{i}-Rluc and D\textsubscript{2L}-GFP\textsuperscript{2} was performed ~120 sec following ligand application. Co-expression of un-tagged CB\textsubscript{1}-pcDNA receptors did not alter BRETEff between Ga\textsubscript{i}-Rluc and D\textsubscript{2L}-GFP\textsuperscript{2} in the presence of vehicle or quinpirole (Fig. 4.3C). A more efficient energy transfer was observed between Ga\textsubscript{i}-Rluc and D\textsubscript{2L}-GFP\textsuperscript{2} in the presence of un-tagged CB\textsubscript{1} and with D\textsubscript{2L} agonist treatment compared to CB\textsubscript{1} agonist treatment (Fig. 4.3C,D). These data indicate that CB\textsubscript{1}/D\textsubscript{2L}/Ga\textsubscript{i} proteins formed functional complexes composed of at least two homodimers each associated with a Ga\textsubscript{i} protein. Agonists of either homodimer activated the Ga\textsubscript{i} protein associated with the cognate receptor pair and the Ga\textsubscript{i} protein associated with the complexed heterodimer (Fig. 4.3D).

To determine the number of Ga\textsubscript{i} proteins a CB\textsubscript{1}/D\textsubscript{2L} complex, we cloned a
blocking peptide that specifically binds to the CB₁ third intracellular loop, CB₁ amino acids 316-344 (CB₁-\(\text{G}_{\alpha_i}\)-BP), and compete for the association between CB₁ with \(\text{G}_{\alpha_i}\), but not the association between D₂L and CB₁ receptors (Mukhopadhyay and Howlett, 2001) (Fig. 4.4A). Co-expression of CB₁-\(\text{G}_{\alpha_i}\)-BP together with \(\text{G}_{\alpha_i}\)-Rluc, CB₁-GFP², un-tagged D₂L-pcDNA significantly reduced BRET\(_{\text{Eff}}\) between \(\text{G}_{\alpha_i}\)-Rluc and CB₁-GFP² in vehicle- and ACEA-treated cells compared to cells co-expressing \(\text{G}_{\alpha_i}\)-Rluc, CB₁-GFP², un-tagged D₂L-pcDNA and empty pcDNA instead of the CB₁-\(\text{G}_{\alpha_i}\)-BP (Fig. 4.4B). The reduction in BRET\(_{\text{Eff}}\) indicated that the CB₁-\(\text{G}_{\alpha_i}\)-BP inhibited the binding of \(\text{G}_{\alpha_i}\) to CB₁ receptors (Fig. 4.4A). There was no difference in the energy transfer between \(\text{G}_{\alpha_i}\)-Rluc and CB₁-GFP² in quinpirole-treated cells in the presence or absence of CB₁-\(\text{G}_{\alpha_i}\)-BP (Fig. 4.4B). Because quinpirole increased the BRET\(_{\text{Eff}}\) between \(\text{G}_{\alpha_i}\)-Rluc and CB₁-GFP² compared to vehicle treatment, we concluded that weak energy transfer was occurring between \(\text{G}_{\alpha_i}\)-Rluc bound to the un-tagged D₂L receptors to the CB₁-GFP² within CB₁/D₂L/\(\text{G}_{\alpha_i}\) complexes (Fig. 4.4B). When BRET² was measured between \(\text{G}_{\alpha_i}\)-Rluc and D₂L-GFP² in the presence of un-tagged CB₁ receptors (Fig. 4.4C), energy transfer between \(\text{G}_{\alpha_i}\)-Rluc and D₂L-GFP² was unaffected by the co-expression CB₁-\(\text{G}_{\alpha_i}\)-BP in vehicle- or quinpirole- treated cells compared to cells not expressing CB₁-\(\text{G}_{\alpha_i}\)-BP (Fig. 4.4D). A weak energy transfer from \(\text{G}_{\alpha_i}\)-Rluc and D₂L-GFP² in ACEA treated cells was detected; however, the expression of CB₁-\(\text{G}_{\alpha_i}\)-BP significantly reduced BRET\(_{\text{Eff}}\) to level that was the same as the level of vehicle-treated cells (Fig. 4.4D). Therefore, limited energy transfer was occurring between \(\text{G}_{\alpha_i}\)-Rluc bound to un-tagged CB₁ receptors and D₂L-GFP² within CB₁/D₂L/\(\text{G}_{\alpha_i}\) complexes (Fig. 4.4D). To confirm that the expression of the CB₁-\(\text{G}_{\alpha_i}\)-BP did not alter the ability of the CB₁ and D₂L receptors to form heterotetramers, we performed SRET² combined with BiFC in the presence of CB₁-\(\text{G}_{\alpha_i}\)-BP. We found that blocking the interaction between CB₁ and \(\text{G}_{\alpha_i}\) using the CB₁-\(\text{G}_{\alpha_i}\)-BP did not alter net SRET² values (Supplementary Fig. 4.3A). Overall, these results are consistent with the hypothesis that CB₁/D₂L formed functional heterotetramers that are coupled to at least two \(\text{G}_{\alpha_i}\) proteins. Application of CB₁ or D₂ agonists activated the \(\text{G}_{\alpha_i}\) protein associated with the cognate homodimer and weakly activated the \(\text{G}_{\alpha_i}\) protein associated with the associated heteromer within the CB₁/D₂L/\(\text{G}_{\alpha_i}\) complex (Fig. 4.4A,B).
Figure 4.4: **CB1/D2L Heterotetramers are Coupled to Two Ga Proteins.** (A) Scheme of BRET$^2$, CB1 was tagged with GFP$^2$ (CB1-GFP$^2$), Ga$_i$ was tagged with Rluc (Ga$_i$-Rluc) while D2L was un-tagged (D2L-pcDNA) expressed together with CB1-Ga$_i$-BP. (B) HEK 293A cells expressing CB1-GFP$^2$, Ga$_i$-Rluc and un-tagged D2L-pcDNA with an empty pcDNA vector or CB1-Ga$_i$-BP. BRET$_{Eff}$ was measured following treatment with vehicle, 1 μM ACEA or 1 μM quinpirole. * P < 0.01 compared to cells expressing Ga$_i$-Rluc, CB1-GFP$^2$, D2L-pcDNA and an empty pcDNA vector and treated with vehicle. ~P < 0.01 compared to cells expressing Ga$_i$-Rluc, CB1-GFP$^2$, D2L-pcDNA and an empty pcDNA vector within the vehicle and ACEA treatment group. (C) Scheme of BRET$^2$, D2L was tagged with GFP$^2$ (D2L -GFP$^2$), Ga$_i$ was tagged with Rluc (Ga$_i$-Rluc) while CB1 was un-tagged (CB1-pcDNA) together with CB1-Ga$_i$-BP. (D) HEK 293A cells expressing D2L-GFP$^2$, Ga$_i$-Rluc and un-tagged CB1-pcDNA with an empty pcDNA vector or CB1-Ga$_i$-BP. BRET$_{Eff}$ was measured following treatment with vehicle, 1 μM ACEA or 1 μM quinpirole. * P < 0.01 compared to cells expressing Ga$_i$-Rluc, D2L-GFP$^2$, CB1-pcDNA and an empty pcDNA vector and treated with vehicle. ~P < 0.01 compared to cells expressing Ga$_i$-Rluc, D2L-GFP$^2$, CB1-pcDNA and an empty pcDNA vector and treated with ACEA. n.s. > 0.05 compared to cells expressing Ga$_i$-Rluc, D2L-GFP$^2$, CB1-pcDNA and an empty pcDNA vector within the vehicle and quinpirole treatment group. Data are presented as mean ± SEM of 3 independent experiments, one-way ANOVA followed by Tukey’s *post-hoc* test.
4.3.3 CB₁ and D₂ Receptor Agonists Allosterically Modulate Interaction Between CB₁/D₂L/Gα Proteins

Co-activation of CB₁ and D₂ receptors with CB₁ and D₂ agonists resulted in an increase in cAMP production while activation of either receptor leads to a decrease in cAMP production. Given that we observed pre-association of CB₁/D₂L/Gα complexes, we hypothesized that co-activation of both CB₁ and D₂ receptor complexes would either uncouple Gα from the complex or switch coupling of CB₁/D₂L complexes from Gα to Gα₄. Our next objective was to determine if CB₁/D₂L/Gα receptor complexes are involved in agonist-dependent Gα protein uncoupling or switching. In cells co-expressing Gα₄-Rluc, CB₁-GFP² and un-tagged D₂L (Fig. 4.5A), increasing concentrations of the CB₁ agonist ACEA resulted in concentration-dependent Gαᵢ activation and an increase in BRETₐeff signals between Gα₄-Rluc and CB₁-GFP² [EC₅₀ = 0.15 μM (0.11-0.23), Eₘₐₓ = 0.51 (0.45-0.56), Hill coefficient= 1.00 (0.88-1.37)] (Fig 4.5.B). This suggests that ACEA promoted conformational changes within the Gα₄-Rluc/CB₁-GFP²/D₂L complexes leading to Gαᵢ protein activation. Treating the cells with quinpirole resulted in a concentration-dependent increase in BRETₐeff signals between Gα₄-Rluc and CB₁-GFP² (Fig. 4.5B) [EC₅₀ = 0.016 μM (0.014-0.019)] as expected given that we had observed energy transfer from the heterodimer partner (Fig. 4.4B). However, quinpirole treatment resulted in significantly lower Eₘₐₓ [0.22 (0.21-0.24)], compared to ACEA treated cells (Fig. 4.5B). Treating the cells with 1 μM ACEA and increasing concentrations of the quinpirole resulted in concentration-dependent inhibition in BRETₐeff between Gα₄-Rluc and CB₁-GFP² (Fig. 4.5B). This observation suggested that quinpirole binding to D₂L inhibited the interaction between Gαᵢ and CB₁. Quinpirole inhibition of this interaction was concentration-dependent (Fig. 4.5B). Higher quinpirole concentrations led to lower BRETₐeff signals between Gα₄-Rluc and CB₁-GFP². In the absence of D₂L, increasing concentrations of quinpirole had no effect on BRETₐeff between Gα₄-Rluc and CB₁-GFP² in the presence of 1 μM ACEA (data not shown). These findings indicate that quinpirole was not acting directly on CB₁ to mediate its effects but rather the effect was dependent on the presence of the D₂L receptor. The influence of different concentrations of quinpirole (0.001-1 μM) on ACEA-induced BRETₐeff between Gα₄-Rluc and CB₁-GFP² was then assessed (Fig. 4.5C; Table 4.1). Quinpirole produced a concentration-
Figure 4.5: Bidirectional AllostERIC Inhibition of CB₁/D₂L Heterotetramer Interactions with Gaᵢ Following CB₁ and D₂L Agonists Treatment. (A) Scheme of BRET², CB₁ was tagged with GFP² (CB₁-GFP²), Gaᵢ was tagged with Rluc (Gaᵢ-Rluc) while D₂L was un-tagged (D₂L-pcDNA). (B) Concentration-response curves of ACEA and quinpirole +/- 1 μM ACEA- induced BRET_{Eff} between Gaᵢ-Rluc and CB₁-GFP² in the presence of D₂L-pcDNA. (C) Concentration-response curves of ACEA- induced BRET_{Eff} between Gaᵢ-Rluc and CB₁-GFP² +/- different concentrations of quinpirole in the presence of D₂L-pcDNA. (D) Scheme of BRET², D₂L was tagged with GFP² (D₂L-GFP²), Gaᵢ was tagged with Rluc (Gaᵢ-Rluc) while CB₁ was un-tagged (CB₁-pcDNA). (E) Concentration-response curves of quinpirole and ACEA ± 1 μM quinpirole-induced BRET_{Eff} between Gaᵢ-Rluc and D₂L -GFP² in the presence of CB₁-pcDNA. (F) Concentration-response curves of quinpirole- induced BRET_{Eff} between Gaᵢ-Rluc and D₂L -GFP² ± different concentrations of ACEA in the presence of CB₁-pcDNA. Data are presented as mean ± SEM of 4 independent experiments.
dependent rightward and downward shift in the ACEA concentration-response curves (Fig. 4.5C). Both the efficacy and the potency of ACEA dependent Gαi-CB1 interaction were diminished by quinpirole. The increase in EC50 and the decrease in EMax for ACEA concentration-response curves were significant at all concentrations of quinpirole tested (Fig. 4.5C; Table 4.1). The Hill coefficient was significantly less than 1 at 0.1 and 1 μM quinpirole for ACEA-concentration-response curves (Table 4.1), suggesting that quinpirole exerts negative cooperativity on CB1 to Gαi interaction within CB1/D2L complexes.

Next, the effects of expression and activation of un-tagged CB1 receptors (CB1-pcDNA) on the interaction and activation of Gαi and D2L was examined (Fig. 4.5D). Quinpirole treatment resulted in concentration-dependent increase in BRETEff between Gαi-Rluc and D2L-GFP2 [EC50 = 0.02 μM (0.01-0.03), EMax = 0.39 (0.36-0.42), Hill coefficient= 1.16 (0.98-1.23)] (Fig. 4.5E). ACEA treatment alone resulted in an EMax of 0.22 (0.19-0.25), which was significantly higher compared to vehicle-treated cells, but lower compared to quinpirole-treated cells. A reduction in BRETEff signals between Gαi-Rluc and D2L-GFP2 was observed in cells treated with 1 μM quinpirole and increasing concentrations of ACEA (Fig. 4.5E). The effects of different concentrations of ACEA on quinpirole concentration-response curve were tested (Fig. 4.5F; Table 4.2). ACEA concentrations higher than 0.1 μM increased the EC50 and reduced both the EMax and the Hill coefficient of quinpirole concentration-response curves (Fig. 4.5F; Table 4.2). This effect was dependent on the co-expression of CB1 receptors (data not shown). ACEA allosterically inhibited the interaction between Gαi and D2L through binding to CB1 only in the presence of quinpirole.

The reduction in BRETEff signals between CB1 and Gαi protein or between D2L and Gαi protein following co-treatment with both ACEA and quinpirole suggested that CB1 and D2L homodimers are dissociated from Gαi proteins within CB1/D2L heterotetramers. First, using BRET2 we examined whether the CB1 couples to Gαs protein (Supplementary Fig. 4.4A). No significant BRETEff signals were observed between Gαs-Rluc and CB1-GFP2 in vehicle-treated cells compared to the negative control obtained from cells transfected with Gαs-Rluc and HERG-GFP2, indicating that CB1 does not interact with Gαs in the absence of ligand (Supplementary Fig. 4.4A). The negative
Table 4.1: The Effects of Quinpirole on BRET$^2$ (Gai-Rluc and CB$_1$-GFP$^2$), Gai-Dependent ERK Phosphorylation, BRET$^2$ (Gas-Rluc and CB$_1$-GFP$^2$), Ga-Dependent CREB Phosphorylation, BRET$^2$ (β-arrestin1-Rluc and CB$_1$-GFP$^2$). Data were determined using nonlinear regression with variable slope (four parameters) analysis. Data are presented as the mean and 95% confidence interval (CI) for four independent experiments. *$P < 0.01$, compared with vehicle; one-way ANOVA with Tukey's multiple comparison test.
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<th>Eₘₐₓ nM (95% CI)</th>
<th>Hill coefficient (95% CI)</th>
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<td>1.14 (1.12-1.23)*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>180 (176-243)*</td>
<td>0.31 (0.28-0.32)*</td>
<td>1.34 (1.20-1.61)*</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>150 (155-223)*</td>
<td>0.39 (0.37-0.42)*</td>
<td>1.77 (1.68-2.13)*</td>
</tr>
<tr>
<td><strong>Gαᵢ-dependent ERK phosphorylation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACEA</td>
<td>0</td>
<td>160 (140-260)</td>
<td>0.81 (0.74-0.85)</td>
<td>1.01 (0.76-1.4)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>180 (160-201)</td>
<td>0.64 (0.61-0.67)</td>
<td>1.02 (0.99-1.05)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>325 (298-356)*</td>
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<td>0.91 (0.65-0.90)</td>
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<tr>
<td></td>
<td>100</td>
<td>540 (490-560)*</td>
<td>0.22 (0.16-0.27)</td>
<td>0.69 (0.57-0.75)</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>711 (590-743)*</td>
<td>0.17 (0.11-0.16)</td>
<td>0.65 (0.51-0.71)</td>
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<tr>
<td><strong>Gαₛ-dependent CREB phosphorylation</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.19 (0.16-0.17)</td>
<td>0.81 (0.78-0.89)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>260 (246-304)*</td>
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<td>1.41 (1.11-1.90)*</td>
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<tr>
<td></td>
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<td>1.80 (1.34-2.13)*</td>
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<tr>
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<td>1.71 (1.53-2.3)*</td>
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<tr>
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<td>150 (135-183)*</td>
<td>0.86 (0.97-0.79)</td>
<td>1.77 (1.68-2.02)*</td>
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<tr>
<td><strong>BRET² (β-arrestin1 – Rluc + CB₁-GFP²)</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.60 (0.58-0.64)</td>
<td>1.20 (0.91-1.41)</td>
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<tr>
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<tr>
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<tr>
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<td>1.65 (1.32-2.07)</td>
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<tr>
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<td>112 (100-160)*</td>
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<td>1.75 (1.32-2.18)</td>
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Table 4.2: The Effects of ACEA on BRET² (Gaᵢ-Rluc and D₂L-GFP²), Gaᵢ-Dependent ERK Phosphorylation, BRET² (Gaₛ-Rluc and D₂L-GFP²), Gaₛ-Dependent CREB Phosphorylation, BRET² (D₂L-Rluc and β-arrestin1-GFP²). Data were determined using nonlinear regression with variable slope (four parameters) analysis. Data are presented as the mean and 95% confidence interval (CI) for four independent experiments. * P < 0.01, compared with vehicle; one-way ANOVA followed by a Tukey's with post-hoc test.
<table>
<thead>
<tr>
<th>Agonist</th>
<th>ACEA nM</th>
<th>EC₅₀ nM (95% CI)</th>
<th>$E_{\text{max}}$ (95% CI)</th>
<th>Hill coefficient (95% CI)</th>
</tr>
</thead>
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<tr>
<td><strong>BRET² (Gai-Rluc + D₂L-GFP²)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Quinpirole</td>
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<td>19 (14-26)</td>
<td>0.39 (0.36-0.42)</td>
<td>1.16 (0.98-1.23)</td>
</tr>
<tr>
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<td>1.13 (0.99-1.05)</td>
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<tr>
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<td>0.78 (0.65-0.90)</td>
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<tr>
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<td>75 (65-81)*</td>
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<td>0.73 (0.70-0.78)*</td>
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<tr>
<td></td>
<td>1000</td>
<td>98 (79-112)*</td>
<td>0.14 (0.12-0.16)*</td>
<td>0.69 (0.58-0.79)*</td>
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<tr>
<td><strong>BRET² (Gas-Rluc + D₂L-GFP³)</strong></td>
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<td></td>
<td></td>
<td></td>
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<td>0.58 (0.51-0.64)</td>
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<td>92 (81-98)</td>
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<td>0.83 (0.71-0.98)*</td>
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<tr>
<td></td>
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<td>0.25 (0.22-0.27)</td>
<td>1.23 (1.00-1.42)*</td>
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<tr>
<td></td>
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<td>26 (24-32)*</td>
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<td>1.64 (1.23-1.85)*</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>21 (12-23)*</td>
<td>0.35 (0.32-0.37)*</td>
<td>1.81 (1.92-2.12)*</td>
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<tr>
<td><strong>Gai-dependent ERK phosphorylation</strong></td>
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<td></td>
</tr>
<tr>
<td>Quinpirole</td>
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<td>22 (15-23)</td>
<td>0.82 (0.79-0.85)</td>
<td>1.01 (0.98-1.23)</td>
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<tr>
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<td>25 (15-28)*</td>
<td>0.75 (0.72-0.79)</td>
<td>0.95 (0.99-1.05)</td>
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<td>0.90 (0.65-0.90)</td>
</tr>
<tr>
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<td>78 (56-82)*</td>
<td>0.43 (0.41-0.46)*</td>
<td>0.82 (0.73-0.90)*</td>
</tr>
<tr>
<td></td>
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<td>92 (98-89)*</td>
<td>0.21 (0.19-0.23)*</td>
<td>0.78 (0.62-0.79)*</td>
</tr>
<tr>
<td><strong>Gas-dependent CREB phosphorylation</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinpirole</td>
<td>0</td>
<td>81 (76-89)</td>
<td>0.19 (0.18-0.21)</td>
<td>0.58 (0.51-0.63)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>71 (57-78)</td>
<td>0.26 (0.25-0.27)</td>
<td>0.69 (0.64-0.79)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50 (35-61)*</td>
<td>0.45 (0.43-0.48)*</td>
<td>0.91 (1.02-0.98)*</td>
</tr>
<tr>
<td></td>
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<td>19 (15-23)*</td>
<td>0.67 (0.63-0.72)*</td>
<td>1.36 (1.12-1.65)*</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>14 (9-18)*</td>
<td>0.76 (0.72-0.82)*</td>
<td>1.62 (1.53-2.11)*</td>
</tr>
<tr>
<td><strong>BRET² (D₂L-Rluc + β-arrestin1-GFP³)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinpirole</td>
<td>0</td>
<td>15 (13-17)</td>
<td>0.13 (0.12-0.14)</td>
<td>1.01 (0.98-1.12)</td>
</tr>
<tr>
<td></td>
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<td>16 (12-17)</td>
<td>0.14 (0.13-0.15)</td>
<td>1.11 (0.10-1.12)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13 (11-15)</td>
<td>0.15 (0.14-0.15)</td>
<td>1.18 (0.11-0.13)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>11(10-12)</td>
<td>0.16 (0.15-0.16)</td>
<td>1.23 (1.21-1.42)*</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>10 (9-11)</td>
<td>0.16 (0.16-0.17)*</td>
<td>1.34 (1.24-1.45)*</td>
</tr>
</tbody>
</table>
control included HERG, a membrane-localized K⁺ channel that does not interact with GPCRs or G proteins (Hudson et al., 2010; Bagher et al., 2016). Consistent with a previous study using BRET (Galés et al., 2005), cells transfected with Gα₃-Rluc and β₂AR-GFP² resulted in a significantly higher BRET$_{\text{Eff}}$ compared to the negative control (Supplementary Fig. 4.4A).

