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Delineation of Domains Within the Cannabinoid CB1 and Dopamine D2 Receptors That Mediate the Formation of the Heterodimer Complex

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Abstract Both the cannabinoid CB1 receptor (CB1) and dopamine D2 receptor (D2R) are G protein-coupled receptors that are linked to inhibitory Gαi/o protein, whereby activation of the receptor leads to the inhibition of cAMP production. Moreover, previous findings have shown evidence of cross-talk between the dopamine and endocannabinoid systems. In this report, we confirm the interaction of CB1 and D2R with co-immunoprecipitation experiments using human embryonic kidney 293T (HEK-293T) cells co-expressing both receptors. We also generated GST and His-tagged fusion proteins of the D2R and CB1 and conducted affinity purification assays and in vitro binding experiments to show that the CB1–D2R complex can be formed by a direct protein–protein interaction. This interaction is mediated by the carboxyl terminus of the CB1 receptor and the third intracellular loop of the D2 receptor. Co-transfection of an inhibitory mini-gene resulted in decreased levels of the CB1–D2R complex. Using a cAMP biosensor, we show that activation of D2R or CB1 alone in HEK-293T cells co-expressing both receptors leads to an inhibition of forskolin-stimulated cAMP accumulation. However, co-activation of both receptors resulted in a loss of this inhibition on cAMP accumulation. Our findings characterize the physical interaction between CB1 and D2R as well as demonstrate the potential functional outcome of the receptor complex.

Keywords Dopamine · Cannabinoid · G protein-coupled receptor · Protein–protein interaction

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Abbreviations

CB1	Cannabinoid CB1 receptor
D2R	Dopamine D2 receptor
DA	Dopamine
coIP	Co-immunoprecipitation
FRET	Förster resonance energy transfer
MBiFC	Multicolor bimolecular fluorescence complementation
PI	Phosphoinositide

Introduction

The dopamine system is involved in many neurological processes including memory, learning, and reward (Wise 2004) that overlaps with the endocannabinoid system, which has also been implicated in physiological processes such as pain perception, mood, memory, and reward (Di Marzo 2008; Solinas et al. 2008). Furthermore, there is evidence for a functional interaction between the dopamine and endocannabinoid systems and specifically between the dopamine D2 receptor (D2R) and the cannabinoid CB1 receptor (CB1). Both the D2R and the CB1 belong to a superfamily of receptors that exert their biological effects via intracellular G protein-coupled signaling cascades (Missale et al. 1998). In mammals, dopamine receptors are encoded by five distinct genes that are divided into D1-like receptors (D1, D5) and D2-like receptors (D2, D3, D4). The D1 and D5 receptors preferentially couple to Gαs proteins while D2-like receptors display a more complex pattern of signal transduction primarily due to their coupling to subtype-specific members of the Gαi/o protein family. D2-like receptors are known to be involved in numerous signal transduction pathways including the inhibition of adenylate cyclase activity, PI turnover, potentiation of arachidonic acid release, inwardly rectifying K+ and Ca^{2+}

channels, and mitogen-activated protein kinases (Picetti et al. 1997). The cannabinoid receptor family consists of the CB1 (Matsuda et al. 1990) and CB2 receptors (Munro et al. 1993). Both receptors mediate signaling through Gαi/o (Howlett et al. 2002; Howlett 2005) which can either inhibit adenylyl cyclase or can potentially activate mitogen-activated protein kinase. The CB1 receptor has also been shown to activate A-type and inwardly rectifying potassium currents and can also inhibit N- and P/Q-type calcium currents (Howlett et al. 2002; Howlett 2005). Furthermore, CB1 receptors are found mainly at the terminals of neurons, where they can inhibit the release of a number of different excitatory and inhibitory neurotransmitters (Howlett et al. 2002; Pertwee and Ross 2002; Szabo and Schlicker 2005). Interestingly, CB1 receptors appear to form oligomers with other G protein-coupled receptors (GPCR). The CB1/opioid receptor heterodimer was detected by BRET in HEK-293 cells co-expressing recombinant YFP-CB1 and luciferase fused with μ-, δ-, or κ-opioid receptors (Rios et al. 2006). However, a functional interaction between CB1 and opioid receptors has thus far been reported only for the CB1/μ-opioid receptor heterodimer (Canals and Milligan 2008). There is also evidence for CB1/orexin-1 (OX1) receptor heterodimers (Hilairet et al. 2003) and an interaction with adenosine receptors (Carriba et al. 2007; Navarro et al. 2008). In addition to these heterodimers, there is evidence of functional cross-talk between CB1 and D2R. Both in situ hybridization assays and immunocytochemistry paired with electron microscopy have demonstrated the co-localization of CB1 with D2R in both the caudate putamen and nucleus accumbens (Hermann et al. 2002; Pickel et al. 2006). Furthermore, one third of the CB1/D2R co-expression has been found in dendrites and cell bodies of neurons while two thirds are found in terminal regions (Hermann et al. 2002; Pickel et al. 2006). Additionally, CB1-null mice appear to exhibit increased binding for the D2R antagonist, raclopride (Hermann et al. 2002; Pickel et al. 2006), while increased dopamine (DA) activity mediated by cocaine administration can lead to increases in the endocannabinoid anandamide (Centonze et al. 2004). Moreover, Kearn et al. have shown co-immunoprecipitation (coIP) of CB1 with D2R in HEK-293 cells co-expressing both receptors and that co-activation promotes the CB1–D2R complex (Kearn et al. 2005). In addition, Marcellino et al. have also shown through Förster resonance energy transfer (FRET) experiments that the CB1–D2R forms a heterodimeric complex (Marcellino et al. 2008). However, in this study, co-activation of receptors did not enhance the CB1–D2R interaction. To clarify the functional implication of the CB1–D2R complex, we aimed to identify specific domains within the CB1 and D2R that mediate this protein–protein interaction and to develop inhibitory mini-genes that disrupt the formation of the CB1/D2R complex. Moreover, we used a cAMP biosensor to identify the functional consequence of the CB1/D2R complex on cAMP production.

Materials and Methods

Materials

Unless otherwise listed, all chemicals were purchased from Bioshop Canada (Burlington, ON, Canada). Reduced L-glutathione, glutathione agarose beads, HIS select nickel affinity gel, forskolin, (*S*)-(−)-propranolol hydrochloride, polyethyleneimine (PEI) solution, and isopropyl β-D-1-thio-galactopyranoside (IPTG) were obtained from Sigma Aldrich (Oakville, ON, Canada). 1-Methyl-3-isobutylxanthine (IBMX) was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Methanandamide and bromocriptine were obtained from Enzo Life Sciences (Farmingdale, NY). For cAMP analysis, ELISA cAMP assay was conducted using cAMP Parameter Assay Kit manufactured by R&D Systems (Minneapolis, MN). CB1 receptor goat polyclonal antibody (sc-5303) and dopamine D2 receptor mouse monoclonal antibody (sc-10066) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). His-tag monoclonal mouse antibody (A00186) was purchased from GenScript (Piscataway, NJ). GST polyclonal rabbit antibody (#2625) was obtained from Cell Signaling Technology (Danvers, MA).

