Opioid receptor dimerisation studied using a functional complementation technique

A thesis presented for the degree of Doctor of Philosophy

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Abstract

In this study, mutated versions of hDOR, hMOR and rKOR fused to $G_{i1\alpha}C^{351}I$ protein were constructed. Mutations were introduced either in the receptor or in the G protein part of the fusion proteins. A highly conserved glycine was mutated into alanine (G202A) in the $G_{i1\alpha}C^{351}I$ protein abolishing GDP/GTP exchange. For the opioid receptors a pair of valines belonging to the 2nd intracellular loop were mutated in glutamic acid and aspartic acid. This double mutation eliminated agonist-induced receptor activation of the $G_{i1\alpha}C^{351}I$ protein. However, when the pair of non-functional fusion proteins were co-expressed agonist induced [^{35}S]-GTPγS binding was recovered for all homo and heterodimers tested including the MOR/KOR combination, which was previously indicated as being unable to dimerise. A cross-talk between hDOR and β_2-adrenoceptor fusion proteins was also observed but the reconstituted signal was two times weaker compared to the hDOR homodimer. This result suggested that the affinity of hDOR to homodimerise is higher than to heterodimerise with β_2-adrenoceptor. Ligand binding affinity for the different fusion proteins was assessed and a loss of ligand binding affinity was observed for all the fusion proteins incorporating the pair of mutated valines. However, upon co-expression with the corresponding fusion protein containg the $G^{202}A$ mutation, the wild-type pharmacology seemed to be recovered. In this study the hDOR N-terminal and/or TM1 were also demonstrated as interacting with the full length hDOR as well as self-associating. Consequently, TM1 is a possible interface for hDOR homodimer formation. Co-expression of membrane tethered hDOR TM1 with full length hDOR did not produce agonist-induced [^{35}S]-GTPγS binding, suggesting that two full length receptors are necessary to generate a functionnal signal.

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Abbreviations

α	alpha subunit of G protein
aa	amino acid
AC	adenylate cyclase
ANOVA	analysis of variance
B_{max}	maximum binding sites
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	Curie
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Trh-Pen-NH$_2$
DADLE	[D-Ala^2, D-Leu^5]enkephalin
DAMGO	[D-Ala^2,N-methyl-Phe^4,Gly^5-ol]enkephalin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DOR	delta opioid receptor
DPDPE	[D-Pen^{2,5}]enkephalin
DPM	desintegration per minute
DTT	dithiothreitol
EC_{50}	concentration of agonist producing half maximal response
E.coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid
Fmol	femtomol
GABA	gamma amino butyric acid
GABAb1	GABAb receptor 1
GABAb2	GABAb receptor 2
G protein	guanine nucleotide binding protein
Gi	inhibitory G protein
Go	other G protein
Gs	stimulatory G protein
GDP	guanosine 5'-diphosphate

GNTI	5'-Guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro4,5-epoxy-3,14-dihydroxy-6,7-2',3'-indolomorphinan
GPCR	G protein coupled receptor
GppNHp	guanylyl 5'-(βγ imido) diphosphate
GTP	guanosine 5'-triphosphate
GTPγS	guanosine-5'-O-(3-thio)triphosphate
^3H	tritium
HCl	hydrochloridric acid
HEK	Human Embryonic Kidney
HEPES	(N- [2-hydroxethyl] piperazine-N'- [2-ethanesulphonic acid])
IC_{50}	concentration of drug which inhibits 50% of an effect
ICI 174 864	N,N-diallyl-Tyr-Aib-Aib-Phe-Leu
kb	kilo base
kDa	kilo dalton
K_d	dissociation constant
K_i	inhibition constant
KO	knock out
KOR	kappa opioid receptor
MAPK	mitogen activated protein kinase
min	minute
MOR	mu opioid receptor
NaCl	sodium chloride
NBCS	newborn Calf Serum
NG 108-15	neuroblastoma X glioma hybrid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pEC_{50}	EC_{50} negative log
pIC_{50}	IC_{50} negative log
PKA	protein kinase A
PKC	protein kinase C
pK_i	K_i negative log
PTX	Pertussis toxin
RGS	regulator of G protein signalling

^{35}S	sulphur 35
SDS	sodium-dodecyl sulphate
SEM	standard error of the mean
SSTr	somatostatin receptor
TIPPΨ	Tyr-Tic$_{\Psi}$(CH_2NH)-Phe-Phe
TM	transmembrane domain
Tris	Tris (hydroxymethyl) aminomethane
U69593	N-methyl-N-[7-(1pyrrolidinyl)-1-oxaspirol[4,5]dec-8-yl]-benzeneacetamide

Fusion protein abbreviations:

$\underline{\beta_2\text{-adrenoceptor-G}_{i1\alpha}C^{351}I}$: the β_2-adrenoceptor fused to $G_{i1\alpha}$ which contains a cysteine mutated in isoleucine in position 351

$\underline{\text{hDOR-G}_{i1\alpha}C^{351}I}$: the human DOR fused to $G_{i1\alpha}$ which contains a cysteine mutated in isoleucine in position 351

$\underline{\text{hDORV}^{150}\text{E,V}^{154}\text{D-G}_{i1\alpha}C^{351}I}$: the human DOR in which a pair of valine belonging to the 2nd intracellular loop has been mutated in glutamic acid and aspartic acid. This mutated receptor is fused to $G_{i1\alpha}$ containing a cysteine mutated in isoleucine in position 351.

$\underline{\text{hDOR-G}_{i1\alpha}G^{202}\text{A,C}^{351}I}$: the human DOR linked with $G_{i1\alpha}$ which contains a glycine mutated in alanine in position 202 and a cysteine mutated in isoleucine in position 351

$\underline{\text{hMOR-G}_{i1\alpha}C^{351}I}$: the human MOR fused to $G_{i1\alpha}$ which contains a cysteine mutated in isoleucine in position 351

$\underline{\text{hMORV}^{169}\text{E,V}^{173}\text{D-G}_{i1\alpha}C^{351}I}$: the human MOR in which a pair of valine belonging to the 2nd intracellular loop has been mutated in glutamic acid and aspartic acid. This mutated receptor is fused to $G_{i1\alpha}$ containing a cysteine mutated in isoleucine in position 351.

$\underline{\text{hMOR-G}_{i1\alpha}G^{202}\text{A,C}^{351}}$: the human MOR fused to $G_{i1\alpha}$ which contains a glycine mutated in alanine in position 202 and a cysteine mutated in isoleucine in position 351

$\underline{\text{rKOR-G}_{i1\alpha}C^{351}I}$: the rat KOR fused to $G_{i1\alpha}$ which contains a cysteine mutated in isoleucine in position 351

$\underline{\text{rKORV}^{160}\text{E,V}^{164}\text{D-G}_{i1\alpha}C^{351}I}$: the rat KOR in which a pair of valine belonging to the 2nd intracellular loop has been mutated in glutamic acid and aspartic acid. This mutated receptor is linked to $G_{i1\alpha}$ containing a cysteine mutated in isoleucine in position 351.

rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$: the human KOR fused to $G_{i1\alpha}$ containing a glycine mutated in alanine in position 202 and a cysteine mutated in isoleucine in position 351

Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$: Flag tagged version of the N-terminal and TM 1 of the DOR and fused to $G_{i1\alpha}$ containing a cysteine mutated in isoleucine in position 351

CHAPTER 1

Introduction

1.1 Cell signalling

The body is made of organs and tissues themselves composed of cells. These cells coordinate all the vital functions and need to communicate. An intercellular network exists, where cells exchange signals either by direct contact or via messenger molecules. The binding of such messenger ligands to cell surface receptors trigger conformational changes within the receptor and initiate a sequence of reactions leading to a specific cellular response. This response is dependent on the nature of both ligand and its receptor. There is a large variety of ligands e.g. hormones, growth factors, neurotransmitters, toxins and four major classes of cell surface receptors are known, namely ion channels, tyrosine-kinase receptors, receptors with intrinsic enzymatic activity and G protein coupled receptors (GPCRs).

The last class, GPCRs, are encoded by some 2-3% of the genes and mediate their actions through intracellular G proteins. GPCRs are key controllers of diverse physiological processes such as neurotransmission, cellular metabolism, secretion, cellular differentiation and growth, inflammatory and immune responses. For this reason GPCRs are important targets for drug discovery with already a majority of commercialised therapeutics acting on them.

1.2 G protein-coupled receptors

1.2.1 Structural features

Many GPCRs have been reported since bovine rhodopsin was first cloned in 1983 and the β_2-adrenoceptor in 1986 (Nathans and Hogness, 1983; Dixon *et al.*, 1986). All GPCRs share a common topology of seven transmembrane helices linked by three extracellular and three intracellular loops (figure 1.1). These receptors have a barrel shape, orientated roughly perpendicular to the plasma membrane and the transmembrane helices have a counter clockwise orientation when viewed from the extracellular membrane surface. The C-terminal tail of the GPCR is intracellular whereas the N-terminal region is extracellular. These regions vary in size depending on the receptor. The N-terminal tail can be composed of 7 to 600 amino acids (aa), the loops from 5 to 230 aa and the C-terminal tail from of 12 to 350 aa.

The extracellular N-terminal domain has been reported to play a role in ligand binding for certain GPCRs (Strader *et al.*, 1994; Wess *et al.*, 1996). The N-terminal domain can be O or

N-glycosylated in order to generate a properly folded receptor as well as providing correct targeting to the plasma membrane. The extracellular regions also contain highly conserved cysteines which can form disulphide bridges that stabilise the receptor's ternary structure.

The intracellular domains and C-terminal tail are known to be important for G protein recognition and activation (Wess *et al.*, 1997). Additionally, the C-terminal tail is rich in serine and threonine residues which can be phosphorylated by second messenger-activated and other kinases consequently inducing receptor desensitisation. The C-terminal domain can also undergo palmitoylation on specific cysteines thus creating an anchor in the plasma membrane which is described as the 4th intracellular loop (Ovchinnikov *et al.*, 1988). Moreover, receptor palmitoylation has been reported to affect G protein coupling and receptor internalisation (Okamoto *et al.*, 1997; Eason *et al.*, 1994).

In attempt to understand GPCRs structure many studies have been performed. Mutagenesis and biochemical experiments provided initial information and recently the rhodopsyn receptor structure, a class A GPCR, was resolved at the atomic level using crystallisation (Palczewski *et al.*, 2000). This crystal structure provides a structural template for other GPCRs as the seven transmembrane helices and the extracellular loops are expected to be similar for class A family members.

Sequence analysis had speculated that the transmembrane domains, predominantly hydrophobic, comprised 20-25 aa and form alpha helical domains. This conjecture was also confirmed by the rhodopsin crystallisation. Other studies have reported that transmembrane domains the most exposed to the membrane lipid bilayer are TM I, TMIV and TMV (Unger *et al.*, 1997) whereas TMIII seems to be deeply buried inside the receptor core. This highly packed core is postulated to be maintained via hydrogen bonds and/or salts bridges between residues of similar or different transmembrane domains (Pebay-Peyroula *et al.*, 1997; Palczewski *et al.*, 2000).

1.2.2 Family subtypes

Initial estimates predicted the existence of about a 1000 GPCRs (Marchese *et al.*, 1999). Detailed analysis of the human genome sequencing programme indicate some 380 genes coding GPCRs likely to respond to endogenously produced ligands and a further 350 that are chemosensory receptors. However, of these many remain orphan receptors for which endogenous ligands have not yet been identified.

Different designations have been used to classify GPCRs depending on their amino acid sequence, ligand structure or G protein coupling properties. Based on sequence homology they are classed as followed:

- The rhodopsin type or Class A receptor, this is by far the largest receptor subfamily with 89% of the known GPCRs and is the most extensively investigated. Ligands acting on these receptors are very diverse and include biogenic amines, peptides and neuropeptides, chemokines and prostanoids as well as sensory stimuli such as light and odours. Class A receptors display two highly conserved motifs, a DRY (Asp/Arg/Tyr) motif situated at the cytoplasmic end of TMIII and a NPXXY (Asn/Pro/X/X/Tyr) motif located in TMVII.
- The secretin/glucagon or Class B receptor family. This group is relatively small with 7% of the known GPCRs. Class B receptors have a relatively large N-terminal extracellular domain that contains six well conserved cysteine residues (Laburthe et al., 1996)
- The metabotropic-glutamate receptor like or Class C family. This is the smallest group with only 4% of the known GPCRs. These have a long extracellular N-terminal segment (500-600aa) which has been reported to contain the ligand binding site (O'Hara *et al.*, 1993; Conn and Pinn, 1997).

A new nomenclature based on phylogenetic analyses of GPCR sequence from the human genome was recently described by Fredriksson et al., (2003). This classification system has been named GRAFS, which is the acronym for the five different groups (glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin) comprising the system.

1.2.3 G protein subunits

A common feature of GPCR family function is the way they transduce signals after receptor activation. All GPCRs activate G proteins which are composed of three different subunits namely α, β and γ.

- G protein activation

In the absence of activation, G proteins are an $\alpha\beta\gamma$ heterotrimer with GDP bound to the G_α subunit. Ligand binding to a GPCR leads to the exchange of GDP for GTP on the G_α subunit. With bound GTP the G_α subunit undergoes a conformational change which triggers its dissociation from the $\beta\gamma$ dimer. At this point both GTP-G_α subunit and the $\beta\gamma$ dimer can

interact with and modulate effector proteins. G_α subunits possess an intrinsic GTPase activity that cleaves the terminal phosphate of GTP, the subsequent GDP-G_α subunit can reassociate with the βγ-dimer resulting in the deactivation of both components. The system is thus returned to basal state (Figure 1.1).

- G_α subunit

The G_α subunit is formed of two domains, a GTPase and an alpha helical domain. Between these two domains there is a cleft where guanine nucleotides bind. The G_α subunit is anchored to the plasma membrane via covalently attached lipids. Twenty different α subunits have been identified and divided in the four major families based on their effector interactions.

- G_s subfamily: named due to its ability to stimulate adenylate cyclase. G_s is ubiquitously expressed in cells and has 4 known splice variants (G_{s1}, G_{s2}, G_{s3} and G_{s4}).
G_{olf} is grouped with G_s due to their high sequence homology. G_{olf} is coupled to olfactory receptors and activates an olfactory specific adenylate cyclase.
The members of this family can be ADP rybosylated on a specific arginine residue by cholera toxin from *Vibrio Cholera*. This results in the G_s subunit being permanently activated.

- $G_{i/o}$ subfamily: inhibits adenylate cyclase and is composed of different isotypes. The family members are G_{i1}, G_{i2} which shares 88% homology with G_{i1} and G_{i3} that stimulates various ion channels.
G_o (other) has two splice variants G_{o1} and G_{o2}. These G proteins are only expressed in neuronal and electrically excitable cells. G_o inhibits the opening of voltage sensitive N-type Ca^{2+} and K^+ channels (Hescheler *et al.*, 1987).
G_t (transducin) has two splice variants G_{t1} and G_{t2} which are major components of retinal rod outer segment (G_{t1}) and cone (G_{t2}). Following activation by opsin G_{t1and2} inhibit cGMP phosphodiesterase.
G_{gust}, (gustducin) is expressed in the taste buds and modulates a cGMP phosphodiesterase.
G_z inhibits adenylate cyclase and is expressed in neuronal cells (Taussig and Gilman, 1995).

All members of this family (except G_z) can be ADP ribosylated on a specific cysteine residue by Pertussis toxin from *Bordetella Pertussis*. This maintains the G_α subunit in the GDP bound state preventing its activation by receptors.

-G_q subfamily: is composed of five different members including G_q and G_{11} which are widely expressed and 88% identical, G_{14} sharing 81% homology with G_q and with a more restricted expression pattern and G_{15} and G_{16} corresponding to the murine and human form of the same G protein. These are only expressed in a subset of haematopoietic cells (Wilkie et al., 1991) These subunits share only 57% homology with G_q. All the members of this family activate phospholipase C-β isoforms.

-$G_{12/13}$ subfamily: regulates small GTP binding proteins of the Rho family (Klages et al., 1999) as well as Na^+/H^+ ion exchange in cells (Hooley *et al.*, 1996) Two members, G_{12} and G_{13}, comprise this family and they are ubiquitously expressed.

C/ βγ subunit

The Gβγ subunit is composed of two polypeptides β and γ. There are six β subunits and 11 γ subunits that have been identified so far.

-β subunit: β1, 2, 3 and 4 share high sequence homology. β5 which has two splices variants (short and long form) shows much less homology with the other member of the family. β5L and β5S are only expressed in the retina and in the central nervous system (Watson *et al.*, 1996)

-γ subunits are more diverse. These subunits are classed in different subfamilies on the basis of their amino acid sequences.

-Class I is composed of γ1, γc and γ11. They undergo different modifications such as farnesylation or geranylgeranylation (Fukada *et al.*, 1990; Mumby *et al.*, 1990). γ1 is expressed in rod photoreceptors, γc in cones and γ11 in several tissues.

-Class II includes γ2, γ3 and γ4, which are abundantly expressed in the nervous system.

-Class III is formed of two members, γ7 and γ12.

-Class IV consists of three members i.e. γ5, γ8 and γ10.

Several approaches have shown that β and γ subtypes associate selectively (Pronin *et al.*, 1992) and with varying affinities for one another (Yan *et al.*, 1996). Experiments have demonstrated that the β subunit is folded improperly in the absence of γ and that γ3 is sensitive to degradation in the absence of the βγ dimer formation (Schmidt and Neer, 1991).
Early studies suggested that βγ complex formation is a requirement for receptor/G protein interaction and that this complex also interacts directly with the receptor (Florio and Sternweis, 1989). Phillips and Cerione, (1992) demonstrated this direct interaction using a peptide mimicking the rhodopsin C-terminal tail. When coexpressed with the βγ subunit and the rhodopsin receptor, the peptide abolished their contact. Further experiments showed that this direct interaction was mainly due to the γ componement of the complex (Kisselev and Gautam, 1993).
When βγ complexes were first discovered their role in signalling was underappreciated. Only after finding that βγ dimers were responsible for activating muscarinic receptor regulated inwardly by rectifying K^+ ion channel in heart were those subunits investigated as modulators of effector functions (Logothetis *et al.*, 1987). Since then, a number of other effectors have been shown to be regulated by βγ complexes; among them are certain adenylate cyclase isoforms (Tang and Gilman, 1991), phospholipase C-β isoforms (Katz *et al.*, 1992), voltage-sensitive Ca^{2+} channels and certain Na^- channels (Ikeda, 1996; Ma *et al* 1997).

1.2.4 Signal transduction and ligand binding

The cascade of events leading to signal transduction through GPCRs is not completely understood. These receptors are believed to exist in an equilibrium between inactivated R and activated R* state that is dependent on ligand binding as well as G protein heterotrimer association. However structural understanding of GPCR activation is still in the early stages and more structural data are awaited.

A/ Ligand binding

Depending on the GPCR class or the receptor considered, ligand binding domains are reported to be different. For example, the ligand binding domain of class C receptors is situated in the extracellular N-terminal region (Hirsch *et al.*, 1996) whereas ligands of many

class A GPCR bind within the transmembrane domains. Moreover, the transmembrane domains involved in ligand binding of class A GPCR appeared to depend on the specific receptor examined. Use of α_2 and β_2-adrenoceptor chimeras showed that TMs VI and VII are essential for agonist and antagonist binding specificity (Kobilka *et al.*, 1988) whereas TMIII was described as giving ligand binding specificity to dopamine D2-4 receptors (Shih *et al.*, 1997). Similarly, residues belonging to TMIII and TMIV of the type 1 angiotensin receptor were reported to play a role in the binding of angiotensin II and various analogues (Monnot *et al.*, 1996).

B/ Signal transduction

Domains and mechanisms responsible for GPCR conformational changes upon ligand binding have been difficult to establish. Early studies described TMIII and TMVI movement as important for activation of rhodopsin as well as the β_2-adrenoceptor (Farrens *et al.*, 1996; Javitch *et al.*, 1997). The activation of these receptors is presumed to cause the "opening" of the intracellular receptor surface thus enabling G protein interaction with residues that were previously inaccessible (Bourne, 1997; Wess, 1997). Rhodopsin crystallisation confirmed this hypothesis by observing that the highly conserved DRY motif situated at the beginning of the 2nd intracellular loop produces critical constraints by creating H-bonds with the surrounding residues. Consequently, this receptor is kept in an inactive state. The authors reported that when the receptor is photo-activated, a rearrangement of TMIII and TMVI takes place resulting in a change of environment of the DRY motif. Associated with these movements, the receptor is switched from an inactive to an active state (Palczewski *et al.*, 2000). A similar mechanism was recently described for β_2-adrenoceptor based on computational studies (Gouldson *et al.*, 2004) as well as for DOR (Decaillot *et al.*, 2003).

C/ Regions of GPCRs involved in interaction with G protein

Different domains are involved in GPCR/G protein interaction/activation including the 2nd and 3rd intracellular loops and at least in some receptors, the C-terminal tail. These domains are important for both receptor/G protein recognition and G protein activation efficacy.
Mutation within the 2nd intracellular loop of one of the most highly conserved residues of the class A family (the arginine residue from the DRY motif) completely abolished or drastically

reduced G protein coupling (Jones *et al.*, 1995; Arora *et al.*, 1997). Substitution of four amino acids of the 2nd intracellular loop of the M3 muscarinic receptor that is coupled to $G_{q/11}$ with into the $G_{i/o}$ coupled M_2 muscarinic receptor is sufficient to confer $G_{q/11}$ coupling selectivity to the M_2 muscarinic receptor (Blin *et al.*, 1995). Moreover, studies on the M5 muscarinic receptor suggested a critical role of intracellular loop 2 in maintaining the G protein in an inactive state (Burstein *et al.*, 1998)

Similar involvements in G protein coupling specificity were observed for the 3rd intracellular loop. Liu and Wess, (1996) described that the substitution of this loop from the G_s coupled V2 vasopressin receptor into the $G_{q/11}$ coupled V1a vasopressin receptor enable the resultant chimera to activate G_s. The role of C-terminal domain in G protein activation has been exemplified for different receptors such as opioid receptor (Georgoussi *et al.*, 1997). These authors observed that in the presence of a peptide mimicking the opioid receptor C-terminal tail, G protein activation by opioid receptors was abolished. Similarly, the C-terminal domain was described as playing a critical role in maintaining β_2-adrenoceptor/G protein coupling (O'Dowd *et al.*, 1988).

D/ Structural features important for G protein coupling with the receptor

A large body of evidence indicate that the C-terminal part of G_α subunits is important for contact with the receptor. A series of residues located in G_α subunit C-terminal domains have been predicted to form contacts with the receptor (Kallal and Kurjan, 1997). Furthermore, the published crystal structure of the G protein heterotrimer suggests that these amino acids are surface exposed and hence can easily make contact with receptors (Lambright *et al.*, 1996). Site directed mutagenesis and use of combinatorial peptide libraries have shown the presence of two leucines in the highly conserved last 7 amino acids of G_α protein C-terminal tail that are essential for receptor and G protein contacts as well as G protein activation (Osawa and Wess., 1995; Martin *et al.*, 1996). To a smaller extent, the N-terminal part of the G_α is also involved in receptor/G protein contact. This was demonstrated by using a synthetic peptide corresponding to the $G_{\alpha t}$ N-terminal part, which was able to prevent rhodopsin/$G_{\alpha t}$ interactions (Hamm *et al.*, 1988).

Similarly, $\beta\gamma$ complexes have also been shown to associate directly with different receptors such as rhodopsin and the β_1-adrenoceptor (Phillips and Cerione, 1992; Kurstjens *et al.*,

1991). This interaction with the receptor seems to be mainly due to the γ subunit tail becoming available when the heterotrimer is formed (Kisselev *et al.*, 1995).

E/ One receptor activates several G proteins

Although most GPCRs are preferentially linked to members of a certain G protein subfamily, it has become clear that they can also couple to other G-protein classes. In fact, members of the secretin/glucagon receptor family which usually interact with G_s were observed to also stimulate $G_{q/11}$ proteins (Gudermann *et al.*, 1996). Another example is the thyrotropin (TSH) receptor which has been described to be coupled with all four major classes of G protein (Laugwitz *et al.*, 1996). Some studies have also demonstrated that one class of coupling can be abolished while the other family remain unaffected. This is exemplified by the LH receptor which activates two pathways i.e. adenylate cyclase and phospholipase C via G_s and G_q respectively. However, when the 3rd intracellular loop of the LH receptor is mutated, the phospholipase C signal is altered but not cAMP production (Gilchrist *et al.*, 1996). This result also highlights the fact that GPCRs interact with G proteins at distinct sites.

1.2.5 GPCR regulation

The binding of an agonist to its receptor not only results in G protein activation but also triggers a series of molecular interactions that allow feedback regulation such as receptor desensitisation and downregulation.

A/ Desensitisation

Desensitisation is defined as a process, which reduces receptor/G protein/2nd messenger coupling. When the receptor is activated by an agonist this may result in the phosphorylation of the serine/threonine of its C-terminal tail. Protein kinases such as PKC, PKA and the G protein coupled receptor kinases (GRKs) are responsible for these phosphorylations. GRK-mediated receptor phosphorylation promotes receptor association with β-arrestins (Krupnick *et al.*, 1998) thus targeting the receptor for internalisation in clathrin-coated vesicles and resulting in receptor/ G protein uncoupling. The receptor can be either recycled at the plasma membrane allowing resensitisation or degraded into lysosomes (Oakley *et al.*, 1999; Zhang *et*

al., 1999). These processes result in a rapid attenuation of responsiveness and occur in the range within seconds for phosphorylation to minutes for endocytosis.

B/ Down-regulation

In contrast to desensitisation, down regulation results from a long (from hours to days), chronic exposure of the receptor to agonist. This leads to an irreversible loss of receptor expression at the plasma membrane. Different regulation mechanisms are triggered to produce a decrease in the number of receptor binding sites. These include reduction in receptor mRNA production and/or protein synthesis as well as receptor internalisation and degradation via lysosomes (Valiquette *et al.*, 1990).

1.3 GPCR dimerisation

1.3.1 History of GPCR dimerisation

For a long time, the conventional assumption was that GPCRs were monomeric entities activating a G protein upon ligand activation. The concept that dimerisation participates in cell surface receptor activation was well accepted for other receptors classes such as growth factor or cytokine receptors (Heldin, 1995). However, studies throughout the 1970s and 1980s had proposed that GPCRs could exist as dimers but it was not before the mid-1990s that this concept started to be accepted. One of the first strong pieces of evidence of dimer formation came from studies on the GABAb receptor, a class C GPCR. The GABAb1 receptor was first cloned as a possible GABAb receptor (Kaupmann *et al.*, 1997) but it was shown that it could not account for the functional activity of the native GABAb receptor. The GABAb2 receptor was then identified and when these two receptors were co-expressed, the pharmacology and functional activity expected for the native GABAb receptor was recovered (Jones *et al.*, 1998, Kaupmann *et al.*, 1998, White *et al.*, 1998, Kuner *et al.*, 1999). This result provided several lines of evidence that the GABAb receptor was a dimer and that this dimer is the functional unit. The observation of rows of rhodopsin receptor dimers in native rod outer-segment disc membranes using atomic force microscopy (Figure 1.2) also provided one of the clearest demonstration that GPCRs can exist as dimers (Fotiadis *et al.*, 2003). However, reservations have been expressed about the preparation procedure employed to observe the rhodopsin dimers in native retinal rods (Chabre *et al.*, 2003). Nevertheless, it is nowadays a widely accepted notion that GPCRs form dimers as well as higher order oligomers (Milligan *et al.*, 2003).

1.3.2 Methods used to study GPCR dimerisation

A range of approaches have been used to study GPCR dimerisation.

-Co-immunoprecipitation

Co-immunoprecipitation was one of the first and still most extensively used approaches to detect GPCR dimers. Differentially epitope tagged GPCRs are co-expressed in heterologous

expression system. Antibodies to one epitope are used to immuno-isolate the receptor in the complex tagged with the corresponding epitope then the second receptor in the complex is visualised by western-blot using antibodies against the second epitope. This technique was used to document homo and hetero-dimerisation of GPCRs including the dopamine D2 receptor (Ng *et al.*, 1996), CCR2 (Rodriguez-Frade *et al*, 1999), 5HT1b and 5HT1d (Xie *et al.*, 1999) and adenosine A1 receptor and metabotropic glutamate 1α receptor ($mGlu_{1\alpha}$) (Ciruela *et al.*, 2001).

Although commonly used to study GPCR oligomerisation, Salim et al., (2002) reported that every GPCR they tested could interact with the $5HT_{1A}$ receptor hence raising questions about the specificity of this technique. One of the issues is that co-immunoprecipitation requires membrane solubilisation. GPCRs being highly hydrophobic, this procedure could induce formation of artefactual GPCR aggregates following solubilisation or apparent dimer pattern due to incomplete solubilisation. To address this concern a combination of detergents as well as cross linking agents have been used (Abdalla *et al.*, 2001a, Jordan and Devi, 1999). Another good control for artefactual aggregation is the use of cell mixtures that individually express each of the differentially epitope-tagged receptors. If after co-immunoprecipitation dimers are not observed from these mixed cells, this would imply that solubilisation did not promote receptor aggregation.

-Resonance Energy Transfer (RET) methods

To provide further evidence of GPCR dimerisation RET approaches such as FRET (Fluorescence Resonance Energy Transfer) or BRET (Bioluminescence Resonance Energy Transfer) have been developed. These techniques measure the energy transfer exchanged between a donor and an acceptor molecule when the distance between these two molecules is <100Å. In FRET the donor molecule is a fluorophore whereas in BRET it is a bioluminescent molecule such as *Renilla* Luciferase, which emits light upon degradation of its substrate, coelenterazine. For both RET methods the acceptor molecule is a fluorophore usually a variant of green fluorescent protein (GFP). Variants of these two techniques have been developed including time resolved FRET, photobleaching FRET and $BRET^2$. As well as monitoring dimer formation, these approaches also have the advantage of detecting dimerisation in living cells, therefore without disrupting the natural environment of the receptors. These approaches have generated further evidence of dimer formation for dopamine D2 and somatostatin SSTr5 receptors (Rocheville *et al.*, 2000b), CCR5 (Issafras *et*

al., 2002) and α_{1a} and α_{1b}-adrenoceptors (Stanasila *et al.*, 2003). Furthermore, they have been used to try to measure the affinity of two receptors to form a dimer (Ramsay *et al.*, 2002; Dinger *et al.*, 2003). Mercier et al., (2002) developed the saturation BRET method to study the ability of the β_2-adrenoceptor to homodimerise. This approach permits determination of two parameters, $BRET_{50}$ and $BRET_{max}$. $BRET_{50}$ reflects the two receptors interaction affinity and $BRET_{max}$ maybe useful in estimating the total number of dimers formed.
However, these strategies have some drawbacks. The signal observed is dependent on the distance and orientation of the donor and the acceptor molecules. BRET does not report GPCR dimer cellular location. Some concerns were also raised about overexpression of the receptors leading to random collisions and consequently artefactual RET signals.

