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**Functional complementation and the analysis of opioid receptor homo-  
dimerization**

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**Abbreviations:** DADLE, D-ala<sup>2</sup>, leu<sup>5</sup>-enkephalin; DAMGO, [D-Ala<sup>2</sup>,NMe-Phe<sup>4</sup>,Gly-ol<sup>5</sup>]-enkephalin; DOP, delta opioid peptide; DPDPE, [D-Pen<sup>2,5</sup>]-enkephalin; GPCR, G protein-coupled receptor; GTPγS, guanosine 5'-(γ-thio) triphosphate; KOP, kappa opioid peptide; MOP, mu opioid peptide; U69593, (+)-(5α,7α,8β)-N-methyl-N-[-7-(1-pyrrolodiny)-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide.

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### Abstract

Complementation of function following co-expression of pairs of non-functional G protein-coupled receptors that contain distinct inactivating mutations supports the hypothesis that such receptors exist as dimers. Chimeras between members of the metabotropic glutamate receptor-like family have been particularly useful because the N-terminal ligand binding and heptahelical transmembrane elements can be considered distinct domains. To examine the utility of a related approach for opioid receptors, fusion proteins were generated in which a Pertussis toxin-resistant (Cys<sup>351</sup>Ile) variant of the G protein G<sub>i1</sub>α was linked to the C-terminal tail of the DOP, KOP and MOP receptors. Each was functionally measured by agonist stimulation of [<sup>35</sup>S]GTPγS binding in G<sub>i</sub>α immunoprecipitates from membranes of Pertussis toxin-treated HEK293 cells. Agonist function was eliminated either by fusion of the receptors to G<sub>i1</sub>αGly<sup>202</sup>Ala,Cys<sup>351</sup>Ile or mutation of a pair of conserved Val residues in intracellular loop 2 of each receptor. Co-expression, but not simple mixing, of the two inactive fusion proteins reconstituted agonist-loading of [<sup>35</sup>S]GTPγS for each receptor. At equimolar amounts, reconstitution of DOP receptor function was more extensive than KOR or MOR. Reconstitution of DOP function required two intact receptors and was not achieved by provision of extra G<sub>i1</sub>αCys<sup>351</sup>Ile membrane anchored by linkage to DOP transmembrane domain 1. Inactive forms of all G protein α subunits can be produced by mutations equivalent to G<sub>i1</sub>αGly<sup>202</sup>Ala. As the amino acids modified in the opioid receptors are highly conserved in most rhodopsin-like receptors this approach should be widely applicable to study the existence and molecular basis of receptor dimerization.

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### **Introduction**

An extensive literature now exists on the capacity of a wide range of G protein-coupled receptors (GPCRs) to form dimers and/or higher-order oligomers (Lee et al., 2003, Breitwieser, 2004, Milligan, 2004). Despite this, many of the reports have been predominantly descriptive and provide limited insights into the proportion of different GPCRs that may exist as dimers, the relative propensity of different GPCRs to oligomerize, the molecular basis of dimerization and whether there are differences in the details of how closely related GPCRs form dimers/oligomers.

The ability of the DOP, KOP and MOP opioid receptor subtypes to form homo-dimers and/or higher-order oligomers has previously been investigated using both co-immunoprecipitation and resonance energy transfer techniques (Cvejic and Devi, 1997, George et al., 2000, McVey et al, 2001, Li-Wei et al., 2002, Ramsay et al., 2002). Despite this, there is little information available on the issues noted above, although informatic analysis has suggested potential interfaces in transmembrane helices that may contribute to opioid receptor subtype homo-dimerization (Filizola and Weinstein, 2002).

If the co-expression of two non-equivalent and non-functional mutants of a GPCR is both able and required to reconstitute receptor ligand binding and/or function this can provide evidence in favour of direct protein-protein interactions and quaternary structure for the active receptor (Milligan and Bouvier, 2005). For example, co-expression of two forms of the angiotensin AT1 receptor that were unable to bind angiotensin II or related ligands due to point mutations in either transmembrane III or transmembrane region V restored ligand binding (Monnot et al., 1996). Such an approach has also been used to explore mechanisms of dimerization. Theoretical

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models of GPCR dimerization include both 'contact' and 'domain swap' dimers.

Using the histamine H1 receptor as a model, Bakker et al., (2004) showed that although single point mutations in both transmembrane region III and transmembrane region VI prevented binding of antagonist radioligands, including [<sup>3</sup>H]mepyramine, co-expression of the two mutants resulted in reconstitution of [<sup>3</sup>H]mepyramine binding sites with the anticipated pharmacological characteristics. Conceptually this should not be possible for a 'contact' dimer in which transmembrane domains are not exchanged but simply appose each other.

In addition to the restoration of ligand binding, studies that have used pairs of non-functional mutants to restore GPCR signalling have produced data consistent with GPCR-GPCR interactions. By generating mutants of the luteinizing hormone receptor that were either unable to bind ligand or unable to signal, although able to bind the agonist, Lee et al., (2002) were able to reconstitute agonist-mediated regulation of cAMP levels following co-expression of the two mutants. The luteinizing hormone receptor, as with other GPCRs with related ligands, has an extended N-terminal region involved in ligand binding. As such, Lee et al., (2002) were able to consider the N-terminal 'exo-domain' and the seven transmembrane element 'endo-domain' as distinct entities in a manner equivalent to the extracellular and transmembrane elements of class C GPCRs that have allowed elegant chimeric receptor approaches to understand the mechanism of signal transduction through obligate hetero-dimers (Pin et al., 2005).

Recently, as a variant of this, functional complementation was observed following the co-expression of pairs of  $\alpha_{1b}$ -adrenoceptor- $G_{11}\alpha$  and histamine H1 receptor- $G_{11}\alpha$  GPCR-G protein fusion proteins that were both inactive when expressed individually

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because they contained specific mutations in either the GPCR or G protein elements (Carrillo et al., 2003). All G protein  $\alpha$  subunits contain a conserved Gly that, when mutated, prevents effective GDP-GTP exchange and hence activation (Milligan et al., 2005). Furthermore, nearly all class A, rhodopsin-like GPCRs have either one, or more usually two, hydrophobic residues in the second intracellular loop homologous to those mutated to generate inactive forms of the  $\alpha_{1b}$ -adrenoceptor and histamine H1 receptor (Milligan et al., 2005). We thus wished to test if equivalent pairs of inactive opioid receptor-G $\alpha$  fusion proteins could be produced and to assess if variations in pharmacology and/or reconstitutive capacity could provide insights into the basis of opioid receptor subtype dimerization.

## **Materials and Methods**

### **Materials/Ligands**

Diprenorphine [15,16- $^3\text{H}$ ] (50 Ci/mmol) and guanosine 5'-( $\gamma$ -thio) triphosphate [ $^{35}\text{S}$ ] (1250 mCi/mmol) were from PerkinElmer life science, Inc. (Boston, USA).

DADLE (D-ala $^2$ , leu $^5$ -enkephalin), DAMGO ([D-Ala $^2$ ,NMe-Phe $^4$ ,Gly-ol $^5$ ]-enkephalin, DPDPE ([D-Pen $^{2,5}$ ]-enkephalin), naloxone and Pertussis toxin were from SIGMA-Aldrich Company Ltd., Poole, Dorset, UK. U69593 ((+)-(5 $\alpha$ ,7 $\alpha$ ,8 $\beta$ )-N-methyl-N-[7-(1-pyrrolodiny)-1-oxaspiro[4,5]dec-8-yl)benzeneacetamide) was from Tocris. Recombinant, myristoylated rat G $_{i1\alpha}$  subunit was from Calbiochem.

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### Antibodies/antisera

The anti-G<sub>α1-2</sub> antiserum (SG) has been described previously (Green et al., 1990). The mouse monoclonal anti-Flag antibody (M2) was from SIGMA-Aldrich Company Ltd., Poole, Dorset, UK. The rabbit polyclonal antiserum anti-c-myc antiserum was from Cell Signalling Technology, Nottingham, UK

### Molecular constructs

hDOP-G<sub>i1α</sub>C<sup>351</sup>I in pcDNA3.1 was generated previously (Moon et al., 2001) and used as a template to introduce mutations in the 2<sup>nd</sup> intracellular loop of the receptor to produce **hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I** using the QuikChange kit (Stratagene) and the following primers:

*Sense primer:* 5'-GAC CGC TAC ATC GCT GAG TGC CAC CCT GAC AAG GCC CTG GAC TTC-3'

*Antisense primer:* 5'-GAA GTC CAG GGC CTT GTC AGG GTG GCA **CTC** AGC GAT GTA GCG GTC-3'

Bold letters indicate bases altered. The PCR product was then digested with *DpnI* and transformed into bacteria.

### **hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I**

In a similar manner hDOP-G<sub>i1α</sub>C<sup>351</sup>I was used to introduce the G<sup>202</sup>A mutation in G<sub>i1α</sub> using the following primers:

*Sense primer:* 5'-G TTT GAC GTG GGA GCC CAG AGA TCA GAG C-3'

*Antisense primer:* 5'-G CTC TGA TCT CTG GGC TCC CAC GTC AAA C-3'

The PCR product was then digested by *DpnI* and was transformed into bacteria.

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### **Flag-hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>ii</sub>αC<sup>351</sup>I**

was constructed using the following primers:

*Sense primer:* 5' ACT AGT GCT AGC ATG GAC TAC AAG GAC GAC GAT GAT AAG  
GAA CCG GCC CCC TCC GCC GGC-3'

*Antisense primer:* 5'-GAA TTT GGA TCC GGC GGC AGC GCC ACC GCC GGG-3'

The sense primer contains a Flag sequence (in italics) and an *NheI* restriction site (underlined) and corresponds to the N-terminal region of hDOP. The antisense primer contains a *BamHI* site (underlined) and corresponds to the C-terminal region of hDOP. The PCR product and pcDNA3.1 vector containing hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>ii</sub>αC<sup>351</sup>I were digested by *NheI* and *BamHI*. The digested products were then ligated.

### **c-myc-hDOP-G<sub>ii</sub>αG<sup>202</sup>A,C<sup>351</sup>I**

was constructed using the following primers:

*Sense primer:* 5'-CCC TTT GCT AGC ATG GAA CAA AAG CTT ATT TCT GAA GAA  
GAT CTG GAA CCG GCC CCC TCC GCC-3'

*Antisense primer:* 5'-GAA TTT GGA TCC GGC GGC AGC GCC ACC GCC GGG-3'

hDOP-G<sub>ii</sub>αG<sup>202</sup>A,C<sup>351</sup>I was amplified by these primers. The sense primer contains a c-myc sequence (in italics) and *NheI* restriction site (underlined), the antisense primer contains a *BamHI* site (underlined). The PCR product and pcDNA3.1 containing hDOP-G<sub>ii</sub>αG<sup>202</sup>A,C<sup>351</sup>I were digested with *NheI* and *BamHI*. The digested products were then ligated.

