

# $\mu$ opioid and CB1 cannabinoid receptor interactions: reciprocal inhibition of receptor signaling and neuritogenesis

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**1** Several studies have described functional interactions between opioid and cannabinoid receptors; the underlying mechanism(s) have not been well explored. One possible mechanism is direct receptor–receptor interactions, as has been demonstrated for a number of G-protein-coupled receptors.

**2** In order to investigate interactions between opioid and cannabinoid receptors, we epitope tagged  $\mu$ ,  $\delta$  and  $\kappa$  opioid receptors with *Renilla* luciferase and CB1 cannabinoid or CCR5 chemokine receptors with yellow fluorescent protein and examined the extent of substrate hydrolysis induced bioluminescence resonance energy transfer (BRET) signal.

**3** We find that coexpression of opioid receptors with cannabinoid receptors, but not with chemokine receptors, leads to a significant increase in the level of BRET signal, suggesting that the opioid–cannabinoid interactions are receptor specific.

**4** In order to examine the implications of these interactions to signaling, we used GTP $\gamma$ S binding and mitogen-activated protein kinase (MAPK) phosphorylation assays and examined the effect of receptor activation on signaling.

**5** We find that the  $\mu$  receptor-mediated signaling is attenuated by the CB1 receptor agonist; this effect is reciprocal and is seen in heterologous cells and endogenous tissue expressing both receptors.

**6** In order to explore the physiological consequences of this interaction, we examined the effect of receptor activation on the extent of Src and STAT3 phosphorylation and neuritogenesis in Neuro-2A cells.

**7** We find that the simultaneous activation of  $\mu$  opioid and CB1 cannabinoid receptors leads to a significant attenuation of the response seen upon activation of individual receptors, implicating a role for receptor–receptor interactions in modulating neuritogenesis.

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**Abbreviations:** AFM, atomic force microscopy; BRET, bioluminescence energy transfer; CCR5, chemokine receptor 5; CHAPS, 3-[3-cholamidopropyl-1]dimethylammonio-1-propanesulfonate; DAMGO, (D-Ala<sup>2</sup>,Nme-Phe<sup>4</sup>,Gly-ol<sup>5</sup>)-enkephalin; DMEM, Dulbecco's modified Eagle's media; GPCR, G-protein-coupled receptor; Luc, luciferase; MAPK, mitogen-activated protein kinase; THC,  $\Delta^9$ -tetrahydrocannabinol; YFP, yellow fluorescent protein

## Introduction

Opioid and cannabinoid receptors share a number of common characteristics; they belong to the rhodopsin subfamily of G-protein-coupled receptor (GPCR) superfamily and transduce signals through activation of G<sub>i/o</sub> proteins that lead to the inhibition of adenylyl cyclase activity, Ca<sup>2+</sup> channel activity and neurotransmitter release (Dhawan *et al.*, 1996; Howlett *et al.*, 2002; Cichewicz, 2004). Activation of these receptors induces a variety of systemic responses such as analgesia, euphoria and decreased intestinal motility (Dhawan *et al.*, 1996; Howlett *et al.*, 2002). Both receptors are targets for drugs of abuse (heroin at  $\mu$  receptors and marijuana at CB1 receptors); therefore, an understanding of the mechanisms modulating their activities is of significant clinical interest.

Interactions between opioid and cannabinoid receptors appear to modulate their activity as evidenced by behavioral studies using selective agonists (Manzanares *et al.*, 1999; Cichewicz, 2004) or mice lacking each of these receptors (Ledent *et al.*, 1999; Ghozland *et al.*, 2002). Although these studies demonstrate functional interactions between opioid and CB1 receptors, the underlying mechanism(s) have not been thoroughly explored. In this study, we examined if these interactions could be due, at least in part, to direct receptor–receptor interactions.

GPCRs have been shown to associate with each other (homodimers) or with members of a related family (heterodimers) and this leads to alterations in receptor function (Rios *et al.*, 2001). Recent atomic force microscopy (AFM) studies with rhodopsin show that these receptors exist in dimeric arrays in native membranes (Fotiadis *et al.*, 2004). X-ray crystallographic studies with the extracellular domain of metabotropic glutamate receptors show that they exist as covalently bonded dimers and this is important for agonist

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binding and receptor activation (Kunishima *et al.*, 2000; Kniazeff *et al.*, 2004). Mass spectrometry studies after chemical crosslinking and neutron scattering in solution with leukotriene B<sub>4</sub> receptors show that the dimeric receptor associates with one G-protein trimer to form a pentameric assembly (Banères & Parello, 2003). Finally, crosslinking studies with D2 dopamine receptors demonstrate that the N-terminal region of transmembrane IV forms a symmetric dimer interface that plays a role in receptor activation (Guo *et al.*, 2003; 2005). Taken together, these studies imply that dimerization is an integral property of many GPCRs.

Heterodimerization between GPCR types has been shown to significantly alter their activities (Rios *et al.*, 2001). This was first demonstrated with GABA<sub>B</sub> receptors where physical associations between the R1 and R2 subunits was found to be a prerequisite for cell surface expression and full receptor activity (White *et al.*, 1998; Gassmann *et al.*, 2004). This was followed by a series of studies showing that associations between closely related as well as distantly related GPCRs could modulate their activity to varying degrees (Angers *et al.*, 2002).

We and others have shown that opioid receptors are also able to heterodimerize with closely related subfamily members and with distantly related family members (adrenergic receptors); these interactions significantly alter their properties (Jordan *et al.*, 2001; 2003; Gomes *et al.*, 2004). Thus, it is possible that opioid receptors associate with other members of Family A GPCRs and such associations modulate receptor activity.

