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CXCR2 chemokine receptor antagonism enhances DOP opioid receptor function via allosteric regulation of the CXCR2-DOP receptor hetero-dimer

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[2]**Abbreviations**: APC, allophycocyanin; eCFP, enhanced cyan fluorescent protein; DADLE, D-ala[2]-D-leu[5] enkepkalin; DPDPE, [D-Pen[2,5]]enkepkalin; FRET, fluorescence resonance energy transfer; GPCRs, G protein-coupled receptors; IL8, interleukin 8; MCP-1, monocyte chemoattractant protein; SB225002, N-(2-hydroxy-4-nitrophenyl)-N'-(2-bromophenyl)urea; SNC 162, 4-[(S)-[(2S,5R) 2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]phenylmethyl]-*N*,*N*-diethylbenzamide; tr-FRET, time-resolved fluorescence resonance energy transfer; eYFP, enhanced yellow fluorescent protein.

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SYNOPSIS

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 alternate 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 hetero-dimer partner for the CXCR2 receptor. These results have novel and important implications for the development and use of small molecule therapeutics.

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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 to 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 G protein-coupled receptors (GPCRs)[2]. 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 interleukin 8 (IL8, 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 IL8-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 hetero-dimers/oligomers (i.e. dimers formed between two different GPCR gene products) [16, 22]. Such hetero-dimers/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 homo-dimerize or to hetero-dimerize 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 homo-dimerize and to show that these two GPCRs were able to form hetero-dimers as effectively as homo-dimers.

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It has recently been demonstrated that selective ligands at pairs of GPCRs that hetero-dimerize have the potential to modulate ligand effects at the partner GPCR via allosterism (see [25] for review). Studies on hetero-dimerization 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 monocyte chemoattractant protein (MCP-1) on cells expressing CCR2b alone, to efficiently prevent 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 current study we use a range of approaches to demonstrate the capacity for hetero-dimerization 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

Diprenorphine [15,16-^{3}H] (50 Ci/mmol) and guanosine 5'-(γ-thio) triphosphate [^{35}S] (1250 Ci/mmol) were from PerkinElmer Life Science, Inc. (Boston, MA). DADLE (D-ala^{2}, leu^{5}-enkephalin), DPDPE ([D-Pen^{2,5}]-enkephalin), and Pertussis toxin were from SIGMA-Aldrich Company Ltd., (Poole, Dorset, U.K.). N-(2-hydroxy-4-nitrophenyl)-N'-(2-bromophenyl)urea (SB225002) was from Calbiochem, and -[(S)-[(2S,5R) 2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]phenylmethyl]-*N,N*-diethylbenzamide (SNC 162) [28] was from Tocris. All reagents for BRET^{2} 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 Company Ltd., Poole, Dorset, UK. The rabbit polyclonal anti-c-myc antiserum was from Cell Signalling Technology, Nottingham, U.K.

Molecular Constructs

h-DOP-Cys^{352}Ile Gα_{i2}, Val^{150}Glu,Val^{154}Asp h-DOP-Cys^{352}Ile Gα_{i2} and h-DOP-Gly^{204}Ala,Cys^{352}Ile Gα_{i2} were constructed as in Pascal and Milligan [30], except that Cys^{352}Ile Gα_{i2} replaced Cys^{351}Ile Gα_{i1}. CXCR2-Cys^{352}Ile Gα_{i2} and Ile^{148}Glu CXCR2-Cys^{352}Ile Gα_{i2} were constructed using similar approaches. FLAG-h-CXCR2, c-myc-h-CXCR2 and forms of the CXCR2 C-terminally tagged with auto-fluorescent proteins or *Renilla* luciferase were described previously [21] as were the equivalent modified forms of h-DOP [31]. See Table 1 for the nomenclature of the molecular constructs employed.

Fluorescent microscopy and FRET imaging in living cells

Cells were grown on poly-D-lysine treated coverslips and transiently transfected with appropriate eCFP/eYFP fusion proteins. Coverslips were placed into a microscope chamber containing physiological saline solution (130mM NaCl, 5mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 20mM HEPES, 10mM D-glucose pH 7.4). Cells were visualized using a Nikon Eclipse TE2000-E fluorescence inverted microscope and images obtained individually for eYFP, eCFP and FRET filter channels using an

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Optoscan monochromator (Cairn Research, Faversham, Kent, U.K.) and a dichroic mirror 86002v2bs (Chroma Inc., Rockingham, VT). The filter sets used were: eYFP (excitation – 500/5nm; emission – 535/30nm), eCFP (excitation 430/12nm; emission – 470/30nm) and FRET (excitation – 430/12nm; emission – 535/30nm). The illumination time was 250ms and binning modes 2x2. 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 – (coefficient B x eCFP) – (coefficient A x 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 varying amounts of donor (eCFP) and acceptor (eYFP), normalized FRET was calculated using the equation FRETn = FRETc / eCFP x eYFP, where FRETc, eCFP and eYFP are equal to the fluorescence values obtained from single cells.

Time resolved (Tr) FRET

$10cm^2$ dishes of HEK293T cells were transfected to express N-terminally c-myc or FLAG-tagged forms of h-CXCR2 and/or h-DOP individually or in combination. 48 h after transfection the cells were harvested. Cell pellets were resuspended in 200µl of ice-cold PBS. Anti-c-myc Eu^{3+} and anti-FLAG allophycocyanin (APC) antibodies [21, 31] were diluted in 50% newborn calf serum: 50% PBS to final concentrations of 5nM and 15nM respectively. Samples were mixed and incubated on a rotating wheel at room temperature for 2 h while covered in aluminium foil to minimize exposure of the fluorophores to light. Samples were centrifuged at 1000 x g for 1 min and the antibody mix removed from the cell pellet. The pellet was then washed 2 x in ice-cold PBS and resuspended in 250µl of PBS. To measure the energy transfer, 40µl 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 340nm and emission filters generated data representing donor (615nm) and acceptor (665nm) fluorescence. Normalized FRET was calculated using the equation: Normalized FRET = $((A_{665}\text{-BLK})/D_{615})\text{-C}$, 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 665nm 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 $A_{665}\text{-BLK}/D_{615}$.

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) construct. BRET^2 was assessed using the luciferase substrate DeepBlue C in intact cells. Cells were also harvested, membranes prepared and diluted to 0.5mg/ml. Luminescence and fluorescence measurements were then obtained to measure construct expression. 50µl of cell membranes were dispensed into white-walled 96-well plates (PerkinElmer) for luminescence measurements and black-walled 386-plates (Costar, Cambridge, MA) for fluorescence measurements. For luminescence measurement h-coelenterazine (5µM) was added and the plate incubated at room temperature for 30 min prior to measurement at 410nm using a

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Mithras LB 940. GFP[2] fluorescence was assessed using a Victor[2] 1420 Multilabel counter (PerkinElmer). Fluorescence readings were corrected for endogenous fluorescence of HEK293T 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.

Cell transfection and treatment

HEK293 cells were transfected transiently using Lipofectamine reagent (Gibco Life Technologies) or Gene Juice (Novagen) and the appropriate cDNA(s) according to the manufacturers' instructions. Where appropriate, cells were treated with Pertussis toxin (25 ng/ml) for 16 to 18 h prior to harvest.

[^{3}H]Diprenorphine binding

The expression of h-DOP-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 100µM naloxone. Samples were incubated for 1h at 25°C and bound ligand separated from free by vacuum filtration through GF/B filters pre-treated with 0.3% polyethyleneimine in TEM (10mM Tris/HCL, 0.1mM EDTA, 10mM $MgCl_2$, pH adjusted to 7.5). Bound ligand was estimated by liquid scintillation spectroscopy. Competition studies were conducted with 1nM [^{3}H]diprenorphine and a range of concentrations of other ligands. Data were analysed using GraphPad Prism software (San Diego, CA). Saturation data were fit to non-linear regression curves.