-Complementation technique

A third technique employed to study GPCR dimerisation is functional complementation. This method involves either the use of chimeric or mutant receptors. This approach can provide further understanding of the region(s) involved in dimerisation as well as giving some clues about the role of dimerisation in cell signalling. Good examples are the studies done by Maggio *et al.*, (1993, 1999) on muscarinic M3 receptor/α_{2c}-adrenoceptor interactions. The authors generated chimeras by exchanging TMVI,VII and the C-terminal tail between both receptors. When transfected alone, neither of the chimeras were able to bind their selective ligands nor signal. However, when co-expressed, ligand binding and signalling properties were rescued, even enhanced. Taken together these results provide evidence of a physical interaction between the receptors and indicate conformational changes upon dimer formation leading to an increase in functional properties. The same authors reported that the functional complementation was no longer observed when the 3rd intracellular loop was deleted but that dimer formation was not altered thus suggesting involvement of the 3rd intracellular loop in receptor function (Maggio *et al.*, 1996). Other groups have also used similar techniques to further understand the purpose of GPCR dimerisation (Monnot *et al.*, 1996; Bai *et al.*, 1999; Carrillo *et al.*, 2003). A limitation of this technique lies in the fact that the receptors physical interaction is not directly demonstrated but this is usually presumed by the fact that functional complementation is observed.

All approaches have their strengths and their limitations and thus are complementary for studying dimerisation.

1.3.3 Homo and Heterodimer/oligomers

Up to now, the term dimer has been used, however, different type of dimers may exist namely homodimers and heterodimers. Furthermore, more than two receptors may associate consequently forming oligomers.

A/ Homodimer and Heterodimer

When two identical proteins associate the term homodimer is used. GPCRs have been widely reported to form homodimers (Herbert *et al.*, 1996; Ng *et al.*, 1996; Romano *et al.*, 1996; Fukushima *et al.*, 1997; Roess *et al.*, 2000) Similarly, when non-identical proteins interact the entity formed is called a heterodimer. Heterodimerisation has been described for various GPCRs and between closely or less-closely related receptors. For example subtypes of the same family can associate such as β1- and β2-adrenoceptors (Mercier *et al.*, 2002), angiotensin II AT1 and AT2 receptors (Abdalla *et al.*, 2001b) and GABAb1 and GABAb2 receptors (Jones *et al.*, 1998). Interactions can also be between less homologous GPCRs e.g. adenosine A2a and dopamine D2 receptors (Hillion *et al.*, 2002) or somatostatin SSTr5 and the dopamine D2 receptor (Rocheville *et al* 2000b). Even heterodimerisation between class A adenosine A1 receptor and class C mGlu1R was observed by Ciruela et al, (2001). This result was unexpected as these two receptors do not share any homology except the 7 transmembrane domain topology.

B/ Oligomers

An oligomer requires the association of more than two identical (homo-oligomer) or non-identical (hetero-oligomer) proteins. Frequently, when dimers have been visualised by co-immunoprecipitation, as well as detecting a band corresponding to the dimer molecular mass, bands of higher molecular size and multiples of the monomer mass could also be visualised. These observations raised the hypothesis that GPCRs not only form dimers but also

oligomers. However, this hypothesis is difficult to validate and little data are available on the subject. However, Park and Wess, (2004) have provided direct evidence that the M2 muscarinic receptor forms at least a trimer by using three differentially epitope-tagged forms of this receptor. Similarly, it was proposed that the complement C5a receptor could form a tetramer (Klco *et al.*, 2003). By contrast, when β_1-and β_2-adrenoceptor interaction was studied and the authors tried to distinguish between dimer and higher-order oligomer formation using saturation BRET, they concluded that these receptors formed dimeric complexes (Mercier *et al.*, 2002). Visualisation of rhodopsin dimer rows by atomic force microscopy also provide further evidence of the dimeric but also the oligomeric status of this GPCR (Fotiadis *et al.*, 2003). Overall, these studies suggest that new approaches will be needed to further investigate the concept of oligomerisation.

The term dimer will thus be used in the following text referring to either dimer or higher-order oligomer.

1.3.4 Ligand modulated or constitutive dimerisation ?

A key question is whether ligands modulate GPCR dimer formation and stability or not.

- ligand modulated dimerisation

Rocheville et al., (2000a, b) studied ligand effects on somatostatin SSTr5 and SSTr1 receptors as well as on somatostatin SSTr5 and dopamine D2 receptor heterodimer formation. They used FRET and/or co-immunoprecipitation assays to monitor ligand-induced dimer formation and concluded that agonists promoted dimer formation. Equally, for the β_2-adrenoceptor and the lutropin receptor agonist-induced dimerisation was observed but the authors also described a certain level of constitutive dimerisation (Herbert *et al.*, 1996; Angers *et al.*, 2000; Tao *et al.*, 2004). Additionally, agonist-dependent dimerisation has been reported for various chemokine receptor homodimers such as CCR2, CCR5 and CXCR4 (Rodriguez-Frade *et al.*, 1999; Vila-Coro *et al.*, 1999-2000). However, more recent studies involving the same chemokine homodimers reported results in complete opposition. The authors observed ligand independent dimerisation of these chemokine receptors (Issafras *et al.*, 2002; Babcock *et al.*, 2003). These contradictory results are confusing and raise questions about the validity of the

approaches used to monitor ligand-dependent dimerisation. Moreover, some constitutive GPCRs dimers have been reported to dissociate upon ligand treatment. Such results were observed for TSH receptor and neuropeptide Y Y4 receptor homodimers as well as for D1 dopamine and A1 adenosine receptor heterodimer (Latif *et al.*, 2002; Berglund *et al.*, 2003; Gines *et al.*, 2000).

- Constitutive dimers

In contrast to the above, a large literature has reported constitutive dimerisation of GPCRs. For example the D2 dopamine receptor, the α_{1b}-adrenoceptor and the yeast α factor receptor were described to be constitutive dimers using various approaches including cross-linking or FRET (Guo *et al.*, 2003; Stanasila *et al.*, 2003; Overton and Blumer, 2000). Recently, Percherancier et al., (2005) have used saturation BRET to study the potential effect of ligand on CCR2-CXCR4 heterodimerisation. They observed no change in $BRET_{50}$ (affinity of the receptors to form a dimer) upon agonist treatment but an increase in $BRET_{max}$ (estimate of number of complex formed) and interpreted these data not as a ligand-induced dimerisation but as a conformational change within the preformed dimer. They argued that an increase in $BRET_{max}$ could not be taken as an increase in dimer formation if the $BRET_{50}$ value is unchanged. Their hypothesis is that the ligand is inducing a conformational change within the dimer consequently bringing the donor and acceptor closer thus an increase in $BRET_{max}$ was observed. A similar analysis was previously reported by Ayoub et al., (2002) for the melatonin receptors. The use of saturation BRET and the interpretation of its parameters ($BRET_{50}$ and $BRET_{max}$) could bring some new insights to ligand-induced dimerisation studies as some of the earliest studies reporting BRET or FRET increases as ligand-dependent dimer formation could have been misinterpreted.

In conclusion, the discrepancies observed suggest that no generality can be made on whether GPCR dimerisation is ligand modulated or not. Each GPCR dimer has to be studied in an independent manner.

1.3.5 Location of GPCR dimer formation

GPCRs have been proposed to dimerise either in the endoplasmic reticulum (ER) during biosynthesis or at the plasma membrane.

- **Plasma membrane dimerisation**

Plasma membrane dimerisation suggests that GPCR monomers are transported from the endoplasmic reticulum to the cell surface where they associate in a ligand-dependent or independent fashion. This mechanism has been reported for several GPCRs such as the somatostatin SSTr5 and SSTr1, SSTr5 and dopamine D2 receptors as well as for the gonadotrophin releasing hormone receptor (GnRH) using co-immunoprecepitation and/or FRET approaches (Rocheville *et al.*, 2000a and b; Cornea *et al.*, 2001).

- **ER dimerisation**

Alternatively, numerous studies have reported that dimers could be assembled in the ER and then be transported as dimeric units to the plasma membrane. Dimerisation prior to plasma membrane expression is described as constitutive dimerisation. One of the first pieces of evidence of ER dimer formation was reported for the class C GPCR, the GABAb receptor heterodimer. GABAb1 was unable to reach the plasma membrane in the absence of GABAb2 receptor expression but trafficked normally upon co-expression with GABAb2 receptor (Margeta-Mitrovic *et al.*, 2000). The authors proposed that the GABAb2 receptor is masking an ER retention motif when dimerising with GABAb1. These observations were comfirmed in a recent study by Villemure *et al*, (2004). Another recent study using cell fractionation provided evidence that the class A β_2-adrenoceptor dimerisation was taking place in the ER. These authors reported that when a putative dimerisation motif was mutated normal trafficking to the plasma membrane was prevented. Hence they concluded that β_2-adrenoceptor homodimerisation was a prerequisite for cell surface targeting (Salahpour *et al.*, 2004). Similar conclusions were drawn for the α_{1b} and α_{1d} adrenoceptors (Hague *et al.*, 2004) and oxytocin and vasopressin receptor heterodimers (Terrillon *et al.*, 2003).

Additionally, the concept that dimerisation occurs prior to cell surface expression is supported by studies involving mutated receptors. Such mutated receptors can act as dominant negatives of the wild type receptor upon dimerisation thus preventing cell surface expression. Such an effect has been reported for the dopamine D2 receptor (Lee *et al.*, 2000), the platelet-activating factor receptor (Le Gouill *et al.*, 1999) and the V2 vasopressin receptor (Zhu and Wess, 1998).

GPCR dimerisation during biogenesis seems to be more predominant in comparison to dimer formation at the plasma membrane.

1.3.6 Domains involved in GPCR dimerisation

Various regions have been implicated in GPCR dimerisation. These include the extracellular, transmembrane and C-terminal domains (Figure 1.3).

- Extracellular domain

Usually when extracellular domains are involved in dimerisation it is via disulphide bridge formation. Studies on the mGluR1 receptor showed that a single disulphide bridge between Cys 140 in the receptor N-terminal domain was implicated in dimer formation (Ray and Hauschild, 2000). In agreement with these data, the extracellular N-terminal domain of mGluR5 and the calcium sensing receptor were also found to be important for dimerisation (Romano *et al.*, 1996; Bai *et al.*, 1998). Furthermore extracellular loop 2 (Cys 140) and 3 (Cys 220) of the muscarinic M3 receptor were described as participating in dimer formation (Zeng and Wess, 1999). However for some receptors the disulphide bond was found not to be the only region involved in dimer formation.

-Transmembrane domains (TM)

Along with disulphide bridges, transmembrane domains have been reported to play a key role in dimerisation. One of the first TM domains suggested to be involved in dimerisation was described for the β2-adrenoceptor, TMVI was proposed as a contact interface between the monomers (Herbert *et al.*, 1996). In a recent study Carrillo et al, (2004) examined all the potential sites of interaction involved in α_{1b}-adrenoceptor homodimerisation instead of testing a limited number of possible interfaces. They concluded that TMI and TMIV were contact points involved in α_{1b}-adrenoceptor dimer formation. Similarly TMI and/or TMIV have been described as being contact interfaces for different GPCRs including the C5a receptor, rhodopsin and the dopamine D2 receptor (Kleo *et al.*, 2003; Liang *et al.*, 2003; Guo *et al.*, 2003). Different TMs, such as TMII and TMV, were also reported to take part in rhodopsin and C5a receptor dimerisation respectively. Additionally a putative dimerisation motif

GXXXG located in TMI or TMVI was reported for the yeast alpha factor receptor (Overton *et al.*, 2003) and the β2-adrenoceptor (Salhapour *et al.*, 2004) respectively. In fact, specific residues have even been identified as crucial for dimerisation such as Ile52 (TMI) and Val150 (TmIV) for the CCR5 receptor (Hernanz-Falcon *et al.*, 2004).

When heterodimers are involved, determination of the dimer interface is even more complicated as transmembrane domains implicated in the dimerisation process must be determined for each of the receptors within the heterodimer. Canals et al, (2003) suggested that TMV and/or TMVI of the dopamine D2 receptor are likely to approach TMIV of the adenosine A2 receptor. In addition to biochemical and biophysical analysis, computational studies have been performed to predict interfaces for GPCR dimerisation (Gouldson *et al.*, 2001; Filizola *et al.*, 2002a and b). For instance, Nemoto and Toh, (2005) have developed a new method which can be applied to different GPCRs as they took into account that the interfaces involved in GPCR interactions differed between receptors. In fact, certain of their predictions seem to agree with what was found previously using biochemical and biophysical experiments, namely for rhodopsin, dopamine D2 and β2-adrenoceptors.

-C-terminal domain

Few studies have reported this domain as playing a role in dimerisation. One of the only GPCRs for which the C-terminal tail has been implicated in dimerisation is the class C GABAb receptor (White *et al.*, 1998). However, this interaction is not essential for GABAb dimerisation as the deletion of the C-terminal tail did not prevent dimer formation (Margeta-Mitrovis *et al.*, 2000). Although, in the case of the $mGluR_{1\alpha}$ and adenosine A1 receptor heterodimer the interaction appears to depend on the $mGluR_{1\alpha}$ C-terminal domain as a splice variant of $mGluR_1$ with a shorter and different C-terminal tail was not observed to interact with the adenosine A1 receptor (Ciruela *et al.*, 2001). Similarly, a recent study by Grant et al, (2004) identified using somatostatin SSTr1 and SSTr5 chimeras the C-terminal tail of SSTr5 as a key determinant for dimerisation

1.3.7 Domain swapped or contact dimers?

The discrepancies observed between GPCRs domains involved in dimer formation make it complicated to propose a general mechanism for GPCR dimerisation. However, two modes of dimerisation have been suggested for the general three-dimensional organisation of GPCR dimers (Figure 1.4). If two monomers interact most likely via hydrophobic interactions and the ligand binding domains of each receptor are maintained, a contact dimer is probably formed. This kind of dimer was first suggested by Herbert *et al*, (1996) for the β_2-adrenoceptor. Studies on V2 vasopressin and D2 dopamine receptors also support the contact dimers hypothesis (Schulz *et al.*, 2000; Lee *et al.*, 2000). However, if transmembrane domains are exchanged between monomers novel binding sites may be generated. These have been described as domain swap dimers (Gouldson *et al.*, 1998). Domain swap dimers have been suggested by studies involving mutant or chimeric receptors. These receptors are unable to function when individually expressed but when the complementary mutant receptors are co-expressed function is rescued. Such functional complementation has been recorded for type 1 angiotensin, dopamine D2, muscarinic M3 and α_{2a}-adrenoceptor and muscarinic M3 and M2 receptor heterodimers (Monnot *et al.*, 1996; Scarselli *et al.*, 2000; Maggio *et al.*, 1993; Barbier *et al.*, 1998). A recent study involving the histamine H_1 receptor has even suggested that contact and swapped dimers can co-exist in the same cells (Bakker *et al.*, 2004). This observation was based on binding data using two different radioligands for the histamine H_1 receptor, one supposedly detecting only histamine H_1 receptor oligomer (Booth *et al.*, 2002). The discrepancy in B_{max} obtained after ligand binding saturation with the two radioligands was interpreted by the authors as reflecting a mix of contact and swapped dimers.

1.3.8 Pharmacology and function alterations due to heterodimerisation

In many cases when GPCR heterodimerisation is observed, changes in pharmacological, functional and/or internalisation properties have been documented.

-Pharmacological property

Heterodimerisation leading to a change in pharmacology has been described for several GPCR pairs such as the M2 and M3 muscarinic receptors. The authors have reported the formation of a new binding site with unique pharmacology when these receptors were coexpressed (Maggio *et al.*, 1999). Likewise, in a recent study, involving β_1 and β_2-

adrenoceptor heterodimers Lavoie and Herbert (2003) have reported that ligand binding to one subtype affected the specific ligand binding of the other subtype. However, when the SSTr2 and SSTr3 receptors were co-expressed a similar pharmacology as the SSTr2 receptor has been observed whereas a 100 fold decrease in SSTr3 selective ligand affinity was noted (Pfeiffer *et al.*, 2001). Ligand binding alteration has also been monitored for the SSTr5 and dopamine D2 receptors (Rocheville *et al.*, 2000b), the adenosine A1 and dopamine D1 (Torniven *et al.*, 2002) and the α_{2A} and β_1-adrenergic receptors (Xu *et al.*, 2003). The pharmacological diversity brought by GPCR interactions could be of great interest for new drug development.

- **Signalling property**

Receptor function has been established as resulting from heterodimerisation for the GABAb1 and GABAb2 and the taste T1R3 and T1R1 receptors (Galvez *et al.*, 2001; Nelson *et al.*, 2001 and 2002). Moreover, alterations of signalling properties such as signalling potentiation were observed when the chemokine CCR5 and CCR2 receptor and the angiotensin AT1 and bradikinin B2 receptors were co-expressed (Mellado *et al.*, 2001; AbdAlla *et al.*, 2001b). In contrast, interactions between the somatostatin SSTr2 and SSTr3 as well as the angiotensin AT1 and AT2 receptors were reported to result in inactivation of the somatostatin SSTr3 or AT1 receptor function respectively (Pfeiffer *et al.*, 2001; AbdAlla *et al.*, 2001a). Alteration in G protein coupling has also been described upon heterodimerisation. For example when the $G_{i/o}$-coupled CCR5 and CCR2 receptors form heterodimers, $G_{q/11}$ pathway has been proposed to be activated as PTX treatment did not block all CCR2/CCR5 signalling (Mellado *et al.*, 2001). A similar alteration in G protein selectivity has been observed when the β_2 and β_3-adrenoceptors were co-expressed (Breit *et al.*, 2004).

- **Internalisation pattern**

Studies have pointed out that heterodimerisation could affect agonist-induced endocytosis. On one hand, it was reported that stimulation of one receptor was sufficient to promote co-internalisation of the two receptors within the heterodimer. Stanasila et al, (2003) showed that an α_{1a}–adrenoceptor selective agonist induced co-internalisation of α_{1a} and α_{1b}-adrenoceptor heterodimers whereas this effect was not observed when the α_{1a}-adrenoceptor was co-

expressed with the tachykinin NK1 or CCR5 receptors thus ruling out any non-specific effect. Similar results were observed for α_{2a} and β_1-adrenoceptors or adenosine A2 and dopamine D2 heterodimers (Xu *et al.*, 2003; Hillion *et al.*, 2002). On the other hand, receptors that do not undergo ligand-promoted endocytosis were documented as having a possible dominant negative effect on the wild-type receptors they were interacting with. Indeed, it was reported that internalisation of the β_2-adrenoceptor upon agonist treatment was prevented by its co-expression with the internalisation resistant β_3-adrenoceptor (Breit *et al.*, 2004).

1.3.9 GPCR-G protein ratio

A common concept was that one GPCR was activating one G protein. However, the acceptance of GPCR dimerisation has jeopardized this idea. The question is asked whether a dimer interacts only with one G protein (Hamm, 2001, Arimoto *et al.*, 2001). The visualisation of rhodopsin by atomic force microscopy (Liang *et al.*, 2003) suggested that the size and geometry of these dimers allow a perfect fit for the binding of one G protein. Early studies on the class C GABAb receptor are also in agreement with this theory (Margeta-Mitrovic *et al.*, 2001; Galvez *et al.*, 2001; Duthey *et al.*, 2002). The authors have demonstrated using chimeras that only GABAb2 intracellular loops were responsible for G protein activation whereas both subunits were taking part in the receptor function and thus supporting the idea that only one G protein was needed for the GABAb receptor dimers to function. Moreover, Banères and Parello, (2003) reported a 2:1 GPCR:G protein ratio for the leukotriene B4 BLT1 receptor homodimer demonstrating that this dimer was able to signal only through one G protein. If this concept of a GPCR dimer activating one G protein is verified for all GPCRs, it would mean that the minimum GPCR functional structure is a dimer.

1.4 Opioid receptor

1.4.1 Opioid history

Opiates have been used for centuries due to their analgesic and euphoric properties. Opium is an extract of the poppy plant, *Papaver somniferum* and its culture goes back to 3400 BC in Mesopotamia. It was reported that opium was used to relieve pain and relax people suffering from asthma or heart failure as far back as ancient Greece, in that Hippocrates, "the father of medicine" acknowledged the usefulness of the opium to treat diseases. In 1806, opium alkaloids were extracted from the poppy plant by the German chemist Fiedrich Serturner. One of these opioids was morphine, named after Morpheus, the god of dreams. Although pure morphine was isolated, it was not until 1833 that chemists at Macfarlane & Co in Edinburgh were able to isolate and purified this molecule on a commercial scale. Since then, morphine has been one of the most widely used opioids in the treatment of pain because of its very high analgesic power. Furthermore, morphine is used as a recreational drug due to its euphoric properties. The use of morphine induces addiction and tolerance as well as other side effects. For these reasons chemists have tried to derivitise morphine hoping to get a compound with no such adverse side effects. In 1874, C.R Wright synthesised diacetylmorphine or heroin by boiling morphine over a stove. Heroin was found to produce the same analgesic effect as morphine without the common side effects raising the possibility that it could be used as a morphine step-down cure. Thus in America a campaign was mounted in which free samples of heroin were distributed through the mail to morphine addicts who were trying to give up their habits. However, it became clear with time that the side effects of heroin were as bad as morphine. Heroin has become one of the most addictive recreational drugs illegally used nowadays. So far few drugs have been developed that are as effective as morphine in the treatment of pain but without the major side effects. A promising compound was etorphine which is about one thousand times more potent than morphine as an analgesic, however, its use is limited to immobilising large animals as it is also a very potent sedative. The development of the ideal opioid analgesic is still awaited.

1.4.2 Endogenous and exogenous opioids

The brain produces its own endogenous analgesic compounds. In mammals they are mainly derived from three precursors i.e. pro-opiomelanocortin, pro-enkephalin and pro-dynorphin (Nakanishi *et al.*, 1979; Kakidani *et al.*, 1982; Noda *et al.*, 1982). These precursors are hydrolysed by specific proteases that recognise basic amino acid sequences positioned just before and after the opioid peptide sequence. The endogenous opioid ligands are characterised by a common tetrapeptide sequence (Tyr-Gly-Gly-Phe) at their N-termini and comprise a dozen or so ligands including β-endorphin, Met/Leu-enkephalin and dynorphin A and B. These peptides have varying affinity for MOR, DOR and KOR but none binds exclusively to one opioid receptor. Two other endogenous ligands, endomorphin 1 and 2, were discovered with a tetrapeptide sequence (Tyr-Pro-X-Phe) differing from that of the classical endogenous opioid peptides. Endomorphin 1 and 2 show high affinity and selectivity for MOR (Zadina *et al.*, 1996).

The discovery of exogenous opioids preceded endogenous ligand identification. The extraction of the natural opioid ligands such as morphine, codeine and thebaine from the poppy plant allowed synthesis of semi-synthetic molecules derived from the natural compounds including buprenorphine, etorphine and methadone. Numerous morphine derivatives have been synthesised in an effort to discover ideal analgesics without the side effects of morphine. Finally, a range of synthetic ligands e.g. DAMGO, DPDPE, 6-GNTI were produced based on the endogenous peptide structures.

1.4.3 Medical applications

Opioid receptor activation results in a multitude of actions including analgesia, respiratory depression, euphoria, feeding, hormone release, inhibition of gastro-intestinal transit, anxiety. Commonly, DOR and MOR agonists produce analgesia and reward effect whereas KOR selective agonists are dysphoric. However, MOR seems to be the primary target of morphine's therefore it is considered as the receptor which mediates the majority of morphine analgesic effects (Matthes *et al.*, 1997)

Morphine, due to its high analgesic property is used clinically. This drug is administrated for anaesthesia but most usually for the relief of pain after surgery or to treat pain resulting from an injury or a disease such as cancer. Morphine, in addition of being a potent analgesic induces adverse effects such as respiratory depression, sedation, nausea and vomiting, constipation, urticaria and, most importantly, tolerance which is a major drawback for

medical use. Severe withdrawal symptoms are also observed when morphine is no longer taken, these includes anorexia, loss of weight, chills, excessive sweating, increase in heart rate and blood pressure as well as cramps, muscle spasms and hyperirritability. Other opioids used therapeutically include heroin, codeine, methadone, buprenorphine, fentanyl and lovarphanol. Codeine has a similar action as morphine but is a less potent analgesic, it is usually used to suppress cough or diarrhoea and in combination with paracetamol and aspirin. Methadone and buprenorphine are given to treat opioid addicts and in cases of opioid overdose naloxone is employed as an antidote. Opioids such as fentanyl and sufentanil are also widely used during delivery to inhibit pain due to contractions. Lovarphenol can be prescribed as a substitute for morphine.

1.4.4 Opioid receptors

Opioids drugs mediate their action through DOR, MOR, and KOR, which belong to the class A GPCR family. For several year opioid pharmacology and physiological effects were reported, however, opioid receptor cDNAs were not cloned before 1992-3. DOR was the first cloned using an expression cloning library (Kieffer *et al.*, 1992; Evans *et al.*, 1992) followed by MOR and KOR. In 1994, NOR was cloned and initially characterised as an orphan receptor termed ORL1 for opioid receptor like-1. It was named ORL1 due to its high homology with the other opioid subtypes (60%) but displayed very low levels of binding to all known opioid ligands. However, a new endogenous peptide able to bind to NOR, was identified soon after cloning of this receptor and named nociceptin (Reinscheid *et al.*, 1995; Meunier *et al.*, 1995). This ligand appeared to be closely related to the KOR ligand dynorphin A so NOR is now recognised as belonging to the opioid receptor family.

Based on pharmacological investigations MOR (μ), DOR (δ) and KOR (κ) subtypes have been further subdivided (μ1, μ2, δ1, δ2, κ1, κ2, κ3) but so far no cDNAs for these subtypes have been cloned. Two subtypes of DOR have been proposed, δ1 which displays high affinity for DPDPE, DADLE and BNTX whereas DSLET, 5'-NTI and naltriben bind selectively to DOR sites designated as δ2 (Noble and Cox, 1995; Xu *et al.*, 1993). Similarly, for KOR the different pharmacologies observed have highlighted three potential binding sites κ1, 2 and 3. Whereas κ1 selectively binds dynorphin but not DADLE, κ2 binds DADLE and Met-enkephalin and κ3 sites are sensitive to naloxone or benzoylhydrazone (Akil and Watson, 1994). Subdivision of MOR into μ1 and μ2 was proposed by Wolozin and Pasternak, (1981)

based on radioligand binding studies. The authors observed two site binding curves when distinct [^{3}H]-radioligands were displaced by various opiates and enkephalins. However, when the same samples were treated with naloxazone the displacement of the [^{3}H]-radioligands by the different ligands appeared to be monophasic. Consequently, the high affinity sites observed were described as corresponding to μ1 sites whereas the low affinity sites detected were named μ2. As the cDNA corresponding to these subtypes have not yet been cloned other hypotheses have been proposed to explain the subdivision of MOR, DOR and KOR. These include different receptor splicing variants, receptor dimerisation and interactions with accessory proteins.

1.4.5 Opioid receptor localisation and function

Opioid receptors are 65% homologous (Reisine and Bell, 1993) with higher homology in the transmembrane domains and the intracellular loops. They are localised mainly in the brain and spinal cord. They have discrete but overlapping distributions. DOR is found in the nucleus accumbens and olfactory bulb where it is highly expressed and with a lower density in the amygdala, cerebral cortex, hypothalamus, thalamus and substantia nigra. Regions which have been described to be rich in MOR receptor are the spinal cord, hypothalamus and amygdala and, in more moderate density, the substantia nigra and the periaqueductal grey. Equally, these regions were shown to express a low density of KOR whereas substantia nigra and cortex display high levels of this receptor (Mansour *et al.*, 1994; Sim and Childers, 1997). All three receptors have also been found in various immune cells e.g. T and B lymphocytes and macrophages (Chuang *et al.*, 1995a and b; Belkowski *et al.*, 1995).
Opioid receptors are coupled predominantly to $G_{i/o}$ proteins and modulate adenylate cyclase activity as well as voltage operated calcium channels, potassium channels, phospholipase Cβ and MAPK. Moreover, opioid receptors have also been showed to modify the immune response to HIV (Peterson *et al.*, 1990; Sharp *et al.*, 2001)

1.4.6 Opioid receptor structure

All four opioid receptors possess two conserved cysteine residues in the first and second extracellular loops and the highly conserved DRY motif specific to the class A GPCR family.

These receptors can be glycosylated on asparagine residues in their N-terminal domains and palmitoylated in the C-terminal domain.

- Ligand binding

Based on biochemical analysis and computational modelling opioid receptors have been postulated to share a common binding pocket formed of TMIII, IV, V, VI and VII. This cavity is partially covered by the extracellular loops (less homologous regions of opioid receptors) which are proposed to be important for ligand interaction (Chavkin *et al.*, 2001). The opioid receptor binding pocket was described as consisting of an inner conserved region between the three opioid receptors and a less homologous peripheral region responsible for DOR, MOR and KOR ligand binding selectivity (Pogozheva *et al.*, 1998). Larger opioid ligands were reported to fill all the available space into the opioid binding pocket whereas smaller agonists such as morphine were reported to bind into the bottom of the cavity thus interacting predominantly with conserved residues. Similarly, small antagonists (e.g. naloxone) were suggested to bind deep into the binding pocket but slightly shifted compare to small agonists thus preventing TMIII and VII movement. This absence of movement is described as blocking the receptor in an inactive state therefore leading to functional antagonism (Pogozheva *et al.*, 1998). In addition to certain transmembrane residues opioid ligands selectivity have been attributed to the 1st and 3rd extracellular loop for MOR, the 2nd extracellular loop for KOR and the 3rd extracellular loop for DOR (Chavkin *et al.*,2001; Wang *et al.*,1995; Zhang *et al.*, 2002). However, MOR ligand binding affinity was reported to be only due to four aminoacids i.e. Asp 128, Asn 150, Lys 103 and Trp 318 (Chavkin *et al.*, 2001). Key residues responsible for DOR ligand binding affinity include Arg 291, Arg 292, Trp 284, Val 296, Val 297 and Trp 284 which belong to TMVI and the 3rd extracellular loop (Quock *et al.*, 1999). For KOR, Asp 138 which belongs to TMIII was described as a key anchoring point for agonist association. Equally, TMVI residues such as His 291, Glu 297 Ile 294, Leu 295, and Ala 298 were reported to be important for KOR ligand binding affinity (Subramanian *et al.*, 1998; Stevens *et al.*, 2000).