We examined the cannabinoid CB1 receptor as a potential partner for association with opioid receptors, as CB1 receptors have been shown to associate with each other to form homodimers (Wager-Miller *et al.*, 2002). Furthermore, they colocalize with  $\mu$  receptors in dendritic spines in the caudate putamen and dorsal horn of the spinal cord (Rodriguez *et al.*, 2001; Salio *et al.*, 2001; Pickel *et al.*, 2004). In this study, we examined interactions between  $\mu$  and CB1 receptors and their functional implication using a variety of assays including GTP $\gamma$ S binding, MAPK phosphorylation and neurite outgrowth. Our results, showing that direct interactions between these receptors impact on signaling, are consistent with a role for heterodimerization in modulating crosstalk between these receptors.

## Methods

### Cell culture and transfections

Human embryonic kidney 293 (HEK-293), Neuro-2A or human neuroblastoma cells (SK-N-SH) were grown in DMEM containing 10% FBS and strepto-penicillin. For BRET studies, HEK-293 cells were transfected, using Lipofectamine as per the manufacturer's specifications (Invitrogen, Carlsbad, CA, U.S.A.), with either (1–5  $\mu$ g) CB1-Luc alone or in combination with (1–5  $\mu$ g)  $\mu$ -,  $\delta$ -,  $\kappa$ -YFP or CCR5-YFP and analyzed approximately 48 h after transfection as described in the next section. For G-protein activation and MAP kinase phosphorylation assays, HEK-293 cells were transfected with cDNAs (3  $\mu$ g) for either Flag-tagged  $\mu$ , *myc*-tagged CB1 or CCR5-YFP receptors alone or in combination and analyzed approximately 48 h after transfection as described previously

(Jordan *et al.*, 2001; Gomes *et al.*, 2003). The level of receptor expression was determined by ligand binding to ensure that each receptor was expressed at comparable levels (see receptor ligand binding). Neuro-2A cells stably expressing mouse  $\mu$  opioid receptors were generated by transfecting the cells with 5  $\mu$ g Flag-tagged  $\mu$  opioid receptor cDNA using Lipofectamine, and colonies with stable expression were selected using geneticin (G418, 500  $\mu$ g ml<sup>-1</sup>). Expression of Flag-tagged  $\mu$  receptors was detected by ELISA using anti-Flag antibodies (Gomes *et al.*, 1999).

### Construction of plasmids and BRET assays

The  $\mu$ ,  $\delta$ , and  $\kappa$  opioid receptors as well as CB1 cannabinoid receptors with mutated stop codons were subcloned into the pLuc-N3 (Perkin-Elmer Life Sciences, Boston, MA, U.S.A.) and pEYFP-N1 (BD Biosciences Clontech, Palo Alto, CA, U.S.A.) plasmids, such that *Renilla* luciferase (Luc) and yellow fluorescent protein (YFP) were present at the C-termini of the receptors. The chemokine CCR5 receptor tagged with YFP (CCR5-YFP) at the C-terminus was a kind gift from Dr Michel Bouvier (University of Montreal). All sequences were confirmed by DNA sequencing. After 48 h, cells (transfected with CB1-Luc alone or in combination with  $\mu$ -,  $\delta$ -,  $\kappa$ - or CCR5-YFP) were washed with PBS, suspended to 1–2  $\times 10^6$  cells ml<sup>-1</sup> and were treated with coelenterazine (5  $\mu$ M final concentration). Light emission was monitored with a closed excitation slit every 0.5 s from 420 to 590 nm at 5 nm intervals by using a FluoroMax-2 spectrometer. A BRET signal is defined as the light emitted by YFP at 530 nm in response to the light emitted at 470 nm upon catalysis of coelenterazine h. For experiments examining the effect of differential expression of CB1-Luc and opioid-YFP receptors on BRET signals, the level of receptor expression was 500 fmol mg<sup>-1</sup> protein for CB1-Luc and 200–5000 fmol mg<sup>-1</sup> protein for  $\mu$ -,  $\delta$ - or  $\kappa$ -YFP as determined by receptor ligand binding (see below).

### Receptor ligand binding

Approximately 5  $\times 10^5$  HEK-293 cells expressing either CB1 or  $\mu$ ,  $\delta$  or  $\kappa$  receptors alone or in combination were incubated with increasing concentrations of [<sup>3</sup>H]-diprenorphine or [<sup>3</sup>H]-SR141716A in 50 mM Tris-Cl pH 7.5. containing 0.1% BSA and in a final volume of 1 ml for 1 h at 37°C. Nonspecific binding was determined in the presence of 1  $\mu$ M diprenorphine or SR141716A. The cells/membranes were collected on Whatman GF/B (Schleicher and Schuell, Keene, NH, U.S.A.) filters with a Brandel cell harvester (Brandel, Gaithersburg, MD, U.S.A.). Filters were washed three times with ice-cold 50 mM Tris-Cl pH 7.5 and radioactivity detected using a scintillation counter. Data were analyzed using Prism 2.0 (Graph Pad, San Diego, CA, U.S.A.).

### [<sup>35</sup>S]-GTP $\gamma$ S binding assay

SK-N-SH or HEK-293 cells expressing  $\mu$  receptors alone or in combination with CB1 receptors were used in a permeabilized [<sup>35</sup>S]-GTP $\gamma$ S binding assay as described previously (Gomes *et al.*, 2003). Briefly, cells were permeabilized with 0.5% 3-(3-cholamidopropyl-1-dimethylammonio)-1-propanesulfonate (CHAPS) in GTP $\gamma$ S assay buffer (50 mM Tris-Cl, pH 7.5, 5 mM