[^{35}S]GTPγS binding studies

Experiments were initiated by adding the assay buffer mix (20 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 100mM NaCl, 10 µM GDP, 0.2 mM ascorbic acid) containing 50 nCi [^{35}S]GTPγS in the presence or absence of ligands to defined amounts of membranes. Non-specific binding was determined in the presence of 100 µM GTPγS. The reaction was incubated for 15 min at 30°C and terminated by adding 1ml of ice-cold stop buffer. The samples were centrifuged for 15 min at 16000 x g at 4°C and the resulting pellets were resuspended in solubilization buffer (100 mM Tris HCl, 200 mM NaCl, 1 mM EDTA, 1.25% NP40, pH adjusted to 7.4) plus 0.2% SDS. Samples were pre-cleared with Pansorbin for 1h at 4°C and centrifuged for 2 min at 16000 x g. Supernatant was added to a mix of protein G and the anti-$G_{i1\alpha}$/$G_{i2\alpha}$ antiserum, SG [29] and left rotating overnight at 4°C for immunoprecipitation. The immunocomplexes were washed twice with ice-cold solubilization buffer and bound [^{35}S]GTPγS measured.

Co-immunoprecipitation

Cells were resuspended in 1ml of 1 x radio-immunoprecipitation assay (RIPA) buffer and rotated for 60 min at 4°C to allow lysis. The samples were centrifuged at 14,000 x g for 10min at 4°C and the supernatant retained. 50µl of a protein G-sepharose/phosphate buffered saline slurry was added to the supernatant and rotated for further 60 min at 4°C to pre-clear. Samples were centrifuged at 14000 x g for 10 min at 4°C. Supernatant was conserved and protein concentration was measured using the BCA assay method. Samples were equalized to 1µg/µl. Target proteins were then immunoprecipitated from 500µl

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samples by incubation with 20µl of protein G-sepharose and the appropriate antibody/antiserum overnight at 4°C on a rotating wheel. Immune complexes were isolated by centrifugation at 14000 x g for 1 min and washed twice with RIPA buffer. Proteins were eluted from the protein G-sepharose by the addition of 30-50µl Laemmli buffer and heated for 4 min at 85°C. The eluates were then loaded onto SDS-PAGE gels.

Construction of Flp-In T-REx HEK293 cell lines

The basic protocol used to generate Flp-In T-REx HEK293 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 Dulbecco's modified Eagle's medium without sodium pyruvate, 4500 mg/litre 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 HEK293 cell lines able to inducibly express c-myc-Ile^{148}Glu CXCR2-Cys^{352}Ile $G\alpha_{i2}$ or VSV-G-CXCR2, 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 manufacturers' 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-h-DOP-Gly^{204}Ala, Cys^{352}Ile $G\alpha_{i2}$ or FLAG-h-DOP 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 to 96 h before assays to induce expression of receptors and receptor constructs cloned into the Flp-In locus.

Immunostaining protocol

Cells were grown onto coverslips, 24 h later medium was removed and the cells incubated with 20mM HEPES/DMEM containing the appropriate dilution of primary antibody for 40 min at 37°C in 5% CO_2. Following 3 washes with PBS cells were fixed by incubating with 4% paraformaldehyde in PBS/5% sucrose for 10 min at room temperature. Following 3 further washes cells were permeabilized with 0.15% Triton-X-100/3% nonfat milk/PBS for 10 min. The coverslips were incubated with a secondary antibody (5µg/ml) conjugated to an Alexa^{594} fluorophore. Following incubation for 1 h cells were washed twice in 0.15% Triton-X-100/3% non-fat milk/PBS and three times in PBS. Coverslips were then mounted onto 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 63x 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 Inc., Thornwood, NY). The excitation laser line for GFP and eYFP was the 488nm argon laser with detection via a 505-530 band pass filter. Alexa^{594} label was excited using a 543nm helium/neon laser and detected via a 560nm long-pass filter. The images were manipulated using MetaMorph imaging software (version 6.1.3; Universal Imaging Corporation, Downing, PA).

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RESULTS

To examine potential interactions between the chemokine CXCR2 and DOP opioid receptors the human (h) 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 homo-dimers/oligomers via co-immunoprecipitation studies [21, 31]. Expression in HEK293 cells of FLAG-h-CXCR2 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-glycosylation [21]. Expression of c-myc-h-DOP resulted in the presence of a c-myc reactive polypeptide of some 60 kDa (**Figure 1**). Only with co-expression of FLAG-h-CXCR2 and c-myc-h-DOP did immunoprecipitation with 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 substantially higher apparent 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 time-resolved fluorescence resonance energy transfer (tr-FRET) studies in intact HEK293 cells [21, 31] to detect protein complexes containing both receptors at the cell surface. Co-expression of c-myc-h-CXCR2 and FLAG-h-DOP followed by the addition of a combination of Eu^{3+}-labeled anti-c-myc, to act as a long-lived energy donor, and APC-labeled 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 HEK293 cell populations individually expressing either c-myc-h-CXCR2 or FLAG-h-DOP were combined prior to addition of the combination of Eu^{3+}-labeled and APC-labeled antibodies (**Figure 2A**). Furthermore, the extent of energy transfer following co-expression of c-myc-h-CXCR2 and FLAG-h-DOP was virtually the same as when c-myc-h-DOP and FLAG-h-DOP were co-expressed to generate tr-FRET competent, cell surface h-DOP homo-dimers (**Figure 2A**). We have also previously shown that in-frame fusion of auto-fluorescent proteins to the C-terminus of each of h-CXCR2 [21] and h-DOP [31, 35] does not significantly alter the function or pharmacology of these receptors. Co-expression of h-CXCR2-enhanced cyan fluorescent protein (eCFP) and h-DOP-enhanced yellow fluorescent protein (eYFP) resulted in a capacity to image eCFP to eYFP fluorescence resonance energy transfer (FRET) in individual single cells (**Figure 2B**), providing further evidence for direct h-CXCR2-h-DOP interactions. Estimates of the relative affinities of GPCRs to interact can be obtained from 'saturation' bioluminescence resonance energy transfer (BRET) studies [36-37]. In such experiments forms of GPCRs C-terminally tagged with *Renilla* luciferase and with an auto-fluorescent protein that is able to act as an energy acceptor of light emitted from substrate oxidation by the luciferase are co-expressed in varying ratios and BRET signal monitored. Co-expression of h-CXCR2-*Renilla* luciferase and h-CXCR2-GFP^2 in HEK293 cells resulted in BRET following addition of the luciferase substrate DeepBlueC (**Figure 2C**). At low energy acceptor (h-CXCR2-GFP^2) to energy donor (h-CXCR2-*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 a [acceptor] to [donor] ratio of 1.6 +/- 0.1. Co-expression of h-DOP-*Renilla* luciferase and h-DOP-GFP^2 also generated BRET signals that

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saturated with increasing [acceptor] to [donor] ratios, in this case with $BRET_{50}$ = 2.2 +/- 0.07. Co-expression of h-DOP-*Renilla* luciferase with h-CXCR2-GFP[2] generated BRET signals that saturated, and in this case $BRET_{50}$ was 0.34 +/- 0.02 (**Figure 2C**). These data are consistent with h-CXCR2-h-DOP hetero-interactions occurring with an even higher affinity than the corresponding h-CXCR2-h-CXCR2 and h-DOP- h-DOP 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 $Cys^{352}Ile$ [40-41], pertussis toxin-insensitive variant of $G\alpha_{i2}$ was linked in-frame to the C-terminal tail of the h-DOP receptor. Following transient expression in HEK293 cells, treatment with pertussis toxin (25 ng/ml, 16 h) to cause ADP-ribosylation of endogenously expressed $G\alpha_i$-G proteins, and membrane preparation, the synthetic enkephalin D-ala[2]-D-leu[5] enkepkalin (DADLE) (10^{-6}M) caused a 4-5 fold increase in the binding of [^{35}S]GTPγS in $G\alpha_{i2}$ immunoprecipitates (**Figure 3A**). By contrast, the non-peptide CXCR2 antagonist N-(2-hydroxy-4-nitrophenyl)-N'-(2-bromophenyl) urea (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 h-DOP-$Cys^{352}Ile$ $G\alpha_{i2}$ fusion protein indicated a pK_i for DADLE = 9.0 +/- 0.09, whilst for SB225002 pK_i was > 4.0 (**data 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 whilst the other contains the inactivating mutation in the G protein [30]. Variants of h-DOP-$Cys^{352}Ile$ $G\alpha_{i2}$ that did not modify the ligand binding domain but failed to significantly increase binding of [^{35}S]GTPγS in response to DADLE were produced by mutation of either $Val^{150}Glu$ + $Val^{154}Asp$ in the receptor element or by introduction of a $Gly^{204}Ala$ mutation into $G\alpha_{i2}$ (**Figure 3B**). As demonstrated previously for the equivalent h-DOP-$Cys^{351}Ile$ $G\alpha_{i1}$ mutants [30], co-expression of $Val^{150}Glu$,$Val^{154}Asp$ h-DOP-$Cys^{352}Ile$ $G\alpha_{i2}$ and h-DOP-$Gly^{204}Ala$,$Cys^{352}Ile$ $G\alpha_{i2}$ reconstituted the ability of DADLE to stimulate binding of [^{35}S]GTPγS in membranes of pertussis toxin-treated HEK293 cells, whilst 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]GTPγS in response to DADLE (**not shown**, but see ref [30]).

