CXCR2 chemokine receptor antagonism enhances DOP opioid receptor function via allosteric regulation of the CXCR2–DOP receptor heterodimer

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Opioid agonists have a broad range of effects on cells of the immune system, including modulation of the inflammatory response, and opioid and chemokine receptors are co-expressed by many white cells. Hetero-oligomerization of the human DOP opioid and chemokine CXCR2 receptors could be detected following their co-expression by each of co-immunoprecipitation, three different resonance energy transfer techniques and the construction of pairs of individually inactive but potentially complementary receptor G-protein α subunit fusion proteins. Although DOP receptor agonists and a CXCR2 antagonist had no inherent affinity for the alternative receptor when either receptor was expressed individually, use of cells that expressed a DOP opioid receptor construct constitutively, and in which expression of a CXCR2 receptor construct could be regulated, demonstrated that the CXCR2 antagonist enhanced the function of DOP receptor agonists only in the presence of CXCR2. This effect was observed for both enkephalin- and alkaloid-based opioid agonists, and the effective concentrations of the CXCR2 antagonist reflected CXCR2 receptor occupancy. Entirely equivalent results were obtained in cells in which the native DOP opioid receptor was expressed constitutively and in which expression of the isolated CXCR2 receptor could be induced. These results indicate that a CXCR2 receptor antagonist can enhance the function of agonists at a receptor for which it has no inherent direct affinity by acting as an allosteric regulator of a receptor that is a heterodimer partner for the CXCR2 receptor. These results have novel and important implications for the development and use of small-molecule therapeutics.

Key words: allosterism, chemokine, G-protein-coupled receptor, heterodimer, opioid.

INTRODUCTION

It has become increasingly clear in recent times that opioid agonists have a broad range of effects on cells of the immune system, including modulation of the inflammatory response [1]. Opioid receptor levels are regulated by peripheral inflammation [2], and it is thought that highly peripherally selective opioid agonists may provide a therapeutic approach for the treatment of inflammation and inflammatory pain (see [3] for review). Opioids function via three molecularly defined receptor subtypes, the DOP, KOP and MOP receptors [4]. These are all members of the rhodopsin-like family of GPCRs (G-protein-coupled receptors). As well as their characterized expression patterns in the central and peripheral nervous systems they are expressed on a wide range of white blood cells, including macrophages and neutrophils [5]. Various opioid agonists are known to produce chemotactic responses in white cells, and to modulate chemotaxis induced by a range of chemokines. Chemokines and their receptors are also integrators of pain and inflammation [6–8] and there is a clear capacity for heterologous modification and desensitization between co-expressed chemokine and opioid receptors [9–11]. Receptors for chemokine ligands also belong to the rhodopsin-like GPCR family, and one further potential mechanism that may contribute to the effects outlined above is direct interactions between chemokine and opioid receptors. At least for the CCR5 chemokine receptor, a capacity to co-immunoprecipitate each of the DOP, KOP and MOP receptors from both human and monkey lymphocytes [12] provided

preliminary evidence of such interactions, and direct protein–protein contacts between CCR5 and the MOP receptor have been confirmed independently [13].

The closely related CXCR1 and CXCR2 receptors [14] share a common agonist ligand in IL-8 (interleukin 8; also called CXCL8). They are widely co-expressed on immune cells, including neutrophils, CD8(+) T-cells and mast cells, and allosteric inhibitors of these receptors have been suggested to offer a general means to inhibit polymorphonuclear cell recruitment *in vivo* [15]. Opioid agonists inhibit IL-8-induced chemotaxis of human neutrophils, and opioid agonists are able to cause phosphorylation of CXCR1 and CXCR2 receptors [9]. Interestingly, CXCR2, but not CXCR1, is expressed by a variety of neurones and astrocytes [14] and may be involved in the directed migration of specific subsets of neurones.

Although it was believed for many years that GPCRs functioned exclusively as monomers, a growing consensus has challenged this view. Indeed, it is now widely believed that the minimal *in situ* functional unit for a GPCR is a dimer and that further, higher-order, oligomeric structures may exist [16–19]. Much evidence suggests that for many GPCRs dimerization/oligomerization occurs during synthesis and maturation within the endoplasmic reticulum [20,21] prior to plasma membrane delivery. This model would explain why many GPCRs appear to be constitutively formed dimers/oligomers. It is also increasingly accepted that at least certain GPCRs have the capacity to form heterodimers/oligomers (i.e. dimers formed between two different GPCR gene products) [16,22]. Such heterodimers/oligomers may have distinct

functional and pharmacological properties and have been suggested to offer novel sets of targets for therapeutic small-molecule drug design [22,23]. Although the CXCR1 receptor was originally reported not to homodimerize or to heterodimerize with the CXCR2 receptor [24], Wilson et al. [21] recently employed a wide range of biochemical and biophysical approaches to demonstrate the capacity of both CXCR1 and CXCR2 to homodimerize and to show that these two GPCRs were able to form heterodimers as effectively as homodimers.

It has recently been demonstrated that selective ligands at pairs of GPCRs that heterodimerize have the potential to modulate ligand effects at the partner GPCR via allosterism (see [25] for review). Studies on heterodimerization between the CCR2b and CCR5 chemokine receptors have demonstrated the ability of CCR5-specific ligands, that are unable to compete for the binding of the CCR2 selective ligand MCP-1 (monocyte chemoattractant protein 1) on cells expressing CCR2b alone, to prevent efficiently MCP-1 binding when the two receptors are co-expressed [26]. Similarly, co-expression of the orphan GPCR, GPR50, along with the melatonin MT1 receptor inhibits binding of [^{125}I]melatonin to the MT1 receptor [27].

In the present study we use a range of approaches to demonstrate the capacity for heterodimerization between the human CXCR2 and DOP opioid receptors and show that a CXCR2 antagonist enhances the function of both peptide and alkaloid-based agonists at the DOP receptor via an allosteric mechanism when the two receptors are co-expressed. This is despite the CXCR2 ligand having no significant affinity to interact directly with the DOP receptor.

EXPERIMENTAL

Materials

[15,16-^{3}H]Diprenorphine (50 Ci/mmol) and [^{35}S]GTP[S] (1250 Ci/mmol) were from PerkinElmer Life Science. DADLE (D-Ala^2,Leu^5-enkephalin), DPDPE ([D-Pen^{2,5}]-enkephalin) and pertussis toxin were from Sigma–Aldrich. SB225002 was from Calbiochem, and SNC162 [28] was from Tocris. All reagents for BRET^2 (bioluminescence resonance energy transfer) studies were from Packard Biosciences.

Antibodies/antisera

The anti-G$_{\alpha i1-2}$ antiserum (SG) has been described previously [29]. The mouse monoclonal anti-FLAG antibody (M2) was from Sigma–Aldrich. The rabbit polyclonal anti-c-Myc antiserum was from Cell Signalling Technology.

Molecular Constructs

hDOP–C352I G$_{\alpha i2}$ (where h indicates the human form), V150E,V154D hDOP–C352I G$_{\alpha i2}$ and hDOP–G204A,C352I G$_{\alpha i2}$ were constructed as in Pascal and Milligan [30], except that C352I G$_{\alpha i2}$ replaced C351I G$_{\alpha i1}$. CXCR2–C352I G$_{\alpha i2}$ and I148E CXCR2–C352I G$_{\alpha i2}$ were constructed using similar approaches. FLAG–hCXCR2, c-Myc–hCXCR2 and forms of the CXCR2 C-terminally tagged with auto-fluorescent proteins or *Renilla* luciferase have been described previously [21], as have the equivalent modified forms of hDOP [31]. The nomenclature for the molecular constructs employed is shown in Table 1.