- G protein activation

As a general mechanism for GPCRs-mediated G protein activation, ligand binding was proposed to induce transmembrane domain movements and lead to the exposure of key residues from the 2nd and 3rd intracellular loops. Residues from the 3rd intracellular loop were proposed to play a key role in G protein coupling whereas amino acids from the 2nd intracellular loop seem to be involved in G protein activation efficacy. More specifically for DOR and MOR, Georgoussi et al, (1997) provided evidence using a series of peptides mimicking the different opioid receptor's intracellular loops that the 3rd intracellular loop and the C-terminal tail were playing a role in G protein activation. Recently, a study based on receptor random mutagenesis provided a ligand-induced activation scheme for DOR (Decaillot *et al.*, 2003). The authors proposed that an opioid agonist would bind to extracellular loop 3 and possibly to the N-terminal of DOR thus destabilising TMVI and TMVII on their extracellular part. This shift would provide the opportunity for the ligand to enter into the binding pocket and produce TMIII, VI and VII movement. Consequent to the TM displacements the cytoplasmic ionic locks would break therefore providing an intracellular anchorage for G protein activation.

1.4.7 Knock out (KO) mice

Genetically modified mice are interesting tools to understand the exact role of each opioid receptor in response to drugs or in nociception. Therefore to complement biochemical and biophysical data, opioid receptor null mutant mice for the three opioid receptor have been generated. Studies of these mice have clarified or revealed roles for MOR, DOR and KOR. To summarise the major results obtained, it was confirmed that MOR is the primary target of morphine. Essentially all responses to morphine, including analgesia and the major side effects (reward, dependence, constipation, respiratory depression) were abolished in mice not expressing MOR (Matthes *et al.*, 1996; Sora *et al.*, 1997). In the absence of DOR expression morphine tolerance did not develop and analgesia was reduced (Zhu *et al.*, 1999). An anti-depressant effect mediated by DOR was also highlighted (Baamonde *et al.*, 1992). Furthermore DOR analgesia appeared to be compromised in MOR ko mice suggesting crosstalk between MOR and DOR (Fuchs *et al.*, 1999; Matthes *et al.*, 1998). KORs' role in visceral chemical pain analgesia and involvement in dysphoria were confirmed by using KOR deficient animals (Simonin *et al.*, 1998). KOR participation in adaptation to long term exposure to morphine was also suggested but no contribution to morphine analgesia and

reward was observed. Additionally, these genetically modified mice as well as a triple ko mouse for all three opioid receptors permitted deduction that the pharmacological sites attributed to further subdivision of the three main opioid receptors (i.e. μ1, μ2, δ1, δ2, κ1, κ2, κ3) are actually encoded by the three cloned opioid genes (Kitchen *et al.*, 1997; Zhu *et al.*, 1999; Simonin *et al.*, 1998; Simonin *et al* 2001). Genetically modified animals are good tools to complement biochemical and biophysical studies to help to further understand the roles mediated by opioid receptors.

1.5 Opioid receptor dimerisation

1.5.1 Homo and Heterodimerisation

All three opioid receptors have been described to form homodimers (Jordan and Devi, 1999; George *et al.*, 2000). Cvejic and Devi, 1997 first demonstrated using co-immunoprecipitation that DOR dimerise. They also reported that its dimerisation was ligand modulated as the number of DOR dimers visualised by Western-blot decreased upon treatment with DOR selective agonists (DADLE, DPDPE) but not with MOR selective agonists (DAMGO and morphine) thus demonstrating the specificity of the dissociation observed. However, different results were observed using FRET and BRET techniques (McVey *et al.*, 2001). The authors reported that no change in FRET or BRET signal was noticed when cells expressing DOR were treated with agonist or inverse agonist ligands and concluded that DOR was a constitutive dimer and that dimerisation was not influenced by the presence of ligands. DOR was also reported to form dimers with the other opioid receptor subtypes KOR and MOR (Jordan and Devi, 1999; George *et al.*, 2000) as well as with less related receptors such as β_2- and the α_{2a}-adrenoceptor (Jordan *et al.*, 2000; Rios *et al.*, 2004). KOR and MOR were first demonstrated as not interacted by co-immunoprecipitation and no dimerisation interface was identified by a computational study, therefore it was concluded that they did not interact (Jordan and Devi, 1999; Filizola *et al.*, 2002b). However, in a recent study these two subtypes were showed as interacting using BRET^2 approach (Wang *et al.*, 2005). KOR was also documented as interacting with less homologous receptors such as the β_2-adrenoceptor and the TRH receptor but its affinity to dimerise with these receptors was lower compared to the KOR homodimer or a KOR/DOR heterodimer (Ramsay *et al.*, 2002). Likewise, heterodimerisation between MOR and the somatostatin SSTr2A was described using co-immunoprecipitation (Pfeiffer *et al.*, 2002) as well as with the α_{2a}-adrenoceptor (Jordan *et al.*, 2003). All three opioid receptor subtypes have also been described as interacting with CCR5 (Suzuki *et al.*, 2002; Chen *et al.*, 2004).

1.5.2 Domains involved in opioid receptor dimerisation

Various domains have been described to be involved in opioid dimerisation, namely C-terminal and transmembrane domains as well as disulphide bridges. The truncation of the DOR C-terminal tail was demonstrated to inhibit DOR homodimerisaton (Cvejic and Devi., 1997) suggesting its involvement in DOR homodimer formation. For the KOR homodimer as well as a DOR/KOR heterodimer Jordan and Devi, (1999) suggested that disulphide bridges were taking part in dimer formation as the immunoprecipitated homo and heterodimers were sensitive to reducing agents. Computational analysis based on the rhodopsin crystal structure was also utilized to try to identify putative transmembrane domains involved in opioid dimerisation. Firstly, for opioid homodimerisation, TMIV and TMV were predicted as being the contact interfaces of the DOR homodimer, for the KOR homodimer only TMV was identified as the domain most likely involved in dimerisation and TMI and TMIII were suggested to participate in MOR homodimer interactions (Filizola and Weinstein, 2002). Secondly, for opioid heterodimers the most likely interfaces were proposed to implicate TMIV, TMV and TMVI of DOR with TMI of MOR (Filizola *et al.*, 2002). However, when the procedure was applied to determine MOR and KOR domains involved in heterodimerisation no TM was predicted. Recently, interactions between G protein and the receptors was suggested to play a role in MOR/DOR dimerisation as in cells co-expressing MOR and DOR treatment with Pertussis toxin (PTX) decreased the number of heterodimers observed (Law *et al.*, 2005).

1.5.3 Pharmacology of heterodimers involving opioid receptor

A large number of studies have reported changes in pharmacological properties associated with formation of opioid receptor heterodimers. Moreover, the pharmacological profiles that have not been explained by the cloning of DOR, MOR and KOR cDNAs and for which different opioid subtypes ($\mu1$, $\mu2$, $\delta1$, $\delta2$, $\kappa1$, $\kappa2$, $\kappa3$) have been postulated might be explained by the original pharmacology representing MOR/DOR or DOR/KOR heterodimerisation. Nonetheless, no significant change in pharmacology was yet reported when opioid receptors heterodimerise with less homologous receptors.

- DOR/KOR heterodimer

The KOR/DOR heterodimer was one of the first for which a change in pharmacology was described (Jordan and Devi, 1999). The authors reported that KOR and DOR selective agonists and antagonists showed no significant affinity for the KOR/DOR heterodimer whereas partially selective ligands such as naloxone, diprenorphine or ethylketocyclazocine displayed a higher affinity for the co-expressed receptors. The authors suggested that this pharmacology was identical to the one reported for the κ2 receptor subtype. Similar suggestion by Bhushan et al., (2004) was based on using a bivalent ligand strategy to target a KOR/DOR heterodimer. The authors observed a pharmacology comparable to that suggested for κ2 sites when their bivalent ligand was tested on cells co-expressing DOR and KOR but not on mixed cells expressing either DOR or KOR. This study also provided evidence for possible development of drugs specifically targeting heterodimers. Jordan and Devi, (1999) observed a synergistic ligand binding when combinations of KOR and DOR ligands (e.g. DPDPE and U69593) were tested on cells co-expressing KOR and DOR. Taken together these results suggest the formation of a new ligand binding site upon KOR/DOR heterodimersation, which could account for the unexplained pharmacological data attributed to the κ2 subtype.

- MOR/DOR heterodimer

Similarly, MOR/DOR heterodimerisation may result in the generation of a novel binding site with distinct pharmacology than that of either individual receptor expressed alone. Several groups have explored these new properties. George et al., (2000) observed a reduced affinity for synthetic agonists such as DADLE, DPDPE or DAMGO whereas endogenous opioid ligands endomorphin 1 and Leu-enkephalin exerted an enhanced affinity for the heterodimer. Additionally, a synergistic ligand binding was reported by Martin and Prather, (2001) as co-exposure of cells co-expressing DOR/MOR to DAMGO and DPDPE resulted in the synergistic displacement of ^{3}H-[DPDPE]. It was also described that occupation of DOR by ligands including deltorphine II, TIPP-ψ, naltriben or ICI 174 864 enhanced MOR agonist binding (Gomes *et al.*, 2003). In a recent report, in which the authors established a 1:1 MOR:DOR ratio, differences in ligand binding affinity between homodimer and heterodimer was confirmed (Law *et al.*, 2005). The authors monitored a decrease in high affinity binding for DAMGO, morphine and endomorphin 1 and an increase of 10 fold for endomorphin 2

whereas no difference in affinity for the tested antagonist was observed upon MOR/DOR co-expression. These results contrast with some of the previous reports, perhaps due to a difference in MOR:DOR ratio.

- **MOR/KOR heterodimer**

As MOR/KOR heterodimer was recently demonstrated as dimerising few data are available on the pharmacology of this homodimer. However, Wang et al, (2005) suggested a KOR binding profile for the MOR/KOR heterodimer.

1.5.4 Functional changes

- DOR/KOR heterodimer

A potential DOR/KOR heterodimer has been reported to exhibit new functional properties (Jordan and Devi, 1999). Adenylate cyclase inhibition was found to be increased by agonists when these two receptors were co-expressed. Furthermore, MAPK kinase phosphorylation was enhanced when a combination of selective agonists of each receptor was used. The trafficking properties of this dimer were also modified as etorphine, a non-selective agonist which provoke internalisation of DOR but not KOR, was unable to induce internalisation of DOR when co-expressed with KOR.

- MOR/DOR heterodimer

Functional changes upon MOR/DOR heterodimer formation have been widely investigated. Firstly, enhanced inhibition of adenylate cyclase activity was documented upon co-exposure to MOR and DOR agonists (Martin and Prather, 2001) as well as when morphine was co-administrated with the DOR antagonist TIPP-ψ (Gomes *et al.*, 2004). As morphine is a clinically relevant drug further studies were conducted to explore the physiological consequences of the observed enhanced activity upon co-exposure to morphine and TIPP-ψ. It was shown that morphine-induced analgesia was increased in the presence of TIPP-ψ therefore the MOR/DOR heterodimer might be used as a model to develop novel drug combinations to treat pain. Secondly, alteration in G protein binding was observed when

DOR/MOR heterodimer was formed. The authors demonstrated that when DOR and MOR were co-expressed a switch from PTX sensitive G_i to PTX insensitive G proteins was occurring (George *et al.*, 2000). Moreover, it was reported that the ability of MOR to inhibit Ca^{2+} release was altered when co-expressed with DOR (Charles *et al.*, 2003). Finally, it was observed that the internalisation pattern of the individual receptors was altered by heterodimer formation. DPDPE-induced internalisation was abolished when MOR was co-expressed with DOR. However, the results of Law et al., (2005) are mainly in opposition with the observations reported by the previous studies. They demonstrated that the ability of MOR agonists to inhibit adenylate cyclase activity was similar in the presence or absence of DOR. No changes in G protein coupling was observed, contrasting with George et al, (2000). DOR and MOR were also suggested as internalising as monomers, contrasting with He et al, (2002) who suggested that MOR receptors were internalising as homodimers.

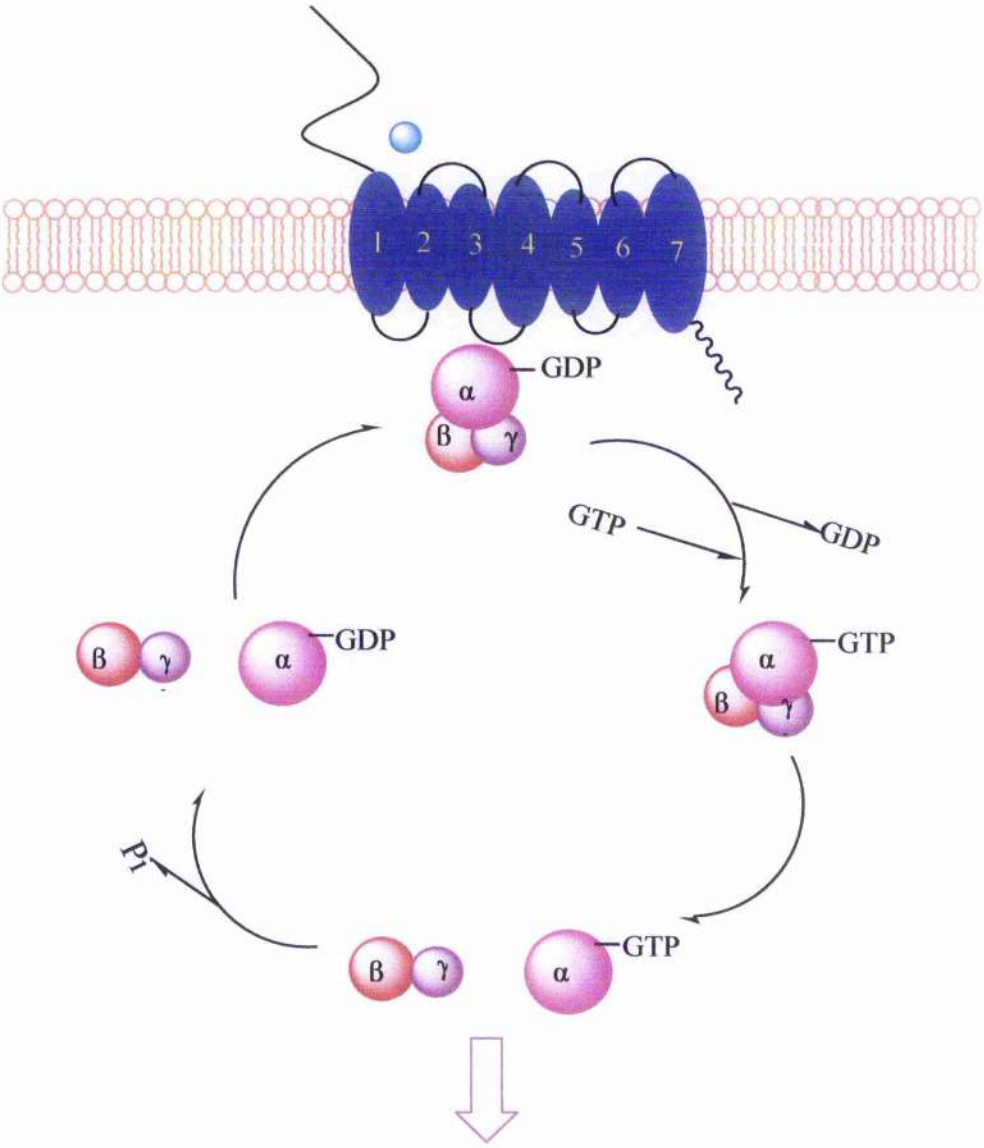
- **Opioid receptor heterodimerisation with other GPCRs**

Opioid receptor heterodimerisation with less related GPCRs has been reported to alter trafficking properties of the receptors. This is exemplified by the heterodimers that β_2-adrenoceptor form with KOR and DOR (Jordan et al., 2000). In cells co-expressing β_2-adrenoceptor and DOR, DOR was described as undergoing endocytosis when cells were treated with a β_2-adrenoceptor agonist. Conversely, the β_2-adrenoceptor in these cells was internalised upon DOR agonist treatment. By contrast, when the β_2-adrenoceptor was co-expressed with KOR, KOR abolished the ability of β_2-adrenoceptor to internalise (Jordan *et al.*, 2000). Trafficking alteration was also observed following SSTr2a and MOR dimerisation, as MOR was observed to undergo internalisation upon SSTr2a agonist exposure (Pfeiffer *et al.*, 2002). Additionally, MOR cross-phosphorylation and desensitisation was reported when the SSTr2a subunit of a SSTr2a/MOR heterodimer was activated and vice versa. Similarly, cross-desensitisation was documented for MOR/CCR5 interactions (Chen *et al.*, 2004). Study of DOR and α_{2c}-adrenoceptor interactions in Neuro 2A cells revealed neurite outgrowth enhancement of the cells when these receptors where co-expressed. The authors suggested that this phenomenon might account for DOR and α_{2c}-adrenoceptor synergy in spinal antinociception (Fairbanks *et al.*, 2002).

In conclusion, a certain amount of data have been collected on opioid receptor dimerisation. Some are complementary whereas others are in contradiction. However, new functional and pharmacological properties have definitively been identified. For these reasons, further studies need to be pursued for a better comprehension of the structure and role of opioid dimer formation. A better understanding of this phenomenon is important to try to improve design of novel compounds targeting those entities.

Figure 1.1: G protein cycle following GPCR activation

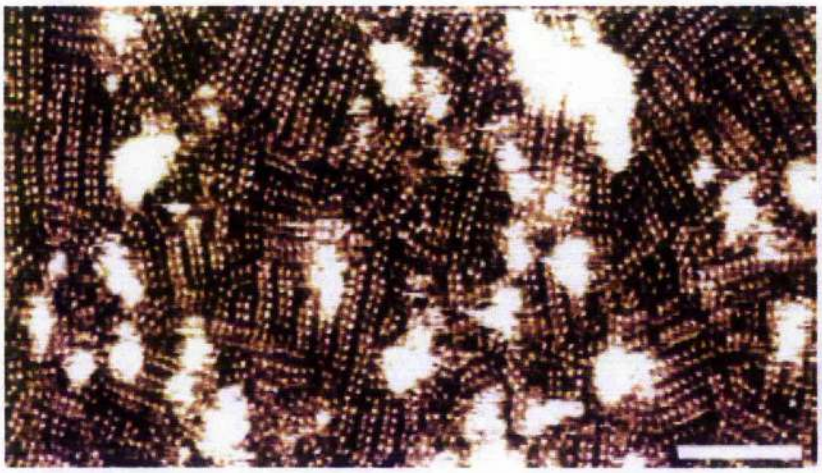
Binding of an agonist induce a conformational change in the receptor triggering its association with the heterotrimeric G protein ($\alpha\beta\gamma$). This association induces a conformational change within the G protein and induces the exchange of GDP for GTP followed by the dissociation of the G protein into G_{α} and $G_{\gamma\beta}$ subunits. These two complexes are then able to activate their effectors. GTP is rapidly hydrolyzed to GDP which leads to the reassembly of the inactive heterotrimer.



Effector activation

Figure 1.2: Rhodopsin organisation in native disc membranes

The organisation of rhodopsin in dimer rows visualised by atomic-force microscopy in mouse disc membranes. From Fotiadis et al., (2003)



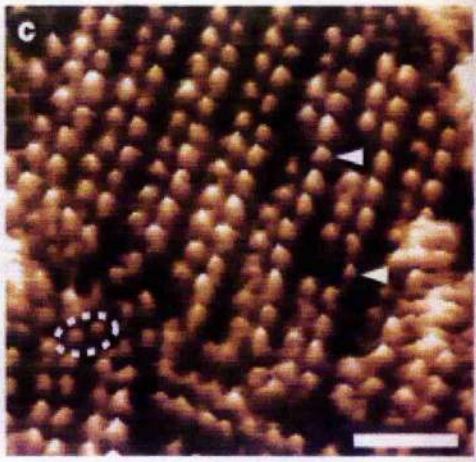
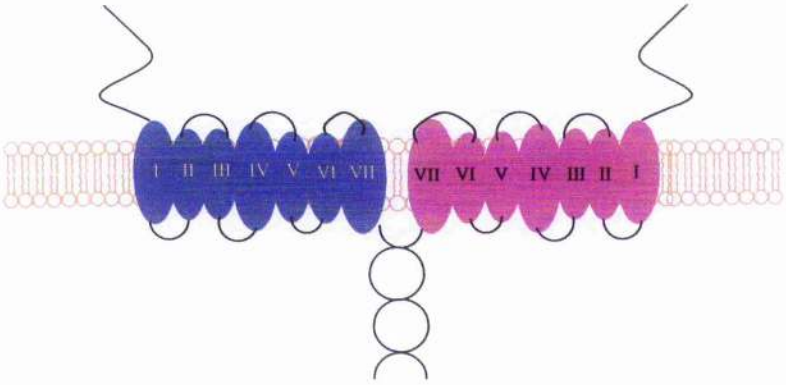

c

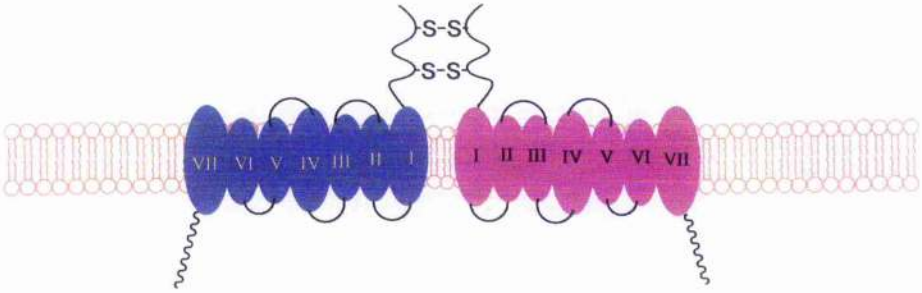
Figure 1.3: Interactions between GPCR monomers

Various domains have been implicated in GPCR dimer formation A. the C-terminal tail via coiled-coil interaction B. by formation of disulphide bridges between two cysteine residues and C. through transmembrane domains.
Adapted from Bouvier (2001)

A


I II III IV V VI VII
VII VI V IV III II I

B


S-S
S-S
VII VI V IV III II I
I II III IV V VI VII

C

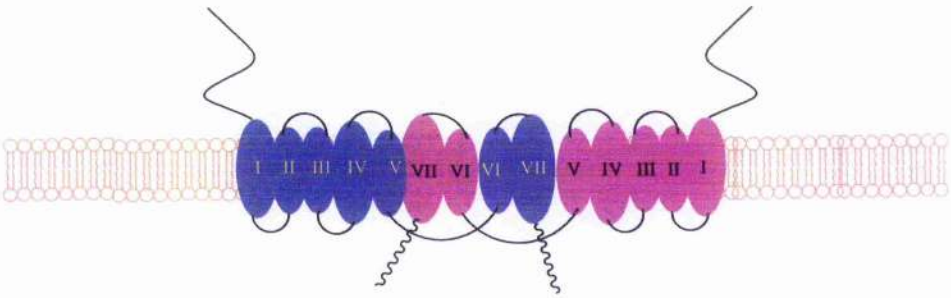
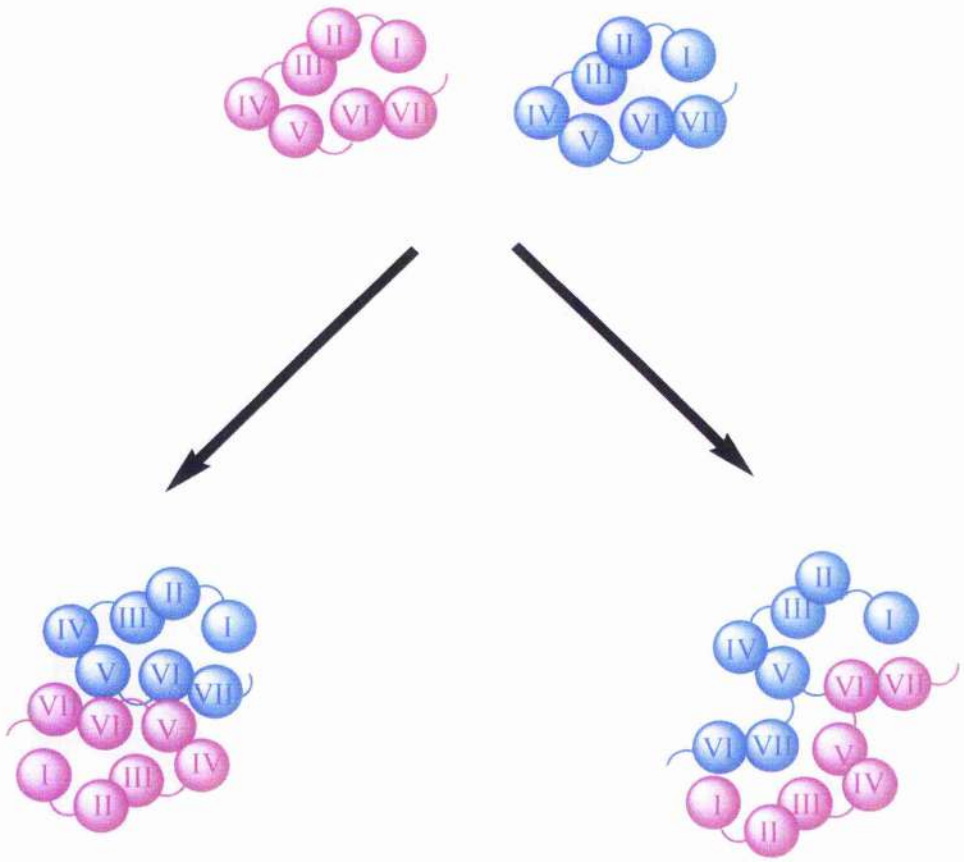

I II III IV V
VII VI VI VII
V IV III II I

Figure 1.4: Contact and domain-swapped dimers

Contact dimers involved two GPCR monomers which are in contact via transmembrane helices. In the example shown this is predominantly TMV and VI. In domain-swapped dimers, these domains are exchanged between the receptor monomers. Adapted from Breitwieser (2004).



contact dimer

swapped dimer

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 General reagents

BDH, Lutterworth, Leicestershire, UK

Sodium di-hydrogen orthophosphate, ethanol, methanol, isopropanol, di-sodium-hydrogen orthophosphate, urea

Calbiochem, CN Biosciences UK, Nottingham, UK

Pansorbin®Cells, $G_{i1\alpha}$ subunit myristoylated rat recombinant, NP40

Duchefa, Haarlem, The Netherlands

Yeast extract, tryptone, agar

Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK

Calcium chloride, glycine, HEPES, sucrose, SDS, HCl, potassium acetate, potassium dihydrogen orthophosphate, sodium hydrogen carbonate, Tris

Interactiva Thermo Hybaid, Ulm, Germany

Oligonucleotides for PCR reactions

Invitrogen BV, Groningen, The Netherlands

NuPage® Novex pre-cast bis-tris gels, Xcell Surelock™ mini-cell tank, Xcell II™ blot module, MES running buffer, MOPS running buffer

Kodak, Kodak Industrie France

MXB X-ray film

Pierce, Perbio Science UK Ltd, Tattenhall, Cheshire, UK

Supersignal® west pico chemiluminescent substrate

Promega UK Ltd., Southampton, UK

Restriction endonucleases, Pfu Polymerase, T4 DNA ligase, calf intestinal alkaline phosphatase, DNA purification kit Wizard™, Plus SV Minipreps and Wizard™ Plus SV Maxipreps systems

Qiagen, Crawley West Sussex, UK

QIAquick gel extraction kit, Maxiprep kit

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Complete mini-protease inhibitor cocktail tablets, 1kb DNA ladder, bovine serum albumin

SIGMA-Aldrich Company Ltd., Poole, Dorset, UK

Agarose, magnesium chloride, sodium chloride, sodium hydroxide, DTT, EDTA, bromophenol blue, deoxycholic acid, Triton X-100, DMSO, ethylene glycol, glycerol, MES, Tween 20, ampicillin, Protein G-sepharose, ethidium bromide, Ponceau S, manganese chloride, sodium hydroxide, polyethylenimine, rubidium chloride, GTPγS, GDP, GppNHp

Stratagene, La Jolla, CA, USA

Quikchange®site directed mutagenesis kit

2.1.2 Tissue culture plastic ware and reagents

American Tissue Culture Collection, Rockville, MD, USA

HEK 293 cells

Bibby Sterelin Ltd., Sone, Staffordshire, UK

15ml and 50ml centrifuge tubes

Costar, Cambridge, MA USA

5ml, 10ml and 25 ml pipettes, 75cm^2 tissue culture flasks, 100mm dishes, cell scrapers

Gibco BRL, Life technologies Ltd., Paisley, UK

OPTIMEM-1, L-glutamine (200mM), NBCS

Novagen, EMD Biosciences Inc., Darmstadt, Germany

Gene juice transfection reagent

SIGMA-Aldrich Company Ltd., Poole, Dorset, UK

Pertussis toxin, DMEM, Trypsin-EDTA

2.1.3 Radiochemicals

Amersham Pharmacia Biotech UK limited, Buckinghamshire, England

l-[*propyl*-2,3-^{3}H] Dihydroalprenolol 61Ci/mmol

PerkinElmer life science, Inc.Boston, MA, USA

Diprenorphine [15,16-^{3}H] 50 Ci/mmol

Guanosine 5'-(γ-thio) triphosphate, [^{35}S] 1250 mCi/mmol

2.1.4 Ligands

SIGMA-Aldrich Company Ltd., Poole, Dorset, UK

DADLE, DAMGO, DPDPE, Naloxone, Isoproterenol, Propanolol

TOCRIS

ICI 174 864, CTOP, U69593

2.1.5 Antisera

Anti-Flag antibody M2

Mouse monoclonal antibody that binds N-terminal Flag protein

Purchased from SIGMA-Aldrich Company Ltd., Poole, Dorset, UK

Anti-Myc antiserum

Rabbit polyclonal antiserum which detects Myc tagged proteins

Purchased from Cell Signalling Technology, NEB, UK

Anti-GFP serum

Sheep antiserum raised against GFP, produced by the Scottish Antibody Production Unit, Lanarkshire, UK

Anti-mouse IgG

Goat polyclonal antiserum conjugated with horseradish peroxidase, purchased from Amersham Pharmacia Biotech, Buckinghamshire, UK

Anti-rabbit IgG

Donkey polyclonal antiserum conjugated with horseradish peroxidase, purchased from Amersham Pharmacia Biotech, Buckinghamshire, UK

Anti-sheep IgG

Donkey polyclonal antiserum conjugated with horseradish peroxidase, purchased from Jackson Immunoresearch, PA., USA

Anti-$G_{\alpha i1-2}$ SG3 #13

From sheep, in house stock

2.2 Methods:

Standard Buffers:

Tris EDTA (TE) (1X)

Tris/HCL 10mM

EDTA 0.1mM

pH adjusted to 7.5

This was made for membrane preparation and protein quantification

Phosphate Buffered Saline (PBS) (1×)

Na_2HPO_4 8.1mM

K_2HPO_4 1.5mM

NaCl 140mM

KCl 2.7mM

pH adjust to 7.4

2.2.1 Molecular Biology

Manipulations were performed with materials that had been autoclaved. Gloves were worn to prevent contamination.