### **hMOPV<sup>169</sup>EV<sup>173</sup>D-G<sub>ii</sub>αC<sup>351</sup>I**



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hMOR-G<sub>11α</sub>C<sup>351</sup>I cDNA in pcDNA3 was generated previously (Massotte et al.,2002) and was used as a template to introduce mutations in the 2<sup>nd</sup> intracellular loop of the receptor using the following primers:

*Sense primer:* 5'-GAT CGA TAC ATT GCA **GAG** TGC CAC CCT GAC AAG GCC TTA  
GAT TTC-3'

*Antisense primer:* 5'-GAA ATC TAA GGC CTT GTC AGG GTG GCA **CTC** TGC AAT  
GTA TCG ATC-3'

The appropriate valines were mutated into glutatmate (GAG) and aspartate (GAC) respectively. Altered bases mutated are in bold. The PCR product was digested by *DpnI* and was transformed into bacteria.

## hMOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I

was produced as for hDOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I but using hMOP-G<sub>11α</sub>C<sup>351</sup>I cDNA as the template .

## rKOP-G<sub>11α</sub>C<sup>351</sup>I

rKOP-G<sub>11α</sub>C<sup>351</sup>I was constructed using the following primers:

*Sense primer:* 5'-CCC AAA AAG CTT ATG GAG TCC CCC ATC CAG ATT TTC C-3'

*Antisense primer:* 5'-GGC ATC GGT ACC TAC TGG CTT ATT CAT CCC ACC CAC  
ATC CCT CAT GGA-3'

Rat KOP was amplified between these primers corresponding to the N and C-termini of rKOP and containing *HindIII* and *KpnI* restriction sites (underlined). The PCR product and pcDNA3 containing G<sub>11α</sub>C<sup>351</sup>I were digested by the above enzymes. Because rKOP

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contains an internal *Hind*III site, a two-way ligation was performed to ligate the vector and the two elements of the digested PCR product.

### **rKOP V<sup>160</sup>E,V<sup>164</sup>D-Gi<sub>1α</sub>C<sup>351</sup>I**

rKOP-Gi<sub>1α</sub>C<sup>351</sup>I cDNA as above was used as a template to introduce mutations in the 2<sup>nd</sup> intracellular loop of the receptor, using the following primers:

*Sense primer:* 5'-GAC CGC TAC ATT GCC **GAG** TGC CAC CCT GAC AAA GCT TTG GAT TTC-3'

*Antisense prime:* 5'-GAA ATC CAA AGC TTT GTC AGG GTG GCA **CTC** GGC AAT GTA GCG GTC-3'

Bases mutated are in bold.

### **rKOP-Gi<sub>1α</sub>G<sup>202</sup>A,C<sup>351</sup>I**

rKOP-Gi<sub>1α</sub>C<sup>351</sup>I cDNA was used as a template to introduce the mutation in Gi<sub>1α</sub> as for hDOP and hKOP.

### **Flag-Nt-TM1-Gi<sub>1α</sub> C<sup>351</sup>I**

Flag-Nt-TM1-Gi<sub>1α</sub> C<sup>351</sup>I was constructed using the following primers:

*Sense primer:* 5' ACT AGT GCT AGC ATG GAC TAC AAG GAC GAC GAT GAT AAG GAA CCG GCC CCC TCC GCC GGC-3'

*Antisense primer:* 5'-CCC ATT GGA TCC GGT GGC CGT CTT CAT CTT AGT GTA CCG-3'

Flag-hDOP-Gi<sub>1α</sub> C<sup>351</sup>I was used as template for PCR. The first 252bp were amplified by PCR and were then digested using *Bam*HI and *Nhe*I (restriction sites underlined). The same

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digestion was used on the template, *NheI* being situated at the end of the receptor sequence.

PCR products and vector were ligated.

### **Cell transfection and treatment**

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

### **[<sup>3</sup>H]Diprenorphine binding**

The expression of GPCR-G protein fusions was assessed by measuring the specific binding of [<sup>3</sup>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<sub>2</sub>, pH adjusted to 7.5). Bound ligand was estimated by liquid scintillation spectroscopy. Competition studies were conducted with 1nM [<sup>3</sup>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.

### **[<sup>35</sup>S]GTPγS binding studies**

Experiments were initiated by adding the assay buffer mix (20 mM HEPES (pH 7.4), 3 mM MgCl<sub>2</sub>, 100mM NaCl, 10 μM GDP, 0.2 mM ascorbic acid) containing 50 nCi [<sup>35</sup>S]GTPγS

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in the presence or absence of agonist to a defined amount of membranes. Non-specific binding was determined in the presence of 100  $\mu$ M GTP $\gamma$ S. 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<sub>11 $\alpha$</sub> /G<sub>12 $\alpha$</sub>  antiserum, SG (Green et al., 1990) and left rotating overnight at 4°C for immunoprecipitation. The immunocomplexes were washed twice with ice-cold solubilization buffer and bound [<sup>35</sup>S]GTP $\gamma$ S measured.

## **Co-immunoprecipitation**

Cells were resuspended in 1ml of 1 x RIPA (radio-immunoprecipitation assay) 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. 50 $\mu$ l 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 1 $\mu$ g/ $\mu$ l. Target proteins were then immunoprecipitated from 500 $\mu$ l samples by incubation with 20 $\mu$ l 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 30-50 $\mu$ l Laemmli buffer and heated for 4 min at 85°C. The eluates were then loaded onto SDS-PAGE gels.

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### Quantitation of Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I expression levels

Varying amounts (12.5-50ng) of recombinantly expressed, myristoylated rat G<sub>i1α</sub> were run on SDS-PAGE alongside membranes of HEK293 cells transfected to co-express Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I and hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I. Following immunoblotting with the anti-G<sub>i1α</sub>/G<sub>i2α</sub> antiserum SG, densitometry indicated that the signal corresponding to the recombinant G<sub>i1α</sub> increased in a linear fashion over this range. Interpolation of the immuno-signal corresponding to Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I (molecular mass 60.57kDa) in different amount of transfected cell membranes allowed estimation of expression levels.

### Results

A fusion protein was constructed between the human DOP (hDOP) receptor and a form of the α subunit of the G protein G<sub>i1</sub> that was rendered resistant to the ADP-ribosyltransferase activity of Pertussis toxin by conversion of Cys<sup>351</sup> to Ile (G<sub>i1α</sub>C<sup>351</sup>I). The hDOP-G<sub>i1α</sub>C<sup>351</sup>I fusion protein was expressed transiently in HEK293 cells that were also treated with Pertussis toxin (25ng/ml, 16h) prior to harvest to cause ADP-ribosylation of the endogenously expressed forms of the G<sub>i</sub>/G<sub>o</sub> group of G proteins. Membranes prepared from these cells were used in saturation [<sup>3</sup>H]diprenorphine ligand binding assays to measure expression levels of the construct and its affinity for the ligand (Table 1). Expression levels were 1816 ± 209 fmol/mg membrane protein and the pK<sub>d</sub> for [<sup>3</sup>H]diprenorphine 9.20 ± 0.03 (n = 4, means ± S.E.M.). The functionality of hDOP-G<sub>i1α</sub>C<sup>351</sup>I was assessed by the capacity of the synthetic opioid peptide D-ala<sup>2</sup>, leu<sup>5</sup> enkephalin (DADLE) to stimulate binding of [<sup>35</sup>S]GTPγS in membranes containing the construct that were subsequently

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immunoprecipitated with the anti-G<sub>i1α</sub>/G<sub>i2α</sub> antiserum, SG (Figure 1a). Virtually no [<sup>35</sup>S]GTPγS was recovered in immunoprecipitates from membranes of mock-transfected cells treated with either DADLE or vehicle (Figure 1a). By contrast, although binding of [<sup>35</sup>S]GTPγS in immunoprecipitates from hDOP-G<sub>i1α</sub>C<sup>351</sup>I-expressing cell membranes was greatly increased by DADLE, the construct was also able to load [<sup>35</sup>S]GTPγS in the absence of agonist (Figure 1a). When membrane amounts corresponding to varying levels of hDOP-G<sub>i1α</sub>C<sup>351</sup>I were used, DADLE stimulation of [<sup>35</sup>S]GTPγS binding was linear with fusion protein amount over the full range tested and up to at least 60fmol (Figure 1b).

We have previously demonstrated that mutation of Gly<sup>208</sup> to Ala in the G protein G<sub>i1α</sub> prevents receptor-mediated guanine nucleotide exchange and hence [<sup>35</sup>S]GTPγS binding (Carrillo et al., 2002). The α subunit of all hetero-trimeric G proteins contains Gly at the equivalent position. To test the general effect of mutating this Gly on the capacity of receptors to enhance guanine nucleotide exchange we thus generated hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I. When this was expressed in HEK293 cells and membranes prepared from Pertussis toxin-treated cells, neither the level of expression of this construct nor the binding affinity for [<sup>3</sup>H]diprenorphine was different from hDOP-G<sub>i1α</sub>C<sup>351</sup>I (Table 1). However, although 10μM DADLE caused a 5.2 +/- 0.3 fold (n = 4 mean ± S.E.M.) increase in levels of [<sup>35</sup>S]GTPγS binding compared to vehicle-treated controls in samples immunoprecipitated from membranes expressing 15 fmol of hDOP-G<sub>i1α</sub>C<sup>351</sup>I (Figure 2), no significant DADLE stimulation of [<sup>35</sup>S]GTPγS binding was observed in immunoprecipitated samples derived from membranes containing 15 fmol of hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (Figure 2). Furthermore,

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[<sup>35</sup>S]GTPγS loading in the absence of DADLE was substantially reduced (Figure 2). Mutation of hydrophobic residues in the second intracellular loop of family A GPCRs can essentially eliminate G protein activation without major effects on antagonist ligand binding (Carrillo et al., 2003, Milligan et al., 2005). To test this for hDOP we thus also generated hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I. This also was expressed as well as hDOP-G<sub>i1α</sub>C<sup>351</sup>I (Table 1) but bound [<sup>3</sup>H]diprenorphine with 3-fold lower affinity than hDOP-G<sub>i1α</sub>C<sup>351</sup>I (Table 1). [<sup>35</sup>S]GTPγS binding studies demonstrated this construct also to have much reduced basal guanine nucleotide exchange and not to produce a statistically significant increase in binding of [<sup>35</sup>S]GTPγS in response to DADLE (Figure 2). When hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I were co-expressed and membranes containing 15 fmol of [<sup>3</sup>H]diprenorphine binding sites were used in [<sup>35</sup>S]GTPγS binding studies, DADLE stimulation was partially reconstituted (Figure 2). With membranes from these cells containing 30 fmol of [<sup>3</sup>H]diprenorphine binding sites, DADLE-stimulated [<sup>35</sup>S]GTPγS binding was 60% of that achieved in membranes expressing 15 fmol of the wild type hDOP-G<sub>i1α</sub>C<sup>351</sup>I fusion construct (Figure 2). Reconstitution of DADLE-stimulated [<sup>35</sup>S]GTPγS binding required the co-expression of hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I and not simply the presence of both in the assay. When membranes containing 15 fmol of individually expressed hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I were simply mixed prior to the assay to provide 30 fmol of fusion proteins in the assay no reconstitution of DADLE-stimulated [<sup>35</sup>S]GTPγS binding was observed (Figure 2). These data are consistent with a requirement for hDOP interactions to generate function.