MgCl<sub>2</sub>, 100 mM NaCl, 0.2 mM EGTA) for 30 min at 37°C. Permeabilized cells (representing ~50 µg of protein) or 10 µg of rat striatal membranes prepared as described by Gomes *et al.* (2003) (3 to 4-month-old Sprague–Dawley rats were used and experimental procedures were carried out according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee) were washed with the GTPγS assay buffer and incubated in the same buffer containing 100 µM GDP, 0.1 nM [<sup>35</sup>S]-GTPγS and 0–10 µM of morphine or DAMGO in the absence or presence of 10 nM WIN 55,212-2 in a final volume of 1 ml. After incubation for 1 h at 30°C, the cells or membranes were collected on Whatman GF/B filters (Schleicher and Schuell, Keene, NH, U.S.A.) with a Brandel cell harvester (Brandel, Gaithersburg, MD, U.S.A.), washed three times with ice-cold 50 mM Tris-Cl pH 7.5 and radioactivity detected using a scintillation counter. Data were analyzed using Prism 2.0 (Graph Pad, San Diego, CA, U.S.A.). For the reciprocal experiment, permeabilized cells were treated with 0–10 µM of WIN in the absence or presence of 10 nM DAMGO. EC<sub>50</sub> and E<sub>max</sub> values were determined by Prism 2.0 (GraphPad, San Diego, CA, U.S.A.).

#### MAP kinase assay

Approximately 1 × 10<sup>5</sup> HEK-293 cells expressing either µ receptors alone or in combination with CB1 or CCR5 receptors were cultured on 24-well plates in DMEM containing 10% FBS. The complete media were replaced with serum-free media (DMEM with no FBS) 24 h before the experiment. Drugs (1 µM) were added to the wells for 5 min and the assay terminated by removal of media and addition of 2% SDS in 50 mM Tris-Cl, pH 6.8. Samples were collected and subjected to SDS–PAGE and Western blot analysis with 1 µg ml<sup>-1</sup> E10-Phospho-MAP kinase (Cell Signaling Technologies, Beverly, MA, U.S.A.) and 1:15,000 dilution of anti-tubulin (Sigma, St Louis, MO, U.S.A.) antibodies as described (Jordan *et al.*, 2001). Both bands corresponding to ERK1 and ERK2 were densitized by the use of NIH image software (version 6.2) and normalized to tubulin.

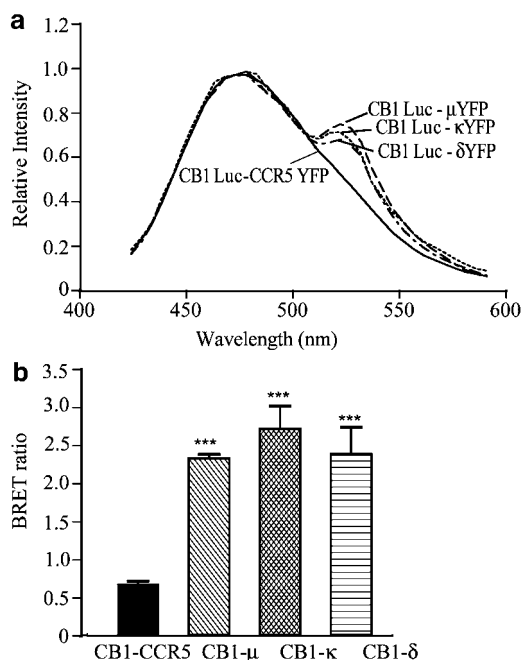
#### Neurite outgrowth assay and Western blotting for phospho-Src and phospho-STAT3

Neuro 2A cells stably expressing Flag-tagged µ opioid receptors (~20,000 cells well<sup>-1</sup>) were plated onto poly-L-lysine-coated 12-well plates in DMEM (+10% FBS). On the following day, the media were substituted by DMEM (-FBS) and cells were treated with 0–10 µM DAMGO, morphine (µ agonists), Hu-210 (CB1 agonist) or a combination for 16 h at 37°C. Three non-overlapping regions of the plate containing 100 cells each were scored under a phase contrast microscope (Nikon TMS). Cells were scored as positive for neurite outgrowth as described by Fricker *et al.* (2005). For the determination of phospho-Src and phospho-STAT3 levels, Neuro 2A cells stably expressing Flag-tagged µ opioid receptors (2 × 10<sup>5</sup> cells well<sup>-1</sup>) were plated in 24-well plates in DMEM containing 10% FBS. After the cells had attached, they were grown in the absence of FBS for 24 h followed by treatment with indicated doses of morphine, Hu-210 or a combination of both for 30 min at 37°C. Cells were lysed in 50 mM Tris-Cl, pH 6.8 containing phosphatase and protease

inhibitor cocktails (Sigma Chemical Co., St Louis, MO, U.S.A.) and aliquots (~30 µg protein) were subjected to Western blotting analysis using anti-phospho Y705 STAT3 (1:2000, Cell Signaling Technology), anti-phospho Y416-Src (1:2000, Cell Signaling Technology), anti-Src (1:1000, Cell Signaling Technology) or anti-STAT3 (1:2000, Cell Signaling Technology) antibodies. Blots were densitized using NIH Image software.

## Results

A number of behavioral studies have previously demonstrated functional interactions between opioid and cannabinoid receptors; the mechanism(s) underlying these are not clearly understood. One possible mechanism could be direct interactions between these two receptor types. To foster such interactions, the receptors have to be in close proximity (<100 Å). We examined this possibility by using the proximity-based BRET assay in HEK-293 cells that were cotransfected with Luc-tagged CB1 receptors and YFP-tagged µ, δ or κ opioid or CCR5 chemokine receptors (Figure 1). We find a significant increase in the BRET signal in cells coexpressing CB1-Luc with µ-, δ- or κ-YFP receptors but not with