A/ Polymerase Chain Reaction (PCR)

PCR was performed with sterile materials. This technique used a modified polymerase enzyme which is resistant to higher temperatures named Pfu polymerase. It permits amplification of specific parts of DNA. It was used to introduce point mutations, tags and new restriction sites in DNA constructs.

PCR reaction mix:

Pfu polymerase buffer (10×)	5µl
DMSO	5µl

Deoxynucleotide tri-phosphates dNTP	1µl
(0.2mM of dATP, dCTP, dTTP, dGTP)	
Primer sense: 25 pmol/µl	1µl
Primer antisense : 25 pmol/µl	1µl
DNA template 50 ng/µl	1µl
Pfu enzyme	1µl
dH_20 to a final volume of	50µl

PCR cycles:

1/Preheating	95°C 5min
2/Denaturation	95°C 1min
3/Annealing	50-60°C (depending on the primer Tm) 1min
4/Extension	72°C 3min
Repeat from step 2/	29 ×
5/End	72°C 10min
6/Hold	4°C

All reactions were performed on a Thermocycler (Mastercycler eppendorf). The annealing temperatures were determined depending on the Tm of the primers used for each PCR.

B/ Introduction of point mutations (QuikChange site-directed point mutagenesis kit)

Point mutations were introduced using QuikChange kit (Stratagene) according to the manufacturers instructions:

Design of primers to introduce point mutations:

Each primer was designed depending on the desired mutation to be introduced. Primers had a length of between 25 to 45 bases, a melting temperature greater than or equal to 78°C and a minimum GC content of 40%.

Reaction mix:

Reaction buffer 10×	5µl
Deoxynucleotide tri-phosphates dNTP	1µl
(0.2mM of dATP, dCTP, dTTP, dGTP)	

Primer sense:	125 ng/µl	1µl
Primer antisense :	125 ng/µl	1µl
DNA template 50ng/µl		1µl
PfuTurbo enzyme (2.5U/µl)		1µl
dH_20 to a final volume of		50µl

PCR program:

The following PCR program was used to introduce mutations.

1/Preheating	95°C	30 sec
2/Denaturation	95°C	30 sec
3/Annealing	55°C	1min
4/Extension	68°C	1min/kb of plasmid length
Repeat from step 2/ 18 cycles		
5/End	72°C	10min
6/Hold	4°C	

Digestion by *DpnI*:

Amplified DNA and a diluted sample of parental DNA (negative control) were digested with 1µl of *Dpn*I restriction enzyme (10U/µl) for 1h at 37°C. This allowed the digestion of the parental (i.e. the non mutated) super coiled dsDNA.

Transformation:

The digested mix and negative control were transformed into XL1-Blue supercompetent cells following the protocol described later in section 2.2.2. No colonies are expected on the negative control plate.

C/ DNA constructions

DOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$

DOR-$Gi_{1\alpha}C^{351}I$ cDNA cloned in pcDNA3.1 was previously generated in the laboratory (Moon *et al.*, 2001) and was used as a template to introduce mutations in the 2nd intracellular loop of the receptor, using 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'

The initial valines (GTC) were mutated into glutamate (GAG) and aspartate (GAC) using QuikChange kit (Stratagene). Bold letters indicate bases mutated. The cDNA was amplified by PCR using the primers containing the mutations and using a specific PCR program as described previously in section 2.2.1B. The PCR product was then digested with *Dpn*I and transformed into bacteria.

DOR-$G_{i1\alpha}$**$G^{202}A$**,$C^{351}I$

DOR-$G_{i1\alpha}C^{351}I$ cDNA was used as a template to introduce the mutation in $G_{i1\alpha}$ 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 initial glycine (GGC) was mutated into alanine (GCC) using QuikChange kit (Stratagene). The cDNA was amplified by PCR. The PCR product was then digested by *Dpn*1 and was transformed into bacteria.

Flag DORV^{150}E,V^{154}D-$Gi_{1\alpha}C^{351}I$

FlagDORV^{150}E,V^{154}D-$Gi_{1\alpha}C^{351}I$ 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'

DORV^{150}EV^{154}D was amplified between these primers The sense primer contained a flag sequence (in italics) and an *Nhe*I restriction site (underlined) and corresponds to the N-terminal region of DOR. The antisense primer, contained a *BamH*I site (underlined) and corresponds to the C-terminal region of DOR. PCR product and pcDNA3.1 containing $Gi_{1\alpha}C^{351}I$ were digested by *Nhe*I and *BamH*I. The digested products were then ligated.

Myc DOR-$Gi_{1\alpha}G^{202}A,C^{351}I$

MycDOR-$Gi_{1\alpha}G^{202}AC^{351}I$ was constructed using the following primers:

Sense primer

5'-CCC TTT GCT AGC ATG *GAA CAA AAA 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'

DOR was amplified between these primers. The sense primer contained myc sequenced (in italics) and *Nhe*I restriction site (underlined) and corresponds to the N-terminal region of DOR. The antisense primer, contained *BamH*I site (underlined) and corresponds to the C-terminal region of DOR. PCR product and pcDNA3.1 vector containing $Gi_{1\alpha}G^{202}A,C^{351}I$ were digested by *Nhe*I and *BamH*I. The digested products were then ligated.

MOR$V^{169}EV^{173}D$-$G_{i1\alpha}C^{351}I$

MOR-$G_{i1\alpha}C^{351}I$ cDNA cloned in pcDNA3 was previously generated in the laboratory (Massote *et al.*, 2002) and was used as a template to introduce mutations in the 2nd intracellular loop of the receptor using the following primers:

Sense primer:

5'-GAT CGA TAC ATT GCA GA**G** TGC CAC CCT GAC AAG GCC TTA GAT TTC-3'

Antisense primer:

5'-GAA ATC TAA GGC CTT GTC AGG GTG GCA **CT**C TGC AAT GTA TCG ATC-3'

The initial valines (GTC) were mutated into glutamate (GAG) and aspartate (GAC) using QuikChange kit (Stratagene). Bases mutated are in bold. The cDNA was amplified by PCR using primers containing the mutations and a specific PCR program. The PCR product was then digested by *Dpn*I and was transformed into bacteria.

MOR-$G_{i1\alpha}G^{202}A,C^{351}I$

MOR-$G_{i1\alpha}C^{351}I$ cDNA was used as a template to introduce the mutation in $G_{i1\alpha}$ 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 initial glycine (GGC) was mutated into alanine (GCC) using QuikChange kit (Stratagene). The cDNA was amplified by PCR. The PCR product was then digested by *Dpn*I and was transformed into bacteria.

KOR-$G_{i1\alpha}C^{351}I$

KOR-$G_{i1\alpha}C^{351}I$ 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'

ratKOR was amplified between these primers corresponding to KOR N and C-termini and containing *Hind*III and *Kpn*I restriction sites (underlined). The PCR products and pcDNA3 vector containing $G_{i1\alpha}$ were digested by the above enzymes. As the rKOR contained an internal *Hind*III site, a two-way ligation was used to ligate the vector and the 2 pieces of digested PCR products.

KOR$\mathbf{V^{160}E,V^{164}D}$-$Gi_{1\alpha}C^{351}I$

KOR$G_{i1\alpha}C^{351}I$ cDNA cloned in pcDNA3.1 was used as a template to introduce mutations in the 2nd intracellular loop of the receptor, using the following primers:

Sense primer

5'-GAC CGC TAC ATT GCC G**A**G TGC CAC CCT G**A**C AAA GCT TTG GAT TTC-3'

Antisense primer

5'-GAA ATC CAA AGC TTT GTC AGG GTG GCA **C**TC GGC AAT GTA GCG GTC-3'

The initial valines (GTC) were mutated into glutamate (GAG) and aspartate (GAC) using QuikChange kit (Stratagene). Bases mutated are in bold. The cDNA was amplified by PCR using the primers containing the mutations and using a specific PCR program. The PCR product was digested by *Dpn*1 and was transformed into bacteria.

KOR-$Gi_{1\alpha}\mathbf{G^{202}A},C^{351}I$

KOR-$G_{i1\alpha}C^{351}I$ cDNA was used as a template to introduce the mutation in $G_{i1\alpha}$ 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 initial glycine (GGC) was mutated into alanine (GCC) using QuikChange kit (Stratagene). Bases mutated are in bold. The cDNA was amplified by PCR. The PCR product was then digested by *Dpn*I and was transformed into bacteria.

β_2-adrenoreceptor-$G_{i1\alpha}$**C^{351}I**

β_2-adrenoreceptor-$G_{i1\alpha}$ cDNA previously generated in the laboratory (Feng *et al.*, unpublished) was used as a template to introduce the mutation in $G_{i1\alpha}$ using the following primers:

Sense primer

5'-GGG ATA TCT TAG AAT GGG CTG CAC ACT GAG C-3'

Antisense primer

5'-GCC ATT CTC GAG TTA GAA GAG ACC GAT GTC TTT TAG GTT-3'

The $C^{351}I$ mutation was introduced by amplifying $G_{i1\alpha}$-$C^{351}I$. The sense primer corresponding to the beginning of the G protein sequence and containing an *Xba*I restriction site (underlined) was used as well as an antisense primer containing the $C^{351}I$ mutation, *Xho*I site (underlined) and corresponding to the end of the G protein. The PCR product and vector were digested. The initial $G_{i1\alpha}$ contained in β_2-adrenoreceptor-$G_{i1\alpha}$ cDNA was digested and extracted from the DNA plasmid. Digested PCR product and plasmid containing β_2-adrenoreceptor were ligated.

Flag-TM1-$G_{i1\alpha}C^{351}I$

Flag-TM1-$G_{i1\alpha}$ $C^{351}I$ 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-DOR-$Gi_{1\alpha}C^{351}I$ 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

digestion was used on the template, *Nhe*I being situated at the end of the receptor sequence. PCR products and vector were ligated.

D/ DNA gel electrophoresis

Agarose gels were run in order to check for the presence of PCR products, for gel extraction, or before a ligation.

Buffers:

Tris acetate EDTA Buffer (TAE) (1X)

Tris-acetate	40mM
EDTA	1mM

pH adjusted to 8

Used to prepare DNA agarose gels and used also as running buffer.

Prepared as a 50X stock solution (242g Tris/HCl, 57.1ml of glacial acetic and 100ml of 10mM EDTA) and was diluted when required to 1X.

Gel loading Buffer 6×

Bromophenol Blue (2%)	1.25ml
Sucrose	4g
dH_2O	10ml

This loading buffer was mixed with the DNA samples to make a final 1X concentration.

Agarose gel

35 ml of 1× TAE was added to 0.35g of agarose for a 1% gel. This percentage depended on the size of the DNA fragment required to be visualized (0.8 to 2% gel). The mix was heated for 1 min in a microwave and 3.5µl of Ethidium Bromide (2.5 mg/ml) was added in order to visualise the DNA under UV light. The gel was poured in the appropriate gel tank of the electrophoresis kit (Life technologies, Gibco, Horizon 58 Model). After solidification the gel was immersed in TAE (1X) and the DNA samples previously mixed with gel loading buffer were loaded. A marker solution (1kb ladder) was also loaded in order to determine the sizes of the band(s) visualized. The samples were run for 20 to 40 min under a voltage of 50 to

100V. The DNA was visualised under UV light using a BioRad Gel Doc 2000. The size of each DNA fragment was evaluated by comparison with the markers.

E/ DNA digestion by restriction endonucleases

cDNAs were digested by restriction enzymes to carry out the subcloning of DNA fragments into plasmids. The digestions were set up as follows:

10X restriction enzyme buffer	2µl
DNA (1µg/µl)	1µl
Enzymes (1unit/µl)	2µl
dH2O up to	20µl

This mix was incubated between 2h and 24h at a temperature dictated by the restriction enzyme used.

F/ Alkaline phosphatase treatment

Digested plasmid was treated with alkaline phosphatase to dephosphorylate the vector ends. This treatment prevents the plasmid religating.

Digested DNA	20µl
Phosphatase Buffer 10X	5µl
Phosphatase alkaline (1U/µl)	5µl
dH2O up to	50µl

This mix was incubated at 37°C for 30 min to 1h. The enzyme was then inactivated by heating the sample for 15 min at 65°C.

G/ Gel extraction (Quiagen QIAquick Kit)

Purification of DNA from agarose gels was performed using Qiaquick Gel Extraction Purification Kit (Quiagen, West Sussex, UK). After running the agarose gel a DNA fragment was excised with a razor blade under a UV lamp and transfered to an Eppendorf tube. The gel slice was weighed and 3 volumes of buffer QG added. The samples were incubated at 50°C for 10 min to dissolve the gel. One volume of isopropanol was added for DNA pieces smaller than 500 bp or bigger than 4kb. The mix was transferred to a DNA binding column and centrifuged for 1min at 13000xg. The column was washed by centrifugation with 750 µl

buffer PE. The column was placed in a clean Eppendorf tube and DNA eluted with 30µl of sterilised water by centrifugation. The eluated DNA was stored at –20°C.

II/ Ligation

Digested cDNA and plasmid were ligated using T4 DNA ligase (Promega). Prior to ligation plasmid and insert quantity were assessed by agarose gel and a plasmid:insert ratio of 1:2 was used for each ligation.

10X T4 DNA buffer	1µl
T4 DNA Ligase (1unit/µl)	1µl
Plasmid	1µl
Insert	2µl
dH2O up to	10µl

This mix was incubated for 4h at room temperature or overnight at 4°C.

2.2.2 Preparation of competent bacteria, transformation, DNA purification

A/ General Materials

Luria Bertani (LB) broth

Trypton	10g
Yeast Extract	5g
NaCl	10g
dH_2O up to	1 l

The solution was sterilised by autoclaving.

LB Ampicillin agar plates

This solution was the same as above but supplemented with agar (1.5% LB).

The solution was autoclaved and left to cool down to 55°C before adding ampicillin to a final concentration of 50µg/ml. 25ml of liquid LB-agar was poured into 10cm dishes and allowed to solidify at room temperature. Plates were stored at 4°C.

B/ Preparation of competent bacteria DH5α

Buffer 1:

Potassium acetate 1M	3ml
RbCl 1M	10ml
$CaCl_2$ 1M	1ml
$MnCl_2$ 1M	5ml
Glycerol (80%)	18.75ml
dH_2O to final volume	100ml

pH adjusted to 5.8 with acetic acid and sterilised.

Buffer 2:

MOPS 100mM, pH 6.5	4ml
$CaCl_2$ 1M	3ml
RbCl 1M	0.4ml
Glycerol (80%)	7.5ml
dH_2O to final volume	40ml

pH adjusted to 6.5 with HCl and sterilised.

A conical flask containing 100ml of LB was innoculated with a 5ml culture of DH5α and allowed to grow at 37°C in a shaker for 90min or until an optical density of 0.48 at 550nm was reached. The culture was then chilled on ice. It was centrifuged at 3000xg for 10 min at 4°C. Each pellet was resuspended in 20ml of buffer 1, chilled on ice and centrifuged as before. They were resuspended in 2ml of buffer 2 and chilled on ice for 15 min. 220 µl aliquots were stored at –80°C for future transformation.

C/ Transformation of DNA

Transformation is the introduction of DNA into bacteria. It allows production of a high amount of DNA by replication of bacteria (such as the DH5α strain of *E.coli*).

This technique was used in order to amplify DNA produced by molecular biology or to make a large amount of a specific DNA construct.

Protocol

1µl of pure DNA or 5 µl for ligated DNA were incubated with 50µl of competent bacteria for 15 min on ice. The mix was heat shocked at 42°C for 45s and returned on ice for 2 min. 450 µl of LB was then added. The samples were allowed to recover by incubation for 45 min at 37°C in a shaking incubator.

200 µl of the mix was spread out on a LB agar ampicillin plate. They were incubated overnight in the incubator at 37°C. The next day random colonies were picked from the plate and cultured in 5ml LB with ampicillin overnight. Glycerol stocks were made of the different DNAs and kept at -80°C.

D/ DNA Purification

Miniprep (Promega Wizard Plus SV Miniprep)

The DNA contained in bacteria from the above cultures were purified with Promega Wizard Miniprep kit according to the manufacturers' instructions:

A 5ml culture was spun down and the cell pellet was resuspended in resuspension buffer. The pellet was lysed using the lysis buffer for 5 min at room temperature. Neutralising solution was added to precipitate the DNA and the sample was spun for 10 min at 13000g. The supernatant was transferred into a DNA purification column. The column was washed twice with wash buffer and the DNA was eluted using sterile water.

Maxiprep (Quiafilter Quiagen Plasmid Purification Kit)

5ml bacterial culture containing the desired DNA was poured into 100ml LB medium containing the appropriate antibiotic and grown overnight at 37°C. Bacterial cells were harvested the next morning by centrifugation at 6000xg for 15 min at 4°C. The bacterial pellet was resuspended in 10ml buffer P1, lysed by addition of 10ml buffer P2 and incubated for 5min at room temperature. The DNA was precipitated by adding 10ml of buffer P3 poured directly into the barrel of the Quiafilter cartridge and incubated for 10 min. The filter cell lysate was transferred into DNA extraction columns. They were washed twice, DNA was eluted and then precipitated by adding 10.5ml of isopropanol. DNA was pelleted by centrifugation at 15000xg for 30min at 4°C. DNA pellet was washed with 5ml of 70% ethanol and recentrifuged at 15000xg for 10min. The pellet was resuspended into sterile water and the absorbance was measured.

E/ Measure of DNA concentration

The concentration of plasmid DNA was determined by UV spectrophotometry at 260nm, using a UV-1201 UV-VIS spectrophotometer (Shimadzu). A 200x dilution was performed and the absorbance read. The OD was transformed into a concentration in μg/μl knowing that an OD of 1 at 260nm corresponds to 50μg/μl of double stranded DNA. For 200x diluted samples, an OD of 0.01 corresponded to 0.1μg/μl.
The absorbance at 280 nm was also measured in order to assess the purity of the DNA. A DNA solution with an A_{260}/A_{280} ratio between 1.7 and 2.0 was considered pure enough for use.

F/ DNA sequencing

DNA constructs were sent for sequencing to BaseClear, 2333C Leinden, The Netherlands. The constructs were fully sequenced to check that the required mutations, tags or DNA were correctly introduced and that no other mutations had appeared during the process of making the constructs. BLAST2 program was used to align the different sequences.

2.2.3 Cell culture

The tissue culture manipulations were performed in a laminar flow hood. Aseptic techniques were used with all manipulations of cells or preparations of dishes. Liquid waste was added to antiseptic before discarding. Solid waste was removed and autoclaved before being disposed.

A/ Routine cell culture

Human Embryonic Kidney cells (HEK 293 T) were used for transient transfection. The HEK 293 T cell line is a derivative of the HEK 293 that give a higher level of expression after transient transfection. The cells were cultured in DMEM supplemented with 10% Newborn Calf Serum and 2mM L-Glutamine. They were grown in monolayers in 75cm^2 tissue culture flasks in humidified atmosphere, at 37°C and under 95% air / 5% CO_2 in cell culture incubators (Jencons Nuaire). The cells were detached using 2ml trypsin solution, followed by

1min incubation at 37°C and neutralisation with 5ml medium. They were then centrifuged for 5min at 1000xg, the supernatant discarded, then resuspended in medium and plated out at 1:10 dilution in 75 cm^3 flasks for routine maintenance or into 10cm dishes for transfection the next day.

B/ Transient Transfection

Cells were transfected using Lipofectamine reagent (Gibco Life Technologies) or Gene Juice (Novagen) and the appropriate cDNA(s) according to the manufacturers' instructions.
Between 1 to 8µg of the required DNA(s) was incubated in 500µl Optimem plus 500µl of Lipomix (Optimem and Lipofectamine 1:30) or 10µl Gene Juice plus 190µl Optimum for 30 min under the laminate air flow cabinet. After 30 min the mix was adjusted to 5 ml by adding Optimum and poured onto 50-70% confluent cells cultured in 10 cm dishes. Dishes were then left 5h in the tissue culture incubators. After this time, the transfection mix was removed and replaced by 10 ml fresh DMEM supplemented with 10% Newborn Calf Serum and 2mM L-Glutamine. The medium was changed the following morning and cells were harvested 48h after tranfection.

Pertussis Toxin treatment:

Pertussis toxin (PTX) is secreted by *Bordella pertussis*. This toxin catalyses the addition of ADP-ribose to the α subunit of G_i and prevents receptor activation of the G protein. The same transient transfection protocol as above was followed, except that the cells were treated with PTX (25ng/ml) for 16 to 18 h prior to harvesting.
1 ml of a 250 ng/ml stock of PTX in medium was added to 9 ml of medium.

C/ Cell Harvesting

Cells were harvested by removing medium followed by addition of 5ml ice cold PBS. The cells were scraped out from the dishes and then centrifuged at 3000xg for 5min at 4°C on a bench top centrifuge. The cells were washed by resuspension in 10ml PBS and centrifuged as above. The PBS was then discarded and the cell pellets were stored at -80°C or used directly.

D/ Cell number determination

Cells were harvested as before and 50µl of the 10ml resuspended cells were diluted 10× in PBS and 15µl was applied to a Neubauer cell to count cell number.

2.2.4 Membrane Preparation

A/ Membrane preparation

Frozen cells were resuspended in ice cold TE supplemented with an anti-protease cocktail. They were transferred into a homogeniser, and broken down by repeated strokes (45 to 50). The resultant homogenate was then centrifuged at 1200xg for 10 min at 4°C. The supernatant was retained and transferred into ultracentrifugation tubes (Beckman). Before centrifugation the rotor was prechilled and the tubes were balanced. The samples were centrifuged at 50000xg for 20 min at 4°C using a Beckman Optima™ TLX Ultrancentrifuge. The supernatant was discarded and the pellet was resuspended in 500µl ice cold TE buffer, first by pipetting up and down and then using a syringe. The protein concentration of the different samples was then measured using the BCA assay method. The samples were frozen at –80°C until required.

B/ Determination of the protein concentration using the BCA assay

Reagents:

Reagent A:	Reagent B
1% BCA	4% $CuSO_4$
2% Na_2CO_3	
0.16% sodium tartrate	
0.4% NaOH	
0.95% $NaHCO_3$	
pH adjusted to 11.25	

Protein concentration of cell lysates or membrane preparations was determined using bincinhononic acid (BCA) and copper sulphate solutions. Proteins reduce Cu(II) ions to Cu(I)

ions in a concentration dependent manner and BCA forms a complex with Cu(I) ions to generate a purple solution.

Standard protein concentrations were prepared using BSA diluted with water in a range of 0.2µg/µl to 2µg/µl. 96 well plates were used to perform the assays. 10 µl of the standards and the different samples were added. Then 200µl of a mix of reagent A and B (1 part reagent B for 49 parts reagent A) was distrubuted in each well. The plate was then incubated for 20min at 37°C. The OD was then measured at 492nm using an SLT spectrophotometer.

2.2.5 Radioligand binding experiments

Membrane preparations were used for all the binding experiments.

A/ Binding buffers

Tris EDTA-$MgCl_2$ (TEM) (1X)

Tris 75mM

EDTA 1mM

$MgCl_2$ 12.5mM

pH adjusted to 7.5

Tris EDTA (TE) (1X)

Tris 75mM

EDTA 1mM

pH adjusted to 7.5

This was used for washing of binding experiments and usually prepared as a 5X stock solution that was diluted as required

B/ Saturation experiments

[^{3}H]-Diprenorphine binding

The expression of opioid receptors was assessed using [^{3}H]-diprenorphine. This was performed in 96 well blocks in triplicate, containing the following mixes:

25µl (0.4µg/µl) membrane + 25µl [^{3}H]-diprenorphine (range of concentrations) + 50µl TEM= Total binding

25µl (0.4µg/µl) membrane + 25µl [^{3}H]-diprenorphine (range of concentrations) + 25µl (400µM) naloxone + 25µl TEM = Non-specific binding

Binding was initiated by adding 10 µg of membrane preparation to TEM containing 0.1nM to 8nM of [^{3}H]-diprenorphine. Non-specific binding was determinated in the same way but in presence of 100µM final concentration of naloxone. Reactions were incubated for 1h at 25°C. Bound ligand was separated from free by vacuum filtration through GF/B filters. The filters were pretreated with 0.3% polyethyleneimine in TEM and washed three times with cold TE buffer. Bound ligand was estimated by liquid scintillation spectroscopy. The specific binding was calculated by subtracting non-specific binding from total binding. Data were analysed using GraphPad Prism software (San Diego, CA). Saturation data were fitted to non-linear regression curves.

[^{3}H]-Dihydroalprenolol binding

The expression of β_2-adrenoceptor was assessed using [^{3}H]-dihydroalprenolol. This was performed in 96 well blocks in triplicates, containing the following mixes:

25µl membrane + 25µl [^{3}H]-dihydroalprenolol (range of concentrations) + 50µl TEM = Total binding

25µl membrane + 25µl [^{3}H]-dihydroalprenolol (range of concentrations) + 25µl propanolol (40µM) + 25µl TEM = Non-specific binding

For [^{3}H]-dihydroalprenolol binding the same protocol as above was followed except that the samples were incubated for 30 min at 30°C and 10µM propanolol was used as competitor to determine non-specific binding.

C/ Competition experiments

[^{3}H]-Diprenorphine competition experiments using different opioid agonists or antagonists

Opioid ligand affinity was measured using competition experiments. Increasing concentrations of unlabelled ligand usually between 10^{-12}M and 10^{-4}M, were used to compete for the binding of 1nM [^{3}H]-diprenorphine. Glass tubes were set up as follows:

25µl membrane + 25µl [^{3}H]-diprenorphine + 200µl TEM = Total binding
25µl membrane + 25µl cold competitor + 25µl [^{3}H]-diprenorphine + 175µl TEM = Binding with competitor
25µl membrane + 25µl naloxone + 25µl [^{3}H]-diprenorphine + 175µl TEM = Non-specific binding

Reactions were initiated by adding membrane preparations into the rest of the reaction mix and incubated for 1h at 25°C. Bound ligand was separated from free by vacuum filtration. The filters were pretreated with 0.3% polyethylenimine in TEM and washed three times with cold TE buffer. Bound ligand was estimated by liquid scintillation spectrometry.
Data were analysed using GraphPad Prism software (San Diego, CA). Competition data with antagonists were fitted using 1 site competition non linear regression curves. For competition with agonists a 1 site competition model was compared with a 2 site model, the preferred model was chosen.
K_i was calculated using Cheng-Prusoff equation $K_i = \frac{IC_{50}}{1 + (L/Kd)}$
Statistics were analysed using pK_i value = -log K_i.
For the 1 way ANOVA, the analysis was followed by a Turkey post test.

D/ [^{35}S]-GTPγS binding experiments

Buffers:

10X Assay Buffer:

Tris 0.5M pH 7.4
$MgCl_2$ 30 mM

NaCl 1 M

pH adjusted to 7.4, and diluted to 1X as stop buffer during the experiment.

Solubilisation Buffer:

Tris 100 mM

NaCl 200 mM

EDTA 1 mM

NP40 1.25%

pH adjusted to 7.4 and 10% SDS added depending on reaction step to 0.2% final.

Beads Buffer:

BSA 2%

NaN_3 0.1%

Protease inhibitor cocktail

pH adjusted to 7.4

Assay Buffer:

HEPES (pH 7.4)	20 mM
$MgCl_2$	3 mM
NaCl	100mM
GDP	10 μM
Ascorbic acid	0.2 mM
[^{35}S]-GTPγS	50 nCi

Protocol

The experiment was initiated by adding the assay buffer mix in the presence or absence of agonist to a defined amount of membranes. The non-specific binding was determined in the same conditions but in the presence of 100 μM GTPγS. The reaction mix was incubated for 15 min at 30°C and was terminated by adding 1ml of ice-cold stop buffer. The samples were centrifuged for 15 min at 16000xg at 4°C and the resulting pellets were resuspended in solubilisation buffer plus 0.2% SDS. Samples were precleared with Pansorbin for 1h at 4°C and centrifuged for 2min at 16000xg. Supernatant was added to a mix of protein G and SG3-13 antiserum (anti-$G_{i\alpha}$Ab) and left rotating overnight at 4°C for immunprecipitation. Finally

the immunocomplexes were washed twice with ice-cold solubilisation buffer and bound [^{35}S]-GTPγS was measured by liquid scintillation spectrometry.

Concentration curves: The same protocol as above was used except that an increasing concentration of the appropriate agonist was added to the samples, usually between 10^{-11}M and 10^{-4}M.

Concentration inhibition curves: The above protocol was also followed. Samples were stimulated with 10^{-7}M agonist and increasing concentrations of antagonist were added usually between 10^{-10}M and 10^{-4}M.

Analysis of results:

Data were analysed using GraphPad Prism. Values were expressed as a percentage of maximum stimulation for concentration curve experiments. For statistics pEC_{50}=-log EC_{50} values were used.

2.2.6 Protein detection

A/ Co-immunoprecipitation

Buffers:

2X RIPA buffer:	
Hepes	100mM
NaCl	300mM
TX100	2% (w/v)
Na-deoxycholate	1% (w/v)
SDS	0.2% (w/v)
pH 7.4	

1X RIPA Buffer:	
2x RIPA	25ml
NaF 0.5M	1ml
EDTA O.5M, pH8	0.5ml
Na_2HPO_4 0.1M	5ml
ethylene glycol	2.5ml
dH_2O up to	50ml
1 complete protease inhibitor cocktail tablet	

Protocol:

Cells were resuspended in 1ml of 1X RIPA buffer and left rotating for 60 min at 4°C to be lysed. The samples were centrifuged at 14000xg for 10min at 4°C and the supernatant was retained. 50µl of a protein G-sepharose and PBS mix was added to the supernatant and left rotating for another 60 min at 4°C. This step allowed the samples to be precleared of non-specific binding proteins. Samples were centrifuged at 14000xg for 10 min at 4°C. Supernatant was conserved and protein concentration was measured using the BCA assay method. The protein samples were equalised to 1µg/µl. The receptors were then immunoprecipitated from 500µl samples by incubation with 20µl of protein G-sepharose and the appropriate antibody overnight at 4°C on a rotating wheel. The immune complex was isolated by centrifugation at 14000xg for 1 min and washed twice with 1 ml RIPA (1X). 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 eluate was then loaded onto SDS-PAGE gels.

