Characterization of the interaction of the dopamine D2 receptor and the cannabinoid CB1 receptor and its effects on signal transduction pathways

by

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B.Sc. (Biology), Simon Fraser University, 2005

Thesis Submitted In Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Master of Science Program
Faculty of Health Sciences

SIMON FRASER UNIVERSITY

Fall 2013

Approval

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Degree: **Master of Science**

Title of Thesis: ***Characterization of the interaction of the dopamine D2 receptor and the cannabinoid CB1 receptor and its effects on signal transduction pathways***

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Date Defended: November 27, 2013

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Abstract

Activation of either the cannabinoid CB1 receptor (CB1) or dopamine D2 receptor (D2R) inhibits cAMP production since both are $G_{\alpha i/o}$ linked G protein-coupled receptors. This study confirms the interaction of CB1 and D2R with co-immunoprecipitation experiments using HEK-293T cells co-expressing both receptors. Moreover, GST and His-tagged fusion proteins of CB1 and D2R were generated and used in affinity purification assays to show that the carboxyl terminus of the CB1 receptor interacts with the third intracellular loop of the D2 receptor to form the CB1-D2R complex. Additionally, the CB1-D2R complex is formed by a direct protein-protein interaction. Furthermore, the activation of either D2R or CB1 in HEK-293T cells co-expressing both receptors leads to inhibition of forskolin stimulated cAMP accumulation. However, co-activation of both receptors results in a loss of cAMP inhibition. This study characterizes the interaction between CB1 and D2R as well as demonstrates the functional outcomes of the receptor complex.

Keywords: dopamine D2 receptor; cannabinoid CB1 receptor; G protein-coupled receptor (GPCR); signal transduction pathway; cyclic adenosine monophosphate (cAMP); protein-protein interaction

To my family: mom, dad, Shania, Shamiza and my husband Ehsan

Acknowledgements

I would like to express my sincerest gratitude to my senior supervisor, Dr. Frank Lee, for providing me with this immense opportunity. I am grateful to him for his guidance, support and motivation in research and in writing this thesis. My gratitude is extended to my committee members Dr. Tim Beischlag and Dr. Gratien Prefontaine for their valuable input and advice throughout my graduate studies. I would also like to thank Dr. Michael Silverman for dedicating his time as an external examiner and Dr. Masa Niikura for chairing the thesis defence.

I would like to thank the past and present members of the Lee lab, especially Beryl Luk for being such a great technician and friend. I would also like to thank the past and present members of Blusson Hall 10750 lab: Shabnam Massah, Mandeep Takhar, Julienne Jagdeo, Kevin Tam, Kel Sakaki, Mark Labrecque, Sam Khakshour, Hadi Esmaeilsabzali and Nicole Bance. I am grateful for the coffee breaks, lunches, dinners, hockey games, bowling, curling, softball and too many more good times to list. I've appreciated the laughs, the good advice, the exchange of ideas and the venting of frustration-research related or otherwise.

I would like to thank my parents who taught me that anything is possible and there is no limit to what can be achieved. I would like to thank my sister Shania for always reminding that the greatest accomplishment is not in never falling but in rising again after you fall and to Shamiza for making me laugh and reminding me to not take things so seriously. Finally, I'm grateful to my husband, Ehsan, for his caring, love and constant support.

Table of Contents

List of Tables

List of Figures

List of Acronyms

CNS	Central nervous system
CB1	Cannabinoid CB1 receptor
D2R	Dopamine D2 receptor
D2S	Dopamine D2Short receptor
D2L	Dopamine D2Long receptor
GPCR	G protein-coupled receptor
2-AG	2-arachidonoylglycerol
EC	endocannabinoid
DA	dopamine
HEK-293T	Human embryonic kidney 293T
CHO	Chinese hamster ovary
CAD	Cath. a differentiated
PLC	Phospholipase C
PKA	Protein kinase A
PKC	Protein kinase C
AC	Adenylate cyclase
MAPK	Mitogen activated protein kinase
ERK	Extracellular signal-regulated kinase
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element binding protein
CRE	cAMP response element
CBP	CREB binding protein
PFC	Prefrontal cortex
VTA	Ventral tegmental area
Epac1	Exchange protein directly activated by cAMP
FRET	Fluorescence resonance energy transfer
MBiFC	Multicolor bimolecular fluorescence complementation
IP	Immunoprecipitation
co-IP	Co-immunoprecipitation
CT	Carboxyl terminus (carboxyl tail)
IL	Intracellular loop
TM	Transmembrane
CFP	Cyan fluorescent protein
YFP	Yellow fluorescent protein

1. Introduction

1.1. Neurotransmission

The neuron is the primary communicating cell in the central nervous system (CNS). The general structure of the neuron includes four defined regions: the cell body, dendrites, axon and presynaptic terminal. Typically, the cell body, which contains the nucleus and other organelles, give rise to multiple dendrites and one long axon. Dendrites generally receive incoming signals from other nerves cells while the axon propagates signals or action potentials to other nerve cells. These specialized structures of the neuron are designed to conduct, receive and transmit signals. Signals are propagated from one neuron to the next through the process of synaptic transmission. The synapse is the site at which communication between neurons occurs. Chemical synaptic transmission is the major mode of communication between neurons. This process is achieved by neurotransmitters that are released from the presynaptic neuron, which then interact with specific receptors on the membrane of postsynaptic neurons. The arrival of an action potential at the presynaptic terminal elicits neurotransmitter release. Depolarization of the presynaptic terminal causes the opening of voltage-gated Ca^{2+} channels and the resulting inward flux of Ca^{2+} leads to a cascade of events that lead to neurotransmitter release form the presynaptic terminal into the synapse. The released neurotransmitters in the synapse can bind to specific receptors on the cell surface of the postsynaptic membrane as well as on the presynaptic membrane. Neurotransmitter receptors are divided into two classes, ionotropic receptors and metabotropic receptors.

Ionotropic receptors are ligand gated ion channels comprised of multiple membrane spanning subunits. The receptors on the postsynaptic membrane can induce flux in electrical currents thereby, changing membrane potential of the post-synaptic neuron. Upon ligand binding, ionotropic receptors change conformation that affects the channel pore size and allow influx of ions into the cell. Ionotropic receptors produce

relatively fast synaptic transmission compared to metabotropic receptor mediated transmission. An example of ionotropic class of receptor is the ionotropic glutamate receptors which are permeable to sodium, potassium and calcium ions. The three classes of ionotropic glutamate receptors are AMPA, kainite and NMDA (Hollmann, et al., 1989; Moriyoshi, et al., 1991; Armstrong, et al., 1998). Kainate and AMPA receptors bind to kainate and AMPA, respectively, as well, both receptors bind glutamate. These receptors contain a channel that is permeable to both sodium and potassium ions. The NMDA receptor which binds NMDA, glutamate and glycine, among other ligands, contains a channel which is permeable to sodium, potassium as well as calcium ions.

Metabotropic receptors are transmembrane G protein-coupled receptors (GPCRs). Upon ligand binding, many GPCRs stimulate G proteins to activate second messengers and signaling pathways. The role of the G protein-coupled receptor is to transmit the information received from the cell surface of the neuron to the appropriate cellular system within the cell, which then establishes a specific cellular response.

This study focuses on the dynamics of the interaction between the G protein-coupled receptors, cannabinoid CB1 receptor (CB1) and dopamine D2 receptor (D2R). CB1 and D2R are coupled to $G_{\alpha i}$ protein and thus inhibit the activation of second messengers upon activation. Previous research suggests that interaction between the D2 receptor and the CB1 receptor changes the signaling outcomes compared to the signaling induced by individual receptor activation. By characterising the dynamics of CB1 and D2R cross-talk, we can identify the nature of the interaction as well as elucidate the signaling consequences of the interaction. G protein-coupled receptor signaling is an important aspect of neuron communication and ultimately, CNS function. Changes in GPCR receptor dynamics can have detrimental consequences on overall function which may contribute to the pathophysiology of diseased states within the CNS. The signaling outcomes of GPCR activation regulate cell proliferation, gene expression, cell survival as well as cell death. Moreover, GPCRs are the target of many therapeutic drugs.

1.2. G protein-coupled receptor (GPCR)

G protein-coupled receptors (GPCRs) are a large class of cell membrane receptors that mediate a diverse array of physiological responses to various stimuli. Human genome analysis indicates that there are over eight hundred identified protein members of the GPCR family (Fredriksson, et al., 2003). Human GPCRs are classified into five families: Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, and Secretin, which forms the GRAFS classification system (Fredriksson, et al., 2003). The largest is the rhodopsin family which is divided into four main groups: alpha, beta, gamma and delta. Both the cannabinoid CB1 receptor and dopamine D2 receptor belong to the alpha group of the rhodopsin family (Fredriksson, et al., 2003).

1.2.1. GPCR structure

G protein-coupled receptors share topology since they are all composed of seven α-helices that span the plasma membrane. They have stretches of 25-35 hydrophobic residues which make up the transmembrane region, in addition to three extracellular loops, three intracellular loops, an extracellular amino terminus and an intracellular carboxyl terminus (Fredriksson, et al., 2003). The intracellular and extracellular regions of GPCRs vary in size and structure which contribute to unique extracellular ligand binding sites and intracellular protein interaction domains (Kobilka, 2007). G protein-coupled receptors have a diverse array of ligands including proteins, lipids, nucleotides, ions and photons (Fredriksson, et al., 2003). Upon binding of a ligand to the GPCR, signaling cascades are activated which then establish specific cellular responses.

1.2.2. GPCR signaling

The binding of a ligand to its respective GPCR on the cell surface of a neuron elicits specific responses within the cell. Activation of G protein-coupled receptors can stimulate cellular enzyme activity which can promote second messenger activity or activate effector channels that allow ionic influx into the neuron. A common effector enzyme is adenylate cyclase which, upon activation, synthesizes cyclic adenosine monophosphate (cAMP) (**Figure 1**). Phospholipase C (PLC), another effector enzyme, produces diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP_3). Production of IP_3

results in the release of intracellular Ca^{2+} stores. The second messenger cAMP activates protein kinase A (PKA) whereas; DAG and Ca^{2+} stimulate protein kinase C (PKC) activity. Activated protein kinases can phosphorylate a broad range of target molecules including transcription factors, ion channels and enzymes.

Signaling of GPCRs is regulated primarily through their coupling with heterotrimeric G proteins comprised of three subunits, G_α, G_β and G_γ. Isoforms of these subunits contribute to diversity within the classified groups of the GPCR families (Radhika and Dhanasekaran, 2001). In the resting or inactive state, the G_α subunit, of the $G_{\alpha\beta\gamma}$ heteromer, is bound to guanosine diphosphate (GDP) and the complex is freely available in the cytoplasm. The $G_{\alpha\beta\gamma}$ heteromer complex binds to a GPCR, which is activated by an agonist, leading to a conformational change of the GPCR and enhancement of the guanine nucleotide exchange factor (GEF) activity. This action causes the exchange of GDP for guanosine triphosphate (GTP) on the G_α subunit (Gilman, 1987). The activated GTP-bound G protein dissociates into two parts whereby, the G_α subunit separates from the $G_{\beta\gamma}$ dimer. The dissociated G_α subunit and $G_{\beta\gamma}$ dimer are then available to influence various effector molecules to initiate signaling cascades. The G_α subunit hydrolyzes GTP to GDP with regulators of G protein signalling proteins (RGS). Regulators of G protein signalling proteins are GTPase-accelerating proteins (GAPs) for G_α proteins. Hydrolysis of GTP leads to a GDP-bound state of the G_α subunit and the re-association of the $G_{\alpha\beta\gamma}$ protein heteromer complex.

G_α mediated signaling

The G_α protein plays an important role in the G protein heteromer since it contains the guanine-nucleotide binding site and regulates GTPase activity (Radhika and Dhanasekaran, 2001). There are four classes of G_α subunit: $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$, which classified based on amino acid homology (Hur and Kim, 2002). Although the GPCR bound G protein heteromers function together to regulate GPCR function, once dissociated, the G_α subunit and $G_{\beta\gamma}$ dimer can initiate separate signaling cascades. Activation of $G_{\alpha s}$ coupled GPCR activates adenylate cyclase (AC) whereas stimulation of the $G_{\alpha i}$ linked GPCR leads to AC inhibition (**Figure 1**). Stimulation of the $G_{\alpha q}$ linked GPCR results in the activation of PLC which can then produce IP_3 and DAG (Hur and Kim, 2002).

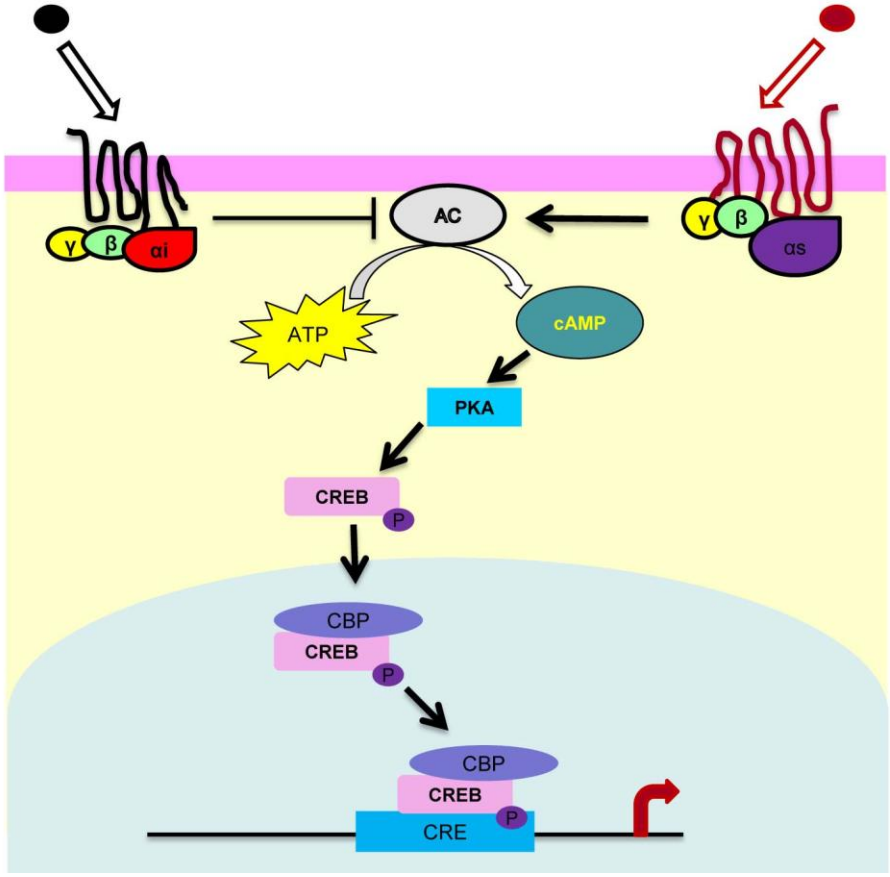

Figure 1. $G_{\alpha i/o}$ and $G_{\alpha s}$ mediated G protein-coupled receptor signaling

Activation of $G_{\alpha i/o}$ or $G_{\alpha s}$ coupled receptor leads to inhibition or activation of adenylate cyclase (AC), respectively. The activated AC produces the second messenger, cyclic adenosine monophosphate (cAMP) which can activate protein kinase A (PKA). PKA activation can lead to phosphorylation of cAMP response element binding protein (CREB). Phosphorylated CREB (pCREB) binds to CREB binding protein (CBP) and together the CBP/CREB complex can activate genes regulated by cAMP response element (CRE) promoter.

$G_{\beta\gamma}$ mediated signaling

The G_{β} and G_{γ} subunits are present as a tightly bound dimer which mediates the coupling of the GPCR complex with other important molecules including kinases (Radhika and Dhanasekaran, 2001). Together, the $G_{\beta\gamma}$ heterodimer regulates multiple signalling outcomes including activation of the extracellular signal regulated kinases (ERK) of the mitogen activated protein kinase (MAPK) family which modulate various actions within the cell including regulation of transcription factors (Crespo, et al., 1994; Faure, et al., 1994). In addition, $G_{\beta\gamma}$ can modulate potassium channels, certain isoforms of adenylate cyclase, including ACI and ACII, phospholipase C and additional kinases (Clapham and Neer, 1993).

β-arrestin

Upon GPCR activation, GPCRs are phosphorylated by G protein-coupled receptor kinases (GRKs). G protein-coupled receptor kinases mediate phosphorylation of GPCRs and subsequent coupling to β-arrestin to desensitize receptor signaling (Pitcher, et al., 1998; Freedman and Lefkowitz, 1996). β-arrestins are recruited from the cytoplasm and transported to the plasma membrane where they bind to the phosphorylated GPCR (Freedman and Lefkowitz, 1996). The GPCR is then uncoupled form the G proteins, preventing further activation, and internalized via endocytosis mediated by clathirin-coated pits (Hanyaloglu and von Zastrow, 2008). The internalized GPCRs are sorted to either the lysosome for proteolytic degradation or into a rapid recycling pathway, where the intact receptor may return to the plasma membrane and re-sensitize the cell to respond to ligand stimulation (Hanyaloglu and von Zastrow, 2008).

Alternatively, the internalized GPCR can activate signaling cascades independent of G proteins. β-arrestin 1 and β-arrestin 2 regulate GPCR mediated intracellular signaling by activating various cytosolic substrates (Luttrell and Lefkowitz, 2002; Lefkowitz and Shenoy, 2005). For example, β-arrestin bound to the internalized GPCR acts as an adaptor or scaffold to promote the activation of ERK1/2 (Azzi, et al., 2003; Shenoy, et al., 2006). Moreover, the β-arrestin bound GPCR can also activate

tyrosine kinases, PI3 kinase and Akt which plays an important role in cell survival pathways to prevent cell death (Lefkowitz and Shenoy, 2005).

1.2.3. GPCR oligomerization

G protein-coupled receptors are able to form homodimers and heterodimers. G protein-coupled receptor homodimers are commonly expressed and it is likely that most GPCRs are found as homodimers (Lee, et al., 2003; Mackie, 2005). Heterodimers of GPCRs can form between receptors of the same class as well as between receptors of different classes. The signaling, transport, localization and ligand binding are different for a GPCR heterodimer when compared to the properties of the individual GPCR participating in the heterodimer complex (Mackie, 2005). The $GABA_B$ receptor is first reported evidence of a functional heterodimer (Jones, et al., 1998). $GABA_B$ receptors function as a heterodimer of $GABA_B$R1 and $GABA_B$R2. Evidence shows that expression and activation of $GABA_B$R1 or $GABA_B$R2 does not result in potassium channel activation. However, co-expression of both receptors results in stimulation of channel activity (Jones, et al., 1998).

Domains responsible for GPCR oligomer formation

Transmembrane (TM) domains mediate GPCR heterodimers. For example, the transmembrane (TM) domains 4 and 5 of glutamate receptor, mGluR2, interacts with serotonin 5-HT(2A) receptor (Gonzalez-Maeso, et al., 2008). Additionally, homodimers of the dopamine D2 receptor (D2R) are formed by interacting regions within transmembrane domain 4, whereas β-adrenergic receptor homodimers are modulated by two separate transmembrane domains, TM1 and TM5 (Guo, et al., 2003; Carrillo, et al., 2004).

In addition to TM regions, intracellular domains of GPCRs can interact with other GPCRs to form heterodimers. Intracellular domains are comprised of the carboxyl terminus (CT) and the intracellular loops (IL). Functional $GABA_B$ receptors are formed by heterodimers of the $GABA_B$-R1 and $GABA_B$-R2 subunits which is regulated by specific regions within the carboxyl terminus of these receptors (Kammerer, et al., 1999). Moreover, the CT of the dopamine D1 receptor (D1R) interacts with the CT of two separate NMDA receptor subunits (Lee, et al., 2002). In a recent study, post-mortem

brain tissue from depressed individuals reveals that D1R and D2R form a complex that is mediated by intracellular loop 3 (IL3) of D2R and the cytosolic CT of the D1 receptor (Pei, et al., 2010). Previously, Dziedzicka-Wasylewska, et al., (2006) and Lukasiewicz, et al., (2009) also demonstrated that the carboxyl terminus of the dopamine D1 receptor interacts with intracellular loop 3 of the dopamine D2 receptor (Dziedzicka-Wasylewska, et al., 2006; Lukasiewicz, et al., 2009). The dopamine D2 receptor also forms heterodimers with serotonin receptor 5-HT(2A) and adenosine A2A receptor. The third intracellular loop of the dopamine D2 receptor mediates the interaction between D2R and serotonin receptor 5-HT(2A) and adenosine A2A receptor (Lukasiewicz, et al., 2009; Lukasiewicz, et al., 2010; Fernandez-Duenas, et al., 2012).

Effects of GPCR oligomerization

Substantial research has been conducted to elucidate the effects of oligomerization of GPCRs. As a complex of multiple receptors, GPCR oligomers behave differently compared to a monomeric GPCR. Oligomerization of GPCR causes changes in signaling outcomes, plasma membrane localization, and dynamics of ligand binding as well as other pharmacological properties (Lee, et al., 2003; Maggio, et al., 2005). Within the CNS, GPCRs are key pharmacological targets for drug discovery. There are many therapeutic drugs that exert their effects on cellular response through the binding of GPCRs. Since the discovery of GPCR oligomers, the outlook on drug development for therapeutics has changed (George, et al., 2002). The study of GPCR oligomerization may give further insight into manifestations of disease states associated with GPCR regulation.

1.3. The endocannabinoid system

The chance discovery of a membrane receptor that mediates the effects of tetrahydrocannabinol (THC), the active ingredient of marijuana, initialized extensive research of the endocannabinoid system in the 1990s. Additional components of the endocannabinoid system have been identified as endocannabinoids which are endogenous lipid-based ligands; enzymes responsible for the regulation of endocannabinoids; and cannabinoid receptors. Cannabinoid CB1 receptor (CB1) and cannabinoid CB2 receptor (CB2) are G protein-coupled receptors. The CB1 receptor is

found abundantly in the central nervous system with some localized to peripheral tissues whereas the CB2 receptor is found in immune tissues and cells (Herkenham, et al., 1990; Bouaboula, et al., 1993; Chakrabarti, et al., 1995). Interestingly, the CB1 receptor is the most abundant GPCR identified in the brain. The amino acid sequence of human CB1 and CB2 receptor is shown in **Figure 2.**

In 1992, *N*-arachidonoylethanolamine (AEA) was the first endogenous endocannabinoid identified (Devane, et al., 1992). This lipophilic compound was named anandamide, based on 'ananda', the Sanskrit word for bliss. Following the discovery of anandamide, a second endogenous endocannabinoid, 2-arachidonoylglycerol (2-AG), was identified (Mechoulam, et al., 1995; Sugiura, et al., 1995). Using mouse brains Liu et al., (2006) show that endocannabinoids, 2-AG and anandamide, are synthesized by phospholipase C and phospholipase D, respectively, from arachidonic acid precursors (Liu, et al., 2006). Alternatively, 2-AG can also be synthesized from arachidonic acid containing diacylglycerol (DAG) precursor by diacylglycerol lipase (Piomelli, 2003). Virodhamine, N-arachidonoyldopamine and noladin ether are also endocannabinoids that bind to cannabinoid receptors (Piomelli, 2003).

Endocannabinoids (ECs) are classified as retrograde messengers which are produced in an "on demand" manner whereby, signal transduction events can elicit endocannabinoid release. Firing of action potentials on the postsynaptic neuron causes voltage gated ion channels to open, allowing an influx of Ca^{2+}. The depolarization of the postsynaptic cell initiates the cleavage of membrane lipids, which is the mode of storage of ECs in the cell, and subsequent synthesis of ECs (Di Marzo, et al., 1994). Since ECs are lipid-based molecules they diffuse from the postsynaptic membrane, travel in a retrograde manner and bind the CB1 receptor on the presynaptic membrane to suppress transmitter release. Once inside the presynaptic cell, anandamide is hydrolyzed by fatty acid amide hydrolase to arachidonic acid and ethanolamine whereas 2-AG is hydrolyzed by monoglyceride lipase. With these actions, the postsynaptic cell can control the activity of the presynaptic neuron. This phenomenon has been deemed depolarization-induced suppression of inhibition or depolarization-induced suppression of excitation in neurons which involves presynaptic inhibition of neurotransmitter release after depolarization of the postsynaptic neuron and release of retrograde messenger. Endocannabinoids are classified as retrograde messengers and thus, on a cellular level,

ECs together with the CB1 receptor regulate synaptic transmission (Maejima, et al., 2001; Ohno-Shosaku, et al., 2002; Robbe, et al., 2001).

On a systemic level, the endocannabinoid system is implicated in physiological processes such as pain perception, mood, learning, memory and reward (Solinas, et al., 2008; Di Marzo, 2008). Endocannabinoids behave like neurotransmitters to modulate memory formation, learning and neural development due to their role in mediating synaptic plasticity (Chevaleyre, et al., 2006). The involvement of endocannabinoids in short-term and long-term synaptic plasticity helps to mediate cognitive functions and emotions in neural circuits (Chevaleyre, et al., 2006). Altered EC function contributes to the pathophysiology of depression and suicidal behaviour (Vinod and Hungund, 2006). In addition, the endocannabinoid system is also involved in the regulation of anxious states and stress (Viveros, et al., 2005). Endocannabinoids also play an important role in the reinforcement of drugs of abuse in the mesolimbic dopaminergic pathway, the dopaminergic pathway responsible for regulating cognition, reward and emotion (Gerdeman, et al., 2003; Parolaro and Rubino, 2002).