Next, we examined whether CB₁ and D₂L homodimers couple to Gα₃ proteins following the activation of both receptors within CB₁/D₂L heterotetramer complexes. The interaction between Gα₃-Rluc and CB₁-GFP² in the presence of un-tagged D₂L receptors was studied (Fig. 4.6A). Treating the cells with increasing doses of ACEA did not significantly increase BRET$_{\text{Eff}}$ between CB₁ and Gα₃ compared to vehicle treatment [$E_{\text{Max}} = 0.10$ (0.09-0.14), $EC_{50} = 0.38$ μM (0.350-0.44) and Hill coefficient = 0.68 (0.59-0.89)]. Similarly, quinpirole treatment did not alter BRET$_{\text{Eff}}$ between CB₁ and Gα₃ compared to vehicle treatment [$E_{\text{Max}} = 0.07$ (0.05-0.08), $EC_{50} = 0.03$ μM (0.02-0.04), Hill coefficient = 1.5 (1.7-1.0)] (Fig. 4.6B). The co-application of 1 μM ACEA and 1 μM quinpirole increased BRET$_{\text{Eff}}$ between Gα₃-Rluc and CB₁-GFP² in the presence of un-tagged D₂L receptors (Supplementary Fig. 4.2B). The increase in BRET$_{\text{Eff}}$ following ACEA and quinpirole co-application was delayed and sustained. BRET$_{\text{Eff}}$ peaked at ~240 sec (4 min) following ligand application and remained significantly elevated for ~ 400 sec (Supplementary Fig. 4.2B; Bagher et al., 2016). Therefore, all BRET$_{\text{Eff}}$ measured between Gα₃-Rluc and CB₁-GFP² was performed ~240 sec (4 min) following ligand application (Supplementary Fig. 4.2B). Treating the cells with 1 μM ACEA and increasing concentrations of quinpirole caused a concentration-dependent elevation in BRET$_{\text{Eff}}$ where $E_{\text{Max}} = 0.40$ (0.37-0.44), $EC_{50} = 0.03$ (0.02-0.04) and Hill coefficient = 1.0 (0.71-1.3) (Fig. 4.6B). We also examined the effects of increasing concentrations of quinpirole on ACEA-induced BRET$_{\text{Eff}}$ between Gα₃-Rluc and CB₁-GFP² (Fig. 4.6C). Quinpirole produced a concentration-dependent leftward and upward shift in the ACEA concentration-response curves. Increasing the concentrations of quinpirole increased the efficacy and the potency of ACEA dependent Gα₃-CB₁ interaction (Fig. 4.6C; Table 4.2) where the Hill coefficient was greater than 1 suggesting that co-treatment with both ACEA and quinpirole exerted positive cooperatively effects on Gα₃-Rluc and CB₁-GFP² interactions within CB₁/D₂L heterotetramers.
Figure 4.6 Bidirectional Allosteric Induction of CB₁/D₂L Heterotetramer Interactions with Ga₅ Following Agonists Treatment. (A) Scheme of BRET², CB₁ was tagged with GFP² (CB₁-GFP²), Ga₅ was tagged with Rluc (Ga₅-Rluc) while D₂L was un-tagged (D₂L-pcDNA). (B) Concentration-response curves of ACEA and quinpirole +/- 1 μM ACEA-induced BRETₐff between Ga₅-Rluc and CB₁-GFP² in the presence of D₂L-pcDNA. (C) Concentration-response curves of ACEA-induced BRETₐff between Ga₅-Rluc and CB₁-GFP² +/- different concentrations of quinpirole in the presence of D₂L-pcDNA. (D) Scheme of BRET², D₂L was tagged with GFP² (D₂L-GFP²), Ga₅ was tagged with Rluc (Ga₅-Rluc) while CB₁ was un-tagged (CB₁-pcDNA). (E) Concentration-response curves of quinpirole and ACEA +/- 1 μM quinpirole-induced BRETₐff between Ga₅-Rluc and D₂L-GFP² in the presence of CB₁-pcDNA. (F) Concentration-response curves of quinpirole-induced BRETₐff between Ga₅-Rluc and D₂L-GFP² +/- different concentrations of ACEA in the presence of CB₁-pcDNA. Data are presented as mean ± SEM of 4 independent experiments.
The interaction between D2L and Gαs protein was also examined using BRET\textsuperscript{2}. In cells expressing Gαs-Rluc and D2L-GFP\textsuperscript{2}, no significant BRET\textsubscript{Eff} signals were detected in the vehicle- or quinpirole-treated cells, compared to cells expressing Gαs-Rluc and β2AR-GFP\textsuperscript{2} (Supplementary Fig. 4.4B). These observations indicate that, similarly to CB\textsubscript{1}, D2L did not interact with Gαs proteins in the absence or presence of D2 agonists.

In order to study the influence of ACEA treatment on Gαs-Rluc and D2L-GFP\textsuperscript{2} interactions, cells were co-transfected with un-tagged CB\textsubscript{1} receptors (Fig. 4.6D). Increasing the concentrations of quinpirole or ACEA did not alter BRET\textsubscript{Eff} values compared to vehicle treatment (Fig. 4.6E). The co-application of 1 μM ACEA and 1 μM quinpirole increased BRET\textsubscript{Eff} between Gαs-Rluc and D2L-GFP\textsuperscript{2} in the presence of un-tagged CB\textsubscript{1} receptors (Supplementary Fig. 4.2D). Similarly to CB\textsubscript{1}, the increase in BRET\textsubscript{Eff} following ACEA and quinpirole co-application was delayed and sustained. BRET\textsubscript{Eff} peaked at ~240 sec (4 min) following co-application of both agonists and remained significantly elevated for ~ 400 sec (Supplementary Fig. 2D). Therefore, all BRET\textsubscript{Eff} measured between Gαs-Rluc and D2L-GFP\textsuperscript{2} was performed ~240 sec (4 min) following ligand application (Supplementary Fig. 4.2D). Significantly higher BRET\textsubscript{Eff} values were observed in cells treated with 1 μM quinpirole and increasing concentrations of ACEA \[E_{\text{Max}} = 0.34 (0.32-0.37), \text{EC}_{50} = 0.19 (0.21-0.31), \text{Hill coefficient} = 1.4 (0.82-2.35)\] (Fig. 4.6E). Increasing ACEA concentrations resulted in a leftward and upward shift in quinpirole concentration-response curves (Fig. 4.6F; Table 4.2), indicating a positive cooperatively effects of ACEA on Gαs and D2L interactions. The co-expression of CB\textsubscript{1}/D2L hetero-oligomer blocking peptide (CB\textsubscript{1}-BP), which inhibited the physical interaction between CB\textsubscript{1} and D2 (Fig. 4.2C), inhibited the switch of CB\textsubscript{1} and D2L coupling from Gα\textsubscript{i} to Gαs proteins following co-activation of both receptors (Supplementary Fig. 4.5A,B). These findings demonstrate that co-activation of CB\textsubscript{1} and D2L with CB\textsubscript{1} and D2 agonists allosterically enhanced the association of CB\textsubscript{1} and D2L receptors with Gαs proteins within CB\textsubscript{1}/D2L heterotetramer complexes. Altogether, co-treatment of CB\textsubscript{1}/D2L heterotetramer complexes led to physical uncoupling of Gα\textsubscript{i} followed by physical coupling of Gαs. All BRET\textsuperscript{2} experiments conducted to measure Gα protein interaction with CB\textsubscript{1} or D2L were performed in the present of excessive Gα protein, which exclude the possibility that competition for a common pool of G protein is the reason for the
observed alteration in Ga protein coupling.

To confirm that the observed changes in coupling between Ga proteins and CB1/D2L complexes following the co-application of both receptor agonists were specific to CB1/D2L heterotetramers, we studied the effect of concurrent activation of CB1 and β2AR receptors by their agonists on the interaction between CB1 and Ga proteins. The CB1 and β2AR can heteromerize when expressed in HEK 293A cells (Hudson et al., 2010). The expression of Ga_i-Rluc and β2-GFP2 resulted in low BRET2 signal similar to cells expressing Ga_i-Rluc and the negative control HERG-GFP2. In addition, treating the cells with the β2AR agonist isoproterenol (1 μM) did not alter BETEff signal between Ga_i-Rluc and β2AR-GFP2 (Supplementary Fig. 4.6A). Treating cells co-expressing Ga_i-Rluc, CB1-GFP2 and un-tagged β2AR with 1 μM ACEA alone or with 1 μM isoprenaline resulted in BRETEff similar to cells treated with 1 μM ACEA and expressing Ga_i-Rluc, CB1-GFP2 (n.s. P > 0.05) relative to cells expressing empty pcDNA within treatment group (Supplementary Fig. 4.6A). Therefore, the co-expression and co-activation of both CB1 and β2AR receptors by their agonists did not alter the interaction between CB1 and Ga_i protein (Supplementary Fig. 4.6A). The interaction between CB1 and Ga_s protein in cells co-expressing un-tagged β2AR was also studied following the co-application of both agonists. Treating cells expressing Ga_s-Rluc, CB1-GFP2 and un-tagged β2AR and treated with 1 μM ACEA alone or with 1 μM isoprenaline resulted in similar BRETEff signals compared to cells treated with only 1 μM ACEA and expressing Ga_i-Rluc, CB1-GFP2 and un-tagged β2AR (Supplementary Fig. 4.6B). These results demonstrate that the co-activation of CB1 and β2AR do not switch CB1 coupling to either Ga_i or Ga_s proteins. Similarly, the co-expression and co-activation of D2L and β2AR receptors did not alter the interaction between D2L and Ga_i protein (Supplementary Fig. 4.5C) or the interaction between D2L and Ga_s proteins (Supplementary Fig. 4.6D).

### 4.3.4 Activation of CB1 and D2 Receptors Allosterically Alter Their Downstream Signaling

To test whether physical uncoupling of CB1 and D2L from Ga_i following treatment with both CB1 and D2L agonists is associated with functional un-coupling from Ga_i proteins, we measured Ga_i-dependent ERK phosphorylation 5 min following drug
application because ERK phosphorylation is transient (Laprairie et al., 2014). Treating cells co-expressing CB1-pcDNA and D2L-pcDNA with increasing concentration of ACEA resulted in concentration-dependent increase in ERK phosphorylation \( [E_{\text{Max}} = 0.81 (0.74-0.85), \text{EC}_{50} = 0.16 \mu M (0.14-0.26) \text{ and Hill coefficient } = 1.01 (0.79-1.4)] \) (Fig. 4.7A). Treating the cells with 1 \( \mu M \) ACEA and increasing concentrations of quinpirole, resulted in an inhibition of ACEA-induced ERK phosphorylation (Fig. 4.7A). Similarly, treating the cells with an increasing concentration of quinpirole led to an increase in ERK phosphorylation \( [E_{\text{Max}} = 0.82 (0.79-0.85), \text{EC}_{50} = 0.022 (0.015-0.028) \text{ and Hill coefficient } = 1.01 (0.98-1.23)] \) (Fig. 7D). Increasing ACEA concentrations inhibited ERK phosphorylation induced by 1 \( \mu M \) quinpirole (Fig. 4.7D). Increasing quinpirole concentrations shifted ACEA concentration-response curves rightward and downward (Fig. 7B; Table 4.1). Similarly, increasing ACEA concentrations shifted quinpirole concentration-response curves rightward and downward (Fig. 4.7E; Table 4.2). These data demonstrate bidirectional negative allosteric effects of ACEA and quinpirole on ERK phosphorylation. The observed ERK phosphorylation following the application of 1 \( \mu M \) ACEA or 1 \( \mu M \) quinpirole was mediated through activation of the PTx-sensitive \( \text{G}_{\alpha_i} \)-dependent pathway (Fig. 4.7C). The inhibition of ERK phosphorylation following the activation of both CB1 and D2L receptors is mediated through CB1/D2L heteromers, as the expression of the CB1/D2L hetero-oligomer blocking peptide (CB1-BP) restored PTx-sensitive ACEA- and quinpirole-dependent ERK activation (Fig. 4.7F).

As co-activation of both CB1 and D2L was associated with CB1 and D2L physical coupling to \( \text{G}_{\alpha_i} \) proteins at the expense of coupling to \( \text{G}_{\alpha_s} \), we next evaluated the effects of co-activation of both CB1 and D2L on \( \text{G}_{\alpha_s} \)-dependent CREB phosphorylation. Cells transfected with un-tagged CB1 and D2L receptors, ACEA (Fig. 4.8A) or quinpirole (Fig. 4.8D) treatment did not alter CREB phosphorylation compared to vehicle-treated cells. Treating the cells with 1 \( \mu M \) ACEA and increasing concentrations of quinpirole led to a concentration-dependent elevation in CREB phosphorylation \( [E_{\text{Max}} = 0.76 (0.71-0.82), \text{EC}_{50} = 0.04 (0.01-0.04) \text{ and Hill coefficient} = 1.7 (1.1-2.3)] \) (Fig. 4.8B). Likewise, treating cells with 1 \( \mu M \) quinpirole and increasing concentrations of ACEA led to an increase in CREB phosphorylation \( [E_{\text{Max}} = 0.72 (0.71-0.82), \text{EC}_{50} = 0.04 \mu M (0.01-0.04) \text{ and Hill coefficient } = 1.8 (1.2-2.5)] \) (Fig. 4.8D). Quinpirole allosterically modulated ACEA-mediated CREB
Figure 4.7: The Co-activation of CB₁/D₂L Heterotetramer Allosterically Inhibited Ga-Mediated ERK Phosphorylation. ERK phosphorylation (pERK1/2(Tyr-205/Tyr-185)/total ERK) concentration-response curves measured at 5 min obtained from HEK 293A expressing un-tagged CB₁ and D₂L receptors and (A) treated with increasing concentration ACEA or with 1 μM ACEA and increasing concentration of quinpirole, or (D) or treated with increasing concentrations of quinpirole or with 1 μM quinpirole and increasing concentration of ACEA. (B) pERK concentration-response curve obtained from cells treated with ACEA alone or in the presence of increasing concentrations of quinpirole, or (E) from cells treated with quinpirole alone or in the presence of increasing concentrations of ACEA. (C) HEK 293A cells expressing un-tagged CB₁ and D₂L receptors and treated with 1 μM ACEA or 1 μM quinpirole or in combination +/- 24 h pre-treatment with 50 ng/ml PTx or CTx. * P < 0.01 compared to vehicle treatment; ~P < 0.01 compared to cells treated with 1 μM ACEA; n.s. P > 0.05 compared to vehicle treated cells. (F) HEK 293A cells expressing un-tagged CB₁ and D₂L receptors together with empty pcDNA vector or CB₁/D₂L hetero-oligomer blocking peptide (CB₁-BP) and treated with 1 μM ACEA or 1 μM quinpirole or in combination for 5 min +/- 24 h pre-treatment with 50 ng/ml PTx. * P < 0.01 compared to vehicle treatment; ~ P < 0.01 compared to cells transfected with empty pcDNA vector and treated with 1 μM ACEA and 1 μM quinpirole; n.s. P > 0.05 compared to vehicle treated cells. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
phosphorylation in a concentration-dependent manner, shifting ACEA concentration-
response curves leftward and upward (Fig. 4.8B; Table 4.1). The same allosteric
modulatory effects were also exerted by ACEA on quinpirole-mediated CREB
phosphorylation (Fig. 4.8C; Table 4.2). The observed CREB-phosphorylation following
the co-application of 1 μM ACEA and 1 μM quinpirole was mediated through the
activation of the CTx-sensitive Gαs-dependent pathway, as pre-treating the cells with CTx
for 24 hr, which suppresses Gαs expression (Milligan et al., 1989), inhibited Gαs-
dependent CREB-phosphorylation (Fig. 4.8C). The induced CREB phosphorylation was
mediated through CB1/D2L heteromers, as the expression of the CB1/D2L hetero-oligomer
blocking peptide (CB1-BP) blocked CREB activation observed following ACEA and
quinpirole co-application (Fig. 4.8F).