We next generated a h-CXCR2-$Cys^{352}Ile$ $G\alpha_{i2}$ fusion construct. Following transient expression in HEK293 cells, pertussis toxin treatment and membrane preparation, IL8 was able to promote binding of [^{35}S]GTPγS in $G\alpha_{i2}$ immunoprecipitates in a concentration-dependent manner with pEC_{50} -7.4 +/- 0.2 (**Figure 4A**). This effect of IL8 was blocked by the co-addition of SB225002 (**Figure 4B**) whilst each of DADLE, the DOP opioid receptor selective enkepkalin [D-Pen[2,5]]enkepkalin (DPDPE) and the highly DOP receptor selective small molecule SNC162 [28] were unable to promote binding of [^{35}S]GTPγS to the h-CXCR2-$Cys^{352}Ile$ $G\alpha_{i2}$ fusion (**Figure 4B**). As noted for the h-DOP-$Cys^{352}Ile$ $G\alpha_{i2}$ construct, introduction of the $Gly^{204}Ala$ mutation into the G protein element of the h-CXCR2-$Cys^{352}Ile$ $G\alpha_{i2}$ fusion protein essentially eliminated response to IL8 (**Figure 4C**). The

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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], a h-Ile^{148}Glu CXCR2-Cys^{352}Ile Gα_{i2} construct was generated and expressed (**Figure 4C**).

FLAG-h-DOP-Gly^{204}Ala, Cys^{352}Ile Gα_{i2} was next expressed stably and constitutively in Flp-In T-REx HEK293 cells that harboured c-myc-Ile^{148}Glu-CXCR2-Cys^{352}Ile Gα_{i2} at the Flp-In locus and individual clones isolated. These cells should allow c-myc-Ile^{148}Glu-CXCR2-Cys^{352}Ile Gα_{i2} expression to be controlled in an entirely tetracycline/doxycycline 'on' fashion. This expectation was confirmed via immunocytochemistry. Whilst 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]GTPγS in Gα_{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 pEC$_{50}$ = -7.2 (**Figure 5C**). This can only reflect guanine nucleotide exchange on the Cys^{352}Ile Gα_{i2} linked to c-myc-Ile^{148}Glu CXCR2 and is entirely consistent with the other observations of h-CXCR2-h-DOP 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]GTPγS 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]GTPγS with pEC$_{50}$ -7.2 for SB225002 (**Figure 5D**). These effects were not restricted to DADLE. Entirely analogous effects of SB225002 were observed when either a second synthetic enkephalin, [D-Pen^{2,5}]enkepkalin (DPDPE) (**Figure 5D**) or the non-peptide ligand SNC 162 (28) (**Figure 5D**) were used as agonists at the DOP receptor.

Because of the ability to control c-myc-Ile^{148}Glu-h-CXCR2-Cys^{352}Ile Gα_{i2} expression in these cells in the face of constitutive expression of FLAG-h-DOP-Gly^{204}Ala,Cys^{352}Ile Gα_{i2} we therefore examined the time-course of induction of c-myc-Ile^{148}Glu-h-CXCR2 Cys^{352}Ile Gα_{i2} expression following addition of doxycycline (1 µg/ml) to these cells. Immunoblotting cell lysates with anti-c-myc confirmed a lack of expression of this polypeptide without doxycycline treatment (**Figure 6A**). c-myc-Ile^{148}Glu h-CXCR2-Cys^{352}Ile Gα_{i2} expression could be detected within 6 hours 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 hours, higher levels of c-myc-Ile^{148}Glu-h-CXCR2-Cys^{352}Ile Gα_{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-h-DOP-Gly^{204}Ala, Cys^{352}Ile Gα_{i2} expression were unaffected by the induction of c-myc-Ile^{148}Glu h-CXCR2-Cys^{352}Ile Gα_{i2} expression (**Figure 6A**). As anticipated, without doxycycline treatment, DADLE could not stimulate binding of [^{35}S]GTPγS in membranes of these cells following pertussis toxin treatment, whilst with time of doxycycline treatment, both the capacity of DADLE to elevate binding of [^{35}S]GTPγS in Gα_{i2} immunoprecipitates and the effect of SB225002 to enhance G

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protein activation in response to DADLE mirrored the time-course of appearance of c-myc-Ile[148]Glu-h-CXCR2-Cys[352]Ile $G\alpha_{i2}$ immunoreactivity (**Figure 6B**).

Although a highly effective means to explore these receptor co-expression-dependent, hetero-dimer 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 HEK293 cell lines in which h-CXCR2 N-terminally tagged with VSV-G was cloned into the inducible locus and in which FLAG-h-DOP was expressed constitutively. As anticipated, anti-FLAG immunocytochemistry demonstrated the presence of FLAG-h-DOP 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- h-CXCR2 and FLAG-h-DOP at the cell surface (**Figure 7A**). In membranes derived from both untreated and doxycycline-treated cells, DADLE stimulated binding of [^{35}S]GTPγS that was blocked by the general opioid receptor antagonist naloxone (**Figure 8B**). However, although DADLE-stimulated binding of [^{35}S]GTPγS 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]GTPγS (**Figure 7B, C)** and did so in a concentration-dependent manner with pEC_{50} = - 8.1 (**Figure 7C**). IL8 stimulated binding of [^{35}S]GTPγS only in membranes of doxycycline-treated cells and in contrast to the effect of DADLE, SB225002 blocked the effect of IL8 whilst 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 to either 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 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 likely integral to function [16, 55]. Although the majority of early studies concentrated on homo-dimerization, i.e. interactions between multiple copies of the same GPCR, there has been a growing base of evidence to

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support the concept that GPCR hetero-dimerization can occur and that it is relevant to physiological function. Given the widespread co-expression of many GPCRs such 'hetero-dimers' 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 homo-dimers, of the molecularly defined DOP, KOP and MOP receptors, there is a large literature on how opioid receptor hetero-dimerization may help to explain 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 hetero-dimer pairings [58].

In a GPCR hetero-dimer, 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 hetero-dimer 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 hetero-dimer is present and would only be detected in assays in which the relevant target GPCR hetero-dimer is present [25].

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 monocyte chemoattractant protein (MCP-1) binding on cells expressing CCR2b alone, and hence presumable do not bind directly to CCR2b, were able to efficiently prevent 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 GPCR50 levels were reduced via a siRNA-based approach [27]. GPR50 is related to the MT1 receptor and these workers also provided a series of observations consistent with their hetero-dimerization [27]. Perhaps even more interestingly from a therapeutic standpoint, the cannabinoid CB1 receptor orthosteric antagonist/inverse agonist rimonabant (marketed as Acomplia^{TM}), 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 hetero-dimer [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 current 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, in very recent studies Pello et al., [59] have shown that interactions between the chemokine CXCR4 receptor and the DOP receptor has functional consequences in immune cells in that co-addition of agonist ligands for these receptors fails to generate function, leading to

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a suppression of signaling potentially because the hetero-dimer produces a dominant negative effect.