B/ Western Blotting

Buffers and solutions

Laemmli Buffer (2×)

DTT	0.4M
SDS	0.17M
Tris/HCl (pH8)	50mM
Urea	5M
Bromophenol Blue	0.01% (w/v)

Gel running Buffer

20× Gel Running Buffer Novagen (MES)	50ml
dH_2O	950ml

Transfer Buffer

Glycine	11.26g
Tris	2.24g
Methanol	200ml
dH_2O	800ml

Gel running buffer and transfer buffer was made fresh each time.

Blocking Buffer :Phosphate buffered Saline, 5% non-fat milk and 0.1% Tween 20

PBS 1X	40 ml
Milk	2g
Tween 20	40µl

Ponceau red:

Ponceau S	0.1%
trichloroacetic acid	3%

Wash 1:

PBS	0.1M
NaCl	0.1M
Tween 20	0.1%

Wash 2:

PBS	0.1M
NaCl	0.5M
Tween 20	0.1%

Protocol

Samples were run on NuPage Novex pre-cast bis-tris gels (Invitrogen BV). The NuPage system is based on a bis-tris-HCl buffered (pH 6.4) polyacrylamide gel, with a separating gel that operates at pH 7. 4-12% acrylamide gels were used to achieve the best seperation of the proteins. NuPage MES or MOPS buffer were used for running the gels. Gels were run at 200V using the Xcell Surelock mini gel tank (Invitrogen BV). Following SDS-PAGE the proteins were transferred onto nitrocellulose using the Xcell II blot module (Invitrogen BV). Gels were transferred at 30V for 1h to 2h in transfer buffer. The transfer onto nitrocellulose was checked using red Ponceau staining. Membranes were then blocked overnight in blocking buffer. The next day membranes were incubated in blocking buffer containing the primary antibody or serum for 1h at room temperature. This incubation was followed by 3 washes of 10 min using alternatively wash 1 and wash 2. The blots were then incubated with a HRP

conjugated secondary antibody in blocking buffer for 1h at room temperature. After 3 washes, the blots were incubated for 5 min in a mix of enhanced chemiluminescent substrate for detection of HRP. This allowed proteins to be visualised after developing on film using a Kodak X-OMAT developer.

For Western blots the following antibody or antiserum dilutions were used:

1st Antibody/Antiserum	Dilution	2nd Antibody	Dilution
Anti-myc	1:1000	Anti-mouse	1:10000
Anti-Flag	1:1000	Anti-rabbit	1:10000
$G_{i1\&2\alpha}$ antiserum	1:1000	Anti-rabbit	1:10000

Chapter 3

Opioid receptor homodimerisation studied using a complementation technique

3.1 Introduction :

The opioid receptor family is composed of three different subtypes, namely the Delta (DOR), Kappa (KOR) and Mu (MOR) opioid receptors which were first cloned only in the early 1990s (Kieffer *et al.*, 1992, Chen *et al.*, 1993, Li *et al.*, 1993). In addition to these three types of opioid receptors, an orphan opioid receptor-like protein ORL1 has been cloned (Lachowicz *et al.*, 1995) and the so-called ε opioid receptor (O'Dowd *et al.*, 1995), still remains poorly characterised. The DOR, KOR and MOR are 65 % homologous with highest homology among the transmembrane domains, intracellular loops and a small portion of the C-terminal tail. The remaining 35% of the opioid receptor confers subtype selectivity (Reisine and Bell, 1993).

Opioid receptors inhibit adenylate cyclase activity and modulate the activity of voltage gated calcium and potassium channels via pertussis-toxin-sensitive G_i/G_o protein activation. They are involved in pain perception by mediating analgesia. They can also modulate endocrine processes (Maggi *et al.*, 1995) and affect immune responses (Mc Carthy *et al.*, 2001; Suzucki *et al.*, 2002). As such opioid receptors are attractive targets for the development of new drugs to control pain. A current problem hindering investigations is that the use of opioid drugs as analgesics also leads to side effects such as addiction and tolerance. One of the principal goals in opioid research, therefore, is to dissociate these effects, and so developing an understanding of the molecular nature of their regulation is essential.

Opioid receptors are found in the central nervous system where they have a discrete but overlapping distribution. They can be found in the nucleus accumbens, olfactory tubercule and bulb, in the cerebral cortex, amygdala, hypothalamus, hippocampus, thalamus or corpus striatum. Diverse types of ligand recognise the opioid receptor family, including peptides, opioid alkaloids and a variety of synthetic non-peptide small molecules. The opioid ligands do not bind exclusively to one specific receptor subtype but frequently have a higher affinity for one particular receptor subtype (Przewlocki and Przewlocka, 2001). The pharmacology of the opioid receptors is complicated, and several subtypes (μ1, μ2, δ1, δ2, κ1, κ2, κ3) have been postulated on the basis of pharmacological studies. The lack of cDNA corresponding to these subtypes and the emergence of receptor dimerisation could suggest that these different pharmacological profiles are due to opioid receptors dimerisation.

The opioid receptors belong to the G Protein Coupled Receptor (GPCR) superfamily. The GPCRs are likely to have a three-dimensional structure with seven transmembrane segments, three extracellular loops and three intracellular loops. The amino terminus is extracellular whereas the C-terminus is intracellular. It has been assumed previously that the GPCRs exist as monomers and interact with G proteins upon ligand activation. More recently, an increasing number of studies have suggested that GPCRs could dimerise. Homodimerisation has been detected for several receptors such as the β2-adrenoceptor (Angers *et al.*, 2000), TRH receptor (Kroeger *et al.*, 2001) and D2 dopamine receptor (Lee *et al.*, 2000; Ng *et al.*, 1996). This has also been demonstrated for DOR (Cvejic *et al.*, 1997), KOR (Jordan and Devi, 1999), and MOR (Li-Wei *et al.*, 2002).

The dimerisation process has been observed using several techniques including Western blotting, immunoprecipitation (Zeng and Wess, 1999), Bioluminescence Resonance Energy Transfer (BRET) and Fluorescence Resonance Energy Transfer (FRET) (McVey *et al.*, 2001 ; Ramsay *et al.*, 2002, Kroeger *et al.*, 2001). However the role of dimerisation in receptor function and the mechanism for the initiation of signal transduction by the dimer has been difficult to investigate.

One of the most informative models so far has been a class C GPCR, the γ-aminobutyric acid type b (GABAb) receptor. This receptor is composed of two subunits GABAb1 and GABab2. The GABAb1 subunit binds the ligand GABA, but is unable to activate G proteins. GABAb2 does not have a GABA binding site but does possess a heptahelical domain which is able to activate the G protein. When these two subunits associate G proteins are activated (Galvez *et al.*, 2001). This observation provides strong evidence that GPCR activation involves transactivation whereby ligand binding to one subunit leads to the activation of the G protein through the second subunit. This is the first model showing the importance of dimerisation for signalling. Mutated versions of those two subunits have also been constructed to try and determine which part of the GABAb1 and GABAb2 were important to activate G proteins (Duthey *et al.*, 2002; Margeta-Mitrovic *et al.*, 2001). It was found that mutations in the GABAb2 prevent formation of the functional dimer, illustrating that a single subunit is important for G protein contact in a dimeric receptor.

Osuga *et al.*, (1997) have also identified a luteinizing hormone (LH) receptor mutant which retained its ability to bind ligand but could not signal. When this mutant was co-expressed with a truncated LH receptor that lacked the ligand binding site, a partial restoration of signal was produced.

Fusion proteins have become an interesting and widely used tool to study GPCR signalling. Fusions between receptor and G protein allow a 1:1 stoichiometry. Their expression level can then be directly assessed by saturation binding (Milligan, 2000; Wurch and Pauwels, 2001). A $C^{351}I$ mutation introduced in the $G_{i\alpha}$ protein subunit gives resistance to Pertussis toxin (PTX). Indeed ADP ribosylation of the G protein normally produced by this toxin is prevented. When cells transfected with such a fusion protein are treated with PTX, endogenous $G_{i/o}$ proteins are inactivated. An exact measurement of the G protein activation by the receptor fusion can then be made.

Mutated fusion proteins consisting of a receptor linked directly to the G protein α subunit have been used to study α_{1b}-adrenoceptor or histamine H1 receptor dimerisation in our group via a protein complementation technique (Carrillo *et al.*, 2003). In this first Results chapter this strategy was employed, whereby pairs of distinct but potentially complementary fusion proteins were generated to analyse opioid receptor homodimerisation.

3.2 Results :

3.2.1 Human DOR homodimerisation studied using a protein complementation technique

A/ Construction of non-functional but potentially complementary fusion proteins: hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$

To study DOR homodimerisation two distinct fusion proteins were constructed. A fusion protein made previously in the laboratory hDOR-$G_{i1\alpha}C^{351}I$ was used as a template (Figure 3.1A). This fusion protein was also utilised as a control in the different experiments. In this construct the human DOR was fused to $G_{i1\alpha}$ subunit, which contains a cysteine residue in position 351 mutated into an isoleucine. This has been shown to induce resistance of the corresponding G protein to the ADP ribosylation activity of PTX. Normally PTX treatment prevents receptor-mediated exchange of GDP for GTP, with $G_{i1\alpha}$ subunit remaining in the GDP-bound inactive conformation. This does not occur when the $C^{351}I$ mutation is incorporated. Cells transfected with such a construct were treated with PTX to inactivate the endogenous pool of $G_{\alpha i/o}$. With this treatment any signal observed must result from the G protein fused to the receptor. In this study all transfected cells were treated with PTX (25 ng/ml) for 16h prior to harvesting.

The first mutated fusion protein, hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (Figure 3.1 B) consisted of the wild type DOR fused with a mutated $G_{i1\alpha}$ subunit. Stratagene point mutation kit was used to introduce the $G^{202}A$ mutation into the $G_{i1\alpha}$ subunit of hDOR-$G_{i1\alpha}C^{351}I$ cDNA. All wild type G protein α subunits have a conserved GGQR sequence (Figure 3.2). The mutation of the initial glycine into alanine generates a form of the G protein which is unable to exchange GDP for GTP and hence become activated. The mutation was introduced to create a non-functional fusion protein. The complementary fusion protein hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (Figure 3.1 C) was also built by point mutation. Two valines near the DRY motif of the 2nd intracellular loop of the receptor were mutated into glutamic acid and aspartic acid, respectively. This time the mutations of highly conserved hydrophobic residues (Figure 3.3) to acidic residues make the interaction between the receptor and the fused G protein non-functional.

B/ Analysis of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ expression and determination of the dissociation constant for [^{3}H]-diprenorphine in comparison with the wild type fusion hDOR-$G_{i1\alpha}C^{351}I$

The mutated fusion proteins, hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$, hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ as well as hDOR-$G_{i1\alpha}C^{351}I$ were transiently transfected into HEK 293 cells which were treated with PTX (25ng/ml) 16h prior to harvesting. A membrane preparation was produced and expression of the fusion proteins was assessed by saturation ligand binding (Figure 3.4). The opioid antagonist [^{3}H]-diprenorphine was used as radioactive ligand to determine total binding and the antagonist naloxone for non-specific binding. Specific binding was calculated by subtracting non-specific binding from total binding. Each of the fusion proteins gave an expression level of approximately 2000 fmol/mg protein (Table 3.1).

The level of expression when the two mutated versions of the fusion were co-transfected was also assessed. The transfection used 1 µg of DNA for each construct so that the final amount used was the same as for each singly transfected construct (2 µg final). The expression observed was 2310 ± 301 fmol/mg (Table 3.1). Although the level of construct expression varied between individual transfections, there was no specific pattern that could be ascribed to the mutations introduced.

Saturation binding also permitted the determination of the dissociation constant (K_d) of the fusion proteins for [^{3}H]-diprenorphine (Figure 3.4). The K_d observed was 0.65 ± 0.05nM (mean ± SEM, n=4) for hDOR-$G_{i1\alpha}C^{351}I$, 1.66 ± 0.06nM for hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$, 0.67 ± 0.07nM for hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and 1.43 ± 0.07nM for the mutated fusions expressed together (Table 3.1). The K_d value for hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ was not significantly different from hDOR-$G_{i1\alpha}C^{351}I$. However, the affinity of hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ for the antagonist [^{3}H]-diprenorphine was diminished. The K_d was significantly increased (1 way ANOVA test, $P<0.001$) compared to the wild type fusion suggesting that the double mutation of the valine in the 2nd intracellular loop altered the binding site of the receptor. Likewise when the mutated pair of fusion proteins were co-transfected a K_d of 1.43 ± 0.07nM was observed. This value was significantly different from hDOR-$G_{i1\alpha}C^{351}I$ and similar to hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$. This could reflect a predominant expression of hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ over hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ when the constructs were co-expressed. This was not obvious from the expression levels obtained when the fusion proteins were individually transfected.

Table 3.1. Expression level (B_{max}) and dissociation constant (K_d) of DOR fusion proteins

Construct	B_{max} (fmol/mg)	K_d (nM)	pK_d
hDOR-$G_{i1\alpha}C^{351}I$	1816 ± 209	0.65 ± 0.05	9.20 ± 0.03
hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$	2181 ± 228	1.66 ± 0.06***	8.78 ± 0.01***
hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1777 ± 285	0.67 ± 0.07	9.19 ± 0.05
hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	2310 ± 301	1.43 ± 0.07***	8.85 ± 0.02***

Data represent n=4 experiments performed on different membrane preparations. Numbers represent means ± SEM.

*** Significantly different from hDOR-$G_{i1\alpha}C^{351}I$, $P<0.001$

C/ Increasing amounts of hDOR-$G_{i1\alpha}C^{351}I$ result in increasing levels of agonist-stimulated [^{35}S]-GTPγS binding

HEK 293 cells were transiently transfected with or without hDOR-$G_{i1\alpha}C^{351}I$ and membranes were prepared. The addition of the agonist DADLE (10μM) to the membranes expressing the fusion protein resulted in a large stimulation of the binding of [^{35}S]-GTPγS (Figure 3.5A). For 10 μg of membrane protein a signal of some 6000 dpm over basal was observed. No significant stimulation was noticed when membranes without hDOR-$G_{i1\alpha}C^{351}I$ were used (10 or 20 μg). This showed that the signal detected in response to agonist stimulation reflected

activation of the fusion protein. [^{35}S]-GTPγS binding could also be observed in the absence of agonist stimulation. This binding increased proportionally with the amount of hDOR-$G_{i1\alpha}C^{351}I$ present. This could reflect some level of constitutive activity of the fusion protein.
When increasing amount of membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ were used [^{35}S]-GTPγS binding induced by DADLE was dependent on the amount of receptors present. A linear increase was observed with the number of fmol of receptor over a range between 0-60 fmol (R^2=0.99).

D/ Reconstitution of function using pairs of distinct non-functional hDOR-$G_{i1\alpha}C^{351}I$ fusion protein mutants

The mutated fusion proteins, hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ were singly or co-transfected into HEK 293 cells. The wild type version of the fusion protein was also transiently transfected into HEK 293 cells. Cells were treated with PTX for 16h prior to harvesting. Expression of the fusion proteins was assessed by the binding of a single, near saturating concentration of [^{3}H]-diprenorphine in membrane preparations. An equal amount of each construct (15 fmol) was used to study their activation as monitored by [^{35}S]-GTPγS binding in the presence or absence of 10 μM DADLE (Figure 3.6). [^{35}S]-GTPγS binding was measured following immunocapture of the fusion proteins using an anti-$G_{i1/2}$ antiserum.
As expected, low [^{35}S]-GTPγS binding was observed in the absence of agonist for hDOR-$G_{i1\alpha}C^{351}I$, described hereafter as basal. In the presence of agonist a large stimulation (520 ±32 %) was produced (Table 3.2). For hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ no stimulation of [^{35}S]-GTPγS binding over basal was detected after DADLE treatment (P>0.05, T test) (Table 3.2). The introduction of the $G^{202}A$ mutation into the $G_{i1\alpha}$ of hDOR-$G_{i1\alpha}C^{351}I$ eliminated DADLE stimulation. A similar effect was observed for hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$. No significant difference (P>0.05, T test) was seen between basal and DADLE-stimulated [^{35}S]-GTPγS binding. Introduction of $V^{150}E,V^{154}D$ in the 2nd intracellular loop of the DOR fusion protein thus also prevented agonist-stimulated [^{35}S]-GTPγS binding by $G_{i1\alpha}$ (Table 3.2). The pair of distinct fusion proteins were non-functional as predicted by their design.
However, the co-expression of the two distinct non-functional mutants reconstituted DADLE-mediated binding of [^{35}S]-GTPγS. When 45 fmol of [^{3}H]-diprenorphine binding sites were used the signal reconstituted upon agonist stimulation of the non-functional pair of fusion

proteins was 484 ± 33% vs. wt basal. This was similar to the stimulation produced by 15 fmol of the wild type fusion protein (520 ± 31% vs. wt basal) (Table 3.2). Three times as many [^{3}H]-diprenorphine binding sites in the co-expression study as hDOR-$G_{i1\alpha}C^{351}I$ were needed to reconstitute a full wild type signal. When membranes containing 15 fmol of [^{3}H]-diprenorphine binding sites of $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ and 15 fmol of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were prepared individually and then combined before the assay, no significant difference ($P>0.05$, T test) in stimulated [^{35}S]-GTPγS was observed in the presence or absence of agonist (Figure 3.6, Table 3.2). This demonstrated that the reconstitution detected when the distinct pair of non-functional fusions were co-expressed was specific and required the proteins to be in the same membrane. Assuming a dimmer as the functional receptor, upon agonist activation, was able to activate the G protein linked to the other receptor in the dimer. This result was consistent with the hypothesis that delta opioid receptor dimerisation is required for agonist function in HEK 293 cells.

[^{35}S]-GTPγS binding could also be observed in the absence of agonist stimulation. This basal binding increased proportionally with the amount of fusion proteins used. This might reflect some level of constitutive activity of the reconstituted fusion proteins (Figure 3.6). It was also noticed that the level of [^{35}S]-GTPγS binding observed in the absence of agonist stimulation for the mutated proteins individually expressed was significantly lower in comparison to the wild type fusion protein ($P<0.05$, T test) (Table 3.2). This could indicate that the mutations introduced decreased the constitutive activity observed for hDOR-$G_{i1\alpha}$ $C^{351}I$.

Table 3.2. [^{35}S]-GTPγS binding of DOR fusion proteins

Construct	Basal	DADLE 10μM % maximum
hDOR-$G_{i1\alpha}$ $C^{351}I$ 15 fmol	100	520 ± 31
$hDORV^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ 15 fmol	27 ± 7*	59 ± 13
hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ 15 fmol	20 ± 4*	31 ± 9
Co-transfection 15 fmol	40 ± 6	179±15
30 fmol	88 ± 30	343 ± 38
45 fmol	137±12	484 ± 33

Mix Membrane hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I 15 fmol + hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I 15 fmol	73 ± 2	105 ± 5

Data represent n=5 experiments performed in triplicate on different membrane preparations. Numbers are the means ± SEM % versus basal wild type fusion protein.
*Significantly different from wild type fusion protein basal $P<0.05$ (1 way ANOVA)

E/ Interactions of hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I with hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I observed by co-immunoprecipitation

The mutated hDOR-G$_{i1\alpha}$C^{351}I fusion proteins were N-terminally tagged. A c-Myc version of hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I and a Flag-tagged version of hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I were constructed. HEK 293 cells were mock transfected or transfected to express either Flag-hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I or c-Myc-hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I or both. Samples were immunoprecipitated using an anti-Flag antibody. They were resolved by SDS-PAGE, and anti-c-Myc antibody was used for protein detection (Figure 3.7A). No band was visualised in the mock-transfected line, or in samples expressing either Flag-hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I or c-Myc-hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I alone. It was observed that bands were only present when the Flag and c-Myc versions of the different fusions proteins were co-expressed. Specific bands were detected at 80, 65 and 35 kDa, as well as higher apparent molecular mass. The expected molecular mass for the fusion protein is around 80 kDa as DOR molecular mass is 42 kDa and G$_{i1\alpha}$ is 40.2 kDa. This was consistent with the 80 kDa band immunodetected corresponding to the monomer form of the DOR fused with G$_{i1\alpha}$. The bands at 65 and 35 kDa may be degradation products, but this was not explored further. Anti-c-Myc reactivity was also seen at the top of the gel which suggests higher order oligomers or aggregated protein. A mixed membrane control was generated where membranes singly expressing Flag-hDORV^{150}EV^{154}D-G$_{i1\alpha}$C^{351}I or c-Myc-hDOR-G$_{i1\alpha}$G^{202}AC^{351}I were mixed and then immunoprecipitated. Nothing was detected in this lane following immunoprecipitation. Re-blot of the gel with anti-Flag antibody revealed bands of the same molecular size in all

samples in which the Flag-tagged version of the fusion were expected to be expressed (Figure 3.7B).

The results demonstrated that the addition of a G protein to the C-terminal tail of the DOR did not prevent GPCR dimerisation. In addition, the introduction of the mutations into the receptor or G protein does not prevent receptor interaction.

F/ The affinity of different DOR selective agonists for hDOR-$G_{i1\alpha}C^{351}I$ fusion proteins

- Competition for [^{3}H]-diprenorphine binding by DADLE

Membranes from HEK 293 cells transfected with hDOR-$G_{i1\alpha}C^{351}I$; hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$; hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ cDNA or co-expressing the mutated pair of distinct fusion proteins (hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$) were used for [^{3}H]-diprenorphine competition experiments using varying concentrations of DADLE (Figure 3.8). Two binding site curves were best fitted in each case, with higher and lower affinity binding sites (K_h, K_l) (Table 3.3). Introduction of the $G^{202}A$ mutation in the G-protein subunit did not alter the DADLE binding properties as similar K_h, K_l values as observed with the wild-type fusion were obtained (Table 3.3). In contrast, the double mutation in the 2nd intracellular loop of DOR did alter binding affinity of DADLE with loss of affinity in both high and low affinity binding sites (56 ± 33 and 21643 ± 17278 nM respectively compared to the wild type fusion protein 1.29 ± 0.48 and 652 ± 372 nM). However, when the distinct but complementary pair of fusion proteins were co-expressed wild-type pharmacology was reconstituted with no significant difference in the percentage of high and low site numbers compared to the wild type fusion protein ($P>0.05$, 1 way ANOVA).

Table 3.3: Binding affinity of DADLE for the different fusion proteins and co-expression of the mutated pair of fusion proteins

Construct	K_h (nM)	% K_h sites	pK_h	K_l (nM)	pK_l	Hill coefficient
hDOR-$G_{i1\alpha}C^{351}I$	1.29 ± 0.48	63 ± 6	9.03 ± 0.18	652 ± 372	6.79 ± 0.42	-0.39 ± 0.03

hDORV^{150}E V^{154}D-G$_{i1\alpha}$C^{351}I	56 ± 33***	57 ± 8	7.40 ± 0.24***	21643±17278*	4.99 ± 0.37*	-0.50 ± 0.02
hDOR-G$_{i1\alpha}$G^{202}AC^{351}I	2.18 ± 0.40	59 ± 3	8.70 ± 0.12	2319 ± 800	5.82 ± 0.23	-0.34 ± 0.04
hDORV^{150}EV^{154}D-G$_{i1\alpha}$C^{351}I + hDOR-G$_{i1\alpha}$G^{202}AC^{351}I	2.7 ± 1.05	45 ± 9	8.69 ± 0.15	1257 ± 842	6.29 ± 0.28	-0.41 ± 0.04

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

Statistics were performed using 1 way ANOVA on pK_h and pK_l numbers.

* Significantly different from hDOR-G$_{i1\alpha}$C^{351}I, P<0.05

*** Significantly different from hDOR-G$_{i1\alpha}$C^{351}I, P<0.001

- Competition for [^{3}H]-diprenorphine binding by DPDPE, a highly selective DOR agonist

Membranes preparation expressing hDOR-G$_{i1\alpha}$C^{351}I, hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I, hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I or co-expressing the pair of mutated fusion proteins were used for [^{3}H]-diprenorphine competition experiments using varying concentrations of DPDPE (Figure 3.9). Competition curves with higher and lower affinity binding sites were observed in each case (Table 3.4). Introduction of the mutation G^{202}A in the G-protein subunit did not alter DPDPE binding properties. Although the double mutation in the 2nd intracellular loop of the receptor significantly modified the binding affinity of DPDPE for the high binding site (40.4 ± 8.8nM) compared to the wild type fusion protein (1.71 ± 0.33nM), the lower affinity site was not affected. As with DADLE, when the pair of distinct fusion protein were co-expressed wild-type pharmacology was reconstituted with no significant difference (P>0.05, 1 way ANOVA) in the proportion of high and low affinity binding sites.

Table 3.4: Binding affinity of DPDPE for the various fusion proteins and co-expression of the mutated pair of fusion proteins.

Construct	K_h (nM)	pK_h	% K_h site	K_l (nM)	pK_l	Hill coefficient
hDOR-$G_{i1\alpha}C^{351}I$	1.71 ± 0.33	8.79 ± 0.09	70 ± 3	2100 ± 1173	5.89 ± 0.25	-0.41 ± 0.005
hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$	40.4± 8.8**	7.42 ± 0.11**	51±20	7658±4250	5.48 ± 0.41	-0.61 ± 0.04
hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1.56± 0.46	8.88 ± 0.15	65 ± 4	3200± 1510	5.82 ± 0.38	-0.40 ± 0.03
hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	7.49± 2.95	8.31 ± 0.28	55 ± 12	1660 ± 888	6.10 ± 0.35	-0.46 ± 0.01

Data represent n=4 experiments performed in triplicate on different membrane preparations. Numbers are the means ± SEM.

Statistics were performed using 1 way ANOVA analysis on pK_h and pK_l numbers

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

G/ The ability of the reconstituted dimer to activate or inactivate G protein function

-Comparison of hDOR-$G_{i1\alpha}C^{351}I$ fusion protein and the reconstituted dimer to activate G protein in response to DADLE

The hDOR-$G_{i1\alpha}C^{351}I$ fusion protein or the pair of mutated fusion proteins were transiently transfected into HEK 293 cells. Cells were treated with PTX for 16h prior to harvesting and membrane preparations were made. Equal amounts of receptor binding sites (15 fmol, based on [^{3}H]-diprenorphine binding) were used to study activation of the wild type versus reconstituted fusion proteins. This was investigated by measuring [^{35}S]-GTPγS binding in the presence of increasing concentrations of DADLE (Figure 3.10). No significant difference was observed between the two samples. EC_{50} for hDOR-$G_{i1\alpha}C^{351}I$ was 273 ± 100 nM and for the co-transfected but individually inactive fusion proteins was 97.2 ± 45.6 nM (Table 3.5). It is

important to note that for the co-transfected sample the [^{35}S]-GTPγS binding observed can only come from the reconstituted dimer as neither of the individual mutants are functional (Figure 3.6). The ability of DADLE at the reconstituted dimer to activate $G_{i1\alpha}$ was similar to the wild-type fusion protein, but as noted previously for equal numbers of [^{3}H]-diprenorphine binding sites the maximal stimulation of [^{35}S]-GTPγS binding was higher for the wild type fusion.

Table 3.5: EC_{50} for hDOR-$G_{i1\alpha}C^{351}I$ versus the reconstituted dimer: [^{35}S]-GTPγS binding studies

Construct	pEC_{50}	EC_{50} (nM)
hDOR-$G_{i1\alpha}C^{351}I$	6.63 ± 0.25	273 ± 100
hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	7.10 ± 0.19	97.2 ± 45.6

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

T test was performed using pEC_{50}.

- Comparison of the potency of naloxone to inhibite the G alpha protein subunit of hDOR-$G_{i1\alpha}C^{351}I$ fusion protein and the reconstituted dimer after DADLE stimulation

Membranes containing equal amounts of [^{3}H]-diprenorphine binding sites (15 fmol) were used to study the inhibition of the $G_{i1\alpha}$ subunit of the hDOR-$G_{i1\alpha}C^{351}I$ or DOR reconstituted dimer by the opioid antagonist naloxone. A concentration of 10^{-7}M DADLE was used to stimulate [^{35}S]-GTPγS binding and increasing concentrations of naloxone applied to compete (Figure 3.11). IC_{50} values of 560 ± 62nM and 270 ± 44 nM were observed for hDOR-$G_{i1\alpha}C^{351}I$ and following co-transfection of the mutated fusion proteins respectively (Table 3.6). These values were not significantly different ($P>0.05$, T test).

Table 3.6: The IC_{50} of naloxone to inhibit hDOR-$G_{i1\alpha}C^{351}I$ fusion and the reconstituted dimer: [^{35}S]-GTPγS binding studies

Construct	pIC_{50}	IC_{50} (nM)
hDOR-$G_{i1\alpha}C^{351}I$	6.26 ± 0.05	560 ± 62
hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	6.58 ± 0.06	270 ± 44

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

T test was performed using pIC_{50}.

3.2.2 Human MOR homodimerisation studied using protein complementation

A/ Construction of non-functional but potentially complementary fusion proteins: hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$

As for DOR, mutated MOR fusion proteins were constructed using hMOR-$G_{i1\alpha}C^{351}I$ as a template (Figure 3.12A). This fusion protein was previously made in the laboratory and contained the $C^{351}I$ mutation that generates a PTX resistant $G_{i1\alpha}$ subunit.
The first fusion protein, hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ was mutated in positions 169 and 173 of the 2nd intracellular loop after the DRY motif (Figure 3.12C). Two hydrophobic valines were substituted with glutamic acid and aspartic acid residues respectively to prevent the receptor from signalling to its G protein. In the second fusion protein hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$, the $G_{i1\alpha}$ subunit was mutated in position 202 whereby the glycine was replaced by alanine (Figure 3.12B) to prevent guanine nucleotide exchange. All transfected cells were treated with PTX 16h prior to harvesting to inactivate endogenous $G_{i/o}$ proteins.