To confirm that the switch in CB1 and D2L coupling and signaling from Gαi to Gαs
proteins following the application of ACEA and quinpirole was not an artifact observed
only in HEK cells or only following of receptor overexpression, we tested the influence
of quinpirole on coupling of CB1 to Gαi and Gαs proteins and downstream signaling using
STHdh<sup>Q7/Q7</sup> cells, a model of striatal medium spiny projection neurons that endogenously
express CB1 and D2L receptors (Trettel et al., 2000; Laprairie et al., 2013) (Supplementary
Fig. 4.7). We observed a reduction in ACEA-dependent BRET<sup>2</sup> signaling between Gαi and
CB1 (Supplementary Fig. 4.5A), followed by an increase in BRET<sup>2</sup> signaling between Gαs
and CB1 (Supplementary Fig. 4.7B) when STHdh<sup>Q7/Q7</sup> cells were treated with 1 μM ACEA
and 1 μM quinpirole. In addition, we measured the effects of co-application of 1 μM ACEA
and/or 1 μM quinpirole on endogenous CB1 and D2L receptor signaling. Similar to our results
using HEK 293A cells, the co-application of 1 μM ACEA and 1 μM quinpirole inhibited
ACEA- and quinpirole-induced Gαi-dependent ERK phosphorylation (Supplementary Fig.
4.7C), followed by induced Gαs-dependent CREB phosphorylation (Supplementary Fig.
4.7D) in STHdh<sup>Q7/Q7</sup> cells. Our findings demonstrated that the observed effects of quinpirole
on CB1 coupling and signaling in HEK 293A could also be replicated in a model of striatal
medium spiny projection neurons that endogenously express both receptors.
**Figure 4.8: The Co-Activation of CB₁/D₂L Heterotetramer Allosterically Induced Gas-Mediated CREB Phosphorylation.** CREB phosphorylation concentration-response curves measured at 30 min obtained from HEK 293A expressing un-tagged CB₁ and D₂L receptors and (A) treated with increasing concentrations of ACEA or with 1 μM ACEA and increasing concentration of quinpirole, or (D) treated with increasing concentrations of quinpirole or with 1 μM quinpirole and increasing concentration of ACEA. pCREB concentration-response curve obtained from cells (B) treated with ACEA alone or in the presence of increasing concentrations of quinpirole, or (E) treated with quinpirole alone or in the presence of increasing concentrations of ACEA. (C) HEK 293A cells were treated with 1 μM ACEA or 1 μM quinpirole alone or in combination for 30 min +/- 24 h pre-treatment with 50 ng/ml PTx or CTx. *P < 0.01 compared to vehicle treatment; ~P < 0.01 compared to cells treated with 1 μM ACEA; n.s. P > 0.05 compared to vehicle treated cells. (F) HEK 293A cells expressing un-tagged CB₁ and D₂L receptors together with empty pcDNA vector or CB₁-BP and treated with 1 μM ACEA or 1 μM quinpirole alone for 30 min or in combination. *P < 0.01 compared to vehicle treatment; ~P < 0.01 compared to cells transfected with empty pcDNA vector and treated with 1 μM ACEA and 1 μM quinpirole for 30 min +/- 24 h pre-treatment with 50 ng/ml CTx; n.s. P > 0.05 compared to vehicle treated cells. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey’s *post-hoc* test.
4.3.5 Quinpirole and ACEA Allosterically Potentiate β-arrestin1 Recruitment to CB₁ and D₂L Receptors, Receptor Co-Internalization and β-arrestin1-Dependent ERK Phosphorylation

CB₁ and D₂L are known to interact with β-arrestin1, which mediates receptor internalization, β-arrestin1-mediated signaling, receptor recycling and degradation (Sim-Selley and Martin, 2003; Laprairie et al., 2014). The effect of simultaneous treatment with CB₁ and D₂L agonists on β-arrestin1 recruitment to CB₁/D₂L receptor complexes and receptors co-internalization was tested. HEK 293A cells were transfected with β-arrestin1-Rluc, CB₁-GFP² and un-tagged D₂L (Fig. 4.9A). β-arrestin1 recruitment to the CB₁ receptors within CB₁/D₂L heterotetramers was measured over 30 min following drug application (Fig. 4.9B). BRETEff signals observed from cells expressing β-arrestin1-Rluc and CB₁-GFP² treated with vehicle were higher than BRETEff between β-arrestin1-Rluc and HERG-GFP² (Fig. 4.9B). Treating the cells with 1 μM ACEA enhanced β-arrestin1 recruitment to CB₁ as demonstrated by increased BRETEff signals compared to vehicle-treated cells starting 5 min post-ACEA application and reaching a plateau at 15 min. The signal was sustained for 30 min (Fig. 4.9B). Treating cells with 1 μM quinpirole increased BRETEff between β-arrestin1-Rluc and CB₁-GFP² compared to vehicle-treated cells. These findings suggest that D₂ agonists induced β-arrestin1-Rluc recruitment to the activated D₂L within D₂L/CB₁-GFP²/β-arrestin1-Rluc complexes. The co-application of both 1 μM ACEA and quinpirole significantly potentiated BRETEff signal between β-arrestin1-Rluc and CB₁-GFP² compared to ACEA-treated cells (Fig. 4.9B). Such an increase in BRETEff signals was not detected in cells co-treated with 1 μM ACEA and quinpirole in the absence of D₂L receptors (data not shown), confirming that the observed induction in BRETEff signals was mediated through the binding of quinpirole to D₂L receptors and not due to its direct effect on CB₁ receptors. Increasing the concentration of ACEA led to a concentration-dependent increase in BRETEff \[ E_{\text{Max}} = 0.60 \ (0.58-0.64), \ EC_{50} = 0.22 \ \mu M \ (0.12-0.25) \text{ and Hill coefficient = 1.20 (0.91-1.41)} \] (Fig. 4.9C). Quinpirole treatment resulted in an increase in BRETEff \[ E_{\text{Max}} = 0.38 \ (0.36-0.41), \ EC_{50} = 0.013 \ \mu M \ (0.008-.02) \text{ and Hill coefficient = 1.00 (0.71-1.29)} \] (Fig. 4.9C). The \( E_{\text{Max}} \) between β-arrestin1-Rluc and CB₁-GFP² was lower compared to \( E_{\text{Max}} \) obtained from ACEA-treated cells. The effect of increasing quinpirole concentrations on ACEA-
Figure 4.9: ACEA Treatment Resulted in Slow and Sustained β-arrrestin1 Recruitment to CB₁, Which was Allosterically Potentiated with Quinpirole Co-Application. (A) HEK 293A cells expressing β-arrrestin1-Rluc CB₁-GFP², and un-tagged D₂L-pcDNA. (B) BRET<sub>Eff</sub> was measured over 30 min in cells expressing β-arrrestin1-Rluc, CB₁-GFP² and un-tagged D₂L-pcDNA and treated with vehicle, 1 μM ACEA, 1 μM quinpirole +/- 1 μM ACEA. As a control, cells were co-transfected with β-arrrestin1-Rluc and HERG-GFP². * P < 0.01 compared to vehicle-treated cells; ~ P < 0.01 compared to cells treated with 1 μM ACEA. (C) BRET<sub>Eff</sub> measured between β-arrrestin1-Rluc and CB₁-GFP² in cells treated with increasing concentrations of ACEA or increasing concentrations of quinpirole in the presence of un-tagged D₂L-pcDNA. (D) Concentration-response curves of ACEA- induced BRET<sub>Eff</sub> between β-arrrestin1-Rluc and CB₁-GFP² +/- increasing concentrations of quinpirole. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
induced BRET\textsubscript{Eff} was evaluated. An increase in the concentration of quinpirole shifted ACEA-concentration-response curves leftward and upward (Fig. 4.9D; Table 4.1). Quinpirole acted as a positive allosteric modulator that potentiated β-arrestin1 requirement to the activated CB\textsubscript{1} receptors within CB\textsubscript{1}/D\textsubscript{2L} heterotetramers. To confirm that the potentiation of BRET\textsuperscript{2} signals following co-application of both ACEA and quinpirole was specific to CB\textsubscript{1}/D\textsubscript{2L} heterotetramers we measured the effect of co-expression and co-activation of CB\textsubscript{1} and mGluR6 receptors on β-arrestin1 recruitment to CB\textsubscript{1} receptors (Supplementary Fig. 4.8A). The expression of β-arrestin1-Rluc and CB\textsubscript{1}-GFP\textsuperscript{2} together with un-tagged mGluR6 and treatment of the cells with 1 μM ACEA resulted in an increase in BRET\textsubscript{Eff} between β-arrestin1-Rluc and CB\textsubscript{1}-GFP\textsuperscript{2} compared to vehicle-treated cells (Supplementary Fig. 4.8A). Treating the cells with the selective mGluR6 agonist L-2-amino-4-phosphonobutyric acid (L-AP4, 1 μM) alone resulted in similar BRET\textsubscript{Eff} signals between β-arrestin1-Rluc and CB\textsubscript{1}-GFP\textsuperscript{2} compared to vehicle-treated cells. The co-application of both 1 μM ACEA and 1 μM L-AP4 resulted in similar BRET\textsubscript{Eff} signal between β-arrestin1-Rluc and CB\textsubscript{1}-GFP\textsuperscript{2} compared to cells treated with 1 μM ACEA (Supplementary Fig. 4.8A). Together these finding show that mGluR6, unlike D\textsubscript{2L}, did not modulate β-arrestin1 recruitment to CB\textsubscript{1}.

β-arrestin1 recruitment to D\textsubscript{2L} receptors was also measured. HEK 293A cells were co-transfected with D\textsubscript{2L}-Rluc, β-arrestin1-GFP\textsuperscript{2}, and un-tagged CB\textsubscript{1} receptors (Fig. 4.10A). BRET\textsubscript{Eff} was measured for 30 min following the addition of different ligands (Fig. 4.10B). Cells expressing D\textsubscript{2L}-Rluc and β-arrestin1- GFP\textsuperscript{2} resulted in higher BRET\textsubscript{Eff} signals compared to the negative control HERG (Fig. 4.10B). Treating the cells with 1 μM quinpirole significantly increased BRET\textsubscript{Eff} between D\textsubscript{2L}-Rluc and β-arrestin1-GFP\textsuperscript{2}. ACEA (1 μM) treatment also resulted in higher BRET\textsubscript{Eff} compared to vehicle-treated cells (Fig. 4.10B). The co-application of both 1 μM quinpirole and ACEA potentiated BRET\textsubscript{Eff} between D\textsubscript{2L}-Rluc and β-arrestin1-GFP\textsuperscript{2} compared to BRET\textsubscript{Eff} obtained from cells treated with 1 μM quinpirole (Fig. 4.10B). Increasing the concentration of quinpirole resulted in a concentration-dependent increase in BRET\textsubscript{Eff} signals between D\textsubscript{2L}-Rluc and β-arrestin1-GFP\textsuperscript{2} \[[E_{\text{Max}} = 0.13 (0.12-0.14), E_{50} = 0.02 \ \mu\text{M} (0.1-0.02) \text{ and Hill coefficient} = 1.06 (0.90-1.21)]\] (Fig. 4.10C). Similarly, increasing the concentration of ACEA led to a concentration-dependent increase in BRET\textsubscript{Eff} signals between D\textsubscript{2L}-Rluc
**Figure 4.10: ACEA Co-Application Allosterically Potentiated Quinpirole-Induced β-arrestin1 Recruitment to D2L.** (A) HEK 293A cells expressing D2L-Rluc and β-arrestin1-GFP² and un-tagged CB1-pcDNA. (B) BRET$_{Eff}$ was measured over 30 min in cells expressing D2L-Rluc and β-arrestin1-GFP² and un-tagged CB1-pcDNA and treated with vehicle, 1 μM quinpirole, 1 μM ACEA alone or with 1 μM quinpirole. As a control, cells were co-transfected with HERG-Rluc and β-arrestin1-GFP². * $P < 0.01$ compared to vehicle-treated cells; ~ $P < 0.01$ compared to cells treated with 1 μM quinpirole. (C) BRET$_{Eff}$ measured between D2L-Rluc and β-arrestin1-GFP² in cells treated with increasing concentrations of quinpirole or increasing concentrations of ACEA in the presence of un-tagged CB1-pcDNA. (D) Concentration-response curves of quinpirole-induced BRET$_{Eff}$ between D2L-Rluc and β-arrestin1-GFP² with increasing concentrations of ACEA. Data are presented as mean ± S.E.M. of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
and β-arrestin1-GFP\(^2\) \([E_{\text{Max}} = 0.1 \ (0.09-0.12), \ EC_{50} = 0.20 \ \mu\text{M} \ (0.12-0.25)\) and Hill coefficient = 1.20 \ (0.98-1.32)\] (Fig. 4.10C). ACEA concentrations higher than 0.01 \ \mu\text{M} shifted quinpirole-concentration-response curves leftward and upward (Fig. 4.10D; Table 4.2). ACEA acted as a positive allosteric modulator that potentiated β-arrestin1 requirement to the activated D\(_{2L}\) within CB\(_1/D_{2L}\) heterotetramers. As a control, we measured the effect of the co-expression and co-activation of D\(_{2L}\) and mGluR6 receptors on β-arrestin1 recruitment to D\(_{2L}\) (Supplementary Fig. 4.6B). The co-expression of un-tagged mGluR6 together with D\(_{2L}\)-Rluc and β-arrestin1-GFP\(^2\) did not alter quinpirole-induced β-arrestin1 recruitment to D\(_{2L}\) receptors in the absence or presence of the mGluR6 agonist (L-AP4, 1 \ \mu\text{M}) (Supplementary Fig. 4.6B).

Next, we tested whether the observed potentiation in β-arrestin1 recruitment following co-activation of both CB\(_1\) and D\(_{2L}\) are mediated through CB\(_1/D_{2L}\) heterotetramers binding to β-arrestin1. Our approach involved measuring the interaction between CB\(_1\) and D\(_{2L}\) with β-arrestin1 signaling in cells co-transfected with a blocking peptide (CB\(_1\)-BP), which interferes with CB\(_1\) and D\(_{2L}\) heterotetramer formation (Khan and Lee, 2014). Cells were transfected with β-arrestin1-Rluc, CB\(_1\)-GFP\(^2\) and un-tagged D\(_{2L}\)-pcDNA together with empty pcDNA or pcDNA expressing CB\(_1\)-BP (Fig. 4.11A). BRET\(_{\text{Eff}}\) was measured at 20 min following ligand application (Fig. 4.11B). A significantly lower energy transfer was observed between β-arrestin1-Rluc and CB\(_1\)-GFP\(^2\) in cells expressing un-tagged D\(_{2L}\)-pcDNA and CB\(_1\)-BP treated with quinpirole alone or co-treated with quinpirole and ACEA compared to cells treated with the same agonist(s) expressing an empty pcDNA (Fig. 4.11B). In contrast, no change in the energy transfer between β-arrestin1-Rluc and CB\(_1\)-GFP\(^2\) was observed in cells expressing the CB\(_1\)-BP following ACEA treatment compared to cells transfected with empty pcDNA (Fig. 4.11B). Our finding demonstrated that the increase in energy transfer between β-arrestin1-Rluc and CB\(_1\)-GFP\(^2\) in the presence of quinpirole was due to the interaction between β-arrestin1-Rluc and D\(_{2L}\) within CB\(_1/D_{2L}/β\)-arrestin1 complexes (Fig. 4.11B).

BRET\(_{\text{Eff}}\) was also measured between D\(_{2L}\)-Rluc and β-arrestin1-GFP\(^2\) in cells expressing CB\(_1\)-pcDNA together with empty pcDNA or CB\(_1\)-BP (Fig. 4.11C). A reduction in energy transfer between D\(_{2L}\)-Rluc and β-arrestin1-GFP\(^2\) was observed in ACEA-treated cells and in cells co-treated with both ACEA and quinpirole when the CB\(_1\)-BP was expressed.
**Figure 4.11: Potentiation of β-arrestin1 Recruitment Following ACEA and Quinpirole Co-Application was Mediated Through CB1/D2L Heterotetramer.**

(A) Scheme of BRET², CB¹ was tagged with GFP² (CB¹-GFP²), β-arrestin1 was tagged with Rluc (β-arrestin1-Rluc) while D₂L was un-tagged (D₂L-pcDNA). Cells were co-transfected with either empty pcDNA or CB¹-BP.

(B) HEK 293A cells expressing β-arrestin1-Rluc, CB¹-GFP², un-tagged D₂L-pcDNA and either empty pcDNA or CB¹-BP. BRET<sub>Eff</sub> was measured 20 min following ligand treatment. *P < 0.01 compared to cells expressing β-arrestin1-Rluc, CB¹-GFP², D₂L-pcDNA and empty pcDNA and treated with vehicle. 

# P < 0.01 compared to cells expressing β-arrestin1-Rluc, CB¹-GFP² and D₂L-pcDNA and empty pcDNA and treated with 1 µM ACEA and 1 µM quinpirole. ~P < 0.01 compared to cells expressing β-arrestin1-Rluc, CB¹-GFP² and D₂L-pcDNA and empty pcDNA and treated with 1 µM quinpirole and ACEA. n.s. compared to cells expressing β-arrestin1-Rluc, CB¹-GFP², D₂L-pcDNA and empty pcDNA treated with 1 µM ACEA.

(C) Scheme of BRET², D₂L was tagged with Rluc (D₂L-Rluc), β-arrestin1 was tagged with GFP² (β-arrestin1-GFP²) while CB¹ was un-tagged (CB¹-pcDNA) and cells were co-transfected with CB¹-BP.

(D) HEK 293A cells were transfected with D₂L-Rluc, β-arrestin1-GFP², CB¹-pcDNA and either empty pcDNA or CB¹-BP. BRET<sub>Eff</sub> was measured 20 min following ligand treatment. *P < 0.01 compared to cells expressing D₂L-Rluc, β-arrestin1-GFP², CB¹-pcDNA and empty pcDNA and treated with vehicle. 

# P < 0.01 compared to cells expressing D₂L-Rluc, β-arrestin1-GFP², CB¹-pcDNA and empty pcDNA and treated with 1 µM ACEA. ~P < 0.01 compared to cells expressing D₂L-Rluc, β-arrestin1-GFP², CB¹-pcDNA and empty pcDNA and treated with 1 µM ACEA and quinpirole. n.s. compared to cells expressing D₂L-Rluc, β-arrestin1-GFP², and CB¹-pcDNA and treated with 1 µM quinpirole.
compared to cells expressing the empty pcDNA (Fig. 4.11D). Treating the cells with quinpirole did not alter BRET \(_{\text{Eff}}\) in cells expressing CB\(_1\)-BP compared to those expressing empty pcDNA (Fig. 11D). Co-application of quinpirole and ACEA potentiated \(\beta\)-arrestin1 recruitment to CB\(_1/D\_2L\) complexes, which is abolished when CB\(_1/D\_2L\) interaction was blocked.

\(\beta\)-arrestin1 recruitment to CB\(_1/D\_2L\) heterotetramers was followed by receptors internalization. CB\(_1\) internalization was measured over 30 min following ligand treatment in cells co-transfected with CB\(_1\)-pcDNA and D\(_{2L}\)-pcDNA (Fig. 4.12A,B). Treating cells with 1 \(\mu\)M ACEA resulted in CB\(_1\) internalization starting at 10 min compared to vehicle-treated cells (Fig. 4.12A,B). As predicted, treating cells with 1 \(\mu\)M quinpirole induced CB\(_1\) internalization compared to vehicle-treated cells only in cells co-expressing both CB\(_1\) and D\(_{2L}\) (Fig. 4.12B). Co-treating the cells with 1 \(\mu\)M ACEA and different concentrations of quinpirole dose-dependently increased CB\(_1\) internalization over 30 min compared to ACEA-treated cells (Fig. 4.12A,B). We also measured D\(_{2L}\) internalization following ligand treatment (Fig. 4.12C,D). D\(_{2L}\) internalization was observed in cells treated with 1 \(\mu\)M quinpirole and 1 \(\mu\)M ACEA; however, ACEA was less efficacious in inducing D\(_{2L}\) internalization compared to quinpirole (Fig. 4.12C,D). Co-application of 1 \(\mu\)M quinpirole with 0.1 or 1 \(\mu\)M ACEA potentiated D\(_{2L}\) internalization compared to quinpirole-treated cells (Fig. 4.12D). Altogether, co-internalization of CB\(_1/D\_2L\) complexes was observed following treatment with either ACEA or quinpirole treatment. Co-application of quinpirole and ACEA potentiated not only \(\beta\)-arrestin1 recruitment to CB\(_1/D\_2L\) complexes but also complex co-internalization.