Our initial studies centred, therefore, 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, whilst 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 chimeric, bi-functional polypeptides to explore many aspects of GPCR and G protein function and pharmacology. We have also used this approach to examine GPCR homo-dimerization 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, whilst 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 IL8 also modulated the function or potency of DOP receptor agonists. However, we were unable to observe such effects (data not shown). The reasons for this are unclear. It could be hypothesised that an agonist ligand might have a greater effect on the structure/function of the partner GPCR of the hetero-dimer 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 hetero-dimer 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 IL8 and the synthetic small molecule CXCR2 blocker employed.

Key experiments employed Flp-In T-REx HEK293 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-Ile^{148}Glu-CXCR2-Cys^{352}Ile Gα_{i2} was harboured at the Flp-In locus, whilst in the same cells FLAG-h-DOP-Gly^{204}Ala, Cys^{352}Ile Gα_{i2} was expressed stably and constitutively. Antibiotic-induced turn-on of c-myc-Ile^{148}Glu-CXCR2-Cys^{352}Ile Gα_{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 hetero-dimer 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.

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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 HEK293 cells were established in which the unfused, wild type h-DOP receptor was expressed constitutively and the unfused, mutant h-CXCR2 receptor could be produced, on demand, from the Flp-In T-REx locus.

The current 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 hetero-dimers 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 hetero-dimer 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 hetero-dimer is expressed [25]. It will be interesting to see if this principle can be exploited therapeutically.

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Table 1
Characteristics of the molecular constructs employed

Construct	Description
FLAG-h-CXCR2	N-terminal FLAG-tagged form of human CXCR2
c-myc-h-CXCR2	N-terminal c-myc-tagged form of human CXCR2
h-CXCR2-eCFP	C-terminal eCFP-tagged form of human CXCR2
h-CXCR2-GFP^2	C-terminal eGFP^2-tagged form of human CXCR2
h-CXCR2-*Renilla* luciferase	human CXCR2 C-terminally tagged with *Renilla* luciferase

h-CXCR2-Cys^{352}Ile Gα_{i2} Fusion protein in which a pertussis toxin-insensitive form of Gα_{i2} is linked to the C-terminus of human CXCR2

Ile^{148}Glu h-CXCR2-Cys^{352}Ile Gα_{i2} Fusion protein in which a pertussis toxin-insensitive form of Gα_{i2} is linked to the C-terminus of human CXCR2 containing a Ile^{148}Glu mutation that prevents receptor-mediated activation of the G protein

c-myc-h-DOP	N-terminal c-myc-tagged form of human DOP receptor
h-DOP-eYFP	C-terminal eGFP^2-tagged form of human DOP receptor
h-DOP-GFP^2	C-terminal eGFP^2-tagged form of human DOP receptor
h-DOP-*Renilla* luciferase	human DOP receptor C-terminally tagged with *Renilla* luciferase

h-DOP-Cys^{352}Ile Gα_{i2} Fusion protein in which a pertussis toxin-insensitive form of Gα_{i2} is linked to the C-terminus the human DOP receptor

Val^{150}Glu,Val^{154}Asp h-DOP-Cys^{352}Ile Gα_{i2} Fusion protein in which a pertussis toxin-insensitive form of Gα_{i2} linked to the C-terminus of a mutant human DOP receptor that can bind ligands but not transduce a signal

h-DOP-Gly^{204}Ala,Cys^{352}Ile Gα_{i2} Fusion protein in which a pertussis toxin-insensitive form of Gα_{i2} that cannot be activated is linked to the C-terminus of the human DOP receptor

Figure Legends

Figure 1
Co-expression of FLAG-h-CXCR2 and c-myc-h-DOP allows their co-immunoprecipitation
HEK293 cells were mock-transfected (**mock**) or transfected to transiently express FLAG-h-CXCR2, c-myc-h-DOP or both (**co-transfected**). Samples containing either FLAG-h-CXCR2 or c-myc-h-DOP 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 (**lower panels**). Cell lysates were subsequently immunoprecipitated using anti-FLAG. Immunoprecipitated samples were resolved by SDS-PAGE and immunoblotted with anti c-myc (**upper panel**). Two further experiments produced similar results.

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Figure 2
FRET and BRET studies confirm hetero-interactions between co-expressed h-CXCR2 and h-DOP receptors

A. **TrFRET**: c-myc-h-DOP and FLAG-h-DOP or c-myc-h-CXCR2 and FLAG-h-DOP were expressed individually in HEK293 cells that were then mixed (**mix**) or the two receptors were co-expressed (**co**). Following addition of a combination of Eu^{3+}-labeled anti-c-myc, to act as a long-lived energy donor, and APC-labeled anti-FLAG, to act as a potential energy acceptor, to intact cells TrFRET was monitored as in Experimental.
B. **FRET imaging**: h-DOP-eYFP (**eYFP**) was transiently expressed in HEK293 cells with (**lower panels**) or without (**upper panels**) h-CXCR2-eCFP (**eCFP**) and fluorescence imaged. Raw FRET (**FRET)** and calculated normalized FRET (**right hand panels**) was then assessed as in Experimental.
C. **Saturation BRET^2 studies**: h-CXCR2-*Renilla* luciferase and h-CXCR2-GFP^2 (**filled squares**), h-CXCR2-*Renilla* luciferase and h-DOP-GFP^2 (**open squares**) or h-DOP-*Renilla* luciferase and h-DOP-GFP^2 (**open circles**) were transiently co-expressed in HEK293 cells. Following addition of the luciferase substrate/ BRET^2 energy donor DeepBlueC BRET measurements were made. Donor and acceptor ratios were assessed as in Experimental.

Each experiment is representative of three.

Figure 3
h-DOP-Gα_{i2} fusion proteins are activated by DADLE but not by SB225002
A: A h-DOP-Cys^{352}Ile Gα_{i2} fusion protein was expressed transiently in HEK293 cells. Following pertussis toxin treatment (25 ng/ml, 16 h) and membrane preparation, basal binding of [^{35}S]GTPγS in Gα_{i2} immunoprecipitates (**Basal**) and its regulation by DADLE (10^{-6}M), concentrations of SB225002 from 10^{-5}M-10^{-9}M and DADLE (10^{-6}M) + SB225002 (10^{-5}M) was assessed.
B: Val^{150}Glu,Val^{154}Asp h-DOP-Cys^{352}Ile Gα_{i2} and h-DOP-Gly^{204}Ala,Cys^{352}Ile Gα_{i2} was co-expressed transiently in HEK293 cells. Following pertussis toxin treatment (25 ng/ml, 16 h) and membrane preparation, binding of [^{35}S]GTPγS in Gα_{i2} immunoprecipitates in response to the same ligands as in **A** was measured
Data represents means +/- SEM, n =3.

Figure 4
Analysis of h-CXCR2-Cys^{352}IleGα_{i2} fusion proteins

A. h-CXCR2-Cys^{352}IleGα_{i2} was expressed transiently in HEK293 cells. Following pertussis toxin treatment (25 ng/ml, 16 h) and membrane preparation, binding of [^{35}S]GTPγS in Gα_{i2} immunoprecipitates was measured in the presence of varying concentrations of IL8.
B. [^{35}S]GTPγS binding studies were performed as in **A** in the presence of 3 x 10^{-7} M IL8, or 10^{-6} M of each of DADLE, DPDPE, or SNC162 (**open bars**). In the **filled bars** the experiments were repeated with the addition of 10^{-6} M SB225002.
C. h-CXCR2-Gly^{204}Ala,Cys^{352}IleGα_{i2} or h-Ile^{148}Glu CXCR2-Cys^{352}IleGα_{i2} were expressed transiently in HEK293 cells. Following pertussis toxin treatment (25 ng/ml, 16 h) and membrane preparation, binding of [^{35}S]GTPγS in Gα_{i2} immunoprecipitates was measured in the absence of ligand (basal) or the presence of 3 x 10^{-7} M IL8 (**IL8**) or 10^{-6} M DADLE (**DADLE**).