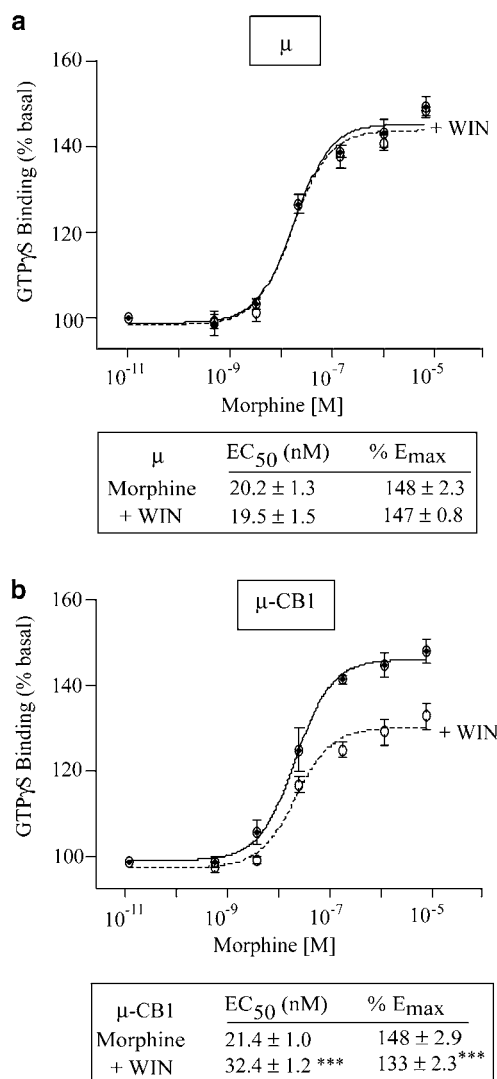
**Figure 1** Opioid and CB1 cannabinoid receptor interactions in live cells. (a) Light emission was monitored from HEK-293 cells cotransfected with 1 µg of CB1-Luc and either 1 µg of µ-YFP (dashed line), κ-YFP (dotted line), δ-YFP (hatched line) or 1 µg of CCR5-YFP (solid line). The BRET assay was carried out as described in Methods. The peak of light emission by luciferase is seen at 470 nm and the peak resulting from the BRET between CB1-Luc and opiate-YFP-tagged receptors is seen at 530 nm. A representative sample of an experiment is shown. (b) The light intensities were expressed as a BRET ratio (ratio of the intensity of light emitted at 530 nm versus the intensity of light emitted at 470 nm) for each experimental paradigm compared to cells expressing only CB1-Luc. Statistically significant differences were determined by one-way ANOVA. \*\*\**P* < 0.001 (*n* = 15–18; *F* = 9.731; *R* squared = 0.3989).

chemokine CCR5-YFP receptors (Figure 1). The BRET signal was not owing to receptor overexpression, as the BRET ratio was not altered when cells were cotransfected with varying levels of CB1-Luc and opioid receptor-YFP cDNAs (data not shown); the level of receptor expression was 200–500 fmol mg<sup>-1</sup> protein for CB1-Luc and 200–5000 fmol mg<sup>-1</sup> protein for opioid receptors, as determined by receptor binding. In addition, agonist treatment did not significantly alter the observed BRET ratios (data not shown). Taken together, the studies support the notion that opioid receptors interact with CB1 receptors in live cells with fairly low levels of receptor expression and that these interactions exhibit receptor type selectivity.

To explore the implications of  $\mu$  and CB1 receptor interactions on signaling, we examined the agonist-mediated G-protein activation in heterologous HEK-293 cells expressing either  $\mu$  or  $\mu$ -CB1 receptors. The agonist-mediated activation of G proteins was monitored using the radiolabeled non-hydrolyzable analog of GTP, [<sup>35</sup>S]-GTP $\gamma$ S (Gomes *et al.*, 2003). We find that morphine treatment leads to a dose-dependent increase in [<sup>35</sup>S]-GTP $\gamma$ S binding in cells that express only  $\mu$  receptors (Figure 2a). The coexpression of CB1 receptors does not alter the magnitude of basal levels or morphine-mediated [<sup>35</sup>S]-GTP $\gamma$ S binding; however, treatment with a low non-signaling dose (10 nM) of the CB1 agonist, WIN, leads to an attenuation (~31%) of morphine-mediated [<sup>35</sup>S]-GTP $\gamma$ S binding (Figure 2b). This effect is not seen in cells expressing only  $\mu$  receptors (Figure 2a). Taken together, these results suggest that the occupancy of CB1 receptors has an antagonistic effect on  $\mu$  receptor-mediated G-protein activation.

Next, we examined if the modulation of receptor activity seen in HEK-293 cells expressing  $\mu$ -CB1 receptors can be observed using another GPCR signaling assay. For this, we monitored the extent of phosphorylation of extracellular signal-regulated kinases (ERK1/2) in response to morphine in the absence or presence of treatment with WIN. We find that treatment with morphine or WIN (1  $\mu$ M) leads to a significant increase in the extent of phosphorylation of ERK1/2 in cells expressing  $\mu$  receptors alone (Figure 3a) or with CB1 receptors, respectively (Figure 3b). Treatment with a combination of agonists (morphine and WIN) leads to a significant decrease in the extent of ERK1/2 phosphorylation in cells coexpressing both receptors (Figure 3b). As a control, we examined the effect of expression of  $\mu$  and CCR5 receptors on ERK1/2 phosphorylation. We find that treatment with morphine or RANTES (a CCR5 receptor agonist) leads to an increase in ERK1/2 phosphorylation (Figure 3c and d). However, treatment with a combination of agonists (morphine and RANTES) does not significantly affect the level of morphine- or RANTES-induced ERK1/2 phosphorylation (Figure 3d). These results together with the results from our BRET and [<sup>35</sup>S]-GTP $\gamma$ S binding assays support the hypothesis that  $\mu$  and CB1 receptors exhibit receptor selectivity in their interactions and these interactions appear to be antagonistic in nature.