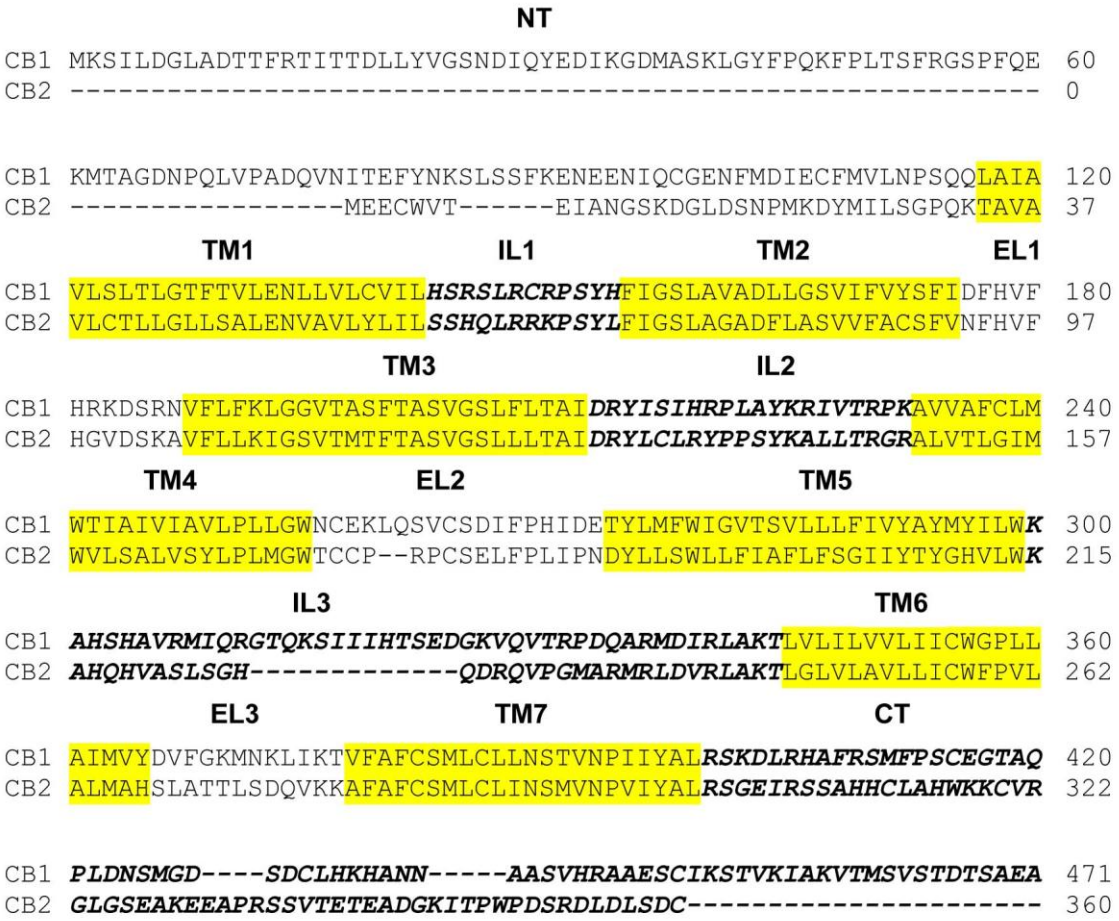

Figure 2. Amino acid sequence of human cannabinoid receptors CB1 and CB2

The amino acid sequence of human CB1 and CB2 receptor showing the amino terminus (NT), extracellular loops (EL), transmembrane (TM) regions, intracellular loops (IL) and the carboxyl terminus (CT).

1.4. Cannabinoid CB1 receptor (CB1)

Cannabinoid CB1 receptors regulate the action of endocannabinoids in the brain. Studies with animals lacking CB1 receptor have elucidated its dynamic roles. For example, Steiner et al., (1999) demonstrate that loss of CB1 receptor leads to reduced locomotion and decreased rearing behaviour in rodents (Steiner, et al., 1999). Moreover, mice lacking functional CB1 show deficits in motor activity, displaying hypomobility and hypotension. Analgesia and hypothermia are also absent in CB1 knockout mice, implicating CB1 in pain and temperature homeostasis (Ledent, et al., 1999; Zimmer, et al., 1999). In addition, endocannabinoids in the hypothalamus play a role in activating CB1 receptors to maintain food intake and form part of the neural circuitry regulated by leptin, a signaling molecule that guides the hypothalamus to modulate food intake (Di Marzo, et al., 2001).

1.4.1. CB1 structure

Cannabinoid CB1 receptor is a 473 amino acid protein originally identified in rats in 1990 (Matsuda, et al., 1990). Following the discovery of rat CB1, the human homolog (Gerard, et al., 1990) and mouse homolog of CB1 were also characterized (Chakrabarti, et al., 1995). The CB1 receptor is a GPCR belonging to the rhodopsin family. CB1 receptors have seven transmembrane regions with an extracellular amino terminus and an intracellular carboxyl terminus.

1.4.2. CB1 expression

CB1 receptor mRNA and protein are found abundantly throughout the central nervous system. Following the cloning of the CB1 receptor, Mailleux et al., (1992) identified CB1 mRNA localization in the human cerebral cortex, dentate gyrus and hippocampus (Mailleux, et al., 1992). Matsuda et al., (1993) studied mRNA localization in rat brain to show CB1 mRNA was present in hypothalamus, thalamus, basal ganglia, cerebellum, amygdala and brainstem (Matsuda, et al., 1993). In addition, Matsuda et al., (1993) confirmed CB1 mRNA presence in the cerebral cortex and hippocampus, as shown earlier by Mailleux et al., (1992). Furthermore, Matsuda et al., (1993) found that

CB1 mRNA showed greatest expression in basal ganglia, hippocampus and cerebral cortex.

CB1 receptor protein is found most abundantly in the cerebellum, hippocampus, brainstem, cerebral cortex and basal ganglia (Herkenham, et al., 1990; Herkenham, et al., 1991; Pertwee, 1997). CB1 receptor is also evident in populations of striatal neurons (Hohmann and Herkenham, 2000; Rodriguez, et al., 2001; Fusco, et al., 2004). Studies with primate brains show that CB1 receptors are found both pre and postsynaptically, on dendritic spines and axon terminals (Ong and Mackie, 1999). In the hippocampus, the CB1 receptor is mostly located presynaptically (Schlicker, et al., 1997) whereas, in the nucleus accumbens CB1 receptor is found on dendrites as well as on axons, where they are co-localized with the dopamine D2 receptor (Pickel, et al., 2006).

1.4.3. CB1 signaling

On a cellular level, the functional role of CB1 receptor is to regulate neurotransmission. On the presynaptic membrane, CB1 receptor activation can inhibit voltage activated calcium channels (Mackie, et al., 1995; Twitchell, et al., 1997) which ultimately depresses neurotransmitter release. Prior reports demonstrate CB1 mediated inhibition of synaptic transmission in the nucleus accumbens and hippocampus; inhibition of glutamate release in the rat striatum; and regulation of glutamatergic and GABAergic cells in the ventral tegmental area (Hoffman and Lupica, 2000; Kim and Thayer, 2000; Gerdeman and Lovinger, 2001; Hoffman and Lupica, 2001; Riegel and Lupica, 2004).

The activation of CB1 receptor on the postsynaptic membrane can elicits various signaling outcomes through G_{α} or $G_{\beta\gamma}$ proteins. For example, activation of CB1 can simultaneously inhibit adenylate cyclase through $G_{\alpha i}$ while the $G_{\beta\gamma}$ dimer can stimulate MAPK activity. Furthermore, G proteins can directly interact with voltage activated Ca^{2+} channels.

Adenylate cyclase (AC) regulation

The activation of $G_{\alpha s}$ coupled GPCRs leads to the activation of adenylate cyclase which results in the production of cyclic adenosine monophosphate (cAMP) levels. Cyclic AMP can activate protein kinase A which can phosphorylate cAMP response

element binding protein (CREB). Downstream effects of phosphorylated CREB include activation of specific genes which are linked to the cAMP response element (CRE) promoter. On the other hand, activation of $G_{\alpha i/o}$ linked GPCRs does not result in activation of adenylate cyclase and therefore cAMP synthesis does not occur (**Figure 1**).

Previously, Howlet and Fleming (1994) demonstrated that delta9-THC activation of neuroblastoma cells inhibited adenylate cyclase (Howlett and Fleming, 1984). Howlett et al., (1986) then demonstrated that these effects are mediated through $G_{\alpha i}$ coupling, since pertussis toxin treatment blocked the effects of THC (Howlett, et al., 1986). After the characterization of CB1 in 1990 by Matsuda et al., Vogel et al., (1993) demonstrated that activating CB1 receptor with endogenous endocannabinoids, anandamide and 2-AG, caused inhibition of forskolin-stimulated cAMP production in mouse neuroblastoma cells (Vogel, et al., 1993). These findings demonstrated that CB1 was coupled to $G_{\alpha i}$ and it also confirmed the earlier findings of Howlett et al. (Howlett and Fleming, 1984; Howlett, et al., 1986). Moreover, Cadogan et al., (1997) showed that synthetic CB1 agonist CP 55940 reduces forskolin-stimulated cAMP accumulation in rat cortical slices, which was reversed in the presence of CB1 antagonist SR 141716 (Cadogan, et al., 1997).

Intracellular Ca^{2+} influx

CB1 stimulation plays a role in establishing intracellular Ca^{2+} influx in mouse neuroblastoma (N18TG) as well in neuroblastoma-glioma hybrid (NG108-15) cell lines (Sugiura, et al., 1996; Sugiura, et al., 1997). Pertussis toxin treatment and PLC inhibition blocked Ca^{2+} influx stimulated by 2-AG. This suggests that the activation of CB1 receptor mediates $G_{\alpha\beta\gamma}$ release from CB1 and subsequent dissociation of $G_{\beta\gamma}$ dimer from G_{α}. The $G_{\beta\gamma}$ is then available to activate the β isoforms of PLC which leads to the release of IP_3 (Sugiura, et al., 1996; Sugiura, et al., 1997). Production of IP_3 results in release of Ca^{2+} stores thereby increasing intracellular Ca^{2+} concentrations. Activation of $G_{\alpha q/11}$ also increases intracellular Ca^{2+} levels and previously it has been demonstrated that activated CB1 receptors can couple to $G_{\alpha q/11}$ (Lauckner, et al., 2005).

Mitogen activated protein kinase (MAPK)

G protein-coupled receptor stimulation can also activate MAPK. Mitogen activated protein kinases play a role in cell death, cell proliferation and cell

differentiation. The three major MAPK cascades are c-Jun NH(2)-terminal protein kinase (JNK), p38 kinase and extracellular signal-regulated kinase (ERK). An *in vitro* study, with Chinese hamster ovary cells stably expressing human CB1, first established both phosphorylation and activation of MAPKs upon cannabinoid treatment (Bouaboula, et al., 1995). These effects were inhibited by SR 141716A, a selective CB1 antagonist, confirming the role of CB1 in mediating this process. Moreover, studies using rodent hippocampal slices show that CB1 activation by endocannabinoids and delta9-THC elicits stimulation of p38, JNK and ERK phosphorylation (Rueda, et al., 2000; Derkinderen, et al., 2001; Derkinderen, et al., 2003). This stimulation of MAPK signaling cascades, upon CB1 receptor activation, is likely mediated by $G_{\beta\gamma}$ heterodimer (Faure, et al., 1994).

Regulation of Ca^{2+} and K^{+} ion channels

CB1 receptors also play a dynamic role in regulating Ca^{2+} and K^{+} ion channels. *In vitro* experiments show that activation of CB1 receptors result in inhibition of calcium channels and activation of inward rectifying potassium channels (Felder, et al., 1995; Mackie, et al., 1995). Inhibitition of N and P/Q types of voltage activated Ca^{2+} channels is likely mediated by $G_{\beta\gamma}$ proteins which interact directly with the channels, after CB1 activation (Twitchell, et al., 1997; Wilson and Nicoll, 2001). CB1 mediated inactivation of calcium channels inhibits synaptic transmission in presynaptic neurons of the hippocampus; striatal neurons including nucleus accumbens; and amygdala (Hoffman and Lupica, 2000; Kim and Thayer, 2000; Hoffman and Lupica, 2001; Huang, et al., 2001; Katona, et al., 2001; Wilson and Nicoll, 2001; Wilson, et al., 2001).

The CB1 receptor stimulates G protein-coupled inwardly rectifying potassium (GIRK) channels (Ho, et al., 1999; Guo and Ikeda, 2004). However, it is unclear whether the activation of GIRK by CB1 is mediated directly by $G_{\beta\gamma}$ or by second messengers generated upon receptor activation (Piomelli, 2003). The activation of GIRK channels by CB1 is a mode of regulating presynaptic function since activated GIRK channels cause hyperpolarization of the presynaptic cell which results in inhibition of neurotransmitter release (Piomelli, 2003).

1.4.4. CB1 interaction with other GPCRs

The function of CB1 receptors can also be modulated by direct protein-protein interactions. CB1 receptors and μ-opioid receptors interact and form heterodimers. CB1 receptor and μ-opioid receptors localize to the same neuronal compartments within the rat caudate putamen nucleus and the nucleus accumbens (Rodriguez, et al., 2001; Pickel, et al., 2004). Furthermore, CB1 and μ-opioid receptors form a complex modulated by a direct protein-protein interaction. The cross-talk between these receptors likely regulates analgesic effects since both the cannabinoid and opioid systems play a role in pain perception (Hojo, et al., 2008; Welch, 2009).

CB1 receptor interacts directly with orexin 1 (OX_1) receptor to form a complex which has unique regulatory properties compared to the individual receptors (Hilairet, et al., 2003; Ward, et al., 2011). Cross-talk between the CB1 receptor and OX_1 receptor may modulate behaviour associated with appetite, feeding and wakefulness (Hilairet, et al., 2003; Ward, et al., 2011).

Carriba et al., (2007) report that the CB1 receptor interacts with the Adenosine A2A receptor. CB1 and adenosine A2A receptors co-localize in the rat striatum where they form heterodimers (Carriba, et al., 2007). The physical and functional interaction between the A2A and CB1 receptors likely mediate the effects of cannabinoids on motor control.

The CB1 receptor also interacts with the β_2-adrenergic receptor (Hudson, et al., 2010). Tissue distribution of β_2-adrenergic receptor overlaps with CB1 receptor particularly in parts of the eye, where the interaction of the CB1 receptor and β_2-adrenergic receptor may play a role in intraocular pressure, the primary risk factor for glaucoma (Hudson, et al., 2010; Hudson, et al., 2011). Cannabinoids reduce intraocular pressure through CB1 receptors within the eye and the β_2-adrenergic receptor interaction with CB1 receptor may mediate this action (Hudson, et al., 2011).

1.5. The dopamine system

In the central nervous system, the dopamine system is involved in various processes including neuroendocrine secretion, locomotion, emotion and cognitive

processes such as memory, learning as well as pleasure and reward (Missale, et al., 1998; Wise, 2004). In the pituitary and peripheral tissues, such as kidney and vasculature, the dopamine system affects hormone secretion, sodium homeostasis and vascular tone, respectively (Missale, et al., 1998).

Within the brain, the dopamine system is divided into four pathways: mesolimbic, mesocortical, nigrostriatal and tuberoinfundibular. Both the mesocortical and mesolimbic pathways originate in the ventral tegmental area (VTA). The mesocortical pathway projects from the VTA to the frontal cortex while mesolimbic pathway projects from the VTA to the nucleus accumbens as well as other regions of the limbic system (Fluxe, et al., 1974). The mesolimbic system plays a role in emotion, learning motivation and reward. The mesocortical system is involved in cognition and control of executive function. The nigrostriatal pathway contains dopaminergic neurons that project from the substantia nigra to the dorsal striatum (Bedard, et al., 1969). This system is involved in motor control and is known to degenerate in Parkinson's disease (Fuxe, et al., 2006). The tuberoinfundibular pathway is involved in the regulation of hormone secretion. This pathway originates from the hypothalamus and projects to the pituitary gland (Bjorklund, et al., 1973). The dopaminergic pathways facilitate many important aspects of central nervous system function, ranging from learning, emotion and reward to motor control and endocrine function.

Dopamine (DA) is produced from tyrosine which is metabolized to L-DOPA by tyrosine hydroxylase. L-DOPA is then decarboxylated to dopamine by L-aromatic amino acid decarboxylase. Dopamine receptors are G protein-coupled receptors which belong to the rhodopsin class. They are grouped into two sub-classes, D1-like and D2-like receptors, based on function, biochemical properties and structure. The D1-like receptors, which include D1 and D5, couple to stimulatory $G_{\alpha s}$ proteins to stimulate adenylate cyclase mediated formation of cAMP (Monsma, et al., 1990; Missale, et al., 1998). Conversely, the D2-like receptors (D2, D3 and D4) are coupled to inhibitory $G_{\alpha i/o}$ proteins and thereby, inhibit adenylate cyclase mediated cAMP production (Missale, et al., 1998). The amino acid sequences of human D2-like receptors, D2S, D2L, D3 and D4 are shown in **Figure 3**.

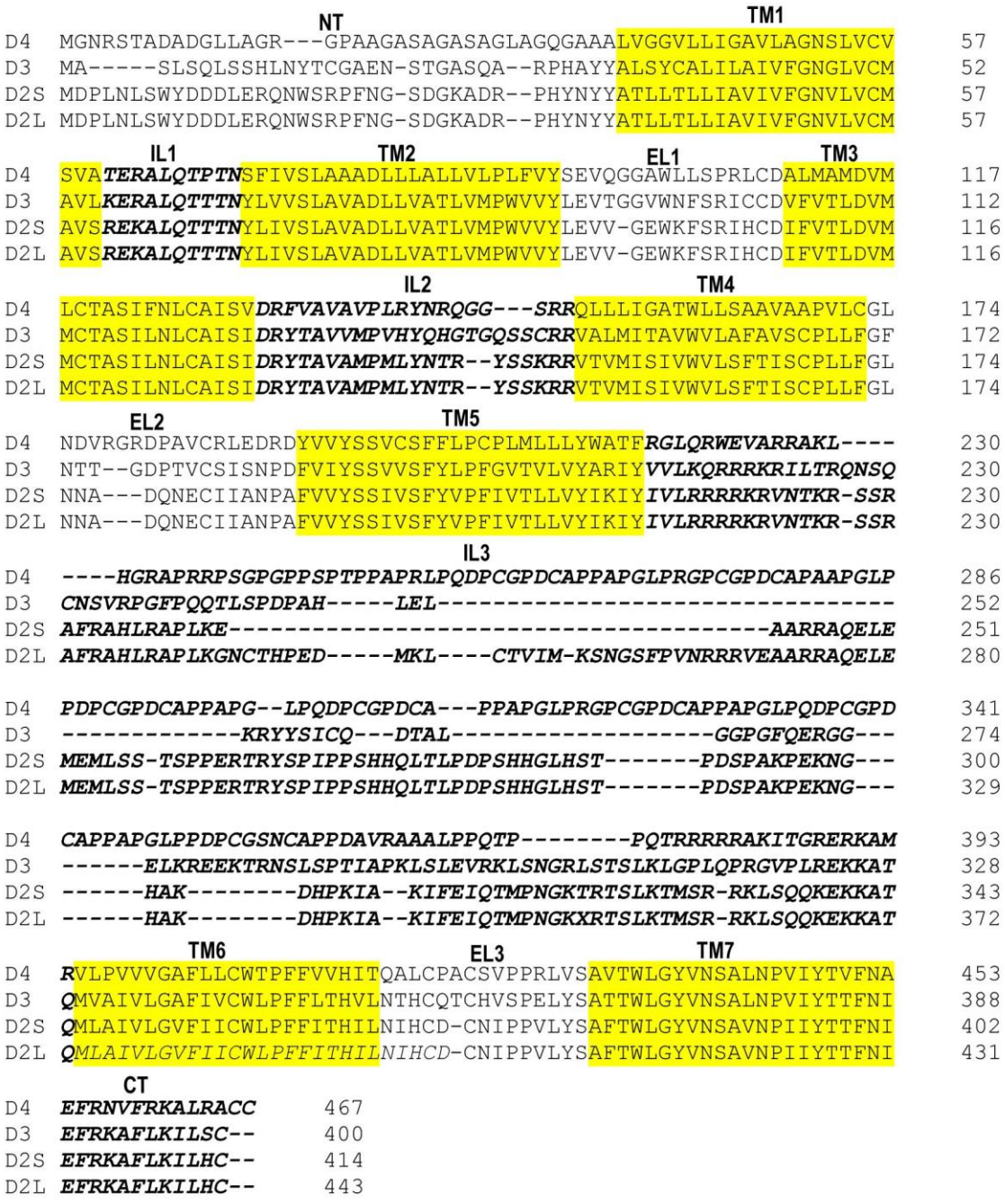

Figure 3. Amino acid sequence of human D2-like receptor D2S, D2L, D3 and D4

The amino acid sequence of human D2-like receptors, D2S, D2L, D3 and D4 showing the amino terminus (NT), extracellular loops (EL), transmembrane (TM) regions, intracellular loops (IL) and the carboxyl terminus (CT).

1.6. Dopamine D2 receptor (D2R)

1.6.1. D2R structure

The dopamine D2 receptor (D2R) was first identified in rats in 1988 (Bunzow, et al., 1988). Subsequent research revealed that D2R existed in two isoforms, D2Long (D2L) and D2Short (D2S), due to alternative splicing resulting in an additional 29 amino acids in the third intracellular loop of D2L (Dal Toso, et al., 1989; Giros, et al., 1989). Further structural analysis revealed that the rat D2L receptor contains 444 amino acids (Bunzow, et al., 1988) while human D2L receptor is comprised of 433 amino acids (Dal Toso, et al., 1989). In addition to D2S and D2L isoforms, D2Longer isoform was identified in a study where the striatum of post-mortem brains from individuals who suffered from psychosis were examined for mutations in D2R mRNA (Seeman, et al., 2000). The D2Longer isoform has two additional amino acids in the third intracellular loop and inhibits adenylate cyclase upon activation (Liu, et al., 2000).

1.6.2. D2R expression

In rat brain, D2R mRNA is present in the pituitary gland, olfactory tubercle, substantia nigra, ventral tegmental area, caudate putamen and nucleus accumbens (Meador-Woodruff, et al., 1989; Mengod, et al., 1989; Le Moine, et al., 1990; Mansour, et al., 1990). Studies of the rat brain show that mRNA localization of the two isoforms of D2R, D2L and D2S, have overlapping localization in the nucleus accumbens and substantia nigra (Fujiwara, et al., 1991).

The highest levels of D2 receptor protein is found in the nucleus accumbens, striatum and in the olfactory tubercle (Missale, et al., 1998; Ennis, et al., 2001). The ventral tegmental area, cortical areas, amygdala, hippocampus, substantia nigra and hypothalamus also show significant D2R protein expression (Missale, et al., 1998; Vallone, et al., 2000). There are differences in cellular localization of D2L and D2S isoforms of D2R (Dal Toso, et al., 1989). The D2S isoform is found predominantly in cell bodies and nerve terminals in dopaminergic neurons of the hypothalamus and mesencephalon whereas the D2L receptor is more abundantly expressed in neurons of the striatum and nucleus accumbens (Khan, et al., 1998; Emilien, et al., 1999). It is

likely that the D2S isoform of D2R functions as the dopamine autoreceptor since the D2S receptor is found presynaptically in contrast to the D2L receptor, which is primarily located on postsynaptic membranes (Khan, et al., 1998; Usiello, et al., 2000; De Mei, et al., 2009).

1.6.3. D2R function

The D2 receptor has many complex roles within the CNS. The location of D2R within the brain provides insight on the specific functional role of the D2R. For example, D2R is abundantly found in the striatum and substantia nigra where it plays an important role in motor control. Mice lacking the D2 receptor exhibit Parkinson-like behavior (Baik, et al., 1995; Fowler, et al., 2002). Furthermore, D2R knockout mice studies indicate increased dopamine turnover which contributes to formation of Lewy-body-like inclusions and axonal degeneration, key hallmarks of Parkinson's disease (Tinsley, et al., 2009). Similar to the CB1 receptor, the D2 receptor also participates in regulation of pain. D2R knockout mice are more sensitive to pain caused by mechanical stimulation (Mansikka, et al., 2005). Moreover, D2 receptor stimulation in the nucleus accumbens inhibits pain in mice (Taylor, et al., 2003).

The D2 receptor has been implicated in learning, memory and reward function. In the nucleus accumbens, D2R mediates reward and spatial learning. D2R knockout mice show reduced motor activity and slower learning which leads to decreased anticipation of reward (Tran, et al., 2002). Moreover, mice lacking D2R show altered reversal learning, a process that requires innervation of the forebrain (De Steno and Schmauss, 2009). The role of D2R in mediating reward, learning and memory also contributes to reinforcing behaviours associated with drug abuse (Missale, et al., 1998; Wise, 2004). In fact, chronic administration of drugs of abuse results in extracellular dopamine release in the limbic system (Di Chiara, 2002).

1.6.4. Signaling of D2R

Presynaptic D2 receptors regulate synaptic transmission by acting as a negative feedback mechanism to inhibit neurotransmitter release. The released dopamine (DA) from the presynaptic membrane can bind to D2R on the postsynaptic membrane as well as D2R autoreceptors located on the presynaptic membrane. Activation of the D2

autoreceptors on the presynaptic membrane results in decreased dopamine release and reduced synaptic transmission (Missale, et al., 1998). In this manner, D2 receptors on the presynaptic membrane can modulate firing rate of the neuron as well as regulate synthesis and release of dopamine. Additionally, binding of DA to D2R can result in various signaling outcomes such as G protein and β-arrestin mediated activation of second messengers; activation of MAPK and CREB; and changes in Ca^{2+} levels.