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Interestingly, the affinity of [<sup>3</sup>H]diprenorphine binding in membranes co-expressing hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I was equivalent to the individually expressed hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I construct (Table 1). Although this observation might indicate the presence of a substantially greater proportion of hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I than hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I in the membranes from co-expressed cells this is not consistent with the functional reconstitution data (Figure 2) or with the equivalent levels of expression of these two constructs when expressed individually (Table 1). However, to examine this further and to confirm direct interactions between hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I we performed co-immunoprecipitation studies using membranes of HEK293 cells transfected to express individually or co-express N-terminally modified Flag-hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I and/or c-myc-hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I. Immunoprecipitation with anti-Flag antibody followed by SDS-PAGE and immunoblotting with anti-c-myc antibody resulted in detection of specific c-myc immunoreactivity only when the two fusion constructs were co-expressed (Figure 3), consistent with a physical interaction between the two variants. To further explore aspects of pharmacology of the fusion proteins, membranes from Pertussis toxin-treated HEK 293 cells transfected to express hDOP-G<sub>i1α</sub>C<sup>351</sup>I; hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I; hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I or the combination of hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I + hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I were used in [<sup>3</sup>H]-diprenorphine/DADLE competition binding experiments (Table 2). Two-site binding curves reflecting higher and lower affinity binding sites for the agonist DADLE were best fitted in each case. Introduction of the G<sup>202</sup>A mutation in the G-protein subunit did not alter DADLE binding properties substantially as similar pK<sub>h</sub> and pK<sub>i</sub> values



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were observed for hDOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I as for hDOP-G<sub>11α</sub>C<sup>351</sup>I (Table 2). In contrast, the double mutation in the 2<sup>nd</sup> intracellular loop of hDOP receptor did alter the binding affinity of DADLE with some 30 fold loss of affinity in both high and low affinity binding sites (hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>11α</sub>C<sup>351</sup>I pK<sub>h</sub> = 7.4 ± 0.2, pK<sub>i</sub> 5.0 ± 0.4, hDOP-G<sub>11α</sub>C<sup>351</sup>I pK<sub>h</sub> = 9.0 ± 0.2, pK<sub>i</sub> 6.8 ± 0.42). In membranes co-expressing hDOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>11α</sub>C<sup>351</sup>I there was no significant difference in the percentage of high and low site numbers compared to the wild type hDOP-G<sub>11α</sub>C<sup>351</sup>I fusion protein (P>0.05, 1 way ANOVA) (Table 2). A similar reduction in affinity of the high affinity site for the DOP selective peptide agonist [D-Pen<sup>2,5</sup>]-enkephalin (DPDPE) was also observed when comparing hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>11α</sub>C<sup>351</sup>I to hDOP-G<sub>11α</sub>C<sup>351</sup>I or hDOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I (Table 3) and although a similar trend was observed for the low affinity site (Table 3), this did not achieve statistical significance due to relatively imprecise estimates of pK<sub>i</sub>. Wild type DPDPE binding characteristics were again restored following co-expression of hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>11α</sub>C<sup>351</sup>I and hDOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I (Table 3). Assuming the predominant form of the hDOP is as a dimer, rather than a higher-order oligomer, co-expression of hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>11α</sub>C<sup>351</sup>I and hDOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I must be expected to generate hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>11α</sub>C<sup>351</sup>I dimers and hDOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I dimers, which as shown in Figure 2 are inactive, as well as the functionally reconstituted hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>11α</sub>C<sup>351</sup>I + hDOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I dimer. Ligand binding studies must reflect the full population of these different hDOP ‘homo-dimers’ in the cell membrane. By contrast, in functional assays, only hDOP-G<sub>11α</sub>C<sup>351</sup>I homo-dimers and hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>11α</sub>C<sup>351</sup>I + hDOP-

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$G_{i1\alpha}G^{202}A,C^{351}I$  'homo-dimers' are reported (Figure 2). The potency of DADLE to stimulate [ $^{35}S$ ]GTP $\gamma$ S binding via the hDOP- $G_{i1\alpha}C^{351}I$  dimer and the reconstituted hDOPV $^{150}E,V^{154}D$ - $G_{i1\alpha}C^{351}I$  + hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  dimer was not different (Figure 4a). Equally, the prototypic opioid receptor antagonist naloxone was equipotent in its ability to prevent DADLE-stimulated [ $^{35}S$ ]GTP $\gamma$ S binding via the hDOP- $G_{i1\alpha}C^{351}I$  dimer and the reconstituted hDOPV $^{150}E,V^{154}D$ - $G_{i1\alpha}C^{351}I$  + hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  dimer (Figure 4b).

To assess if the reconstitution of function observed upon co-expression of hDOPV $^{150}E,V^{154}D$ - $G_{i1\alpha}C^{351}I$  + hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  could possibly be accounted for simply by the provision of the  $G_{i1\alpha}C^{351}I$  attached to the inactive hDOPV $^{150}E,V^{154}D$  receptor rather than specifically requiring interactions between hDOPV $^{150}E,V^{154}D$  and hDOP we generated and expressed a construct (Flag-Nt-TM1- $G_{i1\alpha}C^{351}I$ ) in which  $G_{i1\alpha}C^{351}I$  was linked to a sequence comprising the N-terminal domain, transmembrane region 1 and the first intracellular loop of hDOP. This construct did not bind [ $^3H$ ]diprenorphine (data not show) but its expression as an apparent 48 kDa polypeptide could clearly be detected by immunoblotting transfected HEK293 membranes with the anti- $G_{i1\alpha}/G_{i2\alpha}$  antiserum (Figure 5a). Parallel SDS-PAGE and immunodetection of varying amounts of recombinantly expressed  $G_{i1\alpha}$  followed by densitometry of the signals allowed production of a standard curve for  $G_{i1\alpha}$  expression that was linear over the range (0-50 ng) employed. Based on the anti- $G_{i1\alpha}$  immunological signal in membranes corresponding to Flag-Nt-TM1- $G_{i1\alpha}C^{351}I$  and its calculated molecular mass (60.57 kDa) we estimated levels of this construct to be 11.2 pmol/mg membrane protein. Therefore, this construct was present at some 6

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times the level of the hDOP-G<sub>i1α</sub> fusion proteins. Co-transfection of Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I with hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I resulted in very low, but statistically significant, increases in levels of [<sup>35</sup>S]GTPγS binding in anti-G<sub>i1α</sub>/G<sub>i2α</sub> antiserum immunoprecipitates when DADLE was added to such membranes (Figure 5b). These very small signals did not reflect the possibility that although hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I were co-expressed they were present in distinct membrane compartments. Co-expression of Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I with c-myc-hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I allowed their co-immunoprecipitation (Figure 6a), indicating not only proximity but their capacity for physical interactions. Equally, co-expression of c-myc-Nt-TM1 with the isolated Flag-hDOP allowed their co-immunoprecipitation, indicating interactions were not a reflection of contacts between the two copies of the G protein (Figure 6b).

To extend these reconstitution studies to the other opioid receptors we generated equivalent fusion proteins incorporating the human MOP-1 (hMOP) receptor. hMOP-G<sub>i1α</sub>C<sup>351</sup>I, hMOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>i1α</sub>C<sup>351</sup>I were expressed individually in HEK293 cells and following Pertussis toxin-treatment and membrane preparation, expression levels and affinity for [<sup>3</sup>H]diprenorphine were assessed via saturation binding studies. No significant differences between the three constructs were noted in either parameter (Table 4). Equally, following co-expression of hMOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>i1α</sub>C<sup>351</sup>I the characteristics of [<sup>3</sup>H]diprenorphine binding were equivalent. In functional [<sup>35</sup>S]GTPγS binding studies (Figure 7), the selective MOP receptor agonist [D-Ala<sup>2</sup>,NMe-Phe<sup>4</sup>,Gly-ol<sup>5</sup>]-enkephalin (DAMGO) (10μM) caused a 5.28 ± 0.24 fold (n = 4, mean ± S.E.M.) stimulation in end of assay anti-G<sub>i1α</sub>/G<sub>i2α</sub> antiserum

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immunoprecipitates. As with the related hDOP constructs, membranes expressing equal amounts of either hMOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I or hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>11α</sub>C<sup>351</sup>I did not result in DAMGO stimulation of [<sup>35</sup>S]GTPγS binding (Figure 7). Co-transfection of hMOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>11α</sub>C<sup>351</sup>I did result in partial reconstitution of DAMGO-stimulated [<sup>35</sup>S]GTPγS binding (Figure 7), an effect not achieved by simple mixing of membranes individually expressing hMOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I or hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>11α</sub>C<sup>351</sup>I (Figure 7). In comparison to the 60% reconstitution of hDOP function, membranes expressing twice as many hMOP receptor [<sup>3</sup>H]diprenorphine binding sites following co-expression of the two inactive mutant fusion proteins allowed only 40% of the amount of agonist-stimulated [<sup>35</sup>S]GTPγS binding as generated by the wild type hMOP-G<sub>11α</sub>C<sup>351</sup>I fusion (Figure 7). A potential explanation for this was uncovered on examining the potency of DAMGO to stimulate [<sup>35</sup>S]GTPγS binding in membranes expressing hMOP-G<sub>11α</sub>C<sup>351</sup>I and co-expressing hMOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>11α</sub>C<sup>351</sup>I. The potency of this ligand was reduced ( $p < 0.05$ ) by some 2 fold at the functionally reconstituted dimer ( $pEC_{50} = 6.1 \pm 0.07$ ) compared to the wild type dimer ( $pEC_{50} = 6.5 \pm 0.04$ ). Interestingly, although both hMOP-G<sub>11α</sub>C<sup>351</sup>I and hMOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I displayed both high and low affinity binding sites for DAMGO when this ligand was allowed to compete with [<sup>3</sup>H]diprenorphine (Figure 8, Table 5), only a low affinity binding component could be detected for hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>11α</sub>C<sup>351</sup>I (Figure 8, Table 5) akin to what might be anticipated if GPCR and G protein were uncoupled. When hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>11α</sub>C<sup>351</sup>I and hMOP-G<sub>11α</sub>G<sup>202</sup>A,C<sup>351</sup>I were co-expressed the characteristics of DAMGO binding were akin to a mixture of the two mutant constructs

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(Figure 8, Table 5) and analysis of the binding curves was consistent with the two constructs being present in a close to 1:1 ratio.

Studies were also performed on the rat (r)KOP receptor. rKOP-G<sub>i1α</sub>C<sup>351</sup>I, rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I fusions were generated and expressed. These also all bound [<sup>3</sup>H]diprenorphine with high affinity and expressed to similar levels (Table 6) but, as with the hDOP constructs, a reduction in affinity was recorded for the rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I construct that incorporated mutations into the second intracellular loop of the receptor. As with the equivalent hDOP and hMOP constructs rKOP-G<sub>i1α</sub>C<sup>351</sup>I allowed a large increase in [<sup>35</sup>S]GTPγS binding in response to agonist treatment (Figure 9). Individual expression of neither rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I nor rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I resulted in stimulation of [<sup>35</sup>S]GTPγS binding in the presence of the KOP receptor-selective agonist U69593 whereas co-expression of rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I did (Figure 9). At a maximally effective concentration of U69593 (10μM) membranes expressing twice as many rKOP [<sup>3</sup>H]-diprenorphine binding sites following co-expression of the two inactive mutants, allowed some 50% of the amount of agonist-stimulated [<sup>35</sup>S]GTPγS binding as generated by the wild type rKOP-G<sub>i1α</sub>C<sup>351</sup>I fusion (Figure 9). Akin to the hMOP constructs, in competition studies between [<sup>3</sup>H]diprenorphine and U69593, both rKOP-G<sub>i1α</sub>C<sup>351</sup>I and rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I displayed both high and low affinity binding sites for the agonist. However, rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I displayed only a single, low affinity site for U69593 (Figure 10, Table 7). Also, as with the hMOP constructs, co-expression of rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I and rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I resulted in a pattern of U69593 binding consistent with a mixture of the pharmacology of the two constructs (Figure 10,

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Table 7). The potency of U69593 to activate rKOP-G<sub>i1α</sub>C<sup>351</sup>I (pEC<sub>50</sub> = 7.3 ± 0.08) was higher (p < 0.05) than for the reconstituted rKOP dimer (pEC<sub>50</sub> = 6.8 ± 0.13).