Fluorescent microscopy and FRET (fluorescence resonance energy transfer) imaging in living cells

Cells were grown on poly-D-lysine-treated coverslips and transiently transfected with appropriate eCFP (enhanced cyan

Table 1   Characteristics of the molecular constructs employed

Construct	Description
FLAG–hCXCR2	N-terminal FLAG-tagged form of human CXCR2
c-Myc–hCXCR2	N-terminal c-Myc-tagged form of human CXCR2
hCXCR2–eCFP	C-terminal eCFP-tagged form of human CXCR2
hCXCR2–GFP^2	C-terminal eGFP^2-tagged form of human CXCR2
hCXCR2-*Renilla* luciferase	Human CXCR2 C-terminally tagged with *Renilla* luciferase
hCXCR2–C352I G$_{\alpha i2}$	Fusion protein in which a pertussis-toxin-insensitive form of G$_{\alpha i2}$ is linked to the C-terminus of human CXCR2
I148E hCXCR2–C352I G$_{\alpha i2}$	Fusion protein in which a pertussis-toxin-insensitive form of G$_{\alpha i2}$ is linked to the C-terminus of human CXCR2 containing a I148E mutation that prevents receptor-mediated activation of the G-protein
c-Myc–hDOP	N-terminal c-Myc-tagged form of the human DOP receptor
hDOP–eYFP	C-terminal eGFP^2-tagged form of the human DOP receptor
hDOP–GFP^2	C-terminal eGFP^2-tagged form of the human DOP receptor
hDOP–*Renilla* luciferase	Human DOP receptor C-terminally tagged with *Renilla* luciferase
hDOP–C352I G$_{\alpha i2}$	Fusion protein in which a pertussis-toxin-insensitive form of G$_{\alpha i2}$ is linked to the C-terminus of the human DOP receptor
V150E,V154D hDOP–C352I G$_{\alpha i2}$	Fusion protein in which a pertussis-toxin-insensitive form of G$_{\alpha i2}$ linked to the C-terminus of a mutant human DOP receptor that can bind ligands but not transduce a signal
hDOP–G204A,C352I G$_{\alpha i2}$	Fusion protein in which a pertussis-toxin-insensitive form of G$_{\alpha i2}$ that cannot be activated is linked to the C-terminus of the human DOP receptor

fluorescent protein)/eYFP (enhanced yellow fluorescent protein) fusion proteins. Coverslips were placed into a microscope chamber containing physiological saline solution [130 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 20 mM Hepes and 10 mM D-glucose (pH 7.4)]. Cells were visualized using a Nikon Eclipse TE2000-E fluorescence inverted microscope and images were obtained individually for eYFP, eCFP and FRET filter channels using an Optoscan monochromator (Cairn Research) and a dichroic mirror 86002v2bs (Chroma). The filter sets used were: eYFP (excitation: 500/5 nm; emission: 535/30 nm), eCFP (excitation: 430/12 nm; emission: 470/30 nm) and FRET (excitation: 430/12 nm; emission: 535/30 nm). The illumination time was 250 ms and binning modes 2·2. MetaMorph imaging software was used to quantify the FRET images using the sensitized FRET method. Corrected FRET was calculated using a pixel-by-pixel methodology using the equation:

$$FRETc = FRET - (\text{coefficient B} \times eCFP) \\ - (\text{coefficient A} \times eYFP)$$

where eCFP, eYFP and FRET values correspond to background corrected images obtained through the eCFP, eYFP and FRET channels. B and A correspond to the values obtained for the eCFP (donor) and eYFP (acceptor) bleedthrough co-efficients respectively, calculated using cells singly transfected with either the eCFP or eYFP protein alone. To correct the FRET levels for the various amounts of donor (eCFP) and acceptor (eYFP), normalized FRET was calculated using the equation:

$$FRETn = FRETc/(eCFP \times eYFP)$$

where FRETc, eCFP and eYFP are equal to the fluorescence values obtained from single cells.

Tr (time-resolved) FRET

For tr-FRET, 10 cm^2 dishes of HEK (human embryonic kidney)-293 cells were transfected to express N-terminally c-Myc- or FLAG-tagged forms of hCXCR2 and/or hDOP individually or in combination. At 48 h after transfection, the cells were harvested. Cell pellets were resuspended in 200 μl of ice-cold PBS. Anti-c-Myc Eu^{3+} and anti-FLAG APC (allophycocyanin) antibodies [21,31] were diluted in 50 % newborn calf serum/50 % PBS to final concentrations of 5 nM and 15 nM respectively. Samples were mixed and incubated on a rotating wheel at room temperature (22 °C) for 2 h while covered in aluminium foil to minimize exposure of the fluorophores to light. Samples were centrifuged at 1000 *g* for 1 min and the antibody mix was removed from the cell pellet. The pellet was then washed twice in ice-cold PBS and resuspended in 250 μl of PBS. To measure the energy transfer, 40 μl of each sample was dispensed in triplicate into a black 384-well plate. Blank wells containing PBS were also included. Tr-FRET was determined using a Victor^2 plate reader (Packard Bioscience). Excitation was at 340 nm and emission filters generated data representing donor (615 nm) and acceptor (665 nm) fluorescence. Normalized FRET was calculated using the equation:

$$\text{Normalized FRET} = [(A_{665} - BLK)/D_{615}] - C$$

where A_{665} is the fluorescent emission from the acceptor, D_{615} is the fluorescent emission from the donor and *BLK* represents the background reading at 665 nm from wells containing PBS. *C* represents the cross-talk between the donor and acceptor windows for the samples incubated with only anti-c-Myc Eu^{3+} and is equal to $(A_{665} - BLK)/D_{615}$.

Saturation BRET^2 studies

In saturation BRET^2 experiments, cells were transfected with a constant amount of the energy donor (*Renilla* luciferase) construct and varying amounts of energy acceptor [GFP^2 (green fluorescent protein) construct. BRET^2 was assessed using the luciferase substrate DeepBlueC in intact cells. Cells were also harvested, membranes prepared and diluted to 0.5 mg/ml. Luminescence and fluorescence measurements were then obtained to measure construct expression. Then, 50 μl of cell membranes were dispensed into white-walled 96-well plates (PerkinElmer) for luminescence measurements and black-walled 386-well plates (Costar) for fluorescence measurements. For luminescence measurement 5 μM h-coelenterazine was added and the plate was incubated at room temperature for 30 min prior to measurement at 410 nm using a Mithras LB 940. GFP^2 fluorescence was assessed using a Victor^2 1420 Multilabel counter (PerkinElmer). Fluorescence readings were corrected for endogenous fluorescence of HEK-293 cell membranes alone. BRET^2 readings were corrected for energy transfer resulting from bleedthrough of signal from the *Renilla* luciferase construct expressed alone but detected in the GFP^2 channel. Graphpad Prism 4 was used to analyse data using a one-site binding hyperbola equation yielding BRET$_{MAX}$ and BRET$_{50}$ values (where BRET$_{MAX}$ is the maximal BRET signal and BRET$_{50}$ is half the maximal BRET signal).

Cell transfection and treatment

HEK-293 cells were transfected transiently using Lipofectamine^{TM} reagent (Gibco Life Technologies) or Gene Juice (Novagen) and the appropriate cDNA(s) according to the manufacturer's instructions. Where appropriate, cells were treated with pertussis toxin (25 ng/ml) for 16–18 h prior to harvest.

[^{3}H]Diprenorphine binding

The expression of hDOP–G-protein fusions was assessed by measuring the specific binding of [^{3}H]diprenorphine in cell-membrane preparations. Non-specific binding was assessed by the addition of 100 μM naloxone. Samples were incubated for 1 h at 25 °C and bound ligand was separated from free ligand by vacuum filtration through GF/B filters pre-treated with 0.3 % polyethyleneimine in TEM (10 mM Tris/HCl, 0.1 mM EDTA and 10 mM MgCl$_2$, pH adjusted to 7.5). Bound ligand was estimated by liquid scintillation spectroscopy. Competition studies were conducted with 1 nM [^{3}H]diprenorphine and a range of concentrations of other ligands. Data were analysed using GraphPad Prism software. Saturation data were fitted to non-linear regression curves.

[^{35}S]GTP[S] binding studies

Experiments were initiated by adding the assay buffer mix [20 mM Hepes (pH 7.4), 3 mM MgCl$_2$, 100 mM NaCl, 10 μM GDP and 0.2 mM ascorbic acid] containing 50 nCi of [^{35}S]GTP[S] in the presence or absence of ligands to defined amounts of membranes. Non-specific binding was determined in the presence of 100 μM GTP[S]. The reaction was incubated for 15 min at 30 °C and terminated by adding 1 ml of ice-cold stop buffer [20 mM Hepes (pH 7.4), 3 mM MgCl$_2$ and 100 mM NaCl]. The samples were centrifuged for 15 min at 16 000 *g* at 4 °C and the resulting pellets were resuspended in solubilization buffer (100 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA and 1.25 % Nonidet P40, pH adjusted to 7.4) plus 0.2 % SDS. Samples were pre-cleared with Pansorbin for 1 h at 4 °C and centrifuged for 2 min at 16 000 *g*. Supernatant was added to a mix of Protein G and the anti-G$_{\alpha i1}$/G$_{\alpha i2}$ antiserum [29] and was left rotating overnight at 4 °C for immunoprecipitation. The immunocomplexes were washed twice with ice-cold solubilization buffer and bound [^{35}S]GTP[S] was measured.