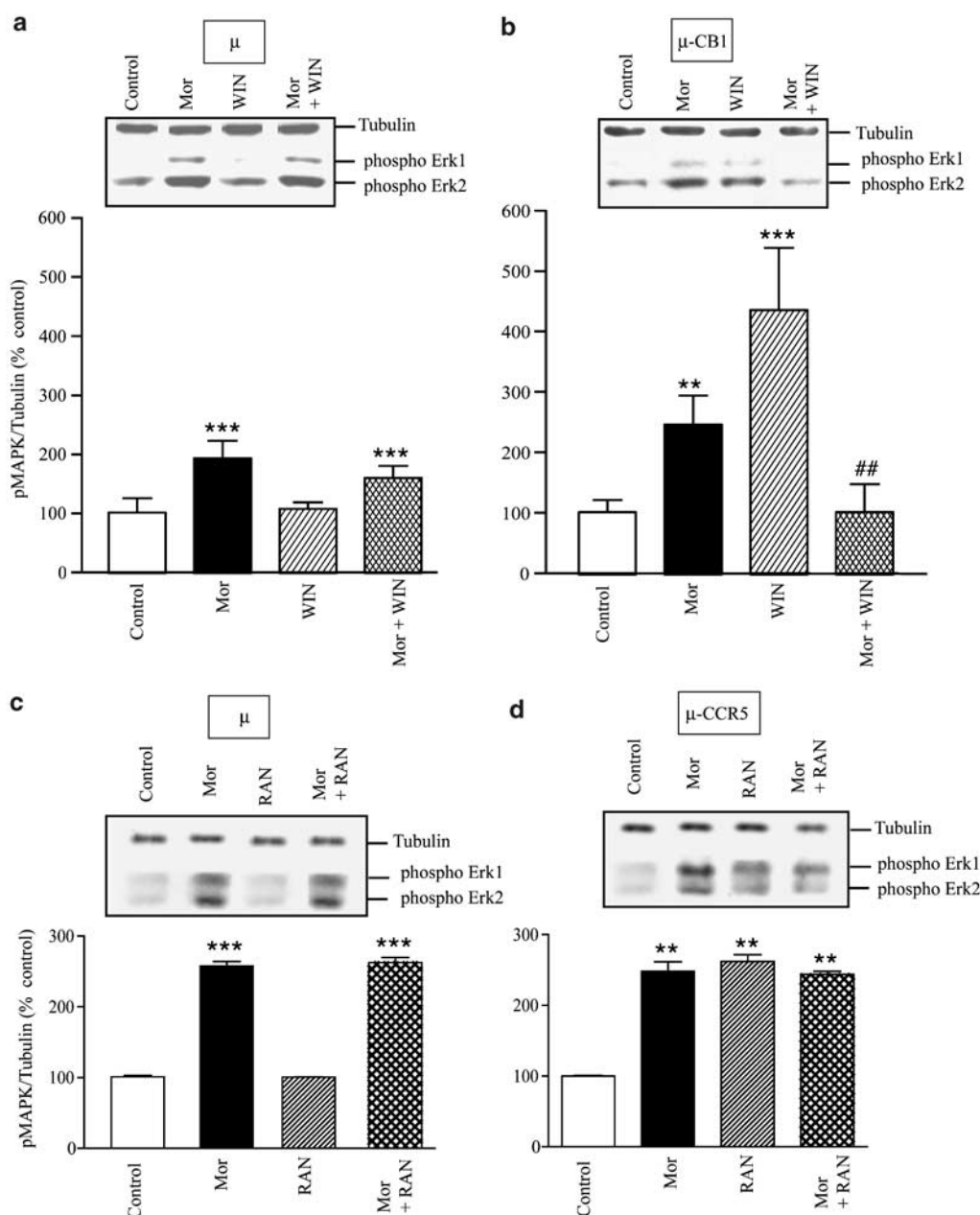
Next, we examined if the  $\mu$ -CB1 interactions could also be seen in cells that endogenously express  $\mu$  and CB1 receptors such as SK-N-SH neuroblastoma cells. In this set of studies, we used the  $\mu$  agonist, DAMGO, to selectively activate  $\mu$  receptors, as these cells also express  $\delta$  receptors; morphine at high doses can activate  $\delta$  receptors (Hochhaus *et al.*, 1986). We



**Figure 2** Signaling in cells expressing  $\mu$  or  $\mu$ -CB1 receptors. HEK-293 cells expressing  $\mu$  receptors alone (a) or coexpressing  $\mu$  and CB1 receptors (b) were permeabilized and subjected to a [<sup>35</sup>S]-GTP $\gamma$ S binding assay (see Methods) with the indicated (0–10<sup>-5</sup> M) concentrations of morphine in the absence or presence of 10 nM WIN 55,212-2. The EC<sub>50</sub> and E<sub>max</sub> changes were determined by GraphPad Prism and are indicated in the box below each graph. The data represent mean ± s.e.m. (*n* = 12). Statistically significant differences were determined by the Student's *t*-test. \*\*\**P* < 0.001 (*n* = 12).

find that DAMGO-mediated increase in G-protein activation is significantly attenuated (~61% as compared to DAMGO alone) by cotreatment with 10 nM WIN (Figure 4a). Reciprocally, WIN-mediated G-protein activation is significantly attenuated (~43% as compared to WIN alone) by cotreatment with 10 nM DAMGO (Figure 4b). As at this low dose (10 nM) the agonists by themselves do not cause detectable G-protein activation, these results suggest that the occupancy of the  $\mu$  receptor could be sufficient to attenuate CB1 receptor signaling and that reciprocal interactions exist between these two receptors.