## Discussion

Fusion proteins between GPCRs and G protein α subunits have been used to examine a wide range of function of these polypeptides (Milligan, 2002, Milligan et al., 2004) with the defined 1:1 stoichiometry of the partner proteins being of particular use in measures of agonist-induced GTPase turnover number (Moon et al., 2001), the regulation, co-ordinated (Stevens et al., 2001) or otherwise (Barclay et al., 2005), of post-translational thio-acylation of GPCR and G protein and the effects of mutations in either partner that alter protein steady-state expression levels (Ward and Milligan, 2002). In the current studies we have generated and explored the function and pharmacology of fusions between each of the DOP, KOP and MOP opioid receptors with G<sub>i1α</sub>. The functionality of each of these mutants was established in [<sup>35</sup>S]GTPγS binding studies in which at assay termination, immunoprecipitation with an anti-G<sub>i1α</sub>/G<sub>i2α</sub> antiserum limited non-specific binding of the nucleotide. All commonly used cell lines express members of the G<sub>iα</sub> G protein family that are substrates for pertussis toxin-catalysed ADP-ribosylation. To ensure agonist-driven [<sup>35</sup>S]GTPγS binding reflected only binding to the fusion proteins under study, these were constructed using G<sub>i1α</sub>C<sup>351</sup>I (Bahia et al., 1998), which is insensitive to the actions of the toxin but able to be effectively activated by receptors, and by treating cells with Pertussis toxin prior to cell harvest to modify the endogenous G<sub>iα</sub> pool. Mutation of Gly<sup>202</sup> to Ala in G<sub>i1α</sub> resulted in a form of the G protein that was unable to exchange guanine nucleotide and bind [<sup>35</sup>S]GTPγS in response to receptor agonists. All G protein α subunits have a Gly residue in the equivalent position and mutation should therefore be anticipated to produce

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equivalent lack of function mutants as previously shown for G<sub>11α</sub> (Carrillo et al., 2002, 2003). Fusion of wild type G<sub>11α</sub> to forms of the α<sub>1b</sub>-adrenoceptor and the histamine H1 receptor containing hydrophobic to acidic residue mutations in intracellular loop 2 also results in lack of agonist-mediated [<sup>35</sup>S]GTPγS binding without destruction of the ligand binding pocket (Carrillo et al., 2003). As most rhodopsin-like GPCRs have a pair of homologous hydrophobic residues (Milligan et al., 2005) and in the DOP, KOP and MOP receptors both are Val, we converted each of these to either Glu or Asp. This did not alter construct expression levels and had either no or only small effects on the binding affinity of [<sup>3</sup>H]diprenorphine. We were thus able to measure and equalise construct expression levels in preparation for functional studies. In each case, co-expression of the pair of non-functional opioid receptor-fusion proteins was able to partially reconstitute agonist-mediated [<sup>35</sup>S]GTPγS binding. Reconstitution did require co-expression, simple mixing of membranes expressing the potentially complementary pairs did not generate agonist function. We have previously argued that such results require receptor dimerization (Carrillo et al., 2003) and provided evidence that the reconstitution reflects an ‘inter-’ rather than ‘intra-’ molecular interaction between GPCR and G protein (Carrillo et al., 2003). Although expression of a single fusion protein, wild type in both GPCR and G protein sequence, allows agonist mediated signal transduction, as with expression of a single GPCR cDNA, this does not allow direct exploration of GPCR quaternary structure. Indeed, the knowledge that a single cDNA was generally sufficient to generate the anticipated function and pharmacology of a GPCR played a significant part in the expectation that GPCRs would be single polypeptide, monomeric structures (Milligan, 2004). Previous studies by Molinari et al. (2003) have also noted a capacity of co-expressed of pairs of inactive DOP-G protein fusions to reconstitute a signal. However, although they also concluded that this

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reflected inter-molecular interactions between the co-expressed forms, they did not specifically suggest this to require dimerization between the pair of DOP receptors. At least in part this may be because they also observed an ability of a DOP-G protein fusion to activate a G protein that was membrane anchored simply by linkage to transmembrane 1 of the vasopressin V2 receptor. In contrast with these observations, we observed only a very limited capacity of the hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I construct to activate co-expressed G<sub>i1α</sub>C<sup>351</sup>I when it was tethered to the membrane by linkage to the N-terminal domain and transmembrane domain 1 of hDOP, even though the G protein was provided at some 5-6 times higher levels in this scenario than when provided by co-expression of the potentially complementary fusion protein. The basis for these differences is unclear but may relate to the high expression levels of the fusion proteins achieved and employed by Molinari et al. (2003) that were in the range in which so called ‘bystander’ interactions and effects have been observed (Mercier et al., 2002) likely due simply to physical proximity rather than direct protein-protein interactions. Although hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I was unable to activate co-expressed Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I to any substantial extent these two constructs were able to interact because they could be co-immunoprecipitated following co-expression. This suggests that interaction between two complete receptors might be required for GPCR function and would support other evidence for conformational alterations in the partner GPCR in a dimer induced by ligand binding (Mesnier and Baneres, 2004, El-Asmar et al., 2005). As Nt-TM1 could also be co-immunoprecipitated with full length hDOP this suggests that TM1 and/or the N-terminal region of hDOP provides a protein-protein interaction interface. Although not explored in detail in these studies, for the α<sub>1b</sub>-adrenoceptor symmetrical TM1-TM1 interactions provide key contributions to the quaternary organization of this GPCR (Carrillo et al., 2004) and a series of other reports



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have supported an important contribution of TM1 to the dimer interface(s) in other GPCRs (Overton and Blumer, 2002, Klco et al., 2003, Stanasila et al., 2003). Because ‘non-specific’ effects, potentially arising from high level expression in heterologous transfection studies, are an inherent concern, in the current experiments we maintained fusion construct expression in the range of 1-2 pmol/mg membrane protein and all ‘functional reconstitution’ experiments were performed under conditions in which agonist-stimulated [<sup>35</sup>S]GTPγS binding increased linearly with construct amount. This was a key requirement for data analysis because if opioid receptors exist and function predominantly as dimers, the reconstitution strategy suggests that with 1:1 expression of the two mutant constructs then, in stochastic terms, 50% of the ligand binding sites should reflect ‘hetero’ interactions that can generate a functional response. A hypothesis was therefore that when using membranes co-expressing a pair of potentially suitable mutants, double the number of binding sites would be required to result in the same level of agonist-stimulated [<sup>35</sup>S]GTPγS binding as with the wild type fusion. In all cases this was not achieved, with the level of reconstitution ranging from 40% for the MOP receptor to 60% for the DOP receptor. This may imply that not all cellular copies of a particular GPCR are present within dimers. This has been an extremely difficult issue to assess quantitatively. The proportion of a GPCR that migrates through SDS-PAGE as an SDS-resistant dimer is almost certainly a lower limit for the native state and although resonance energy transfer-based estimates of ‘dimer’ proportions have ranged from 25-85% (Mercier et al., 2002, Dinger et al, 2003) a considerable number of assumptions are required to allow such calculations (Milligan and Bouvier, 2005). Equally, there is growing evidence for a requirement of GPCR dimerization for productive signal transduction that is not restricted to the examples of the GABA<sub>B</sub> and other family C receptors and for greater than dimeric, higher-order quaternary structure (Klco et al., 2003,

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Carrillo et al., 2004, Fotiadis et al., 2004). Equally, however, the basic strategy used herein might be restrictive as a pair of hydrophobic residues from the second intracellular loop were mutated to acidic residues and this might compromise the effectiveness of GPCR dimerization. It is worth noting, however, that the cytoplasmic face of the opioid receptor subtypes is very highly conserved between DOP, KOP and MOP and despite making the equivalent mutations in each, significant differences in reconstitutive effectiveness were observed. This may imply differences in the details of the homo-dimerization process. Although homo-dimerization of each of these three receptors has previously been recorded (Cvejic and Devi, 1997, George et al., 2000, McVey et al, 2001, Li-Wei et al., 2002, Ramsay et al., 2002) there is no useful information on the similarities or differences in mechanisms of these interactions that have involved direct experimental study, although this topic has been considered via an informatic approach (Filizola and Weinstein, 2002). Although the mutation of hydrophobic residues in intracellular loop 2 may have limitations in producing an inactive GPCR, a marked advantage over certain other reconstitutive studies (Monnot et al., 1996, Bakker et al., 2004) is that the orthosteric GPCR ligand binding site was not destroyed. This allowed antagonist binding studies to confirm not only expression of each construct but that each inactive mutant was expressed at the same level as the wild type fusion. This was central to the 'stochastic' calculations of the potential makeup of the GPCR dimer population generated following co-expression of different proteins. Importantly, the complete conservation in G protein  $\alpha$  subunits of the Gly residue modified herein to generate one of the pair of inactive fusions and the very high conservation of the pair of GPCR intracellular loop hydrophobic residues suggest that this strategy should be widely applicable (Milligan et al., 2005). It is likely, for example, to be of considerable use in mutational studies designed to identify key residues involved in the

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dimerization interface(s) (Hernanz-Falcon et al., 2004). Equally, there is no reason to limit such studies to GPCR homo-dimerization and the effectiveness of functional reconstitution may provide quantitative data on the propensity of GPCRs to hetero-dimerize. Indeed, this has been initiated by studies showing that the histamine H1 receptor and the  $\alpha_{1b}$ -adrenoceptor are very poor interaction partners (Carrillo et al., 2003). Finally, as only the reconstituted 'hetero-dimer' is an active signalling unit, then in true GPCR hetero-dimerization studies the functional pharmacology of the hetero-dimer could be examined without interfering signals generated by the corresponding co-expressed homo-dimers, which, as shown herein, are essentially inactive.

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### Footnotes

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### Legends for Figures

#### Figure 1. A hDOP- $G_{i1}\alpha$ C<sup>351</sup>I fusion protein is functional

**1A.** 10 (1,3) or 20 (2,4)  $\mu$ g of Pertussis toxin-treated, HEK 293 cell membranes expressing (3,4) or not (1,2) hDOP- $G_{i1}\alpha$ C<sup>351</sup>I were used to measure the binding of [<sup>35</sup>S]-GTP $\gamma$ S in the absence (open bars) or presence (filled bars) of 10 $\mu$ M DADLE. At assay termination samples were immunoprecipitated with an anti- $G_{i1}\alpha$ / $G_{i2}\alpha$  antiserum and counted.