Co-immunoprecipitation

Cells were resuspended in 1 ml of 1 · RIPA [radio-immunoprecipitation assay; 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % Triton X-100, 100 mM NaF, 5 mM EDTA, 0.1 mM NaPO$_4$ and 5 % ethylene glycol] buffer and rotated for 60 min at 4 °C to allow lysis. The samples were centrifuged at 14 000 *g* for 10 min at 4 °C and the supernatant was retained. A 50 μl aliquot of a Protein G–Sepharose/PBS slurry was added to the supernatant and rotated for a further 60 min at 4 °C to pre-clear. Samples were centrifuged at 14 000 *g* for 10 min at 4 °C. The supernatant was conserved and the protein concentration was measured using the BCA (bicinchoninic acid) assay method. Samples were equalized to 1 μg/μl. Target proteins were then immunoprecipitated from 500 μl aliquots of samples by incubation with 20 μl of Protein G–Sepharose and the appropriate antibody/antiserum overnight at 4 °C on a rotating wheel. Immune complexes were isolated by centrifugation at 14 000 *g* for 1 min and washed twice with RIPA buffer. Proteins were eluted from the Protein G–Sepharose by the addition of 30–50 μl of Laemmli buffer and heated for 4 min at 85 °C. The eluates were then loaded on to SDS/PAGE gels.

Construction of Flp-In T-REx HEK-293 cell lines

The basic protocol used to generate Flp-In T-REx HEK-293 cell lines that constitutively express one GPCR and can be induced

to express a second has been described previously [32–34]. In brief, cells were maintained in DMEM (Dulbecco's modified Eagle's medium) without sodium pyruvate, 4500 mg/l glucose and L-glutamine supplemented with 10 % (v/v) fetal calf serum, 1 % antibiotic mixture and 10 μg/ml blasticidin at 37 °C in a humidified atmosphere of air/CO$_2$ (19:1). To generate Flp-In T-REx HEK-293 cell lines able to inducibly express c-Myc–I148E CXCR2–C352I G$_{i2\alpha}$ or VSV-G–CXCR2 (where VSV-G is vesicular-stomatitis-virus glycoprotein), cells were transfected with a mixture containing the desired receptor cDNA in pcDNA5/FRT/TO vector and the pOG44 vector (1:9) using Lipofectamine™ (Invitrogen) according to the manufacturer's instructions. After 48 h, the medium was changed to medium supplemented with 200 μg/ml hygromycin B to initiate selection of stably transfected cells. To constitutively express FLAG–hDOP–G204A, C352I G$_{\alpha i2}$ or FLAG–hDOP in cell lines already capable of the induction of expression of a second construct, the appropriate cells were further transfected with the desired receptor cDNA in pcDNA3 as described above, and resistant cells were selected in the presence of 1 mg/ml G418. Resistant clones were screened for receptor expression by immunocytochemical analysis. Cells were treated with 1 μg/ml doxycycline 6–96 h before assays to induce expression of receptors and receptor constructs cloned into the Flp-In locus.

Immunostaining protocol

Cells were grown on to coverslips, and 24 h later medium was removed and the cells were incubated with 20 mM Hepes/DMEM containing the appropriate dilution of primary antibody for 40 min at 37 °C in 5 % CO$_2$. Following three washes with PBS, cells were fixed by incubating with 4 % paraformaldehyde in PBS/5 % sucrose for 10 min at room temperature. Following three further washes, cells were permeabilized with 0.15 % Triton X-100/3 % non-fat dried skimmed milk/PBS for 10 min. The coverslips were incubated with a secondary antibody (5 μg/ml) conjugated to an Alexa Fluor®-594 fluorophore. Following incubation for 1 h, cells were washed twice in 0.15 % Triton X-100/3 % non-fat dried skimmed milk/PBS and three times in PBS. Coverslips were then mounted on to microscope slides with 40 % glycerol in PBS.

Confocal laser scanning microscopy

Cells were imaged using a laser scanning confocal microscope (Zeiss LSM 5 Pascal) equipped with a 63× oil-immersion Plan Fluor Apochromat objective lens with a numerical aperture of 1.4. A pinhole of 20 and an electronic zoom of 1 or 2.5 was used (Carl Zeiss). The excitation laser line for GFP and eYFP was the 488 nm argon laser with detection via a 505–530 band pass filter. Alexa Fluor®-594 label was excited using a 543 nm helium/neon laser and detected via a 560 nm long-pass filter. The images were manipulated using MetaMorph imaging software (version 6.1.3; Universal Imaging).

RESULTS

To examine potential interactions between the chemokine CXCR2 and DOP opioid receptors, the human forms of these receptors were modified to incorporate either the FLAG or c-Myc epitope tag sequences at the N-terminus. We have previously shown the capacity of each of these receptors to form homodimers/oligomers via co-immunoprecipitation studies [21,31]. Expression in HEK-293 cells of FLAG–hCXCR2 resulted in immunological detection in lysates of these cells of a 34 kDa polypeptide with a degree of micro-heterogeneity (Figure 1) representing differential N-
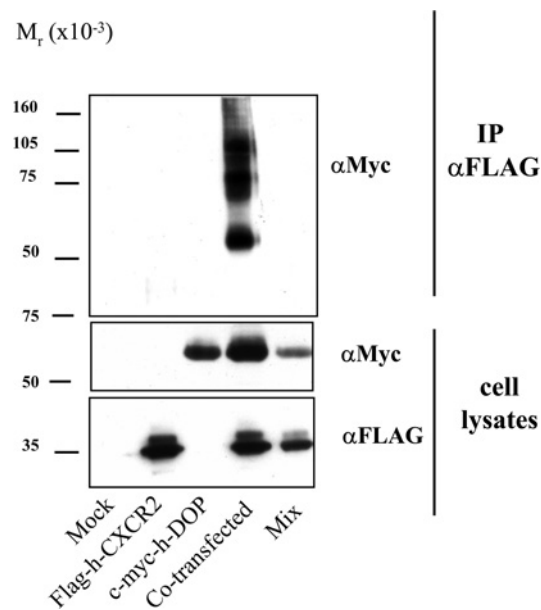

Figure 1   Co-expression of FLAG–hCXCR2 and c-Myc–hDOP allows their co-immunoprecipitation

HEK-293 cells were mock-transfected (Mock) or transfected to transiently express FLAG–hCXCR2, c-Myc–hDOP or both (Co-transfected). Samples containing either FLAG–hCXCR2 or c-Myc–hDOP were also mixed (Mix). Confirmation of expression of the appropriate constructs was obtained by immunoblotting cell lysates with either anti-c-Myc or anti-FLAG antibodies (lower panels). Cell lysates were subsequently immunoprecipitated using anti-FLAG. Immunoprecipitated samples were resolved by SDS/PAGE and immunoblotted with an anti-c-Myc antibody (upper panel). Two further experiments produced similar results. IP, immunoprecipitation. The molecular mass in kDa is indicated on the left-hand side.