Cell Culture and Transfection

Human embryonic kidney 293T (HEK-293T) cells were grown in DMEM (Invitrogen, Burlington, ON) supplemented with 10 % FBS and maintained at 37 °C with 5 % CO_2. Cells were seeded on PEI-coated plates and were transfected the following day. Calcium phosphate transfections were done with cDNA ratios of 1:1 (CB1/D2L). For coIP experiments with mini-genes, the cDNA ratio was 1:1:1 (CB1/D2L/pcDNA3 or CB1/D2L/CB1-CT2B mini-gene). For FRET experiments, all cDNA was at 1:1:0.2 ratio where the amount of epac1-camp plasmid used was 1/10 of the total cDNA transfected. For all experiments, cells were utilized 48 h post-transfection.

Generation of Fusion Protein and Mini-gene Constructs

All glutathione-S-transferase (GST) fusion protein, His-tagged, and mini-gene constructs were generated through PCR amplification and subcloned into appropriate plasmids. All recombinant plasmids were sequenced to verify correct orientation and nucleotide sequence. Domains encoding CB1-$IL2_{G214\text{-}K234}$, CB1-$IL3_{K302\text{-}T346}$, CB1-$CT_{R402\text{-}L474}$, $CT1_{R402\text{-}N426}$, $CT2_{C417\text{-}T441}$, $CT3_{D432\text{-}V456}$, $CT4_{A447\text{-}L474}$, CB1-$CT2A_{C417\text{-}N426}$, CB1-$CT2B_{C417\text{-}S431}$, and CB1-$CT4A_{K460\text{-}L474}$ were PCR-amplified from full-length rat CB1 cDNA and subcloned into the *Bam*HI and *Xho*I restriction sites of the pGEX-4T3 plasmid. Intracellular loop 3 of D2 long

($D2LI3_{I211\text{-}Q373}$) and D2 short ($D2SI3_{I211\text{-}Q344}$) regions were PCR-amplified from full-length D2L or D2S receptor cDNA and subcloned into the *Bam*HI/*Xho*I restriction sites of pET-28a. For the CB1-CT2B mini-gene, the C417-S431 region of CB1-CT was PCR-amplified with an insertion of a 5′ start codon and cloned into the *Bam*HI/*Xho*I restriction sites of pcDNA3.

Fusion Protein Generation and Purification

For protein expression in bacteria, BL21 *Escherichia coli* were transformed with either recombinant pGEX-4T3 or pET-28a. Briefly, bacterial cultures were grown at 37 °C for 2 h followed by induction with 500 μM IPTG and then returned to 37 °C for 2 h. Cultures were resuspended in ice-cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) with 1 % Triton X-100, allowed to mix for 10 min at 4 °C before being sonicated for 45 s. Samples were centrifuged to pellet the insoluble fraction. For GST fusion protein purification, 50 μl of 50 % glutathione agarose beads in PBS was added to 1 ml of bacteria lysates, mixed overnight at 4 °C, and washed three times for 5 min with cold PBS at room temperature. The protein was eluted with elution buffer (20 mM reduced L-glutathione, 100 mM Tris pH 8, 120 mM NaCl). His-tagged protein was purified according to the manufacturer's instructions (Sigma-Aldrich, Oakville, ON). In brief, 50 μl of HIS-select nickel affinity gel was equilibrated with equilibration buffer (150 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and then added to 1 ml of bacterial lysate and mixed at room temperature for 2 h and washed three times with wash buffer (150 mM NaCl, 50 mM NaH_2PO_4, pH 8.0). The His-tagged protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The Quick Start Bradford kit from Bio-Rad Laboratories (Richmond, CA) was used to determine protein concentrations by reading absorbance values at 595 nm on a Victor Plate Reader (PerkinElmer, Woodbridge, ON, Canada).

Affinity Purification Assay and Co-immunoprecipitation

Transfected HEK-293T cells were homogenized in modified RIPA buffer (50 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 2 mM EDTA, 1 mM sodium orthovanadate, 0.1 % Triton X-100) and complete protease inhibitor cocktail (Roche, Indianapolis, IN) and centrifuged at 16,100 × *g* for 15 min at 4 °C. Five hundred micrograms of protein extracts were incubated with 15–40 μg of GST fusion protein overnight at 4 °C. The next day, 25–50 μl of 50 % glutathione agarose beads (in PBS) were added and incubated at room temperature for 2 h. Beads were washed three times with 500 μl PBS containing 0.01 % Triton and boiled for 10 min in SDS sample buffer. Samples were resolved by SDS–PAGE followed by western blotting. For co-immunoprecipitation experiments, 15 μl Protein A/G magnetic beads were incubated in the presence or absence of primary D2 antibody (2.5 μg) for 2 h at room temperature, followed by the addition of 750 μg of HEK-293T cell lysates (homogenized in modified RIPA buffer) for 2 h. Magnetic beads were washed three times with PBS-T (0.05 % Tween) and eluted with 30 μl of 100 mM glycine (pH 2.8). After the addition of 3 μl of 1 M Tris–HCl (pH 8.0) and SDS sample buffer, the eluate was boiled for 5 min and subjected to SDS–PAGE and western blotting.

Western Blotting

Solubilized proteins were subjected to electrophoresis on 10 % gels (SDS–PAGE). Gels were transferred to polyvinylidenedifluoride (PVDF) membrane (Bio-Rad Laboratories, Mississauga, ON), blocked with 5 % nonfat milk in TBS-T buffer (10 mM Tris–HCl, 150 mM NaCl, and 0.1 % Tween) for 1 h at room temperature, washed three times, and incubated with primary antibody, at appropriate dilution in TBS-T, overnight at 4 °C. Following three TBS-T washes, the blot was incubated with appropriate secondary antibody (diluted 1:12,000 in TBS-T with 0.5 % milk) for 1 h at room temperature. The blots were visualized with SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Ottawa, ON) with Dyversity image analysis system and GeneSnap image acquisition software (Syngene, Frederick, MD).

Direct In Vitro Binding Experiments

For affinity purification using His-tagged protein as bait, 10 μg His-tagged protein and 0.5 μg GST fusion protein were incubated overnight at 4 °C. Five microliters of equilibrated HIS-select nickel affinity gel was added at room temperature for 2 h. Beads were washed three times with cold PBS with 0.05 % Triton X-100 (centrifuged at 4 °C at 400 × *g*), boiled for 10 min in SDS sample buffer, and 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 GST fusion protein as bait, 1 μg His-tagged protein and 20 μg GST fusion protein were incubated overnight at 4 °C. Ten microliters of 50 % glutathione agarose beads in PBS were added at room temperature for 2 h. Beads were washed three times with cold PBS with 0.05 % Triton X-100 (centrifuged at 400 × *g* between washes), boiled for 10 min in SDS sample buffer, and resolved by SDS–PAGE followed by western blotting with His-tag primary antibody (1/3,000 dilution) and goat anti-mouse IgG secondary antibody.