B/ Analysis of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ expression and determination of the dissociation constant for [^{3}H]-diprenorphine in comparison with the wild type hMOR-$G_{i1\alpha}C^{351}I$ fusion protein

Receptor expression was assessed using membrane preparations of HEK 293 cells transfected to express hMOR-$G_{i1\alpha}C^{351}I$, hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or to co-express the two mutated fusion proteins. [^{3}H]-diprenorphine saturation binding assays were performed (Figure 3.13). The expression levels obtained were not significantly different between membranes expressing the individual fusion proteins or the co-transfection, with a B_{max} of approximately 1200 fmol/mg of protein (Table 3.7). [^{3}H]-diprenorphine had a high affinity for the different fusion proteins and the co-transfected pair of mutated fusion proteins with K_d values of 0.36 ± 0.07; 0.46 ± 0.15; 0.32 ± 0.05 and 0.31 ± 0.07nM (mean ± SEM, n=3) for hMOR-$G_{i1\alpha}C^{351}I$, hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$; hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and the co-

transfection, respectively. The pK_d values were not significantly different (1 way ANOVA, $P>0.05$). None of the mutations affected diprenorphine binding, which contrasts with the observation for the DOR fusion proteins.

Table 3.7: Expression level (B_{max}) and dissociation constant (K_d) of hMOR-$G_{i1\alpha}C^{351}I$ fusion proteins

Construct	B_{max} (fmol/mg)	K_d (nM)	pK_d
hMOR-$G_{i1\alpha}C^{351}I$	1217 ± 72	0.36 ± 0.07	9.47 ± 0.08
hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$	901 ± 110	0.46 ± 0.15	9.39 ± 0.17
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1251 ± 20	0.32 ± 0.05	9.52 ± 0.08
Co-transfection	1285 ± 120	0.31 ± 0.07	9.56 ± 0.10

Data represent n=3 experiments performed in triplicate on different membrane preparations and numbers are the means ± SEM.
Statistics were performed using 1 way ANOVA on B_{max} and pK_d numbers.

C/ Reconstitution of function using pairs of distinct non-functional hMOR-$G_{i1\alpha}C^{351}I$ fusion protein mutants

Membrane preparations expressing hMOR-$G_{i1\alpha}C^{351}I$, hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or co-expressing the pair of distinct mutated fusion proteins were used to study guanine nucleotide exchange on the G protein α subunit by [^{35}S]-GTPγS binding (Figure 3.14). When membranes expressing 15 fmol of hMOR-$G_{i1\alpha}C^{351}I$ binding sites were treated with 10 μM of the MOR specific agonist DAMGO, a stimulation of 528 ± 23 % over basal was observed. In contrast, following expression of either hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ or hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ fusion proteins, DAMGO did not cause a significant stimulation of nucleotide binding (Table 3.8). As expected by their design the two mutated fusion proteins

were non-functional. Interestingly, subjecting membranes containing 15 fmol or 30 fmol of [^{3}H]-diprenorphine binding sites of the co-expressed mutated fusion proteins to agonist treatement resulted in reconstitution of [^{35}S]-GTPγS binding. Approximately two times as many [^{3}H]-diprenorphine binding sites as hMOR-$G_{i1\alpha}C^{351}I$ were required to reconstitute half of the full wild-type signal when the mutated fusion proteins were co-expressed. When membranes individually expressing hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ or hMOR-$G_{i1\alpha}G^{202}AC^{351}I$ were mixed and agonist added, no significant stimulation was noted. This indicates that the reconstitution observed was specific and that MOR dimerisation was required for agonist function. These observations were consistent with those obtained for the DOR fusion proteins. [^{35}S]-GTPγS binding was observed in absence of agonist stimulation. This basal activity increased proportionally with the amount of fmol of fusion proteins used. This may reflect some level of constitutive activity of the fusion proteins.

Table 3.8: [^{35}S]-GTPγS binding of hMOR-$G_{i1\alpha}C^{351}I$ fusion proteins

Construct	Basal	DAMGO 10μM
hMOR-$G_{i1\alpha}C^{351}I$	100	528 ± 23
hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$	79 ± 41	84 ± 25
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	66 ± 24	82 ± 9
Co-transfection 15fmol	35 ± 5	149 ± 7
30fmol	106 ± 7	277 ± 14
Mix membrane 30fmol	124 ± 58	137 ± 38

Data represent n=3 experiments performed in triplicate on different membrane preparations and numbers are the means ± SEM % versus basal compared to the wild type fusion protein.

D/ The affinity of MOR selective agonist DAMGO for hMOR-$G_{i1\alpha}C^{351}I$ fusion proteins

Membranes from HEK 293 cells expressing hMOR-$G_{i1\alpha}C^{351}I$, $hMORV^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or the pair of mutated fusion proteins were used to compete [^{3}H]-diprenorphine binding using increasing concentrations of DAMGO (Figure 3.15). Two binding site curves with high and low affinity sites were observed, except when $hMORV^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ was expressed alone which provided a Hill coefficient of -0.86 ± 0.07 (Table 3.9). In this case a single binding site was observed with a K_i value of 961 ± 50 nM. However when both mutated fusion proteins were co-transfected wild type pharmacology was reconstituted. The percentage of high affinity sites were similar as if the co-expression of the mutated fusion proteins rescued the original binding sites (P>0.05, 1 way ANOVA). This could also reflect the mix of $hMORV^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ and hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ pharmacology.

Table 3.9: Binding affinity of DAMGO for MOR fusion proteins and the co-expressed pair of mutated fusion proteins

Construct	K_h (nM)	pK_h	% high affinity site	K_l (nM)	pK_l	Hill coefficient
hMOR-$G_{i1\alpha}C^{351}I$	2.37 ± 1.1	8.71 ± 0.18	47 ± 8	182 ± 116	6.91 ± 0.27	-0.55 ± 0.007
$hMORV^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$				961 ± 50*	6.02 ± 0.02*	-0.86 ± 0.07
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	2.73 ± 1.3	8.68 ± 0.23	54 ± 9	268 ± 135	6.69 ± 0.23	-0.54 ± 0.03
Co-transfection	3.5 ± 0.5	8.47 ± 0.06	40 ± 2	675 ± 110	6.18 ± 0.07	-0.44 ± 0.05

Data represent n=3 experiments performed in triplicate on different membrane preparations and numbers are the means ± SEM.

Statistics were performed using 1 way ANOVA on pK_h and pK_l numbers.

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

E/ The ability of the reconstituted dimer to activate G protein function

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$ or the pair of mutated fusion proteins were used to compare DAMGO potency to activate the G_α protein subunit by measuring [^{35}S]-GTPγS binding. Equal amounts of [^{3}H]-diprenorphine binding sites were used. Increasing concentrations of DAMGO were utilised to build a concentration response curve (Figure 3.16). A significant difference was observed between the two samples with EC_{50} values of 345 ± 29 nM and 830 ± 124 nM for hMOR-$G_{i1\alpha}C^{351}I$ and the co-transfected but individually inactive fusion proteins respectively (Table 3.10). A reduction in potency to activate the G protein was observed for the reconstituted dimer and as noted previously for equal numbers of [^{3}H]-diprenorphine binding sites the maximal stimulation of [^{35}S]-GTPγS binding was higher for the wild type fusion.

Table 3.10: The EC_{50} for hMOR-$G_{i1\alpha}C^{351}I$ versus the reconstituted dimer: [^{35}S]-GTPγS binding studies

Construct	pEC_{50}	EC_{50} (nM)
hMOR-$G_{i1\alpha}C^{351}I$	6.46 ± 0.04	345 ± 29
Co-transfection	6.09 ± 0.07*	830 ± 124*

Data represent n=3 experiments performed in triplicate on different membrane preparations and the numbers are the means ± SEM.

T test was performed using pEC_{50}.

*Significantly different $P<0.05$

3.2.3 Human KOR homodimerisation studied by protein complementation

A/ Construction of non-functional but potentially complementary fusion proteins: rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$

To complete the study on opioid receptor homodimers, similar fusion proteins to those incorporating DOR and MOR were constructed for KOR. The rat KOR was first fused with the PTX resistant $G_{i1\alpha}$ subunit to generate rKOR-$G_{i1\alpha}C^{351}I$ (Figure 3.17A). Secondly, two distinct but potentially complementary pair of fusions were produced. The first of these, rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$, incorporated the same mutation in the G_α protein subunit as used previously (Figure 3.17B), wherein the second glycine of the GGQR motif was mutated into alanine to prevent guanine nucleotide exchange and hence activation. To generate the second fusion protein rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$, the highly conserved valines in the 2nd intracellular loop of rKOR were mutated to glutamic acid and aspartic acid respectively, hopefully to abolish receptor signalling to the G protein (Figure 3.17C). All transfected cells were treated 16h with PTX prior harvesting to inactivate endogenous $G_{i/o}$ proteins.

B/ Analysis of rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ expression and determination of the dissociation constant for [^{3}H]-diprenorphine in comparison with rKOR-$G_{i1\alpha}C^{351}I$

Expression of the different fusion proteins as well as the co-transfection of the distinct but potentially complementary pair of fusion proteins was similar with a B_{max} of some 2200 fmol/mg of protein (Table 3.11 and Figure 3.18). The K_d of rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and the co-transfected, mutated, fusion proteins were comparable to the wild type construct. However, rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ displayed a significantly different K_d compared to rKOR-$G_{i1\alpha}C^{351}I$ with a reduction in affinity for the antagonist [^{3}H]-diprenorphine. These results were similar to those previously noted for the DOR fusion proteins.

Table 3.11: Expression level (B_{max}) and dissociation constant (K_d) of rKOR-$G_{i1\alpha}C^{351}I$ fusion proteins

Construct	B_{max} (fmol/mg)	K_d	pK_d
rKOR-$G_{i1\alpha}C^{351}I$	2355 ± 193	0.51 ± 0.07	9.30 ± 0.06
rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$	2391 ± 177	1.35 ± 0.13**	8.88 ± 0.04**
rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$	2191 ± 148	0.49 ± 0.07	9.32 ± 0.06
Co-transfection	2417 ± 187	0.70 ± 0.09	9.17 ± 0.06

Data represent n=3 experiments performed in triplicate on different membrane preparations and the numbers are the means ± SEM.

Statistics were performed using 1 way ANOVA on B_{max} and pK_d numbers.

**Significantly different $P<0.01$

C/ Reconstitution of function using pairs of distinct non-functional rKOR-$G_{i1\alpha}C^{351}I$ mutants

Membranes containing 15 fmol of rKOR-$G_{i1\alpha}C^{351}I$ [^{3}H]-diprenorphine binding sites were treated with 10 µM or 100 nM of the highly selective KOR agonist U69593. A large stimulation of 1193 ± 319 and 868 ± 226% over basal was observed respectively (Table 3.12). Similar to the DOR and MOR fusion proteins, mutations introduced in the 2nd intracellular loop of the receptor of the first fusion and in the G protein of the other fusion protein resulted in the absence of signal upon agonist treatment. These results confirmed that both rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ and rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were non-functional as expected from their design (Figure 3.19). Once again, when the pair of distinct but complementary fusions were co-transfected, the wild type signal was reconstituted. Three times the number of binding sites of co-expressed mutated fusion proteins were necessary to reconstitute a full wild type signal. No significant difference was observed when membranes singly expressing both mutated fusion protein were mixed and agonist-stimulated ($P>0.05$, T test).

This demonstrates that for opioid receptor homodimers (MOR, DOR and KOR) the reconstitution produced by the co-expression of the two non-functional but potentially complementary fusion proteins must result from a transactivation in the dimer in which the wild type receptor activated the wild type G protein even though it was linked to the inactive receptor (Figure 3.20).

Similarly as to the DOR and MOR fusion proteins [^{35}S]-GTPγS binding was observed for KOR fusion proteins in the absence of agonist stimulation. This binding increased proportionally with the amount of fmol of mutanted fusion proteins used. This basal binding likely reflects some level of constitutive activity of the fusion proteins.

Table 3.12: [^{35}S]-GTPγS binding of rKOR-$G_{i1\alpha}C^{351}I$ fusion proteins

	Basal	U69593 10 μM	U69593 100 nM
rKOR-$G_{i1\alpha}C^{351}I$	100	1193 ± 319	868 ± 226
rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$	72 ± 12	95 ± 7	96 ± 9
rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$	120 ± 77	61 ± 15	71 ± 13
Co-transfection 15fmol	60 ± 5	384 ± 102	223 ± 45
30fmol	110 ± 17	688 ± 150	417 ± 68
45 fmol	145 ± 47	1193 ± 534	615 ± 180
Mix membrane 30fmol	155 ± 35	226 ± 8	212 ± 12

Data represent n=4 experiments performed in triplicate on different membrane preparations and numbers are the means ± SEM % versus basal of the wild type fusion protein.

D/ The affinity of the KOR selective agonist U69593 for rKOR-$G_{i1\alpha}C^{351}I$ fusion proteins

The affinity of the highly selective KOR agonist U69593 for single KOR fusion proteins or following co-transfection of the pair of mutated fusion proteins was investigated by competition experiments using [^{3}H]-diprenorphine (Figure 3.21). Competition curves were best fitted to a two binding sites model for the different fusion proteins except for rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$. For this construct, one binding site was the preferred model (Table 3.13). The double mutation introduced in the 2nd intracellular loop affected the receptor affinity for U69593 in a similar manner as for hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$. Only a low affinity binding site (Hill coefficient = -0.92 ± 0.07) was observed with a K_i=1007 ± 133 nM which was significantly lower compared to the wild type fusion protein (K_i=115 ± 41 nM). When the mutated pair of fusion proteins were expressed wild type pharmacology was

reconstituted, with no significant difference in the percentage of high affinity sites (P>0.05). To investigate these findings further, competition experiments were performed both in the presence or in the absence of 100 μM GppNHp for rKOR-$G_{i1\alpha}C^{351}I$ and $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$. As shown in Figure 3.22 in the presence of GppNHp the competition curve for rKOR-$G_{i1\alpha}C^{351}I$ was still best fitted to a two binding site model with similar K_h and K_l values (Table 3.14) as in the absence of GppNHp. The percentage of the high affinity binding site was not affected by the presence of GppNHp (P>0.05, T test). Likewise the K_i value for $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ was not altered in presence of GppNHp. This was not surprising because this fusion protein already exhibited only a low affinity binding site.

Table 3.13: Binding affinity of U69593 for the different fusion proteins and co-expression of the mutated pair of fusion proteins

Construct	K_h (nM)	pK_h	% high affinity site	K_l (nM)	pK_l	Hill coefficient
rKOR-$G_{i1\alpha}C^{351}I$	1.75 ± 0.54	8.85 ± 0.19	57 ± 7	115 ± 41	7.10 ± 0.18	-0.60 ± 0.02
$rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$				1007 ± 133*	6.00 ± 0.06*	-0.92 ± 0.07
rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1.71 ± 0.77	8.91 ± 0.21	50 ± 6	121 ± 59	7.12 ± 0.26	-0.57 ± 0.01
Co-transfection	2.00 ± 0.79	8.92 ± 0.31	48 ± 5	336 ± 127	6.62 ± 0.23	-0.45 ± 0.01

Data represent n=4 experiments performed in triplicate on different membrane preparations and are the means ± SEM.

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

Table 3.14: Binding affinity of U69593 in membranes expressing rKOR-$G_{i1\alpha}C^{351}I$ or $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ in the presence or absence of GppNHp

	rKOR-$G_{i1\alpha}C^{351}I$		rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$	
	Control	100μM GppNHp	Control	100μM GppNHp
K_h (nM)	0.82 ± 0.12	2.1 ± 2		
pK_h	9.1 ± 0.06	8.97 ± 0.47		
% high affinity site	37 ± 7	27 ± 10		
K_l (nM)	48 ± 10	107 ± 29	798 ± 293**	440 ± 70**
pK_l	7.34 ± 0.09	7.01 ± 0.11	6.16 ± 0.17	6.37 ± 0.06

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

Statistics were performed using 1 way ANOVA on pK_h and pK_l numbers.

**Significantly different P<0.01 compare to rKOR-$G_{i1\alpha}C^{351}I$ control in presence or absence of GppNHp

E/ The ability of rKOR-$G_{i1\alpha}C^{351}I$ and the reconstituted dimer to activate G protein in response to U69593

Increasing concentrations of U69593 were used to build a concentration response curve to compare its potency to activate G protein (Figure 3.23). Equal amounts of receptor [^{3}H]-diprenorphine binding sites were used in this study (15 fmol). Membranes expressing the reconstituted dimer exhibited an EC_{50} value of 168 ± 52 nM which was significantly different from the wild type fusion protein 54 ± 11 nM (Table 3.15).

Table 3.15: rKOR-$G_{i1\alpha}C^{351}I$ versus the reconstituted dimer: [^{35}S]-GTPγS binding studies

Construct	pEC_{50}	EC_{50} (nM)
rKOR-$G_{i1\alpha}C^{351}I$	7.29 ± 0.08	54 ± 11
Co-transfection	6.80 ± 0.13*	168 ± 52*

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

T test was performed using pEC_{50}.

*Significantly different $P<0.05$

3.3 Discussion:

Many GPCRs have now been described to form homodimers or higher order oligomers (Milligan, 2001; Angers *et al.*, 2002). This is also true for the different opioid receptors (Cvejic and Devi, 1997; Jordan and Devi, 1999), which have been reported to dimerise using different techniques such as immunoprecipitation and resonance energy transfer (McVey *et al.*, 2001; Ramsay *et al.*, 2002). These techniques were mainly qualitative and did not report anything about the function or mechanisms of dimerisation. In this study I used a complementation technique to further investigate the importance of opioid dimerisation for signalling and pharmacology.

Mutated DOR, MOR and KOR fusion proteins were constructed. Fusion proteins have become widely used tools (Wurch and Pauwels, 2001; Seifert *et al.*, 1999). The fusion of receptor to G protein has been shown to facilitate observation of agonist-induced signalling and owing to the 1:1 stoichiometry, the level of fusion protein expression can easily be monitored by saturation ligand binding assays. The effects of mutations in the receptor or the G protein can also be analysed.

In the complementation study the first fusion protein used contained a functional receptor linked to a non-functional G protein. The second fusion protein was composed of a non-functional receptor fused to a functional G protein. As the binding of [^{3}H]-diprenorphine was little affected by the mutations used, the expression of these fusion proteins was assessed by saturation binding experiments (Figures 3.4; 3.13 and 3.18). A B_{max} of approximately 2000 fmol/mg was obtained. This number was slightly lower for the hMOR-$G_{i1\alpha}C^{351}I$ fusion proteins. Opioid receptors are endogenously expressed in the brain at around 300 to 500 fmol/mg (Szekeres and Traynor, 1997; Zhao *et al.*, 2003) so significant overexpression of these fusions was produced by transient expression in HEK 293 cells. It has been suggested that dimerisation can be forced by overexpression of receptors. However, a study on β_2- and β_1-adrenoceptors showed that in a range of 0.44 to 46.6 pmol/mg dimerisation was independent of receptor density but above that, random collision events could be observed (Mercier *et al.*, 2002). Molinari et al., (2003) showed that the co-transfecton of a membrane targeted G protein and a non-fused receptor produced a similar agonist-induced [^{35}S]-GTPγS

binding as a receptor fused to a G protein. They concluded that a G protein only needs to be anchored to the membrane to interact with a nearby receptor suggesting that G protein activation in a fusion protein results from a close proximity with a receptor and not from dimer formation. However, in the work of Molinari et al., (2003) fusion proteins were expressed at very high levels, within the range of potential random collision events. In my study approximately 2 pmol/mg of receptor was used so the dimerisation observed should not simply reflect receptor proximity, but this point will be futher investigated in Chapter 4. Moreover, other groups have worked on opioid receptor dimerisation using similar receptor expression levels (e.g. Jordan and Devi, 1999). The K_d for [^{3}H]-diprenorphine of the different mutated fusion proteins was measured and compared to the respective wild type fusion proteins hDOR-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}C^{351}I$ and rKOR-$G_{i1\alpha}C^{351}I$ (Table 3.1, 3.7 and 3.11). The parental fusion proteins displayed similar K_d values to those that have been previously reported for opioid receptors (Szekeres and Traynor, 1997; Ramsay *et al.* 2002; Valenzano *et al.*, 2004). rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ and hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ displayed a reduction in affinity for [^{3}H]-diprenorphine of some 3 fold compare to their parental fusion proteins. Although the opioid receptor domains with the greatest homology are the transmembrane domains and the intracellular loops, this difference in K_d was not noted for rMORV^{169}EV^{173}D-$G_{i1\alpha}C^{351}I$. Diprenorphine has been described as a MOR antagonist but also to have some partial agonist effects on KOR and DOR (Szekeres and Traynor, 1997; Lewis and Husbands, 2004). This may explain the change of affinity observed for KOR and DOR but not the corresponding MOR fusion protein. The K_d values for [^{3}H]-diprenorphine of the fusion proteins mutated in the G protein part and the different co-transfections was not significantly different from the wild type proteins except for the co-expressed pair of DOR fusion proteins. One possible explanation might be that in the co-expression hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ was highly expressed compared to hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$. The observed K_d would predominantly reflect the hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ construct. However, this was not obvious from the expression levels measured in individual transfections which exhibited similar B_{max} levels, 2181 ± 228 and 1777 ± 285 fmol/mg of protein respectively. Although it is impossible to measure what level of each construct was present when they were co-transfected from analysis of binding studies, the immunoblots of Figure 3.7 do not suggest different levels of hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ in the singly or co-transfected samples.

Immunoprecipitation studies demonstrated that neither adding G protein α subunit to the end of the receptor nor introducing mutations prevented DOR from dimerising (Figure 3.7). Co-immunoprecipitation has been widely used to study GPCR dimerisation (Herbert *et al.*, 1996; Ng *et al.*, 1996; Jordan and Devi, 1999) but certain studies have suggested that it is maybe a non-specific process (Salim *et al.*, 2002) raising the possibility that incomplete receptor solubilisation may lead to biological artefacts. Studies using resonance energy transfer techniques BRET or FRET have been used to complement immunoprecipitation data (Angers *et al.*, 2000; Rocheville *et al.*, 2000a). These techniques have allowed observation of dimerisation in living cells but also have their limitations. The signal observed is dependent on the distance between the receptors or energy donor and acceptor molecules and is subject to the orientation of the electromagnetic dipoles. These limitations, as well as the success of other receptor complementation studies, are one of the reasons why complementation was employed to study dimerisation in my work.

In addition to using pairs of distinct but complementary fusion proteins, [^{35}S]-GTPγS binding was measured to monitor complementation. This permitted the activation of the G protein to be monitored by measuring exchange of GDP for GTP using an analogue of GTP that is resistant to GTPase activity. Guanine nucleotide exchange is a very early event in the signal transduction cascade. Therefore it is an attractive level to study as it is less subjected to amplification or regulation by other cellular processes and thus directly reflects ligand-induced signalling. It was demonstrated that a linear rise in agonist stimulated [^{35}S]-GTPγS binding was observed when increasing amounts of wild type fusion proteins were used (Figure 3.5). In each experiment expression of the fusion proteins were measured and equal amounts of each construct employed. When the various mutated fusion proteins were transfected alone no substantial agonist-induced stimulation was observed. The mutations introduced therefore successfully abolished any information transfer between receptor and its fused G protein and vice versa. This was anticipated from the conservation of sequences in the targeted region between GPCRs and G proteins used herein and the previous studies of Carrillo *et al.*, (2003). Moreover, introduction of the C^{351}I mutation in the $G_{i1\alpha}$ subunit confers PTX resistance and successfully prevented activation of the endogenous $G_{i/o}$ pool by the receptors of the fusion proteins. However, when the contrasting pairs of non-functional mutants were co-transfected a signal was detected after agonist stimulation (Figures 3.6, 3.14 and 3.19). Receptor function was therefore rescued. The functional receptor was able to

activate the functional G protein fused to the non-functional receptor thus generating a signal (Figure 3.20).

Although opioid receptors are class A rhodopsin-like GPCRs, the transactivation observed within the dimer is similar to results monitored from class C receptors. Galvez *et al.*, (2001) showed that the GABAb receptor where the GABAb1 subunit contains the GABA binding site is unable to activate G protein. However when co-tranfected with GABAb2, which cannot bind GABA, function is generated.

Studies involving chimeras of muscarinic M3 and α_{2c}-adrenoceptors have also shown restoration of function (Maggio *et al.*, 1993a). When individually expressed such chimeras could not bind muscarinic or adrenergic radioligands nor transmit a signal. After co-transfection, however, a significant number of muscarinic and α_{2c}-adrenoceptor binding sites were detected. An agonist-dependent increase in phosphoinositide breakdown was also produced, reflecting a recovery of muscarinic receptor signalling ability. These results described the importance of dimerisation in receptor signalling and are in agreement with that observed in my study.

The complementation approach I have employed was used previously in our group for α_{1b}-adrenergic and histamine H1 receptors. Similar results to those I have described for opioid receptors were reported (Carrillo *et al.*, 2003). In the case of the histamine H1 receptor when complementary fusion proteins were co-expressed, the presence of twice the number of receptor binding sites were required to generate as large a signal as from the wild type fusion protein. It was argued that if the functional receptor is a dimer then when the complementary fusion proteins are co-expressed half of the dimers formed should be non-functional. Dimers of fusion proteins mutated both in the receptor part or in the G protein would be non-functional. The other 50% represent combinations of the different fusion proteins that subsequently signal in the presence of agonist. A similar ratio was not observed in the case of the α_{1b}-adrenoreceptor (Carrillo *et al.*, 2003). Monnot et al, (1996), also witnessed intermolecular complementation using two ligand binding-deficient type 1 angiotensin II receptors. When singly expressed those mutated receptors were unable to bind different ligands, but upon co-expresssion 5% of the binding sites were restored compared to the wild type receptor. These studies suggest that class A GPCRs differ in their ability to form dimers. For opioid receptors a signal corresponding to wild type was generated when three times the number of [^{3}H]-diprenorphine binding sites of the co-transfected fusion proteins were used. A first hypothesis to explain these data might be that opioid receptors are present as dimers but

also as monomers at the plasma membrane. This might allow different pharmacology or systems of regulation to be observed between monomers and dimers. If the fusions were present as a mixture of dimers and monomers, the chance of forming the functional dimer would decrease.

The second explanation for the ratio observed is that one non-functional construct was under-expressed compared to the other, reducing the chances of forming a functional dimer. It was not possible to test this idea directly as the fusion proteins contained the same receptor but individual expression of each fusion protein did not result in markedly different expression level (Tables 3.1, 3.7 and 3.11). This hypothesis is therefore considered less likely.

This difference in ratio could also be due to the mutations introduced diminishing the ability of the receptor to form a dimer, but, in general, domains described to be involved in homo-dimerisation of opioid receptors appear to be the transmembrane domains (Filizola and Weinstein, 2002) or the C-terminal tail (Cvejic and Devi, 1997) and these regions were not mutated in my studies.

One commonly proposed purpose for GPCR dimerisation is that the cytoplasmic surface area of a GPCR monomer is not large enough, 42Å for the inactive rhodopsin monomer, to be in contact with both α and $\beta\gamma$ elements of a G protein heterotrimer. This supports the idea that the activating platform for G protein activation is a dimer (Bouvier, 2001; Hamm, 2001, Arimoto *et al.*, 2001). In the case of the class C GPCR, the GABAb receptor Margeta-Mitrovic et al, (2001) demonstrated that only a single GPCR monomer was necessary for the activation of G protein within a dimeric receptor by using receptor chimeras. The crystallisation of the rhodopsin receptor (Palczewski *et al.*, 2000) in combination with visualisation of these molecules by atomic force microscopy suggested that rhodopsin forms dimers and oligomers and that the size and geometry of such dimers allow a perfect fit for the binding of one G protein (Liang *et al.*, 2003). These observations are consistent with those of Banères and Parello, (2003) on the leukotriene B4 receptor BLT1. Using a combination of chemical cross- linking followed by size-exclusion chromatography and mass spectroscopy they established that only one G protein $\alpha\beta\gamma$ trimer binds to a receptor dimer. In my study the functional receptor of the dimer unit was able to activate the G protein linked to the non-functional receptor under agonist stimulation (Figure 3.20). This result suggests that one G protein per receptor dimer is sufficient to transduce a signal.

Basal, constitutive activity was also measured for all the active fusion proteins. A linear rise in such activity was seen as the amount of fusion protein was increased. This basal activity is

likely to reflect constitutive activity of the fusion proteins as the introduction of the $C^{351}I$ mutation in the G protein has previously been shown to optimise basal activity (Kellett *et al.*, 1999; Welsby *et al.*, 2002).

Ligand affinity for the different mutated fusion proteins was studied as it was important to check that alterations introduced would not cause substantial changes to their ability to bind ligands. Competition binding experiments were performed for the different opioid receptor fusion proteins using a variety of opioid agonists (Figures 3.8; 3.9; 3.15; 3.21). The parental fusion proteins were used as references to compare the agonist binding affinity of the modified constructs. All [^{3}H]-diprenorphine versus agonist competition curves for the wild type fusion proteins were preferentially fitted by a two binding site model. No significant differences in agonist binding were noticed when the $G^{202}A$ mutation was introduced into the $G_{i1\alpha}$ subunit fused to the different opioid receptors. This may reflect that GDP bound G protein forms a relatively high affinity complex with these receptors. However the scenario was different in the case of the double modifications introduced in the 2nd intracellular loop of the receptors. Each opioid receptor demonstrated a change in its ability to bind agonist. This difference was most obvious in the case of $rKORV^{160}E,V^{164}D\text{-}G_{i1\alpha}C^{351}I$ and $hMORV^{169}E,V^{173}D\text{-}G_{i1\alpha}C^{351}I$ with a complete loss of high affinity binding sites resulting in a single site curve. For $hDORV^{150}E,V^{154}D\text{-}G_{i1\alpha}C^{351}I$ by contrast only a shift in the affinity for both high and low binding sites for DADLE and DPDPE was witnessed (Figures 3.8 and 3.9). The 2nd and 3rd intracellular loops and the C-terminal tail of GPCRs have been described to be involved in the activation and selectivity of G protein coupling (Liu and Wess, 1996; Wess, 1998, Verrall *et al.*, 1997, Konig *et al.*, 1989). Peptides mimicking these three regions in MOR and DOR were used to identify which part of the receptor was able to activate G protein (Merkouris *et al.*, 1996, Georgoussi *et al.*, 1997). The 3rd intracellular loop and C-terminal tail were found to be involved in both MOR and DOR G protein coupling. Although described as a non-specific effect by the authors, the peptide mimicking a part of MOR 2nd intracellular loop was found to decrease 40% of the wild type [^{35}S]-GTPγS binding suggesting some role in G protein activation. One of the possibilities, in my study is that mutations may have abolished the association between receptor and the G protein, leading to the loss of the high affinity binding site.