Next, we wanted to test the influence of co-application of CB\(_1\) and D\(_2\) agonists on \(\beta\)-arrestin1-dependent ERK phosphorylation. Ga\(_i\)-dependent PTx-sensitive ERK phosphorylation was observed at 5 min following treatment with either 1 \(\mu\)M ACEA or quinpirole (Fig. 4.7C, 4.13A). As expected based on earlier experiments, co-application of both 1 \(\mu\)M ACEA or quinpirole did not lead to Ga\(_i\)-dependent PTx-sensitive ERK phosphorylation (Fig. 4.7C, 4.13B). However, the co-application of both agonists resulted in a delayed and sustained potentiation in ERK phosphorylation, which peaked at 15 min (Fig. 4.13A). Such an elevation in pERK was mediated through Ga\(_i\) -independent (PTx-insensitive) pathways (Fig. 4.13B). To test whether the observed increase in pERK was \(\beta\)-arrestin1-dependent, we co-transfected the cells with plasmid encoding \(\beta\)-arrestin1
Figure 4.12: Quinpirole and ACEA Co-Application Potentiated CB₁/D₂L Heterotetramer Internalization. (A) Time-course analysis of CB₁ receptors cell surface expression and total protein levels over 30 min measured using On-Cell Western™ and In-Cell Western™ in cells treated with ligands. (B) Cell surface expression of CB₁ receptors measured at 30 min following ligand treatment. * $P < 0.01$ compared with vehicle-treated cells. ~ $P < 0.01$ compared to ACEA-treated cells (C) Time-course analysis of D₂L receptors cell surface expression and total protein levels over 30 min measured using On-Cell Western™ and In-Cell Western™ in cells treated with ligands. (D) Cell surface expression of D₂L receptors measured at 30 min following ligand treatment. * $P < 0.01$ compared with vehicle-treated cells. ~ $P < 0.01$ compared to quinpirole-treated cells. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
Figure 4.13: Quinpirole and ACEA Co-Application Resulted in β-arrestin1-Dependent Sustained ERK Phosphorylation. (A) ERK phosphorylation (pERK1/2(Tyr-205/Tyr-185)/total ERK) was measured over 60 min in cells treated with vehicle, 1 μM ACEA, 1 μM quinpirole or both agonists. Vehicle or drug was added to cells at time 0; * $P < 0.001$ compared to vehicle-treated cells; ~ $P < 0.001$ compared to ACEA-treated cells. (B) ERK phosphorylation was measured at 15 min in cells treated with 1 μM ACEA, 1 μM quinpirole or both agonists with or without pre-treatment with 50 ng/ml PTx, 50 ng/ml CTx or in the presence of a β-arrestin1 dominant negative mutant (β-arrestin1 V53D). * $P < 0.01$ compared to vehicle-treated cells. ~ $P < 0.001$ compared to No Toxin treatment within the treatment group. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
dominant negative mutant (β-arrestin1 V53D) that has previously been shown to block sustained pERK signaling (Daaka et al., 1998). Co-expressing β-arrestin1 V53D with CB₁-blocked ACEA and quinpirole mediated pERK at 15 min (Fig. 4.13B). Based on these data, ACEA and quinpirole co-application potentiated a delayed and a sustained ERK phosphorylation via β-arrestin1-mediated signaling.

4.4 Discussion

The first objective of the current study was to understand the stoichiometry of CB₁/D₂L/Gα protein complexes. The second objective was to understand the allosteric interactions among the components of the CB₁/D₂L/Gα protein complex following the co-application of CB₁ and D₂ agonists. Our results from SRET² experiments combined with BiFC in addition to BRET² saturation experiments provide strong evidence that the basic functional unit is a CB₁/D₂L heterotetramer composed of CB₁ and D₂L homodimers coupled to a minimum of two Gα proteins. While the minimum functional unit appears to be a heterotetramer plus at least two Gα proteins, it is possible that multiple units associate to form higher order hetero-oligomers. The co-application of CB₁ and D₂ agonists led to changes in receptor- Gα units association from Gαᵢ to Gαₛ, which influenced signaling and trafficking of CB₁/D₂L heterotetramer via bidirectional allosteric mechanism (Summarized in Fig. 4.14).

4.4.1 CB₁/D₂L Receptors Form Heterotetramers Consisting of CB₁ and D₂L Homodimers

Our current study and other studies provide evidence that supports the hypothesis that two GPCR homodimers associate to form a heterotetramer (Guitart et al., 2014; Bonaventura et al., 2015; Navarro et al., 2016). Specifically, Guitart et al. (2014) reported that the dopamine receptor type 1 (D₁) and dopamine receptor type 3 (D₃) receptors form heterotetramers composed of D₁ and D₃ homodimers as demonstrated using BRET and BiFC and bimolecular luminescence complementation (BiLC) assays. The same approach has also been used to uncover the tetrameric structure of A₂A and D₂ heteromers (Bonaventura et al., 2015). A more recent study, using microscope-based single-particle tracking and molecular modeling, reported that A₁ and A₂A form mainly
Figure 4.14: Allosteric Interactions Within CB₁/D₂L Heterotetramers. (A) CB₁/D₂L receptors form heterotetramers consisting of CB₁ and D₂L homodimers. CB₁/D₂L heterotetramers are coupled to at least two Gα proteins. Treating cells with the CB₁ agonist [arachidonyl-2-chloroethylamide (ACEA)] (A), or the D₂ agonist quinpirole (B) resulted in Gα₁-dependent ERK phosphorylation, β-arrestin1 recruitment, and receptor co-internalization. (C) Treating cells co-expressing CB₁ and D₂L with both ACEA and quinpirole, switched CB₁/D₂L heterotetramers coupling and signaling from Gα₁ to Gα₅ proteins, enhanced β-arrestin1 recruitment, and receptor co-internalization.
heterotetramers composed of two homodimers, while A1 and A2A homomers, homotrimers and homotetramers were scarce (Navarro et al., 2016). The application of the Veatch and Stryer model (Vrecl et al., 2006; Drinovec et al., 2012) to the BRET2 saturation data suggests that CB1 and D2L heterotetramers were the main species in cells that expressed CB1 and D2L. SRET2 combined with BiFC further confirmed that CB1 and D2L heterotetramers were composed of CB1 and D2L homodimers. However, these findings do not rule out the possibility that a mixed population of CB1 and D2L homomers, heterodimers and higher oligomeric complex may simultaneously exist.

Even though monomeric GPCRs can activate G-proteins (Ernst et al., 2007; Kuszak et al., 2008), recent evidence suggests that a single protein binds to a GPCR homodimer (Navarro et al., 2016). It follows then that hetero-oligomeric complexes would be composed of multimers of homodimers each with an associated protein (reviewed in Ferré, 2015). Using complemented donor-acceptor resonance energy transfer (CODA-RET), Guitart et al., (2014) found that D1 and D3 heterotetramers are composed of two interacting D1 and D3 homodimers coupled to one Gαs and one Gαi protein, respectively. The same scheme has also been reported for A1/A2A heterotetramers (Navarro et al., 2016). BRET and computer modeling was used to demonstrate that A1 and A2A homodimers form a heterotetrameric complex with two G proteins. Gαi couples with an A1 homodimer and Gαs couples with an A2A homodimer (Navarro et al., 2016). Our result using BRET2 experiments fit with the proposed model of receptor heterotetramers/G protein stoichiometry where CB1 and D2L homodimers each associate with one Gαi protein. Even in the presence of a peptide that specifically blocks the interaction between CB1 receptors and Gαi, we were able to detect energy transfer from Gαi-Rluc to CB1-GFP2 following D2 agonist treatment. This energy transfer would be observed only if another Gαi protein was bound to the D2L homodimer within the CB1/D2L/Gαi protein complex. There was no energy transfer in the presence of the Gαi blocking peptide when only CB1 was expressed. However, our experimental design does not exclude the possibility that CB1 and D2L monomers interact with the Gαi protein. Although several powerful tools are available and have been used to identify GPCR heteromerization in recombinant heterologous systems, it remains a challenge to detect and quantify the stoichiometry and distribution of GPCR complexes in native cells.
4.4.2 Bidirectional Allosteric Interactions Within CB₁/D₂L Heterotetramers Modulate G Protein Coupling

CB₁/D₂L heterotetramers elicit distinct signaling properties compared with receptor homodimers (Kearns et al., 2005; Glass and Felder, 1997; Khan and Lee, 2014). Activation of either CB₁ or D₂L homodimers by their respective selective agonists, within the CB₁/D₂L heterotetramers, activated Gαᵢ proteins and resulted in Gαᵢ-dependent signaling. Simultaneous co-activation of CB₁ and D₂L altered the coupling of each homodimer within the CB₁/D₂L/Gα heterotetrameric complex. This effect was specific to CB₁/D₂L heterotetramers as the effect was not observed when the interaction between CB₁ and D₂L was blocked. In addition, the co-expression CB₁ and β₂ in HEK 293A cells, which are known to form heteromers, and the co-application of both receptor agonists did not alter the interaction between Gαᵢ and CB₁. We speculate that binding of both CB₁ and D₂ agonists to CB₁ and D₂L, respectively, leads to agonist dose-dependent conformational changes within CB₁/D₂L/Gαᵢ complexes. This conformational change induces bidirectional allosteric modulation to reduce coupling of both receptors to Gαᵢ, while inducing each CB₁ and D₂L homodimer within the CB₁/D₂L complex to couple to Gαₛ. In such a situation, D₂-selective agonists, through D₂L receptor binding within CB₁/D₂L/Gαᵢ complexes, acted as allosteric modulators that altered the efficacy and potency of CB₁ to couple and activate different Gα protein pathways only in the presence of CB₁ agonist. At the same time, CB₁-selective agonists, binding to CB₁, acted as allosteric modulators that altered the efficacy and potency of D₂L receptors to couple and activate different G proteins in the presence of D₂ agonist. The allosteric mechanisms exert negative and positive cooperatively with respect to Gαᵢ and Gαₛ. Changes in GPCR and G-protein coupling following ligand application was also observed by Rashid et al., (2007). D₁ and D₂ homomers are coupled to Gαₛ and Gαᵢ, respectively. Heterodimerization between D₁ and D₂ results to a drastic shift of G protein coupling, where D₁/D₂ heterodimer is mainly coupled to a Gα₉/₁₁ protein (Rashid et al., 2007).

4.4.3 Co-Activation of Both CB₁ and D₂L Potentiated CB₁/D₂L Heterotetramers β-Arrestin Recruitment

CB₁ and D₂L are known to interact with β-arrestin1, which mediates receptor
internalization, β-arrestin1-mediated signaling, receptor recycling and degradation (Laprairie et al., 2014; Sim-Selley and Martin, 2003; Wu et al., 2008). Within CB1/D2L heterotetramers, the D2 agonist acted as a positive allosteric modulator that potentiated the efficacy and potency of β-arrestin1 interaction with CB1 receptors following the application of the CB1 agonist. Similarly, the CB1 agonist potentiated the interactions between β-arrestin1 and D2L. These finding suggest bidirectional allosteric interactions between CB1 and D2L within CB1/D2L heterotetrameric complexes positively modulate β-arrestin1 recruitment to CB1/D2L complexes paralleled CB1/D2L complex co-internalization. Unlike the D2 agonist quinpirole used in the current experiments, the high-affinity D2 antagonist haloperidol acts as a negative allosteric modulator that reduced β-arrestin1 recruitment to CB1 receptors and subsequently inhibited CB1 receptor internalization (Przybyla and Watts, 2010). Quinpirole did not alter β-arrestin1-CB1 interaction in the absence of D2L. Similarly, the CB1 agonist ACEA did not alter β-arrestin1-D2L interactions in the absence of CB1. Therefore, expression and activation, and not simply ligand binding, of both CB1 and D2L is required for the potentiation β-arrestin1 recruitment to CB1/D2L complexes. As was observed for CB1/D2L heterotetramers, agonist co-activation of other GPCR heteromers have been shown to alter agonist-induced β-arrestin recruitment to receptor complexes (Borroto-Escuela et al., 2011). In A2A/D2L co-expressing cells, A2A/D2L form heterotetramers and the A2A agonist CGS21680 was found to enhance the D2 agonist-induced β-arrestin1 recruitment to D2L receptors with subsequent co-internalization of A2AR/D2L complexes (Borroto-Escuela et al., 2011).

In addition to modulating β-arrestin1 binding and receptor internalization, co-treatment of CB1/D2L heterotetramers with CB1 and D2 agonists significantly augmented β-arrestin1-dependent ERK phosphorylation compared to cells treated with either CB1 agonist or D2 agonist alone. β-arrestin1-dependent ERK phosphorylation was insensitive to Ptx treatment, but was significantly reduced in cells expressing a β-arrestin1 dominant negative mutant. The potentiation of β-arrestin1-dependent ERK phosphorylation can be explained by the potentiation of β-arrestin1 binding to CB1/D2L complexes. Similarly, the co-activation of both D1 and D3 with their agonists, 7-OH-PIPAT and SKF 38393, respectively, increased recruitment of β-arrestin1 to D1/D3 heterotetramers and
potentiated β-arrestin1-dependent ERK phosphorylation compared to levels observed when single agonist was applied (Guitart et al., 2014).

4.5 Conclusion

Taken together, the results presented here demonstrated bidirectional allosteric interactions between CB₁ and D₂L within CB₁/D₂L heterotetramers, which modulate both G protein-coupling and G protein-dependent signaling as well as β-arrestin1 recruitment and G-protein-independent ERK signaling. The concept of bidirectional allosteric interaction between CB₁/D₂L heterotetramers has important implications for understanding the activity of receptor complexes in native tissues and the potential for altered drug response under pathological conditions. For example, patients with Parkinson’s disease, which is characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta and dopaminergic denervation of the striatum (Pisani et al., 2011; El Khoury et al., 2012), may have altered responses to their prescribed Parkinson’s disease medication if they are prescribed cannabinoids or choose to expose themselves to cannabinoids. Treatment of Parkinson’s disease frequently involves the administration of levodopa to increase striatal dopamine levels or administration of direct dopamine agonists. The half-life of levodopa is relatively short requiring multiple daily dosing leading to peak and trough values throughout the day (Brooks, 2008). The timing of exposure to cannabinoids in relation to levodopa or dopamine agonists could influence drug response and the pool of receptors at the membrane. In addition, the dose, potency, combination of cannabinoids (such as levels of THC relative to cannabidiol, and half-life of specific cannabinoids within marijuana may not be consistent such that the response to the combination of drugs may be variable. On the other hand, understanding the interaction within CB₁/D₂L heterotetramers may assist in the design, identification and use of novel combinations of ligands. Ligands specifically targeting CB₁/D₂L heterotetramers within restricted neuronal populations may be beneficial in central nervous disorders associated with dopaminergic and/or endocannabinoid signaling dysregulation.
4.6 Supplementary Figures

**Supplementary Figure 4.1: CB₁ and β₂AR Receptors form Homodimers When Expressed in HEK 293A Cells Demonstrated Using BiFC.** (A) HEK 293A cells were transfected with CB₁-VC, CB₁-VN, or CB₁-VC and, CB₁-VN with or without CB₁/D₂L hetero-oligomer blocking peptide (CB₁-BP). *P < 0.01 compared to cells expressing CB₁-VN. (B) HEK 293A cells were transfected with β₂AR-VC, β₂AR-VN or β₂AR-VC, and β₂AR-VN. Fluorescence was measured using an EnVision plate reader with excitation at 515 nm and emission at 528 nm. *P < 0.01 compared to cells expressing β₂AR-VN. Data are presented as mean ± SEM of 4 independent experiments, significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
Supplementary Figure 4.2: Kinetic Interaction of CB1 and D2L with Ga\textsubscript{i} and Ga\textsubscript{s} Proteins. BRET\textsubscript{Eff} was measured over 540 s in cells expressing Ga\textsubscript{i}-Rluc, CB1-GFP\textsuperscript{2}, and D\textsubscript{2L}-pcDNA (A), Ga\textsubscript{s}-Rluc, CB1-GFP\textsuperscript{2}, and D\textsubscript{2L}-pcDNA (B), Ga\textsubscript{i}-Rluc, D\textsubscript{2L}-GFP\textsuperscript{2}, and CB1- pcDNA or (C) or Ga\textsubscript{s}-Rluc, D\textsubscript{2L}-GFP\textsuperscript{2}, and CB1-pcDNA (D). Cells were treated with vehicle, 1 µM ACEA, 1 µM quinpirole alone or in combination added at 50 sec after the addition of Coelenterazine 400a. Arrows indicate the times of drug(s) application. Data are presented as mean ± SEM of 4 independent experiments.
Supplementary Figure 4.3: The Expression of CB1-Ga_i-BP Does Not Alter the Ability of the CB1 and D2L to Form Heterotetramers. Cells were transfected with D2 Rluc, D2-GFP, CB1-VN and CB1-VC together with an empty pcDNA, CB1-BP or CB1- Ga_i-BP. SRET2 combined with BiFC was performed. * P < 0.01 compared to cells expressing empty pcDNA; n.s. P > 0.05 relative to cells expressing empty pcDNA. Data are presented as mean ± SEM of 4 independent experiments, significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
Supplementary Figure 4.4: CB₁ and D₂L Do Not Interact With Gα₅ Proteins. BRETₜwas measured following the addition of vehicle or 1 µM ACEA +/- 24 h pre-treatment with 50 ng/ml CTx (A) in cells expressing Gα₅-Rluc and CB₁-GFP² or (B) cells expressing Gα₅-Rluc and D₂L-GFP². Controls included cell transfected with Gα₅-Rluc and β₂AR-GFP² (positive control) or HERG-GFP² (negative control) treated with vehicle or β₂AR agonist isoprenaline. * P < 0.01 compared to cells expressing Gα₅-Rluc and HERG-GFP²; ~ P < 0.01 compared to cells expressing Gα₅-Rluc and β₂AR-GFP² and treated with vehicle; n.s. P > 0.05 relative to cells expressing Gα₅-Rluc and HERG-GFP². Data are presented as mean ± SEM of 4 independent experiments; significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
Supplementary Figure 4.5: Blocking the Interaction Between CB1 and D2L Inhibited the Switch of CB1 and D2L Coupling from Ga\textsubscript{i} to Ga\textsubscript{s} Proteins Following Co-Application of Both Receptor Agonists. BRET\textsubscript{Eff} was measured in cells treated with vehicle, 1 μM ACEA, 1 μM quinpirole alone or in combination. (A) Cells expressing Ga\textsubscript{i}-Rluc, CB1-GFP\textsuperscript{2}, D2L-pcDNA together with an empty vector pcDNA or CB1-B (B) Cells expressing Ga\textsubscript{i}-Rluc, CB1-GFP\textsuperscript{2}, D2L-pcDNA together with an empty vector pcDNA or CB1-BP. * P < 0.01 compared to cells expressing empty pcDNA and treated with quinpirole; ~ P < 0.01 compared to cells expressing empty pcDNA and treated with ACEA and quinpirole; # P < 0.01 compared to cells expressing empty pcDNA and treated with ACEA; n.s. P > 0.05 relative to cells expressing empty pcDNA within treatment group. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
Supplementary Figure 4.6: The Co-Expression and Co-Activation of Either CB₁ and β₂AR or D₂L and β₂AR Did Not Alter CB₁ or D₂ Coupling to G proteins. BRET<sub>Eff</sub> was measured in cells expressing G<sub>α</sub><sub>i</sub>-Rluc (A, C) or G<sub>α</sub><sub>s</sub>-Rluc (B, D) and either CB₁-GFP<sup>2</sup>, D₂L-GFP<sup>2</sup>, β₂-GFP<sup>2</sup> or the negative control HERG-GFP<sup>2</sup> and treated with vehicle, 1 μM ACEA, 1 μM quinpirole, 1 μM isoprenaline alone or in combination. * P < 0.01 compared to cells expressing G<sub>α</sub><sub>i</sub>-Rluc or G<sub>α</sub><sub>s</sub>-Rluc and HERG-GFP<sup>2</sup>. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
Supplementary Figure 4.7: The Co-Application of ACEA and Quinpirole Switched CB1 Coupling and Signaling From Ga to Ga proteins in STHdhQ7/Q7 Cells. BRET_{Eff} was measured in STHdhQ7/Q7 cells expressing Ga_{i}-Rluc and CB1-GFP2 +/- D2L-pcDNA (A) or Ga_{s}-Rluc and CB1-GFP2 +/- D2L-pcDNA (B) and treated with vehicle, 1 μM ACEA, 1 μM quinpirole or both agonists. * P < 0.01 compared to cells treated with vehicle; ~ P < 0.01 relative to cells expressing Ga_{i}-Rluc and CB1-GFP2 and treated with 1 μM ACEA and 1 μM quinpirole; n.s. P > 0.05 relative to cells expressing Ga_{i}-Rluc and CB1-GFP2 or Ga_{i}-Rluc and CB1-GFP2. HEK 293A cells were treated with 1 μM ACEA, 1 μM quinpirole or both agonists +/- 24 h pretreatment with 50 ng/ml PTx or CTx; ERK phosphorylation was measured 5 min following ACEA, quinpirole or the combination (C), while CREB phosphorylation was measured following 30 min treatment (D). * P < 0.01 compared to vehicle treatment; ~ P < 0.01 compared to cells treated with 1 μM ACEA. Data are presented as mean ± SEM of 4 independent experiments, one-way ANOVA followed by Tukey's post-hoc test.
Supplementary Figure 4.8: The Potentiation of β-arrestin1 Recruitment to CB₁ and D₂L Following ACEA and Quinpirole Co-Application is Specific to CB₁/D₂L Heteromer. HEK 293A Cells were transfected with β-arrestin1-Rluc + CB₁-GFP² + mGluR6-pcDNA (A) or D₂L-Rluc + β-arrestin1-GFP² + mGluR6-pvDNA (B). BRET Eff signals were measured over 60 min following the application of vehicle, 1 µM ACEA, 1 µM quinpirole or 1 µM L-AP4 alone or in combination. Data are presented as mean ± SEM of 4 independent experiments.
CHAPTER 5