glycosylation [21]. Expression of c-Myc–hDOP resulted in the presence of a c-Myc-reactive polypeptide with a molecular mass of 60 kDa (Figure 1). Only with co-expression of FLAG–hCXCR2 and c-Myc–hDOP did immunoprecipitation with an anti-FLAG antibody result in co-immunoprecipitation of c-Myc immunoreactivity (Figure 1) and, even in SDS/PAGE, such immunoreactivity migrated with sizes ranging from 60 kDa to complexes with a substantially higher apparent molecular mass. Because the N-terminal region of GPCRs that are effectively delivered to the cell surface is expected to be extracellular, we also took advantage of the introduced N-terminal tags to perform tr-FRET studies in intact HEK-293 cells [21,31] to detect protein complexes containing both receptors at the cell surface. Co-expression of c-Myc–hCXCR2 and FLAG–hDOP followed by the addition of a combination of Eu^{3+}-labelled anti-c-Myc, to act as a long-lived energy donor, and APC-labelled anti-FLAG, to act as a potential energy acceptor, resulted in strong tr-FRET and output of light at 665 nm when samples were illuminated with 320 nm light (Figure 2A). This did not occur when HEK-293 cell populations individually expressing either c-Myc–hCXCR2 or FLAG–hDOP were combined prior to addition of the combination of Eu^{3+}-labelled and APC-labelled antibodies (Figure 2A). Furthermore, the extent of energy transfer following co-expression of c-Myc–hCXCR2 and FLAG–hDOP was virtually the same as when c-Myc–hDOP and FLAG–hDOP were co-expressed to generate tr-FRET competent, cell-surface hDOP homodimers (Figure 2A). We have also previously shown that in-frame fusion of auto-fluorescent proteins to the C-terminus of each of hCXCR2 [21] and hDOP [31,35] does not significantly alter the function or pharmacology of these receptors. Co-expression of hCXCR2–eCFP and hDOP–eYFP resulted in a capacity to
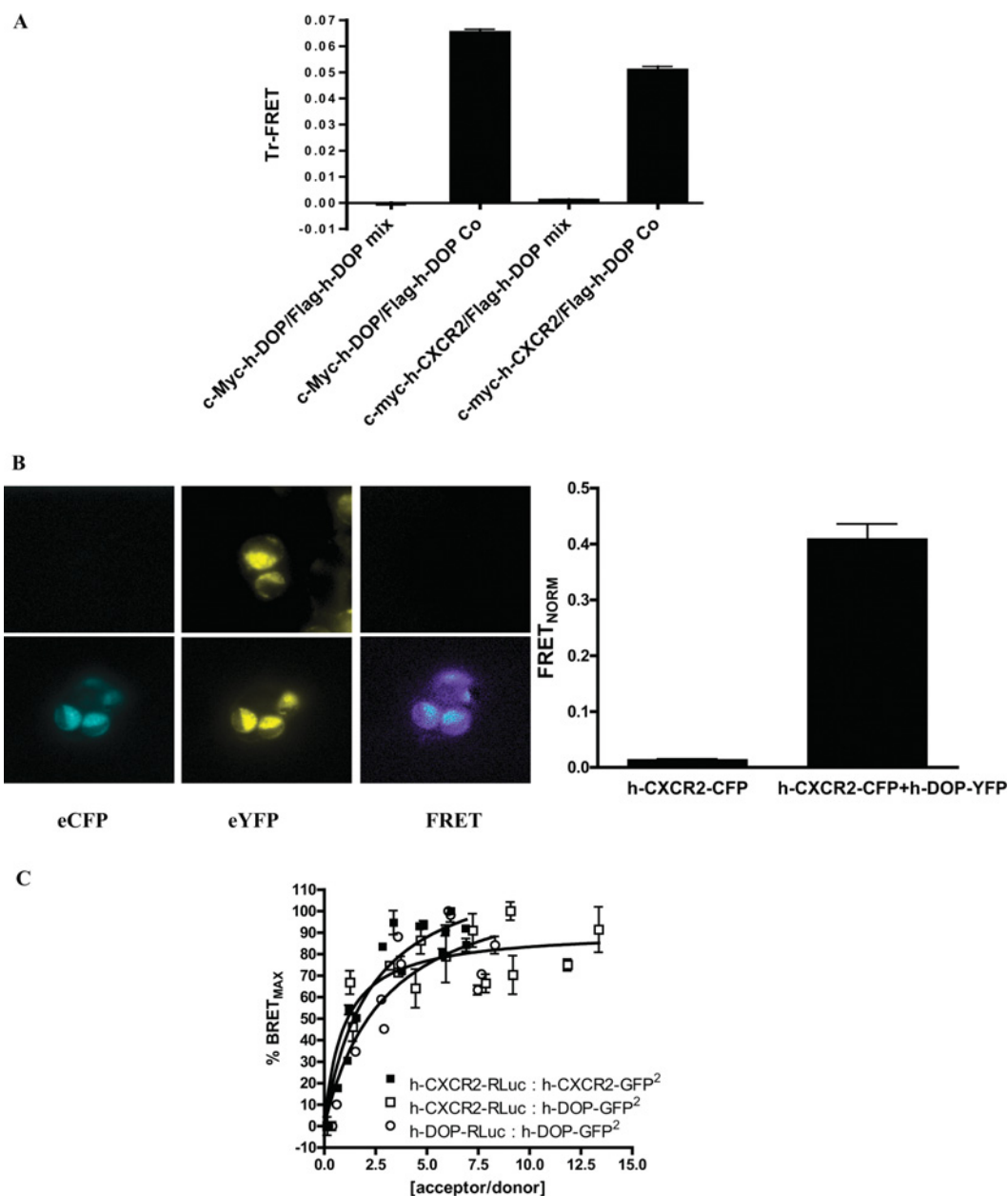
Figure 2   FRET and BRET studies confirm hetero-interactions between co-expressed hCXCR2 and hDOP receptors

(**A**) shows tr-FRET. c-Myc–hDOP and FLAG–hDOP or c-Myc–hCXCR2 and FLAG–hDOP were expressed individually in HEK-293 cells that were then mixed (mix) or the two receptors were co-expressed (Co). Following addition of a combination of Eu^{3+}-labelled anti-c-Myc, to act as a long-lived energy donor, and APC-labelled anti-FLAG, to act as a potential energy acceptor, to intact cells tr-FRET was monitored as described in the Experimental section. (**B**) shows FRET imaging. hDOP–eYFP (eYFP) was transiently expressed in HEK-293 cells with (lower panels) or without (upper panels) hCXCR2–eCFP (eCFP) and fluorescence imaged. Raw FRET (FRET) and calculated normalized FRET (right-hand panels) was then assessed as described in the Experimental section. (**C**) shows saturation $BRET^2$ studies. hCXCR2–*Renilla* luciferase and hCXCR2–GFP^2 (■), hCXCR2–*Renilla* luciferase and hDOP–GFP^2 (□) or hDOP–*Renilla* luciferase and hDOP–GFP^2 (○) were transiently co-expressed in HEK-293 cells. Following addition of the luciferase substrate/$BRET^2$ energy donor DeepBlueC, BRET measurements were made. Donor and acceptor ratios were assessed as described in the Experimental section. Each experiment is representative of three.

image eCFP to eYFP FRET in individual single cells (Figure 2B), providing further evidence for direct hCXCR2–hDOP interactions. Estimates of the relative affinities of GPCRs to interact can be obtained from 'saturation' BRET studies [36,37]. In such experiments, forms of GPCRs C-terminally tagged with *Renilla* luciferase and with an autofluorescent protein that is able to act as an energy acceptor of light emitted from substrate oxidation by the luciferase are co-expressed in various ratios and the BRET signal is monitored. Co-expression of hCXCR2–*Renilla* luciferase and hCXCR2–GFP^2 in HEK-293 cells resulted in

BRET following addition of the luciferase substrate DeepBlueC (Figure 2C). At low energy acceptor (hCXCR2–GFP^2) to energy donor (hCXCR2–*Renilla* luciferase) ratios the BRET signal increased with increasing [acceptor] to [donor] ratios, but this asymptotically approached a maximal value at higher [acceptor] to [donor] ratios (Figure 2C). Half-maximal BRET signal ($BRET_{50}$) was achieved at an [acceptor] to [donor] ratio of 1.6 ± 0.1. Co-expression of hDOP–*Renilla* luciferase and hDOP–GFP^2 also generated BRET signals that saturated with increasing [acceptor] to [donor] ratios, in this case with $BRET_{50} = 2.2 \pm 0.07$.
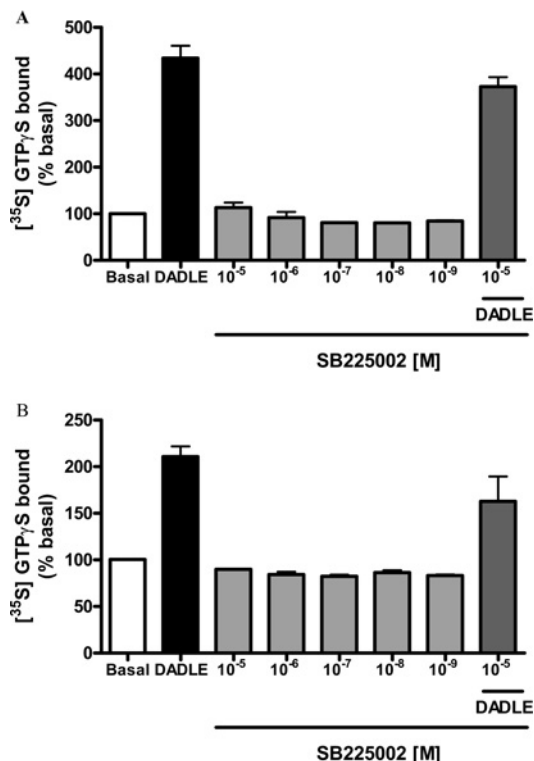
Figure 3   hDOP–$G_{\alpha i2}$ fusion proteins are activated by DADLE but not by SB225002

(**A**) A hDOP-C352I $G_{\alpha i2}$ fusion protein was expressed transiently in HEK-293 cells. Following pertussis-toxin treatment (25 ng/ml for 16 h) and membrane preparation, basal binding of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates (Basal) and its regulation by DADLE (10^{-6} M), concentrations of SB225002 from 10^{-5}–10^{-9} M and DADLE (10^{-6} M) + SB225002 (10^{-5} M) was assessed. (**B**) V150E,V154D hDOP–C352I $G_{\alpha i2}$ and hDOP–G204A,C352I $G_{\alpha i2}$ were co-expressed transiently in HEK-293 cells. Following pertussis-toxin treatment (25 ng/ml for 16 h) and membrane preparation, binding of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates in response to the same ligands as in (**A**) was measured. Values represent means $\pm$ S.E.M. ($n = 3$).