**1B.** Membranes, as above, expressing different amounts of hDOP- $G_{i1}\alpha$ C<sup>351</sup>I were used to measure DADLE (10  $\mu$ M) stimulation of [<sup>35</sup>S]-GTP $\gamma$ S binding.

Data are means +/- S.E.M. of triplicate assays. 2 further experiments produced similar data.

#### Figure 2. Reconstitution of hDOP- $G_{i1}\alpha$ C<sup>351</sup>I function by co-expression of two non-functional mutants

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Membranes of Pertussis toxin-treated HEK 293 cells expressing 15fmol of hDOP- $G_{i1\alpha}C^{351}I$  (1), hDOPV<sup>150</sup>E,V<sup>154</sup>D- $G_{i1\alpha}C^{351}I$  (2), hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  (3) or hDOPV<sup>150</sup>E,V<sup>154</sup>D- $G_{i1\alpha}C^{351}I$  + hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  (4) were used to measure basal (open bars) and 10 $\mu$ M DADLE (filled bars) binding of [<sup>35</sup>S]GTP $\gamma$ S as in Figure 1A. Membranes co-expressing a total of 30 fmol hDOPV<sup>150</sup>E,V<sup>154</sup>D- $G_{i1\alpha}C^{351}I$  + hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  (5) were also analysed as were membranes expressing 15fmol of hDOPV<sup>150</sup>E,V<sup>154</sup>D- $G_{i1\alpha}C^{351}I$  or 15fmol of hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  that were mixed prior to assay (6). Data represent n = 5 experiments performed in triplicate. \* significant (p < 0.05) stimulation by DADLE.

### Figure 3. Interactions between co-expressed hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$ and hDOPV<sup>150</sup>E,V<sup>154</sup>D- $G_{i1\alpha}C^{351}I$ monitored by co-immunoprecipitation

**3A.** Membranes from control HEK 293 cells (1) and cells transiently expressing Flag-hDOPV<sup>150</sup>E,V<sup>154</sup>D- $G_{i1\alpha}C^{351}I$  (2), c-myc-hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  (3), Flag-hDOPV<sup>150</sup>E,V<sup>154</sup>D- $G_{i1\alpha}C^{351}I$  + c-myc-hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$  (4) or a mix of membranes from lanes 2 and 3 (5) were immunoprecipitated with anti-Flag antibody and after resolution by SDS-PAGE were immunoblotted to detect c-myc immunoreactivity.

**3B.** Samples equivalent to 3A were directly resolved by SDS-PAGE and immunoblotted to detect Flag immunoreactivity.

### Figure 4. Similar functional pharmacology of hDOP- $G_{i1\alpha}C^{351}I$ and the reconstituted dimer

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- 4A.** Membranes of Pertussis toxin-treated HEK 293 cells expressing 15fmol of hDOP-G<sub>i1α</sub>C<sup>351</sup>I (open symbols) or hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I + hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (closed symbols) were used to measure the ability of increasing concentrations of DADLE to enhance [<sup>35</sup>S]GTPγS binding as in Figure 1A. Because the absolute amount of [<sup>35</sup>S]GTPγS bound was less per [<sup>3</sup>H]diprenorphine binding site in membranes expressing the functionally reconstituted dimer (see Figure 2) data are shown as % maximal signal.
- 4B.** The ability of varying concentrations of naloxone to inhibit [<sup>35</sup>S]GTPγS binding stimulated by 100nM DADLE is shown. Data are means +/- S.E.M. of n = 3 experiments.

### **Figure 5. Provision of Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I does not reconstitute substantial function to hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I**

- 5A.** Membranes from control, pertussis toxin-treated HEK 293 cells (1) and those transfected to express Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I (2) or Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I + hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (3, 4) were resolved by SDS-PAGE and immunoblotted using the anti-G<sub>i1α</sub>/G<sub>i2α</sub>antiserum. The polypeptide(s) migrating with apparent M<sub>r</sub> close to 48 kDa is Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I whilst the polypeptide(s) with apparent M<sub>r</sub> close to 40kDa is endogenously expressed G<sub>i1α</sub>/G<sub>i2α</sub>. Previous studies of HEK293 cells has shown this to be predominantly G<sub>i2α</sub> which is expressed at some 50pmol/mg membrane protein (McClue et al., 1992).

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5B. Membranes expressing 15 fmol of hDOP-G<sub>i1α</sub>C<sup>351</sup>I (1), hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (2), 10 μg of membranes expressing Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I (estimated to contain 112 fmol of this construct (see Results) (3), membranes co-expressing 15 (4), or 30 (5) fmol of hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I + (estimated 116.5 fmol (4) or 233 fmol (5)) Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I or a mixture of 10 μg of membranes expressing Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I (112 fmol) + 30 fmol of hDOR-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (6) were used to measure the binding of [<sup>35</sup>S]GTPγS in the absence (open bars) or presence of 10 μM (filled bars) or 100nM (checkered bars) DADLE. Data represent means ± S.E.M. of n = 5 experiments performed in triplicate. \* significant (p < 0.05) stimulation by DADLE.

### **Figure 6. Co-expressed hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I interact and can be co-immunoprecipitated**

**6A.** Membranes from Pertussis toxin-treated HEK 293 cells (1) and equivalent cells transiently expressing c-myc-hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (2), Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I (3), or Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I + c-myc-hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (4), were immunoprecipitated with anti-Flag antibody and anti-c-myc immunoreactivity detected after separation of the samples by SDS-PAGE (**Upper panel**). The expression of Flag-Nt-TM1-G<sub>i1α</sub>C<sup>351</sup>I in the appropriate samples was confirmed by immunoblotting membranes with anti-Flag antibody (**Lower panel**).

**6B.** Membranes from Pertussis toxin-treated HEK 293 cells (1) or those transiently expressing Flag-hDOP (2), c-myc-Nt-TM1 (3), or Flag-hDOP + c-myc-Nt-TM1 (4) were immunoprecipitated with anti-Flag antibody and detected with anti-c-myc antibody after being resolved by SDS-PAGE.

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### **Figure 7. Reconstitution of hMOP function by co-expression of two non-functional hMOP-G<sub>i1α</sub> mutants**

Membranes of Pertussis toxin-treated HEK 293 cells expressing 15fmol of hMOP-G<sub>i1α</sub>C<sup>351</sup>I (1); hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>i1α</sub>C<sup>351</sup>I (2), hMOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (3), and either 15 (4) or 30 (5) fmol of co-transfected hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>i1α</sub>C<sup>351</sup>I + hMOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I were used to measure [<sup>35</sup>S]-GTPγS binding in the absence (open bars) or presence (filled bars) of 10 μM DAMGO as in Figure 2. A control was provided by mixing membranes expressing 15 fmol hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>i1α</sub>C<sup>351</sup>I and 15 fmol hMOPG<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I prior to assay (6). Data represent means +/- S.E.M. of n = 4 experiments performed in triplicate. \* Significant (p < 0.05) stimulation by DAMGO.

### **Figure 8. The characteristics of binding of DAMGO to individually expressed and co-expressed hMOP-G<sub>i1α</sub> fusion proteins**

Membranes expressing hMOP-G<sub>i1α</sub>C<sup>351</sup>I (squares); hMOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (inverted triangles), hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>i1α</sub>C<sup>351</sup>I (triangles) or both hMOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I and hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>i1α</sub>C<sup>351</sup>I (circles) were used to measure the ability of varying concentrations of DAMGO to compete for binding with 1nM [<sup>3</sup>H]diprenorphine. Data represent n = 4 experiments performed in triplicate.

### **Figure 9. Reconstitution of rKOP function by co-expression of two non-functional rKOP-G<sub>i1α</sub> mutants**

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Membranes of HEK 293 cells expressing 15fmol of rKOP-G<sub>i1α</sub>C<sup>351</sup>I (1); rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I (2) , rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (3) and 15 (4) or 30 (5) fmol of [<sup>3</sup>H]diprenorphine binding sites following co-expression of rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I+ rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I were used to measure [<sup>35</sup>S]GTPγS binding in the absence (open bars) or presence of 10 μM (filled bars) or 100 nM (checked bars) U69593. A control was performed by mixing membranes expressing 15 fmol of rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I and 15 fmol of rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (6). Data represent means +/- S.E.M. of n = 4 experiments performed in triplicate. \* Significant (p < 0.05) stimulation by U69593.

### **Figure 10. The characteristics of binding of U69593 to individually expressed and co-expressed rKOP-G<sub>i1α</sub> fusion proteins**

Membranes expressing rKOP-G<sub>i1α</sub>C<sup>351</sup>I (squares); rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (inverted triangles), rKOPV<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I (triangles) or both rKOP-V<sup>160</sup>E,V<sup>164</sup>D-G<sub>i1α</sub>C<sup>351</sup>I and rKOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I (circles) were used to measure the ability of varying concentrations of U69593 to compete for binding with 1nM [<sup>3</sup>H]diprenorphine. Data represent n = 4 experiments performed in triplicate.

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**Tables**

**Table 1. Expression levels and [<sup>3</sup>H]diprenorphine binding affinity of hDOP-G<sub>i1α</sub>C<sup>351</sup>I fusion proteins**

<b>Construct</b>	<b>B<sub>max</sub> (fmol/mg)</b>	<b>pK<sub>d</sub></b>
<b>hDOP-G<sub>i1α</sub>C<sup>351</sup>I</b>	<b>1816 ± 209</b>	<b>9.20 ± 0.03</b>
<b>hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I</b>	<b>2181 ± 228</b>	<b>8.78 ± 0.01***</b>
<b>hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I</b>	<b>1777 ± 285</b>	<b>9.19 ± 0.05</b>
<b>hDOPV<sup>150</sup>E,V<sup>154</sup>D-G<sub>i1α</sub>C<sup>351</sup>I + hDOP-G<sub>i1α</sub>G<sup>202</sup>A,C<sup>351</sup>I</b>	<b>2310 ± 301</b>	<b>8.85 ± 0.02***</b>

Data represent means ± SEM of n=4 experiments performed on different membrane preparations. \*\*\* Significantly different from hDOP-G<sub>i1α</sub>C<sup>351</sup>I, P<0.001

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**Table 2**

**Binding affinity of DADLE for individually expressed and co-expressed hDOP- $G_{i1\alpha}C^{351}I$  fusion proteins.**

Construct	$pK_h$	% $K_h$ sites	$pK_i$	Hill number
hDOP- $G_{i1\alpha}C^{351}I$	9.03 ± 0.18	63 ± 6	6.79 ± 0.42	-0.39 ± 0.03
hDOPV <sup>150</sup> E,V <sup>154</sup> D- $G_{i1\alpha}C^{351}I$	7.40 ± 0.24**	57 ± 8	4.99 ± 0.37*	-0.50 ± 0.02
hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$	8.70 ± 0.12	59 ± 3	5.82 ± 0.23	-0.34 ± 0.04
hDOPV <sup>150</sup> E,V <sup>154</sup> D- $G_{i1\alpha}C^{351}I$ + hDOP- $G_{i1\alpha}G^{202}AC^{351}I$	8.69 ± 0.15	45 ± 9	6.29 ± 0.28	-0.41 ± 0.04

Data represent means ± SEM of n=4 experiments performed in triplicate on different membrane preparations