CHRONIC CANNABINOID AND TYPICAL ANTIPSYCHOTIC TREATMENT REDUCE CANNABINOID RECEPTOR TYPE 1 (CB\(_1\)) AND THE DOPAMINE RECEPTOR TYPE 2 (D\(_2\)) HETEROMER EXPRESSION IN THE GLOBUS PALLIDUS OF MICE

Copyright Statement

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Contribution Statement

The manuscript used as the basis for this chapter was written with guidance from Dr. Eileen Denovan-Wright. Data were collected and analyzed by myself. Data were collected by myself with assistance from Dr. Robert B. Laprairie, Dr. James T. Toguri, and Adel Zrein. Critical reagents were provided by Drs. Eileen Denovan-Wright and Melanie Kelly.
5.1 Abstract

The cannabinoid receptor type 1 (CB₁) and the dopamine receptor type (D₂L) are co-localized on medium spiny neuron terminals in the globus pallidus where they play an important role in modulating voluntary movement. Physical interactions between the two receptors (heteromerization) have been shown to alter receptor coupling and signaling in cell culture. The main objectives of the current study were to examine whether CB₁ and D₂L heteromers can be detected in the globus pallidus of C57BL/6J mice and to determine whether CB₁/D₂L heteromer levels are altered following chronic treatment with cannabinoids and antipsychotic alone or in combination. By using in situ proximity ligation assays, we observed CB₁ and D₂L heteromer-specific signals in the globus pallidus of C57BL/6J mice. An increase in CB₁/D₂L heteromer-specific signal was observed in the globus pallidus of C57BL/6J mice following chronic CP 55,940 treatment. In contrast, haloperidol treatment reduced CB₁/D₂L heteromer-specific signals. Olanzapine treatment did not affect CB₁/D₂L heteromer-specific signals relative to vehicle treatment. Chronic co-treatment with CP 55,940 and haloperidol resulted in CB₁/D₂L heteromer-specific signals similar to those observed in the haloperidol-treated group. Chronic co-treatment with CP 55,940 and olanzapine resulted in a similar distribution of heteromers as the CP 55,940-treated group. The alteration in CB₁/D₂L heteromer-specific signals following persistent ligand exposure was due to alteration in the mutual affinity of CB₁ and D₂L receptors and was not due to changes in CB₁/D₂L protein expression or receptor co-localization. Chronic exposure to cannabinoid and antipsychotics alone or in combination alters CB₁/D₂L heteromerization and affects movement.

5.2 Introduction

The endocannabinoid system (ECS) and dopaminergic system (DS) play important roles modulating voluntary movement under the control of the basal ganglia (reviewed in Fernández-Ruiz et al., 2010; El Khoury et al., 2012; Bloomfield et al., 2016; García et al., 2016). Stimulating dopaminergic transmission in the basal ganglia results in hyperkinesia (Gershanik et al., 1983; Kelly et al., 1998; reviewed in Iversen and Iversen, 2007), whereas blocking normal dopamine function leads to hypolocomotion (Hauber and Lutz, 1999; Schindler and Carmona, 2002). In contrast, activation of the
ECS has been associated with motor inhibition, although effects on locomotion are dose-dependent (McGregor et al., 1996, reviewed in Giuffrida and Piomelli, 2000; Fernández-Ruiz and Gonzáles 2005; Fernández-Ruiz, 2009; Kluger et al., 2015). Interactions between the ECS and DS have been described. For example, cannabinoid agonists block both dopamine agonist-induced hyperlocomotion (Marcellino et al., 2008) and amphetamine-induced hyperactivity (Gorriti et al., 1999). Interactions between the ECS and DS may occur indirectly through the independent modulation of GABA- and/or glutamate release (reviewed in Fernández-Ruiz et al., 2010; El Khoury et al., 2012; Bloomfield et al., 2016; García et al., 2016). The interactions can also occur at the synapse via depolarization-induced suppression of excitation (DSE) and inhibition (DSI) involving receptors located on both sides of the synaptic cleft (reviewed in Fernández-Ruiz et al., 2010; El Khoury et al., 2012). In addition, recent evidence indicates that the cannabinoid receptor type 1 (CB1) is able to physically interact with the dopamine receptor 2 long (D2L) to form heteromers (Kearn et al., 2005; Marcellino et al., 2008; Przybyla and Watts, 2010; Bagher et al., 2016, 2017). Heteromers composed of CB1 and D2L might represent an additional pharmacological target for the combined effects of cannabinoids and dopaminergic ligands. Both receptors are co-localized in GABAergic medium spiny projection neuron (MSN) terminals located in the globus pallidus of rodents and primates (Herkenham et al., 1991; Levey et al., 1993; Ong and Mackie, 1999; Pickel et al., 2006). Heteromerization between CB1 and D2L has been detected in the striatum of Macaca fascicularis using in situ proximity ligation assays (PLA), demonstrating that the association between CB1/D2L receptors occurs in native tissues (Bonaventura et al., 2014).

Physical and functional interactions between CB1 and D2L receptors have been observed in cell culture. Physical interactions between CB1 and D2L receptors have been observed using co-immunoprecipitation, Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET) and bimolecular fluorescence complementation (BiFC) (Kearn et al., 2005; Marcellino et al., 2008; Przybyla and Watts 2010; Bagher et al., 2016). Functional interactions have been observed in cells co-expressing both CB1 and D2L receptors. Stimulation of either CB1 or D2L receptors by receptor-specific agonists resulted in the activation of the Ga_i protein, while simultaneous
co-activation of both receptors switched coupling and signaling from Ga to Ga, protein (Glass and Felder, 1997; Kearn et al., 2005; Marcellino et al., 2008; Bagher et al., 2016). The co-ligand dependent switch in signaling is dependent on reciprocal allosteric modulation of G protein coupling, which results in CB1 and D2L heteromer-specific signaling and co-internalization that differs from independent CB1 or D2 receptor signaling (Bagher et al., 2017). It has been suggested that ligands that bind CB1 and/or D2L might modulate receptor expression and the proportion of CB1 and D2L receptors existing in homo- versus heteromers (Bonaventura et al., 2014). For example, CB1/D2L heteromer expression was lower in the striatum of Macaca fascicularis following chronic administration of the dopamine precursor levodopa (L-DOPA) (Bonaventura et al., 2014).

Given the documented interactions between CB1 and D2L receptors in cultured cells and brain tissue, receptor-specific ligands must be considered in the context of their effects on the cognate receptor, and on interacting receptors within heteromeric complexes. Drugs that act on the D2 receptors such as typical- and atypical-antipsychotics are prescribed for the management of movement disorders such as Tics, Tourette syndrome and Huntington's disease (Videnovic, 2013; Gilberta and Jankovicb, 2014; Wyant et al., 2017). Typical and atypical antipsychotics, however, have been shown to have different clinical, biochemical and behavioral profiles (reviewed in Seeman and Ulpian, 1988; Lowe et al., 1988; Blin, 1999; Rummel-Kluge et al., 2012). Patients prescribed typical- or atypical- antipsychotics are sometimes exposed to cannabinoids for therapeutic or recreational purposes. Based on the co-localization of CB1 and D2L in MSN terminals in the globus pallidus, we hypothesized that chronic cannabinoid and antipsychotics administration alone or in combination differentially affects CB1/D2 heteromerization and protein expression, which in turn affects motor output. In the current study, in situ PLA was utilized to detect CB1/D2 heteromerization and to measure changes in CB1/D2 heteromer-specific PLA signals following chronic drug administration of either cannabinoid or antipsychotics alone or in combination. Heteromer distribution was measured in the globus pallidus of C57BL/6J mice and in a cell culture model of MSN that endogenously expresses both CB1 and D2L receptors. Haloperidol and olanzapine were chosen as representative typical and atypical
antipsychotics, respectively. Haloperidol acts primarily as a D₂ dopamine receptor antagonist. In contrast, olanzapine is an antagonist at many receptors, including 5-HT₂₄, H₁, D₂, D₄, and M₅ receptors (reviewed in Murray et al., 2017). The CB₁ agonist CP 55,940 was used in the current study; this synthetic cannabinoid has similar tetrad effects and ligand bias compared to the phytocannabinoid delta-9-tetrahydrocannabinol (THC) found in Cannabis (Glass and Northup, 1999; Mukhopadhyay and Howlett 2005; Laprairie et al., 2016; reviewed in Laprairie et al., 2017).

5.3 Results
5.3.1 CB₁ and D₂ Heteromers are Found in the Globus Pallidus of C57BL/6J Mice, and Chronic Cannabinoid and/or Antipsychotic Treatment Alters CB₁/D₂ Heteromer-Specific PLA Signals

The first aim of the current study was to examine whether CB₁ and D₂ receptors physically associate in the globus pallidus of C57BL/6J mice. In situ PLA detects endogenous receptors that are in close proximity (< 16 nm). In PLA, closely associated receptors allow two different receptor-specific antibody-DNA probes to form a ligation complex resulting in a punctate fluorescent signal (PLA signal) that can be detected by fluorescence microscopy. By incubating mouse brain slices with two primary antibody-DNA probes directed against the N-terminal of CB₁ and D₂L receptors, we observed CB₁/D₂L heteromer-specific PLA signals in the globus pallidus (Fig. 5.1A, B). PLA signals were not observed when brain slices were incubated with CB₁ or D₂ antibody/probe alone (data not shown). These results indicate that CB₁ and D₂L can physically associate in the globus pallidus.

Our second aim was to investigate whether chronic exposure to cannabinoid or antipsychotic treatment alone or in combination alters the number of CB₁/D₂L heteromer-specific PLA signals in the globus pallidus of C57BL/6J mice. C57BL/6J mice were treated with vehicle or 0.01 mg/kg/d CP 55,940, 0.3 mg/kg/d haloperidol, 1.5 mg/kg/d olanzapine, or co-treated with 0.01 mg/kg/d CP 55,940 and 0.3 mg/kg/d haloperidol or 0.01 mg/kg/d CP 55,940 and 1.5 mg/kg/d olanzapine. Dosages used in this study were based on previous studies and were chosen for pharmacological and behavioral effects (Arjona et al., 2004, Huang et al., 2006; Han et al., 2009). The dosages of haloperidol
Figure 5.1: Chronic Haloperidol Treatment Inhibited CB₁/D₂ Heteromer-Specific PLA Signals in the Globus Pallidus of C57BL/6J Mice, Unlike CP 55,940 Which Increased CB₁/D₂ Heteromer-Specific PLA Signals. **(A)** *In situ* PLA in the globus pallidus following treatment for 21 days with vehicle or 0.01 mg/kg/d CP 55,940, 0.3 mg/kg/d haloperidol or 1.5 mg/kg/d olanzapine i.p. alone or in combination and primary antibodies for CB₁ and D₂L receptors. Microscopy images (superimposed sections) are shown in where heteromers appear as red dots, while cell nuclei were stained with DAPI (blue). Scale bars: 10 μm. **(B)** PLA signals were presented as the number of the red dot per 1000 μM² from three different fields within globus pallidus from five different animals per group. * P < 0.01 compared to vehicle-treated group. # P < 0.01 compared to CP 55,940-treated group. Data are presented as mean ± SEM of 15 different fields. Significance was determined via one-way ANOVA followed by Tukey's *post-hoc* test.
and olanzapine result in 70-80% D2 receptor occupancy in rats (Kapur and Mamo, 2003, Natesan et al., 2006). The dose of CP 55,940 was chosen based on the preliminary studies of Marcellino et al. (2008). Daily drug injection began when mice were 7 weeks of age and continued for 3 weeks (21 days). At the end of the study, mouse brains were collected and brain sections were prepared. In situ PLA assays were performed to detect changes in the number of CB1/D2L heteromers-specific PLA signals for each treatment. The numbers of CB1/D2L heteromer-specific PLA signals was reduced in the globus pallidus of haloperidol-treated mice compared with vehicle (Fig. 5.1A, B). CP 55,940 increased the number of CB1/D2L heteromer-specific PLA signals (Fig. 5.1A, B) in the globus pallidus compared to vehicle treatment. However, co-treatment with both CP 55,940 and haloperidol resulted in lower CB1/D2L heteromer-specific PLA signals compared to either CP 55, 940 or vehicle treatment suggesting that the haloperidol effect blocked CP 55, 940-dependent increases in heteromer formation (Fig. 5.1A, B). No alteration in CB1/D2L heteromer-specific PLA signals was observed in the globus pallidus of olanzapine-treated mice. Co-treatment of mice with CP 55, 940 and olanzapine resulted in CB1/D2L heteromer-specific PLA signals similar to that observed in CP 55, 940-treated mice (Fig. 5.1A, B). Taken together, these results indicate that chronic cannabinoid and typical, but not atypical, antipsychotics differentially altered the CB1 and D2L heteromer population in the globus pallidus of C57BL/6J mice.

5.3.2 Persistent Treatment with Cannabinoid and/or Antipsychotics Modulates CB1 / D2L Heteromerization in STHdhQ7/Q7 Cells

We also tested whether the observed alteration in CB1/D2L heteromer-specific PLA signals in the globus pallidus of C57BL/6J mice following chronic drug treatment also occurred in STHdhQ7/Q7 cells endogenously expressing CB1 and D2L receptors that model striatal MSN. Co-localization of CB1 and D2L receptors in STHdhQ7/Q7 has been reported previously (Bagher et al., 2016) suggesting that the two endogenous receptors might form heteromers. STHdhQ7/Q7 cells were subjected to in situ PLA. CB1/D2L heteromer-specific PLA signals were observed in cells when both CB1 and D2L primary antibodies were applied (Fig. 5.2A), whereas no PLA signal was detected if CB1 or D2-specific primary antibodies were applied alone (data not shown). These observations
**Figure 5.2: Persistent Treatment with Cannabinoid and/or Antipsychotics Modulates Endogenous CB₁ and D₂L Heteromers in STHdh<sup>Q7/Q7</sup> Cells Demonstrated Using PLA.** (A) Cells were treated with vehicle or cannabinoid and/or antipsychotics for 20 hr, fixed, blocked and exposed to antibodies against CB₁ and D₂L. Interacting complexes were visualized following PLA. Immunofluorescence microscopy images (merged images) are shown in which CB₁/D₂L heteromers appear as red dots and cell nuclei were stained with DAPI (blue). Scale bars 100 μm. (B) PLA signals are presented as the average number of red dots per cell. *P < 0.01 compared to cells treated with vehicle. #P < 0.01 compared to cells treated with CP 55, 940. Data are represented as mean ± SEM for 10-20 cells from three independent experiments. Significance was determined via one-way ANOVA followed by Tukey's *post-hoc* test.
indicate that endogenous CB1 and D2L receptors form heteromers in STHdh<sup>Q7/Q7</sup> cells. The effect of persistent treatment with CB1 and/or D2 ligands on CB1/D2L heteromer-specific PLA signals in STHdh<sup>Q7/Q7</sup> cells was evaluated (Fig. 5.2A, B). STHdh<sup>Q7/Q7</sup> cells were treated with 1 μM CP 55,940, haloperidol, olanzapine or combinations of each antipsychotic with CP 55,940 for 20 hr followed by in situ PLA. Treating STHdh<sup>Q7/Q7</sup> cells with haloperidol alone or in combination with CP 55,940 decreased the number of CB1/D2L heteromer-specific PLA signals compared to vehicle-treated cells (Fig. 5.2A,B his finding might suggest that haloperidol alone or in the presence of CP 55,940 reduced the affinity of the two receptors, reduced expression of CB1 and D2L, or changed the cellular localization of the two receptors. Olanzapine treatment alone did not alter PLA signals (Fig. 5.2A, B). The application of CP 55,940 alone or in combination with olanzapine significantly increased CB1/D2L heteromer-specific PLA signals compared to vehicle-treated cells (Fig. 5.2A, B) indicating that CP 55,940 either increased the affinity of the two receptors, increased expression of the CB1 and D2L proteins, or altered the cellular localization of the two receptors.

5.3.3 Persistent Treatment with Cannabinoid and/or Antipsychotics Modulates Inter-Receptor CB1/D2L Affinity and the Probability of Heteromer Formation

BRET<sup>2</sup> saturation assays were generated to measure the interaction between C-terminally tagged CB1 and D2L receptors in HEK 293A cells. BRET<sup>2</sup> assays were conducted using HEK 293A cells, instead of STHdh<sup>Q7/Q7</sup> cells, because HEK 293A cells do not express endogenous CB1 or D2L receptors and therefore no endogenous CB1 or D2L receptors were available to interfere with the observed BRET<sub>Eff</sub> values generated by exogenous expression of each receptor. BRET<sup>2</sup> saturation assays provide information about the affinity of tagged receptors and provide information about conformational changes within tagged receptor complexes (Ramsay et al., 2002; James et al., 2006). HEK 293A cells were co-transfected with a constant amount of CB1-Rluc with increasing amounts of D2L -GFP<sup>2</sup> and ligands were added 5 hr following transfection. Cells were exposed to ligand treatment for 20 hours. The combination of CB1-Rluc with D2 -GFP<sup>2</sup> resulted in a hyperbolic increase in BRET<sup>2</sup> saturation curve as previously observed (Bagher et al., 2016). The BRET<sup>2</sup> saturation curve in the presence of vehicle resulted in a
BRET$_{50}$ of 0.41 ± 0.03 and a BRET$_{\text{Max}}$ of 0.32 ± 0.01 (Fig. 5.3A,B). Negative controls included a plasmid expressing GFP$^2$-linked mGluR6 (mGluR6-GFP$^2$), a GPCR that is not known to have an affinity for CB$_1$ or D$_{2L}$ (Hudson et al., 2010). The BRET$^2$ saturation curve obtained from cells expressing CB$_1$-Rluc and mGluR6-GFP$^2$ (Fig. 5.3A) resulted in very weak BRET$^2$ signals. Consistent with earlier reports, the BRET$_{\text{Eff}}$ signal resulting from the interaction between CB$_1$ and D$_{2L}$ was specific and saturable (Fig. 5.3A).