Co-expression of hDOP–*Renilla* luciferase with hCXCR2–GFP^2 generated BRET signals that saturated, and in this case BRET$_{50}$ was 0.34 ± 0.02 (Figure 2C). These results are consistent with hCXCR2–hDOP hetero-interactions occurring with an even higher affinity than the corresponding hCXCR2–hCXCR2 and hDOP–hDOP homo-interactions.

Over time, we have generated a wide range of GPCR–G-protein fusion constructs [38,39]. These allow production, from single open reading frames, of bi-functional polypeptides containing the sequence of both a receptor and a G-protein. A C352I [40,41] pertussis-toxin-insensitive variant of $G_{\alpha i2}$ was linked in-frame to the C-terminal tail of the hDOP receptor. Following transient expression in HEK-293 cells, treatment with pertussis toxin (25 ng/ml for 16 h), to cause ADP-ribosylation of endogenously expressed $G_{\alpha i}$ G-proteins, and membrane preparation, the synthetic enkephalin DADLE (10^{-6} M) caused a 4–5-fold increase in the binding of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates (Figure 3A). By contrast, the non-peptide CXCR2 antagonist SB225002 [42] had no effect at concentrations from 10^{-9}–10^{-5} M and, indeed, at 10^{-5} M it had a limited capacity to inhibit the stimulatory effect of DADLE (Figure 3A). The capacity of both DADLE and SB225002 to compete with [^{3}H]diprenorphine for binding to the hDOP–C352I $G_{\alpha i2}$ fusion protein indicated a pK_i for DADLE of 9.0 ± 0.09, whereas for SB225002 the pK_i was > 4.0 (results not shown). Pairs of GPCR–G-protein fusions that are individually both inactive due to the introduction of mutations

can reconstitute function when co-expressed if the GPCRs form a dimer and if one of the fusion proteins is mutated in the receptor whereas the other contains the inactivating mutation in the G-protein [30]. Variants of hDOP–C352I $G_{\alpha i2}$ that did not modify the ligand-binding domain but failed to increase significantly binding of [^{35}S]GTP[S] in response to DADLE were produced by mutation of either V150E + V154D in the receptor element or by introduction of a G204A mutation into $G_{\alpha i2}$ (Figure 3B). As demonstrated previously for the equivalent hDOP–C351I $G_{\alpha i1}$ mutants [30], co-expression of V150E,V154D hDOP–C352I $G_{\alpha i2}$ and hDOP–G204A,C352I $G_{\alpha i2}$ reconstituted the ability of DADLE to stimulate binding of [^{35}S]GTP[S] in membranes of pertussis-toxin-treated HEK-293 cells, whereas SB225002 was again unable to replicate this effect (Figure 3B). The effect of DADLE required co-expression of the two individually non-responsive fusion constructs. Simple mixing of membranes expressing each construct individually did not result in a substantial increase in binding of [^{35}S]GTP[S] in response to DADLE (results not shown, but see [30]).

We next generated a hCXCR2–C352I $G_{\alpha i2}$ fusion construct. Following transient expression in HEK-293 cells, pertussis-toxin treatment and membrane preparation, IL-8 was able to promote binding of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates in a concentration-dependent manner with a pEC$_{50}$ of -7.4 ± 0.2 (Figure 4A). This effect of IL-8 was blocked by the co-addition of SB225002 (Figure 4B), whereas each of DADLE, the DOP opioid receptor selective DPDPE and the highly DOP receptor selective small molecule SNC162 [28] were unable to promote binding of [^{35}S]GTP[S] to the hCXCR2–C352I $G_{\alpha i2}$ fusion (Figure 4B). As noted for the hDOP–C352I $G_{\alpha i2}$ construct, introduction of the G204A mutation into the G-protein element of the hCXCR2–C352I $G_{\alpha i2}$ fusion protein essentially eliminated the response to IL-8 (Figure 4C). The same was true when, based on sequence comparisons and a central role for hydrophobic residues in the second intracellular loop of class A GPCRs in agonist activation of G-proteins [43], an I148E hCXCR2–C352I $G_{\alpha i2}$ construct was generated and expressed (Figure 4C).

FLAG–hDOP-G204A,C352I $G_{\alpha i2}$ was next expressed stably and constitutively in Flp-In T-REx HEK-293 cells that harboured c-Myc–I148E-CXCR2-C352I $G_{\alpha i2}$ at the Flp-In locus and individual clones were isolated. These cells should allow c-Myc–I148E-CXCR2-C352I $G_{\alpha i2}$ expression to be controlled in an entirely tetracycline/doxycycline 'on' fashion. This expectation was confirmed via immunocytochemistry. Although plasma-membrane-localized anti-FLAG immunoreactivity was observed in these cells both with and without treatment with doxycycline (Figure 5A), anti-CXCR2 reactivity was only observed following doxycycline treatment (Figure 5A) and this overlapped strongly with the anti-FLAG signal (Figure 5A). DADLE (10^{-6} M) promoted binding of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates from membranes of pertussis-toxin-treated cells of this clone that had been exposed to doxycycline, but not in those that had not been treated with doxycycline (Figure 5B). This occurred in a concentration-dependent manner with a pEC$_{50} = -7.2$ (Figure 5C). This can only reflect guanine-nucleotide exchange on the C352I $G_{\alpha i2}$ linked to c-Myc–I148E CXCR2 and is entirely consistent with the other observations of hCXCR2–hDOP opioid receptor hetero-oligomerization (Figures 1 and 2). Although without effect in the absence of DADLE, co-addition of SB225002 (10^{-6} M) resulted in higher levels of DADLE-stimulated [^{35}S]GTP[S] binding without altering the potency of the opioid agonist (pEC$_{50} = -7.1$) (Figure 5C). When similar experiments were performed with a single concentration of DADLE (10^{-6} M), increasing concentrations of SB225002 resulted in an increase in DADLE-stimulated binding of [^{35}S]GTP[S] with a pEC$_{50}$ of -7.2
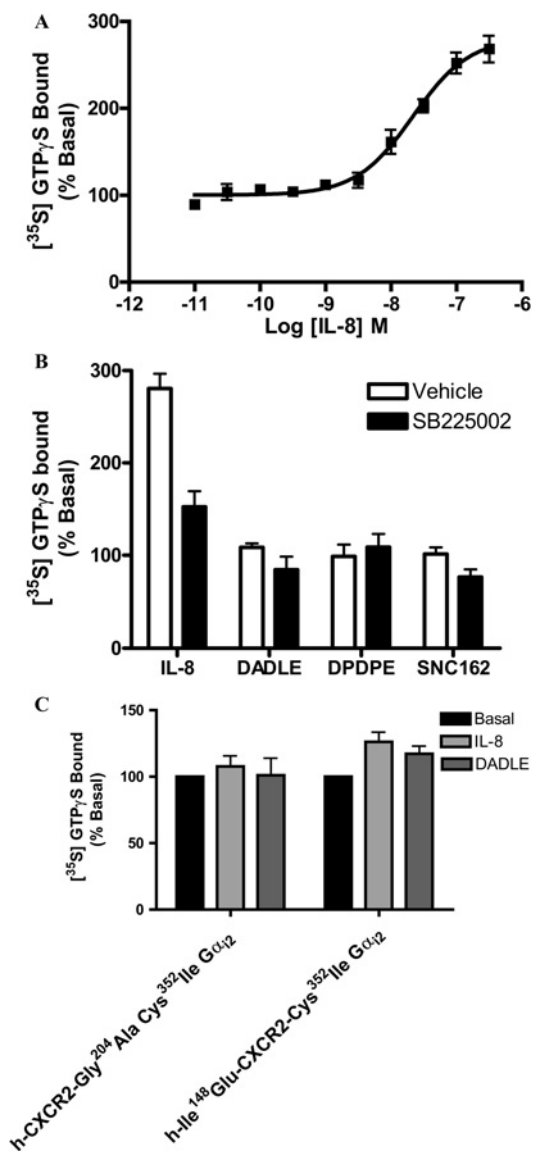
Figure 4   Analysis of hCXCR2–C352I G$_{\alpha i2}$ fusion proteins

(**A**) hCXCR2–C352I G$_{\alpha i2}$ was expressed transiently in HEK-293 cells. Following pertussis-toxin treatment (25 ng/ml for 16 h) and membrane preparation, binding of [^{35}S]GTP[S] in G$_{\alpha i2}$ immunoprecipitates was measured in the presence of various concentrations of IL-8. (**B**) [^{35}S]GTP[S] binding studies were performed as in (**A**) in the presence of 3×10^{-7} M IL-8, or 10^{-6} M of each of DADLE, DPDPE or SNC162 (open bars). In the solid bars the experiments were repeated with the addition of 10^{-6} M SB225002. (**C**) hCXCR2–G204A,C352I G$_{\alpha i2}$ or hI148E CXCR2–C352I G$_{\alpha i2}$ were expressed transiently in HEK-293 cells. Following pertussis-toxin treatment (25 ng/ml for 16 h) and membrane preparation, binding of [^{35}S]GTP[S] in G$_{\alpha i2}$ immunoprecipitates was measured in the absence of ligand (Basal) or the presence of 3×10^{-7} M IL-8 (IL-8) or 10^{-6} M DADLE (DADLE). Values are means $\pm$ S.E.M. ($n = 3$).

for SB225002 (Figure 5D). These effects were not restricted to DADLE. Entirely analogous effects of SB225002 were observed when either a second synthetic enkephalin, DPDPE (Figure 5D), or the non-peptide ligand SNC162 [28] (Figure 5D) were used as agonists at the DOP receptor.