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Data represent means +/- S.E.M. n = 3.

Figure 5
Inducible expression of c-myc-Ile^{148}Glu-CXCR2-Cys^{352}Ile Gα_{i2} in the presence of FLAG-h-DOP-Gly^{204}Ala, Cys^{352}Ile Gα_{i2} results in their co-localization at the surface of Flp-In T-REx HEK293 cells and G protein activation by DADLE
A. Flp-In T-REx HEK293 cells were generated that harboured c-myc-Ile^{148}Glu-CXCR2- Cys^{352}Ile Gα_{i2} at the Flp-In locus and constitutively expressed FLAG-h-DOP-Cys^{352}Ile Gα_{i2}. Cells were treated with (**bottom panels**) or without (**upper panels**) doxycycline (1 µg/ml, 48 h). FLAG-h-DOP-Cys^{352}Ile Gα_{i2} (**green**) was detected using anti-FLAG and c-myc-Ile^{148}Glu-CXCR2-Cys^{352}Ile Gα_{i2} (**red**) with anti-CXCR2. Merged images are also shown (**right hand panels**).
B. Flp-In T-REx HEK293 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]GTPγS in Gα_{i2} immunoprecipitates in the absence (**open bars**) and presence (**filled bars**) of DADLE (10^{-6}M). ** Significantly greater after treatment with doxycycline, $p < 0.001$, paired t-test.
C. Membranes of the above doxycycline and pertussis toxin-treated Flp-In T-REx HEK293 cells were used to measure binding of [^{35}S]GTPγS in Gα_{i2} immunoprecipitates in response to varying concentrations of DADLE (**squares**) and DADLE + 10^{-6}M SB225002 (**circles**). ** Significant enhancement in the presence of SB225002, $p < 0.001$, One way ANOVA.
D. Membranes as above were used to measure binding of [^{35}S]GTPγS in Gα_{i2} immunoprecipitates in the presence of 10^{-6}M DADLE (**filled circles**), DPDPE (**open squares**) or SNC162 (**filled squares**) and varying concentrations of SB225002. Data represents means +/- SEM, n = 3.

Figure 6
The effect of SB225002 requires expression of h-CXCR2
Flp-In T-REx HEK293 cells that harbored c-myc-Ile^{148}Glu-CXCR2-Cys^{352}Ile Gα_{i2} at the Flp-In locus and constitutively expressed FLAG-h-DOP-Cys^{352}Ile Gα_{i2} were treated for varying 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-Ile^{148}Glu-CXCR2-Cys^{352}Ile Gα_{i2} (**upper panel**) or FLAG-h-DOP-Cys^{352}Ile Gα_{i2} (**lower panel**). **B**. Membranes of these cells were then used to assess basal binding (**open bars**) of [^{35}S]GTPγS in Gα_{i2} immunoprecipitates and the effects of DADLE (10^{-6}M) (**light bars**) or DADLE + SB225002 (both at 10^{-6}M) (**dark bars**). * $p < 0.01$, ** $p < 0.001$, One way ANOVA.

Figure 7
Production and characterization of Flp-In T-REx HEK293 cells harbouring VSV-G-h-CXCR2 and constitutively expressing FLAG-h-DOP: SB225002 enhances the function of an opioid agonist in cells co-expressing h-DOP and h-CXCR2
A. Flp-In T-REx HEK293 cell lines were created in which h-CXCR2 N-terminally tagged with VSV-G was cloned into the inducible locus and in which FLAG-h-DOP

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was expressed constitutively. Cells were treated with **(bottom panels**) or without (**upper panels**) doxycycline (1 μg/ml, 48 h). Anti-FLAG immunocytochemistry (**red**) demonstrated the presence of FLAG-h-DOP 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- h-CXCR2 and FLAG-h-DOP (**yellow**) at the cell surface when both constructs were expressed.
B. Membranes of Flp-In T-REx HEK293 cells as in **A** were used both without (**filled bars**) or following doxycycline induction of VSV-G- h-CXCR2 expression (**open bars**) to measure the ability of DADLE (10^{-6}M) , DADLE (10^{-6}M) + SB225002 (10^{-6}M), DADLE (10^{-6}M) + naloxone (10^{-5}M), IL8 (10^{-8}M), IL8 (10^{-8}M) + SB225002 (10^{-6}M) or IL8 (10^{-8}M) + naloxone (10^{-5}M) to enhance basal binding of [^{35}S]GTPγS.
** SB225002 enhances the effect of DADLE $p < 0.001$; = SB225002 does not have a significant effect.
C. Following doxycycline induction of VSV-G- h-CXCR2 expression, membranes as above, were used to measure binding of [^{35}S]GTPγS in response to DADLE (10^{-6}M) in the presence of varying concentrations of SB225002.

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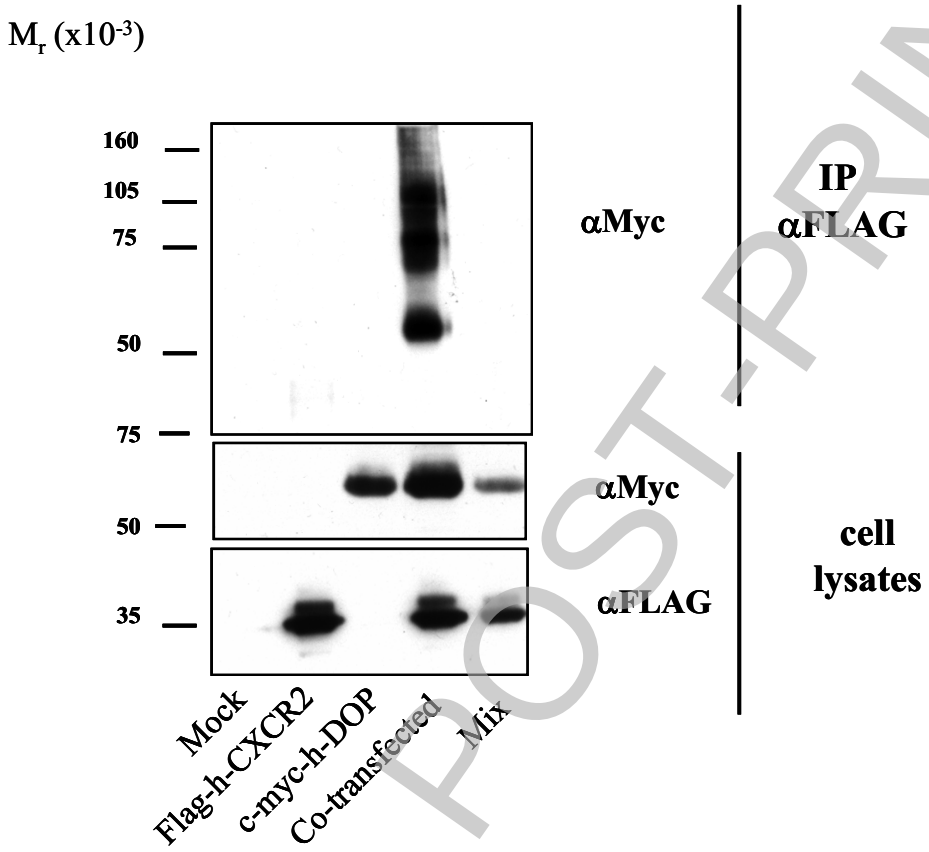