\* Significantly different from hDOP- $G_{i1\alpha}C^{351}I$ , P<0.05

\*\* Significantly different from hDOP- $G_{i1\alpha}C^{351}I$ , P<0.01



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**Table 3**

**Binding affinity of DPDPE for individually expressed and co-expressed hDOP-  
 $G_{i1\alpha}C^{351}I$  fusion proteins**

Construct	$pK_h$	% $K_h$ site	$pK_I$	Hill number
hDOP- $G_{i1\alpha}C^{351}I$	$8.79 \pm 0.09$	$70 \pm 3$	$5.89 \pm 0.25$	$-0.41 \pm 0.005$
hDOPV <sup>150</sup> E,V <sup>154</sup> D- $G_{i1\alpha}C^{351}I$	$7.42 \pm 0.11^{**}$	$51 \pm 20$	$5.48 \pm 0.41$	$-0.61 \pm 0.04$
hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$	$8.88 \pm 0.15$	$65 \pm 4$	$5.82 \pm 0.38$	$-0.40 \pm 0.03$
hDOPV <sup>150</sup> E,V <sup>154</sup> D- $G_{i1\alpha}C^{351}I$ + hDOP- $G_{i1\alpha}G^{202}A,C^{351}I$	$8.31 \pm 0.28$	$55 \pm 12$	$6.10 \pm 0.35$	$-0.46 \pm 0.01$

Data represent means  $\pm$  SEM of n=4 experiments performed in triplicate on different membrane preparations

\*\* Significantly different from hDOR- $G_{i1\alpha}C^{351}I$ ,  $P < 0.01$ , 1 way ANOVA

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**Table 4**

**Expression levels and [<sup>3</sup>H]diprenorphine binding affinity of hMOP-G<sub>iiα</sub>C<sup>351</sup>I fusion proteins**

<b>Construct</b>	<b>B<sub>max</sub> (fmol/mg)</b>	<b>pK<sub>d</sub></b>
<b>hMOP-G<sub>iiα</sub>C<sup>351</sup>I</b>	<b>1217 ± 72</b>	<b>9.47 ± 0.08</b>
<b>hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>iiα</sub>C<sup>351</sup>I</b>	<b>901 ± 110</b>	<b>9.39 ± 0.17</b>
<b>hMOP-G<sub>iiα</sub>G<sup>202</sup>A,C<sup>351</sup>I</b>	<b>1251 ± 20</b>	<b>9.52 ± 0.08</b>
<b>hMOPV<sup>169</sup>E,V<sup>173</sup>D-G<sub>iiα</sub>C<sup>351</sup>I + hMOP-G<sub>iiα</sub>G<sup>202</sup>A,C<sup>351</sup>I</b>	<b>1285 ± 120</b>	<b>9.56 ± 0.10</b>

Data represent means ± SEM from n=3 experiments performed in triplicate on different membrane preparations. Statistics were performed using 1 way ANOVA on B<sub>max</sub> and pK<sub>d</sub> numbers.

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**Table 5**

**Binding affinity of DAMGO for individually expressed and co-expressed hMOP- $G_{i1\alpha}C^{351}I$  fusion proteins**

<b>Construct</b>	<b>pK<sub>h</sub></b>	<b>% high affinity site</b>	<b>pK<sub>i</sub></b>	<b>Hill number</b>
<b>hMOP-<math>G_{i1\alpha}C^{351}I</math></b>	<b>8.71 ± 0.18</b>	<b>48 ± 4</b>	<b>6.91 ± 0.27</b>	<b>-0.53 ± 0.02</b>
<b>hMOPV<sup>169</sup>E,V<sup>173</sup>D- <math>G_{i1\alpha}C^{351}I</math></b>			<b>6.02 ± 0.02*</b>	<b>-0.82 ± 0.05*</b>
<b>hMOP-<math>G_{i1\alpha}G^{202}A,C^{351}I</math></b>	<b>8.68 ± 0.23</b>	<b>60 ± 2</b>	<b>6.69 ± 0.23</b>	<b>-0.52 ± 0.02</b>
<b>hMOPV<sup>169</sup>E,V<sup>173</sup>D- <math>G_{i1\alpha}C^{351}I</math> + hMOP- <math>G_{i1\alpha}G^{202}A,C^{351}I</math></b>	<b>8.47 ± 0.06</b>	<b>40 ± 2</b>	<b>6.18 ± 0.07</b>	<b>-0.34 ± 0.01</b>

Data represent means ± SEM from n=3 experiments performed in triplicate on different membrane preparations.

Statistics were performed using 1 way ANOVA on pK<sub>h</sub> and pK<sub>i</sub> numbers.

\* Significantly different from hMOP- $G_{i1\alpha}C^{351}I$ , P<0.05

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**Table 6**

**Expression levels and [<sup>3</sup>H]diprenorphine binding affinity of rKOP-G<sub>iiα</sub>C<sup>351</sup>I fusion proteins**

Construct	B <sub>max</sub> (fmol/mg)	pK <sub>d</sub>
rKOP-G <sub>iiα</sub> C <sup>351</sup> I	2355 ± 193	9.30 ± 0.06
rKOPV <sup>160</sup> E,V <sup>164</sup> D-G <sub>iiα</sub> C <sup>351</sup> I	2391 ± 177	8.88 ± 0.04**
rKOP-G <sub>iiα</sub> G <sup>202</sup> AC <sup>351</sup> I	2191 ± 148	9.32 ± 0.06
rKOPV <sup>160</sup> E,V <sup>164</sup> D-G <sub>iiα</sub> C <sup>351</sup> I + rKOP-G <sub>iiα</sub> G <sup>202</sup> AC <sup>351</sup> I	2417 ± 187	9.17 ± 0.06

Data represent means ± SEM from n=3 experiments performed in triplicate on different membrane preparations.

Statistics were performed using 1 way ANOVA on B<sub>max</sub> and pK<sub>d</sub> numbers.

\*\* significantly different P<0.01

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**Table 7**

**Binding affinity of U69593 for individually expressed and co-expressed rKOP-  
 $G_{i1\alpha}C^{351}I$  fusion proteins**

Construct	$pK_h$	% high affinity site	$pK_l$	Hill number
rKOP- $G_{i1\alpha}C^{351}I$	$8.85 \pm 0.19$	$61 \pm 2$	$7.10 \pm 0.18$	$-0.56 \pm 0.06$
rKOPV <sup>160E,V<sup>164</sup>D</sup> - $G_{i1\alpha}C^{351}I$			$6.00 \pm 0.06^*$	$-0.82 \pm 0.12$
rKOP- $G_{i1\alpha}G^{202}A,C^{351}I$	$8.91 \pm 0.21$	$57 \pm 3$	$7.12 \pm 0.26$	$-0.54 \pm 0.06$
rKOPV <sup>160E,V<sup>164</sup>D</sup> - $G_{i1\alpha}C^{351}I$ + rKOP- $G_{i1\alpha}G^{202}A,C^{351}I$	$8.92 \pm 0.31$	$53 \pm 2$	$6.62 \pm 0.23$	$-0.40 \pm 0.04$

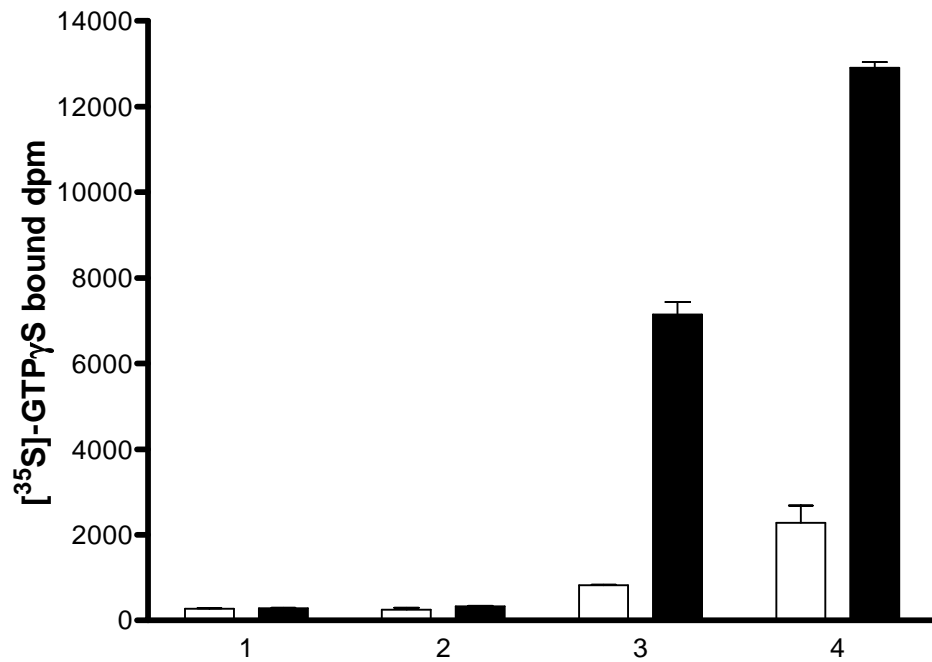
Data represent means  $\pm$  SEM of n=4 experiments performed in triplicate on different membrane preparations.

Statistics were performed using 1 way ANOVA on  $pK_h$  and  $pK_l$  numbers and on high affinity site numbers.