cAMP Assay Using the epac1-camps Biosensor

HEK-293T cells transfected with the epac1-camps biosensor in cells co-transfected either CB1, D2L, pcDNA3, or CB1, D2L, or CB1-CT2B mini-gene cDNA 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 pretreated with 50 nM bromocriptine, 5 μM methanandamide or co-treated with both agonists for 15 min at room temperature in tyrode buffer. At 2.5 min mark from the start of the imaging session, 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 min at 20 s intervals under ×40 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 (472/30 for CFP, 542/27 for YFP) operated in a filter wheel (Sutter Instruments, Novato, CA) were used. To determine the cAMP levels in a given experiment, 10 individual cells were manually outlined and the average CFP/FRET ratio was determined.

cAMP Immunoassay

HEK-293T cells transfected with CB1 and D2L were treated with either 1 μM methanandamide or 10 μM bromocriptine or co-treated with both for 15 min in serum-free DMEM supplemented with 0.5 mM IBMX and 1 μM propanolol at 37 °C. Following the agonist pretreatment, 10 μM of forskolin was added and the cells were incubated for 15 min. The cells were washed with cold PBS and incubated at 4 °C in 1× cold lysis buffer (cAMP Parameter Assay Kit R&D Systems, Minneapolis, MN) for 1 h. 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 1/10,000 dilution of mAb cAMP antibody on a microplate shaker at 500 rpm for 1 h at room temperature. Following four washes with PBS-T (0.05 % Tween), cAMP standards, lysis buffer, and samples were added to appropriate wells. To all wells, 1/10,000 dilution of cAMP-HRP was added and the plate was incubated at room temperature for 2 h at 500 rpm. After washing four times with PBS-T, the substrate solution was added and incubated at room temperature for 5 min. The reaction was stopped with Stop solution and the absorbance was measure at 450–550 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.

Statistical Analysis

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

Results

The CB1 and D2L Receptor Complex Involves the CB1 Carboxyl Terminus

We first conducted coIP experiments to verify a potential interaction between the D2 receptor and CB1 receptor. As shown in Fig. 1a, immunoprecipitation of the D2Long (D2L) receptor from HEK-293T cells co-expressing CB1 and D2L resulted in coIP of the CB1 receptor confirming that both receptors can be found in the same complex. We next sought to determine the region within the CB1 receptor that mediates the formation of the CB1–D2R complex by creating glutathione-S-transferase (GST) fusion proteins of intracellular loop 2 (GST-$CB1_{IL2}$ [G214-K234]), intracellular loop 3 (GST-$CB1_{IL3}$ [K302-T346]), and carboxyl tail (GST-$CB1_{CT}$ [R402-L474]) of the CB1 receptor to be used in affinity purification assays (Fig. 1b). As demonstrated in Fig. 1c, only GST-$CB1_{CT}$ but not GST-$CB1_{IL2}$, GST-$CB1_{IL3}$, or GST alone precipitated the D2L receptor from HEK-293T cell lysates expressing D2L receptor. These results suggest that the interaction between the CB1 and D2 receptor is mediated by the carboxyl tail (CT) region of the CB1 receptor. To delineate the region within CB1 CT involved in the CB1–D2R interaction, the CT was divided into four domains to produce GST fusion proteins that were truncations of the CB1 CT (GST-$CB1_{CT1}$ [R402-N426], GST-$CB1_{CT2}$ [C417-T441], GST-$CB1_{CT3}$ [D432-V456], GST-$CB1_{CT4}$ [A447-L474]). These GST fusion proteins were then purified and utilized in pull-down assay using lysates from D2L-transfected HEK-293T cells. As shown in Fig. 2a, GST-$CB1_{CT1}$ and GST-$CB1_{CT2}$ but not GST-$CB1_{CT3}$, GST-$CB1_{CT4}$, or GST alone precipitated the D2L receptor from cell lysates of HEK-293T expressing the D2L receptor. Both $CB1_{CT1}$ and $CB1_{CT2}$ are 25 amino acids in length and share identity with an overlapping domain of 10 amino acids. To verify that this overlapping domain is indeed involved in the CB1–D2R interaction, we created GST fusion protein that encodes the first 15 amino acids of $CB1_{CT2}$ (GST-$CB1_{CT2B}$ [C417-T431]), which includes the overlapping domain and 5 additional amino acids found within $CB1_{CT2}$. We also created a negative control GST fusion protein that consisted of the last 15 amino acids of the CT4

region (GST-$CB1_{CT4A}$ [K460-L474]). As shown in Fig. 2b when these peptides were purified and utilized in pull-down assays with protein extracts from D2L-transfected HEK-293T cells, the western blots show that GST-$CB1_{CT2B}$ but not 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 critical role in mediating the CB1–D2L receptor interaction. To provide an index for the relative amounts of GST protein used in each sample, blots were stained with Ponceau S prior to immunoblotting.

Direct Protein–Protein Interaction Between CB1 and D2L Receptors

The affinity purification assays indicate that there is an interaction between CB1 and D2L receptors; however, it is not clear whether it is a direct protein–protein interaction or whether there may be additional accessory proteins involved. To clarify whether the complex is formed by a direct or indirect interaction, His-tagged fusion proteins encoding the third intracellular loop of the long and short isoform of D2 receptor (HIS-D2LI3 [I211-Q373] and HIS-D2SI3 [I211-Q344]) were purified and used for in vitro binding experiments with purified GST-$CB1_{CT}$. In Fig. 3a, a western blot using GST antibody shows that HIS-D2LI3 and HIS-D2SI3 are both able to pull-down the purified GST-$CB1_{CT}$. Furthermore, this is specific to the GST-$CB1_{CT}$ as there was no precipitation of purified GST alone when used as prey (Fig. 3a, right panel). Together, this suggests that the alternative splice region within the D2R IL3 is not critical for the CB1–D2R interaction and that the interaction is not D2R isoform specific. We also attempted the in vitro binding experiment in reverse whereby the GST fusion protein was used as bait and the purified His-tagged protein was used as prey. Figure 3b (top panel) shows that only GST-$CB1_{CT}$ but not GST-$CB1_{IL2}$, GST-$CB1_{IL3}$, or GST alone is able to affinity-purify HIS-D2LI3. In addition, HIS-D2LI3 has greater affinity for GST-$CB1_{CT2B}$ compared to GST-$CB1_{CT2A}$, GST-$CB1_{CT4A}$, or GST alone. Together these data confirm that the third intracellular loop of the D2 receptor and $CB1_{CT2B}$ facilitate the CB1–D2R interaction as well as demonstrate that the complex formed between CB1 and D2L receptor can be mediated by a direct protein–protein interaction.