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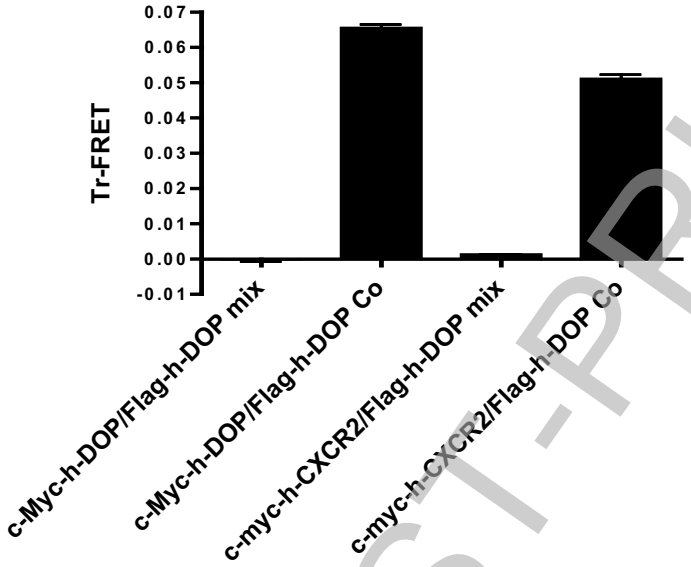

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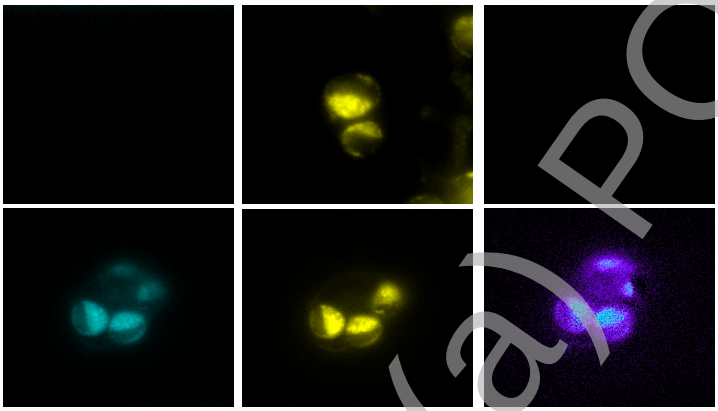

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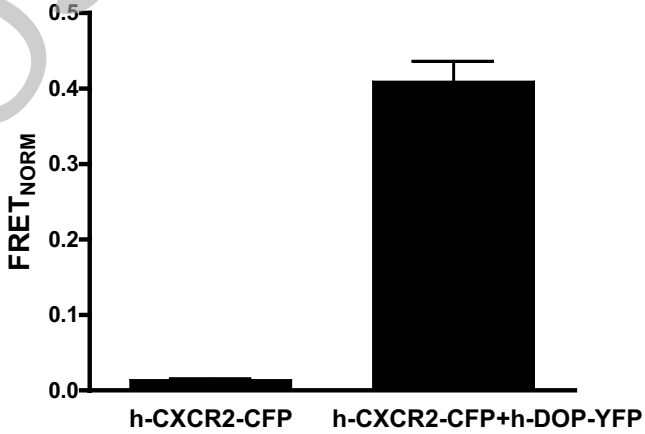

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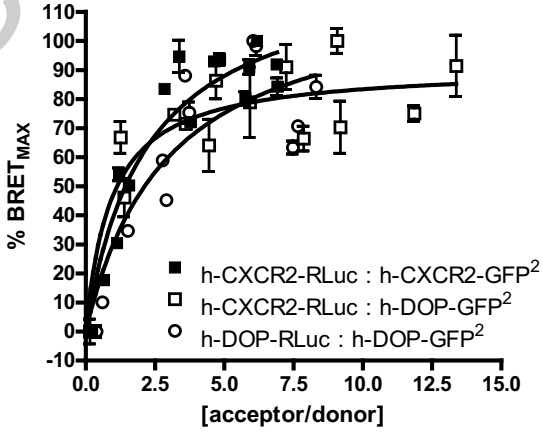

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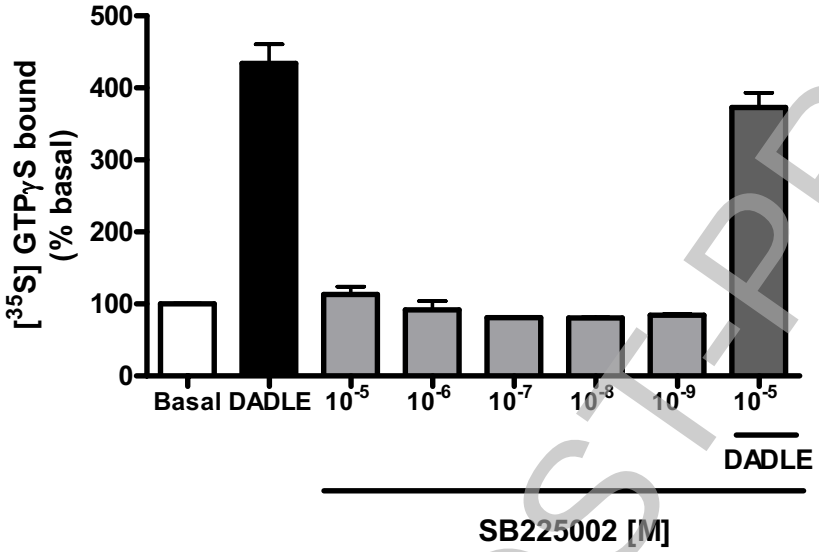

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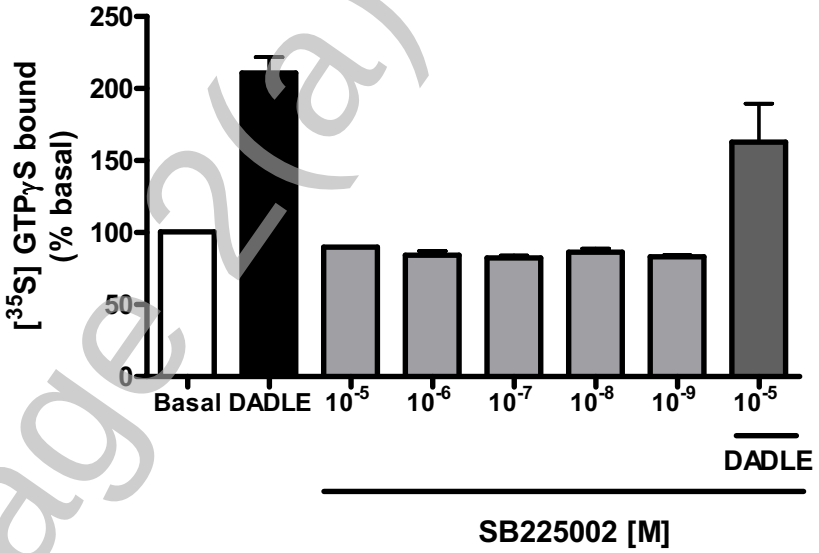

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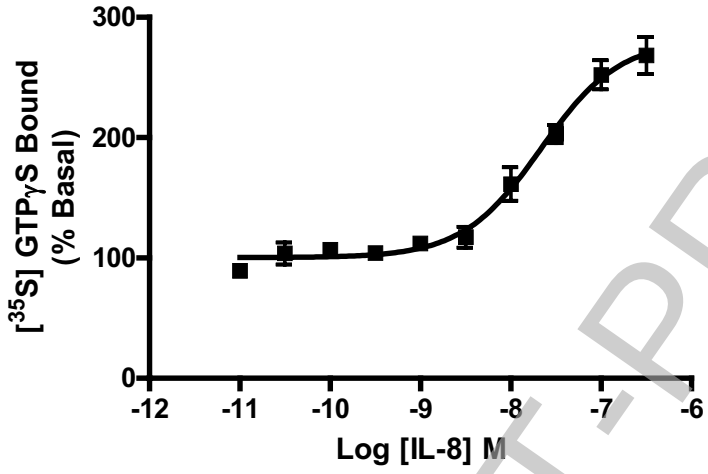