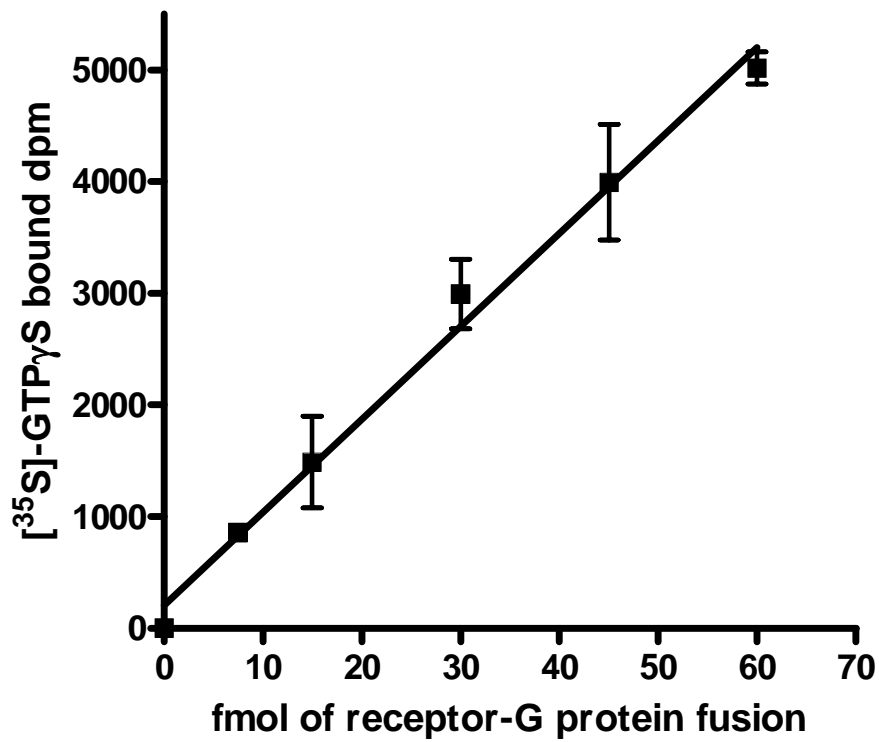
\* Significantly different  $P < 0.05$

## Figure 1

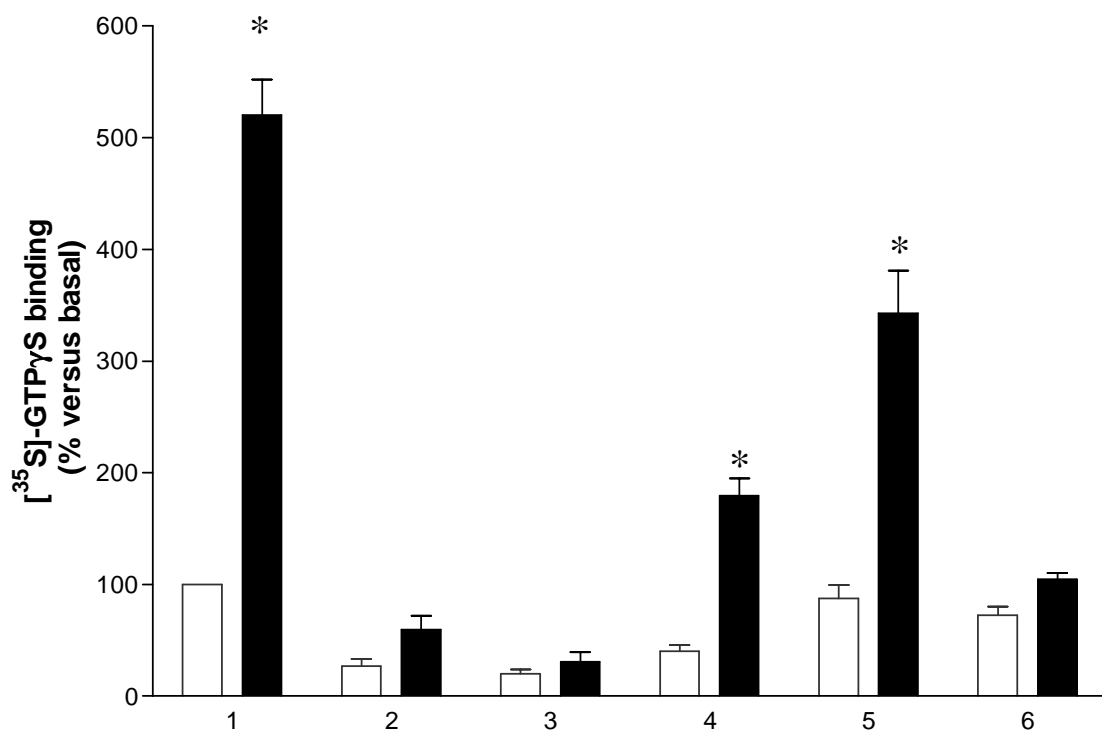
1A.



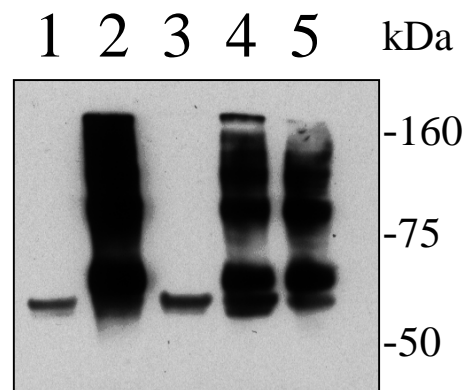
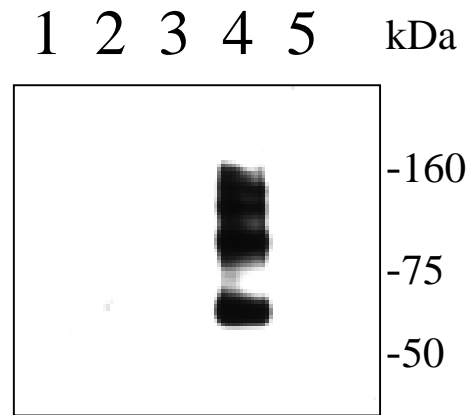
1B.



**Figure 2**



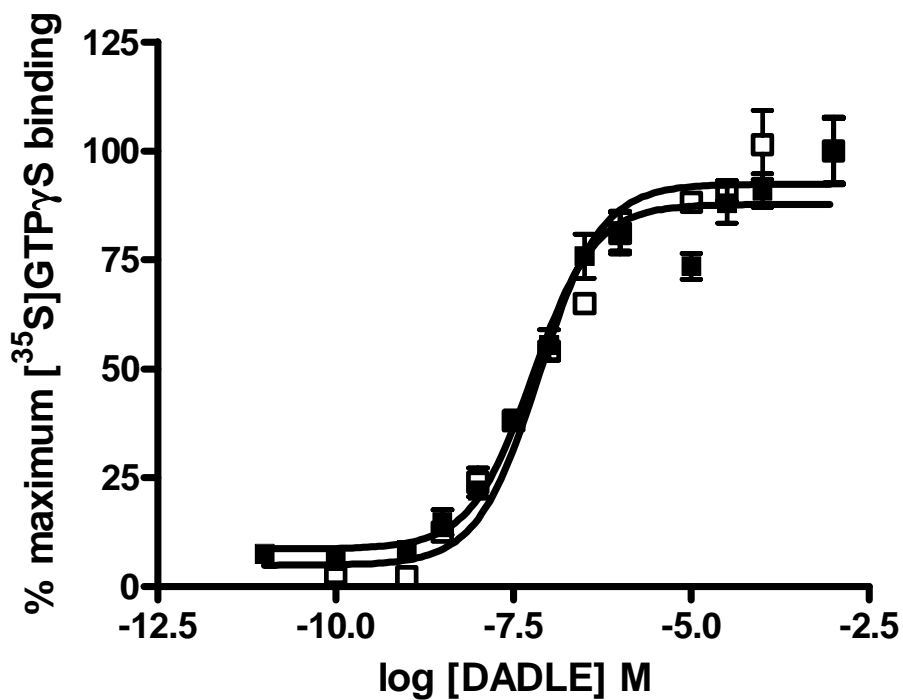
## Figure 3



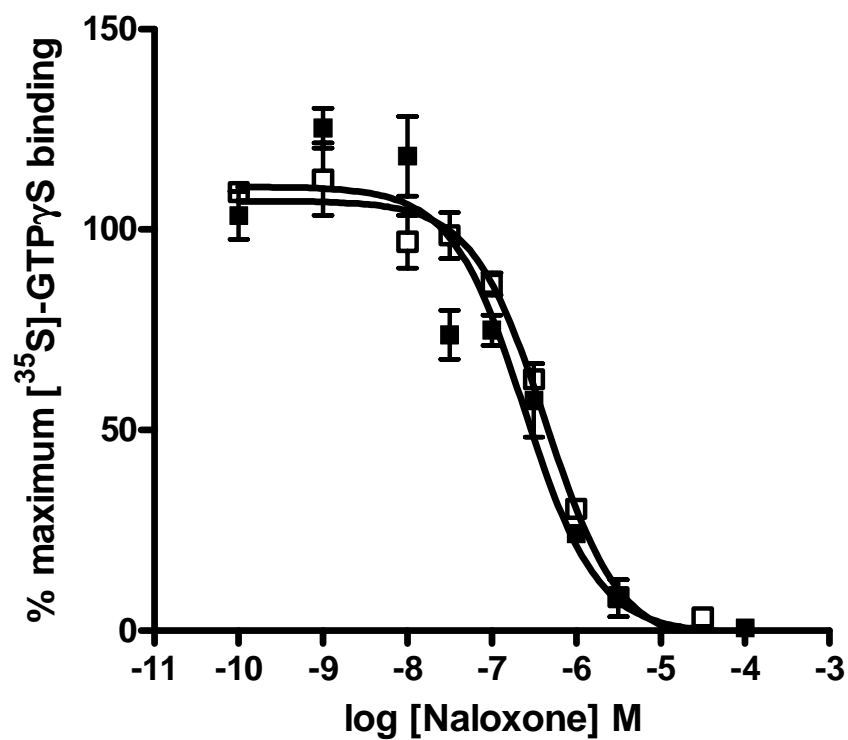


## Figure 4

4A.

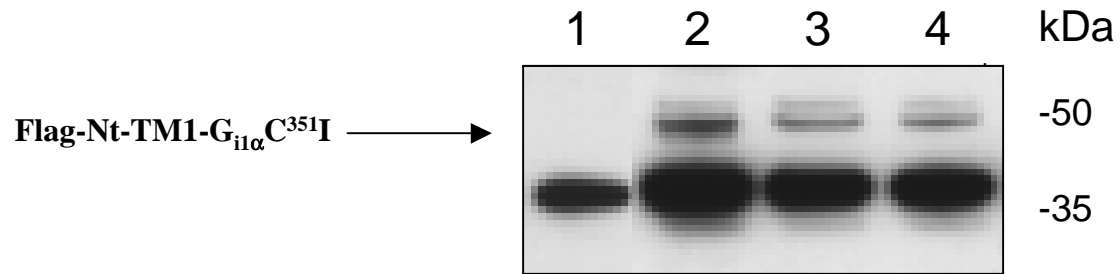


4B.

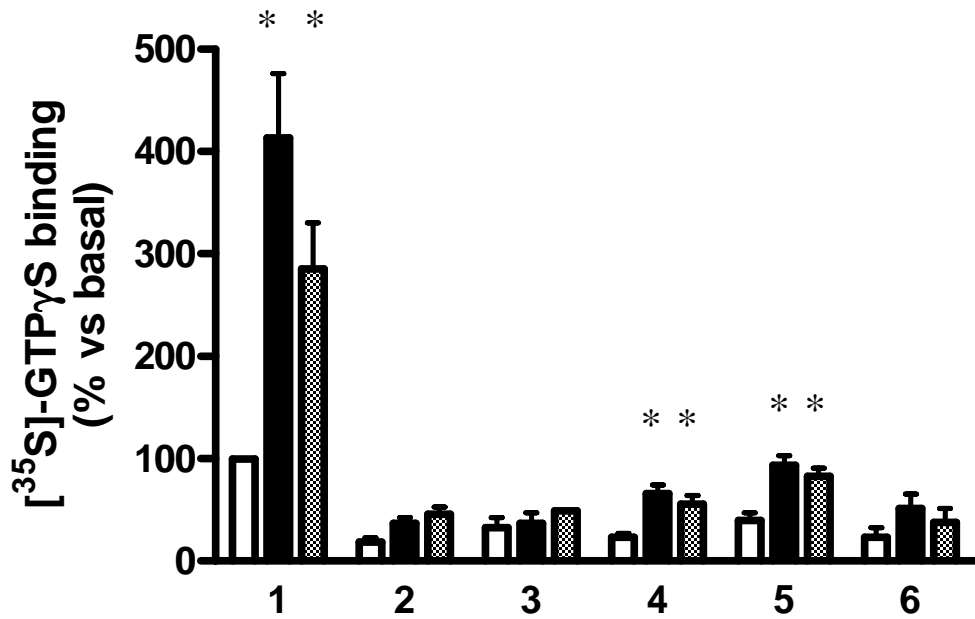


## Figure 5

5A.