G protein mediated signaling

Upon activation, D2R can couple to the heteromer complex of G proteins, G_{α}, G_{β} and G_{γ}. Following binding of agonists to the D2 receptor, the G protein heteromer is separated from D2R. The $G_{\alpha i/o}$ inhibits adenylate cyclase activity (Beaulieu and Gainetdinov, 2011; Missale, et al., 1998) (**Figure 1**) while the $G_{\beta\gamma}$ dimer complex can activate G protein coupled inwardly rectifying potassium channels (GIRKs) and inhibit L/N-Type calcium channels (Hernandez-Lopez, et al., 2000; Beaulieu and Gainetdinov, 2011). Moreover, the $G_{\beta\gamma}$ dimer is also able to stimulate PLC which can lead to activation of DAG and IP_3 transduction pathways (Yan, et al., 1999).

β-arrestin mediated signaling

Arrestins play a key role in GPCR inactivation and internalization (Freedman and Lefkowitz, 1996). However, analysis of GPCR signaling shows that β-arrestin is also able to activate GPCR signaling independent of G proteins (Azzi, et al., 2003; Shenoy, et al., 2006). D2R activation leads to β-arrestin mediated regulation of protein kinase Akt which plays an important role in cell survival pathways to prevent cell death (Beaulieu, et al., 2005; Beaulieu and Gainetdinov, 2011).

Activation of MAPK and CREB

Multiple studies demonstrate that agonist stimulation of D2R leads to activation of MAPK pathways (Luo, et al., 1998; Yan, et al., 1999; Kim, et al., 2004). In brain slices, the phosphorylation and activation of MAPK is mediated by PKC (Yan, et al., 1999). Additionally, the activation of D2R results in the phosphorylation of CREB which is regulated by PKC and calmodulin dependent protein kinase (CaMK) (Yan, et al., 1999).

Intracellular Ca^{2+}

Previous research with anterior pituitary cells shows that D2R activation inhibits Ca^{2+} influx into the cell as well as prevents the increase of Ca^{2+} through release from intracellular stores by IP_3 (Malgaroli, et al., 1987; Vallar, et al., 1988). Experiments with neurons from rat brain demonstrate that dopamine can inhibit calcium channels through activation of the D2 receptor (Cardozo and Bean, 1995). Calcium channel inhibition results in decreased dopamine release (Watanabe, et al., 1998). In addition, the D2 receptor is able to modulate potassium levels in the neurons of the substantia nigra (Lacey, et al., 1987; Lacey, et al., 1988). Increased influx of K^+ into the neurons leads to hyperpolarization and decreased intracellular Ca^{2+} levels. In addition to inhibitory effects on dopamine release, D2 receptors also prevent the release of other neurotransmitters, such as GABA, through regulation of calcium channels (Momiyama and Koga, 2001). Intracellular calcium levels are mediated by $G_{\beta\gamma}$, upon agonist activation of D2 receptor (Canti, et al., 1999; Hernandez-Lopez, et al., 2000). However, D2R regulation of calcium channel by signal cascades independent of G proteins is also likely (Beaulieu and Gainetdinov, 2011).

1.6.5. D2R interactions with other GPCRs

There is evidence of dopamine D2 receptor interaction with adenosine A2A receptor. The D2R-A2A complex has been studied in cultured striatal cells as well as in specific regions of the brain such as the dorsal and ventral striatum where the activation of A2A receptors reduces D2R receptor signaling (Fuxe, et al., 2007). Fuxe et al., (2007) suggest that A2A receptor antagonist may be a viable treatment of Parkinson's disease where D2 receptor signaling is reduced, whereas A2A receptor agonist may be a possible treatment for schizophrenia where D2 receptor signaling is increased (Fuxe, et al., 2005; Fuxe, et al., 2007).

Somatostatin receptors (SSTRs) are $G_{\alpha i/o}$ coupled GPCRs which mediate the effects of somatostatin and cortistatin. Somatostatin and cortistatin have dynamic roles including endocrine regulation and neurotransmission. There is evidence of oligomerization of SSTR and D2R. For instance, SSTR and D2R complexes are evident in lung cancer and prostate cancer cell lines. This interaction is ligand mediated and may play a role in modulating cell proliferation (Arvigo, et al., 2010). In addition, D2R

and SSTR2 heterodimer has been observed in cultured striatal neurons (Baragli, et al., 2007).

In addition to GPCRs, various other proteins interact with D2R, collectively termed dopamine receptor interacting proteins (DRIPs). Many DRIPs such as calmodulin, Ca^{2+} binding protein S100B, actin-binding protein 280 (ABP-280) and protein kinase C- ζ interacting protein (ZIP) bind to D2R to regulate function (Bofill-Cardona, et al., 2000; Stanwood, 2008; Li, et al., 2000; Kim, et al., 2008). Moreover, Lee et al., (2007) report D2R interaction with the dopamine transporter (DAT). The cross-talk between DAT and D2R is mediated by a direct protein-protein interaction and the outcomes of this interaction include enhanced localization of DAT to the cellular membrane and greater dopamine uptake into the cell from the extracellular space (Lee, et al., 2007).

D2R interaction with other dopamine receptors

Dopamine D1 receptor (D1R), a $G_{\alpha s}$ coupled GPCR co-localizes with D2R in rat brain and the two receptors form a complex in striatal cultured cells (Lee, et al., 2004). Moreover, the receptors form a complex in live cells and the consequence of the D1R and D2R forming a complex is altered signal transduction outcomes. D1R and D2R co-activation of cells co-expressing both receptors results in PLC mediated calcium signaling which is not observed upon activation of the individual receptors (Lee, et al., 2004). This altered calcium signaling is linked to neuronal growth (Hasbi, et al., 2009). Furthermore, D1R-D2R heterodimer co-stimulation leads to $G_{\alpha q/11}$ signaling (Rashid, et al., 2007). Additionally, the D1R-D2R interaction results in internalization of the heterodimer upon co-activation which is not observed when receptors are individually stimulated and therefore interaction of the two receptors also influence receptor localization (So, et al., 2005). In a recent study, D1R and D2R receptor complex was observed in postmortem brain of patients who suffered from depression (Pei, et al., 2010). Pei et al., (2010) disrupted the direct protein-protein interaction between D1R and D2R in rats to demonstrate anti-depressant effects which was indexed by rat behavioural tests.

Dopamine D5 receptor (D5R) is a D1-like receptor and thus is linked to $G_{\alpha s}$ protein. Similar to the D1R-D2R heterodimer signaling, the D2R and D5R interaction

mediates calcium signaling modulated by $G_{\alpha q/11}$ proteins linked to PLC (So, et al., 2009). However, the D2R-D5R heterodimer signaling is dependent on extracellular calcium levels, which was not observed in D1R-D2R heterodimer signaling (So, et al., 2009). This mechanism of signaling is not observed in D5R alone.

1.7. Cross-talk of the endocannabinoid and dopamine systems

Multiple *in vitro* studies demonstrate that endocannabinoids regulate dopamine release in the brain. For example, rat striatal slices treated with CB1 receptor agonist, CP 55940 and anandamide show decreased dopamine release (Cadogan, et al., 1997). Moreover, CB1 receptor activation inhibits NMDA-stimulated dopamine release in the striatum (Kathmann, et al., 1999). Furthermore, O'Neil et al., (2009) demonstrate that the release of dopamine in the striatum is modulated by the CB1-D2R interaction (O'Neill, et al., 2009).

CB1 receptor activation by delta9-THC and other agonists increase the activity of dopamine neurons in the mesolimbic pathway (Gessa, et al., 1998). Furthermore, in the nucleus accumbens, CB1 stimulation increases extracellular dopamine concentration which is inhibited with CB1 antagonist treatment (Tanda, et al., 1997). Additionally, CB1 receptor agonist treatment increases dopaminergic neuron activity in the VTA as well as the substantia nigra (French, et al., 1997). Melis et al., (2000) demonstrate that intravenous injection of delta9-THC increases firing rate of meso-accumbens and nigro striatial dopaminergic neurons when measured in unanaesthetized rats (Melis, et al., 2000). In humans, delta9-THC inhalation stimulates dopamine neurotransmission in the ventral striatum (Bossong, et al., 2009). In a recent study, *in vivo* observations in anaesthetized rats reveal that CB1 agonist administration significantly increases extracellular dopamine levels in the striatum and prefrontal cortex (Polissidis, et al., 2013).

There is also evidence of dopamine and D2 receptor regulating endocannabinoid signaling. Reports indicate that administration of D2R agonist, quinpirole, elicits anandamide release in the dorsal striatum of rats (Giuffrida, et al., 1999). Moreover, experiments with mice indicate that activation of postsynaptic D2 receptors results in the

release of endocannabinoids, which then act on presynaptic CB1 receptors (Andre, et al., 2010). These actions result in decreased excitatory currents in the striatum. Analysis of whole-cell patch-clamp recordings of brain slices from rats show that D2R activation results in decreased excitatory transmission in striatal cells. These effects were lost in the presence of CB1 antagonist treatment which indicates that the D2 receptor mediated synaptic regulation is dependent on CB1 activation (Yin and Lovinger, 2006). Similarly, in the prefrontal cortex, dopamine suppresses GABA release in addition to regulating CB1mediated endocannabinoid signaling (Chiu, et al., 2010).

Receptor knockout studies provide further evidence of cross-talk between dopamine and endocannabinoid systems. Activation of D2 receptor controls synaptic transmission in striatal cells (Yin and Lovinger, 2006). Using patch-clamp techniques, Yin and Lovinger (2006), demonstrate that in CB1 knockout mice, D2R mediated inhibition of synaptic transmission was lost, indicating that CB1 and D2R cross-talk may regulate synaptic transmission in striatal neurons. Furthermore, the loss of CB1 receptor expression in mice leads to increased D2R expression in the striatum (Houchi, et al., 2005). Conversely, loss of functional D2 receptor causes increased activity of the CB1 receptor in the nucleus accumbens and striatum (Thanos, et al., 2011). In a recent study, loss of CB1 receptor resulted in decreased D2R mRNA and protein expression in the dorsal striatum. Similarly, knockdown of D2 receptor results in significantly lower protein expression of CB1 receptor in striatal cells (Blume, et al., 2013). Interestingly, the localization and distribution of D2R in the prefrontal cortex is also dependent on CB1 expression. The prefrontal cortex of CB1 knockout mice show decreased levels of D2R in the cytoplasm compared to wild type mice (Fitzgerald, et al., 2012).

1.7.1. Overlapping expression of CB1 and D2R

Within the striatum, the D2S isoform of the D2 receptor is found presynaptically whereas D2L is found primarily on postsynaptic membranes (Khan, et al., 1998). There is evidence of CB1 receptor localization in the dendrites of the dorsal striatum (Rodriguez, et al., 2001) which suggests overlapping expression of CB1 and D2R. In situ hybridization experiments with mouse brain sections show that CB1 co-localizes with the D2 receptor in the striatum, olfactory tubercle and hippocampus (Hermann, et al., 2002). Electron microscopy studies of the ventral striatum confirm overlapping

distribution of CB1 and D2L both pre- and postsynaptically (Pickel, et al., 2006). Studies of primate brains also indicate co-localization of CB1 with D2R in striatal cells (Meschler and Howlett, 2001). In addition, CB1 and D2L receptor show co-localization in the prefrontal cortex of rats and mice (Pickel, et al., 2006; Chiu, et al., 2010).

1.7.2. CB1 and D2R form a complex

In vitro studies report that the CB1 receptor and D2L receptor form a complex (Kearn, et al., 2005; Marcellino, et al., 2008; Przybyla and Watts, 2010). Kearn et al., (2005) demonstrate, through co-immunoprecipitation (co-IP) experiments, that the CB1 and the D2 receptor are found in a heterodimer complex. Moreover, CB1 and D2L agonist treatment favours receptor heterodimer formation whereas treatment of cells with individual CB1 or D2R agonists does not affect receptor complex formation. This suggests that agonist treatment and receptor activation can potentially influence the dynamics of CB1 and D2L receptor heterodimer formation.

The CB1 and D2L receptor heterodimer complex is also evident in live cells (Marcellino, et al., 2008; Przybyla and Watts, 2010). Marcellino et al., (2008) use fluorescent tagged CB1 and D2L to conduct fluorescence resonance energy transfer (FRET), a common technique used to study the physical association between GPCR heterodimers. Excitation of a fluorescent tagged donor receptor results in transfer of energy to an acceptor receptor. The efficiency of the energy transferred is proportion to the distance between the two receptors. In their study, Marcellino et al., (2008) utilize CB1 and D2L receptor co-expressing HEK-293 cells to demonstrate that application of either CB1 or D2R agonist alone or co-treatment with both agonists did not affect FRET signal thus agonist treatment did not influence CB1-D2R heterodimer formation. Multicolor bimolecular fluorescence complementation (MBiFC) is technique in which the fluorescent protein is divided into two fragments and the fragments are fused to CB1 and D2L receptor. If the receptors interact with each other, the fluorescent protein fragments will form a functional fluorescent protein. Przybyla and Watts (2010) show, through MBiFC assays with Cath.a differentiated (CAD) cells which are derived from a mouse brain tumor, that agonist treatment with either CB1 or D2R agonist favours the formation of CB1-D2L heterodimer complex.

1.7.3. CB1 and D2R interaction causes altered cAMP signaling

Both the CB1 and D2L receptor are coupled to $G_{\alpha i/o}$ proteins and therefore inhibit cAMP formation. However, studies show that upon co-activation of D2L and CB1 receptors in striatal cultures and transfected HEK-293 cells, cAMP accumulation increases (Glass and Felder, 1997; Kearn, et al., 2005). Glass and Felder, (1997) demonstrate that cAMP accumulation, in Chinese hamster ovary (CHO) co-expressing D2L and CB1 receptor, is due to the switch of CB1 receptor coupling from $G_{\alpha i/o}$ to $G_{\alpha s}$ caused by activation of D2L receptor. However, a later finding suggests that co-expression of D2L receptor with CB1 receptor in HEK-293 cells induces CB1 receptor to switch to $G_{\alpha s}$ coupling and activation of D2L receptor is not necessary (Jarrahian, et al., 2004).

1.8. Rationale of the study

Both the CB1 and D2 receptor are separately implicated in addictions, specifically in ethanol abuse. A growing body of evidence suggests that cross-talk between these receptors are mediating ethanol addictions related behaviour (Houchi, et al., 2005; Cheer, et al., 2007; Thanos, et al., 2011). With this notion, it is important to study the role of CB1-D2R direct interaction and subsequent changes in signaling with respect to disease states including ethanol addictions. Furthermore, this study establishes that CB1-D2R complex can be disrupted with inhibitory mini-genes and peptides. This concept can provide preliminary information in developing therapeutic molecules to treat potential disease states associated with CB1-D2R interaction.

1.9. Hypothesis and objectives of the study

1.9.1. Hypothesis

The CB1 and D2 receptors form a complex which is mediated by specific domains within each receptor. The consequences of the CB1 and D2R interaction is changes in signal transduction, specifically changes in cAMP signaling. Moreover, altered cAMP signaling may have downstream changes such as phosphorylation of CREB.

1.9.2. Specific aims of the project:

The purpose of this study was to elucidate the nature of the interaction between CB1 and D2 receptor and explore the consequences of this interaction on signaling pathways associated with these receptors. Specifically, two main objectives were addressed in this study:

A. Characterize the interaction between CB1 and D2 receptor:

1. To confirm the interaction between CB1 receptor and D2 receptor
2. Identify specific motifs within both receptors that mediate the heterodimer complex formation
3. To determine if the interaction between CB1 and D2R is a direct protein-protein interaction
4. To generate mini-genes to disrupt the interaction

B. Investigate the effects of CB1 and D2 receptor interaction on signal transduction pathways:

1. To determine if co-activation of receptors can modify cAMP outcomes
2. Utilize mini-genes to disrupt cAMP signaling patterns
3. To determine the effects of receptor activation on CREB phosphorylation

2. Materials and Methods

2.1. Materials

Chemicals were purchased from Bioshop Canada (Burlington, ON, Canada), unless otherwise listed. Reduced L-glutathione, glutathione agarose beads, HIS select nickel affinity gel, forskolin, (*S*)-(−)-propranolol hydrochloride, poly(ethyleneimine) (PEI) solution and isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from Sigma Aldrich (Oakville, ON, Canada). 1-Methyl-3-Isobutylxanthine (IBMX) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Methanandamide and bromocriptine were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Restriction endonucleases were obtained from New England Biolabs (Whitby, ON, Canada). High fidelity PCR enzyme mix, T4 DNA ligase, 100 mM dNTP mix, GeneJET gel extraction kit, GeneJET plasmid miniprep kit, O'GeneRuler 1 kb DNA ladder, PageRuler prestained protein ladder, nuclease-free water, Bradford reagent, Protein A/G magnetic beads, SuperSignal West Dura Chemiluminescent Substrate and HyClone fetal bovine serum were purchased from Thermo Scientific (Ottawa, ON, Canada). DMEM Dulbecco's Modification of Eagle's Medium and trypsin EDTA (0.25%) were ordered from Corning Cellgro (Manassas, VA, USA). For cAMP analysis, ELISA cAMP assay was conducted with the Parameter Assay Kit from R&D Systems (Minneapolis, MN, USA).

2.1.1. Antibodies

CB1 receptor goat polyclonal antibody (Cat. No. sc-5303), dopamine D2 receptor mouse monoclonal antibody (Cat. No. sc-10066) and alpha-tubulin mouse monoclonal antibody (Cat. No. sc-8035) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). His-tag monoclonal mouse antibody (Cat. No. A00186) was purchased from GenScript (Piscataway, NJ, USA). GST polyclonal rabbit antibody (Cat. No. 2625), phospho-CREB mouse monoclonal antibody (Cat. No. 9196) and CREB rabbit monoclonal antibody (Cat. No. 9197) were obtained from Cell Signaling Technology

(Danvers, MA, USA). Peroxidase conjugated AffiniPure Goat Anti-Mouse IgG, Goat Anti-Rabbit IgG and Donkey Anti-Goat IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

2.1.2. Expression vectors and receptor cDNA

The pGEX-4T3 plasmid used to produce GST fusion proteins was manufactured by GE Healthcare (Mississauga, ON, Canada). The pET28a(+) vector used to generate His-tagged constructs was manufactured by Novagen (EMD Chemicals, Gibbstown, NJ, USA). The pcDNA3 plasmid was manufactured by Invitrogen (Burlington, ON, Canada). The human CB1 receptor encoding plasmid was provided by Dr Mary Abood (Temple University, Philadelphia, PA, USA). The epac1-camps vector (Borner, et al., 2011; Nikolaev, et al., 2004) was provided by Dr. Martin Lohse (University of Wurzburg, Wurzburg, Germany). Rat CB1 receptor encoding cDNA was provided by Dr. Ken Mackie (Indiana University, Bloomington, IN, USA). The plasmids encoding D2L and D2S receptors were originally cloned by Stormann et al. in pcD-PS (Stormann, et al., 1990).

2.2. Cloning methods

The procedure for generating GST fusion proteins, His-tagged proteins and mini-genes involved multiple steps. To begin, the DNA of interest was amplified through polymerase chain reaction (PCR) from template cDNA using oligonucleotides designed with specific restriction sites. The amplified DNA and expression vector were digested with appropriate restriction endonucleases and then subjected to gel electrophoresis on an agarose gel. The bands of the digested amplified DNA of interest and linearized expression vector were then extracted from the agarose gel. Gel extracted DNA was incubated with the gel extracted vector in the presence of ligase. Following incubation, the ligation reaction was transformed into *E.coli* cells and plated onto LB agar plate, supplemented with antibiotics, and incubated at 37°C. Positive transformant colonies were cultured, mini-prepped and digested to identify recombinant vectors containing the insert DNA. Positive recombinant vectors were sequenced to confirm correct insert and orientation.

2.2.1. Polymerase chain reaction (PCR)

The following PCR protocol served as a general guideline for amplification of the DNA of interest. The components were added together for a final reaction volume of 50 µL:

41.5 µL	Nuclease free H_2O
5 µL	10X buffer with $MgCl_2$
0.5 µL	Forward primer
0.5 µL	Reverse primer
1 µL	100mM dNTP
1 µL	Template cDNA
0.5 µL	High fidelity PCR enzyme mix

The PCR reaction was conducted using Eppendorf Mastercylcer personal thermocycler (Mississauga, ON, Canada). The annealing temperature was adjusted according to the melting temperature of the oligonucleotides (primers) used in the reaction. The reactions were added to the thermocycler and incubated at 94°C for 5 minutes followed by 30 cycles of amplification with the following settings:

Denaturing:	94°C for 30 seconds
Annealing:	65°C for 30 seconds
Extension:	72°C for 30 seconds

The samples were then incubated at 72ºC for 10 minute and then held at 4°C. To visualize the amplification products, a 5 µL aliquot of the PCR product was added to 1µL 6X loading dye (10 mM Tris-HCl (pH 7.6), 0.15% orange G, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA) and the sample was loaded onto a 1% agarose gel which was prepared with ethidium bromide. Molecular weight standard, 1 kb DNA ladder, was also loaded onto the gel. The gel was resolved at 100 V for approximately 30 minutes. Following electrophoresis, the gel was visualized under UV light using Dyversity image analysis system and GeneSnap image acquisition software (Syngene, Frederick, MD).

2.2.2. Restriction digest

Once the amplification PCR product was visualized by agarose gel electrophoresis, 5 µL aliquot of the amplified DNA was digested with the BamHI and XhoI restriction endonucleases using the appropriate buffer (New England Biolabs). The total reaction volume was 20 µL:

12 µL	Nuclease free H_2O
5 µL	Amplified DNA
2 µL	NEBuffer 3
0.5 µL	XhoI
0.5 µL	BamHI

In addition, the target expression vector was linearized by digesting with BamHI and XhoI in a total volume of 20 µL:

12 µL	Nuclease free H_2O
5 µL	Expression vector
2 µL	NEBuffer 3
0.5 µL	XhoI
0.5 µL	BamHI

The restriction digest reaction was incubated at 37°C for 1 hour. To visualize the samples, 4 µL of 6X loading dye was added to the above restriction digest reaction and the total volume was loaded onto a 1% agarose gel. The gel was resolved at 100 V for 30 minutes and following visualization under UV light, the appropriate bands were excised from the agarose gel and the DNA was purified from the gel.

2.2.3. Gel extraction

After the restriction digested DNA and expression vector were cut from the gel, GeneJET gel extraction kit (Thermo Scientific) was used to extract the DNA from the agarose gel. Briefly, Binding Buffer was added to the excised gel and the gel mixture was incubated at 55°C for 10 minutes. The solution was transferred to the GeneJET Purification Column and centrifuged at 16,800 x *g* for 1 minute. The flow-through was discarded and the column was washed with Wash Buffer and centrifuged at 16,800 x *g*

for 1 minute. After the flow-through was discarded, the empty column was centrifuged at 16,800 x *g* for 1 minute. The column was transferred to a clean microcentrifuge tube. Elution Buffer was added and the column was centrifuged at 16,800 x *g* for 1 minute to elute the purified DNA. To determine the DNA yield, Nanovue Spectrophotometer (GE Healthcare, Mississauga, ON, Canada) was used. Once the concentration was determined, the purified DNA was ready for the ligation step.

2.2.4. Ligation of insert and expression vector

The next step of creating recombinant DNA molecules is to ligate the expression vector to the insert DNA. For this process, the gel extracted DNA and expression vector are incubated together using T4 DNA ligase with the provided buffer (Thermo Scientific) in a total volume of 40 µL:

15.5 µL	Nuclease free H_2O
4 µL	10X buffer
15 µL	Insert DNA
5 µL	Expression vector
0.5 µL	T4 DNA Ligase

The ligation reaction was incubated overnight at room temperature. The following day, the ligation reaction was transformed into competent DH5α *E.coli* cells.

2.2.5. Transformation

The total 40 µL volume of the ligation reaction was added to 100 µL DH5α competent *E.coli* cells and left on ice for 30 minutes. Following heat shock at 42°C for 1 minute, the cells were returned to ice for 15 minutes. The bacterial cells were then plated onto agar LB plates containing a selective antibiotic, for which the expression vector contains resistance. The plates were then incubated overnight at 37°C. The next day, transformant colonies were used to inoculate starter cultures to identify colonies containing correct recombinants.

2.2.6. Identification of recombinants

The DH5α cells transformed with the vector will have resistance to the antibiotics and therefore will successfully grow colonies. To distinguish between colonies containing recombinant vectors from those containing self-ligated empty expression vectors, multiple colonies were selected to inoculate cultures which were grown in LB supplemented with antibiotics. The cultures were grown overnight at 37°C while shaking at 250 RPM. The following day, the cultures were collected and mini-prepped using the GeneJET Plasmid Miniprep Kit (Thermo Scientific). Briefly, 1.5 mL of the bacterial culture was added to a microcentrifuge tube and centrifuged at 16,800 x *g* for 1 minute. The supernatant was removed and the cell pellet was re-suspended in 250 µL Resuspension Solution. To lyse the cells, 250 µL Lysis Solution was added and the tubes were inverted 6 times followed by addition of 350 µL Neutralization Solution. The tubes were inverted 6 times and centrifuged at 16,800 x *g* for 5 minutes. The supernatant was added to the GeneJET spin column and centrifuged for 1 minute to bind the DNA to the column. The flow-through was discarded and the column was washed twice with 500 µL Wash Solution. Following washes, the empty column was centrifuged at 16,800 x *g* for 1 minute. The purified DNA was eluted from the column with 50 µL Elution Buffer.

To determine if the purified plasmid contained the correct recombinant DNA, the samples were digested with BamHI and XhoI and subjected to gel electrophoresis, as previously described. The samples containing the correct band sizes for insert DNA and expression vector were sent for sequencing to determine if the DNA sequence was correct. Following confirmation of the DNA sequence, the recombinant expression vector containing DNA of interest was ready for experimentation.