We next examined the extent of  $\mu$ -CB1 interactions in endogenous tissue. For this, we used striatal tissue that has been shown to express both  $\mu$  and CB1 receptors by electron microscopy (Rodriguez *et al.*, 2001). Treatment of rat striatal

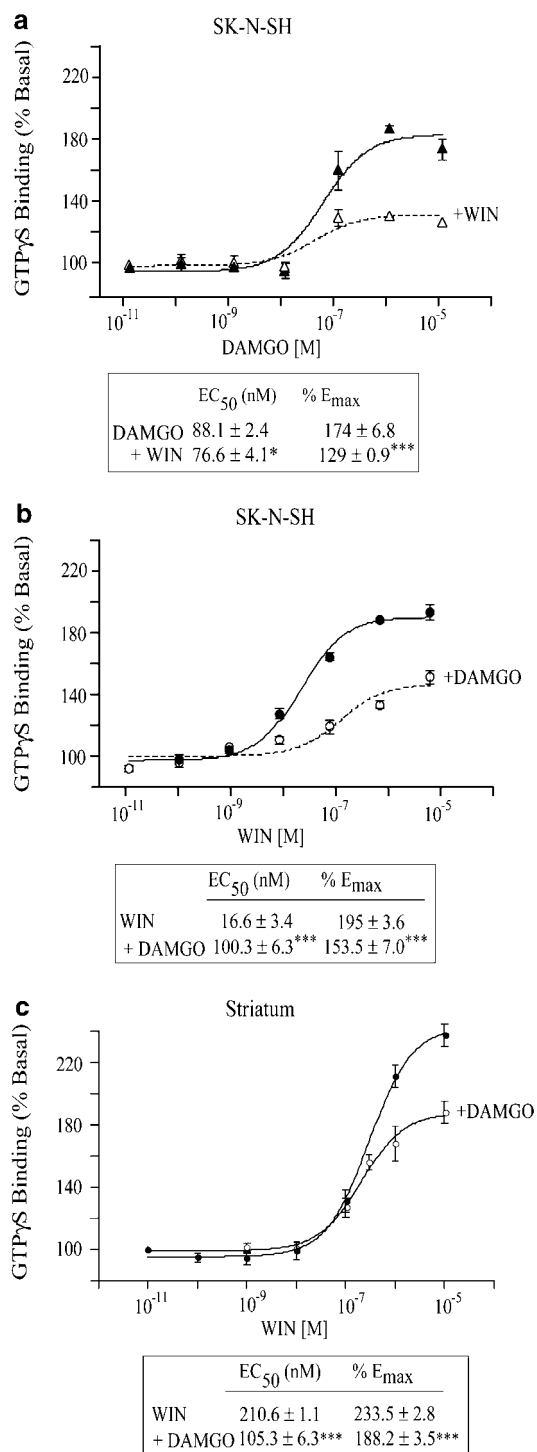


**Figure 3** MAPK phosphorylation in cells expressing  $\mu$ ,  $\mu$ -CB1 or  $\mu$ -CCR5 receptors. HEK-293 cells expressing  $\mu$  receptors alone (a and c), coexpressing  $\mu$  and CB1 (b) or  $\mu$ -CCR5 (d) receptors were treated with 1  $\mu$ M morphine (MOR), WIN 55,212-2 (WIN) or RANTES (RAN) for 5 min and subjected to SDS-PAGE and Western blot analysis as described in Methods. Results are the mean  $\pm$  s.e.m. ( $n=9$ ). Statistically significant differences were determined by one-way ANOVA ( $n=9$ ). \*\* $P<0.01$ , \*\*\* $P<0.001$  between control and drug treatment, ## $P<0.01$  between morphine and morphine + WIN. Representative blot is shown in the figure.

membranes with increasing concentrations of WIN induces a robust increase in [ $^{35}$ S]-GTP $\gamma$ S binding and this is significantly attenuated ( $\sim 34\%$  as compared to WIN alone) by treatment with 10 nM DAMGO (Figure 4c), suggesting that the antagonistic interactions between  $\mu$  and CB1 receptors exist in endogenous tissue.

In order to explore the physiological relevance of this interaction, we used the  $G_{\alpha i/o}$ -mediated neuritogenesis as an assay and examined the effect of activation of individual or combination of receptors on the extent of neurite outgrowth. For this, we used Neuro-2A cells as in a recent set of studies we have shown that activation of the endogenous cannabinoid

receptors in these cells leads to a significant increase in the number of neurites and this is dependent on the Src-STAT3 pathway (He *et al.*, 2005; Jordan *et al.*, 2005). In order to examine if  $\mu$  receptor activation leads to neuritogenesis and if coactivation of  $\mu$ -CB1 receptors modulates this response, we generated Neuro-2A cell lines stably expressing mouse  $\mu$  receptors. In these cells, activation of  $\mu$  receptors leads to dose-dependent increase in the number of neurites (Figure 5a) and activation of the endogenous CB1 receptors also leads to a dose-dependent increase, consistent with previous observations (Jordan *et al.*, 2005). Interestingly, coactivation of both  $\mu$  and CB1 receptors leads to a significant attenuation of the response