Treating cells co-expressing CB$_1$-Rluc and D$_{2L}$-GFP$^2$ for 20 h with 1 μM CP 55,940 resulted in a BRET$_{50}$ of 0.32 ± 0.02, which indicated that CP 55,940 increased the affinity between CB$_1$-Rluc and D$_{2L}$-GFP$^2$ (Fig. 5.3A,B). In contrast, 1 μM haloperidol-treatment resulted in a BRET$_{50}$ value of 0.51 ± 0.01, which indicated that haloperidol reduced the affinity of CB$_1$-Rluc and D$_{2L}$-GFP$^2$ relative to vehicle treatment (Fig. 5.3A,B). There was no difference in the BRET$_{50}$ values in cells treated with the vehicle or 1 μM olanzapine indicating that olanzapine did not alter the interaction between CB$_1$-Rluc and D$_{2L}$-GFP$^2$ (Fig. 5.3A,B).

The effect of co-treating cells with CP 55,940 together with haloperidol or olanzapine on the interaction between CB$_1$ and D$_{2L}$ were also evaluated. Co-treating the cells with 1 μM CP 55,940 and haloperidol yielded a BRET$_{50}$ of 0.51 ± 0.01, which was similar to the value observed in the presence of haloperidol alone (Fig. 5.3A,B). Co-treating the cells with 1 μM CP 55,940 and olanzapine yielded a BRET$_{50}$ of 0.35 ± 0.02, which was similar to the value observed in the presence of CP 55,940 alone (Fig. 5.3A,B). When CP 55,940 was co-applied with haloperidol, the destabilizing influences of haloperidol on CB$_1$/D$_{2L}$ heteromerization predominated. When CP 55,940 was co-applied with olanzapine, the stabilizing influences of CP 55,950 on CB$_1$ and D$_2$ was unopposed.

BRET$_{\text{Max}}$ reflects the relative orientations of the Rluc Donor and the GFP$^2$ acceptor (Guan et al., 2009). Although BRET$_{\text{Max}}$ values can change if levels of the donor and acceptor are altered by ligand treatment, this is unlikely to have occurred in the current experiments; both donor and acceptor molecules were under the control of the CMV promoter within expression plasmids. Elevation in BRET$_{\text{Max}}$ values relative to vehicle treatment was observed in all treatment groups with the exception of olanzapine (Fig. 5.3B). The increase in BRET$_{\text{Max}}$ indicated that ligand binding altered and stabilized
Figure 5.3: Persistent Treatment with Cannabinoid and/or Antipsychotics Modulates CB₁ and D₂L Receptors Heteromerization When Expressed in HEK 293A Cells Demonstrated Using BRET². (A) BRET² saturation curves obtained from cells transiently transfected with CB₁-Rluc and D₂L-GFP². As a negative control, cells were co-transfected with CB₁-Rluc and mGluR6-GFP². BRET Eff is plotted against the ratio of GFP²/ Rluc. Data were fit to a rectangular hyperbolic curve. Cells were treated for 20 hr with vehicle or 1 µM CP 55,940, haloperidol, olanzapine alone or in combination. (B) BRET Max and BRET 50 parameters derived from BRET² saturation curves. Data are presented as mean ± SEM of 4 independent experiments. Significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
A)

![Graph showing BRET values for different treatments.](image)

B)

<table>
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<th>Treatment</th>
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<th>$BRET_{\text{MAX}}$</th>
</tr>
</thead>
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<td>$0.32 \pm 0.01$</td>
</tr>
<tr>
<td>1 $\mu$M CP 55,940</td>
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<td>$0.46 \pm 0.02^*$</td>
</tr>
<tr>
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<td>$0.41 \pm 0.01^*$</td>
</tr>
<tr>
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<td>$0.34 \pm 0.01$</td>
</tr>
<tr>
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<td>$0.47 \pm 0.01^*$</td>
</tr>
<tr>
<td>1 $\mu$M CP 55,940 + 1 $\mu$M Olanzapine</td>
<td>$0.35 \pm 0.02^*$</td>
<td>$0.44 \pm 0.02^*$</td>
</tr>
</tbody>
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the conformation of the CB₁ and D₂L heteromer, which enhanced the energy transfer between CB₁ and D₂L. Therefore, the observed changes in CB₁/D₂L heteromer-specific PLA signals following chronic exposure to ligand was most likely due to ligand-dependent changes in the affinity of the two receptors within the heteromeric complex.

5.3.4 Chronic Cannabinoid and/or Antipsychotic Treatment in C57BL/6J Mice Alters the Expression of CB₁ and D₂ in the Globus Pallidus

CB₁ and D₂L protein expression were measured in the globus pallidus to determine whether the ligand-dependent changes in CB₁/D₂L heteromer-specific PLA signals in the globus pallidus of C57BL/6J mice was due to alterations in the pool of receptors available to form heteromeric complexes. To measure the effects of chronic ligand treatment on CB₁ and D₂ protein expression in the globus pallidus of C57BL/6J mice, brain sections (Bregma - 0.82 mm) were subjected to dual-labeled QF-IHC and scanned using a LI-COR Odyssey IR scanner. The use of LI-COR Odyssey IR scanner allows for the determination of the relative CB₁ and D₂ protein-immunoreactivity in defined anatomic regions. Mice treated for 21 days with CP 55,940 had lower CB₁ levels compared to vehicle treatment (Fig. 5.4A, B). In contrast, mice treated with haloperidol showed higher CB₁ expression relative to vehicle treatment. Olanzapine-treated mice showed no change in CB₁ levels in the globus pallidus compared to vehicle-treated mice (Fig. 5.4A, B). CB₁ levels in the globus pallidus of C57BL/6J in mice co-treated with CP 55,940 and haloperidol were similar to vehicle treatment (Fig. 5.4A, B). CP 55,940 and olanzapine co-treatment resulted in CB₁ levels that were similar to CP 55,940-treated mice (Fig. 5.4A, B). We did not detect significant changes in D₂L protein levels following ligand treatment although there were similar trends in the patterns of drug-dependent protein changes compared to CB₁ (Fig. 5.4A, C).

5.3.5 Persistent Treatment with Haloperidol Increased the Steady-State Level of CB₁ and D₂ at the Plasma Membrane. CP 55, 940 Treatments Decreased the Level of Both Receptors at the Plasma Membrane

To confirm that the observed changes in CB₁/D₂L heteromer-specific PLA signals in STHdh<sup>Q7/Q7</sup> cells following chronic exposure to ligand might be due to changes in the
Figure 5.4: Chronic Haloperidol Treatment Increases CB₁ Expression in the Globus Pallidus of C57BL/6J Mice. (A) IR images showing CB₁ receptor (IRDye 800; green) and D₂ receptor (IRDye 700; red) labeling in C57BL/6J mice brain sections. Images were captured on the LI-COR Odyssey IR scanner at maximum quality, 21μm resolution. Graphical representation of the raw arbitrary abundance units of both CB₁ (B) and D₂ (C) expression. * $P < 0.01$ compared to vehicle-treatment group. ~ $P < 0.01$ compared to haloperidol-treated group. # $P < 0.01$ compared to CP 55,940 treatment. Data are presented as mean ± SEM of 5 independent experiments. Significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
affinity of the two receptors to interact with each other rather than changes in the steady-state levels of protein or localization of the receptor. In- and On- Cell Western™ analyses were used to estimate receptor densities and plasma membrane localization of CB₁ and D₂L in an effort to determine if changes in heteromer numbers were due to differential receptor expression and/or plasma localization. In- and On- Cell Western™ analyses were performed after 20 hr persistent drug treatment in STHdhQ7/Q7 cells. Treating STHdhQ7/Q7 cells with 1 μM CP 55,940 resulted in decreased CB₁ levels compared with vehicle treatment (Fig. 5.5A). In contrast, 1 μM haloperidol increased CB₁ levels and 1 μM olanzapine did not change CB₁ protein levels (Fig. 5.5A). Co-treating cells with 1 μM CP 55,940 and 1 μM haloperidol resulted in CB₁ protein levels similar to vehicle-treated cells (Fig. 5.5A). In contrast, co-treatment with olanzapine and CP 55,940 reduced CB₁ levels similar to that observed when cells were treated with CP 55,940 alone (Fig. 5.5A).

The fraction of CB₁ receptors at the membrane following 20 hr ligand treatment was measured using On- Cell Western™ analysis (plasma membrane) relative to In-Cell Western™ (total protein) analysis. The fraction of CB₁ receptors at the cell membrane following 20 hr treatment with CP 55, 940 was significantly lower compared to vehicle-treated cells (Fig. 5.5B). An increase in the fraction of CB₁ receptors at the membrane was observed in haloperidol-treated cells compared to vehicle-treated cells (Fig. 5.5B). Treatment with olanzapine did not alter the fraction of CB₁ receptors at the membrane relative to vehicle-treated cells (Fig. 5.5B). Co-treatment with both CP 55,940 and haloperidol resulted in a lower fraction of CB₁ at the membrane compared to cell treated with haloperidol alone, but a higher fraction of CB₁ at the membrane compared to cells treated with CP 55,940 (Fig. 5.5B). However, CP 55,940 co-treatment with olanzapine yielded a similar fraction of CB₁ receptors as was observed when CP 55, 940 was applied alone. CP 55,940-induced CB₁ internalization, this effect was not opposed by olanzapine (Fig. 5.5B). Haloperidol stabilized CB₁ receptors at the plasma membrane. Haloperidol reduced but did not abolish CP 55,940-dependent CB₁ receptor internalization. Olanzapine did not affect the relative distribution of CB₁ receptor relative to vehicle treatment.

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**Figure 5.5:** Persistent Treatment with Cannabinoid and/or Antipsychotics Modulates Endogenous CB$_1$ and D$_{2L}$ Receptor Expression and Membrane Localization in STHdh$^{Q7/Q7}$ Cells. (A) CB$_1$ and, (C) D$_{2L}$ receptor total protein levels measured at 20 hr measured using In-Cell Western™ in cells treated with vehicle or 1 µM CP 55,940, haloperidol, olanzapine alone or in combination. * $P < 0.01$ compared with vehicle-treated cells. ~ $P < 0.01$ compared to haloperidol-treated cells. (B) CB$_1$ and, (D) D$_{2L}$ cell surface expression at 20 hr measured using On-Cell Western™ and In-Cell Western™ in cells treated with vehicle or 1 µM CP 55,940, haloperidol, olanzapine alone. * $P < 0.01$ compared with vehicle-treated cells. ~ $P < 0.01$ compared to haloperidol-treated cells. Significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
Overall, CP 55,940 treatments reduced both CB1 protein expression and CB1 membrane localization (Fig. 5.5A, B). Haloperidol treatment increased CB1 expression and CB1 membrane localization. CP 55,940 and haloperidol co-treatment resulted in CB1 protein levels and CB1 membrane distribution similar to that observed in vehicle-treated cells (Fig. 5.5A, B). In contrast, olanzapine treatment did not alter either CB1 protein expression or CB1 membrane localization; CP 55,940 co-treatment with olanzapine yielded both CB1 protein expression and CB1 membrane localization similar to CP 55,940 treated cells (Fig. 5.5A, B).

D2L expression and membrane localization were also measured in STHdhQ7/Q7 cells following 20 hr ligand treatment. CP 55, 940 treatment reduced D2L levels compared with vehicle-treated cells (Fig. 5.5 C). Treatment with 1 µM haloperidol increased D2L protein levels, while olanzapine treatment did not alter D2L compared to vehicle-treated cells (Fig. 5.5C). Co-treatment with CP 55,940 together with haloperidol resulted in D2L protein levels similar to vehicle-treated cells (Fig. 5.5C), unlike cells co-treated with CP 55,940 and olanzapine, which had lower D2L protein levels compared with vehicle-treated cells (Fig. 5.5C).

D2L membrane localization following ligand treatment for 20 hr was also analyzed. A decrease in the fraction of D2L receptors at the membrane was observed in CP 55,940-treated cells compared with vehicle-treated cells (Fig. 5.5D). The fraction of D2L at the membrane was increased following haloperidol treatment compared with vehicle-treated cells (Fig. 5.5D). Treatment with olanzapine did not alter the fraction of D2L receptors at the membrane relative to vehicle-treated cells (Fig. 5.5D). Co-treatment with CP 55, 940 and haloperidol resulted in levels of D2L at the membrane similar to vehicle-treated cells (Fig. 5.5D). Cells co-treated with CP 55, 940 and olanzapine showed similar D2L receptors at the membrane compared to cells treated with CP 55,940 (Fig. 5.5D). Overall, haloperidol treatment increased D2L at the plasma membrane, while CP 55,940 treatment alone or together with olanzapine reduced D2L localization at the plasma membrane (Fig. 5.5D). CP 55,940 reduced the haloperidol-dependent increase in D2L at the plasma membrane (Fig. 5.5D).
5.3.6 CP 55,940 Blocks GABA Release and This Effect is Not Altered by Co-Administration of Haloperidol or Olanzapine

GABA levels in the cell culture medium were measured at 30 min and at 20 h following ligand treatment in STHdh<sup>Q7/Q7</sup> cells. GABA levels in the cell culture medium were determined using a GABA-specific enzyme-linked immunosorbent assay (Novatein Bio, Woburn MA). GABA release was inhibited by 1 μM CP 55,940 treatment for 30 min or 20 h compared to vehicle treatment (Fig 5.6A, B). No change in GABA levels was observed in both haloperidol- and olanzapine-treated cells at either 30 min or 20 h relative to vehicle treatment (Fig. 5.6A, B). Co-treating STHdh<sup>Q7/Q7</sup> cells with CP 55,940 and haloperidol for 30 min resulted in GABA levels similar to vehicle-treated cells, however co-treating STHdh<sup>Q7/Q7</sup> cells with both drugs for 20 hr resulted in significantly lower GABA levels compared to vehicle-treated cells (Fig. 5.6A, B). Cells co-treated with CP 55,940 and olanzapine for 30 min or 20 hr yielded GABA levels similar to cells treated with CP 55,940 alone (Fig. 5.6A, B). Overall, acute treatment (30 min) with haloperidol inhibited CP 55,940-induced inhibition of GABA release; this effect was not observed following persistent treatment at 20 hr suggesting that over 20 hr the effect of CP 55, 940 over GABA release was unopposed by haloperidol. Neither typical nor atypical antipsychotics directly affected GABA levels and CP 55, 940-decreased GABA release even in the presence of haloperidol or olanzapine.

5.3.7 CP 55,940 Attenuated Haloperidol-Induced Hypolocomotion and Catalepsy in C57BL/6J Mice

The effect of acute and chronic administration of CP 55,940, haloperidol and olanzapine alone or in combination on the locomotor activities of C57BL/6J mice was studied. Total distance travelled and time spent immobile in the open field were recorded for C57BL/6J mice treated with vehicle or 0.01 mg/kg/d CP 55,940, 0.3 mg/kg/d haloperidol, 1.5 mg/kg/d olanzapine, or co-treated with 0.01 mg/kg/d CP 55,940 and 0.3 mg/kg/d haloperidol and or 0.01 mg/kg/d CP 55,940 and 1.5 mg/kg/d olanzapine. Daily drug injection began when mice were 7 weeks of age and continued for 3 weeks (21 days). Twenty-four hours after the first injection, we observed that CP 55,940 did not have an effect on total distance traveled as expected for the low-dose of drug chosen (Marcellino
Figure 5.6: Changes in GABA Release in STHdhQ7/Q7 Cells Treated with Cannabinoids and/or Antipsychotics. STHdhQ7/Q7 cells were treated with ligands for 30 min (A) or 20 hr (B) and change in GABA release was measured from cell culture media using a GABA enzyme-linked immunosorbent assay. * $P < 0.01$ compared with vehicle-treated cells. $\sim P < 0.01$ compared to CP 55,940-treated cells. Data are presented as mean ± SEM of 4 independent experiments. Significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
Haloperidol- and olanzapine-treated mice showed reduced total distance traveled compared to vehicle-treated mice (Fig. 5.7A). The total distance traveled by C57BL/6J mice co-treated with both CP 55,940 and haloperidol was significantly higher compared to that observed for haloperidol-treated mice (Fig. 5.7A). C57BL/6J mice co-treated with CP 55,940 and olanzapine displayed similar total distance traveled compared to olanzapine-treated mice (Fig. 5.7A). Twenty-four hours after the first injection, we observed that CP 55,940 treatment did not affect immobility time, while haloperidol and olanzapine-treated mice showed increased immobility in the open field compared to vehicle treatment (Fig. 5.7B). Mice co-treated with CP 55,940 and haloperidol spent significantly less immobile time compared to haloperidol-treated mice; these mice had similar levels of immobility as vehicle-treated mice (Fig. 5.7B). In contrast, mice co-treated with CP 55,940 and olanzapine spent more time immobile compared to the vehicle-treated mice (Fig. 5.7B).

Open field tests were also performed after 21 daily drug treatments (Fig. 7C, D). C57BL/6J mice treated with CP 55,940 showed no change in either total distance traveled (Fig. 7C) nor time spent immobile compared to vehicle-treated mice (Fig. 5.7D). In contrast, mice treated with haloperidol or olanzapine alone or in combination with CP 55,940 showed a reduction in total distance traveled (Fig. 5.7C) and spent more time immobile (Fig. 5.7D). Therefore, acute co-treatment of CP 55,940 with haloperidol blocked reduced haloperidol-dependent decreases locomotor activities; such effect was not observed in mice following chronic exposure to both drugs or in mice treated with CP 55,940 together with olanzapine. This suggests that intermittent, but not chronic exposure, to low dose cannabinoids might alter locomotor effects of haloperidol.

5.4 Discussion

The main objective of this study was to examine whether the heteromeric CB₁/D₂ receptor population change following chronic exposure to cannabinoid alone or in combination with typical- or atypical- antipsychotics. We observed alterations in CB₁/D₂L heteromer-specific PLA signals both in the globus pallidus of C57BL/6J mice and STHdh<sup>Q7/Q7</sup> cells following chronic exposure to cannabinoid and/or antipsychotics (Summarized in Fig. 5.8).
Figure 5.7: CP 55,940 Attenuated Haloperidol-Induced Hypolocomotion in C57BL/6J Mice. Mice were treated with vehicle or 0.01 mg/kg/d CP 55,940, 0.3 mg/kg/d haloperidol or 1.5 mg/kg/d olanzapine i.p. alone or in combination for 3 weeks and total distance travelled (cm) and time spent immobile (s) was measured in the open field test. Total distance traveled was measured at Day 1 (A) and day 21 (C) and time spend immobile was measured at Day 1 (B) and Day 21 (D) post drug treatment. * $P < 0.01$ compared to vehicle-treatment. ~ $P < 0.01$ compared to haloperidol-treated group. Data are presented as mean ± SEM of 10 independent experiments. Significance was determined via one-way ANOVA followed by Tukey's post-hoc test.
The cannabinoid, CP 55,940, increased the number of detectable CB1/D2L heteromeric complexes. The typical antipsychotic haloperidol reduced the population of CB1/D2 heteromeric complexes when administered alone or in combination with CP 55,940. The atypical antipsychotic, olanzapine, did not alter CB1/D2 heteromeric complexes population when administrated alone, whereas co-administration of CP 55,940 and olanzapine increased CB1/D2L heteromeric complexes population. The alteration in the CB1/D2L heteromer population observed in our study probably involves different mechanisms not reflected in shorter drug treatment or acute studies (Kearns et al., 2005; Marcellino et al., 2008; Przybyla and Watts, 2010; Bagher et al., 2016). The alteration in CB1/D2L heteromeric complexes population following ligand treatment could have been caused by alteration(s) in: (1) the affinity of CB1 and D2 receptors to form homo- versus heteromeric complexes, (2) the expression of either CB1 and/or D2 receptors, or (3) the localization of CB1 and/or D2L receptors.