2.3. Cell culture and transfections

Human embryonic kidney 293T (HEK-293T) cells were grown in DMEM supplemented with 10% FBS and maintained at 37°C with 5% CO_2. For transfections, culture plates were coated with PEI in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) at 37°C for one hour. The PEI coating was removed and cells were seeded at a density of 25%-33% confluency in

complete media (DMEM with 10% FBS). The following day, complete media was replaced with serum-free DMEM and calcium phosphate transfections were done with CB1:D2L cDNA ratios of 1:1. For co-immunoprecipiation experiments with mini-genes, the cDNA ratio was 1:1:1 CB1:D2L:pcDNA3 and CB1:D2L:CB1-CT2B mini-gene. For FRET experiments, all cDNA was at 1:1 ratio except for epac1-camps vector which was 1/10 of total cDNA transfected. Representative amounts of H_2O, 2 M $CaCl_2$, and cDNA for transfecting HEK-293T cells in a 100 mm culture dish with CB1 and D2L receptor cDNA:

36 µL	2 M $CaCl_2$
20 µg	CB1 cDNA
20 µg	D2L cDNA
	H_2O to 300 µL

The volume of H_2O was adjusted according to the volumes of DNA used, which was dependent on the concentrations of the DNA. The final volume of the mix was 300 µL. Then, 300 µL 2X HEPES buffered saline (HBS) (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.05) was added to the mix while vortexing. The total 600 µL mix was added to the plate of cells in a drop-wise manner. The cells were incubated in a CO_2 incubator at 37°C for 4 hours before the serum-free media was changed to complete media. Cells were utilized 48 hours post transfection.

2.4. Mini-gene construct

The CB1-CT2B region was PCR amplified from rat CB1 cDNA template using oligos designed with BamHI and XhoI restriction sites (**Table 1**). PCR products and pcDNA3 expression vector were restriction digested with BamHI and XhoI. Digested amplified DNA and digested pcDNA3 vector were ligated using T4 DNA ligase. Ligated vectors were transformed into DH5α competent *E.coli* and plated onto LB agar plates supplemented with 50 µg/mL ampicillin. Colonies were cultured and vector DNA was isolated using GeneJET Plasmid Miniprep Kit and then digested with BamHI and XhoI restriction endonucleases. Correct recombinant vectors were sequenced for verification.

2.5. Protein measurement

To determine the protein concentration of samples, protein standards were first created from 2 mg/mL bovine serum albumin (BSA) (Bio-Rad, Richmond, CA, USA). The standard concentrations were 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL and 250 µg/mL. Using a 96-well microplate, 90 µL of distilled water was added to a row of wells. Then 10 µL of each of the standards was added in duplicate to rows containing water. Protein samples were diluted in water to a final volume of 100 µL and added to the plate in duplicate. To all the wells, 100 µL of Bradford reagent was added. The absorbance of standards and the samples were recorded at 595 nm using Victor Plate Reader with Workout 2.5 Data Analysis software (PerkinElmer, Woodbridge, ON, Canada). The known concentration of the BSA standards and the absorbance readings were used to generate a standard graph. The protein concentrations of the samples were then calculated based on the absorbance of the samples and the equation of the line of the standard curve.

2.6. SDS-PAGE

To prepare protein for SDS-PAGE (sodium dodecyl sulfate poly-acrylamide gel electrophoresis), SDS sample buffer (0.0625 M Tris [pH 6.8], 2% SDS, 10% glycerol, 2% β-Mercaptoethanol, 0.01% bromophenol blue) was added to the protein and the samples were boiled for 5 minutes. The samples were resolved on a 10% SDS-polyacrylamide gel (10% acrylamide/bis, 1.5 M Tris [pH 8.8], 0.4% SDS, 0.1% ammonium persulfate, 0.01% TEMED) in electrophoresis running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 125 V for 1.5 hours using Mini-PROTEAN Electrophoresis System (Bio-Rad Laboratories, Richmond, CA, USA)

2.7. Coomassie staining

Gels resolved by SDS-PAGE were fixed at room temperature for 30 minutes in fixing solution (50% methanol, 10% acetic acid) and stained at room temperature for 1 hour in Coomassie blue solution (50% methanol, 0.05% Coomassie brilliant blue, 10% acetic acid). Gels were de-stained for 2 hours in de-staining solution (5% methanol, 7%

acetic acid). After acceptable de-staining, gel images were captured with Dyversity image analysis system and GeneSnap image acquisition software (Syngene, Frederick, MD, USA).

2.8. Immunoblot (western blot)

Protein samples were prepared by adding SDS sample buffer and boiling for 5 minutes; the samples were then resolved by SDS PAGE. Following SDS-PAGE, gels were transferred to polyvinylidenedifluoride (PVDF) membrane (Bio-Rad Laboratories, Richmond, CA). To prepare PVDF membrane for transfer, the PVDF membrane was soaked in methanol for 1 minute, rinsed in distilled H_2O and then soaked in transfer buffer (50 mM boric acid, 2 mM EDTA, pH 8.8). The resolved gel was equilibrated in transfer buffer for 10 minutes and then transferred to the PVDF membrane in the presence of transfer buffer at 400 mA for 1 hour using Mini-PROTEAN Electrophoresis System.

Following transfer of the gel, the PVDF was dried, re-activated with methanol and then rinsed in distilled H_2O. The membrane was then blocked with 5% non-fat milk in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl and 0.1% Tween) for 1 hour at room temperature. The membrane was washed with TBS-T and incubated with primary antibody, at appropriate dilution in TBS-T, overnight at 4°C. The following day, the membrane was washed three times with TBS-T for 5 minutes at room temperature. The blot was incubated with appropriate secondary antibody (diluted 1:12,000 in TBS-T with 0.5% milk) for 1 hour at room temperature. After incubation with secondary antibody the blots were washed three times with TBS-T and were visualized with SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Ottawa, ON) using Dyversity image analysis system and GeneSnap image acquisition software.

2.8.1. Ponceau S stain

Following imaging of the blots, the SuperSignal West Dura was rinsed in distilled water and the blot was incubated with Ponceau S stain (0.1% ponceau S, 5% acetic acid) for 10 minutes at room temperature. After rinsing in distilled water, the blots were

imaged with Dyversity image analysis system and GeneSnap image acquisition software.

2.9. GST fusion proteins

2.9.1. Creation of GST fusion proteins

Intracellular loops 2 and 3 of the CB1 receptor, CB1-IL2 [G214-K234], CB1-IL3 [K302-T346], and carboxyl tail, CB1-CT [R402-L474], as well as truncations of the carboxyl tail of the CB1 receptor, CB1-CT1 [R402-N426], CB1-CT2 [C417-T441], CB1-CT3 [D432-V456], CB1-CT4 [A447-L474], GST-CB1-CT2A [C417-N426], GST-CB1-CT2B [C417-S431], GST-CB1-CT4A [K460-L474] were PCR amplified from rat CB1 template cDNA using oligonucleotides designed with BamHI and XhoI restriction sites (**Table 1**). PCR products and pGEX-4T3 plasmid were both digested with BamHI and XhoI. Digested PCR product was ligated to linearized pGEX-4T3 vector using T4 DNA ligase. Ligated vectors were transformed into DH5α competent *E.coli* and plated onto ampicillin supplemented LB agar plates. Colonies were cultured and plasmid DNA was isolated using GeneJET Plasmid Miniprep Kit and then digested with BamHI and XhoI restriction endonucleases to identify correct recombinants. Correct constructs were further verified with sequencing. Correct recombinants were transformed into BL21 competent *E.coli* cells and plated onto ampicillin (50 μg/mL) supplemented LB plates and incubated overnight at 37°C. The next day, a colony from the plate was used to inoculate 3 mL LB media supplemented with 50 μg/mL ampicillin. The cultures were grown overnight at 37°C while shaking at 250 RPM. The following day, 800 μL of culture was supplemented with 200 μL sterile glycerol and stored at -80°C. Subsequent starter cultures were inoculated from these glycerol stocks.

2.9.2. Purification of GST fusion proteins

Starter cultures were grown overnight and 1 mL was added to 20 mL LB media supplemented with 50 μg/mL ampicillin. Bacterial cultures were grown at 37°C for two hours while shaking at 250 RPM followed by induction with 500 μM IPTG and then returned to 37°C shaker for 2 hours. The cultures were centrifuged at 2400 x *g*; the cell pellet was re-suspended in 1 mL cold PBS with 1% Triton X-100 and mixed at 4°C for 10

minutes. The samples were then sonicated for 45 seconds followed by centrifugation at 16,100 x *g* at 4°C for 15 minutes, to pellet the cell debris.

For GST fusion protein purification, 50 µL of 50% glutathione agarose beads in PBS was added to 1 mL of bacteria lysates and mixed overnight at 4°C. The following day, the beads were centrifuged for 10 minutes at 400 x *g* at 4°C and after removal of the supernatant; the beads were washed three times for 5 minutes with cold PBS at room temperature (centrifuged at 400 x *g* at 4°C between washes). After the third wash, the beads were incubated at room temperature with 50 µL of GST elution buffer (20 mM reduced L-glutathione, 100 mM Tris [pH 8], 120 mM NaCl). The eluted protein was collected and the elution process was repeated. The protein concentration was determined and SDS sample buffer was added to 1 µg purified GST fusion protein. The sample was boiled and resolved by SDS-PAGE followed by Coomassie staining of the gel to visualize the purified protein.

2.10. His-tagged proteins

2.10.1. Creation of His-tagged proteins

Intracellular loop 3 of the D2L (D2LI3 [I211-Q373]) and D2S (D2SI3 [I211-Q344]) receptor were PCR amplified from full-length D2L and D2S template cDNA with oligos designed with BamHI and XhoI restriction sites (**Table 2**). PCR products and pET-28a(+) were both digested with BamHI and XhoI restriction enzymes. Digested PCR products were ligated to digested pET-28a(+) vector using T4 DNA ligase. Ligated vectors were transformed into DH5α competent *E.coli* and plated onto LB agar plates supplemented with 25 µg/mL kanamycin. Colonies were cultured and plasmid DNA was isolated using GeneJET Plasmid Miniprep Kit and then digested with BamHI and XhoI restriction enzymes to identify potential recombinants. Correct constructs were sequenced to verify insert sequence and orientation. Recombinant pET-28a(+) vector containing the correct insert was transformed into BL21 competent *E.coli* cells, plated onto LB agar plates supplemented with 25 µg/mL kanamycin and incubated overnight at 37°C. The next day, a colony from the plate was used to inoculate 3 mL LB media supplemented with 25 µg/mL kanamycin. The cultures were grown overnight at 37°C while shaking at 250 RPM. The following day, 800 µL of culture was supplemented with

200 µL sterile glycerol and stored at -80°C. Subsequent starter cultures were inoculated from these glycerol stocks.

2.10.2. Purification of His-tagged proteins

Starter cultures were grown overnight and the following day, 1 mL was added to 20 mL LB media supplemented with kanamycin (25 µg/mL). Bacterial cultures were grown at 37°C shaker for two hours shaking at 250 RPM followed by induction with 500 µM IPTG and then returned to 37°C shaker for 2 hours. The cultures were centrifuged at 2400 x *g* and the cell pellet was re-suspended in 1 mL cold PBS with 1% Triton X-100. After mixing at 4°C for 10 minutes, the samples were sonicated for 45 seconds. The lysates were centrifuged for 15 minutes at 16,100 x *g* at 4°C to pellet the cell debris.

For His-tagged protein purification, 50 µL HIS-select nickel affinity gel was washed three times with equilibration buffer (150 mM, NaCl, 50 mM NaH_2PO_4, pH 8.0) and then added to 1 mL of bacterial lysates. After mixing with the lysates for 1 hour at room temperature, the affinity gel was washed three times with wash buffer (150 mM NaCl, 50 mM NaH_2PO_4 pH 8.0) at room temperature and centrifuged at 5,000 x *g* between washes. His-tagged protein was eluted twice with 50 µL elution buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl; 250 mM imidazole). Following elution of purified His-tagged protein, 1 µg was added to SDS sample buffer and boiled. The samples were subjected to SDS-PAGE followed by western blotting with His-tag antibody (1:3,000 dilution) and goat anti-mouse IgG secondary antibody to visualize the purified His-tagged protein.

2.11. Affinity purification assays (pull-down assays)

HEK-293T cells expressing D2L receptor were homogenized in modified RIPA buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40 [IgepalCa630], 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM sodium orthovanadate, 0.1 % Triton X-100) and complete protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After mixing at 4°C for 1 hour, the samples were centrifuged at 16,100 x *g* for 15 minutes at 4°C and Bradford protein assay was conducted to determine protein concentration. 500 µg of protein extracts were incubated with 15 - 40 µg of GST fusion protein (CB1 truncations),

overnight, at 4°C in PBS. The following day, 25 - 50µL of 50% glutathione agarose beads (in PBS) was added and incubated at room temperature for 2 hours, while rocking. Beads were washed three times with 500 µL PBS containing 0.01% Triton and centrifuged at 400 x *g* between washes. After the final wash, the beads were boiled for 10 minutes in SDS sample buffer, resolved by SDS-PAGE followed by western blotting with D2 primary antibody (1:200 dilution) and goat anti-mouse IgG secondary antibody.

For affinity purification assay using purified His-tagged protein as bait, 10 µg His-tagged protein and 0.5 µg GST fusion proteins were mixed in PBS and incubated overnight at 4°C, on a nutator. 5 µL of equilibrated HIS-select nickel affinity gel was added at room temperature for 2 hours. Beads were washed three times with cold PBS with 0.05% Triton X-100 (centrifuged at 4°C at 400 x *g* between washes) and boiled for 10 minutes in SDS sample buffer. The eluate was resolved by SDS-PAGE followed by western blotting with GST primary antibody (1:2,000 dilution) and goat anti-rabbit IgG secondary antibody.

For affinity purification using purified GST fusion protein as bait, 1 µg His-tagged protein and 20 µg GST fusion proteins were mixed in PBS and incubated overnight on a nutator at 4°C. The following day, 10 µL of 50% glutathione agarose beads in PBS was added and incubated at room temperature for 2 hours. Beads were washed three times with cold PBS containing 0.05% Triton X-100 (centrifuged at 400 x *g* between washes) and boiled for 10 minutes in SDS sample buffer. The eluate was resolved by SDS-PAGE followed by western blotting with His-tag primary antibody (1:3,000 dilution) and goat anti-mouse IgG secondary antibody.

2.12. Co-immunoprecipitation

For co-immunoprecipitation experiments, Protein A/G magnetic beads were utilized. To separate the beads from the supernatant, the microcentrifuge tubes containing the beads were placed in a magnetic rack (GenScript, Piscataway, NJ, USA) to pellet the beads along one side of the tube while the supernatant was carefully pipetted out without disturbing the beads.

The 15 µL aliquot of Protein A/G magnetic beads was washed with 175 µL PBS-T (0.05% Tween). The beads were then washed with 500 µL PBS-T while gently vortexing for 1 minute. The supernatant was removed and the beads were incubated in the presence or absence of primary D2 antibody (2.5 µg) in PBS-T for 2 hours at room temperature. Following incubation, the unbound antibody was removed from the beads and 750 µg of HEK-293T cell lysates (homogenized in modified RIPA buffer) were added to the beads and incubated for 2 hours at room temperature. Magnetic beads were then washed three times with PBS-T and eluted with 30 µL of 100 mM glycine (pH 2.8) for 10 minutes at room temperature. After the addition of 3 µL 1 M Tris-HCl (pH 8.0) and SDS sample buffer, the samples were boiled for 5 minutes and subjected to SDS–PAGE on a 10% gel followed by western blotting with CB1 primary antibody (1:500 dilution) and donkey anti-goat IgG secondary antibody.

2.13. Blot overlay

PVDF membrane was activated with methanol, rinsed with distilled water and washed with TBS-T for 10 minutes. 0.5 µg GST fusion protein was blotted onto the PVDF. The blot was dried, re-activated with methanol and rinsed with distilled water. The blot was then overlayed with 20 µg purified HIS-D2LI3 protein at room temperature for 2 hours. Following three washes with TBS-T (5 minutes at room temperature), the blot was incubated with His-tag antibody (diluted 1:3,000) in TBS-T at 4°C, overnight on a rocker. The following day, the blot was washed three times with TBS-T and incubated with goat anti mouse IgG secondary antibody (diluted 1:12,000 in TBS-T with 0.5% milk) for 1 hour at room temperature. Following three washes with TBS-T, the blots were visualized with SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Ottawa, ON) with Dyversity image analysis system and GeneSnap image acquisition software.

2.13.1. Blot overlay using GST-CB1-CT2B as a blocking peptide

The initial step of this experiment was to pre-incubate 50 µg HIS-D2LI3 was with 50 µg GST-CB1-CT2B in 1 mL of PBS, overnight at 4°C. As a control, 50 µg HIS-D2LI3 was pre-incubated with 50 µg GST alone. Following incubation, the mix was added to

TBS-T (final volume of 10 mL with 0.5% milk). GST fusion proteins were blotted onto 2 PVDF membranes, as described above. The PVDF membranes were dried, re-activated with methanol and washed with TBS-T. The two membranes were then overlayed with either HIS-D2LI3 and GST-CB1-CT2B pre-incubated mix or HIS-D2LI3 and GST pre-incubated mix. The remaining steps were as described above. After image capture, the blots were analyzed with GeneTools software (Syngene, Frederick, MD) to determine the level of interaction.

2.13.2. Re-probe with GST antibody

Blots were stripped with strip buffer (200 mM glycine, 0.1% Tween 20, 0.1% SDS, pH 2.4) at room temperature for 30 minutes, washed twice with TBS-T for 15 minutes and blocked with TBS-T with 5% milk at room temperature rocker for 1 hour. The blots were incubated with GST antibody (diluted 1:2,000) in TBS-T at 4°C, overnight. The next day, the blot was washed three times with TBS-T, incubated with goat anti-rabbit IgG secondary antibody (diluted 1:12,000 in TBS-T with 0.5% milk) for 1 hour at room temperature. Following three washes with TBS-T, the blots were visualized as described in the previous section.

2.14. cAMP immunoassay

HEK-293T cells transfected with CB1 and D2L were treated with 1 µM methanandamide or 10 µM bromocriptine or co-treated with both agonists for 15 minutes in serum free DMEM supplemented with 0.5 mM IBMX and 1 µM propanolol at 37°C. Following agonist pre-treatment, 10 uM of forskolin was added and the cells were incubated for 15 minutes at 37°C. The cells were washed with cold PBS and incubated at 4°C in 1X cold lysis buffer (part 895890, cAMP Parameter Assay Kit R&D Systems, Minneapolis, MN, USA) for one hour. The cells were collected and centrifuged at 4°C for 15 minutes at 16,100 x *g*. The supernatant was collected and the cAMP assay was conducted with cAMP Parameter Assay Kit, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, a microplate, coated with goat anti-mouse polyclonal antibody, was incubated with 50 µL of 1:10,000 dilution of monoclonal mouse cAMP antibody in PBS-T (0.05% Tween) at 500 RPM on a microplate shaker for 1 hour

at room temperature. During the incubation, the 2400 pmol/mL cAMP standard was diluted to 240 pmol/mL, 120 pmol/mL, 60 pmol/mL, 30 pmol/mL, 15, pmol/mL, 7.5 pmol/mL and 3.75 pmol/mL standards. Following four washes with PBS-T, 100 µL cAMP standards, lysis buffer and samples were added to appropriate wells of the microplate. To all wells, 50 µL cAMP-HRP (1:10,000 dilution in PBS-T) was added and the plate was incubated at room temperature for 2 hours at 500 RPM on a microplate shaker. After washing four times with PBS-T, 100 µL of substrate solution (3,3', 5,5" tetramethylbenzidine) was added and incubated at room temperature for 5 minutes. The reaction was stopped with 100 µL stop solution and the absorbance was measured at 450 nm - 550 nm using Victor plate reader (PerkinElmer, Woodbridge, ON, Canada). The cAMP values of the samples were calculated based on the absorbance of the cAMP standards.

2.15. Fluorescence resonance energy transfer (FRET)

Fluorescence resonance energy transfer (FRET) experiments were conducted with HEK-293T cells transfected with CB1, D2L, CB1-CT2B mini-gene and epac1-camps. As a control, HEK-293T cells were transfected with CB1, D2L, pcDNA3 and epac1-camps. Epac1-camps vector contains, Epac1 flanked by cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) tags. In the absence of cAMP, the CFP and and YFP tags are in close proximity whereby excitation of CFP results in energy transfer to YFP and positive FRET signal is observed. Forskolin treatment results in adenylate cyclase mediated cAMP production. As shown in **Figure** 4, the binding of cAMP to Epac1 changes the conformation of the complex and disrupts FRET between CFP and YFP tags. Forty eight hours post transfection, cells were washed three times with tyrode buffer (129 mM NaCl, 2.5 mM $CaCl_2$, 5 mM KCl, 3 mM $MgCl_2$, 30 mM glucose, 25 mM Hepes, 2 mM EGTA, 0.1% BSA, pH 7.4) and pre-treated with 50 nM bromocriptine or 5 µM methanandamide or co-treated with both agonists for 15 minutes at room temperature in tyrode buffer. At 2.5 minute mark after the start of the imaging, 25 µM forskolin was added to the cells. The cells were examined using an inverted epifluorescence microscope (Olympus IX81, Richmond Hill, ON, Canada), and images were captured by CoolSNAP HQ2 CCD camera (Photometrics, Tucson, AZ). Images were collected for 20 minutes at 20-second intervals under 40X objective lens using

Metamorph software (Molecular Devices, Sunnyvale, CA). For ratio imaging, a 427/10 excitation filter, a 455-dichroic mirror and two emission filters (427/30 for CFP, 542/37 for YFP) were implemented in a filter wheel (Sutter Instruments, CA, USA). To determine the cAMP levels in a given experiment, 10 individual cells were manually outline and the average CFP/FRET ratio was determined. The CFP/FRET ratio represented cAMP level.

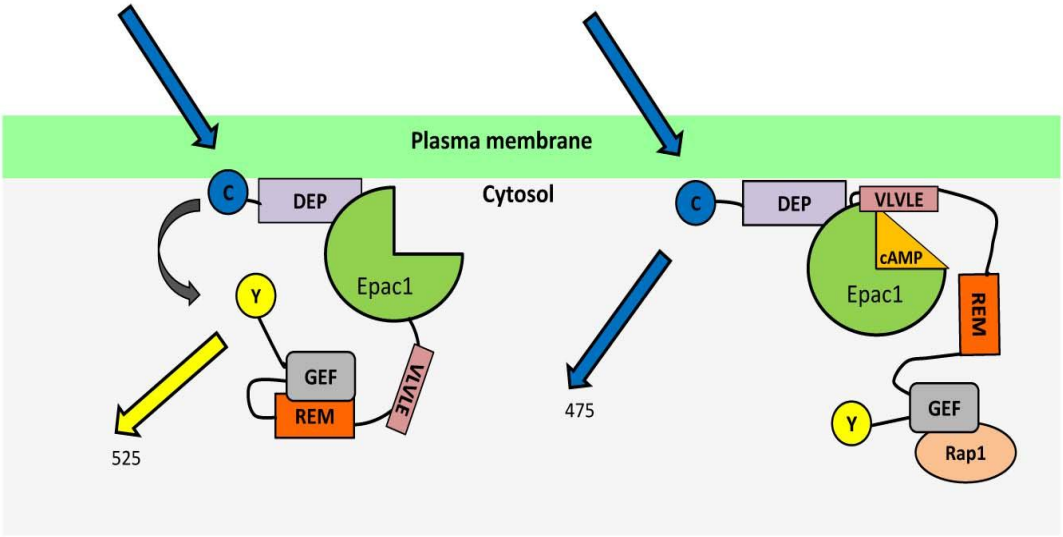

Figure 4. cAMP-induced conformational change in Epac1 detected by FRET

Forskolin treatment of HEK-293T cells transfected with epac1-camps vector results in adenylate cyclase mediated cAMP accumulation. Epac1 (for exchange proteins directly activated by cAMP) is a guanine nucleotide exchange factor (GEF) for Rap1 regulatory proteins. In the inactive state, the GEF domain is inhibited by the Ras exchange motif (REM). Following cAMP binding to the N-terminal regulatory domain of Epac1, the VLVLE sequence binds the regulatory domain of Epac1 which releases REM from the GEF domain and allows Rap1 to bind GEF. The DEP domain which is the membrane localization domain has a CFP (C) tag and the GEF domain contains a YFP (Y) tag. The binding of cAMP to Epac1 changes the conformation of the molecule and disrupts FRET between the CFP and YFP tags. Increase in cAMP accumulation is represented by the increase in FRET ratio (CFP/FRET). Figure adapted from *EMBO*, 2004 5(12):1176.

2.16. Phosphorylation of CREB

HEK-293T cells were transfected with CB1 and D2L, CB1 and pcDNA3 or D2L with pcDNA3. Cells were maintained in DMEM with 10% FBS for 24 hours post transfection and then the media was changed to serum free DMEM. The cells were incubated in serum free DMEM for an additional 24 hours before treating with drugs. Cells were washed with room temperature PBS and treated with drugs diluted in serum free DMEM at 37°C for 30 minutes. CB1/D2L co-transfected cells were treated with 10 μM forskolin, 1 nM bromocriptine, 50 nM methanandamide, or co-treated with both bromocriptine and methanandamide. CB1/pcDNA3 cells were treated with 50 nM methanandamide and cells co-transfected with D2L/pcDNA3 were treated with 1 nM bromocriptine. Following treatment, cells were collected in RIPA DOC (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS), complete protease inhibitor cocktail (Roche, Indianapolis, IN), 1 mM sodium orthovanadate and 1 mM sodium fluoride. Cells were homogenized and mixed at 4°C for 1 hour and centrifuged at 16,100 x *g* for 15 minutes at 4°C, then the lysates were collected and protein was quantified. The samples were prepared with the addition of SDS sample buffer and boiled for 5 minutes. The samples were then resolved on a 10% gel (SDS-PAGE) in triplicate followed by western blotting with pCREB antibody (1:1,000 dilution), CREB antibody (1:1000 dilution) and alpha tubulin (1:200 dilution).