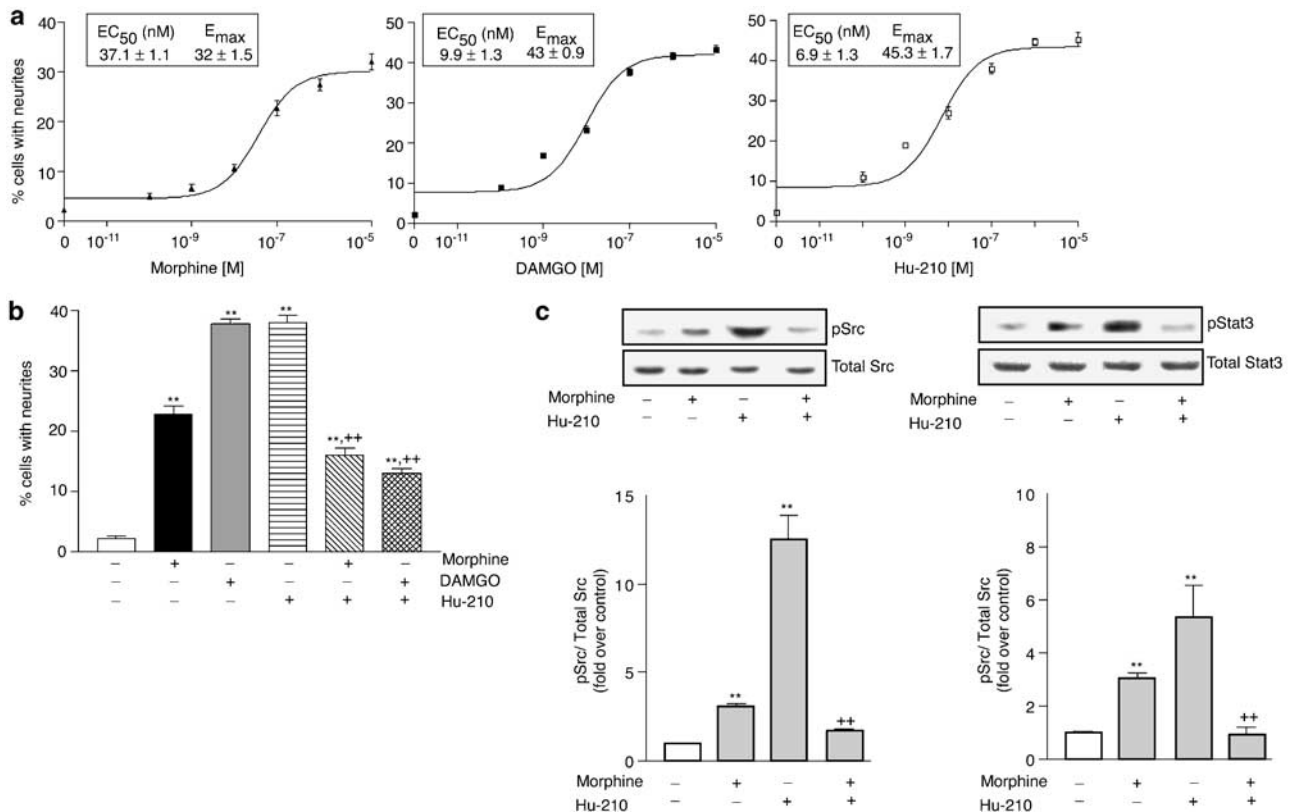
**Figure 4** Signaling in SK-N-SH cells and striatal membranes endogenously expressing  $\mu$  and CB1 receptors. (a) SK-N-SH cells were permeabilized and used in the [<sup>35</sup>S]-GTP $\gamma$ S binding with the indicated concentrations of DAMGO in the absence or presence of 10 nM WIN or (b) WIN in the absence or presence of 10 nM DAMGO as described in Methods. (c) Striatal membranes were treated with the indicated concentrations of WIN in the absence or presence of 10 nM DAMGO and [<sup>35</sup>S]-GTP $\gamma$ S binding was measured as described in Methods. The EC<sub>50</sub> and E<sub>max</sub> were determined using GraphPad Prism and are indicated in the box below each graph. The data represent mean  $\pm$  s.e.m. ( $n = 16$ ). Statistically significant differences for EC<sub>50</sub> and E<sub>max</sub> were determined using the Student's  $t$ -test. \* $P < 0.05$ , \*\*\* $P < 0.001$  ( $n = 16$ ).

(Figure 5b and Table 1). Examination of the extent of phosphorylation of Src and STAT3 showed an increase in phosphorylation of these proteins upon activation of individual receptors and substantial decrease in phosphorylation upon activation of both these receptors (Figure 5c). These results support the notion that Src–STAT3 pathway is involved in  $\mu$  opioid and CB1 cannabinoid receptor-mediated neuritegenesis and that receptor–receptor association leads to an attenuation of signaling involving this pathway.

## Discussion

A major finding of this study is that coactivation of  $\mu$  and CB1 receptors results in attenuation of signaling by either receptor. These results are similar to those previously reported in the case of  $\mu$ - $\alpha_{2A}$  receptors (Jordan *et al.*, 2003). The molecular mechanism for this effect is not clear. A possibility is that the close proximity of these receptors could lead to competition for the pool of G proteins. The results from our BRET studies show that when expressed at near endogenous levels, the receptors are in close enough proximity for efficient energy transfer. Such proximity would allow for interactions at the level of sharing G-protein pools. Previous studies have suggested that CB1 receptors have a high affinity for G<sub>i/o</sub> proteins and can sequester them from common G-protein pools thereby preventing signaling by neighboring  $\alpha_2$ -adrenergic and somatostatin receptors (Vasquez & Lewis, 1999). Our finding that  $\mu$  receptor signaling is attenuated by the CB1 receptor ligand supports such a notion. However, it is unlikely that this is the sole mechanism as we find that the reciprocal is also true (i.e. CB1 receptor signaling is attenuated by  $\mu$  receptor ligands). Therefore, it is likely that additional mechanisms are involved in the observed antagonistic cross-talk between these two receptors.

It is possible that the agonist occupied  $\mu$  receptor functions as an allosteric modulator of the partner receptor and *vice versa*. Support for such a possibility comes from studies examining the allosteric modulation of GPCRs; ligands that are positive or negative allosteric modulators of GPCRs have been identified in the case of adenosine and muscarinic receptors (May & Christopoulos, 2003). For example, 2-amino-3-benzoylthiophenes and its analogs have been found to be positive allosteric modulators of adenosine A1 receptors (May & Christopoulos, 2003). In contrast, *N*-chloromethyl brucine was found to be a negative allosteric modulator of M1, M2 and M5 muscarinic receptors (May & Christopoulos, 2003). Opiate ligands have been shown to negatively modulate adrenergic receptors in competition assays (Ballesta & Orts, 1992). Interestingly, cannabinoid ligands have been shown to negatively modulate opioid receptors in binding assays using rat brain membranes (Vaysse *et al.*, 1987). These results suggest that  $\mu$  and CB1 receptors are able to undergo allosteric modulation. Thus, it is possible that occupancy of the CB1 receptor is sufficient to modulate the activity of a closely interacting  $\mu$  receptor and *vice versa*. Such a notion is consistent with data from recent modeling studies based on the X-ray crystal structure and the AFM of rhodopsin on native disk membranes (Fotiadis *et al.*, 2004). These studies show that heterotrimeric G proteins form a complex with two rhodopsin dimers and that efficient coupling requires the activation of one rhodopsin monomer in this complex (Filipek