Prolonged exposure to cannabinoids and/or antipsychotics, during the time of ongoing receptor biosynthesis and oligomerization, can alter the affinity of the receptors to form CB1/D2L heteromers (Przybyla and Watts, 2010). Changes in the relative affinity of CB1 and D2L receptors to interact was determined by comparing BRET50 values obtained from BRET2 saturation curves of cells co-expressing CB1-Rluc and D2L-GFP2. CP 55,940 increased the affinity between CB1 and D2L, while haloperidol reduced the affinity of CB1 and D2L relative to vehicle treatment. CP 55,940 and haloperidol co-treatment reduced the affinity between CB1 and D2L. Olanzapine did not alter the affinity between CB1 and D2L. The changes in the affinity of CB1- and D2L receptors to each other following drug treatment was consistent with the observed changes in the number CB1/D2 heteromeric complexes in vivo and in vitro. Ligand-dependent changes in the relative affinity of receptors within the heteromeric complex population might shift the ratio of CB1 and D2L homomers versus heteromers. Consistent with our finding, multicolor BiFc was used to examine the regulation of CB1 and D2L homo- and heteromers in neuronal cells (Przybyla and Watts 2010). Persistent treatment for 20 hr with CP 55,940 increased the CB1/D2 heteromeric population relative to CB1 and D2 homomers. This effect was CB1-dependent as pre-treating cells with the CB1 receptor antagonist AM281 attenuated the CP55,940-induced increase in CB1/D2 heteromers.
Figure 5.8: Chronic Cannabinoid and Typical Antipsychotic Alter CB₁ and D₂L Localization, Expression and Heteromerization. (A) CB₁ and D₂L receptors are localized at the plasma membrane and intracellular. CB₁ and D₂L receptors form monomers, homomers and heteromers. Chronic treatment with olanzapine did not alter CB₁ and D₂L protein levels, the fraction of the receptors at the membrane or CB₁/D₂L heteromer expression. (B) The typical antipsychotic haloperidol increases CB₁ and D₂L protein levels and the fraction of the receptors at the membrane, while reduces CB₁/D₂L heteromer. (C) Chronic treatment with CP 55,940 alone or in combination with olanzapine reduces both CB₁ and D₂L protein levels and the fraction of the receptors at the membrane, but increases CB₁/D₂L heteromer expression. (D) Co-treatment with CP 55,940 and haloperidol results in CB₁ and D₂L protein levels similar to vehicle treatment, the fraction of CB₁ and D₂L receptors at the plasma membrane and CB₁/D₂L heteromer are reduced.
A. Vehicle (or Olanzapine)

B. Haloperidol

↑ CB₁ and D₂₅ protein levels
↑ Fraction of CB₁ and D₂₅ at plasma membrane
↓ CB₁/D₂₅ heteromeric complexes

C. CP 55,940 (+/- Olanzapine)

D. CP 55,940 + Haloperidol

↓ CB₁ and D₂₅ protein levels
↓ Fraction of CB₁ and D₂₅ at plasma membrane
↑ CB₁/D₂₅ heteromeric complexes
No change in CB₁ and D₂₅ protein levels
↓ Fraction of CB₁ and D₂₅ at plasma membrane
↓ CB₁/D₂₅ heteromeric complexes
(Przybyla and Watts, 2010). There are no tools available to directly determine the proportion of monomeric versus heteromeric species that coexist in vivo. The current in vivo work can only determine the relative change in heteromeric complex number. While it is likely that there was a shift in the distribution of CB$_1$/D$_{2L}$ hetero-versus homodimers, it is also possible that the reduction in the CB$_1$/D$_{2L}$ heteromeric population might be due to CB$_1$ or D$_{2L}$ interacting with other GPCRs expressed in the same cells as a result of ligand treatment. For example, In MSNs, CB$_1$ and D$_{2L}$ are known to interact with adenosine A$_{2A}$ receptors (Carriba et al., 2007; Bonaventura et al., 2015), which might compete with CB$_1$ and D$_{2L}$ receptors.

Alteration in CB$_1$ and D$_{2L}$ total protein expression following chronic cannabinoid and/or antipsychotic treatment is another possible mechanism by which these drugs might influence the relative CB$_1$/D$_{2L}$ heteromeric population. Induction or suppression of either CB$_1$ or D$_2$ protein expression would alter the steady-state levels of receptors available for heteromeric receptor complex formation. Changes in CB$_1$ and D$_2$ expression following chronic exposure to exogenous cannabinoids and dopamine antagonists have been reported previously both in vivo and in vitro. Subchronic or chronic exposure to exogenous cannabinoids, such as THC, decreases CB$_1$ receptor binding in the mice caudate-putamen and the globus pallidus (Breivogel et al., 1999; McKinney et al., 2008; Falenski et al., 2010). Moreover, chronic exposure to marijuana decreases the expression of D$_2$ receptors in rat brain (Walter and Carr, 1986). Consistent with previous studies, we have observed a reduction in both CB$_1$ (Laprairie et al., 2014) and D$_{2L}$ protein expression following 20 h treatment with CP 55,940 in STHdh$^{Q7/Q7}$ cells. In addition, we observed a reduction in CB$_1$ expression in the globus pallidus of C57BL/6J mice following chronic CP 55,940 treatment. In the current study, we found that persistent haloperidol treatment, but not olanzapine, increased CB$_1$ and D$_2$ protein levels in cell culture model. Likewise, chronic treatment with haloperidol, but not olanzapine, increased CB$_1$ protein expression in the globus pallidus. Consistent with our findings, an increase in CB$_1$ protein expression following haloperidol treatment was previously reported by Andersson et al. (2005). Specifically, chronic treatment with haloperidol (1 mg/kg) for 14 days increased $[^{3}H]$ CP 55,940 binding in the striatum of male Sprague-Dawley rats (Andersson et al., 2005). Even though previous studies have found that chronic treatment with high dose
haloperidol (10 mg/kg/d) for 3 weeks increases D2 receptor levels in the striatum (Muller and Seeman, 1977; Fox et al., 1994; Andersson et al., 2005), we did not observe a significant increase in D2 protein expression in the globus pallidus following chronic haloperidol treatment, which could be due to the lower dose of haloperidol (0.3 mg/kg/d) used in the current study. The co-administration of CP 55,940 reduced haloperidol ability to increase CB1 and D2 protein expression STHdhQ7/Q7 cells, and CB1 in the globus pallidus. The endocannabinoids anandamide (AEA) and its synthetic analogues can alter CB1 gene transcription by modulating CB1 promoter activity, mRNA, and protein expression through Akt- and NF-κB-dependent mechanism (Laprairie et al., 2013). The mechanism by which haloperidol as an antagonist can alter CB1 expression is still not known, but the additive effects of CP 55,940 and haloperidol co-administration on CB1 protein expression suggest that both drugs might modulate gene transcription and/or mRNA translation(s). A previous study by Blume et al., (2013) found that chronic reduction of CB1 or D2 expression in the rat globus pallidus using RNA interference resulted in deficits in gene and protein expression of the alternative receptor. Our study also indicates a reciprocal influence of the levels of CB1 or D2 receptors; together these data suggest that CB1 and D2 receptors are tightly coupled at the level of transcription and translation. Overall, alteration in CB1/D2L heteromer expression did not correlate with the observed alteration in CB1 and D2 protein expression following chronic ligand treatment. For example, even though haloperidol reduced the relative level of CB1/D2L heteromers, haloperidol induced both CB1 and D2L protein expression. Therefore, the observed loss of CB1/D2L receptor heteromers in both STHdhQ7/Q7 cells and in the globus pallidus was unlikely to be caused by a reduction in the pool of available CB1 and D2L receptors.

We studied the influence of persistent ligand application on CB1 and D2 receptor localization in STHdhQ7/Q7 cells to determine if chronic exposure to these agents affected the population of CB1/D2L heteromers by changing CB1 and/or D2L receptor localization. We observed similar distribution pattern of CB1 and D2L receptors following ligand treatment(s) in STHdhQ7/Q7 cells. Treating cells with CP 55,940 induced CB1 and D2L receptor internalization suggesting that D2L receptors were co-internalized with CB1 receptor as heteromeric complexes in response to CP 55,940. In contrast, haloperidol
increased the ratio of CB1 and D2L receptors localized at the cell membrane and reduced CP 55,940-dependent CB1 and D2L receptor co-internalization. Olanzapine did not alter CB1 nor D2L receptors localization. Given the fact that persistent exposure to CB1 agonists and the D2L antagonist produced similar effects on the localization of both receptors, it is unlikely that these ligands differentially altered the location of CB1 and D2L receptors preventing or promoting association. The receptors appeared to respond to ligand binding as a complex. Co-internalization of GPCR heteromers has previously been reported for several GPCRs following ligand-receptor binding at both receptors of the GPCR heteromer. Additionally, ligand-receptor binding at one of the receptors in a GPCR heteromer can also induce receptor co-internalization (reviewed in Terrillon and Bouvier, 2004; Milligan, 2009; Ferré et al., 2014; Franco et al., 2016). Further studies will be required to determine whether both CB1 and D2L receptors are localized to the same subcellular compartments following ligand exposure.

The effects of chronic treatment with cannabinoid and/or antipsychotics on mice locomotor activities were examined in the current study. Both haloperidol and olanzapine reduced locomotor activities in mice on day 1 and day 21 after daily drug administration. No changes in locomotion activities were observed at day 1 and day 21 in mice treated with low dose of CP 55,940. Interestingly, co-administration of CP 55,940 and haloperidol blocked the haloperidol-dependent reduction in locomotor activities on day 1 after drug administration. An in vitro study showed that CP 55,940 reduces the affinity of D2 receptor agonist binding to the D2 receptors in both the dorsal and ventral striatum including the nucleus accumbens shell (Marcellino et al., 2008). Cannabinoid-dependent reduction on D2 receptor agonist affinity might explain the observed change in locomotor activities in mice co-treated with both CP 55,940 and haloperidol compared to haloperidol-treated mice. Alternatively, concurrent activation of both CB1 and D2 within heteromeric complexes switched CB1/D2 heteromer coupling from Ga\textsubscript{i} to Ga\textsubscript{s} proteins, which could cause the observed disinhibition of movement (Glass and Felder, 1997; Bagher et al., 2016). In contrast to the effect observed 24 hours after a single dose of each drug, the ability of CP 55,940 to block haloperidol-dependent inhibition of locomotion was not observed in mice chronically co-treated with both CP 55,940 and haloperidol. The chronic treatment with CP 55,940 and haloperidol significantly reduced
the CB₁/D₂L heteromeric complexes population in the globus pallidus of C57BL/6J mice thereby removing the inhibitory effect exerted by CB₁ receptors on D₂L receptors. Variation in CB₁/D₂L heteromeric expression might influence GABA transmission in the globus pallidus. As expected, activation of the CB₁ receptor by CP 55,940 resulted in inhibition of GABA release (Manzoni and Bockaert, 2001; Szabo et al., 2002; D'Amico et al., 2004), while the D₂ antagonist haloperidol and olanzapine did not alter GABA release in STHdh<sup>Q7/Q7</sup> cells. Co-treating the cells for 30 min with CP 55,940 and haloperidol blocked CP 55,940-induced inhibition of GABA release, while persistent (20 h) co-treatment with CP 55,940 and haloperidol abolished the antagonistic effect of haloperidol on cannabinoid-induced inhibition of GABA release, which is consistent with the reduction in the expression of CB₁/D₂ heteromers.

5.5 Conclusion

This is the first study to our knowledge that reports alteration in CB₁ and D₂ heteromer expression in vivo following cannabinoid and/or antipsychotic exposure. The following conclusions may be drawn from our data. First, CB₁/D₂L receptor heteromers are expressed in the globus pallidus of C57BL/6J mice and STHdh<sup>Q7/Q7</sup> cells, as demonstrated using in situ PLA. Second, the expression of CB₁/D₂L receptor heteromers is altered in both STHdh<sup>Q7/Q7</sup> cells and in mouse globus pallidus following chronic exposure to cannabinoids and/or typical antipsychotic. Third, alterations in CB₁/D₂L heteromer expression following chronic ligand treatment(s) might disturb the negative cross-talk between the CB₁ and D₂L receptor in the globus pallidus, which can affect movement. Typical and atypical antipsychotics differently altered CB₁/D₂L heteromer population, CB₁ and D₂L protein expression and localization when applied alone or in combination with cannabinoids. Overall, drugs that target CB₁ and D₂ receptors must be considered in the context of their interactions and effect on their cognate receptor and for their actions within allosteric heteromeric complexes. Pharmacodynamic drug-drug interactions are likely.
CHAPTER 6
GENERAL DISCUSSION

6.1. Objectives of the Research

The overall objective of my thesis was to understand the allosteric interactions within CB₁/D₂L heteromers. My hypothesis was that co-localization of CB₁ and D₂L receptors in the basal ganglia allows for bidirectional allosteric interactions within CB₁/D₂L heterotetramers following the applications of CB₁ and D₂L ligands, which may be physiologically and clinically relevant.

6.2. Summary of Research

Given that allosteric communication within heteromeric GPCR complexes is known to result in unique pharmacology (reviewed in Smith and Milligan, 2010; Ferré et al., 2015; Jonas et al., 2016), the pharmacology of CB₁/D₂L heteromers was investigated in the current thesis. Using BRET² saturation curves, we confirmed that CB₁ and D₂L receptors physically interact to form homomeric and heteromeric complexes when these receptors were co-expressed in HEK 293A cells and STHdh⁹⁷/⁹⁷ cells. The interaction was observed at low levels of expression and was specific and saturable. To improve the understanding of the functional consequences of the CB₁ and D₂L interaction and given the clinical importance of D₂ antagonists, the effects of D₂ antagonists on CB₁ pharmacology was investigated, and the finding was presented in chapter three and published in the Journal of Molecular of Pharmacology as “Antagonism of dopamine receptor 2 long (D₂L) affects cannabinoid receptor 1 (CB₁) signaling in a cell culture model of striatal medium spiny projection neurons”. In this study, the effects of a D₂ antagonist haloperidol on CB₁ coupling to Gaᵢ and Gaₛ proteins and β-arrestin₁ recruitment to CB₁ receptors were investigated using STHdh⁹⁷/⁹⁷ cells. Also, CB₁-dependent ERK1/2, CREB phosphorylation and CB₁ internalization following co-applications of CB₁ agonist and D₂ antagonist were quantified. We confirmed that CB₁ was pre-assembled with Gaᵢ protein in the absence of CB₁ agonist. The application of the selective CB₁ agonist ACEA resulted in a rapid and transient increase in BRETₐf
between Ga\textsubscript{i} - Rluc and CB\textsubscript{1}-GFP\textsuperscript{2} due to conformational changes within pre-assembled heteromeric complexes. The co-application of ACEA and haloperidol caused a rapid uncoupling of CB\textsubscript{1} from Ga\textsubscript{i} protein followed by a delayed and sustained interaction of the CB\textsubscript{1}/D\textsubscript{2L} with Ga\textsubscript{s} protein. In addition, haloperidol treatment reduced ACEA-induced \(\beta\)-arrestin1 recruitment to CB\textsubscript{1} receptor and receptor internalization. Overall, our first study suggested that a high-affinity D\textsubscript{2} antagonist allosterically modulated cannabinoid-induced CB\textsubscript{1} coupling, signaling and \(\beta\)-arrestin1 recruitment through binding to CB\textsubscript{1}/D\textsubscript{2L} heteromers.

Next, we tested whether a D\textsubscript{2} agonist could also modulate CB\textsubscript{1} pharmacology via allosteric interactions within CB\textsubscript{1}/D\textsubscript{2L} heteromeric complexes. D\textsubscript{2} agonists can modulate CB\textsubscript{1} coupling to Ga\textsubscript{i} protein, \(\beta\)-arrestin1 recruitment, and internalization when co-applied with the CB\textsubscript{1} agonist, but not if applied as single agents. Similarly, CB\textsubscript{1} agonists modulated D\textsubscript{2L} coupling to Ga\textsubscript{i} protein, \(\beta\)-arrestin1 recruitment, and internalization in the presence of a D\textsubscript{2} agonist. The co-application of both CB\textsubscript{1} and D\textsubscript{2L} agonists potentiated \(\beta\)-arrestin1 recruitment to CB\textsubscript{1}/D\textsubscript{2L} heteromeric complexes and resulted in CB\textsubscript{1}/D\textsubscript{2L} co-internalization. Since we observed bidirectional allosteric interactions within CB\textsubscript{1}/D\textsubscript{2L} heteromeric complexes, we aimed to define the stoichiometry of CB\textsubscript{1}/D\textsubscript{2L}/Ga\textsubscript{i} protein complexes. Using BRET\textsuperscript{2} saturation curves, we observed that CB\textsubscript{1} and D\textsubscript{2L} homodimers were the predominant species when either receptor was expressed alone; however heterotetramers were the predominant species when the receptors were co-expressed. Using mathematical models and SRET\textsuperscript{2} combined with BiFC, we predicted that one CB\textsubscript{1} homodimer interacts with one D\textsubscript{2L} homodimer to form a CB\textsubscript{1}/D\textsubscript{2L} heterotetrameric complex. Each homodimer, within a heterotetrameric complex, was coupled to at least one Ga\textsubscript{i} protein. Higher order oligomeric complexes might also form although our data suggested that the minimal functional unit was a heterotetramer. This work is presented in chapter four and was submitted to the European Journal of Pharmacology for publication with the title “Bidirectional Allosteric Interactions Between Cannabinoid Receptor 1 (CB\textsubscript{1}) and Dopamine Receptor 2 Long (D\textsubscript{2L}) Heterotetramers” (in press).

The main objective of the fifth chapter was to examine whether CB\textsubscript{1} and D\textsubscript{2L} form heteromers in defined nuclei of the basal ganglia in C57BL/6J mice and to determine whether CB\textsubscript{1}/D\textsubscript{2L} heteromer levels were altered following chronic treatment with
cannabinoids and antipsychotic alone or in combination. By using in situ PLA, we observed CB₁ and D₂L heteromer-specific PLA signals in the globus pallidus, but not the striatum, of C57BL/6J mice. An increase in CB₁/D₂L heteromer-specific PLA signals was observed in the globus pallidus of C57BL/6J mice following chronic CP 55,940 treatment alone or in combination with olanzapine. In contrast, haloperidol treatment alone or in combination with CP 55,940 reduced CB₁/D₂L heteromer-specific PLA signals. Olanzapine treatment did not affect CB₁/D₂L heteromer-specific PLA signals relative to vehicle treatment. This finding demonstrated that typical and atypical antipsychotics differentially alter CB₁/D₂L heteromerization in the globus pallidus when applied alone or in combination with cannabinoid, which might have a different impact on the control of movement. Overall, the studies presented within this body of work improve understanding of allosteric interactions within GPCR heteromeric complexes and provide a better understanding of the effects of cannabinoids administration on the therapeutic effects of antipsychotics.