2.17. Statistical analysis

For comparison of more than two groups, GraphPad Prism software (San Diego, CA, USA) was used for one-way ANOVA followed by Student–Newman–Keuls and Tukey's multiple comparison *post hoc* analysis. For comparisons between two groups, *t*-test (two-tailed) was performed. All values are stated as means with standard error of means. The significance level of the values was 0.05.

Table 1. List of oligonucleotide pairs for PCR amplification of CB1 receptor truncations for generating GST fusion proteins

CB1 receptor region	**Oligonucleotide pair**
CB1-IL2	Forward: 5' TACGGATCCATGGACAGGTACATATCCATTCAC 3' Reverse: 5' CATCTCGAGTCACTTGGGCCTGGTGACGATCCT 3'
CB1-IL3	Forward: 5' CATGGATCCATGAAGGCTCACAGCCACGCGGTC 3' Reverse: 5' CATCTCGAGTCAGGTTTTGGCCAGCCTAATGTC 3'
CB1-CT	Forward: 5' GATGGATCCATGAGGAGCAAGGACCTGAGACAT 3' Reverse: 5' CATCTCGAGTCACAGAGCCTCGGCGGACGTGTC 3'
CB1-CT1	Forward: 5' GATGGATCCATGAGGAGCAAGGACCTGAGACAT 3' Reverse: 5' TATCTCGAGTCAGTTGTCTAGAGGCTGTGCGGT 3'
CB1-CT2	Forward: 5' TATGGATCCATGTGCGAAGGCACCGCACAGCCT 3' Reverse: 5' GCGCTCGAGTCATGTGTTGTTGGCGTGCTTGTG 3'
CB1-CT3	Forward: 5' TATGGATCCATGGACTGCCTGCACAAGCACGCC 3' Reverse: 5' GCGCTCGAGTCAAACGGTGCTCTTGATGCAGCT 3'
CB1-CT4	Forward: 5' GATGGATCCATGGCCGCGGAGAGCTGCATCAAG 3' Reverse: 5' TATCTCGAGTCACAGAGCCTCGGCGGACGTGTC 3'
CB1-CT2A	Forward: 5' GATGGATCCATGTGCGAAGGCACCGCACAGCCT 3' Reverse: 5' GGCCTCGAGTCAGTTGTCTAGAGGCTGTGCGGT 3'
CB1-CT2B	Forward: 5' GATGGATCCATGTGCGAAGGCACCGCACAGCCT 3' Reverse: 5' GGCCTCGAGTCATGAGTCCCCCATGCTGTTGTC 3'
CB1-CT4A	Forward: 5' GGCGGATCCATGAAGGTGACCATGTCTGTGTCC 3' Reverse: 5' TATCTCGAGTCACAGAGCCTCGGCGGACGTGTC 3'

Table 2. ***List of oligonucleotide pair for PCR amplification of intracellular loop 3 of the D2L and D2S receptor for generating His-tagged proteins***

D2 receptor region	**Oligonucleotide pair**
Intracellular Loop 3	Forward: 5’ AGAGGATCCATGAAGATCTACATTGTCCTC 3’ Reverse: 5’ TATCTCGAGTTACTGAGTGGCTTTCTTCTC 3’

3. Results

3.1. CB1 and D2L receptor form a complex mediated by the carboxyl tail of CB1 receptor

Previous studies have employed co-immunoprecipitation (co-IP), fluorescence resonance energy transfer (FRET) and multicolor bimolecular fluorescence complementation (MBiFC) techniques to demonstrate the interaction between CB1 and D2 receptor (Kearn, et al., 2005; Marcellino, et al., 2008; Przybyla and Watts, 2010). The first step of the present study was to confirm CB1 and D2L receptor interaction by conducting co-IP experiments. Co-immunoprecipitation is a simple procedure employed to study protein-protein interactions. This technique is commonly used to determine whether two proteins of interest interact. For the present study, HEK-293T cells were co-transfected with CB1 and D2L receptor and the cells were utilized for co-IP experiment 48 hours post transfection. Protein A/G magnetic beads were incubated with 2.5 µg D2 receptor antibody and 750 µg of cell lysates solubilized from HEK-293T cells co-transfected with CB1 and D2L receptor. The co-IP was assayed with SDS-PAGE and western blotting with CB1 receptor antibody. As depicted in **Figure 5**, immunoprecipitation of the D2L receptor, using cell lysates from CB1-D2L over-expressing HEK-293T cells, results in co-immunoprecipitation of CB1 receptor which is approximately 43 kDa in size; confirming presence of CB1 and D2L receptor presence in the same complex. Both the 43 kDa monomer and 95 kDa dimer forms of D2L receptor are present in the lysates (input) used in the co-IP experiments. The results of the western blot clearly indicate that there is an interaction between the CB1 and D2L receptor; however the nature and specific domains of the receptor mediating the interaction remained unclear.

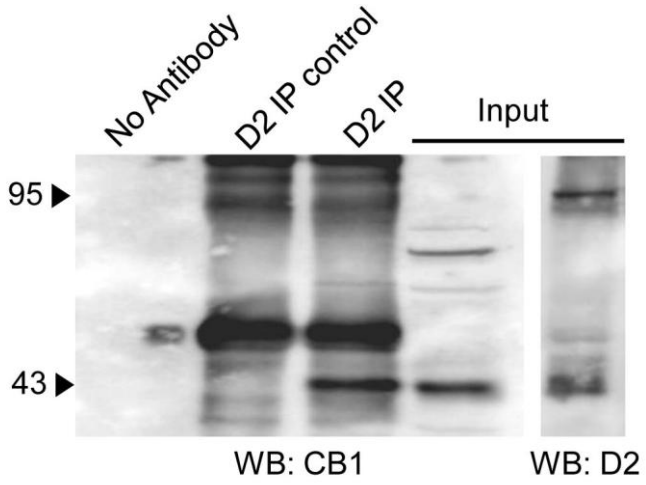

Figure 5. Identification of an interaction between CB1 and D2L receptor

Immunoprecipitation (IP) of the D2L receptor using 2.5 µg of the D2R antibody leads to the co-precipitation of CB1 receptor from 750 µg of HEK-293T cell lysates co-expressing D2L and CB1 receptors. Controls included IP with lysates in the absence of D2R antibody (No antibody) and IP in the absence of lysates (D2 IP control). Gels were loaded with 50 µg cell lysates (Input) to confirm CB1 and D2L receptor expression. D2R western blot shows 95 kDa and 43 kDa bands which represent the dimeric and monomeric forms of D2L receptor, respectively. This is a representative blot of 3 independent experiments.

The next goal of the study was to identify specific domains of the CB1 receptor involved in mediating the interaction between CB1 and D2L receptor. The intracellular domains of the CB1 receptor interact with other proteins to mediate many functional aspects such as signaling, membrane trafficking and receptor internalization (Jin, et al., 1999; Mukhopadhyay, et al., 2000; Nie and Lewis, 2001; Azzi, et al., 2003). Therefore, to determine the regions of CB1 involved with D2R, intracellular domains of the CB1 receptor were PCR amplified and cloned into pGEX-4T3 vector to generate glutathione-S-transferase (GST) fusion proteins.

Glutathione-S-transferase fusion proteins are efficiently purified from *E.coli* with high level of protein expression. Purified GST fusion proteins are used in various biochemical analyses such as studying protein-protein interactions (Sambrook and Russell, 2006). To determine the region within the CB1 receptor that mediates the CB1-D2L receptor interaction, intracellular loop 2 (CB1-IL2 [G214-K234]), intracellular loop 3 (CB1-IL3 [K302-T346]) and carboxyl tail (CB1-CT [R402-L474]) of the CB1 receptor, depicted in **Figure 6B**, were PCR amplified from full-length rat CB1 cDNA. Amplified DNA, shown in **Figure 6C**, was cloned into BamHI and XhoI sites of the pGEXT-4T3 plasmid. After confirmation of sequence and orientation, plasmids were transformed into BL21 *E.coli* cells. The purified GST fusion proteins, GST-CB1-IL2, GST-CB1-IL3 and GST-CB1-CT, shown in **Figure 6D**, were utilized in affinity purification assays.

To assess whether GST fusion proteins interact with D2L receptor, HEK-293T cells over-expressing D2L receptor were solubilized and 500 µg of the cell lysates were first incubated with 40 µg of purified GST fusion proteins, followed by the addition of 50 µL 50% glutathione agarose beads. The beads were washed, boiled and the eluates were subjected to SDS-PAGE and immunoblotted with D2 receptor antibody. As demonstrated in the western blot in **Figure 6E**, GST-CB1-CT but not GST-CB1-IL2, GST-CB1-IL3 or GST alone precipitated D2L receptor from HEK-293T cell lysates expressing D2L receptor. These results suggest that the interaction between the CB1 and D2L receptor is mediated by the carboxyl tail (CT) region of the CB1 receptor. The blot was stained with Ponceau S to reveal the relative amounts of GST fusion proteins employed for the pull-downs.

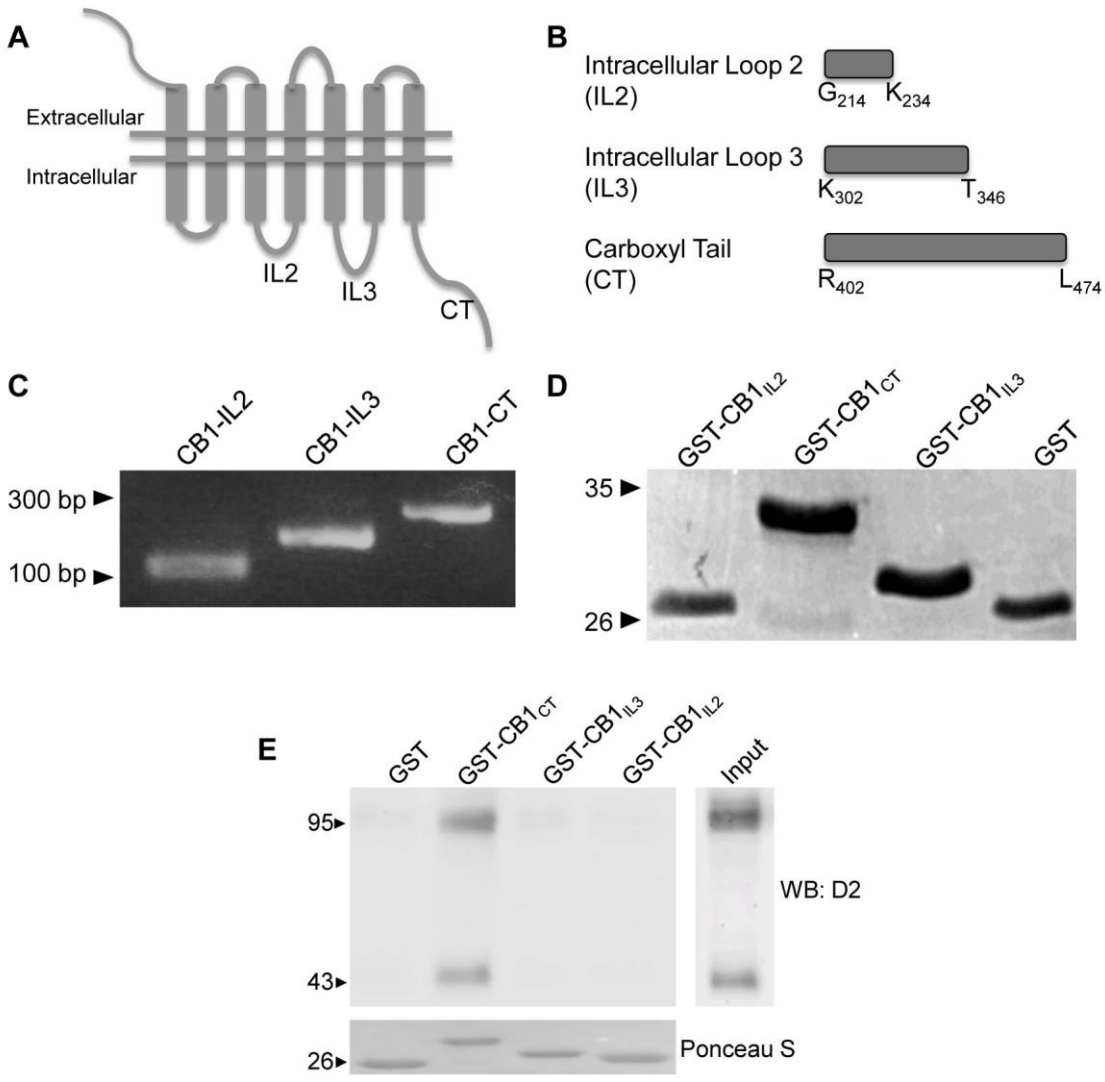

Figure 6. Affinity purification of D2L receptor by carboxyl tail region of CB1 receptor

(A) Structure of CB1 receptor. The second and third intracellular loops (CB1-IL2 [G214-K234] and CB1-IL3 [K302-T346]) and the carboxyl tail (CB1-CT [R402-L474]) of the CB1 receptor shown in **(B)** were PCR amplified and resolved on an agarose gel. Sizes shown are DNA base pairs (bp) **(C)**. PCR amplified regions were cloned into pGEX-4T3 vector and transformed into BL21 cells to generate purified GST fusion proteins. Purified GST-CB1-1L2, GST-CB1-1L3 and GST-CB1-CT proteins were subjected to SDS-PAGE and gels were Coomassie stained shown in **(D)** (Sizes indicated in kDa). **(E)** Affinity purification assays were conducted with 500 μg cells lysates solubilized from HEK-293T cells expressing D2L receptor and 40 μg GST fusion protein. Representative western blot (of 5 independent experiments) shows GST-CB1-CT was able to affinity purify D2L receptor. 30 μg of D2L protein extracts were loaded as input. 95 kDa and 43 kDa bands represent the dimeric and monomeric forms of D2L receptor, respectively.

The carboxyl tail domain of the CB1 receptor consists of 73 amino acid residues. To further delineate the interacting region within the carboxyl tail, the domain was divided into three truncations of 25 amino acids, CB1-CT1 [R402-N426], CB1-CT2 [C417-T441], CB1-CT3 [D432-V456] and a fourth truncation of 28 amino acids, CB1-CT4 [A447-L474] **(Figure 7A)**. Each of these regions has a 10 amino acid overlap with the adjacent region to ensure that the interaction domain was detected. The four truncations of the carboxyl tail of the CB1 receptor were PCR amplified **(Figure 7B)** and sub-cloned into BamHI and XhoI sites of the pGEXT-4T3 plasmid. Correct plasmids were transformed into BL21 *E.coli* cells and GST fusion proteins were purified **(Figure 7C)**. The purified GST fusion proteins, GST-CB1-CT1, GST-CB1-CT2, GST-CB1-CT3 and GST-CB1-CT4 (50 µg) were incubated with 750 µg cell lysates solubilised from HEK-293T cells expressing D2L receptor to allow the complex formation; followed by addition of 50 µL 50% glutathione agarose beads. The beads were washed to remove any non-specific binding and boiled with SDS sample buffer. The eluates were resolved by SDS-PAGE followed by western blotting with D2 receptor antibody. The blots were stained with Ponceau S to visualize the amounts of GST fusion proteins in each pull-down assay. The results of the affinity purification assay, depicted in **Figure 7D**, indicate that GST-CB1-CT1 and GST-CB1-CT2 but not GST-CB1-CT3, GST-CB1-CT4 or GST alone precipitated D2L receptor from solubilised HEK-293T cell lysates expressing D2L receptor. Both CB1-CT1 and CB1-CT2 are 25 amino acids in length which have an overlap of 10 amino acids. Since both GST-CB1-CT1 and GST-CB1-CT2 were able to precipitate D2L receptor shown in **Figure 7D**, it was predicted that interacting region was within the overlap regions between CB1-CT1 and CB1-CT2.

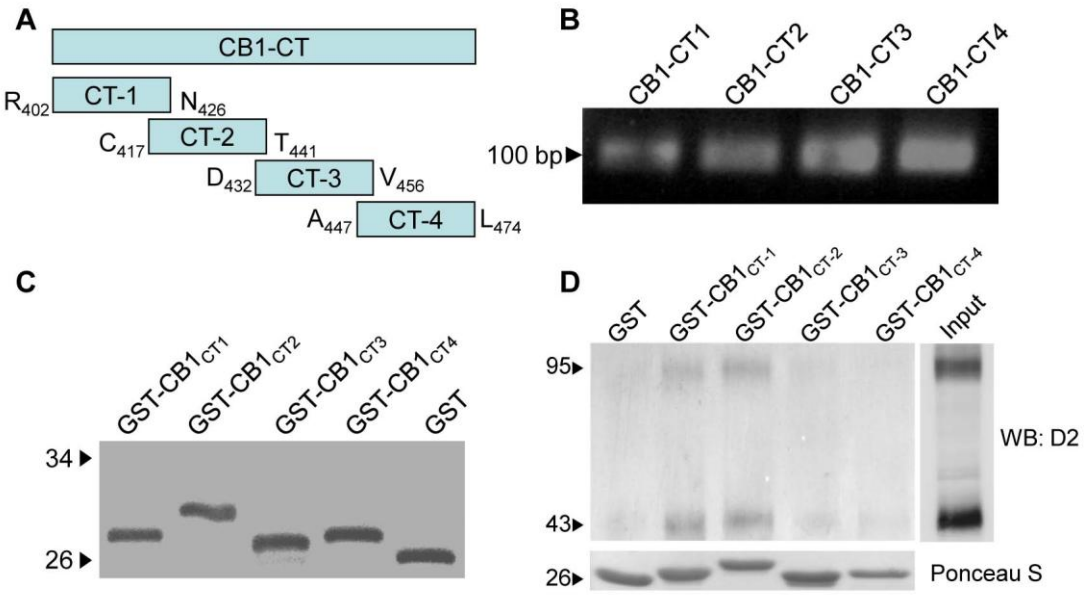

Figure 7. Delineation of the interaction domain within the CT region of CB1 receptor

Truncations of the CT region of the CB1 receptor (CB1-CT1 [R402-N426], CB1-CT2 [C417-T441], CB1-CT3 [D432-V456], CB1-CT4 [A447-L474]) shown in **(A)** were PCR amplified and resolved on an agarose gel to determine correct size in DNA base pairs (bp) **(B)**. PCR amplified regions were cloned into pGEX-4T3 vector to generate GST fusion proteins. **(C)** Coomassie gel showing the purified GST fusion proteins utilized in affinity purification assay with lysates from D2L transfected HEK 293T cells. Size indicated in kDa. **(D)** Representative D2L receptor western blot (experiment was repeated 4 times) identifying the region within the CB1-CT that interacts with D2L receptor. 750 µg of D2L lysates and 50 µg of GST fusion proteins were used in affinity purification assay. 50 µg of D2L protein extracts were loaded as input. 95 and 43 kDa bands present the dimeric and monomeric forms of D2L receptor, respectively.

To determine if the overlap region was involved in mediating the interaction between CB1 and D2L receptor, GST fusion proteins were created encoding the 10 amino acid overlap region of CB1-CT1 and CB1-CT2 (GST-CB1-CT2A [AC417-N426]) and the first 15 amino acids of CB1-CT2 (GST-CB1-CT2B [C417-S431]) which includes the overlap region with an additional 5 amino acids of the CB1-CT2 domain. Since the interaction domain within the CT was identified within the first two truncations, CB1-CT1 and CB1-CT2, GST fusion protein of the last 15 amino acids of the CT4 region (GST-CB1-CT4 [AK460-L474)] was also generated as a negative control **(Figure 8A)**. To generate the GST fusion proteins, CB1-CT2A, CB1-CT2B, CB1-CT4A regions were PCR amplified **(Figure 8B)** and cloned into BamHI and XhoI restriction sites of the pGEX-4T3 vector and correct plasmids were transformed into BL21 *E.coli* cells. GST fusion proteins were purified **(Figure 8C)** and 15 µg the purified protein was utilized in affinity purification assay with 500 µg protein extracts from D2L transfected HEK-293T cells and 25 µL of 50% glutathione agarose beads. After washes, the beads were boiled with SDS sample buffer. The eluted samples were subjected to SDS-PAGE and western blotting with D2 receptor antibody. **Figure 8D** demonstrates that GST-CB1-CT2B but not GST-CB1-CT2A, GST-CB1-CT4A or GST alone was able to affinity purify D2L. These results indicate that the CB1-CT2B region within the CB1 receptor plays a role in mediating the CB1-D2L receptor interaction. Following visualization of the western blots, Ponceau S was added to the blot to reveal the relative amounts of GST protein used in each pull-down.

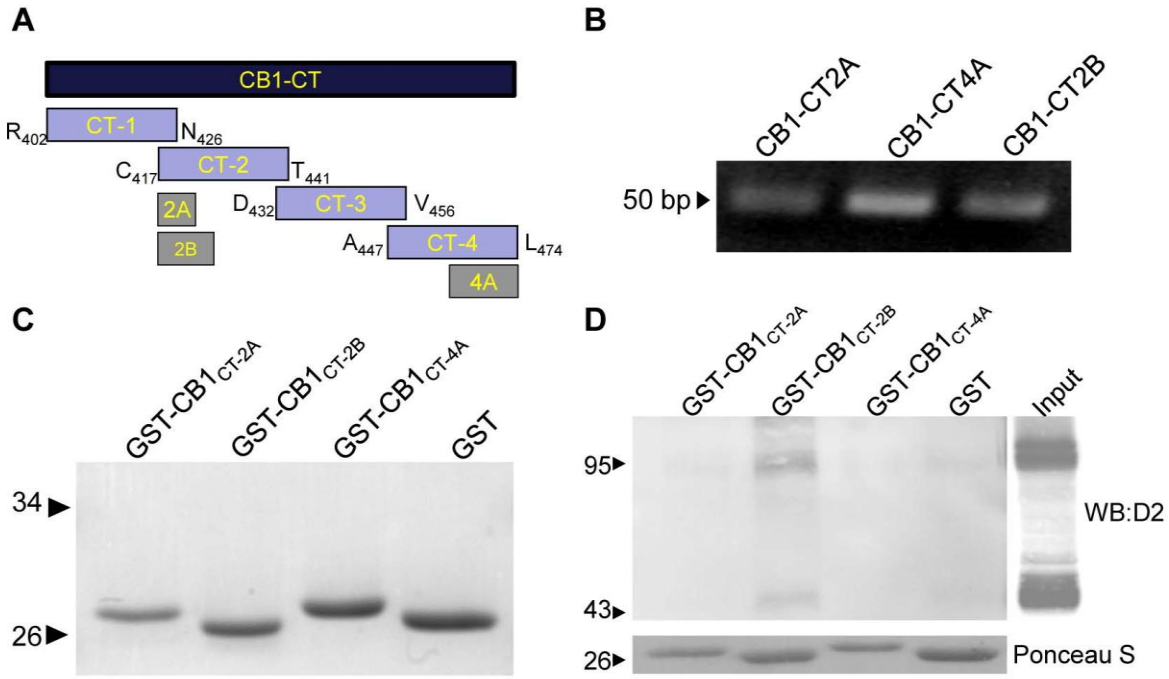

Figure 8. Delineation of the interaction domain within the CT1 and CT2 region of CB1 receptor

To further delineate the interaction region, GST fusion proteins truncated of the 10 amino acid overlap region of CB1-CT1 and CB1-CT2 (CB1-CT2A [C417-N426]); the first 15 amino acids of CB1-CT2 (CB1-CT2B [C417-S431]); and the last 15 amino acids of the CT4 region (CB1-CT4A [K460-L474]) shown in (**A**) were PCR amplified and resolved on an agarose gel to determine correct size in DNA base pairs (bp) **(B)**. PCR amplified regions were cloned into pGEX-4T3 vector to generate GST fusion proteins. **(C)** Coomassie gel showing the purified GST fusion proteins utilized in affinity purification assay with lysates from D2L transfected HEK-293T cells. Size indicated in kDa. **(D)** D2 receptor western blot identifying the CB1-CT2B region that interacts with D2L receptor. 500 µg of protein extracts from HEK293T cells transfected with D2L receptor and 15 µg of GST fusion proteins were used in affinity purification assay. 50 µg of D2L protein extracts were loaded as input. 95 kDa and 43 kDa bands present the dimeric and monomeric forms of D2L receptor, respectively. Immunoblot is representative of 4 independent experiments.