Interaction Between CB1-CT and D2LI3 Is Blocked by CT2B Region of the CB1 Receptor

Next, we wanted to develop a method that could disrupt the CB1–D2R interaction. Towards that goal, we created an inhibitory mini-gene encoding the $CB1_{CT2B}$ region of the CB1 receptor. The peptide expressed from this mini-gene should compete with the full-length CB1 receptor for binding to the D2 receptor and thereby inhibit the formation of the CB1–

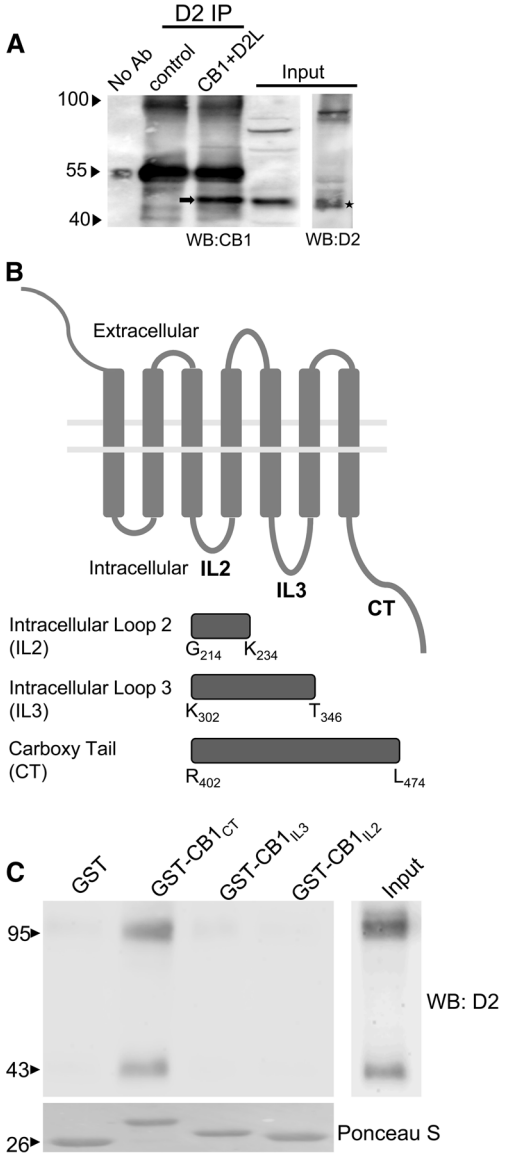

Fig. 1 Identification of an interaction between CB1 and D2L receptors. **a** Immunoprecipitation of the D2L receptor using 2.5 μg of the D2R antibody leads to the co-precipitation of CB1 receptor from 750 μg of solubilized HEK-293T cell lysates co-expressing D2L and CB1 receptors. Negative controls included immunoprecipitation in the absence of D2R antibody (No Ab) or in the absence of lysates (control). The *arrow* indicates the monomeric CB1 receptor, while the monomeric D2R is marked by a *star*. Gels were loaded with 50 μg of cell lysates to confirm the expression of both the D2L and CB1 receptors. In the D2 western blots, the 95- and 43-kDa bands represent the dimeric and monomeric forms of the D2L receptor, respectively. **b** Schematic of the CB1 receptor and an illustration of the various domains that were targeted for the creation of GST fusion proteins. GST fusion proteins encoding the second and third intracellular loops (GST-$CB1_{IL2}$, GST-$CB1_{IL3}$) and the carboxyl tail (CT) of the CB1 receptor (GST-$CB1_{CT}$) were generated and used in affinity purification assay. **c** For the affinity purification assay, solubilized lysates of HEK-293T cells expressing D2L (500 μg) were incubated with 40 μg of GST fusion proteins. Western blots for the D2L receptor revealed that the GST-$CB1_{CT}$ was able to purify both the dimeric (95 kDa) and monomeric (43 kDa) forms of D2L receptor. Immunoblots are representative of three to five independent experiments

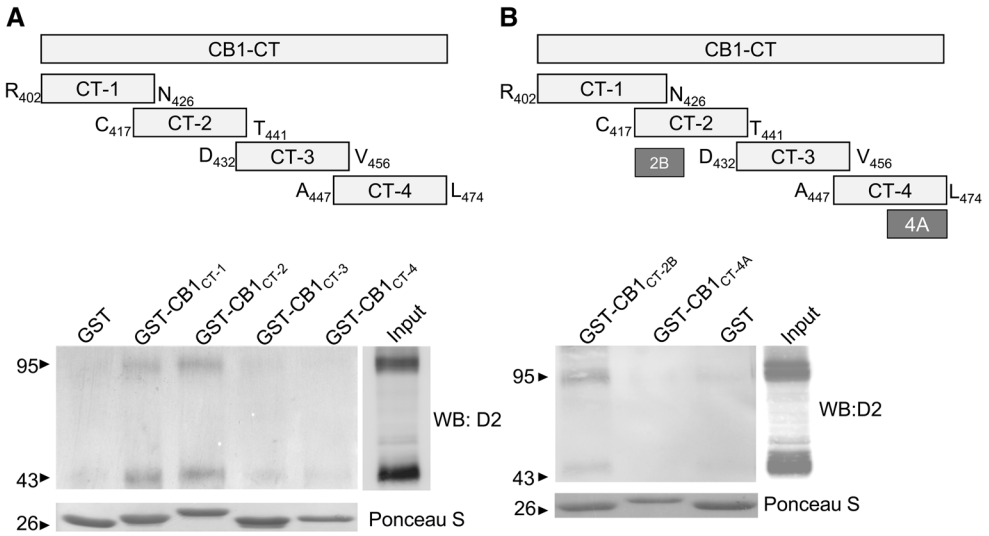

Fig. 2 Delineation of the interaction domain within the CT region of the CB1 receptor. **a** GST fusion proteins that are truncations of the CT region of the CB1 receptor were generated and utilized in affinity purification assay with lysates from D2L-transfected HEK-293T cells. Representative D2 receptor western blot identifies the region within the $CB1_{CT}$ that interacts with the D2L receptor; 750 μg of D2L lysates and 50 μg of GST fusion proteins were used in affinity purification assay; 50 μg of cell lysates were loaded as input. Ninety-five and 43 kDa bands present the dimeric and monomeric forms of the D2L receptor, respectively. **b** To further delineate the interaction region, GST fusion proteins containing the 10 amino acids overlap the region of $CB1_{CT1}$ and $CB1_{CT2}$ ($GST\text{-}CB1_{CT2B}$); and the last 15 amino acids of the CT4 region ($GST\text{-}CB1_{CT4A}$) were generated and utilized in affinity purification assay with protein extracts from D2L-transfected HEK-293T cells. Representative D2 receptor western blot identifies the region within the CB1-CT2 that interacts with D2L receptor; 500 μg of protein extracts from HEK-293T 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. Ninety-five and 43 kDa bands present the dimeric and monomeric forms of the D2L receptor, respectively. Immunoblots are representative of four independent experiments

D2R complex. To determine whether co-expressing the $CB1_{CT2B}$ mini-gene will block the CB1–D2R interaction, we performed coIP of CB1 with the D2 receptor using cells that had been co-transfected with the inhibitory mini-gene. Figure 4a shows that immunoprecipitation of the D2L receptor leads to the co-precipitation of CB1 receptor from HEK-293T cell lysates that were co-transfected with the empty expression vector pcDNA3. However, the coIP is significantly reduced in samples where the cells were co-transfected with the $CB1_{CT2B}$ mini-gene. Blots were also probed with mouse IgG antibodies to show equivalent amounts of antibody were used in each IP. Western blot of lysates shown in Fig. 4b demonstrates that the coIP results were not due to variations in the expression of the D2L and CB1 receptors. Analysis of the coIP western blots of three independent experiments indicates that the interaction between the CB1 and D2L receptors was decreased to 55 % of the control with the expression of the $CB1_{CT2B}$ mini-gene (Fig. 4c). The results indicate that the CB1–D2R interaction can be inhibited using a peptide that encodes the $CB1_{CT2B}$ region of the CB1 receptor.