Because of the ability to control c-Myc–I148E hCXCR2–C352I G$_{\alpha i2}$ expression in these cells in the face of constitutive expression of FLAG–hDOP–G204A,C352I G$_{\alpha i2}$, we therefore examined the time course of induction of c-Myc–I148E hCXCR2–C352I G$_{\alpha i2}$ expression following addition of doxycycline (1 μg/ml) to these cells. Immunoblotting cell lysates

with an anti-c-Myc antibody confirmed a lack of expression of this polypeptide without doxycycline treatment (Figure 6A). c-Myc–I148E h CXCR2–C352I G$_{\alpha i2}$ expression could be detected within 6 h of doxycycline treatment, but at that time the fusion protein was present largely as an immature form that lacked terminal N-glycosylation (Figure 6A). By 24 h, higher levels of c-Myc–I148E hCXCR2–C352I G$_{\alpha i2}$ were present and a substantial amount of this polypeptide was terminally N-glycosylated. This pattern was maintained in the presence of doxycycline for up to at least 96 h (Figure 6A). Levels of FLAG–hDOP–G204A, C352I G$_{\alpha i2}$ expression were unaffected by the induction of c-Myc–I148E hCXCR2–C352I G$_{\alpha i2}$ expression (Figure 6A). As anticipated, without doxycycline treatment, DADLE could not stimulate binding of [^{35}S]GTP[S] in membranes of these cells following pertussis-toxin treatment, whereas with time of doxycycline treatment, both the capacity of DADLE to elevate binding of [^{35}S]GTP[S] in G$_{\alpha i2}$ immunoprecipitates and the effect of SB225002 to enhance G-protein activation in response to DADLE mirrored the time course of appearance of c-Myc–I148E hCXCR2–C352I G$_{\alpha i2}$ immunoreactivity (Figure 6B).

Although a highly effective means to explore these receptor co-expression-dependent heterodimer-specific [25,44] allosteric effects of CXCR2 antagonists on DOP receptor function, the fusion proteins are artificial constructions [45,46]. Hence, to explore these effects further we generated extra Flp-In T-REx HEK-293 cell lines in which hCXCR2 N-terminally tagged with VSV-G was cloned into the inducible locus and in which FLAG–hDOP was expressed constitutively. As anticipated, anti-FLAG immuno-cytochemistry demonstrated the presence of FLAG–hDOP both without and with treatment of the cells with doxycycline (Figure 7A). In contrast, anti-VSV-G immunoreactivity was only detected following treatment of the cells with doxycycline (Figure 7A) and merging of the images indicated co-localization of VSV-G–hCXCR2 and FLAG–hDOP at the cell surface (Figure 7A). In membranes derived from both untreated and doxycycline-treated cells, DADLE-stimulated binding of [^{35}S]GTP[S] was blocked by the general opioid receptor antagonist naloxone (Figure 7B). However, although DADLE-stimulated binding of [^{35}S]GTP[S] in membranes of untreated cells was unaffected by SB225002 (Figure 7B), in doxycycline-treated cells SB225002 again enhanced the capacity of DADLE (10^{-6} M) to promote binding of [^{35}S]GTP[S] (Figures 7B and 7C) and did so in a con-centration-dependent manner with a pEC$_{50} = -8.1$ (Figure 7C). IL-8 stimulated binding of [^{35}S]GTP[S] only in membraness of doxycycline-treated cells and, in contrast with the effect of DADLE, SB225002 blocked the effect of IL-8, whereas naloxone was without effect (Figure 7C).

DISCUSSION

GPCRs are the most tractable class of therapeutic targets for the design and development of small-molecule therapeutic medicines and it has been estimated that some 30–50 % of clinically available drugs target the function of GPCR family members [47,48]. All but two of the medicines that interact directly with a GPCR do so via the orthosteric binding site, i.e. the binding site for the natural ligand, and function either to mimic or block the action of the natural ligand [25]. Despite this, and due in part to the clinical effectiveness of cinacalcet, that acts as a positive allosteric modulator of the Ca^{2+}-sensing receptor [49,50], and the recent development of the anti-HIV medicine maraviroc that acts as a CCR5 receptor-directed negative allosteric regulator [25,51], there is now considerable interest in understanding the basis and mode of action of ligands that bind to allosteric sites on
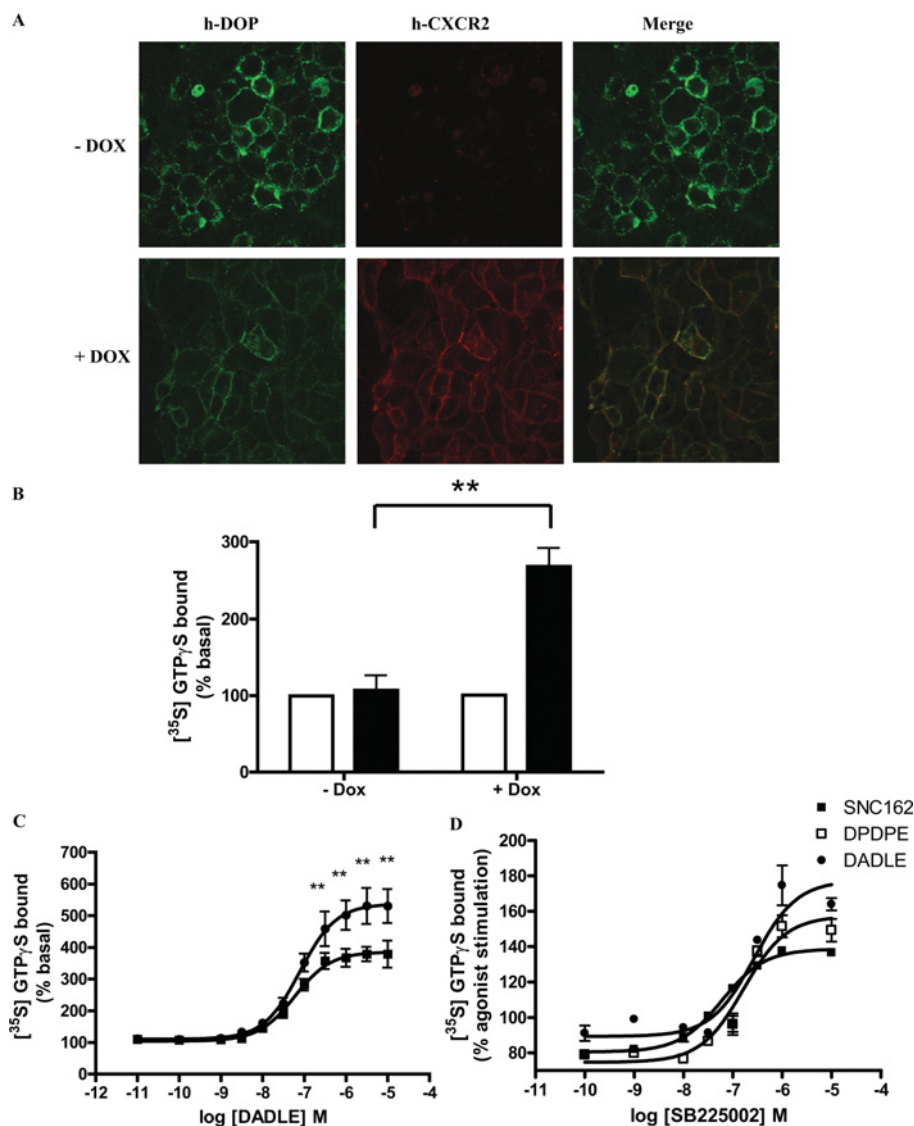
Figure 5   Inducible expression of c-Myc–I148E–CXCR2–C352I $G_{\alpha i2}$ in the presence of FLAG–hDOP-G204A,C352I $G_{\alpha i2}$ results in their co-localization at the surface of Flp-In T-REx HEK-293 cells and G-protein activation by DADLE