3.2. Direct protein-protein interaction between CB1 and D2R mediated by third intracellular loop of D2R

The affinity purification assays conducted thus far have identified CB1-CT2B region of the carboxyl tail of CB1 as a mediator of the interaction observed between CB1 and D2 receptor. Since the carboxyl tail of CB1 is found intracellularly, it was predicted that the interacting region of the D2 receptor is likely an intracellular domain. The D2 receptor contains three intracellular loops and a cytosolic carboxyl terminus, similar to CB1 receptor. Prior studies have demonstrated that protein interactions with the dopamine D2 receptor are mediated by the third intracellular loop of D2R. G protein-coupled receptor interactions with D2R including the adenosine A2A receptor, serotonin receptor 5-HT(2A) and the dopamine D1 receptor are modulated by the third intracellular loop of the dopamine D2 receptor (Lukasiewicz, et al., 2009; Lukasiewicz, et al., 2010; Fernandez-Duenas, et al., 2012). In addition to GPCRs, various dopamine receptor interacting proteins (DRIPs) interact with D2R. Many of these DRIPs such as the dopamine transporter, calmodulin, Ca^{2+} binding protein S100B, actin-binding protein 280 (ABP-280) and protein kinase C- ζ interacting protein (ZIP) bind the third intracellular loop of D2R (Lee, et al., 2007; Bofill-Cardona, et al., 2000; Stanwood, 2008; Li, et al., 2000; Kim, et al., 2008). With these considerations, the predicted region of D2R mediating the interaction with CB1 was the third intracellular loop, the largest intracellular domain of the dopamine D2 receptor (**Figure 9A**). D2 receptor expression has a long and short isoform and intracellular loop 3 (IL3) of D2Long (D2L) has an addition of 29 amino acids, alternative splice (AS) region, which is not present in the IL3 of D2short (D2S) isoform (Missale, et al., 1998) (**Figure 9B**). The third intracellular loop regions of the D2S and D2L receptor were PCR amplified from full length D2S and D2L cDNA, respectively, using oligos with BamHI and XhoI cut sites. The amplified DNA, presented in **Figure 9C**, was sub-cloned into pET28a(+) vector to generate His-tagged proteins. His-tagged recombinant proteins are efficiently purified using pET28a(+) expression vector and BL21 *E.coli* cells for protein expression. Following purification, HIS-D2LI3 (I211-Q373 and HIS-D2SI3 (I211-Q344) were resolved with SDS-PAGE and subjected to western blotting with His-tag antibody **(Figure 9D)**.

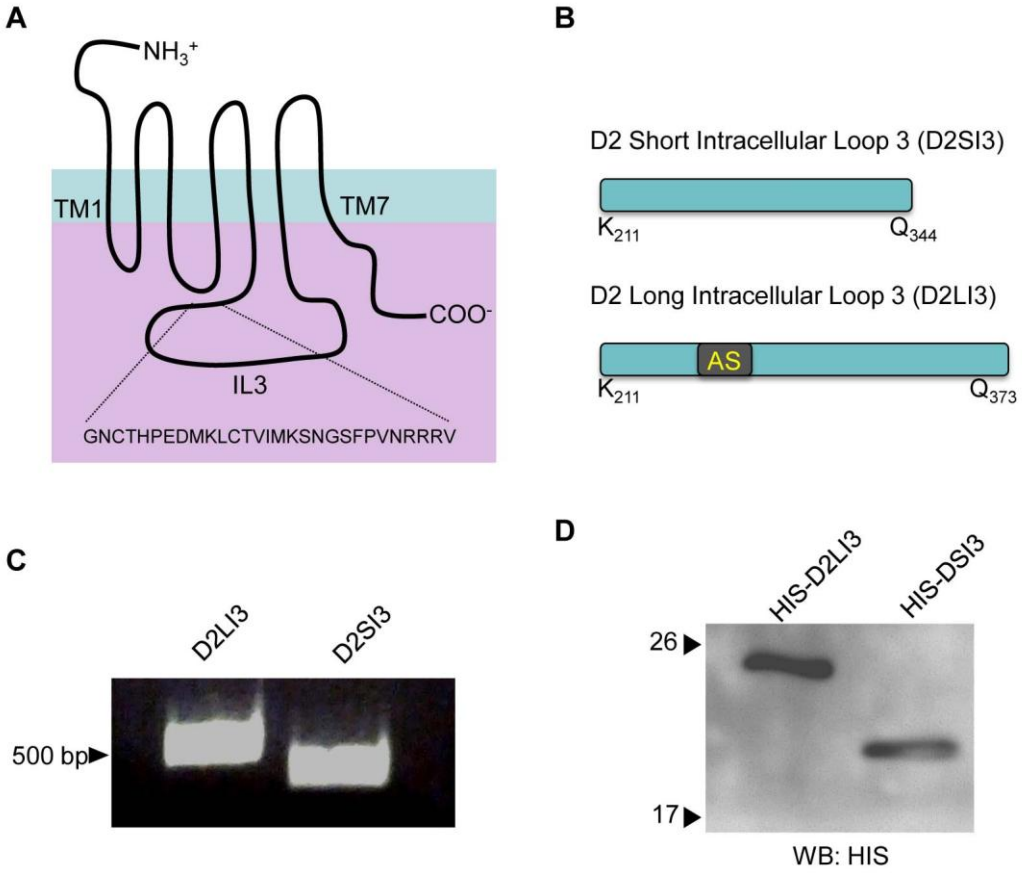

Figure 9. Generation of His-tagged protein of intracellular loop three of D2 receptor

(A) Protein structure of D2 receptor. Intracellular loop three of D2 long (D2L) and D2 short (D2S) isoform of the D2 receptor, which differ by 29 amino acids shown as alternate splice (AS) region in **(B)** were PCR amplified and resolved on an agarose gel to determine correct size in DNA base pairs (bp) **(C)**. PCR amplified regions were cloned into pET-28a (+) vector to generate His-tagged proteins, HIS-D2LI3 and HIS-D2SI3. **(D)** His-tag antibody western blot of purified HIS-D2LI3 and HIS-D2SI3. Sizes are indicated in kDa.

The purified His-tagged proteins were utilized in affinity purification assays. For *in vitro* affinity purification experiments using purified His-tagged protein as bait, 0.5 μg of purified GST-CB1-CT was utilized with 10 μg of HIS-D2LI3 and 10 μg HIS-D2SI3 with 5 μL of equilibrated HIS-select nickel affinity gel. Upon completion of pull-down assay, the HIS-select nickel affinity gel was boiled and the eluate was subjected to SDS-PAGE and western blotting with GST primary antibody. The results indicate that both HIS-D2LI3 and HIS-D2SI3 were able to pull-down GST-CB1-CT (**Figure 10A**). Additionally, the His-tagged proteins did not interact with GST alone. The blots were stripped and re-probed with His-tag antibody to determine relative amounts of HIS-D2LI3 and HIS-D2SI3 prtoeins used in the assay. The His-tag western blot in **Figure 10A** indicates less HIS-D2SI3 compared to HIS-D2LI3, however, both were able to affinity purify GST-CB1-CT. These results suggest that the interaction domain of the third intracellular loop of D2 receptor is in a region which is common to both D2LI3 and D2SI3 and not the 29 amino acid, alternative splice region of D2LI3. Therefore, subsequent experiments were carried out using only purified HIS-D2LI3 protein. To further demonstrate interaction between the identified regions, the assay was also conducted in reverse where purified GST fusion protein was used as bait. For this experiment, 1 μg of purified His-tagged protein and 20 μg purified GST-CB1-CT, GST-CB1-IL2, GST-CB1-IL3 fusion proteins were implemented with 10 μL of 50% glutathione agarose beads in PBS for affinity purification. Western blot analysis of the eluates from the glutathione agarose beads indicate that only GST-CB1-CT but not GST-CB1-IL2, GST-CB1-IL3 or GST alone are able to affinity purify HIS-D2LI3 (**Figure 10B**). Furthermore, the pull-down assays were also conducted with purified GST-CB1-CT2A, GST-CB1-CT2B and GST-CB1-CT4A and HIS-D2LI3. The western blot in the lower panel in **Figure 10B** indicates that HIS-D2LI3 has greater affinity for GST-CB1-CT2B compared to GST-CB1-CT2A, GST-CB1-CT4A or GST alone.

Together, these experiments provide evidence that the interaction between CB1 and D2L receptor is mediated by the carboxyl tail region of the CB1 receptor and the third intracellular region of the D2L receptor. Since these experiments were conducted *in vitro* with purified His-tagged proteins of the D2 receptor and GST fusion proteins of the CB1 receptor, the results suggest that the interaction between CB1 and D2L receptor can potentially be mediated by a direct protein-protein interaction.

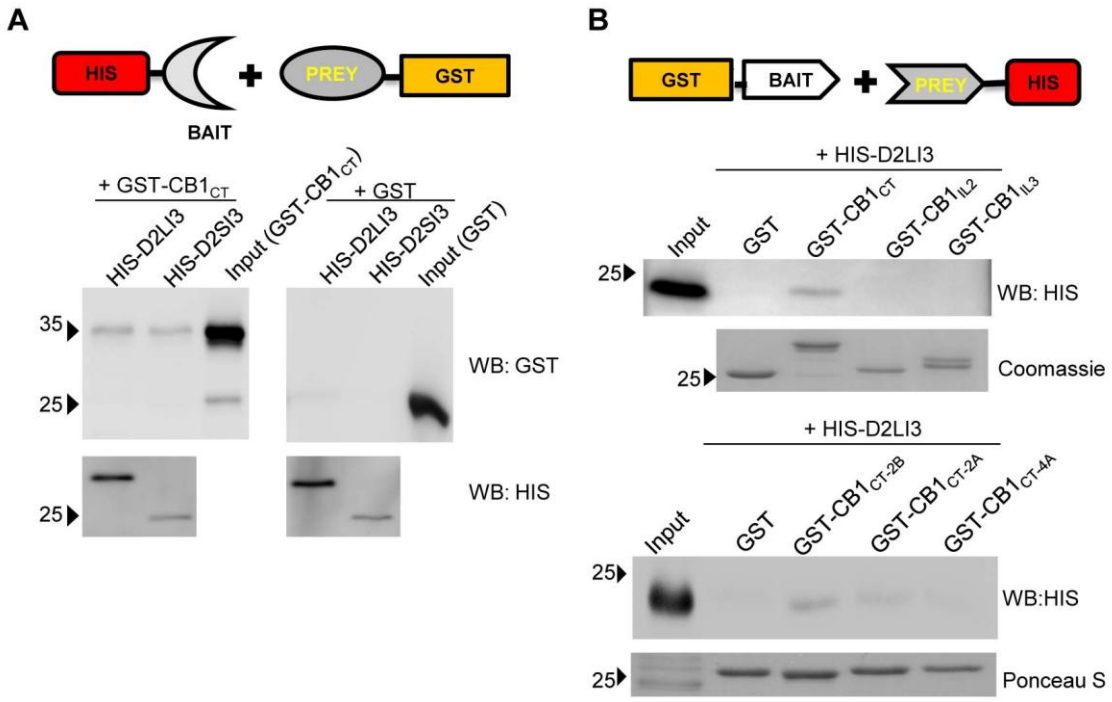

Figure 10. Intracellular loop 3 of the D2 receptor interacts with carboxyl terminus of CB1 receptor

(**A**) GST antibody western blot showing CT region of the CB1 receptor interacts with the third intracellular loop of the D2L and D2S receptor. 0.5 μg of GST fusion protein and 10 μg His-tagged protein were used in the affinity purification assay. 0.5 μg of GST fusion protein was loaded as input. **(B)** His-tag antibody western blot showing CT region of the CB1 receptor interacts with the intracellular loop 3 of the D2L receptor (D2LI3). 20 μg of GST fusion protein and 1 μg HIS-D2LI3 fusion protein were used in the affinity purification assay. 0.5 μg of HIS-D2LI3 fusion protein was loaded as input. His-tag antibody western blot showing CT-2B region of the CB1 receptor interacts with the third intracellular loop of the D2L receptor. 25 μg of GST fusion protein and 20 μg HIS-D2LI3 fusion protein were used in the affinity purification assay. 3 μg of HIS-D2LI3 fusion protein was loaded as input. All sizes indicated are in kDa.

3.3. Disruption of CB1 and D2L receptor interaction

3.3.1. Blot overlay with GST-CB1-CT2B

The results of the study thus far have identified regions within the CB1 and D2 receptor that mediate the formation of the heterodimer complex. In addition, *in vitro* assays using GST fusion proteins and His-tagged proteins demonstrate that the interaction between CB1 and D2L receptor is potentially mediated by a direct protein-protein interaction. To further verify that the heteromer of CB1 and D2L was modulated by a direct protein-protein interaction as well as investigate the potential of CB1-CT2B peptide to disrupt the interaction, blot overlay experiments were conducted. Blot overlay methods can be utilized to detect protein-protein interactions. There are various ways of conducting blot overlay experiment studies. For this study, the GST fusion prey proteins were blotted onto PVDF and probed with His-tagged bait protein. The His-tagged protein interacts with GST fusion protein which is detected by a His-tag primary antibody and peroxidase conjugated secondary antibody, followed by visualization with chemiluminescent substrate.

For the blot overlay experiments, 0.5 µg purified GST fusion proteins, GST-CB1-CT, GST-CB1-IL2 and GST alone were blotted onto PVDF membrane which prepared by activating with methanol. The membrane was allowed to dry and after re-activation with methanol, the membrane was incubated with 20 µg HIS-D2LI3. After incubation to allow the bait and prey to interact, unbound HIS-D2LI3 was removed and the membrane was incubated with His-tagged antibody followed by incubation with secondary antibody. Visualization of the blot revealed that HIS-D2LI3 was able to bind GST-CB1-CT with greater affinity than with GST-CB1-IL2 or GST alone **(Figure 11A)**. The PVDF membrane was stripped and the re-probed with GST antibody to visualize the amounts of GST fusion proteins blotted **(Figure 11A)**.

The next step of the study was to explore the potential to disrupt the CB1-D2L receptor interaction using the blot overlay technique. First, two PVDF membranes were blotted with 2 µg of purified GST fusion proteins: GST-CB1-CT and GST alone. Next, 50 µg HIS-D2LI3 was pre-incubated with 50 µg of GST-CB1-CT2B before addition to the PVDF membrane. As a control, HIS-D2LI3 was pre-incubated with GST alone and then

added to second PVDF membrane blotted with the GST fusion proteins. The pre-incubation allowed GST-CB1-CT2B to bind HIS-D2LI3 and thus prevented HIS-D2LI3 from interacting with the blotted GST-CB1-CT on the PVDF membrane. The blot shown in **Figure 11B** demonstrates that the pre-incubation of HIS-D2LI3 with GST-CB1-CT2B decreases the level of interaction of HIS-D2LI3 with GST-CB1-CT compared to HIS-D2LI3 pre-incubated with GST alone. With the presence of GST-CB1-CT2B, the interaction between HIS-D2LI3 and GST-CB1-CT was decreased to 62.9% of the control (*t*-test, $^{*}P<0.001$, $n = 3$) **(Figure 11C)**. The blots were stripped and probed with GST antibody to show the relative amounts of GST fusion proteins blotted **(Figure 11B)**. The outcomes of this experiment suggest that GST-CB1-CT2B forms a complex with HIS-D2LI3 and thus prevents HIS-D2LI3 from binding to the blotted GST-CB1-CT protein. The blot overlay experiments verify the regions of interaction as well as further demonstrate that interaction is a direct protein-protein interaction.

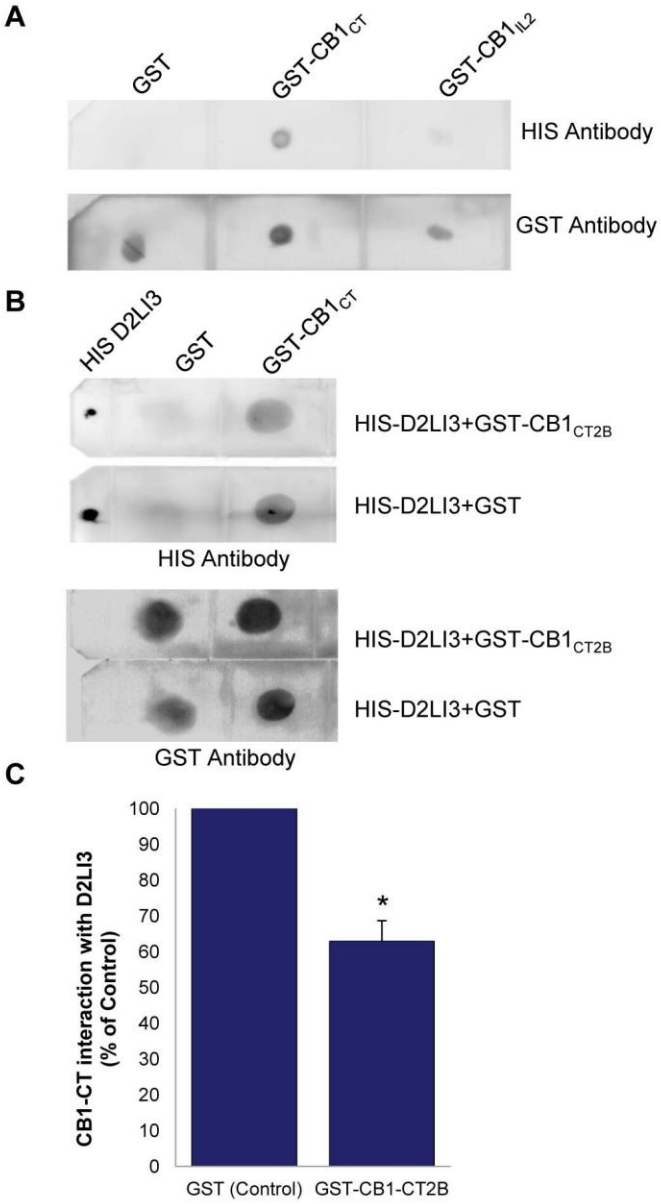

Figure 11. Direct protein-protein interaction between CB1-CT and D2LI3 is disrupted by CT2B region of CB1 receptor

(A) 0.5 μg of GST-CB1-1L2, GST-CB1-CT and GST alone were blotted and overlayed with 20 μg HIS-D2LI3 protein followed by His-tag antibody incubation. Blots were stripped and re-probed with GST antibody. **(B)** 2 μg of GST-CB1-CT and GST were blotted and overlayed with either 50 μg HIS-D2LI3 and 50 μg GST-CB1-CT2B pre-incubated mix or 50 μg HIS-D2LI3 and 50 μg GST pre-incubated mix (control), followed by His-tag antibody incubation. 0.5μg of HIS-D2LI3 was blotted as control. Blots were then stripped and re-probed with GST antibody. The pre-incubation of HIS-D2LI3 with GST-CB1-CT2B decreased the interaction between HIS-D2LI3 and the blotted GST-CB1-CT to 62.9% of the control where HIS-D2LI3 was pre-incubated with GST alone (*t*-test, $^{*}P<0.001$, $n = 3$) **(C)**.

3.3.2. Co-IP with mini-gene encoding CB1-CT2B

The blot overlay experiments show *in vitro* disruption of the interaction between HIS-D2LI3 and GST-CB1-CT using GST-CB1-CT2B as a blocking peptide. The next step of the study was to investigate whether the CB1-CT2B region of CB1 was able to block the interaction of CB1 and D2L receptors in HEK-293T cells. To this end, mini-gene encoding the CB1-CT2B region was generated and HEK-293T cells were co-transfected with the mini-gene, CB1 and D2L cDNA to conduct co-immunoprecipitation experiments.

To generate the mini-gene, the CB1-CT2B region was PCR amplified from full-length rat CB1 cDNA and sub-cloned into the BamHI and XhoI cloning site of pcDNA3 plasmid. The mini-gene plasmid was then co-transfected with CB1 and D2L receptor into HEK-293T cells. As a control, HEK-293T cells were co-transfected with CB1, D2L receptor and pcDNA3 empty expression vector. Transfected cells were collected 48 hours post transfection and solubilised. For the co-immunoprecipitation experiment, 2.5 µg of D2 receptor antibody was utilized with 15 µL Protein A/G magnetic beads and 750 µg of lysates from HEK-293T cells. The co-IP was assessed by SDS-PAGE and western blotting with CB1 receptor antibody.

Immunoprecipitation of D2L receptor leads to the co-IP of CB1 receptor when cells are co-expressing CB1, D2L and pcDNA3, as depicted in **Figure 12A**. However, the interaction between CB1 and D2L receptor is significantly decreased when cells are co-expressing CB1, D2L and the mini-gene encoding the CB1-CT2B region of the CB1 receptor. Immunoprecipitation (IP) with lysates in the absence of D2R antibody was used as IP control. In addition, 2.5 µg D2R antibody was utilized in the absence of lysates as D2R IP control. The blots were also probed with mouse IgG antibody to show that equivalent amounts of D2 receptor antibody were employed in each IP reaction. Lysates shown in the western blot in **Figure 12B** demonstrate that differences in the co-IP results were not due to variations in the expression of the D2L and CB1 receptor. Quantification of the co-IP western blots of three independent experiments demonstrate that the presence of the CB1-CT2B mini-gene decreased the interaction between CB1 and D2L to 55% of the control (*t*-test, $^{*}P<0.01$, $n = 3$) **(Figure 12C)**. These results indicate that the interaction between CB1 and D2L receptor can be inhibited with the presence of mini-gene encoding CB1-CT2B region of the CB1 receptor. Furthermore,

these outcomes also indicate that CB1 and D2L receptor interaction is mediated by a direct protein-protein interaction.

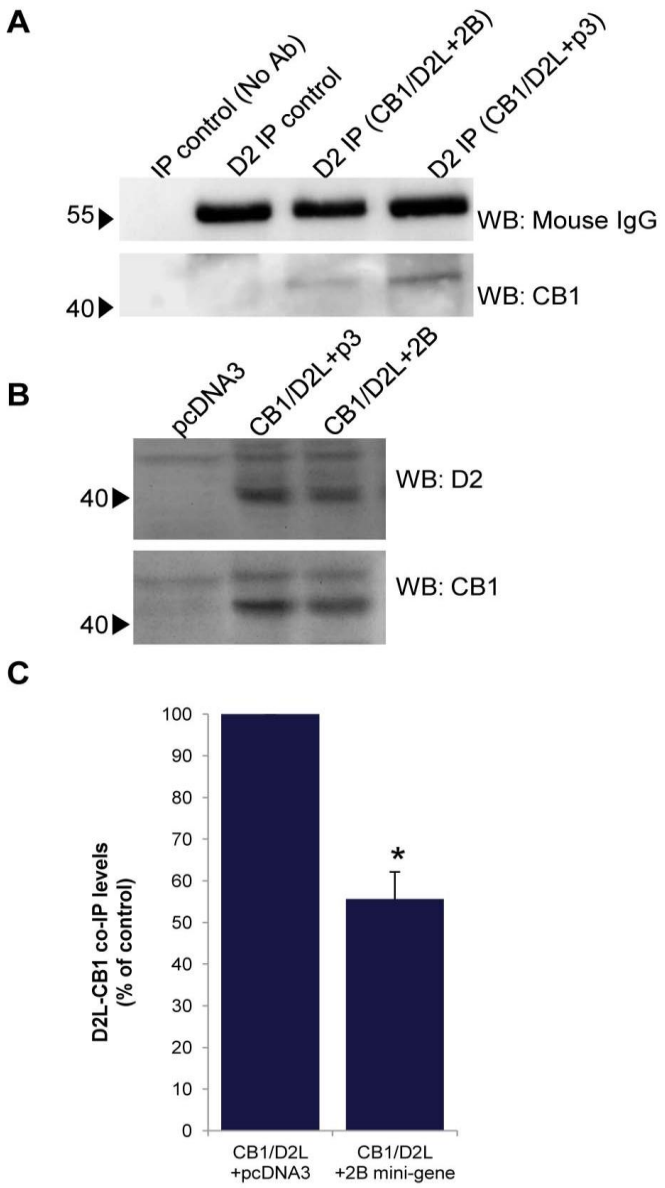

Figure 12. Disruption of co-IP interaction between CB1 and D2L receptor

(A) Immunoprecipitation (IP) of the D2L receptor leads to the co-precipitation of CB1 receptor from 750 μg of HEK-293T cell lysates co-transfected with CB1, D2L and pcDNA3 (CB1/D2L+p3). The interaction is disrupted when cells are co-transfected with CB1, D2L and CB1-CT2B mini-gene encoding the 2B region of the carboxyl tail of the CB1 receptor (CB1-CT2B [C417-S431]) (CB1/D2L+2B mini-gene). IP with lysates in the absence of D2R antibody was used as IP control (No Ab). D2R antibody (2.5μg) in the absence of lysates was used as D2 IP control. Following CB1 western blot, blots were re-probed with goat anti-mouse antibody. **(B)** Western blot of cell lysates used in IP experiment to confirm CB1 and D2 receptor expression. Gel was loaded with 50 μg of cell lysates. **(C)** Interaction between the CB1 and D2L receptors was decreased to 55% of control (CB1/D2L+pcDNA3) with the presence of CB1-CT2B mini-gene (*t*-test, $^{*}P<0.01$, $n = 3$).

3.4. Co-activation of CB1 and D2L receptor increases cAMP accumulation

The activation of either CB1 or D2L receptor when expressed alone and not in a complex result in inhibition of adenylate cyclase and therefore cAMP production is inhibited (Howlett, et al., 1986; Missale, et al., 1998). However, previous studies, with striatal cultures and transfected HEK-293 cells show that co-activation of CB1 and D2L receptor results in accumulation of cAMP which is not observed with activation of the either CB1 or D2L receptor alone (Glass and Felder, 1997; Jarrahian, et al., 2004; Kearn, et al., 2005) .

To explore the effects of D2L and CB1 receptor interaction on cAMP signaling, HEK-293T cells were co-transfected with CB1 and D2L receptor and cAMP levels were assessed following activation with CB1 and D2L receptor agonists, methanandamide and bromocriptine, respectively. Cells were treated with forskolin which activates adenylate cyclase activity to increase cAMP levels. The activation of either CB1 or D2L with their respective agonists will lead to a decrease in forskolin stimulated cAMP accumulation since both receptors are linked to $G_{\alpha i}$. Forty eight hours post-transfection, cells were either treated with individual agonist, 1 μM methanandamide or 10 μM bromocriptine, or co-treated with both agonists for 30 minutes at 37°C. Following agonist treatments, 10 μM forskolin was added and the cells were returned to 37°C for 15 minutes. After treatments, the cells were collected in lysis buffer and cAMP levels were assessed with cAMP immunoassay.

Analysis of compiled experiments show that treatment of HEK-293T cells co-transfected with CB1 and D2L receptor with either methanandamide or bromocriptine in the presence of forskolin resulted in a significant decrease of cAMP (*$p<0.05$ vs forskolin; one-way ANOVA with post-hoc SNK analysis, $n = 4$). However, co-treatment with both agonists did not decrease forskolin stimulated cAMP accumulation **(Figure 13)**. Cells treated with individual agonist or co-treated with both agonists in the absence of forskolin showed little change in cAMP accumulation compared to vehicle treated control cells **(Figure 13)**.