cAMP Accumulation Inhibited by Co-activation of CB1 and D2L Receptors with the Presence of CB1-CT2B Mini-gene

Both CB1 and D2R can couple to Gαi proteins that inhibit cAMP production. To determine the functional effects of the CB1 and D2L receptor interaction, we measured cAMP levels in response to agonist treatment. To measure cAMP levels, we co-transfected cells with the epac1-camps plasmid that has previously been used as a biosensor to measure real-time cAMP levels in live cells (Nikolaev et al. 2004). Using HEK-293T cells co-transfected with CB1, D2L, and the epac1-camp plasmid, we imaged cells under a fluorescence microscope for a period of 20 min at room temperature. For these experiments, the amount of epac1-camps plasmid used was one tenth of the total DNA used in the transfection to increase the probability that any cells exhibiting fluorescence would also be co-expressing both the CB1 and D2L receptors. The epac1-camps biosensor exhibits FRET fluorescence when uncoupled to cAMP and the signal intensity is inversely related to cAMP levels. The FRET experiments were analyzed as a ratio between CFP/FRET intensities. Figure 5a shows the ratio images of an HEK-293T cell before and after the addition of 25 μM forskolin. Forskolin was added 2.5 min, after the start of imaging and the resulting increase in cAMP accumulation is represented by an increase in the FRET ratio (CFP/FRET). Figure 5b shows a scatter plot of a representative FRET experiment of HEK-293T cells co-expressing CB1, D2L, and epac1-camps. Cells pretreated with 5 μM methanandamide (M) or 50 nM bromocriptine (B) exhibit a significant decrease in cAMP levels in response to forskolin treatment. However, when cells were co-treated with 5 μM methanandamide and 50 nM bromocriptine (M/B), no

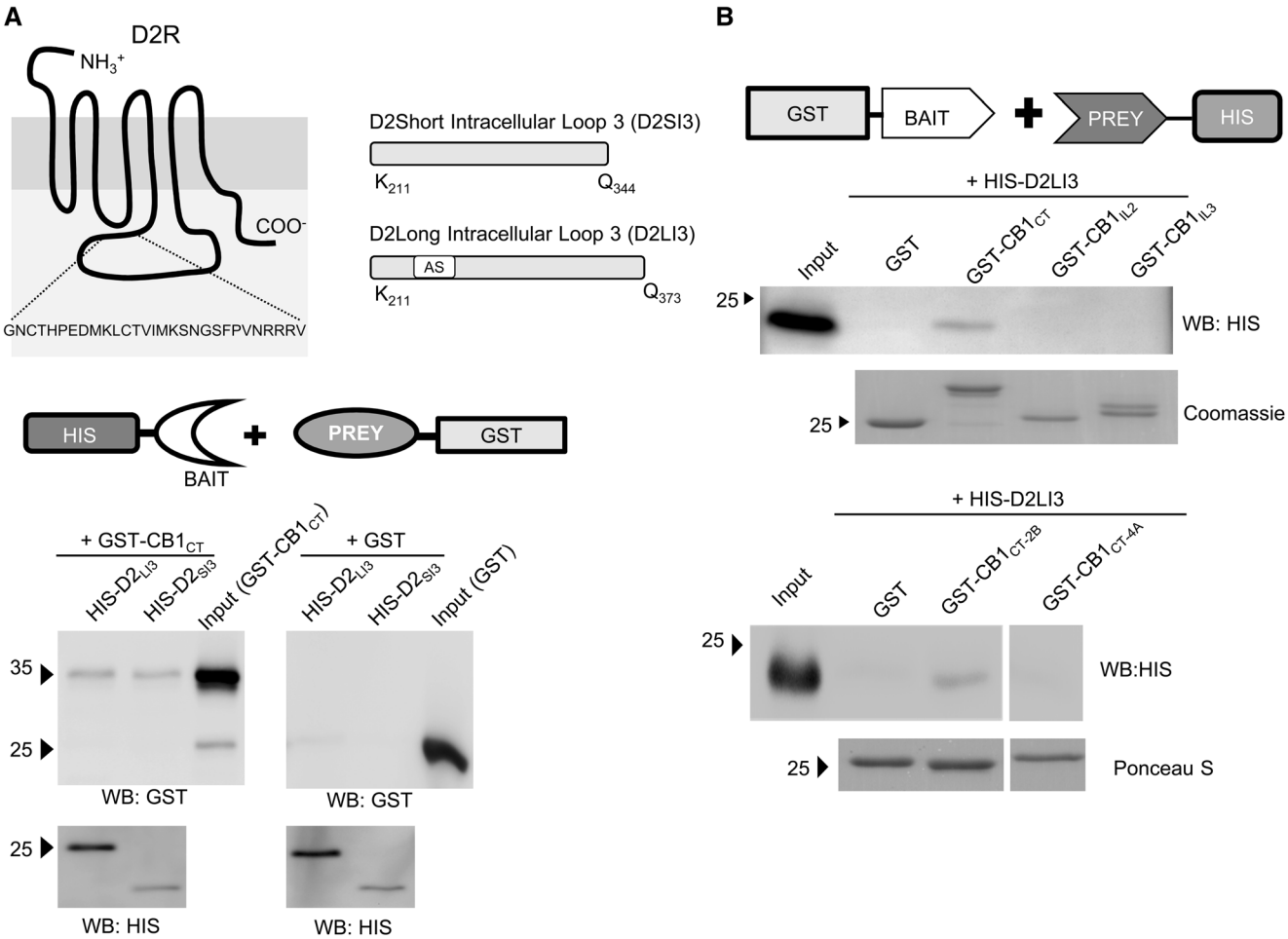

Fig. 3 Affinity purification of the CT region of the CB1 receptor by HIS-tagged intracellular loop 3 of the D2 receptor. **a** His-tagged fusion proteins encoding the third intracellular loop of the D2Long (D2LI3) or D2Short (D2SI3) isoforms were generated and used for in vitro binding experiments. Representative GST antibody western blot reveals that GST-$CB1_{CT}$ is precipitated by His fusion proteins that encode the third intracellular loop of the D2L and D2S receptor. In these in vitro binding experiments, 0.5 μg of purified GST fusion protein was incubated with 10 μg His fusion protein. Purified GST-$CB1_{CT}$ (0.5 μg) was loaded as positive control. **b** Representative His antibody western blot that indicates pull-down of HIS-D2LI3 by the GST-$CB1_{CT}$ (*upper panel*). For these in vitro binding experiments, 20 μg of purified GST fusion protein was incubated with 1 μg of purified HIS-D2LI3 fusion protein. Purified HIS-D2LI3 fusion protein (0.5 μg) was loaded as positive control. In addition, GST-$CB1_{CT2B}$ is also able to pull-down-purified HIS-D2LI3 (*lower panel*). In these in vitro binding experiments, 25 μg of GST fusion protein was incubated with 20 μg HIS-D2LI3 fusion protein. Immunoblots are representative of four to five independent experiments