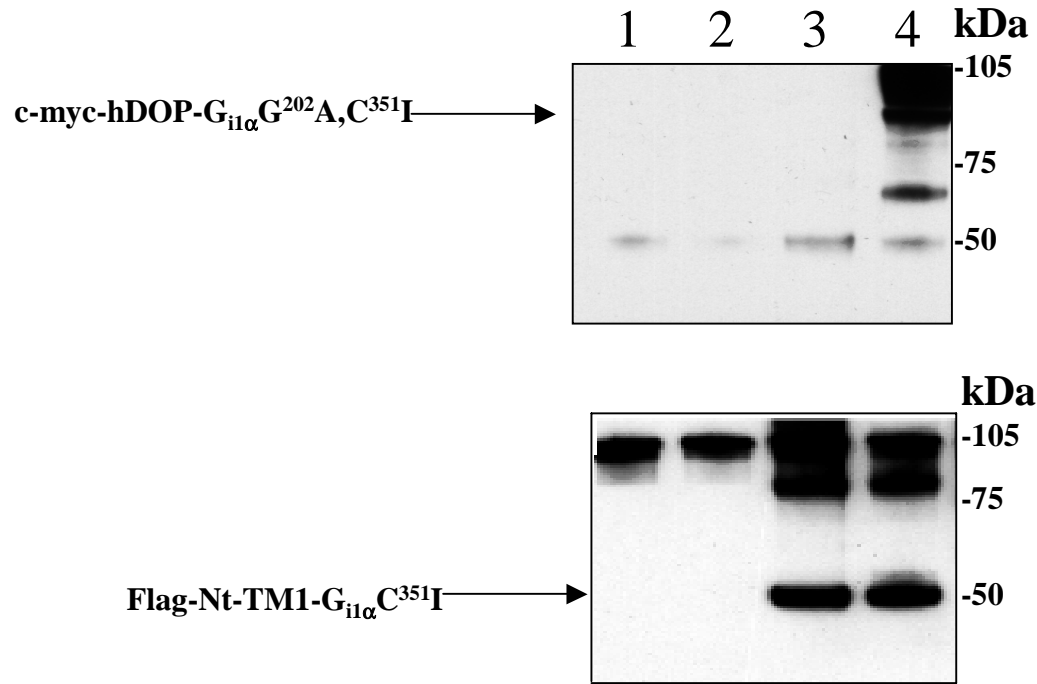


5B.

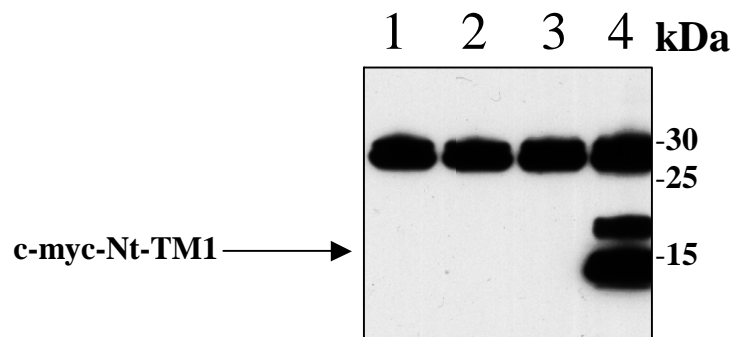


## Figure 6

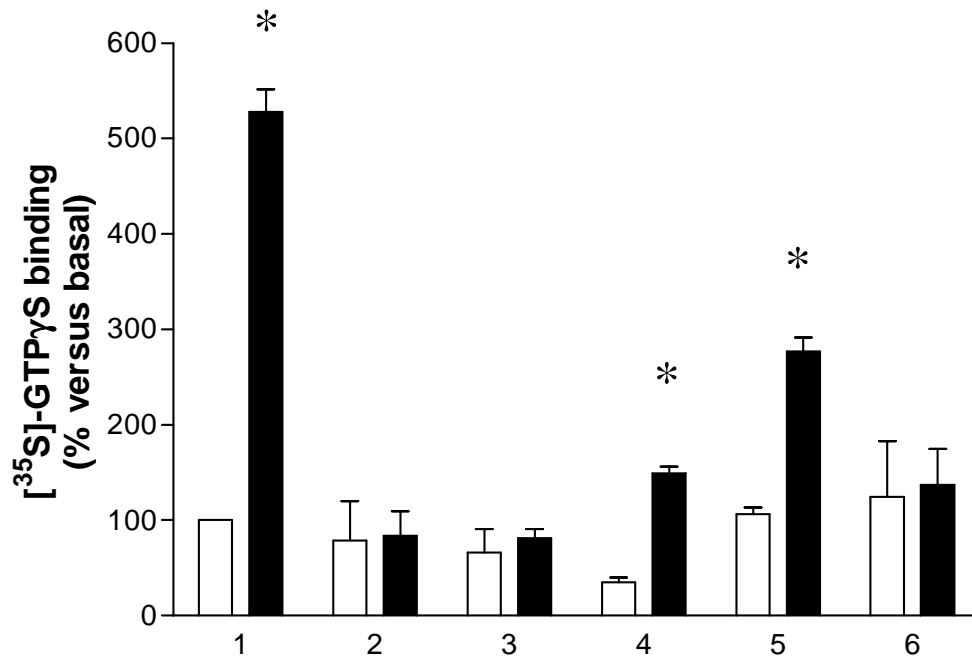
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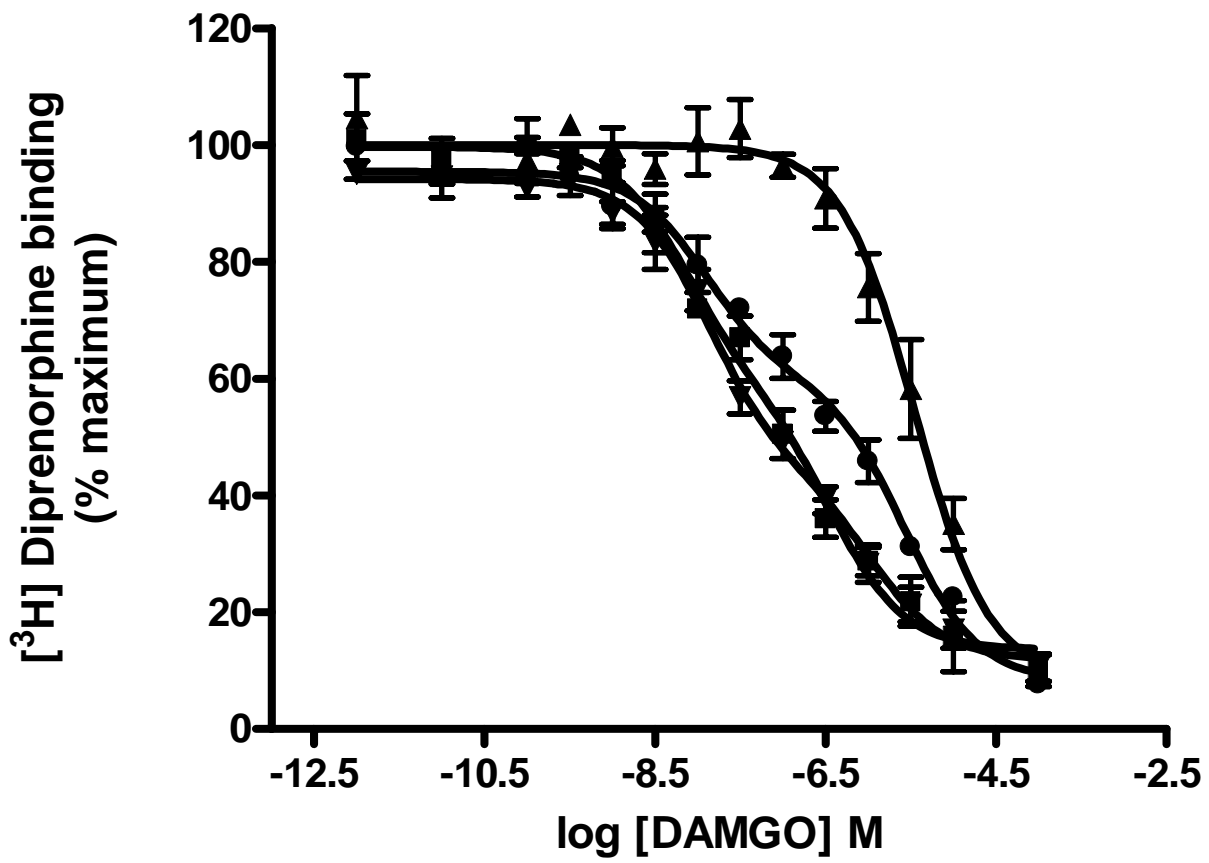
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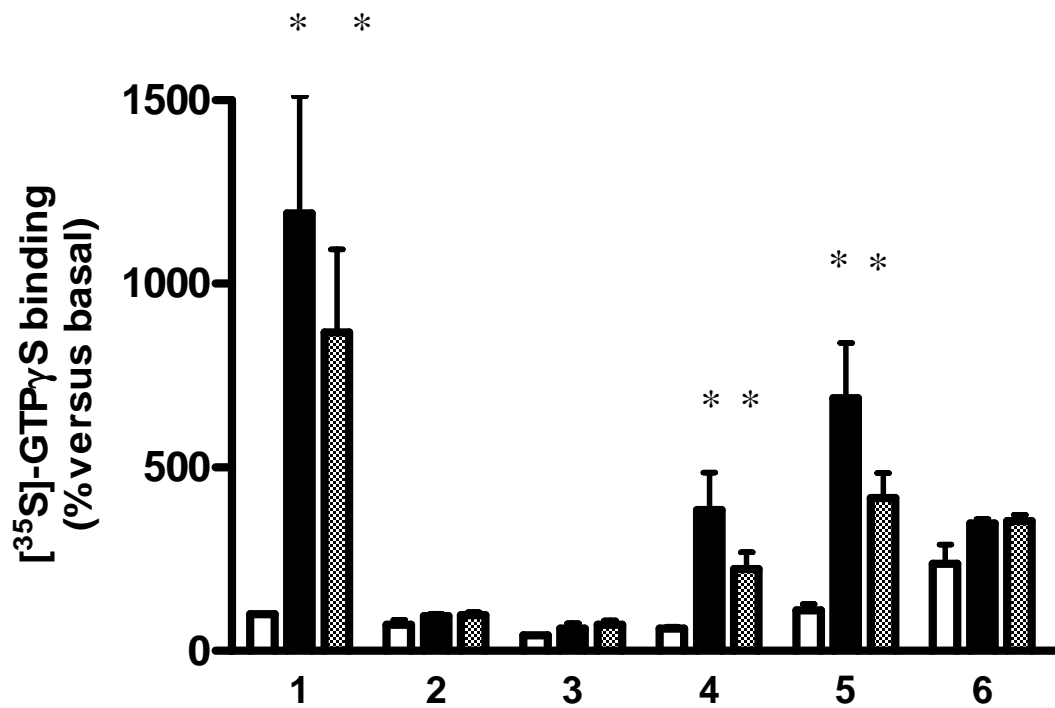
**Figure 7**



**Figure 8**



**Figure 9**



**Figure 10**