6.3 Allosteric Interactions Within CB₁/D₂L Heteromeric Complexes in Cell Culture

Bidirectional allosteric interactions within CB₁/D₂L heteromers were ligand-dependent as has been observed for other GPCR heteromers (Kenakin and Miller, 2010; Ferré et al., 2015). In this model, CB₁/D₂L heteromeric complexes act as a conduit of the allosteric modulator. CB₁ agonists act as allosteric modulators influencing the efficacy of D₂ ligands. Conversely, D₂ ligands act as allosteric modulators of ligand efficacy of CB₁ agonists. The co-expression of D₂ receptors with CB₁ receptors, in the absence of D₂ ligands, did not alter G protein coupling to CB₁. In contrast to our finding, Jarrahian et al., (2004) reported that co-expression of the D₂ receptors with the CB₁ receptors in HEK 293 cells led to increased levels of cAMP instead of the expected decrease in levels of cAMP following CB₁ agonist treatment. Based on these finding, these authors suggested that the co-expression of the D₂ receptor was sufficient to change CB₁-dependent signaling from Gαᵢ to Gαₛ proteins. In the same paper, they proposed that D₂ receptors sequester the available Gαᵢ pool, preventing the binding of the CB₁ receptor to Gαᵢ, which promotes CB₁ to interact with the Gαₛ protein. Overexpression of Gαₛ, but not Gαᵢ, restored coupling of the CB₁ with Gαᵢ protein in the presence of D₂L (Jarrahian et al.,
Our results strongly suggest that the coupling of CB₁/D₂L heteromeric complexes to Gαᵢ proteins following the application of CB₁ agonist and D₂ ligands is a result of allosteric interactions within CB₁/D₂L heteromeric complexes and not due to the competition between CB₁ and D₂L receptors for the Gαᵢ-protein pool. This finding was confirmed by the fact that preventing the interaction between CB₁ and D₂ receptors using a blocking peptide was able to block the switching in G protein coupling following ligands CB₁/D₂ co-application. Importantly, we observed these effects in the presence of excess Gαᵢ protein. Even though the expression of D₂L receptors did not alter BRET_Eff between CB₁ and Gαᵢ-protein, in the absence of D₂ ligands, it is important to acknowledge that there is the possibility that the expression of D₂L receptor might induce conformational changes within CB₁/Gαᵢ that may be undetectable using BRET².

Our data suggest that the overall functional receptor unit is composed of CB₁ and D₂L homodimers that interact to form heterotetramers coupled to at least two Gαᵢ proteins. One might argue that the reduction in BRET_Eff signals between Gαᵢ-Rluc and CB₁-GFP² or between Gαᵢ-Rluc and D₂L-GFP² following the co-application of both CB₁ and D₂ ligands is due to conformational changes within the complexes that resulted in a reduction in the energy transfer from Rluc to GFP² and not due to uncoupling of CB₁ and D₂L homodimers from Gαᵢ proteins (Szalai et al., 2014; Lan et al., 2015). Based on crystal structures of GPCR homodimers and computer modeling, the width of one G heterotrimer is larger than the width of one GPCR receptor (Han et al., 2009; Wu et al., 2010; Manglik et al., 2012, Wu et al., 2012; Haung et al., 2013; Jastrzebska et al., 2013; Navarro et al., 2016). This observation suggests that it is not possible for CB₁/D₂L heterotetramers to couple simultaneously to two Gαᵢ proteins and two Gαₛ proteins and uncoupling of CB₁/D₂L heterotetramers from Gαᵢ proteins is required before coupling to Gαₛ proteins.

Previous studies have reported asymmetric structural arrangements within homo- or heterodimeric complexes, wherein individual protomers in a receptor dimer may interact with a shared heterotrimeric G protein through distinct interfaces. These studies suggest that structural asymmetries may result in asymmetric allosteric interactions (Damian et al., 2006; Han et al., 2009; Zylbergold and Hébert; 2009; Jonas et al., 2015; Mishra et al., 2016; Levitz et al., 2016; Sleno et al., 2017). In our study, we have
observed that D2 agonists can modulate CB1 coupling to Gα protein and β-arrestin1 recruitment when co-applied with the CB1 agonist. Similarly, CB1 agonists modulated D2L coupling to Gα protein and β-arrestin1 recruitment in the presence of a D2 agonist. Based on our findings, we concluded that the allosteric communications between CB1/D2L heterotetramer are symmetrical and ligand-dependent. Asymmetric binding of G proteins may occur within CB1 homodimers and D2 homodimers and still produce symmetrical reciprocal allosteric interactions with the CB1/D2L heterotetramer. The precise conformational changes within of CB1 homodimer induced by the co-expression and activation of D2L receptors are yet to be determined. Alternative techniques such as GPCR conformation–sensitive biosensors might be useful to measure intramolecular conformational dynamics of CB1/D2L receptors within heteromeric complexes in response to agonist (Zurn et al., 2009; Maier-Peuschel et al., 2010; Ziegler et al., 2011; Bourque et al., 2017; Devost et al., 2017; Sleno et al., 2017).

6.4. Allosteric Interactions Within CB1/D2 Heteromic Complexs in the Basal Ganglia

While there is extensive in vitro evidence for heteromerization, there is currently considerably less evidence for allosteric interactions in vivo or an understanding of the functional consequences of heteromerization. In our studies, we observed that allosteric interactions within CB1/D2 heterooligomeric complexes occurred at relatively high concentrations of CB1 and D2 (chapters three and four). Endocannabinoids are released from depolarized postsynaptic neurons into the synapse. The levels of endogenous 2-AG in rat striatum ranges from 3 to10 nM, while AEA levels in rat striatum ranges from 0.5 to 5 nM (Giuffrida et al., 1999; Walker et al., 1999; Béquet et al., 2007; Alvarez-Jaimes et al., 2009; Orio et al., 2009; reviewed in Buczynski and Parsons, 2010). The reported endogenous levels of both 2-AG and AEA are much lower than the concentrations that induced allosteric interactions within CB1/D2 heteromeric complexes in vitro although the local synaptic levels of endocannabinoids may be higher than those measured by microdialysis (reviewed in Buczynski and Parsons, 2010). On the other hand, the concentration of dopamine in the striatum varies during the tonic (baseline spike activity) and phasic (burst-spike firing pattern) dopamine release states. Dopamine concentrations measured locally in the vicinity of tonically firing neurons ranges from 10 to 20 nM,
while dopamine concentrations during phasic dopamine release are much higher and ranges from 100 μM to 1 mM (Ross and Jackson, 1989; Ross, 1991; Keef et al., 1993; Floresco et al., 2003). The phasic dopamine release state is transient as dopamine is immediately taken up via selective transporters into pre-synaptic terminals (Grace, 1991; Chergui et al., 1994; Floresco et al., 2003; Goto et al., 2007). We concluded that during phasic dopamine release, the levels of dopamine in the synapse would be transiently high while endocannabinoids levels would be relatively low; therefore bidirectional allosteric interactions between the two receptors might not occur in vivo in the absence of exogenous cannabinoids. It is possible that transient increases in dopamine could influence the production of endocannabinoids postsynaptically and influence presynaptic dopamine receptor function and indirectly affect cannabinoid signaling.

Direct and indirect dopamine agonists are used clinically to treat symptoms of Parkinson’s disease (reviewed in Brooks, 2000; Stowe et al., 2008; Tomlinson et al., 2010; Stocchi et al., 2016), while D₂ antagonists are used to treat schizophrenia, Huntington’s disease, and Tourette’s syndrome (Seeman, 2010; Eddy and Rickards, 2011; Frank, 2014). Cannabinoid CB₁ orthosteric ligands have been proposed as pharmacotherapeutics for treating neurodegenerative diseases, spasticity, chronic pain, substance use disorders, and managing energy intake (Pacher et al., 2006; Vemuri et al., 2008; Pertwee, 2012; Aizpurua-Olaizola, 2017). Also, patients might be exposed to drugs such as marijuana or stimulants that modulate the ECS and DS. Several clinical scenarios are likely for patients receiving combinations of drugs that target the CB₁ and D₂ receptors.

In the first scenario, patients taking drugs that lead to increased activation of dopamine receptors, such as D₂ agonists, levodopa (L-DOPA) or dopamine transporters reuptake inhibitors, such as cocaine, amphetamine, and methamphetamine. These patients would experience an increase in dopaminergic neurotransmission in the basal ganglia and an increase in locomotor activity (reviewed in Iversen and Iversen, 2007). In this case, an increase in endocannabinoids release in the dorsal striatum is predicted as a negative feedback mechanism to compensate for sustained over-activation of dopaminergic transmission (Giuffrida et al., 1999; Melis et al., 2004; Centonze et al., 2004; Pan et al., 2008). Signaling through CB₁ and D₂L homodimers and heteromers could occur leading
to complex regulation of the ECS and DS pathways depending on the concentration of agents and duration of action.

In the second scenario, patients exposed to prescribed drugs that act as CB₁ agonists such as Sativex® (extract containing equimolar THC and cannabidiol), or the combination of cannabinoids in marijuana may influence dopaminergic transmission in addition to affecting the ECS. *In vivo* microdialysis showed that acute THC administration increases dopamine efflux in the striatum in rodents (Cheong *et al*., 1988; Chen *et al*., 1990; Pistis *et al*., 2002). Similarly, using positron emission tomography scanning, it was reported that THC causes an increase in dopamine release in the ventral striatum in the human brain (Bossong *et al*., 2015). In such case, the concentrations of both cannabinoid and dopamine will be relatively high in the synapse and may induce allosteric interactions between CB₁/D₂ heteromers. Allosteric interactions will result in switching G protein coupling from Gαᵢ to Gαₛ proteins. High concentrations of cannabinoid and dopamine are predicted to exert negative cooperativity on CB₁/D₂L interaction within CB₁/D₂L complexes. The negative cooperativity effects on Gαᵢ protein coupling could be a modulatory mechanism to protect the system from acute over elevation of endocannabinoids and dopamine resulting in hyperactivation of CB₁/D₂L receptors. Also, we might expect to see positive cooperativity effects on β-arrestin recruitment to CB₁/D₂L heteromer, which potentiates heteromer co-internalization and termination of signaling protecting the system from receptor over-activation. There is evidence that acute and chronic THC exposure have differing effects on the dopaminergic system. Chronic THC treatment reduces the expression of CB₁ in the striatum of both rodents and human, which is consistent with our finding presented in chapter 5 (Sim-Selley, 2002; Hirvonen *et al*., 2012). Moreover, chronic THC treatment increases the formation of CB₁/D₂L heteromers (chapter 5). Elevation in CB₁/D₂ heteromeric complexes will further potentiate those allosteric interactions within the two receptors and further increase the complexity of interactions between ECS and DS.

In the third scenario, patients taking drugs acting on D₂ receptors (agonist or antagonists) may be simultaneously exposed to CB₁ agonists. Acute administration of Δ⁹-THC was reported to counteract the motor effect induced by ligands that increase synaptic dopamine concentration (Aulakh *et al*., 1980; Moss *et al*., 1981; Anderson *et al*.,
1996; Giuffrida et al., 1999; Andersson et al., 2005; Marcelino et al., 2008). For example, a single low-dose of the cannabinoid agonist CP 55940, which did not affect locomotor activity when administered alone, was able to reduce quinpirole-induced hyperactivity; this effect was counteracted by the CB₁ receptor antagonist rimonabant at a dose that did not change basal locomotor activity (Marcelino et al., 2008). In our in vitro study, we found that application of high-affinity D₂ receptor antagonists as haloperidol-induced allosteric interactions within CB₁/D₂L heteromeric complexes (chapter three). Altogether, acute co-administration of cannabinoids along with D₂ agonists or antagonists might result in an allosteric interaction within CB₁/D₂ heteromers in the globus pallidus. Our study suggests that the administration of cannabinoid and/or antipsychotic can modulate the expression of CB₁/D₂L heteromeric complexes, which might can an effect on the control of movement and have clinical implications.

In addition to CB₁ and D₂L, GABAergic MSNs projecting to the globus pallidus express other GPCRs including the adenosine 2A (A₂A) receptor. CB₁/D₂L/A₂A heteromerization has been confirmed both in rodent MSNs and cell cultures (Marcellino et al., 2008; Carriba et al., 2008; Navarro et al., 2008; Pinna et al., 2014; Bonaventura et al., 2014). Linking the observations available in the literature and the present study suggests a scenario where striatal neurons expressing CB₁/D₂/A₂A heteromers would be subject to a very complicated receptor regulation scheme. For example, persistent exposure to CB₁ agonist would reduce CB₁ and D₂L receptor expression and promote the interaction between CB₁ and D₂ while the level of A₂A receptor would be lower resulting in the disturbance in the formation of the CB₁/D₂L/A₂A heteromer. A more complicated scenario would be expected in patients being exposed to antipsychotic medications. As mentioned before, when CB₁ and D₂ receptors co-expressed in the same cells and co-stimulated by both agonists they couple to Ga₅ proteins (Glass and Felder, 1997; Jarrahi et al., 2004; Kearns et al., 2005; Bagheri et al., 2016). Whereas when A₂A and D₂ receptors are co-expressed in the same cells and co-activated by agonists, they couple to Ga₅ proteins (Ferré et al., 1992; Bonaventura et al., 2015). It is still unknown whether CB₁/D₂/A₂A heteromeric complexes coupled to Ga₅, Ga₅, and/or Ga₅ proteins. G protein coupling to the CB₁/D₂/A₂A heteromeric complex might depend on which protomers are stimulated in the receptor heteromeric complexes.
6.5 CB$_1$/D$_2$L Allosteric Interactions in the Context of Huntington’s Disease

CB$_1$/D$_2$L interactions may be of particular interest during the current drive to develop therapeutics for the management of HD. Despite the loss of CB$_1$ receptors early in Huntington’s Disease (HD) progression, there is evidence that cannabinoids may reduce hyperkinetic movement, striatal atrophy, and peripheral inflammation in HD animal models (Sagredo et al., 2007, 2011; Blázquez et al., 2011; Bari et al., 2013; Valdeolivas et al., 2012, 2015). In addition, cannabinoids can increase appetite and affect energy utilization, which has the potential to normalize weight loss that occurs during HD progression (Petersé et al., 2005; van der Burg et al., 2008; Casteels et al., 2011; Chiarlione et al., 2014). Several clinical trials have been conducted to investigate cannabinoid-based medicines as a treatment for HD. In an early trial, cannabidiol was found to be safe and well tolerated in HD patients, but did not reduce abnormal choreic movement (Consroe et al., 1991). Cesamet® (nabilone), a synthetic THC analog, was evaluated in two clinical trials (Müller-Vahl et al., 1999; Curtis et al., 2009). The Unified Huntington’s Disease Rating Scale (UHDRS) was used to evaluate total motor score, chorea, cognition and neuropsychiatric outcomes (Müller-Vahl et al., 1999; Curtis et al., 2009). In both trials, there was evidence of improvement in cognitive outcomes, but no reduction of chorea (Müller-Vahl et al., 1999; Curtis et al., 2009). In 2011, a double-blind, randomized, crossover, phase 2 clinical trial was conducted to assess the neuroprotective effects of Sativex® in HD. Although Sativex® in HD was found to be safe, no differences in motor, cognitive or, behavioral outcomes were detected during treatment with Sativex® compared to placebo (López-Sendón et al., 2016). To date, all cannabinoid-based clinical trials have only enrolled symptomatic HD patients and trials had relatively short duration. For future trials, treatments with cannabinoid-based therapeutics might be administered earlier during HD progression and for a longer duration.

Tetrabenazine and deutetetrabenazine, specifically approved as an antichoreic agent for HD, inhibit the vesicular monoamine transporter (VMAT), decrease levels of dopamine and act as indirect D$_2$ antagonists. Patients who do not tolerate tetrabenazine, or have other contraindications to its use such as depression, may be prescribed antipsychotics to control chorea, aggression, agitation, impulsivity, delirium, and
psychosis (Hayden et al., 2009; Frank and Jankovic, 2010; Mestre and Ferreira, 2012; Frank et al., 2016). There is no consensus based on evidence for selection of one antipsychotic over another for HD patients (Canadian Huntington’s Physician Guide, Huntington Society of Canada, 2013). Patients prescribed tetrabenazine or antipsychotics may also be exposed to cannabinoids via prescribed cannabinoids or self-medication. The overall effects of these drugs on symptom management and disease progression are currently unknown.

Are typical-antipsychotics a favorable treatment strategy for HD or atypical-antipsychotics? Typical antipsychotics such as haloperidol have high affinity to block D2L receptors; therefore the use of typical antipsychotics can result in extrapyramidal side effects (akathisia, dystonia and tardive dyskinesia). In a study of 10 patients with HD using haloperidol, oral doses of 1.5 to 10.0 mg/day resulted in at least a 30 % reduction in chorea compared with baseline (Barr et al., 1988). Other common side effects of typical antipsychotics are related to their potent antimuscarinic actions such as dry mouth, nervousness, urinary retention, and constipation. Atypical antipsychotic agents such as olanzapine are known to cause sedation (blocking the H1 histamine receptors), and weight gain (possibly due to blocking H1 histamine and 5-HT2 serotonin receptor) (reviewed in Gerlach, 1991; Kapur and Mamo, 2003; Meltzer, 2013; Murray et al., 2017). HD patients suffer from severe weight loss and using olanzapine might be beneficial for them (Ross 2010; Ross and Tabrizi 2011; Labbadia and Morimoto, 2013). In two open-label studies of patients with HD, treatment with olanzapine (10 to 30 mg/day) resulted in significant improvement in anxiety, irritability, depression, and choreic movements (Paleacu et al., 2002, Bonelli et al., 2002; reviewed in Adam et al., 2008). The new atypical antipsychotic aripiprazole is a partial agonist at D2 receptors and, thus, has a unique profile compared to other atypical antipsychotics (Leung et al., 2012). In one trial, aripiprazole was found to be as beneficial in reducing chorea having an equivalent effect to that of tetrabenazine (Ciammola et al., 2009). Aripiprazole is associated with tardive dyskinesia (Ciammola et al., 2009) therefore; particular attention has to be taken when prescribing antipsychotics to HD patients. The effects of partial agonists on CB1/D2L heteromer function have yet to be tested.
Based on studies presented in this thesis, I speculate that acute exposure to cannabinoid while taking typical or atypical- antipsychotics drugs will differentially affect CB₁/D₂L function. Exposure to exogenous cannabinoids and haloperidol, but not olanzapine, was able to allosterically modulated CB₁/D₂L functions and altered CB₁/D₂ heteromer expression in the basal ganglia. However, further studies are required to test whether typical- or atypical antipsychotics might be beneficial when co-administrated with cannabinoids. Since CB₁ and D₂L are co-expressed and co-localized selectively in the GABAergic MSNs, it may be possible to develop novel therapeutic compounds capable of recognizing and binding to the oligomeric arrangement of CB₁/D₂L, rather than individual receptors, thereby selectively regulating oligomer-related signaling and function and reducing unwanted side effects. Furthermore, it has been proposed that alterations in GPCR heteromer formation may be associated with neurological disorders such as schizophrenia and Parkinson’s disease (reviewed in Borroto-Escuela et al., 2017). Thus, being able to measure the relative population of CB₁/D₂L heteromers in HD using in situ PLA will increase understanding of normal and pathological states. Overall, a better understanding of the relationship between the ECS and DAS especially in respect to the pharmacology of heteromeric complexes is not only critical in and of itself, but it is also applicable to the design of therapies for HD.
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