**Figure 5** Neurite outgrowth in Neuro-2A cells expressing  $\mu$  opioid and CB1 cannabinoid receptors. (a) Neuro-2A cells stably expressing Flag-tagged  $\mu$  opioid receptors were treated with different concentrations of agonists (0– $10^{-5}$  M) and the number of cells with neurites determined as described in Methods. Data represent mean  $\pm$  s.e.m. ( $n = 3$ ). (b) Agonist-induced neurite outgrowth in Neuro 2A cells treated with a combination of 100 nM DAMGO or morphine, and 100 nM CB1 agonist HU 210. Cells were scored for neurites as described above. Data represent mean  $\pm$  s.e.m. from triplicate determinations of two independent experiments. (c) Cells were treated with 100 nM morphine or 100 nM Hu-210 or a combination of the two for 30 min followed by lysis and Western Blot analysis as described in Methods. A representative blot is shown (upper) and data from densitization of autoradiograms are shown (lower). Values represent mean  $\pm$  s.e.m. ( $n = 9$ ). Statistically significant differences from control cells are indicated, \*\* $P < 0.001$  versus no drug; +++ $P < 0.001$  versus morphine/Hu-210, one-way ANOVA ( $n = 9$ ).

**Table 1** Effect of co-activation of  $\mu$  opioid and CB1 cannabinoid receptors on neurite outgrowth

	% cells with neurites with drugs at		
	$1 \times 10^{-7}$ M	$1 \times 10^{-6}$ M	$1 \times 10^{-5}$ M
Morphine	22.7 $\pm$ 1.5**	27.3 $\pm$ 1.2**	32.0 $\pm$ 1.5**
DAMGO	37.7 $\pm$ 0.9**	41.7 $\pm$ 1.0**	43.3 $\pm$ 0.5**
Hu-210	38.0 $\pm$ 1.2**	44.7 $\pm$ 0.9**	45.3 $\pm$ 1.7**
Morphine + Hu-210	16.0 $\pm$ 1.3***,++	12.4 $\pm$ 0.3***,++	8.7 $\pm$ 0.3***,++
DAMGO + Hu-210	13.0 $\pm$ 0.9***,++	9.9 $\pm$ 0.3***,++	7.2 $\pm$ 0.7***,++

Neuro 2A cells stably expressing Flag-tagged  $\mu$  opioid receptors were treated with different concentrations of morphine, DAMGO ( $\mu$  agonists), Hu-210 (CB1 agonist) or combination of these drugs as described in Methods. Control cells were not exposed to any drug treatment ( $\sim 2.3 \pm 0.33\%$  cells had neurites). Cells were scored as positive for neurite outgrowth when the length of the neurite was more than twice the diameter of the cell. Results are the mean  $\pm$  s.e.m. ( $n = 6$ ). \*\* $P < 0.001$  versus control; +++ $P < 0.001$  versus morphine/DAMGO/Hu-210; one-way ANOVA ( $n = 6$ ).

*et al.*, 2004). According to this model, the rhodopsin monomer undergoes a conformational change upon ligand binding and passes this information to the second monomer, which signals through the heterotrimeric G protein. The second rhodopsin

dimer appears to serve as a docking platform (Filipek *et al.*, 2004), which could, in turn, act as an allosteric modulator. Based on this model, the opioid receptor would serve as an allosteric modulator of the CB1 receptor and *vice versa*. Coactivation of both receptors could lead to destabilization of receptor–G-protein interactions, leading to decreased efficacy in G protein activation and ultimately signal attenuation. This is further supported by the lack of functional interaction seen between the  $\mu$  opioid and CCR5 chemokine receptors.

Interactions between  $\mu$  opioid and CB1 cannabinoid receptors have a significant impact on important physiologic processes such as neuritogenesis. Recent studies show that activation of the CB1 cannabinoid receptor leads to neurite outgrowth in Neuro 2A cells *via* activation of the  $G_{\alpha i}$ , Rap 1, Src and STAT3 pathway (He *et al.*, 2005; Jordan *et al.*, 2005). We have also shown that activation of  $\delta$  opioid or serotonin 5-HT<sub>1A</sub> receptors also leads to neurite outgrowth in Neuro 2A cells (Rios *et al.*, 2004; Fricker *et al.*, 2005). Thus, receptor-mediated neuritogenesis serves as a useful assay to monitor a physiological response to receptor–receptor interactions. We have found that  $\delta$  receptor-mediated neuritogenesis could be modulated by the presence of  $\alpha_{2A}$  adrenergic receptors (Rios *et al.*, 2004). In the present study, we observe an attenuation in neurite outgrowth as well as in the levels of phosphorylated Src and STAT3

upon coactivation of  $\mu$  opioid and CB1 cannabinoid receptors, supporting the involvement of  $G_{\alpha i}$ –Rap–Src–STAT3 pathway in this event. In addition to this pathway, it is possible that other signaling pathways are involved in opioid- or cannabinoid-mediated neurite outgrowth such as those involving MAPK and CREB (Xiao & Liu, 2003; Zhao *et al.*, 2003). In the case of dopamine D2 receptors, the phosphoinositide 3-kinase pathway has been shown to be involved (Nair & Sealton, 2003; Nair *et al.*, 2003). Thus, it appears that multiple pathways are involved in  $G_{\alpha i}$ -coupled receptor-mediated neurite outgrowth and this suggests additional roles for these receptors during neuronal development and maturation.

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