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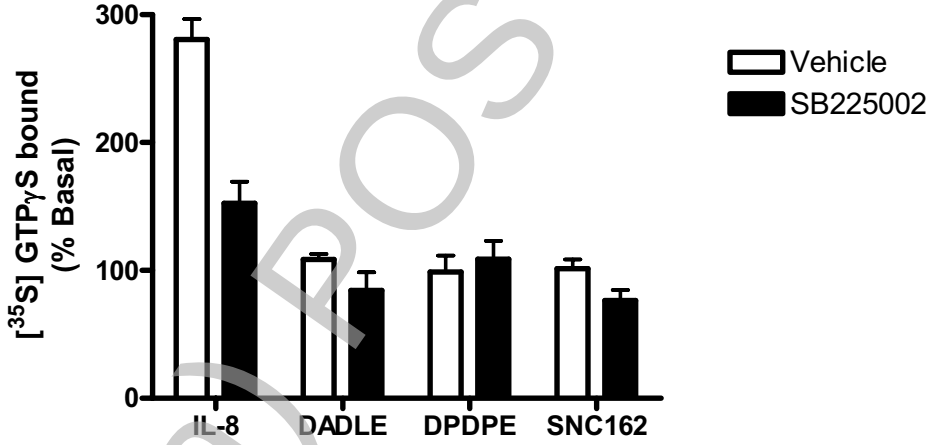

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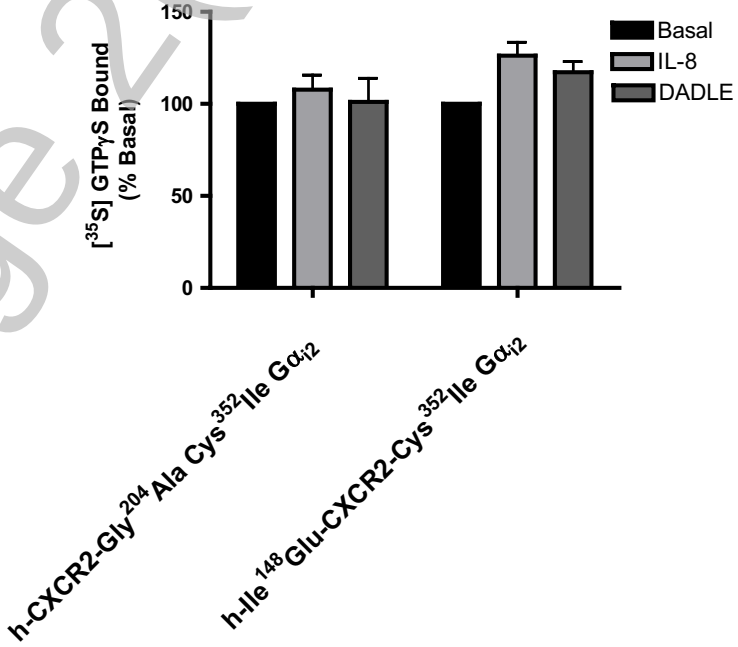

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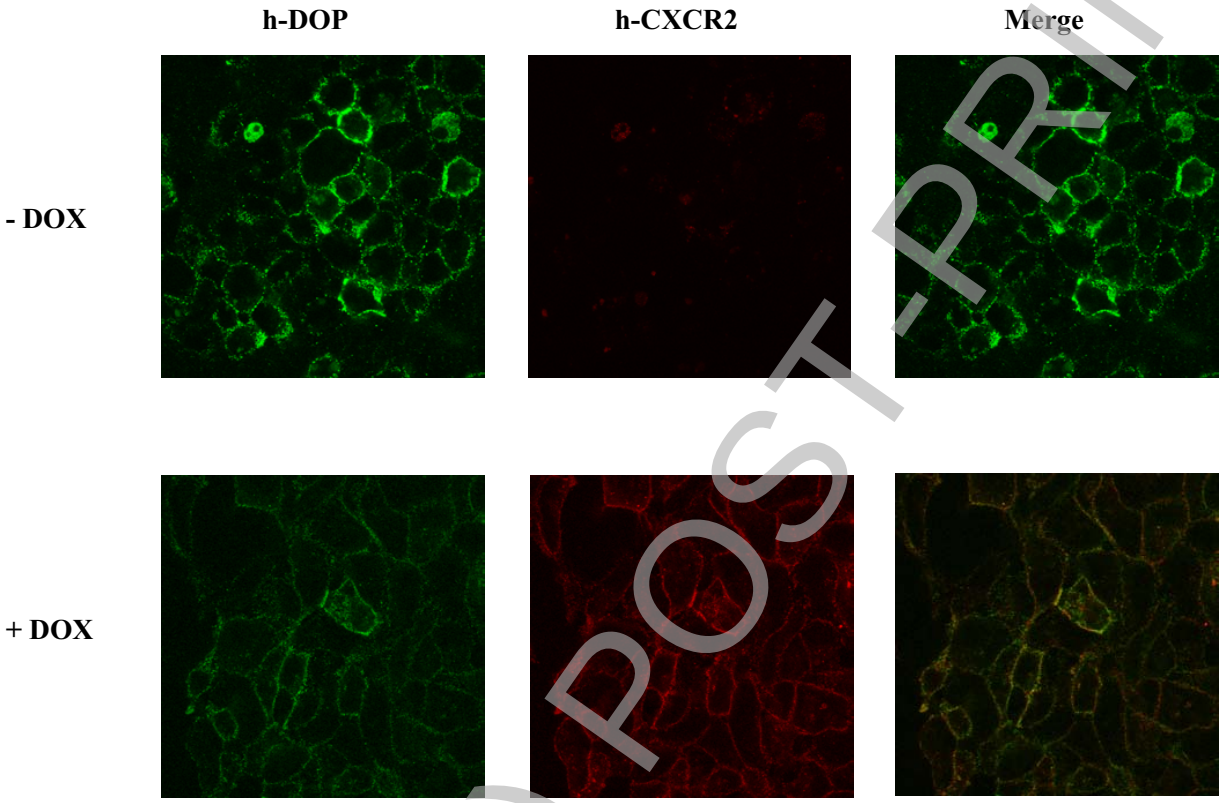

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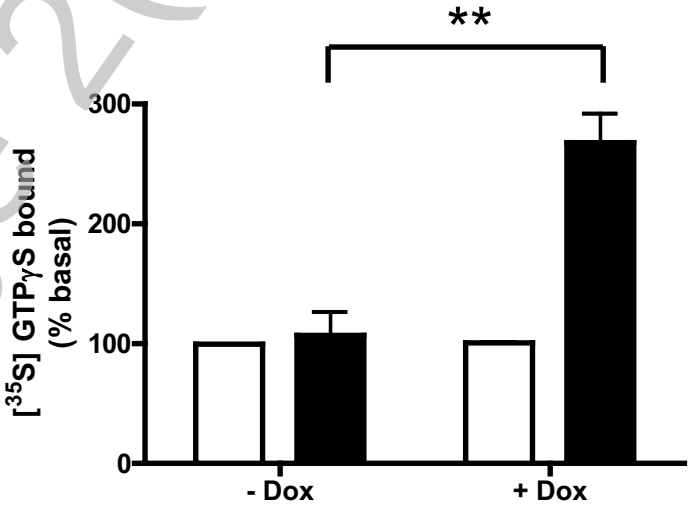

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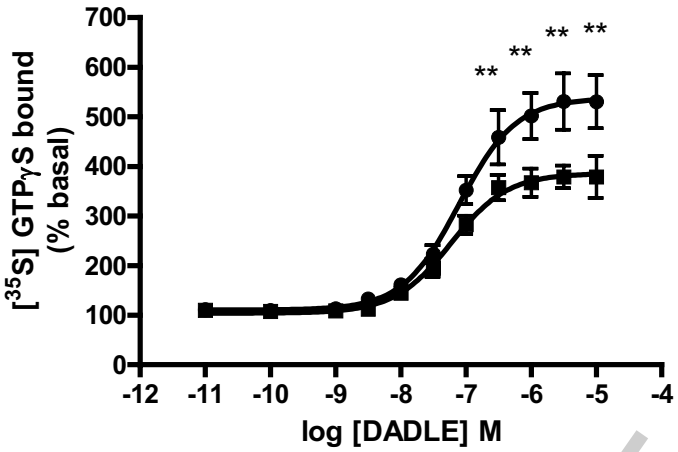
C.