This idea was tested for the $rKOR\text{-}G_{i1\alpha}C^{351}I$ fusion proteins (Figure 3.22). Competition experiments using $rKOR\text{-}G_{i1\alpha}C^{351}I$ and $rKORV^{160}E,V^{164}D\text{-}G_{i1\alpha}C^{351}I$ were carried out in the presence or in the absence of GppNHp, a non-hydrolysable analogue of GTP which binds

permanently to the G protein. However, GppNHp failed to convert the rKOR-$G_{i1\alpha}C^{351}I$ agonist binding curve to a single low affinity state. Ugur et al, (2003) studied binding characteristics of the β_2-adrenoceptor fused to a wild type or mutated G_s protein agonist in the absence or presence of GTPγS. In this study the membranes expressing the wild type fusion protein displayed high and low affinity binding sites whereas when the G_s subunit of the fusion protein was mutated only a single binding site was detected. However, in their case, in the presence of GTPγS the wild type β_2-adrenoceptor-G_s fusion protein high affinity binding site was abolished and a similar low affinity site as for the mutated version of the fusion protein was observed. This was not the case in my study where in the presence of GppNHp the wild type fusion protein still displayed both a high and low affinity binding site. This absence of shift to a single binding site in the presence of GppNHp was also witnessed by other groups for the dopamine D_2 or the β_2-adrenoceptor fused to G_α subunits (Gazi *et al.*, 2003, Seifert *et al.*, 1999). One possible explanation may be related to the fact that the KOR was fused to the $G_{i1\alpha}$ protein, leading to a spatial constraint due to the proximity of the C-terminus of the receptor and N-terminus of the G protein (Wurch and Pauwels, 2001). In this case, the modifications introduced in the 2nd intracellular loop of opioid receptors may have abolished or created different spatial contraints.

For all three receptors the co-transfection of the distinct but complementary pairs of fusion proteins reconstituted the pharmacology of the wild type receptor with no significant differences observed between the respective K_h and K_l values for agonist ligands or percentage observed for high and low affinity sites. These results reflect a physical interaction between the two receptors forming a dimer leading to a rescued binding pocket that other groups have also recorded. Galvez *et al.*, (2001) described that even though the extracellular binding domain of the GABAb1 subunit of the GABAb receptor was sufficient to bind GABA, the extracellular domain of the GABAb2 subunit had increased agonist affinity when they formed a dimer. Although more extreme, two angiotensin II receptor (AT_1) mutants where agonist binding was abolished by different mutations, lead to restoration of a normal binding site when co-expressed (Monnot *et al.*, 1996). Two structural models of dimer formation have emerged in order to explain changes in functions and pharmacology observed upon dimerisation, namely contact dimers (Herbert *et al.*, 1996) and the domain-swapped theories (George *et al.*, 2002). In contact dimers, certain transmembrane domains are though to interact via hydrophobic interactions whereas in the domain-swapped theory, transmembrane domains of both receptors are likely to be exchanged. Until recently receptor

dimerisation was described by either of these two theories. In a recent study, two histamine H1 receptor mutants unable to bind radioligands, showed a reconstituted radioligand binding site when co-expressed (Bakker *et al.*, 2004). The authors suggested that the ligand binding sites were reconstituted upon mutual exchange of transmembrane domains from both receptors suggesting a domain-swapped dimer, however the difference observed between [^{3}H]-mepyramine and [^{3}H]-PAT B_{max} values also suggested the presence of contact dimers, implying that a mix of contact and domaim swapped dimers could co-exist at the plasma membrane.

One of the advantages of complementation studied by [^{35}S]-GTPγS binding was that only dimers are anticipitated to be functional. For this reason it was interesting to note what happened to the potency of opioid ligands to activate or inactivate the G protein of the reconstituted dimer. In the case of DOR, no significant difference in EC_{50} or IC_{50} was observed between the wild type fusion protein and the reconstituted dimer when they were stimulated by the DOR agonist DADLE or inhibited by the opioid antagonist naloxone (Figures 3.10 and 3.11). This could suggest that DOR is only expressed in a dimeric form at the plasma membrane.

In the case of KOR and MOR, a decrease of EC_{50} for agonist was noticed for the reconstituted dimers (Tables 3.10 and 3.15) in comparison to the parental fusion proteins. A first hypothesis to explain this reduction in agonist affinity is related to the observations witnessed in the competition binding experiments. In these experiments when either rKORV^{160}E,V^{164}D-$G_{i1\alpha}$C^{351}I or hMORV^{169}E,V^{173}D-$G_{i1\alpha}$C^{351}I were expressed a loss of agonist affinity was observed for DAMGO and U69593 (Figure 3.15 and 3.21). These agonists were used to study the ability of the reconstituted dimer to activate G protein function, thus the loss of agonist affinity observed in the competition experiment might affect G protein coupling. However, this does not seem likely as the wild type pharmacology for DAMGO and U69593 was reconstituted when the mutated fusion proteins were co-expressed, suggesting that in the reconstituted dimer a high affinity binding site was rescued. However, the mutation in the 2nd intracellular loop could have impaired G protein coupling of the homodimer based on the hypothesis that MOR and KOR formed a swapped dimer. Consequently the mutations introduced in KOR could affect the potency of the dimer to activate the G protein. A second hypothesis is that MOR and KOR were expressed as a mixed population of monomers and dimers at the plasma membrane. In this case the reduction in potency observed for the

reconstituted dimer could reflect monomers being more potent to activate G protein compared to dimers.

Figure 3.1: Graphic representation of hDOR-$G_{i1}C^{351}I$; hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$

A. hDOR-$G_{i1\alpha}C^{351}I$ fusion protein

This fusion protein consisted of DOR fused to $G_{i1\alpha}C^{351}I$. The $G_{i1\alpha}$ contained a cysteine mutated into isoleucine to prevent ADP ribosylation by PTX.

B. hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ fusion protein

This fusion protein consisted of DOR fused to the mutated $G_{i1\alpha}C^{351}I$ subunit. The glycine at position 202 of $G_{i1\alpha}C^{351}I$ was mutated into an alanine.

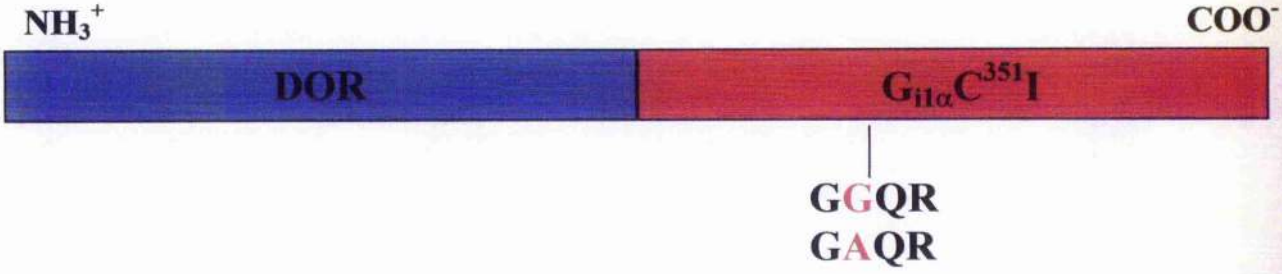
C. hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ fusion protein

This fusion protein consisted of DOR mutated in the 2nd intracellular loop of the receptor. Two valines in positions 150 and 154 were mutated into glutamic acid and aspartic acid respectively.

A


NH3+
COO-
DOR
Gi1αC351I

B


NH3+
COO-
DOR
Gi1αC351I
GGQR
GAQR

C

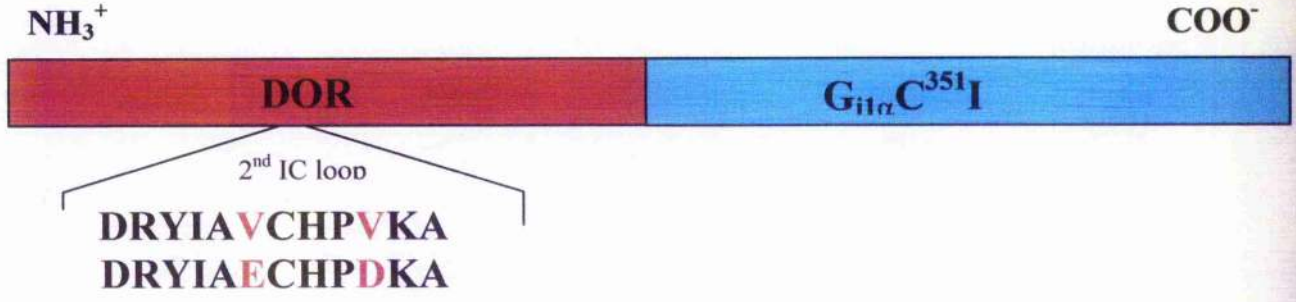

NH3+
COO-
DOR
Gi1αC351I
2nd IC loop
DRYIAVCHPVKA
DRYIAECHPDKA

Figure 3.2: Conserved GGQR sequence in G protein α subunits

Comparison of G protein α subunit sequences belonging to different subfamilies and/or species. The alignment of the sequences highlights the conserved glycine contained in the GGQR motif that was mutated.

G alpha subunit	Species	sequence
G_s	Human	VGLQRDERRK
G_{olf}	Human	VGLQRDERRK
G_{i1}	Human	VGLQRSERKK
	Rat	VGLQRSERKK
G_{i2}	Human	VGLQRSERKK
	Mouse	VGLQRSERKK
G_{i3}	Human	VGLQRSERKK
	Rat	VGLQRSERKK
G_{o1}	Human	VGLQRSERKK
	Bovine	VGLQRSERKK
G_{o2}	Human	VGLQRSERKK
G_z	Human	VGLQRSERKK
	Rat	VGLQRSERKK
Gt_1	Bovine	VGLQRSERKK
Gt_2	Human	VGLQRSERKK
Ggust	Rat	VGLQRSERKK
Gq	Human	VGLQRSERRK
	Mouse	VGLQRSERRK
G_{11}	Human	VGLQRSERRK
	Rat	VGLQRSERRK
G_{12}	Human	VGLQRSQRQK
	Rat	VGLQRSQRQK
G_{13}	Human	VGLQRSERKR
G_{14}	Human	VGLQRSERRK
G_{15}	Mouse	VGLQRSERRK
G_{16}	Human	VGLQKSERKK

From Milligan et al, (2004)

Figure 3.3: Highly conserved hydrophobic residues in the 2nd intracellular loop of the rhodopsin-like, class A GPCRs

Comparison of amino acid sequences situated downstream of the DRY motif in the 2nd intracellular loop of different class A GPCRs. The alignment of the sequences highlights the conserved hydrophobic residues that were mutated.

GPCR	specie	G protein	sequence
5HT1A receptor	**human**	**Gi/Go**	DRYWAITDPID
5HT1B receptor	**mouse**	**Gi/Go**	DRYWAITDAVE
5HT1D receptor	**rabbit**	**Gi/Go**	DRYWAITDALE
5HT2A receptor	**rat**	**Gq/G11**	DRYVAIQNPIH
5HT2C receptor	**rat**	**Gq/G11**	DRYVAIRNPIE
5HT4 receptor	**mouse**	**Gs**	DRYYAICCQPL
5HT6 receptor	**rat**	**Gs**	DRYLLILSPLR
α1a adrenergic receptor	**bovine**	**Gq/G11**	DRYIGVSYPLR
α1b adrenergic receptor	**hamster**	**Gq/G11**	DRYIGVRYSLQ
α2b adrenergic receptor	**rat**	**Gi/Go**	DRYWAVSRALE
β1 adrenergic receptor	**human**	**Gs**	DRYLAITSPFR
β2 adrenergic receptor	**bovine**	**Gs**	DRYLAITSPFK
β3 adrenergic receptor	**mouse**	**Gs**	DRYLAVTNPLR
A1 adenosine receptor	**human**	**Gi/Go**	DRYLRVKIPLR
A3 adenosine receptor	**human**	**Gi/Go**	DRYLRVKLTVR
M1 receptor	**mouse**	**Gq/G11**	DRYFSVTRPLS
M2 receptor	**human**	**Gi/Go**	DRYFCVTKPLT
M3 receptor	**mouse**	**Gi/Go**	DRYFSITRPLT
Melanocortin2 receptor	**human**	**Gs**	DRYITIFHALR
AT1A receptor	**human**	**Gq/G11**	DRYLAIVHPMK
AT1B receptor	**rat**	**Gq/G11**	DRYLAIVHPMK
B2 bradikinin receptor	**human**	**Gq/G11**	DRYLAIVKTMS
CXCR3	**mouse**	**Gi/Go**	DRYLSIVHATQ
CXCR4	**human**	**Gi/Go**	DRYLAIVHATN
D2 receptor	**mouse**	**Gi/Go**	DRYTAVAMPML
D3 receptor	**rat**	**Gi/Go**	DRYTAVVMPVH
FSHR	**bovine**	**Gs**	ERWHTITHAMQ
GRHR	**mouse**	**Gq/G11**	DRSLAITQPLA
H1 receptor	**mouse**	**Gq/G11**	DRYRSVQQPLR
H2 receptor	**human**	**Gs**	DRYCAVMDPLR
LSHR	**mouse**	**Gs**	ERWHTITYAVQ
δ opioid receptor 1	**rat**	**Gi/Go, Gz**	DRYIAVCHPVK
κ opioid receptor 1	**mouse**	**Gi/Go**	DRYIAVCHPVK
μ opioid receptor 1	**rat**	**Gi/Go, Gz**	DRYIAVCHPVK
rhodopsin	**bovine**	**Gt**	ERYVVVCKPMS
Oxytocin receptor	**rat**	**Gq/G11**	DRCLAICQPLR
P2U purinoceptor 1	**rat**	**Gq/G11**	HRCLGVLRPLH
Prostaglandin D2 receptor	**mouse**	**Gs**	ECWLSLGHPFF
Prostaglandin E2 receptor	**rat**	**Gq/G11**	ERCVGVTQPLI
Somatostatin receptor 2	**human**	**Gi/Go Gq**	DRYLAVVHPIK
TRH receptor	**rat**	**Gq/G11**	ERYIAICHPIK

From Milligan et al, (2004)

Figure 3.4: [^{3}H]-Diprenorphine saturation binding assays following expression of DOR-$G_{i1\alpha}$ fusion proteins

Membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ (dark blue squares); hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green dots); hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (light blue dots) and of the co-transfection of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the specific binding of different concentrations of [^{3}H]-diprenorphine. Data are representative of n=4 experiments performed in triplicate. Data points represent means ± SEM.

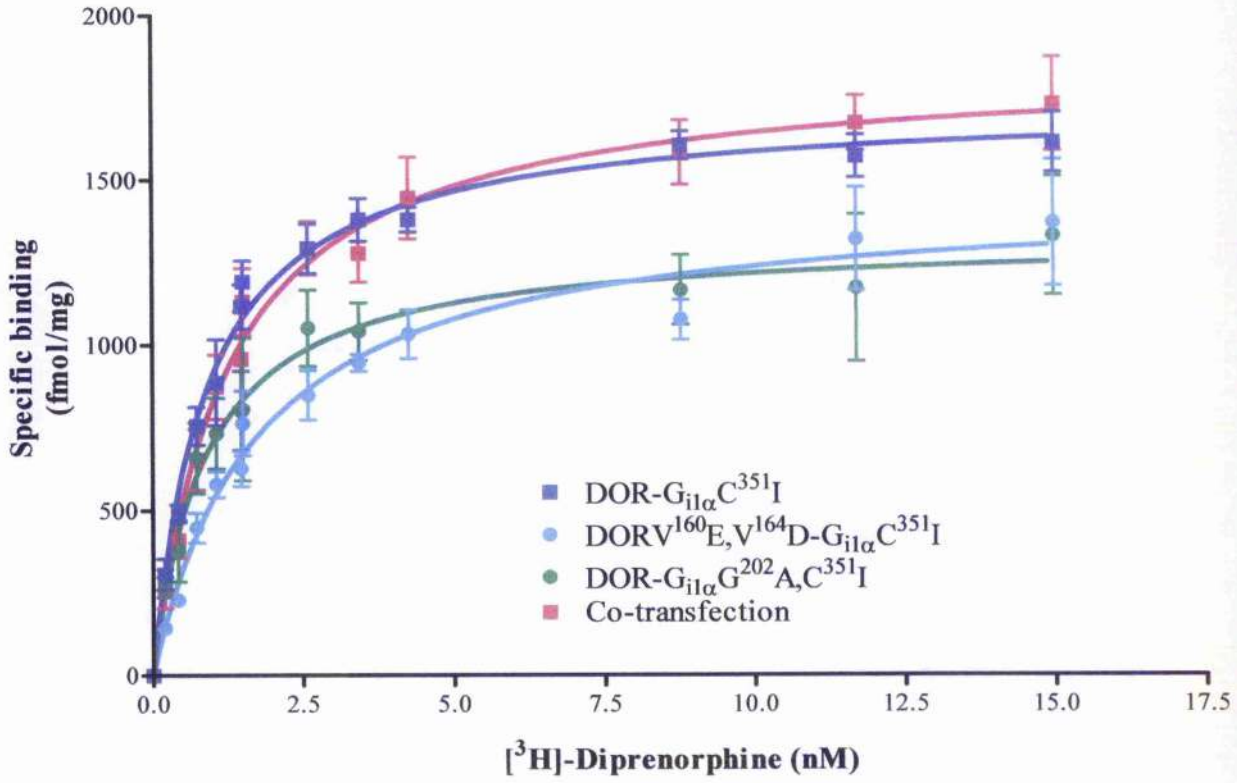

2000
1500
1000
500
0
Specific binding (fmol/mg)
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
[3H]-Diprenorphine (nM)
DOR-Gi1αC351I
DORV160E,V164D-Gi1αC351I
DOR-Gi1αG202A,C351I
Co-transfection

Figure 3.5: DADLE activates hDOR-$G_{i1\alpha}C^{351}I$

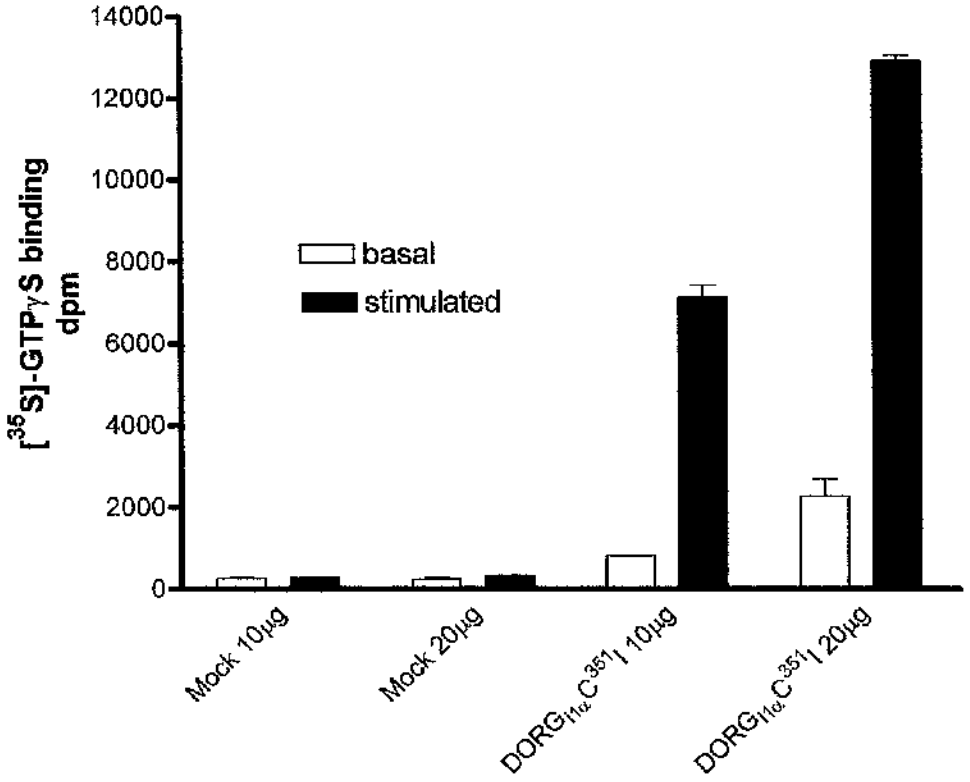
A. Specific activation of hDOR-$G_{i1\alpha}C^{351}I$

Membranes of HEK 293 cells expressing or not hDOR-$G_{i1\alpha}C^{351}I$ were used to measure the binding of [^{35}S]-GTPγS in the absence (open bars) or presence (filled bars) of 10μM DADLE. Data are representative of n=3 experiments performed in triplicate.

B. Increasing levels of hDOR-$G_{i1\alpha}C^{351}I$ proportionally increase agonist stimulated [^{35}S]-GTPγS binding

Increasing amounts of membranes of HEK 293 cell expressing hDOR-$G_{i1\alpha}C^{351}I$ were used to measure the binding of [^{35}S]-GTPγS in the presence of 10 μM DADLE. Numbers represent stimulation over basal. Data are representative of n=3 experiments performed in triplicate.

A


14000
12000
10000
8000
6000
4000
2000
0
[35S]-GTPγS binding dpm
basal
stimulated
Mock 10μg
Mock 20μg
DORG$_{i1\alpha}$C^{351}] 10μg
DORG$_{i1\alpha}$C^{351}] 20μg

B

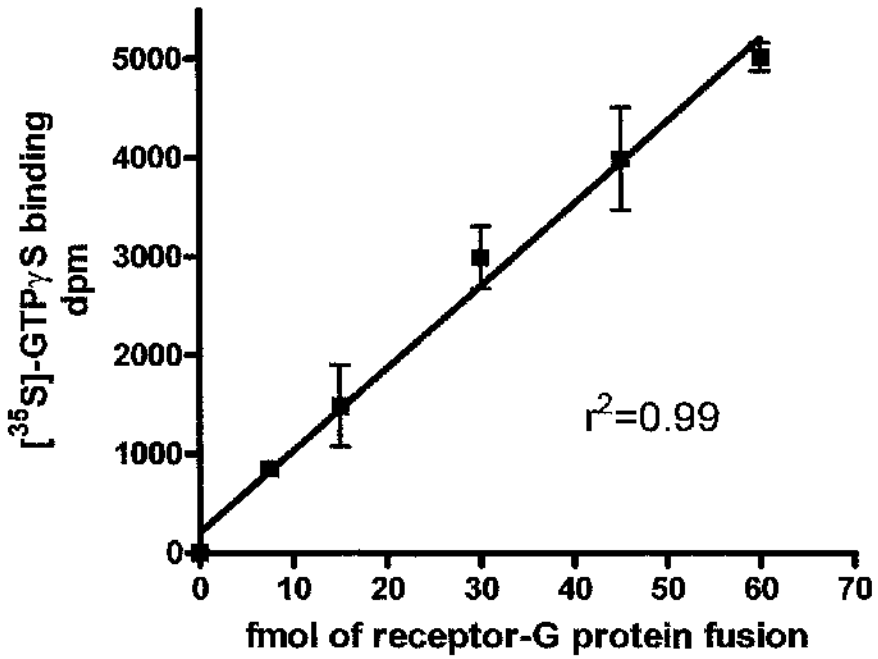

5000
4000
3000
2000
1000
0
[35S]-GTPγS binding dpm
r2=0.99
0
10
20
30
40
50
60
70
fmol of receptor-G protein fusion

Figure 3.6: Co-expression of a pair of distinct non-functional mutants of hDOR-$G_{i1\alpha}C^{351}I$ fusion proteins reconstitute function

Membranes of HEK 293 cells expressing 15fmol of hDOR-$G_{i1\alpha}C^{351}I$, hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$, hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or 30 or 45 fmol in the case of hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$+ hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ co-transfection were used to measure [^{35}S]-GTPγS binding in the absence (open bars) or presence (filled bars) of 10 μM DADLE. Membrane expressing 15 fmol of hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ and 15 fmol of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were also mixed and stimulated with DADLE. Data represent of n=5 experiments performed in triplicate. Data points represent means ± SEM.

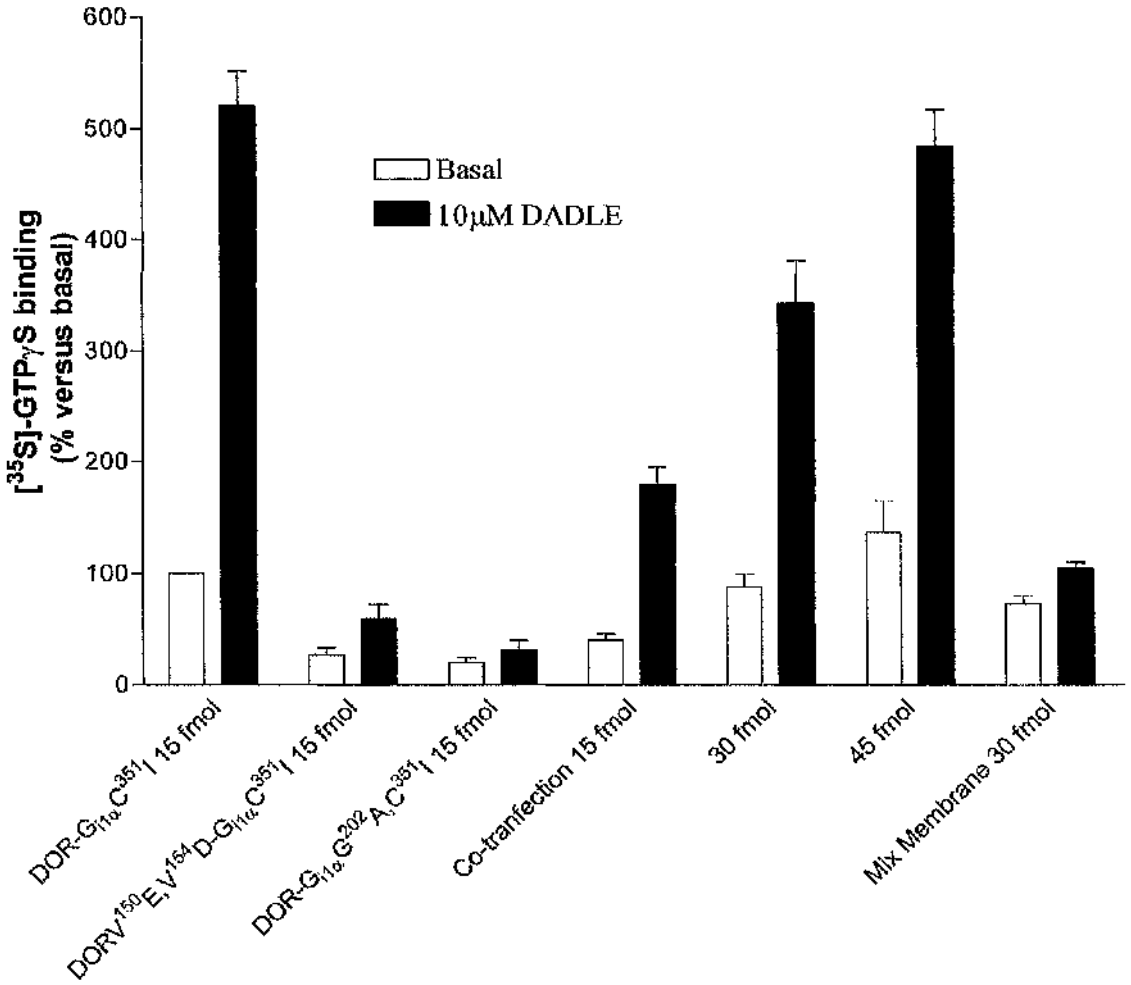

600
500
400
300
200
100
0
[35S]-GTPγS binding
(% versus basal)
Basal
10μM DADLE
DOR-G$_{i1\alpha}$C^{351}] 15 fmol
DORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}] 15 fmol
DOR-G$_{i1\alpha}$G^{202}A,C^{351}] 15 fmol
Co-tranfection 15 fmol
30 fmol
45 fmol
Mix Membrane 30 fmol

Figure 3.7: Co-immunoprecipitation of a pair of epitope tagged non-functional fusion proteins

A. Co-immunoprecipitation of differentially epitope-tagged forms of the mutated DOR fusion proteins

Membranes from HEK 293 cells (1) and cells transiently expressing Flag-hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I (2), Myc-hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I (3), Flag-hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I + Myc-hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I (4) or mix membrane of Flag-hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I and Myc-hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I (5) were immunoprecipitated with Flag antibody and detected with Myc after being resolved by SDS-PAGE. Data are representative of n=3 experiments.

B. Anti-Flag antibody was used to detect anti-Flag reactive patterns

The same membranes as in panel A were rebloted using anti-Flag antibody.

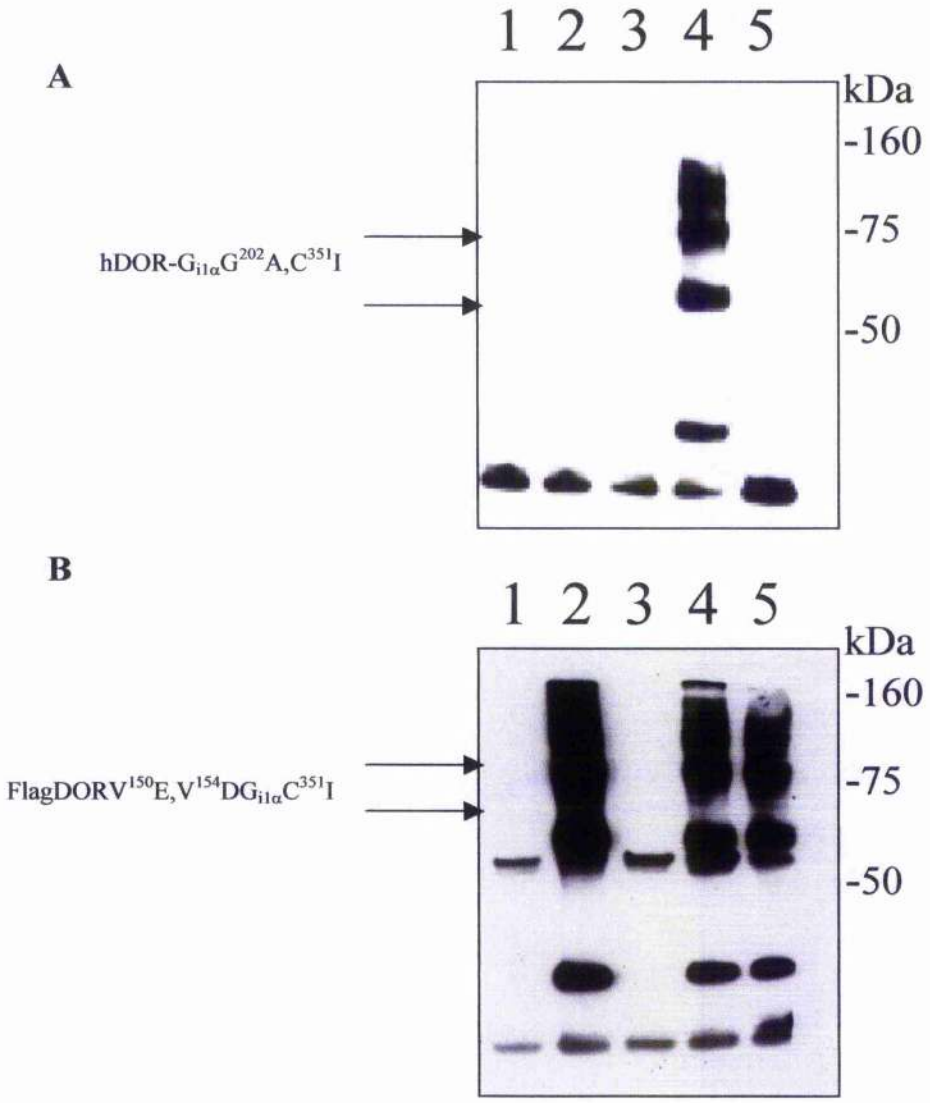

A
1 2 3 4 5
kDa
-160
-75
-50
hDOR-Gi1αG202A,C351I
B
1 2 3 4 5
kDa
-160
-75
-50
FlagDORV150E,V154DGi1αC351I

Figure 3.8: Effect of mutation and co-expression of hDOR-$G_{i1\alpha}C^{351}I$ fusion proteins on competition between [^{3}H]-Diprenorphine and DADLE

Membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ (dark blue squares), hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green dots), hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (light blue dots) and both hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the ability of varying concentrations of DADLE to compete with 1nM [^{3}H]-diprenorphine. Data are representative of n=4 experiments performed in triplicate. Data points represent means ± SEM.