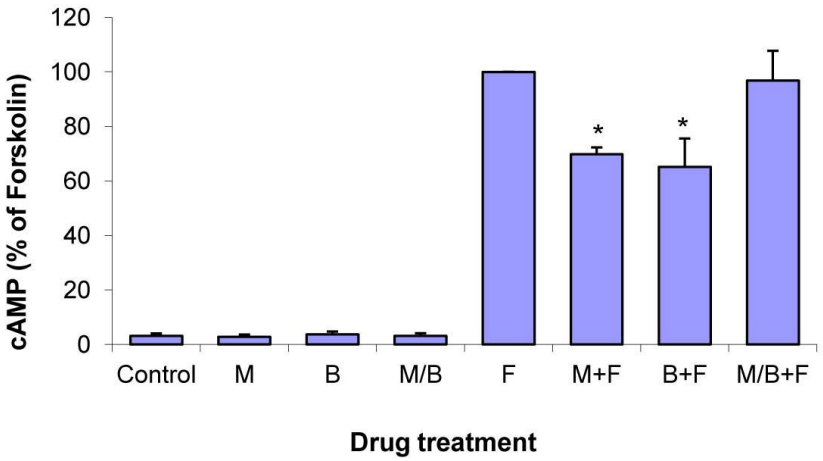

Figure 13. Co-activation of CB1 and D2L receptor alters cAMP signaling

Co-treatment of HEK-293T cells co-transfected with CB1 and D2L receptor with1 μM methanandamide (M) and10 μM bromocriptine (B) for 30 min at 37°C did not decrease forskolin (F) (10 μM) stimulated cAMP accumulation as seen in individual agonist treated cells. Individual agonist treatment as wells co-treatment with both agonists in the absence of forskolin did not show changes in cAMP response (*$p<0.05$ vs forskolin; one-way ANOVA with post-hoc SNK analysis, n = 4. Error bars represent S.E.M.)

3.5. cAMP accumulation inhibited by co-activation of CB1 and D2L receptor in the presence of CB1-CT2B mini-gene

To measure cAMP levels in live cells, epac1-camps FRET vector was utilized (Nikolaev, et al., 2004). Epac1-camps vector contains Epac1 (Exchange protein activated by cAMP), flanked by cyan fluorescent protein tag (CFP) and yellow fluorescent protein tag (YFP). The binding of cAMP to Epac1 changes the conformation of Epac1 and disrupts FRET between the CFP and YFP tags and thus an increase in FRET ratio (CFP/FRET) is observed which is indicative of increase in cAMP levels (**Figure 4**).

Thus far, we've established that immunoprecipitation of D2 receptor results in co-immunoprecipitation of CB1 receptor in cell lysates of HEK-293T cell co-expressing CB1 and D2L (**Figure 5**). However, the presence the CB1-CT2B mini-gene disrupted the interaction between CB1 and D2L, as shown in **Figure 12A**. The next goal was to investigate the effects of mini-gene expression on cAMP signaling. Towards this goal, HEK-293T cells were co-transfected with CB1, D2L, CB1-CT2B mini-gene and epac1-camps plasmid. As a control, HEK 293T cells were co-transfected with CB1, D2L, pcDNA3 and epac1-camps. The ratio of CB1, D2L, pcDNA3 (or CB1-CT2B mini-gene) cDNA used in the transfections was 1:1:1. The amount of epac1-camps vector transfected was 1/10 of the total DNA and therefore any cells exhibiting fluorescence had a high probability of also having CB1 and D2L receptor co-expression. Forty eight hours post transfection; cells were washed and treated in tyrode buffer with 5 μM methanandamide or 50 nM bromocriptine or co-treated with both agonists for 15 minutes at room temperature. Following agonist treatment, the cells were imaged under a fluorescence microscope for 20 minutes. **Figure 14A** shows the ratio image of cells before and after the addition of 25 μM forskolin. Forskolin addition, 2:25 minutes after the start of imaging, resulted in cAMP accumulation and thus an increase in FRET ratio observed.

Individual agonist treatment of cells transfected with CB1, D2L, and pcDNA3 with 5 μM methanandamide or 50 nM bromocriptine resulted in a decrease of forskolin stimulated cAMP accumulation however co-treatment with both agonists did not show a

decrease in cAMP which is shown in the scatter plot of a representative FRET experiment in **Figure 14B** (left panel). Compilation of 5 independent experiments indicate that the FRET ratio of bromocriptine and methanandamide co-treated cells was comparable to forskolin only treated cells and individual agonist treated cells show a significant decrease in forskolin stimulated cAMP accumulation at the 20 minute time point (**p<0.01 vs Forskolin, ***p<0.001 vs Forskolin; one-way ANOVA with post-hoc Tukey's multiple comparison test, n = 5) **(Figure 14C)**. The findings of these experiments using live cells agree with the outcome of the cAMP immunoassay whereby, individual agonist treatment resulted in reduced cAMP response whereas co-treatment with both receptor agonists did not show decreased cAMP accumulation.

The cAMP signaling response was changed when HEK-293T cells were co-transfected with CB1, D2L and CB1-CT2B mini-gene. Co-activation with 5 μM methanandamide and 50 nM bromocriptine exhibited a decrease in forskolin stimulated cAMP accumulation, shown in representative scatter plot in **Figure 14B** (right panel). Analysis of FRET ratios at the 20 minute time point of 5 independent experiments indicate the FRET ratio of bromocriptine and methanandamide co-treated cells was significantly different from forskolin only treated cells (*p<0.05 vs Forskolin; one-way ANOVA with post-hoc Tukey's multiple comparison test, n = 5) **(Figure 14C)**. Moreover, individual agonist treatment of CB1, D2L and CB1-CT2B transfected cells, with either methanandamide or bromocriptine, showed a significant decrease in forskolin stimulated cAMP accumulation (***p<0.001 vs Forskolin; one-way ANOVA with post-hoc Tukey's multiple comparison test, n = 5) **(Figure 14C)**. Cells co-transfected with CB1, D2L and CB1-CT2B mini-gene showed a decrease in cAMP levels with individual agonist treatment indicating that the presence of the mini-gene did not interfere with CB1 or D2L receptor function.

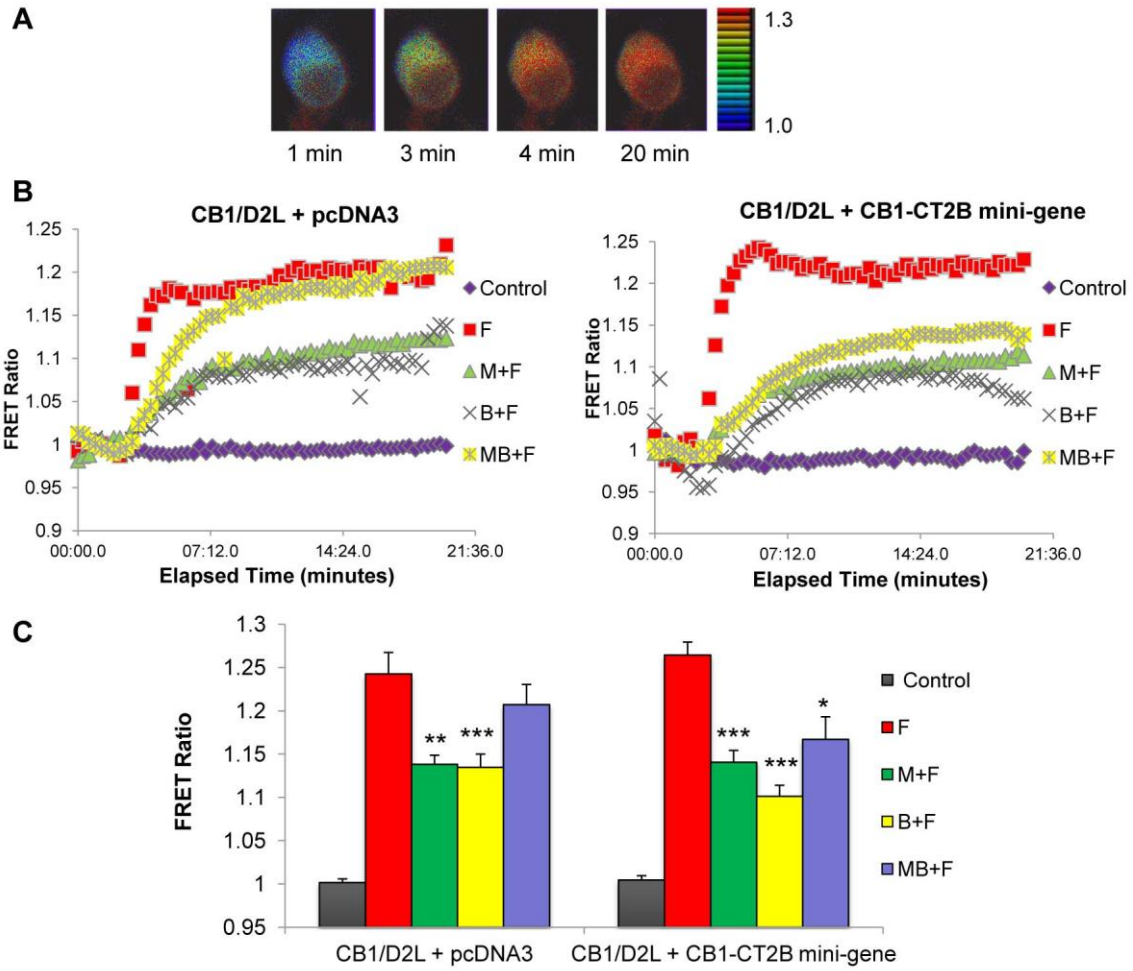

Figure 14. cAMP accumulation inhibited by co-activation of CB1 and D2L receptor with the presence of CB1-CT2B mini-gene

(A) FRET ratio image of HEK-293T cells co-transfected with CB1, D2L and epac1-camps vector. The cells were imaged at 20 second intervals for 20 minutes. The addition of 25 μM forskolin, 2:25 minutes after imaging was started, resulted in increased cAMP accumulation which is represented by the increase in FRET ratio (CFP/FRET). **(B)** Scatterplot of representative FRET experiment with HEK-293T cells co-transfected with either CB1, D2L, epac1-camps and pcDNA3 or CB1, D2L, epac1-camps and CB1-CT2B mini-gene. Pre-treatment of cells with either 5 μM methanandamide (M) or 50 nM bromocriptine (B), for 15 minutes at room temperature in Tyrode buffer prior to imaging, decreases forskolin (F) (25 μM) stimulated cAMP. Co-treatment of HEK-293T cells co-transfected with CB1, D2L, epac1-camps and pcDNA3, with bromocriptine and methanandamide, leads to increased forskolin stimulated cAMP accumulation. Whereas, co-treatment of HEK-293T cells co-transfected with CB1, D2L, epac1-camps and CB1-CT2B mini-gene leads to a decrease in forskolin stimulated cAMP accumulation. **(C)** Bar plot summary of 5 independent FRET experiments. **p<0.01 vs Forskolin, ***p<0.001 vs Forskolin with CB1/D2L/pcDNA3; *p<0.05 vs Forskolin, ***p<0.001 vs Forskolin with CB1/D2L/CB1-CT2B mini-gene; one-way ANOVA with post-hoc Tukey's multiple comparison test, n = 5. Error bars represent S.E.M.

3.6. Effects of CB1 and D2L receptor activation on CREB phosphorylation

One of the downstream signaling outcomes of cAMP accumulation is the phosphorylation of CREB through the activation of PKA. Our previous experiments have established that co-activation CB1 and D2L receptor complex results in altered cAMP responses. To determine whether these changes in cAMP signaling have downstream effects, phosphorylation of CREB was investigated. Human embryonic kidney-293T cells were co-transfected with CB1 and D2L; CB1 and pcDNA3 or D2L with pcDNA3. Forty eight hours post-transfection, CB1 and D2L transfected cells were treated with 1 nM bromocriptine or 50 nM methanandamide or co-treated with both agonists while the CB1/pcDNA3 and D2L/pcDNA3 co-transfected cells were treated with 50 nM methanandamide and 1 nM bromocriptine, respectively, for 30 minutes at 37°C. Cells co-transfected with CB1 and D2L were also treated with vehicle and 10 μM forskolin, as a negative and positive control, respectively. Following the treatments, cells were collected, solubilized and lysates were resolve by SDS-PAGE followed by western blotting with pCREB, CREB and tubulin antibodies. Western blot signals were quantified and the level of pCREB was determined by normalizing with the CREB expression. Blots were probed with tubulin antibody to show relative amounts of protein loaded for each sample.

Treatment of CB1/D2L co-transfected cells with forskolin resulted in significantly enhanced levels of pCREB compared to vehicle treated cells, shown in representative immunoblots in **Figure 15A**. Individual agonist treatment, of CB1/D2L co-transfected cells, with 1 nM bromocriptine or 50 nM methanandamide or co-treatment with both agonists showed an increasing trend in pCREB levels compared to control, however levels of pCREB were not significantly different from control. Moreover, methanandamide treatment of CB1/pcDNA3 co-transfected cells and bromocriptine treatment of D2L/pcDNA3 co-transfected cells did not significantly enhance pCREB levels compared to control cells. However, pCREB levels of methanandamide treatment of CB1/pcDNA3 and bromocriptine treatment of D2L/pcDNA3 co-transfected cells were significantly different from the pCREB levels of CB1/D2L transfected cells co-treated with bromocriptine and methanandamide. The pCREB values were normalized with CREB values for each treatment group to account for differences in CREB expression levels.

The compiled results of 3 independent experiments are depicted in **Figure 15B** (***p<0.001 vs control; #p<0.05 vs M/B(CB1/D2L), #p<0.01 vs M/B(CB1/D2L); one-way ANOVA with post-hoc SNK analysis, n = 3. Error bars represent S.E.M.)

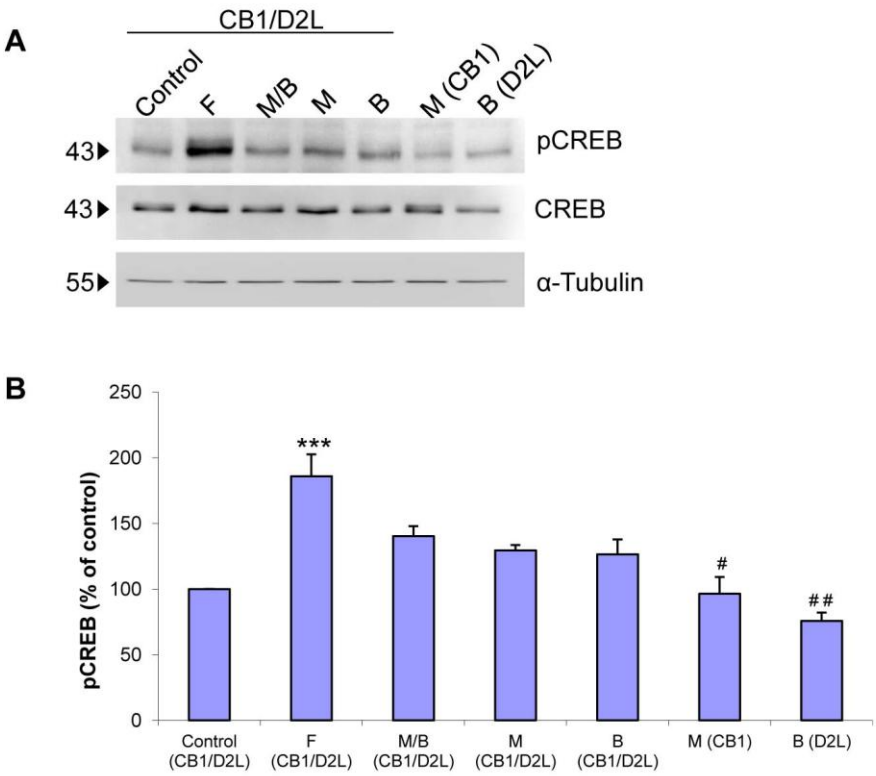

Figure 15. CB1 and D2L receptor activation and phosphorylation of CREB

(**A**) Western blot showing treatment of HEK-293T cells co-transfected with CB1/D2L with 10 μM forskolin (F) for 30 min at 37°C resulted in significant increase of phosphorylated CREB (pCREB) levels compared to untreated Control. Treatment of HEK-293T cells co-transfected with CB1/D2L with 50 nM methanandamide (M) or 1 nM bromocriptine (B) or co-treatment with both agonists showed slight increase in pCREB which was not significantly different from Control. Methanandamide treatment of CB1 transfected cells and bromocriptine treatment of D2L transfected cells did not significantly enhance pCREB levels. pCREB levels of CB1 transfected cells treated with methanandamide and D2L transfected cells treated with bromocriptine were significantly different from CB1/D2L co-transfected cells co-treated with bromocriptine and methanandamide. pCREB levels were normalized with CREB levels. Compiled quantification of normalized pCREB levels of western blots are shown in (**B**) (***p<0.001 vs control, #p<0.05 vs M/B(CB1/D2L), ##p<0.01 vs M/B(CB1/D2L); one-way ANOVA with post-hoc SNK analysis, n = 3. Error bars represent S.E.M).

4. Discussion

4.1. CB1-D2R form a complex mediated by a direct protein-protein interaction

Co-immunoprecipitation, fluorescence resonance energy transfer (FRET) and multicolor bimolecular fluorescence complementation (MBiFC) techniques provide evidence of CB1-D2R heterodimer (Kearn, et al., 2005; Marcellino, et al., 2008; Przybyla and Watts, 2010). In this study, the presence of CB1 and D2L receptor complex was first established by co-immunoprecipitation (co-IP) experiment using CB1 and D2L co-transfected HEK-293T cells. These results indicate that there is an interaction between CB1 and D2L receptor; however the nature of the interaction and the domains of the receptors involved in mediating the interaction remained unclear. To elucidate the motifs of CB1 receptor involved in the interaction, GST fusion proteins of the CB1 receptor were generated and utilized with HEK-293T cell lysates expressing D2L receptor for affinity purification assays. As a result of these experiments, a 15 amino acid region (CB1-CT2B [C417-S431]) within the carboxyl terminus (CT) of CB1 receptor was identified to mediate the interaction with D2L receptor.

A previous report shows that the CT region of CB1 receptor also mediates the interaction between CB1 and the A2A receptor (Navarro, et al., 2010). Moreover, the CT region of the CB1 receptor interacts with β-arrestin which modulates receptor desensitization and internalization (Jin, et al., 1999; Freedman and Lefkowitz, 1996). Interestingly, the region of CB1 that interacts with D2R overlaps with region that interacts with β-arrestin (Jin, et al., 1999). Additionally, the CT domain of CB1 is also the localization region of specific isomers of G proteins (Nie and Lewis, 2001; Mukhopadhyay, et al., 2000). Mukhopadhyay et al., (2000) show that $G_{\alpha o}$ and $G_{\alpha i3}$ bind to CB1 on 401 - 417 amino acid residues of the CT region whereas the intracellular loop 3 of CB1 is important for interacting with $G_{\alpha i1}$ and $G_{\alpha i2}$ subunits. With this overlap in

binding domain on the CT, prospective studies should investigate whether D2R binding to CB1 interferes with CB1 interacting with specific G_α subunits or with β-arrestin and whether the formation of CB1-D2L complex alters CB1 desensitization and internalization.

Like CB1, the dopamine D2 receptor has three intracellular loops and a cytosolic carboxyl tail. The third intracellular loop of D2R mediates the heterodimer between D2R and dopamine transporter (Lee, et al., 2007). Moreover, the D2 receptor couples directly to the adenosine A2A receptor (Canals, et al., 2003; Agnati, et al., 2005; Fuxe, et al., 2005) and mutations of specific arginine residues within the third intracellular loop of D2R disrupts this interaction (Fernandez-Duenas, et al., 2012). Furthermore, intracellular loop 3 (IL3) of D2R mediates the interaction between D2R and the dopamine D1 receptor as well as serotonin 5-HT(2A) receptor (Lukasiewicz, et al., 2009; Lukasiewicz, et al., 2010). Together, these reports implicate the importance of the IL3 domain in mediating the interaction of D2R with other GPCRs and therefore, we predicted that the interaction between D2R and CB1 was likely modulated by the third intracellular loop of D2R. To explore this notion, His-tagged fusion proteins of IL3 of both the long (D2LI3) and short (D2SI3) isoforms of D2R were generated, purified and utilized for *in vitro* binding experiments with GST fusion proteins of the CB1 receptor. The results indicate that the third intracellular loop of D2 receptor couples directly to the carboxyl terminus of the CB1 receptor. Furthermore, outcomes of the *in vitro* assay demonstrated that both His-tagged D2LI3 and D2SI3 interact with CT region of the CB1 receptor (Figure 10A) and therefore the site of interaction on the IL3 of D2 receptor is in a region which is common to both D2LI3 and D2SI3 and not the, 29 amino acid, alternative splice region of D2LI3. Previously, CB1 and D2R interaction studies were conducted with D2L isoforms of the D2 receptor (Glass and Felder, 1997; Jarrahian, et al., 2004; Kearn, et al., 2005; Marcellino, et al., 2008; Przybyla and Watts, 2010). Therefore, for this study, subsequent experiments with His-tagged fusion proteins were carried out with HIS-D2LI3, to show D2LI3 was able to bind the CT region of the CB1 receptor (Figure 10B).

Blot overlay experiments establish further evidence of direct protein-protein interaction between CB1 and D2L receptor. The results of these experiments indicated direct binding between HIS-D2LI3 and GST-CB1-CT. To disrupt the interaction between

CB1-CT and D2LI3, HIS-D2L3 was pre-incubated with GST-CB1-CT2B before adding to the blotted proteins, GST-CB1-CT and GST alone. The pre-incubation of GST-CB1-CT2B with HIS-D2LI3 prevented HIS-D2LI3 from interacting with GST-CB1-CT on the blot. These experiments confirmed that there is a direct protein-protein interaction between the third intracellular loop of D2R and the carboxyl terminus of CB1, however; specific domains of interaction within IL3 of D2R have not been identified. A previous study shows that specific arginine residues, 217–222 and 267–269, of the third intracellular loop of D2R are involved in protein-protein interactions with the CT of the A2A receptor (Fernandez-Duenas, et al., 2012) and it is likely that these arginine rich domains of D2R also mediate the CB1-D2R interaction.

With the identification of CB1-CT2B region, the next step of the study was to explore the effects of disrupting the interaction between CB1 and D2L by generating a mini-gene encoding this interacting region of CB1. Mini-gene encoding the CB1-CT2B was generated and co-transfected with CB1 and D2L receptor in HEK-293T cells. Results of co-IP experiments indicate that the presence of CB1-CT2B mini-gene decreases the interaction between CB1 and D2R compared to control cells co-transfected with CB1, D2L and empty expression vector, pcDNA3 (Figure 12A). This suggests that the translation of the CB1-CT2B mini-gene produces a peptide that interacts with D2R thereby, preventing D2R from forming a complex with CB1. The outcomes of co-IP experiment with mini-genes further implicates that the CB1-D2R complex is formed by a direct protein-protein interaction.

4.2. CB1-D2R complex activation and cAMP signaling

CB1 and D2R are coupled to $G_{\alpha i}$ and thus inhibit adenylate cyclase mediated cAMP production upon activation (Howlett, et al., 1986; Missale, et al., 1998). Prior *in vitro* studies establish that the interaction of CB1 and D2R results in changes in cAMP signalling. Co-activation of CB1 and D2R in striatal cultures and transfected HEK-293 cells results in cAMP accumulation which was not observed with activation of either CB1 or D2R alone (Glass and Felder, 1997; Jarrahian, et al., 2004; Kearn, et al., 2005). Glass and Felder, (1997) and Jarrahian et al., (2004) suggest that the accumulation in cAMP is a result of a switch of CB1 receptor coupling from $G_{\alpha i}$ to $G_{\alpha s}$ which was

demonstrated in Chinese hamster ovary (CHO) cells as well as HEK-293 cells co-expressing D2L and CB1 receptor. The switch in CB1 receptor coupling from $G_{\alpha i}$ to $G_{\alpha s}$ is enhanced in the presence of pertussis toxin treatment. Pertussis toxin treatment ADP ribosylates $G_{\alpha i}$ thereby, uncoupling it from G_{β} and G_{γ} and subsequently prevents $G_{\alpha i}$ from associating with the CB1 receptor. Moreover, Glass and Felder, (1997) suggested that the switch in CB1 receptor coupling from $G_{\alpha i}$ to $G_{\alpha s}$ was observed with persistent activation of D2L receptor. Conversely, Jarrahian et al., (2004) reported that that co-expression of D2L receptor with CB1 receptor in HEK-293 cells induces CB1 receptor to switch to $G_{\alpha s}$ coupling and activation of D2L receptor is not necessary (Jarrahian, et al., 2004).