decrease in forskolin-stimulated cAMP levels was observed. These concentrations of agonists were used since at higher concentrations, although individually they exhibited greater inhibition of forskolin-mediated cAMP production, the co-treatment effects were lost. When we measured cAMP levels using a cAMP immunoassay (R&D Systems), we observed the same decrease in cAMP levels when cells were treated with either 1 μM methanandamide or 10 μM bromocriptine (Fig. 5c) and a similar loss in this cAMP inhibition upon agonist co-treatment. To verify that this loss in receptor-mediated reduction in cAMP production is mediated by the CB1–D2R, we co-expressed the $CB1_{CT2B}$ mini-gene in cells transfected with CB1 and D2L. We have previously shown that co-expression of the $CB1_{CT2B}$ mini-gene leads to a disruption of the CB1–D2R complex (Fig. 4). As shown in Fig. 6a, in cells co-expressing the inhibitory $CB1_{CT2B}$ mini-gene, treatment with either methanandamide or bromocriptine alone leads to a reduction in forskolin-stimulated cAMP production. However, in contrast to cells co-expressing CB1 and D2L in the absence of the inhibitory mini-gene in which co-activation of both receptors leads to a loss in cAMP inhibition (Fig. 5b, c), agonist co-treatment in cells co-expressing the inhibitory $CB1_{CT2B}$ mini-gene continues to exhibit inhibition of forskolin-stimulated cAMP levels. Figure 6b shows the compiled results of five independent experiments taken at the 20-min time point to illustrate the loss of receptor-mediated cAMP inhibition upon co-activating the CB1 and D2L receptors and that this loss of receptor-mediated signaling can be rescued by disrupting the CB1–D2R complex.

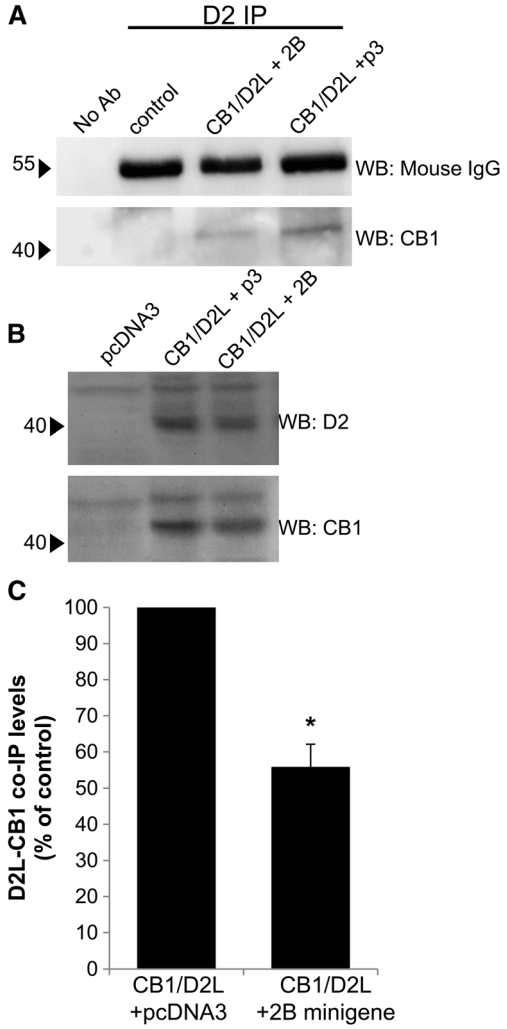

Fig. 4 Disruption of interaction between CB1 and D2L receptors. **a** Immunoprecipitation of the D2L receptor leads to the co-precipitation of CB1 receptor from 750 μg of HEK-293T cell lysates transfected with D2L and CB1 cDNA along with the empty expression plasmid pcDNA3 (CB1/D2L+p3). The interaction is blocked when cells are co-transfected with mini-gene encoding the $CB1_{CT2B}$ region of the carboxyl tail of the CB1 receptor (CB1/D2L+2B). Immunoprecipitation in the absence of D2R antibody (No Ab) or in the absence of lysates (control) was used as negative controls. After immunoblotting for CB1, membranes were stripped and re-probed with goat ant-mouse IgG antibody. **b** Western blot of cell lysates used in the IP experiment to confirm expression of CB1 and D2L. Gel was loaded with 50 μg of cell lysate. **c** Interaction between the CB1 and D2L receptors was decreased to 55 % of control (CB1/D2L+pCDNA3) with the presence of 2B mini-gene (*t* test, $*P<0.01$, $n=3$). Immunoblot is representative of three independent experiments

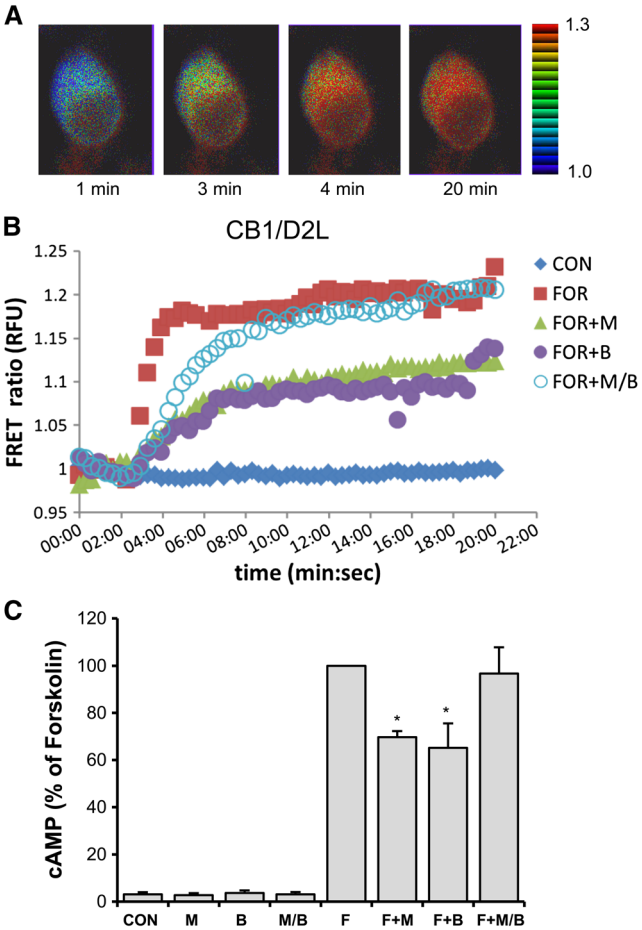

Fig. 5 Effects of CB1 or D2L receptor co-activation on cAMP accumulation. **a** Measurement of relative cAMP levels in HEK-293T cells co-transfected with the CB1, D2L, and the epac1-camps cAMP biosensor. Epac1-camps exhibits a FRET signal that is inversely related to the level of cAMP present in the cell (Nikolaev et al. 2004). Cells were imaged at 20 s intervals for 20 min and forskolin (25 μM final concentration) was added after imaging for 2–2.5 min. The addition of forskolin (FOR) resulted in an increase in cAMP accumulation which is represented by the increase in FRET ratio (CFP/FRET). **b** Scatterplot of representative FRET experiment of HEK-293T cells co-transfected with plasmids containing cDNA encoding CB1, D2L, and epac1-camps. Prior to the imaging session, cells were pretreated for 15 min at room temperature with either 5 μM methanandamide (M), 50 nM bromocriptine (B), or both agonists (M/B). Single activation of either the D2L or CB1 receptor led to decreased levels of forskolin-stimulated cAMP accumulation, which was absent when cells were treated with both agonists. **c** Loss of receptor-mediated cAMP inhibition by co-activation of CB1 and D2L receptor detected by cAMP immunoassay. Co-treatment with 1 μM methanandamide and 10 μM bromocriptine (30 min at 37 °C) does not decrease forskolin (10 μM)-stimulated cAMP accumulation in HEK-293T cells co-transfected with CB1 and D2L receptors as seen in individual agonist-treated cells ($*P<0.05$ vs forskolin; one-way ANOVA with post hoc SNK analysis, $n=4$, *error bars* represent SEM)