(**A**) Flp-In T-REx HEK-293 cells were generated that harboured c-Myc–I148E–CXCR2–C352I $G_{\alpha i2}$ at the Flp-In locus and constitutively expressed FLAG–hDOP–C352I $G_{\alpha i2}$. Cells were treated with (lower panels) or without (upper panels) doxycycline (1 μg/ml, 48 h). FLAG–hDOP–C352I $G_{\alpha i2}$ (green) was detected using anti-FLAG and c-Myc–I148E-CXCR2–C352I $G_{\alpha i2}$ (red) with anti-CXCR2. Merged images are also shown (right-hand panels). (**B**) Flp-In T-REx HEK-293 cells as in (**A**) were treated (+ Dox) or not (−Dox) with doxycycline (1 μg/ml, 48 h) and with pertussis toxin (25 ng/ml) for the last 16 h. Membranes of these cells were used to measure binding of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates in the absence (open bars) and presence (solid bars) of DADLE (10^{-6}M). **$P < 0.001$, significantly greater after treatment with doxycycline (measured using the paired t test. (**C**) Membranes of the above doxycycline- and pertussis-toxin-treated Flp-In T-REx HEK-293 cells were used to measure binding of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates in response to varying concentrations of DADLE (■) and DADLE + 10^{-6}M SB225002 (●). **$P < 0.001$, significant enhancement in the presence of SB225002 (measured using one-way ANOVA). (**D**) Membranes as above were used to measure binding of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates in the presence of 10^{-6}M DADLE (●), DPDPE (□) or SNC162 (■) and various concentrations of SB225002. Values are means $\pm$ S.E.M. ($n = 3$).

GPCRs [52–54]. This reflects, in part, that true allosteric ligands are anticipated to function only in the presence of an orthosteric agonist and to modulate the effectiveness of signal transduction. To date, virtually all studies on allosteric regulators of GPCRs have focused on modulators that bind to the same GPCR as the orthosteric ligand but at a separate location. This reflects a combination of the traditional view that GPCRs exist and function as non-interacting monomeric species and that ligand screening strategies concentrate on analysis of the function of a single GPCR expressed in isolation.

It is now widely accepted that GPCRs can form dimers and/or higher-order oligomers and that dimerization is probably integral

to function [16,55]. Although the majority of early studies concentrated on homodimerization, i.e. interactions between multiple copies of the same GPCR, there has been a growing base of evidence to support the concept that GPCR heterodimerization can occur and that it is relevant to physiological function. Given the widespread co-expression of many GPCRs, such 'heterodimers' are being considered as novel sets of therapeutic targets. Indeed, as the multiplicity of opioid receptor pharmacologies *in vivo* are far too complex to be explained by individual monomers, or indeed homodimers, of the molecularly defined DOP, KOP and MOP receptors, there is a large literature on how opioid receptor heterodimerization may help to explain
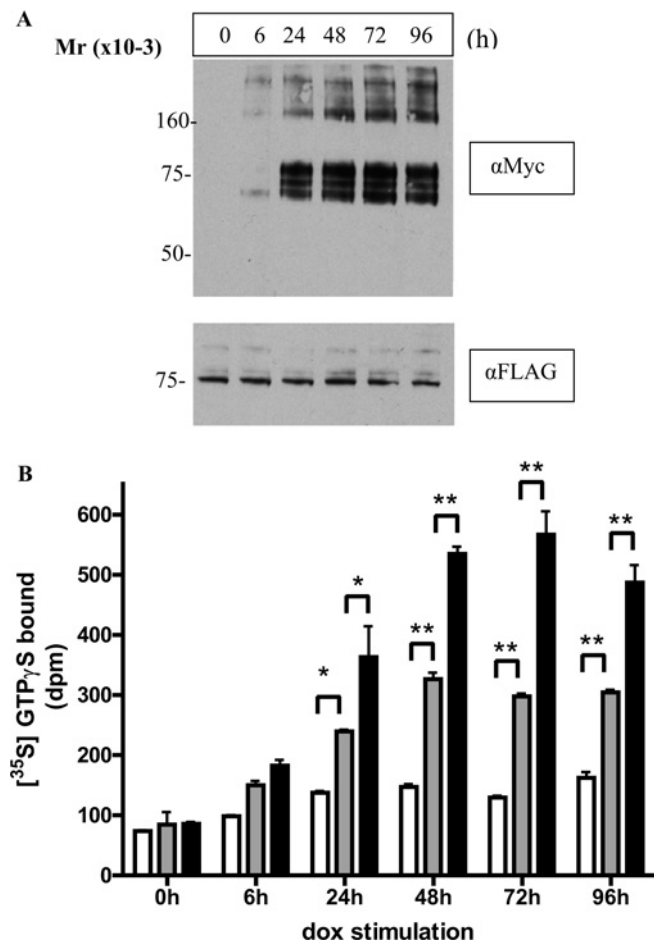
Figure 6   The effect of SB225002 requires expression of hCXCR2

Flp-In T-REx HEK-293 cells that harboured c-Myc–I148E-CXCR2–C352I $G_{\alpha i2}$ at the Flp-In locus and constitutively expressed FLAG–hDOP–C352I $G_{\alpha i2}$ were treated for various times with doxycycline (1 μg/ml). Following pertussis-toxin treatment and membrane preparation, samples were resolved by SDS/PAGE and immunoblotted (**A**) to detect c-Myc–I148E-CXCR2–C352I $G_{\alpha i2}$ (upper panel) or FLAG–hDOP–C352I $G_{\alpha i2}$ (lower panel). (**B**) Membranes of these cells were then used to assess basal binding (open bars) of [^{35}S]GTP[S] in $G_{\alpha i2}$ immunoprecipitates and the effects of DADLE (10^{-6} M; light-grey bars) or DADLE + SB225002 (both at 10^{-6} M; black bars). *$P < 0.01$, **$P < 0.001$ (as compared with control; measured using one-way ANOVA).

this complex pharmacology [56,57]. Furthermore, based on the co-expression patterns of opioid receptor subtypes, apparently orthosteric ligands have been reported to show considerable selectivity for specific opioid receptor heterodimer pairings [58].

In a GPCR heterodimer, it might be anticipated that the binding of a ligand to the orthosteric site of one GPCR could alter the pharmacology and function of the orthosteric binding site of the partner GPCR [25]. In such a situation the ligand at the first GPCR would act as an allosteric agent for the orthosteric agonist of the second GPCR and this would be a heterodimer-specific effect [25] because the ligand would display no direct effect on the second GPCR in assays in which the second GPCR was expressed alone. This concept has important implications for the design, identification and use of novel small-molecule regulators of GPCRs. For example, the allosteric effect of such ligands would be restricted to cells and tissues in which the GPCR heterodimer is present and would only be detected in assays in which the relevant target GPCR heterodimer is present [25].
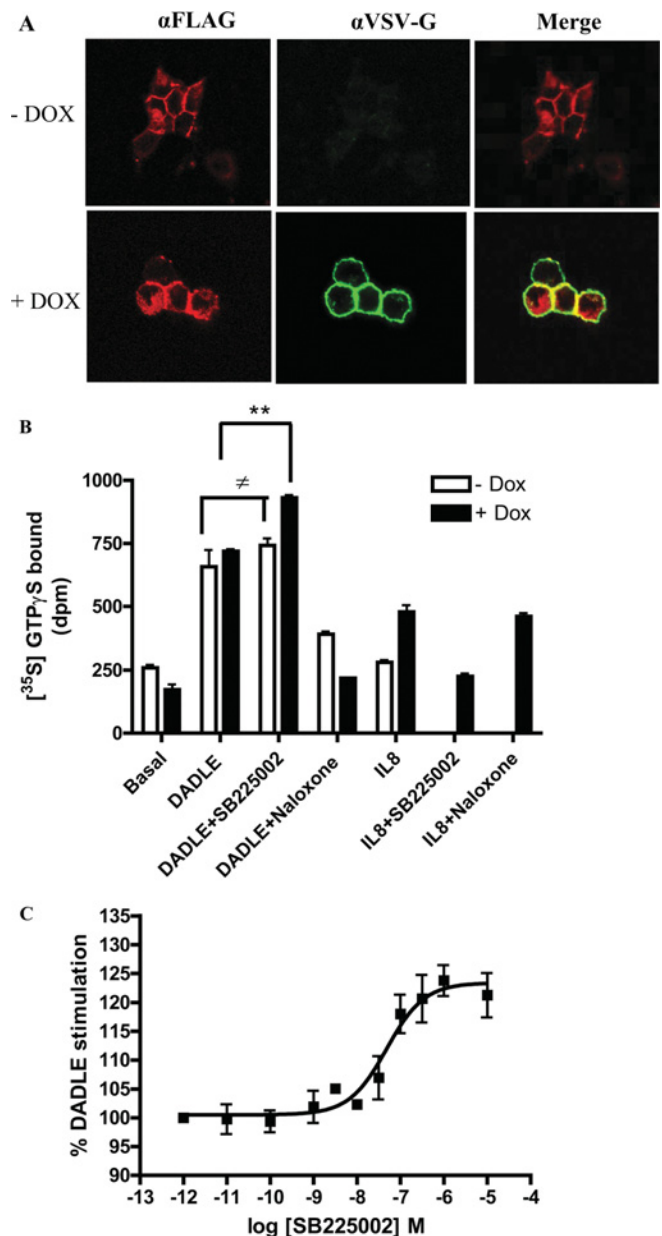