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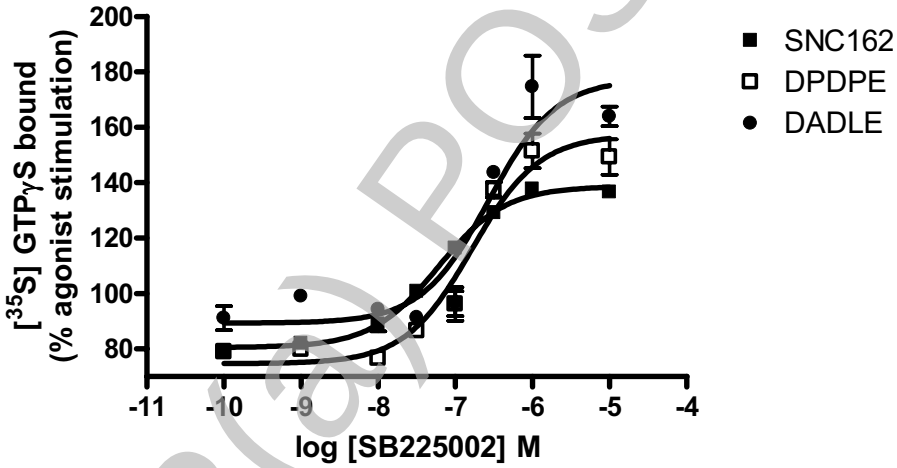

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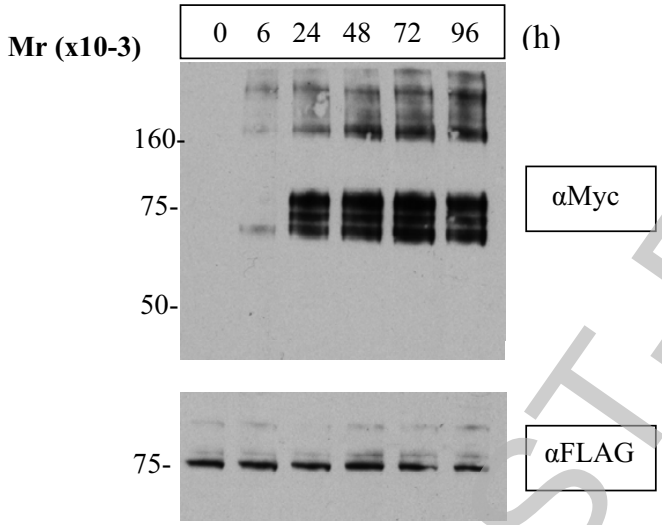
Figure 6

A



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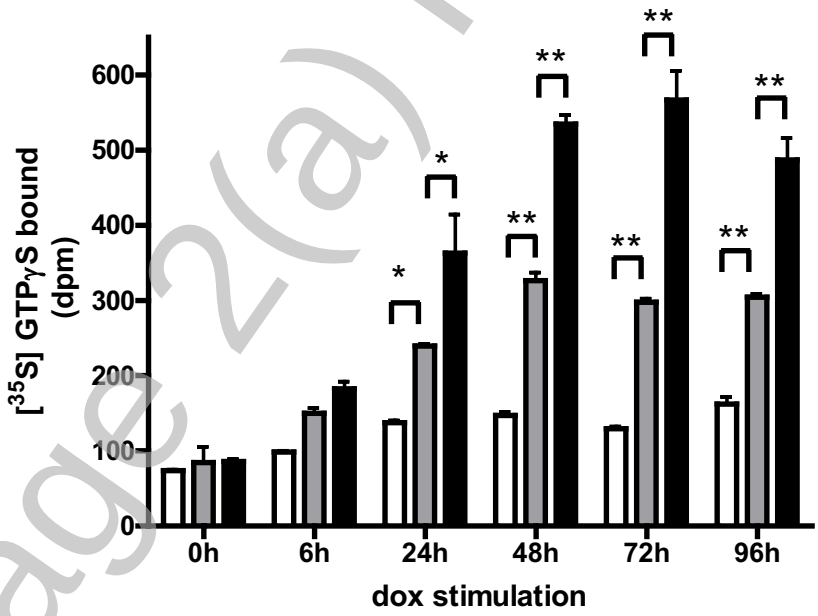

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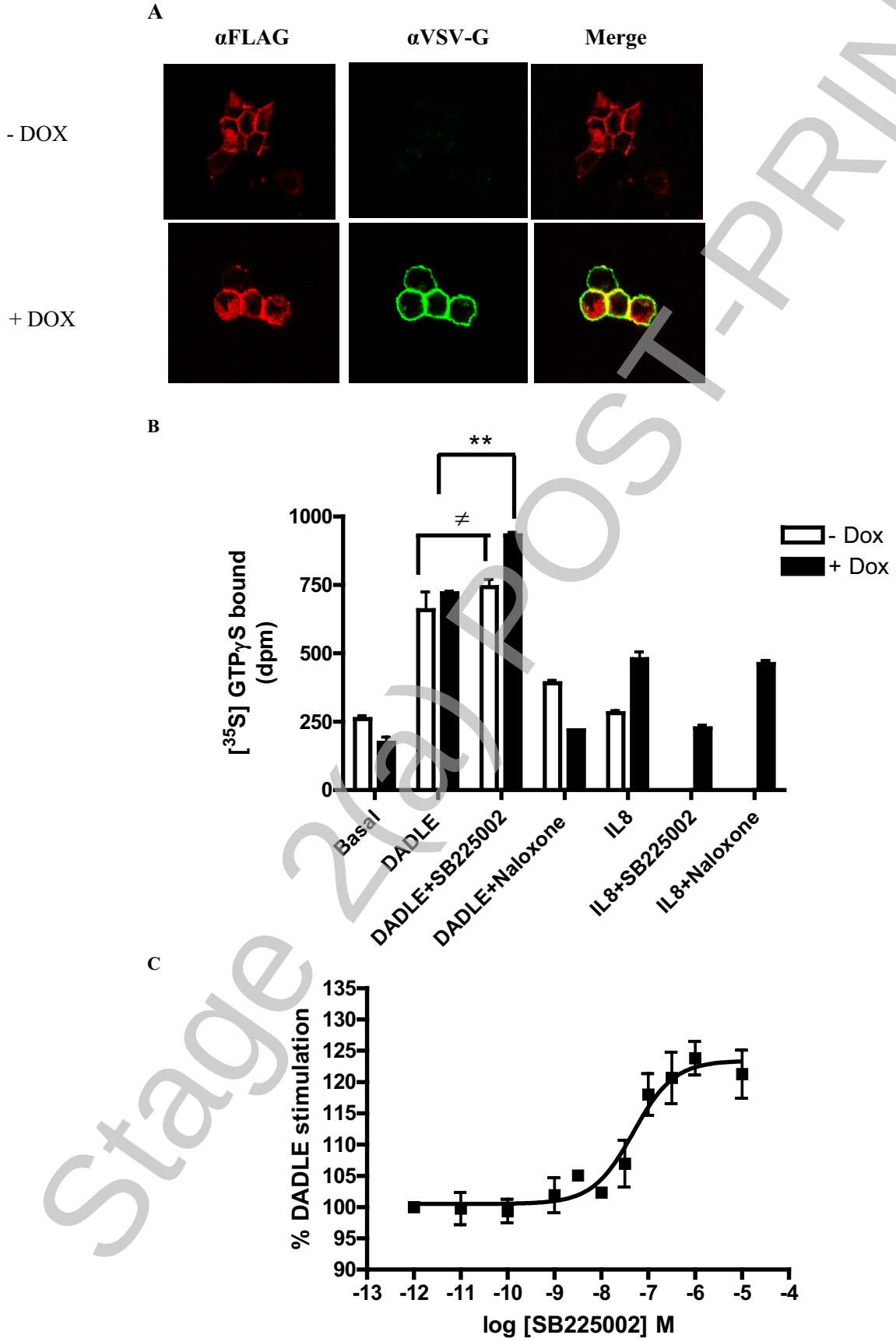
Figure 7



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