Discussion

Cross-talk of the cannabinoid and dopamine systems has important consequences as both systems play a role in learning, memory, and reward (Wise 2004; Riedel and Davies 2005; Zhu 2006; Solinas et al. 2008; Marsicano and Lafenetre 2009; Volkow et al. 2012). Several studies have shown that cannabinoids can induce DA release in the brain (Hattendorf et al. 1977; Bloom and Kiernan 1980; Diana et al. 1998). Furthermore, there is evidence that CB1 receptor stimulation can increase dopamine turnover and release in the striatum (Romero et al. 1995; Szabo et al. 1999) and that cannabinoid exposure can lead to changes in both dopamine receptor protein and mRNA levels (Higuera-

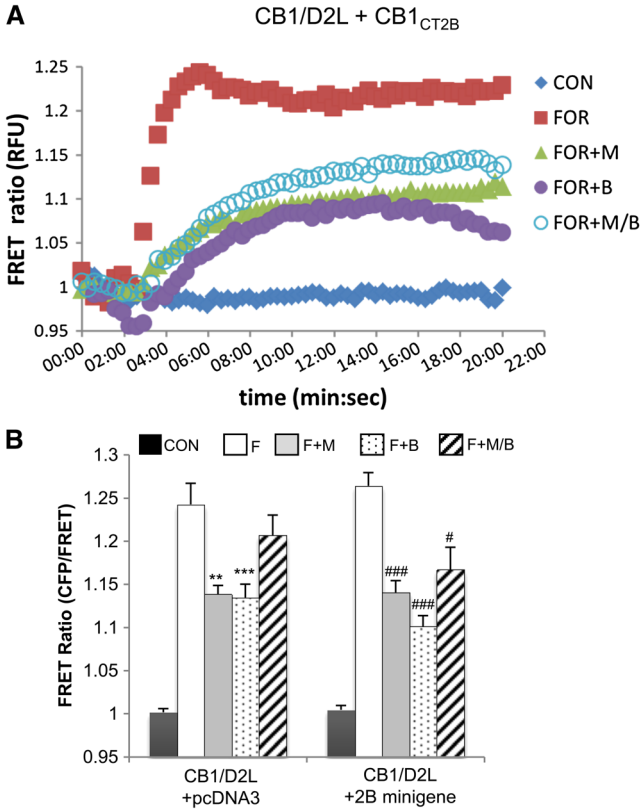

Fig. 6 cAMP accumulation inhibited by co-activation of CB1 and D2L receptors with the presence of $CB1_{CT2B}$ mini-gene. **a** Scatterplot of representative FRET experiment of HEK-293T cells co-transfected with CB1, D2L, and the $CB1_{CT2B}$ mini-gene. Co-treatment with both agonists (5 μM methanandamide (M) and 50 nM bromocriptine (B)) led to a similar decrease in forskolin-stimulated cAMP accumulation compared to cells treated with either bromocriptine or methanandamide alone. Adenylate cyclase activity was induced with 25 μM forskolin (F). cAMP levels were detected by co-transfecting with the epac1-camps cAMP biosensor at 1/10 of the total DNA for FRET analysis. FRET ratios (CFP/FRET) represent relative cAMP levels. **b** Bar plot summary of the cAMP biosensor data to compare the effects of $CB1_{CT2B}$ mini-gene co-expression compared to control cells (transfected with empty expression vector pcDNA3). In both conditions, single receptor activation led to a significant decrease in 25 μM forskolin-stimulated cAMP levels. However, in control cells, co-activation led to a loss of this cAMP inhibition. In cells co-transfected with $CB1_{CT2B}$ mini-gene, receptor co-activation continued to show inhibition of 25 μM forskolin-stimulated cAMP levels (**$P<0.01$, ***$P<0.001$ vs forskolin CB1/D2L/pcDNA3; $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs forskolin CB1/D2L/$CB1_{CT2B}$ mini-gene; one-way ANOVA with post hoc Tukey's multiple comparison test, $n=5$, *error bars* represent SEM)

Matas et al. 2010; Ginovart et al. 2012). Conversely, dopaminergic agonists have also been shown to increase endocannabinoid synthesis or release (Giuffrida et al. 1999; Patel et al. 2003; Centonze et al. 2004; Pan et al. 2008; Solinas et al. 2010). Moreover, a functional interaction between CB1 and D2 receptors has been implicated through behavioral studies that examined motor function (Meschler et al. 2000), learning (Zarrindast et al. 2010; Rodrigues et al. 2011), and social behavior (Zenko et al. 2011). At the cellular level, Glass and Felder described that co-activation of CB1 and D2R leads to the loss of cAMP inhibition that is normally observed with single receptor activation (Glass and Felder 1997). They further suggest that this may be facilitated by a switch in G protein-coupling from Gαi to Gαs, an observation that becomes more pronounced with pertussis toxin treatment. Similarly, chronic activation of D2 receptors appears to switch CB1 receptor coupling to Gαs (Jarrahian et al. 2004). Interestingly, there is evidence that the functional interaction between CB1 and D2 may be mediated by a direct protein–protein interaction between the two receptors (Kearn et al. 2005; Marcellino et al. 2008). Kearns et al. have shown co-immunoprecipitation of the CB1 and D2R in transfected cells and that agonist pretreatment leads to an increase in the coIP of the receptors. Through the use of fluorescently tagged CB1 and D2 receptors, Marcellino et al. observed FRET signals in cells co-expressing both receptors, providing evidence for a direct protein–protein interaction, although in this instance no changes in the CB1–D2R interaction could be observed with agonist treatment. In contrast, Przybyla and Watts have found that either CB1 or D2 receptor activation led to increases in the CB1–D2R interaction as measured through multicolor bimolecular fluorescence complementation (MBiFC) experiments (Przybyla and Watts 2010). Moreover, depending on which receptor was activated, this increase in the CB1–D2R interaction was accompanied by changes in subcellular localization of the receptor heterodimer. In our study, we have confirmed that the CB1–D2R interaction can be mediated by a direct protein–protein interaction. In addition, we have identified distinct domains within the CB1 and D2R that mediate this direct protein–protein interaction. However, in contrast to previous reports, we did not see a switch from Gαi to Gαs as a consequence of the CB1–D2R interaction. Although cell line differences could explain this discrepancy (Glass and Felder 1997), it would not explain it entirely as we used the same cell line employed by other groups (Kearn et al. 2005; Jarrahian et al. 2004). In our hands, co-expression of CB1 and D2L did not lead to any appreciable increase in cAMP levels measured through either the cAMP immunoassay or using the cAMP biosensor. Nonetheless, while single receptor activation (either CB1 or D2L) led to decreases in forskolin-mediated cAMP levels, we observed a loss in cAMP inhibition upon receptor co-activation, similar to the results observed in previous studies (Glass and Felder 1997). As shown in Fig. 5b, using the cAMP biosensor epac1-camps to index relative cAMP levels, we observed a loss in cAMP inhibition with agonist co-treatment in cells co-expressing CB1 and D2L. Moreover, we provide additional evidence that the CB1–D2R interaction is facilitating the loss in cAMP inhibition by using inhibitory mini-genes that can disrupt the coIP of CB1 and D2R (Fig. 4c). Co-expression of the inhibitory mini-gene ($CB1_{CT2B}$), which can disrupt the CB1–D2R protein–protein interaction, led to a loss in the functional effects seen by receptor co-activation and facilitates a decrease in