In the present study, cAMP immunoassay was implemented to show that co-activation of CB1 and D2L receptors in HEK-293T leads to forskolin stimulated cAMP accumulation which agrees with previous studies using rat striatal cultures as well as HEK-293 cells (Glass and Felder, 1997; Jarrahian, et al., 2004; Kearn, et al., 2005). Using CB1 and D2L co-transfected HEK-293T cells, the findings of the present study also show that individual agonist activation of D2L or CB1 receptor with bromocriptine or methanandamide, respectively, decreases forskolin stimulated cAMP response (Figure 13). In the absence of forskolin, cAMP changes were not observed and were comparable to vehicle treated control cells. Earlier studies suggest that the forskolin stimulated cAMP accumulation upon CB1 and D2L receptor co-activation can be attributed to CB1 receptor switching from $G_{\alpha i}$ to $G_{\alpha s}$ coupling (Glass and Felder, 1997; Jarrahian, et al., 2004). However, in this study, the coupling of CB1 to $G_{\alpha s}$ was not observed which is inconsistent with previous findings. Glass and Felder (1997) used CHO cells and therefore some inconsistencies can be attributed to difference in cell lines. However, Jarrahian et al., (2004) and Kearn et al., (2005) utilized HEK-293 cells and thus disparities in findings cannot be attributed entirely to differences in cell lines. Pertussis toxin treatment prevents the $G_{\alpha i/o}$ heteromer from coupling with the CB1 receptor and therefore blocks the effects of CB1 agonist (Glass and Felder, 1997; Jarrahian, et al., 2004); however the evidence of CB1 receptor coupling to $G_{\alpha s}$ is not convincing. The weak coupling of CB1 to $G_{\alpha s}$ has only been observed in the presence of pertussis toxin perhaps due to the lack of availability of $G_{\alpha i}$ and not as a functional outcome of CB1 and D2L receptor interaction. Moreover, if co-activation of CB1 and D2L leads to CB1 coupling to $G_{\alpha s}$, co-activation of CB1-D2L co-transfected cells with

CB1 and D2L agonists in the absence of forskolin and pertussis toxin would result in amplified cAMP response. However, this was not observed. Results of cAMP immunoassays (Figure 13) demonstrate that in the absence of forskolin, co-activation of CB1 and D2L receptor co-transfected cells did not result in increased cAMP accumulation and therefore we cannot conclude that the CB1 receptor was coupled to $G_{\alpha s}$. In the studies conducted by Glass and Felder (1997), Jarrahian et al., (2004) and Kearn et al., (2005), there is a possibility that treatment with pertussis toxin may switch CB1 receptor coupling from $G_{\alpha i}$ to $G_{\alpha q}$ to indirectly activate adenylate cyclase. Previous research shows that CB1 receptor can couple to $G_{\alpha q/11}$ (Lauckner, et al., 2005). However, co-IP studies conducted with rat brain lysates, to examine the types of G_{α} coupling to CB1, showed no evidence of CB1 coupling to either $G_{\alpha q}$ or $G_{\alpha s}$ (Mukhopadhyay, et al., 2000).

To observe the signaling effects CB1 and D2L receptor co-activation in individual, live cells, further investigation of cAMP signaling was conducted with the epac1-camps FRET vector (Nikolaev, et al., 2004). HEK-293T cells co-transfected with CB1, D2L, epac1-camps and CB1-CT2B mini-gene showed a reduction in forskolin mediated cAMP production with treatment of either bromocriptine or methanandamide demonstrating that the presence of CB1-CT2B mini-gene did not affect the function of the receptors when activated individually (Figure 14B). Furthermore, co-activation of CB1, D2L, and CB1-CT2B mini-gene co-transfected cells resulted in loss of cAMP accumulation that was observed in cells co-transfected with CB1, D2L, epac1-camps and pcDNA3. The presence of the mini-gene is these cells disrupted the formation of the CB1-D2L complex as shown in co-IP experiments (Figure 12A) and this disruption of CB1-D2L complex formation resulted in cAMP inhibition similar to cells treated with methanandamide or bromocriptine alone (Figure 14B). With these outcomes, we conclude that the loss of cAMP inhibition with co-activation of CB1-D2L is dependent on the formation of CB1-D2L complex. Figure 16 shows a summary of the findings of cAMP response with respect to CB1-D2L activation. It is plausible that the co-activation of CB1-D2L receptor complex in HEK-293T cells leads to the uncoupling of $G_{\alpha i/o}$ from either CB1, D2L or both and therefore co-treatment with both agonists in the presence of forskolin results in cAMP accumulation. The interaction domain in the CT region of CB1 receptor also mediates $G_{\alpha i}$ coupling (Mukhopadhyay, et al., 2000) and thus the presence of D2L complex with CB1 may interfere with CB1 coupling to $G_{\alpha i}$. Although, single

receptor activation of the cells co-transfected with CB1 and D2L receptor resulted in expected functional outcome; nonetheless, CB1 and D2L receptor $G_{\alpha i}$ coupling in the CB1-D2L complex should be investigated. The epac1-camps FRET experiments were conducted with transiently transfected HEK-293T cells and FRET ratios were determined by manually outlining individual cells expressing fluorescence. Although the cells were transiently transfected, the amount of epac1-camps cDNA was one tenth of the total cDNA used in the transfection to increase the probability that any cells exhibiting CFP and YFP fluorescence would be co-expressing CB1, D2L and CB1-CT2B mini-gene.

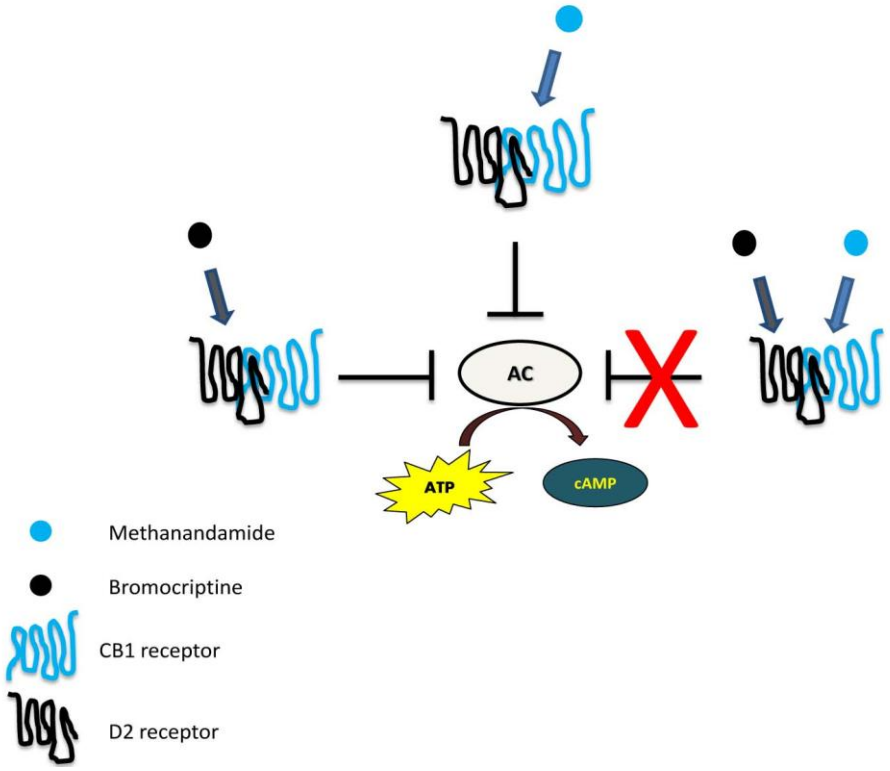

Figure 16. Schematic model of CB1-D2L receptor complex activation and cAMP response

Diagram shows summary of cAMP signaling findings. Activation of either CB1 or D2L receptor in the CB1-D2L heterodimer complex with CB1 agonist, methanandamide or D2R agonist, bromocriptine, results in inhibition of adenylate cyclase (AC) mediated cAMP production. Co-activation of both receptors in the CB1-D2L heterodimer complex results in cAMP accumulation.

The changes in functional effects of CB1-D2L interaction are observed when CB1 and D2R are present as a heterodimer and are co-activated. CB1 and D2L receptor oligomers are evident in HEK-293 cells as well as in Cath.-a- differentiated (CAD) cells which are a variant of a CNS catecholaminergic cell line established from a mouse brain tumor. Once differentiated, CAD cells have neuronal-like properties. As discussed previously, the presence of CB1-D2L receptor complex has been demonstrated by co-IP, FRET and MBiFC techniques (Kearn, et al., 2005; Marcellino, et al., 2008; Przybyla and Watts, 2010). There is some conflicting evidence that treatment with either CB1 or D2R agonist or co-treatment with both agonists promotes CB1 and D2L receptor complex formation. For example, Kearn et al., (2005) utilize *in vitro* experiments to demonstrate that acute CB1 and D2L receptor agonist co-treatment favours receptor heterodimerization whereas treatment of cells with individual CB1 or D2L receptor agonists does not affect receptor complex formation. On the other hand, Przybyla and Watts (2010) show, through MBiFC assays, that agonist treatment with either CB1 or D2R agonist favours the formation of CB1-D2L heterodimer in live cells. Furthermore, Marcellino et al., (2008) exhibited CB1-D2R interaction in transfected HEK-293 cells using FRET techniques. In their studies, Marcellino et al., (2008) demonstrated that application of either CB1 or D2R agonist alone or co-treatment of both agonists did not affect FRET signal thus agonist treatment did not affect CB1-D2R heteromer formation. It should be noted that Kearn et al., (2005) and Marcellino et al., (2008) utilized HEK-293 cells acutely treated with agonists to conduct co-IP and FRET experiments, respectively; whereas Przybyla and Watts (2010) employed MBiFC with CAD cells pre-treated with either agonist for 20 hours before analysis.

The findings of the present study suggest that the formation and subsequent co-activation of the CB1 and D2L oligomer complex alters cAMP signaling outcomes. It is likely that the co-activation of CB1 and D2L receptor may promote the formation of the CB1-D2L complex which has been demonstrated previously (Kearn, et al., 2005). The co-IP experiments conducted by Kearn et al., (2005) suggest that CB1 and D2L receptor co-activation promotes the formation of CB1-D2L complex. The results of the co-IP experiment in this study with HEK-293T cells co-expressing CB1 and D2L suggest that receptor activation is not necessary for CB1-D2L complex formation; although, co-IP

experiments with agonist treated cells were not thoroughly explored. Further analysis of the CB1-D2L complex with agonist treatment as well as in the presence of mini-genes will provide greater understanding of the dynamics of CB1-D2L receptor complex formation with respect to receptor activation. Co-immunoprecipitation experiments were conducted with HEK-293T cells over-expressing CB1 and D2L receptors and thus co-IP in the absence of agonist treatment was observed. Ideally, co-IP experiments should be conducted with neurons endogenously expressing CB1 and D2L receptors and treated with agonists to determine whether agonist treatment promotes CB1-D2L complex formation.

4.2.1. CB1-D2R complex activation and phosphorylation of CREB

The activation of adenylate cyclase leads to increased cAMP levels which in turn can activate PKA. Once activated, PKA can phosphorylate specific serine residues of the transcription factor CREB (Andrisani, 1999). Phosphorylated CREB (pCREB) interacts with CREB binding protein (CBP), together the CBP/CREB complex is able to activate gene transcription by coupling to CREB response element (CRE); found on the promoter of cAMP responsive genes (Andrisani, 1999). CREB dependent gene expression is implicated in various functional processes including learning and memory as well in neurological diseases (Lamprecht, 1999; Lonze and Ginty, 2002; Kida, 2012).

To explore whether cAMP accumulation has effects on phosphorylation of CREB, HEK-293T cells co-transfected with CB1/D2L were individually treated with bromocriptine or methanandamide or co-treated with both agonists. Although pCREB levels in these treatment groups showed an increasing trend, the levels of pCREB were not significantly different from vehicle treated control cells. In addition, individual agonist treatment of CB1/pcDNA3 and D2L/pcDNA3 co-transfected cells, with either methanandamide or bromocriptine, respectively, did not show significant increase in pCREB levels compared to the untreated control cells. Interestingly, pCREB levels of CB1/D2L co-transfected cells co-activated with bromocriptine and methanandamide were significantly different from CB1/pcDNA3 co-transfected cells treated with methanandamide and D2L/pcDNA3 co-transfected cells treated with bromocriptine. These preliminary results suggest that co-activation of CB1-D2L complex may result in increased cAMP levels compared to individual activation of either CB1 in CB1/pcDNA3

transfected cells or activation of D2L in D2L/pcDNA3 transfected cells, which in turn promote phosphorylation of CREB levels, although further experiments should be conducted. Ideally, levels of pCREB should be examined after cells are treated with agonists in the presence of forskolin to increase cAMP levels. The outcomes of experiments in the presence of forskolin may provide clarification on whether changes in pCREB are a result of cAMP accumulation. Additional signaling pathways can also lead to phosphorylation of CREB. For example, calmodulin-dependent protein kinase (CaMK) and ERK of the MAPK pathway can phosphorylate CREB. Furthermore, it is possible that the $G_{\beta\gamma}$ linked to either CB1 or D2R or both may stimulate the MAPK pathway which then can phosphorylate CREB, independent of adenylate cyclase activity and cAMP (Faure, et al., 1994).

4.3. Implications of CB1-D2R cross-talk

4.3.1. Regulation of neurotransmission at the cellular level

The CB1 receptor plays an important role in regulation of neurotransmission. Endocannabinoids released from the postsynaptic neuron can activate presynaptic CB1 receptors to inhibit further neurotransmitter release (Maejima, et al., 2001; Ohno-Shosaku, et al., 2002; Robbe, et al., 2001). The D2 autoreceptors found on the presynaptic membrane have inhibitory effects on neuron excitability which can lead to a decrease in DA release (Lacey, et al., 1987; Lacey, et al., 1988; Hernandez-Lopez, et al., 2000). Thus, the modulation of neurotransmission of dopamine or other neurotransmitter is a critical role of the D2 receptor (Momiyama and Koga, 2001). Separately, both receptors have crucial roles in modulating neurotransmission and as a consequence of forming a heterodimer, either CB1 or D2R or both receptors may lose its individual regulatory roles. The extent of neurotransmission disruption is dependent on the specific region of the brain affected. On a cellular level, formation of the CB1 and D2R complex on the presynaptic neuron may interfere with neurotransmission, as a result dopamine accumulation in the synapse will likely occur. Another possibility is over excitation of the presynaptic and postsynaptic neuron, which will contribute to disruptions in normal synaptic transmission. Although the specific consequences are speculative, the general outcome of CB1-D2R interaction is potential to disrupt neurotransmission

regulation. The D2S receptor localizes to the presynaptic membrane in dopaminergic neurons and thus, we can predict that presynaptic CB1 will likely interact with D2S receptor while postsynaptic CB1 is more likely to form a complex with D2L receptor (Khan, et al., 1998; Usiello, et al., 2000; De Mei, et al., 2009).

The previous reports studying D2R and CB1 interaction demonstrate that activation of either CB1 or D2L or co-activation of both receptors may enhance the formation of CB1 and D2L receptor complex (Kearn, et al., 2005; Przybyla and Watts, 2010). With this notion, it is tempting to speculate that activation of either CB1 or D2R or co-activation of both receptors may encourage the formation of the CB1-D2R complex in neurons. Such conditions with excess dopamine or endocannabinoid activation of D2R and CB1, respectively, may exist in disease states. For example, in the mesolimbic system, dopamine (DA) release is increased in response to drugs of abuse (Di Chiara and Imperato, 1988). The surge in DA release may activate D2R to couple with CB1 or alternatively, activate endocannabinoid signaling as seen in striatal cells by Giuffrida et al., (1990), which may also modulate CB1-D2R complex formation. Further research must be conducted to clarify the conditions which promote CB1-D2R complex formation and whether it is in response to disease state or a mode of regulating neuron function.

4.3.2. Cross-talk of D2R and CB1 at the systemic level

There is significant evidence of endocannabinoids mediating dopamine transmission in the mesocortical and nigrostriatal pathways (Fitzgerald, et al., 2012). Cross-talk of the cannabinoid and dopamine systems has important consequences since both systems play a role in learning, memory and reward (Wise, 2004; Solinas, et al., 2008). The mesocortical dopaminergic pathways, which project from the ventral tegmental area (VTA) to the prefrontal cortex (PFC) and the amygdala, modulate learning and emotion. The PFC regulates dopamine transmission and mediates emotion processing and learning (Kienast, et al., 2013). In addition, the amygdala, hippocampus and dorsal striatum circuitry regulate long-term memory, conditioning and declarative memory (Mishkin, 1978; Squire and Zola-Morgan, 1991; McDonald and White, 1993). The D2 receptor in the amygdala is involved in the formation and retrieval of emotional memory which mediates emotions such as fear (Nader and LeDoux, 1999; de Oliveira, et al., 2011). The cannabinoid system is also implicated in learning and memory. In

rodents, the loss of CB1 receptor results in loss of memory acquisition (Marsicano, et al., 2002) whereas disruption of CB1 signaling leads to suppression of learned behaviour (Varvel, et al., 2005). The limbic system modulates behaviour associated with reward and motivation. In particular the mesolimbic dopaminergic pathway has been the subject of extensive research with particular interests in its role of modulating the rewarding effects of environmental stimuli. The mesolimbic pathway includes dopaminergic neurons that project from the VTA of the midbrain to the nucleus accumbens (NAc) in the forebrain (Wise, 2004). There is evidence that the specific cross-talk between CB1 and D2 receptor modulates reward, memory and learning. For example, the endocannabinoid signaling system is involved in modulating dopamine regulated reward function (Gardner, 2005). In addition, CB1 receptor regulates D2 receptor in hippocampal cells to modulate learning (Zarrindast, et al., 2010) and blocking D2 receptors in the prefrontal cortex of mice disrupted CB1 mediated learning and memory (Rodrigues, et al., 2011).

The reward, learning and memory circuitry is activated in response to natural stimuli such as food, sex and other pleasures. Drugs of abuse activate this circuitry in much the same manner but with greater intensity and persistency than natural rewards. After chronic exposure to drugs of abuse, the brain reward circuitry becomes desensitized and drug users are unable to attain feelings of reward in the absence of drugs. For example, ethanol abuse causes increased DA release in the VTA and NAc, increased levels of extracellular DA and greater firing rate of DA neurons in the mesolimbic circuit (Di Chiara and Imperato, 1988; Brodie, et al., 1990). The CB1 and D2 receptor have been separately implicated in ethanol addictions. Furthermore, there is evidence that cross-talk between these receptors may contribute to the behaviours associated with ethanol addictions (Houchi, et al., 2005; Cheer, et al., 2007; Thanos, et al., 2011).

4.4. The role of CB1 and D2R in ethanol addictions

4.4.1. CB1 and ethanol abuse

A number of studies show that CB1 receptor plays a role in alcohol addiction. For instance, CB1 receptor agonist administration in male Wistar rats results in

increased alcohol consumption (Gallate, et al., 1999). These effects are also observed in ethanol preferring rats, where administration of CB1 agonists also showed a significant increase in alcohol consumption (Colombo, et al., 2002). Furthermore, CB1 receptor knockout mice show reduced voluntary ethanol consumption, increased ethanol sensitivity, greater withdrawal severity and decreased conditioned place preference for alcohol (Hungund, et al., 2003; Naassila, et al., 2004; Houchi, et al., 2005). Moreover, activation of CB1 receptor results in greater alcohol relapse behaviour in rats (Alen, et al., 2008). Together, these results demonstrate that CB1 receptor is involved in ethanol addictions; specifically, ethanol self-administration and reinforcement of ethanol preference.

4.4.2. D2R and ethanol abuse

Alcohol preferring rats have decreased expression of D2R mRNA in the nucleus accumbens and hippocampus compared to mice with less alcohol preference (Bice, et al., 2008). Such trends are evident in human studies in which alcoholics show significant reductions in striatal D2R levels when compared with non-alcoholics (Volkow, et al., 1996). D2R knockout mice show decreased ethanol self administration and reduced alcohol preference (Phillips, et al., 1998; Risinger, et al., 2000). On the other hand, over-expression of D2R in the nucleus accumbens of wild type mice leads to decreased alcohol intake and preference, further implicating the role of D2R in alcohol abuse (Thanos, et al., 2005).

4.4.3. CB1 and D2R interaction and ethanol abuse

Since both CB1 and D2R play important roles in learning and reward which are important factors contributing to alcohol addictions, it is likely that the cannabinoid system facilitates the effects of positive reinforcement by modulating D2R mediated dopamine neurotransmission. There is substantial evidence of cannabinoid and dopamine system cross-talk within specific regions in the brain. Cannabinoids, delta9-THC and CB1 agonist, WIN 55,212-2, increase firing of dopaminergic neurons in ventral tegmental area and substantia nigra (French, et al., 1997). Moreover, administration of delta9-THC and WIN 55,212-2 increased extracellular dopamine concentrations in the nucleus accumbens; which was prevented with CB1 receptor antagonist, SR141716A

(Tanda, et al., 1997). These interactions of endocannabinoid and dopamine systems likely play a role in mediating addictions. Administration of addictive substance such as nicotine, alcohol and cocaine result in marked increase of dopamine in the nucleus accumbens. However, treatment with CB1 receptor antagonist, SR141716 (rimonabant), blocks these effects which suggests that the CB1 receptor may be mediating the effects of addictive drugs on dopaminergic neurons. Additionally, CB1 and D2 receptor are important modulators of alcohol consumption. Mice lacking CB1 receptor show decreased conditioned place preference for alcohol and increased levels of D2R in the striatum (Houchi, et al., 2005). Moreover, the absence of D2R causes upregulation of CB1 receptor in the cerebral cortex, the caudate-putamen and the nucleus accumbens of mice. These effects in mice are reversed with chronic ethanol intake (Thanos, et al., 2011). Additionally, CB1 receptor activation promotes alcohol relapse behaviour. Conversely, inactivation of D2R contributes to alcohol abuse related behaviour. Together, these studies suggest that the interaction and cross-talk between CB1 and D2R mediate the behaviours associated with alcohol abuse (Alen, et al., 2008).

The dynamics of CB1 and D2L receptor interaction provide further insight on cross-talk between cannabinoid and dopaminergic systems. The CB1 receptor and its interaction with the D2 receptor may play a significant role in disorders associated with regulation of the dopaminergic system including alcohol addictions. Prospective studies will focus on the effects of CB1-D2R complex and the outcomes on alcohol symptoms implicated by the cross-talk of CB1 and D2R (Alen, et al., 2008).

5. Concluding remarks and future directions

This study has identified the domains of the CB1 and D2R that mediate the protein-protein interaction between these receptors. The outcomes of this direct interaction between CB1 and D2R result is altered cAMP signaling. These results add to what is known about the dynamics of the CB1-D2R receptor heterodimer. However, many questions remain unanswered and thus further investigations should be conducted. Subsequent studies should identify the specific domains within the third intracellular loop of D2R that mediates the interaction between D2R and CB1.

The CB1 and D2R heterodimer complex are likely found in nucleus accumbens and caudate putamen of the striatum where there is evidence of co-localization of CB1 and D2R (Pickel, et al., 2006). Co-IP experiments with brain tissue from these regions will indicate the presence of CB1-D2R heterodimers. Furthermore, co-localization of CB1 and D2R has been shown in dendrites, cell bodies as well as axon terminals in these regions (Pickel, et al., 2006). It will be interesting to investigate whether the CB1-D2R heterodimers are also present in the same regions where co-localization is observed or perhaps some regional conditions within neurons favor heterodimerization more than others. In addition, cellular localization of the heterodimer should be considered. It is unclear if the CB1-D2R receptor complex is localized to the plasma membrane. If there is evidence of plasma membrane localization, future studies should determine whether the complex forms at the plasma membrane, before it is transported to the plasma membrane or immediately following post-translational processing.

The conditions in which CB1 and D2R form a complex also remain unclear, and thus the regulation of heterodimer formation should be investigated. The observations of this study demonstrate that CB1-D2R is present as a heterodimer when both receptors are over-expressed; however, the nature of receptor complex in brain tissue needs further exploration. G protein-coupled receptors are commonly found as homodimers (Mackie, 2005) and thus the conditions that promote the formation of heterodimers of CB1 and D2R require further investigation. The formation of homodimers is likely

favoured over the formation of heterodimers and therefore, specific cellular conditions may contribute to CB1-D2R heterodimer formation. Experiments with neurons will provide insight on the location of CB1-D2R complex formation as well as the conditions which promote CB1-D2R heterodimer formation.

Establishing the disruption of CB1 and D2R complex formation opens up avenues for studying the dynamics of other oligomers involving CB1 and D2R or both (Navarro, et al., 2010). Inhibitory peptides and mini-genes can be employed to study the dynamics of CB1 and D2R oligomers with other proteins and how CB1-D2R complex contributes to other oligomer formations including heteromers of three receptors (Navarro, et al., 2010). Moreover, future studies should explore whether CB1 oligomerization with other GPCRs are also mediated by the identified regions of the carboxyl terminus of CB1. For example, the orexin and μ-opioid receptors both interact with CB1, though it's not clear which domains of CB1 are mediating these interactions. It is plausible that the CT of CB1 may mediate the cross-talk between CB1 and orexin and μ-opioid receptors.

Further research is needed to not only explore the outcomes of aberrant cAMP signaling and its downstream effects but to also clarify what causes the change in cAMP signaling within the CB1-D2R heterodimer. The change in cAMP response is observed only when CB1-D2R is present in a complex and both receptors are co-activated. The regulation of these dynamics remains unclear. The CT region of CB1 regulates binding of G proteins (Mukhopadhyay, et al., 2000) and thus the presence of D2R coupling with CT of CB1 may disrupt G protein coupling of CB1, however further clarification is needed. Furthermore, as a result of the changes in cAMP signaling, other downstream effects, in addition to CREB phosphorylation, should be examined. Activation of either CB1 or D2R can stimulate MAPK pathways (Bouaboula, et al., 1995; Kim, et al., 2004; Luo, et al., 1998) and thus interaction of CB1 and D2R with respect to MAPK signaling may also be significant. Furthermore, delta9-THC induced ERK activation, via CB1, in dorsal striatum and nucleus accumbens, is linked to dopamine transmission (Valjent, et al., 2001). In a recent study, activation of either CB1 or D2R increased phosphorylation of ERK1/2 and co-stimulation of both CB1 and D2R resulted in an additive effect on the phosphorylated ERK1/2 signal in striatal neurons (Chiang, et al., 2013).

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