Figure 7   Production and characterization of Flp-In T-REx HEK-293 cells harbouring VSV-G–hCXCR2 and constitutively expressing FLAG–hDOP: SB225002 enhances the function of an opioid agonist in cells co-expressing hDOP and hCXCR2

(**A**) Flp-In T-REx HEK-293 cell lines were created in which hCXCR2 which was N-terminally tagged with VSV-G was cloned into the inducible locus and in which FLAG–hDOP was expressed constitutively. Cells were treated with (lower panels) or without (upper panels) doxycycline (1 μg/ml, 48 h). Anti-FLAG immunocytochemistry (red) demonstrated the presence of FLAG–hDOP both without and with treatment of the cells with doxycycline. In contrast, anti-VSV-G immunoreactivity (green) was only detected following treatment of the cells with doxycycline. Merging of the images (right-hand panels) indicated co-localization of VSV-G–hCXCR2 and FLAG–hDOP (yellow) at the cell surface when both constructs were expressed. (**B**) Membranes of Flp-In T-REx HEK-293 cells as in (**A**) were used both without (open bars) or following doxycycline induction of VSV-G–hCXCR2 expression (solid bars) to measure the ability of DADLE (10^{-6} M), DADLE (10^{-6} M) + SB225002 (10^{-6} M), DADLE (10^{-6} M) + nal-oxone (10^{-5} M), IL-8 (10^{-8} M), IL-8 (10^{-8} M) + SB225002 (10^{-6} M) or IL-8 (10^{-8} M) + naloxone (10^{-5} M) to enhance basal binding of [^{35}S]GTP[S]. **SB225002 enhances the effect of DADLE ($P < 0.001$); ≠, SB225002 does not have a significant effect. (**C**) Following doxycycline induction of VSV-G–hCXCR2 expression, membranes as above were used to measure binding of [^{35}S]GTP[S] in response to DADLE (10^{-6} M) in the presence of various concentrations of SB225002.

Although this concept has not been explored widely, there are a number of reports consistent with such a mechanism. For example, in cells co-expressing the CCR2b and CCR5 chemokine receptors CCR5-specific ligands that are unable to compete for MCP-1 binding on cells expressing CCR2b alone, and hence presumable do not bind directly to CCR2b, were able to prevent efficiently MCP-1 binding when the two receptors were co-expressed [26]. In a similar vein, binding of the agonist [^{125}I]melatonin to the MT1 receptor was eliminated when the orphan GPCR GPR50 was co-expressed along with the melatonin MT1 receptor [27]. Furthermore, in cells natively co-expressing GPR50 and the MT1 receptor, specific [^{125}I]melatonin binding could not be observed until GPR50 levels were reduced via a siRNA (small interfering RNA)-based approach [27]. GPR50 is related to the MT1 receptor and these workers also provided a series of observations consistent with their heterodimerization [27]. Perhaps even more interesting from a therapeutic standpoint, the cannabinoid CB1 receptor orthosteric antagonist/inverse agonist rimonabant (marketed as Acomplia™), which has been approved in Europe for the treatment of obesity, decreases the potency of the orexigenic peptide orexin A only when the cannabinoid CB1 receptor is co-expressed with the orexin-1 receptor [33] and these two GPCRs effectively form a heterodimer [33].

As noted earlier, opioid receptors are widely expressed by white blood cells and there is a substantial literature on functional interactions between opioid ligands and chemokines in the regulation of white-cell chemotaxis. Prior to the present studies there have also been reports of direct physical interactions between opioid and chemokine receptors. Previously, these have concentrated on the CCR5 receptor due to its role as a co-receptor for HIV-1 entry into CD4$^+$ white cells and the susceptibility of opiate addicts to succumb to HIV-1 infection. Furthermore, very recent studies by Pello et al. [59] have shown that interactions between the chemokine CXCR4 receptor and the DOP receptor have functional consequences in immune cells in that co-addition of agonist ligands for these receptors fail to generate function, leading to a suppression of signalling potentially because the heterodimer produces a dominant-negative effect.

Therefore our initial studies centred on obtaining evidence for physical interactions between the CXCR2 receptor and the DOP opioid receptor. This was achieved by a combination of co-immunoprecipitation and various intact cell resonance energy transfer techniques, whereas the use of so-called 'saturation' BRET studies [36,37] suggested that CXCR2 receptor–DOP opioid receptor hetero-interactions may display higher avidity than either corresponding homo-interaction. This concept is not without parallel. Work on melatonin receptor subtypes has indicated greater avidity of hetero-interactions between the MT1 and MT2 receptors than the corresponding MT1–MT1 or MT2–MT2 homo-interactions [60].

For a number of years we [38,39] and others (see [61,62] for review) have employed fusion proteins, in which a G-protein α subunit is linked in-frame to the C-terminal tail of a GPCR to generate chimaeric, bi-functional polypeptides to explore many aspects of GPCR and G-protein function and pharmacology. We have also used this approach to examine GPCR homodimerization by demonstrating reconstitution of function only upon co-expression of pairs of GPCR–G-protein fusions that are individually inactive [30,63]. When we co-expressed pairs of such fusion proteins in which, in the first, the CXCR2 receptor was not signal-transduction-competent because of the introduction of a mutation into the second intracellular loop, whereas in the second the DOP receptor was wild-type but the linked G-protein was modified to prevent guanine-nucleotide exchange, DOP agonists were able to cause activation of the wild-type G-protein linked to the CXCR2

receptor in a concentration-dependent manner. Furthermore, following co-expression of this pairing, a CXCR2 antagonist was able to enhance the maximal activity of a range of DOP receptor agonists with distinct chemotypes. We also attempted to explore whether the CXCR2 agonist IL-8 also modulated the function or potency of DOP receptor agonists. However, we were unable to observe such effects (results not shown). The reasons for this are unclear. It could be hypothesized that an agonist ligand might have a greater effect on the structure/function of the partner GPCR of the heterodimer because agonist ligands must alter the conformation of their own target receptor to initiate signal transduction. Such an effect might then alter the orthosteric binding pocket or function of the GPCR heterodimer partner. However, it is impractical at this stage to speculate on the basis or implications of our inability to detect such an effect, not least because of the size and structural differences between the peptide agonist IL-8 and the synthetic small-molecule CXCR2 blocker employed.

Key experiments employed Flp-In T-REx HEK-293 cells. These contain a single site of chromosomal integration from which constructs cloned into this locus can be expressed in a completely inducible fashion. In these studies c-Myc–I148E CXCR2–C352I G$_{\alpha i2}$ was harboured at the Flp-In locus, whereas in the same cells FLAG–hDOP–G204A,C352I G$_{\alpha i2}$ was expressed stably and constitutively. Antibiotic-induced turn-on of c-Myc–I148E CXCR2–C352I G$_{\alpha i2}$ expression was required to observe both DOP receptor function and the positive allosteric effects of the CXCR2 antagonist on this. Initial preliminary studies had confirmed that the DOP ligands had no direct effect at the CXCR2 receptor and that the CXCR2 antagonist had no direct effects at the DOP receptor, indicating the allosteric effects to be heterodimer specific. Importantly, as well as requiring expression of CXCR2, the allosteric effects of the CXCR2 antagonist were produced in a concentration-dependent fashion and with affinity consistent with its occupancy of the CXCR2 orthosteric binding site.

Although these key experiments were performed using pairs of GPCR–G-protein fusions it is important to note that entirely equivalent results were produced when Flp-In T-REx HEK-293 cells were established in which the unfused wild-type hDOP receptor was expressed constitutively and the unfused mutant hCXCR2 receptor could be produced, on demand, from the Flp-In T-REx locus.

The present studies provide clear biochemical, biophysical and now pharmacological evidence to indicate the capacity of CXCR2 and DOP opioid receptors to interact physically. They also clearly indicate the capacity of GPCR heterodimers to provide novel allosteric pharmacology. Detection of such activities in ligand screening campaigns will be a challenge because they will be identified by neither primary screens, nor secondary counter screens, in which individual GPCRs are expressed and studied 'one target at a time' [25]. However, such heterodimer specific allosteric ligands offer obvious opportunities, modulating the function of the partner GPCR only in the presence of the orthosteric ligand for that receptor and doing so only in cells and tissues in which the relevant heterodimer is expressed [25]. It will be interesting to see if this principle can be exploited therapeutically.

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