forskolin-mediated cAMP levels, similar to cAMP levels observed with single receptor activation (Fig. 6a, b). Although the effect of the $CB1_{CT2B}$ mini-gene is statistically significant from the forskolin group, when the compiled data is compared to the co-treated samples transfected with CB1, D2L, and pcDNA3, the difference is marginal. Although we made efforts to increase the probability that the cells we chose for our analysis would be expressing the inhibitory $CB1_{CT2B}$ mini-gene, there is the possibility that a proportion of the cells chosen in the image analysis may not be expressing the mini-gene peptide at all or at very low levels, which would mask the functional effect of the $CB1_{CT2B}$. In fact, when we examined the disruption of the CB1–D2R complex by the mini-gene through coIP, the mini-genes decrease the coIP of D2R with CB1 by ~45 % (Fig. 4). We predict that using alternative methods to express or deliver the inhibitory peptide at a greater efficiency, or to selectively choose cells that are expressing high levels of the mini-gene, would lead to a more profound effect of the inhibitory peptide on cAMP production. However, with this caveat in mind, we also found that mini-gene co-expression does not affect forskolin-mediated cAMP levels after single receptor activation, which indicates that the $CB1_{CT2B}$ mini-gene does not disrupt normal interactions between Gαi with either the CB1 or D2R. We therefore conclude that that this loss in cAMP inhibition is dependent, at least in part, by the formation of the CB1–D2R heterodimer. Moreover, it appears that both receptor co-expression and co-activation is required to induce this functional uncoupling to cAMP inhibition since cells co-expressing both receptors but only treated with a single agonist maintains normal inhibition of forskolin-mediated increases in cAMP levels. We speculate that receptor occupancy of both CB1 and D2R leads to conformational changes in the CB1–D2R complex that leads to reduced coupling with Gαi. While receptor activation may not be absolutely required for the formation of the CB1–D2R complex, receptor co-activation may promote the formation of the heterodimer. In fact, Kearn et al. have shown that co-treatment with the CB1 agonist CP55,940 and D2 agonist quinpirole leads to an increase in the levels of the CB1–D2R complex as indexed through coIP (Kearn et al. 2005). Similarly, Przybyla and Watts (2010) have also shown through MBiFC assays that receptor activation increases heterodimer formation. Nevertheless, we cannot definitively rule out alternative explanations as to why co-activation is required to observe the functional effects of the CB1–D2R complex. One possibility is that the CB1–D2R complex may lead to changes in agonist affinities, and thus, single agonist treatment may preferentially activate populations of receptors that are not found in the heterodimer complex. The report by Kearn et al. would suggest that it is unlikely since they demonstrate no change in affinities for CB1 or D2 ligands in cells co-transfected with both receptors (Kearn et al. 2005). However, another report suggests that there may be slight changes in agonist low and high affinity sites upon receptor co-activation (Marcellino et al. 2008). Regardless, it will be important to determine whether changes in Gαi coupling occur in environments that favor the formation of the CB1–D2R complex.

The functional cross-talk between CB1 and D2R has also been implicated in receptor knockout studies. Houchi et al. have shown that CB1 knockout mice have elevated levels of D2 receptor as indexed by radioligand binding experiments (Houchi et al. 2005). Moreover, Fitzgerald et al. suggest that D2R trafficking is affected by the absence of CB1 receptors as CB1 knockout mice exhibit changes in subcellular localization of the D2R (Fitzgerald et al. 2012). Similarly, D2-null mice have also shown changes in CB1 function. Thanos et al. have shown a ~2-fold increase in CB1 levels in various brain regions of the D2 knockout mice (Thanos et al. 2011). In addition to the D2R, there have been other proteins that have been identified to interact with CB1 including β-arrestin, CRIP1a/CRIP1b, and GASP1 (Jin et al. 1999; Bakshi et al. 2007; Martini et al. 2007; Niehaus et al. 2007). β-Arrestin facilitates the internalization of the CB1 after activation and subsequent receptor phosphorylation (Jin et al. 1999; Bakshi et al. 2007). Interestingly, the domain involved in the CB1/β-arrestin interaction overlaps with the domain identified in this study that is important for the CB1–D2R interaction. CRIP1a appears to reduce the constitutive activity of CB1 receptors by altering CB1 subcellular localization (Niehaus et al. 2007), while the interaction with GASP1 modulates CB1 receptor post-endocytic sorting (Martini et al. 2007). Furthermore, the CB1 receptor has been shown to form heterodimers with other GPCR proteins, including adenosine A2A (Carriba et al. 2007; Navarro et al. 2008), μ-, κ-, and δ-opioid (Rios et al. 2006; Hojo et al. 2008), and orexin-1 (Ellis et al. 2006) receptors. Similarly, the D2R has also shown to form heterodimers with other GPCR including SST5, A2A, and D1 receptors (Rocheville et al. 2000; Canals et al. 2003; So et al. 2005). In addition, the D2R has also shown to interact with other non-GPCR proteins including spinophilin, calmodulin, β-arrestin, NCS-1, GASP, N-ethylmaleimide-sensitive factor, DAT, and ZIP, to name a few (Smith et al. 1999; Bofill-Cardona et al. 2000; Kim et al. 2001, 2008; Kabbani et al. 2002; Bartlett et al. 2005; Zou et al. 2005; Lee et al. 2007). Therefore, future studies involving the CB1–D2R complex may investigate the role of other protein–protein interactions in regulating the cross-talk between CB1 and D2R.

The functional cross-talk between the dopamine and endocannabinoid systems has been suggested to be involved in numerous brain disorders including Parkinson's disease, Huntington's disease, and drug addiction (van der Stelt and Di Marzo 2003; Gardner 2005; Laviolette and Grace 2006; Maccarrone et al. 2007; Pisani et al. 2011; El Khoury et al. 2012). Thus, characterization and delineation of the domains involved in the CB1–D2R interaction may prove to be important in determining how this interaction contributes to the pathophysiology of various brain diseases.

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