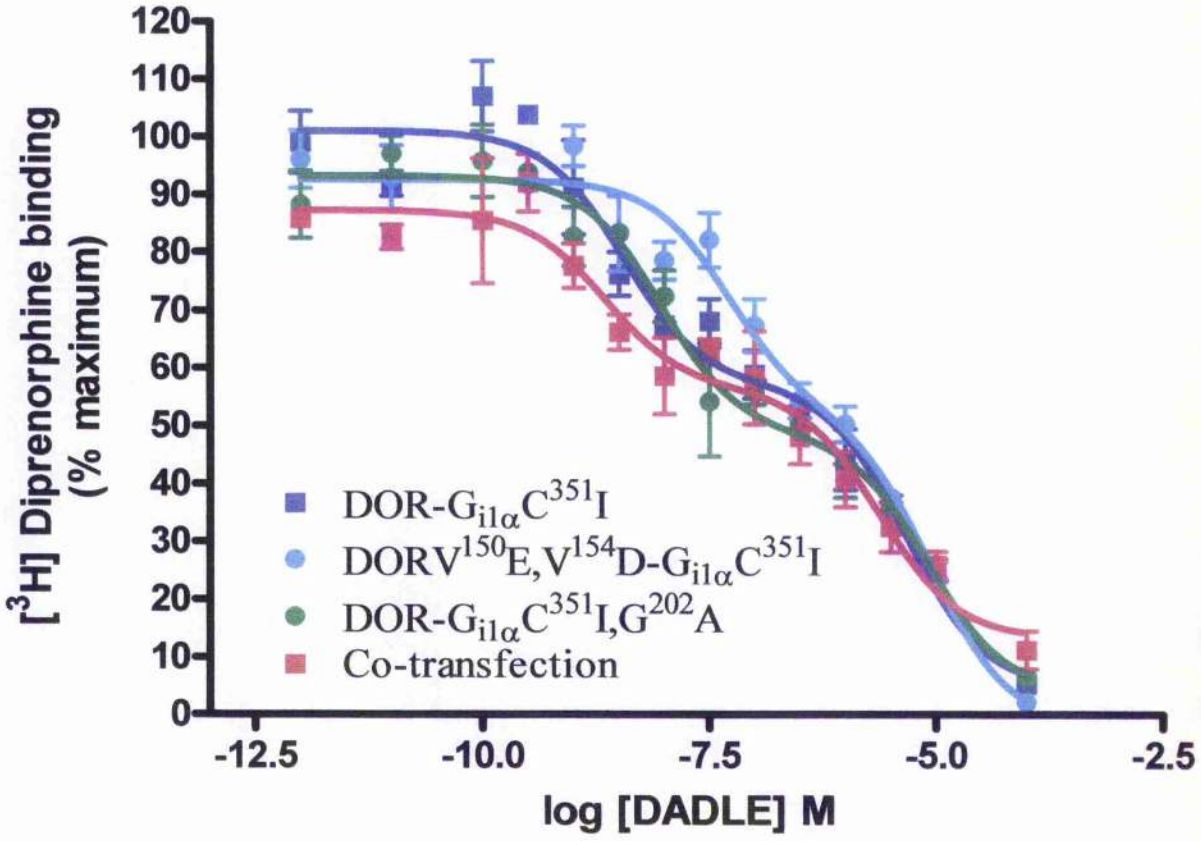

[3H] Diprenorphine binding (% maximum)
120
110
100
90
80
70
60
50
40
30
20
10
0
-12.5
-10.0
-7.5
-5.0
-2.5
log [DADLE] M
DOR-Gi1αC351I
DORV150E,V154D-Gi1αC351I
DOR-Gi1αC351I,G202A
Co-transfection

Figure 3.9: Effect of mutation and co-expression of hDOR-$G_{i1\alpha}C^{351}I$ fusion proteins on competition between [^{3}H]-Diprenorphine and DPDPE

Membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ (dark blue squares), hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green dots), hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (light blue dots) and both hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the ability of varying concentrations of DPDPE to compete with 1nM [^{3}H]-diprenorphine. Data are representative of n=4 experiments performed in triplicate. Data points represent means ± SEM.

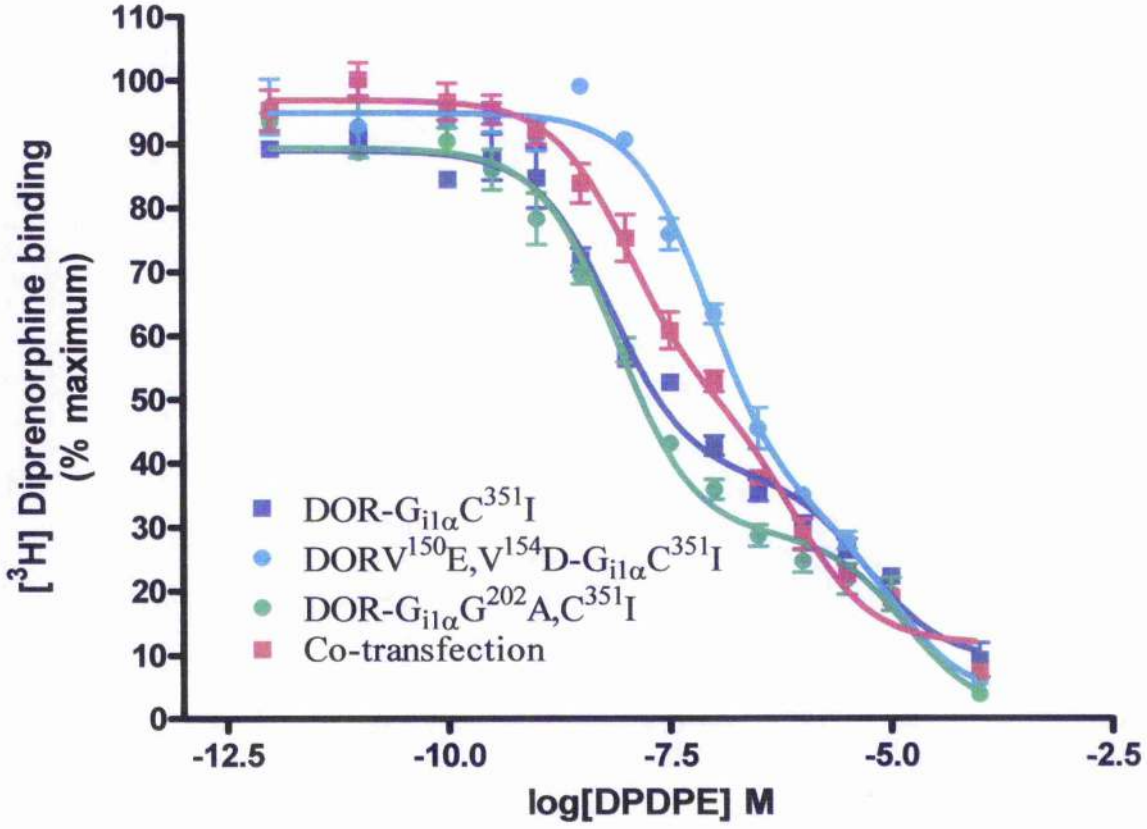

[3H] Diprenorphine binding (% maximum)
110
100
90
80
70
60
50
40
30
20
10
0
-12.5
-10.0
-7.5
-5.0
-2.5
log[DPDPE] M
DOR-Gi1αC351I
DORV150E,V154D-Gi1αC351I
DOR-Gi1αG202A,C351I
Co-transfection

Figure 3.10: Comparaison of agonist stimulated [^{35}S]-GTPγS binding of hDOR-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ reconstituted dimer

Membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ (blue squares) or co-transfected with hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the ability of increasing concentrations of DADLE to activate [^{35}S]-GTPγS binding. Data represents n=3 experiments performed in triplicate. Data points represent means ± SEM.

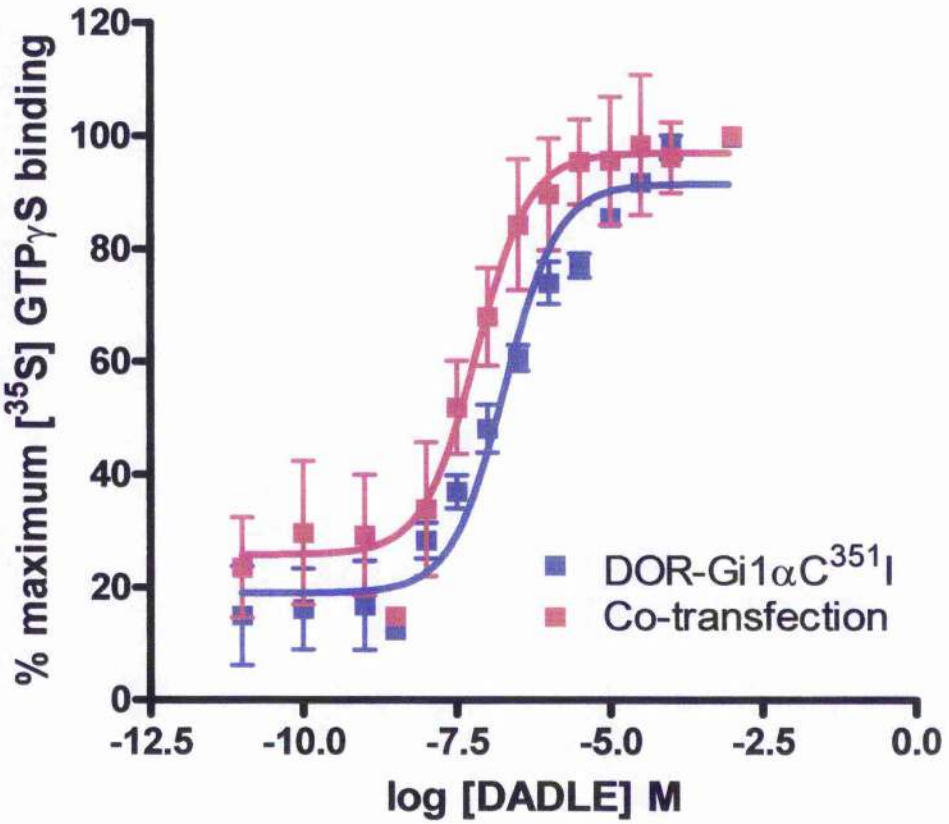

% maximum [35S] GTPγS binding
120
100
80
60
40
20
0
-12.5
-10.0
-7.5
-5.0
-2.5
0.0
log [DADLE] M
DOR-Gi1αC351I
Co-transfection

Figure 3.11: Comparaison of the ability of naloxone to reverse DADLE stimulated [^{35}S]-GTPγS binding of hDOR-$G_{i1\alpha}C^{351}I$ and the reconstituted dimer

Membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ (blue squares) or co-transfected with hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the ability of increasing concentrations of naloxone to inactivate [^{35}S]-GTPγS binding produced by 10^{-7}M DADLE. Data represents n=3 experiments performed in triplicate. Data points represent means ± SEM.

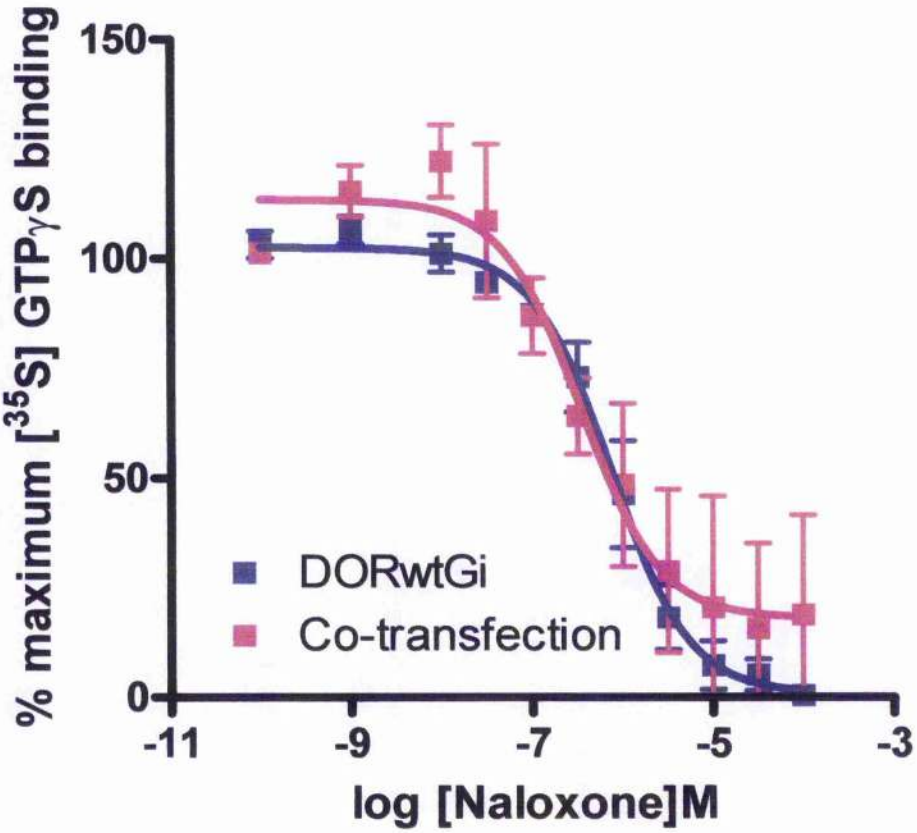

% maximum [35S] GTPγS binding
150
100
50
0
-11
-9
-7
-5
-3
log [Naloxone]M
DORwtGi
Co-transfection

Figure 3.12: Graphic representation of hMOR-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$

A. hMOR-$G_{i1\alpha}C^{351}I$ fusion protein

This fusion protein consisted of MOR fused to $G_{i1\alpha}C^{351}I$. The $G_{i1\alpha}$ contained a cysteine mutated into isoleucine to prevent ADP ribosylation by PTX.

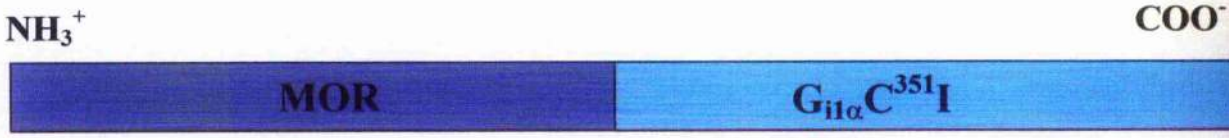
B. hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ fusion protein

This fusion protein consisted of MOR fused to the $G_{i1\alpha}C^{351}I$ subunit. The glycine at position 202 of $G_{i1\alpha}C^{351}I$ was mutated into an alanine.

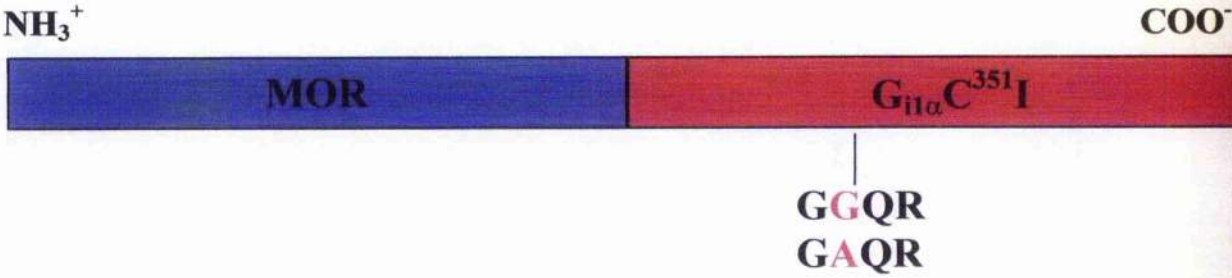
C. hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ fusion protein

This fusion protein consisted of MOR mutated in the 2nd intracellular loop of the receptor. Two valines in positions 169 and 173 were mutated into glutamic acid and aspartic acid respectively.

A


NH3+
COO-
MOR
Gi1αC351I

B


NH3+
COO-
MOR
Gi1αC351I
GGQR
GAQR

C

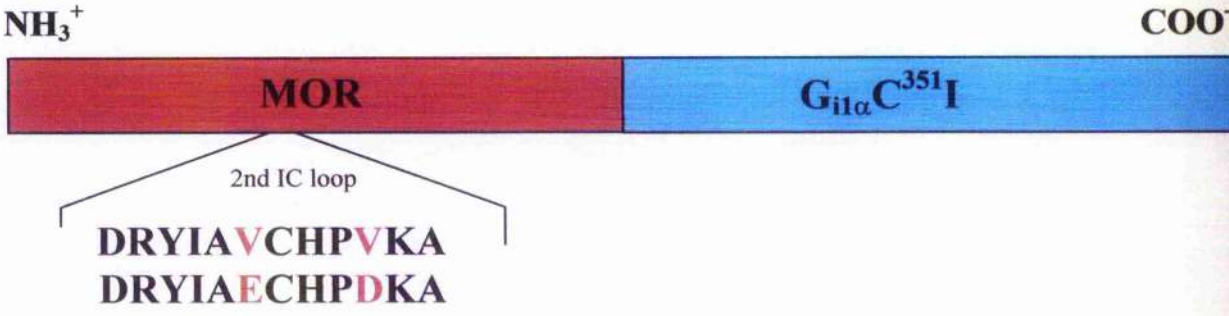

NH3+
COO-
MOR
Gi1αC351I
2nd IC loop
DRYIAVCHPVKA
DRYIAECHPDKA

Figure 3.13: [^{3}H]-Diprenorphine saturation binding assays following the expression of hMOR-$G_{i1\alpha}C^{351}I$ fusion proteins

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$ (dark blue squares), hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green dots), hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ (light blue dots) and co-expressing hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ (pink dots) were used to measure the specific binding of different concentrations of [^{3}H]-diprenorphine. Data are representative of n=3 experiments performed in triplicate.Data points represent mean ± SEM.

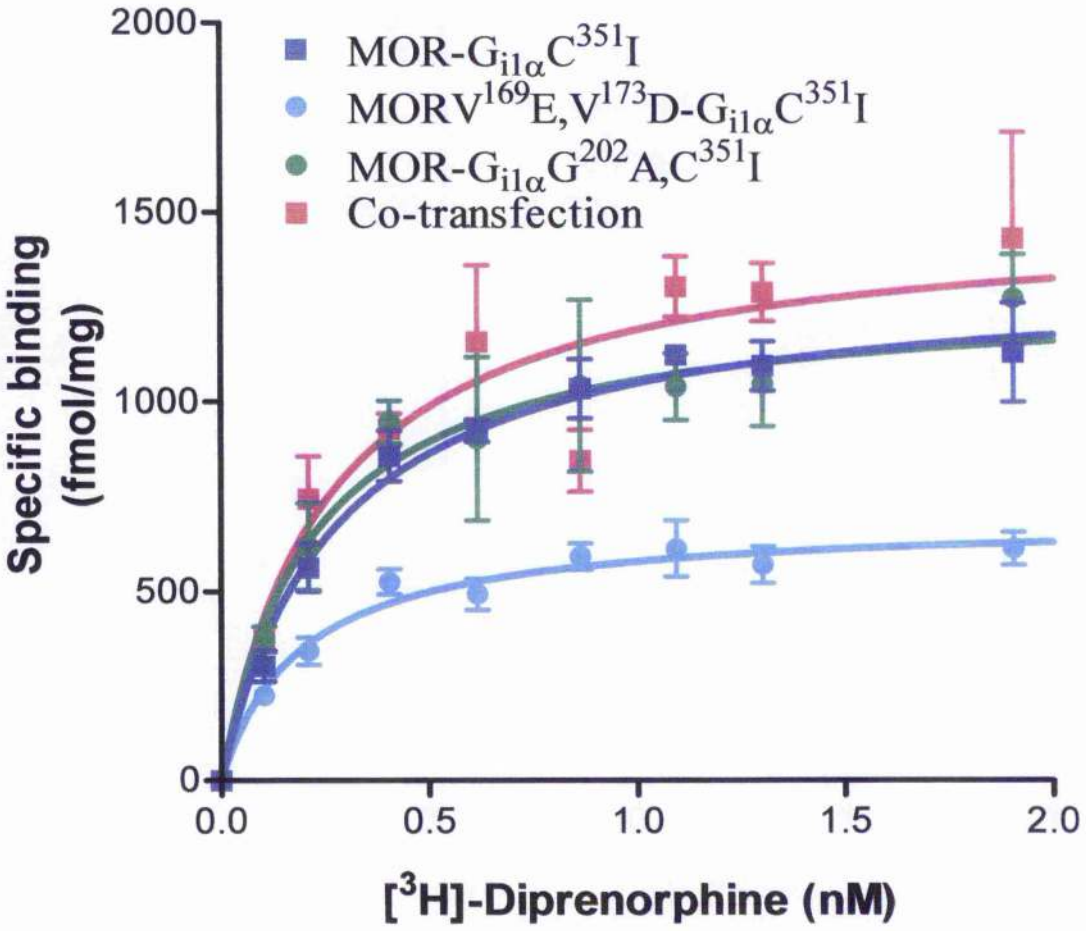

MOR-$G_{i1\alpha}C^{351}I$
MOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$
MOR-$G_{i1\alpha}G^{202}A,C^{351}I$
Co-transfection
Specific binding (fmol/mg)
2000
1500
1000
500
0
0.0
0.5
1.0
1.5
2.0
[^{3}H]-Diprenorphine (nM)

Figure 3.14: Co-expression of a pair of distinct non-functional mutants of hMOR-$G_{i1\alpha}C^{351}I$ fusion proteins reconstitute function

Membranes of HEK 293 cells expressing 15fmol of hMOR-$G_{i1\alpha}C^{351}I$; $hMORV^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or 30fmol in the case of $hMORV^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ + hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ co-transfection were used to measure [^{35}S]-GTPγS binding in the absence (open bars) or presence (filled bars) of 10 μM DAMGO. Membranes expressing 15 fmol $hMORV^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ and 15 fmol hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were also mixed and stimulated with DPDPE. Data represent n=3 experiments performed in triplicate. Data points represent means ± SEM.

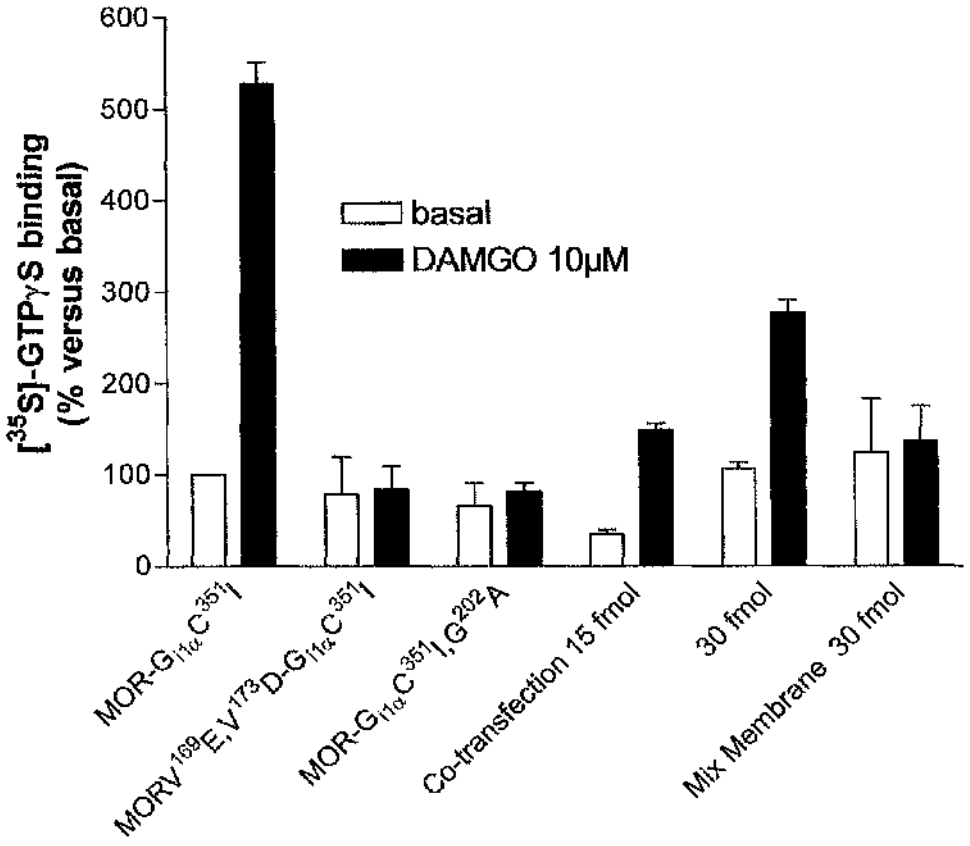

600
500
400
300
200
100
0
[35S]-GTPγS binding
(% versus basal)
basal
DAMGO 10μM
MOR-G11α C351I
MORV169E,V173D-G11α C351I
MOR-G11α C351I,G202A
Co-transfection 15 fmol
30 fmol
Mix Membrane 30 fmol

Figure 3.15: Effect of mutation and co-expression of hMOR-$G_{i1\alpha}C^{351}I$ fusion proteins on competition between [^{3}H]-Diprenorphine and DAMGO

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$ (dark blue squares); hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green dots), hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ (light blue dots) and both hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the ability of varying concentrations of DAMGO to compete with 1nM [^{3}H]-diprenorphine. Data are representative of n=4 experiments performed in triplicate. Data points represent means ± SEM.

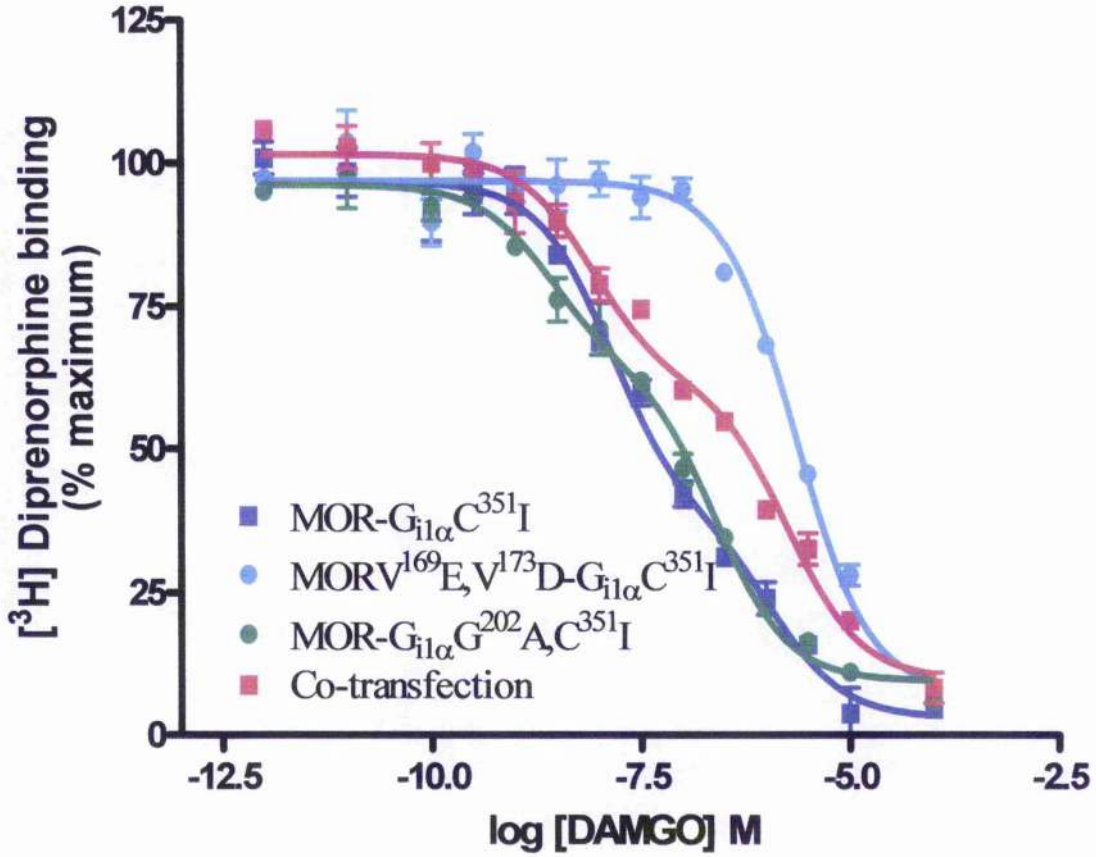

125
100
75
50
25
0
[3H] Diprenorphine binding (% maximum)
MOR-Gi1αC351I
MORV169E,V173D-Gi1αC351I
MOR-Gi1αG202A,C351I
Co-transfection
-12.5
-10.0
-7.5
-5.0
-2.5
log [DAMGO] M

Figure 3.16: Comparaison of agonist-stimulated [^{35}S]-GTPγS binding of hMOR-$G_{i1\alpha}C^{351}I$ and hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ reconstituted dimer

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$ (blue squares) or co-transfected with hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hMOR$V^{169}E,V^{173}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the ability of increasing concentrations of DAMGO to activate [^{35}S]-GTPγS binding. Data represents n=3 experiments performed in triplicate. Data points represent means ± SEM.

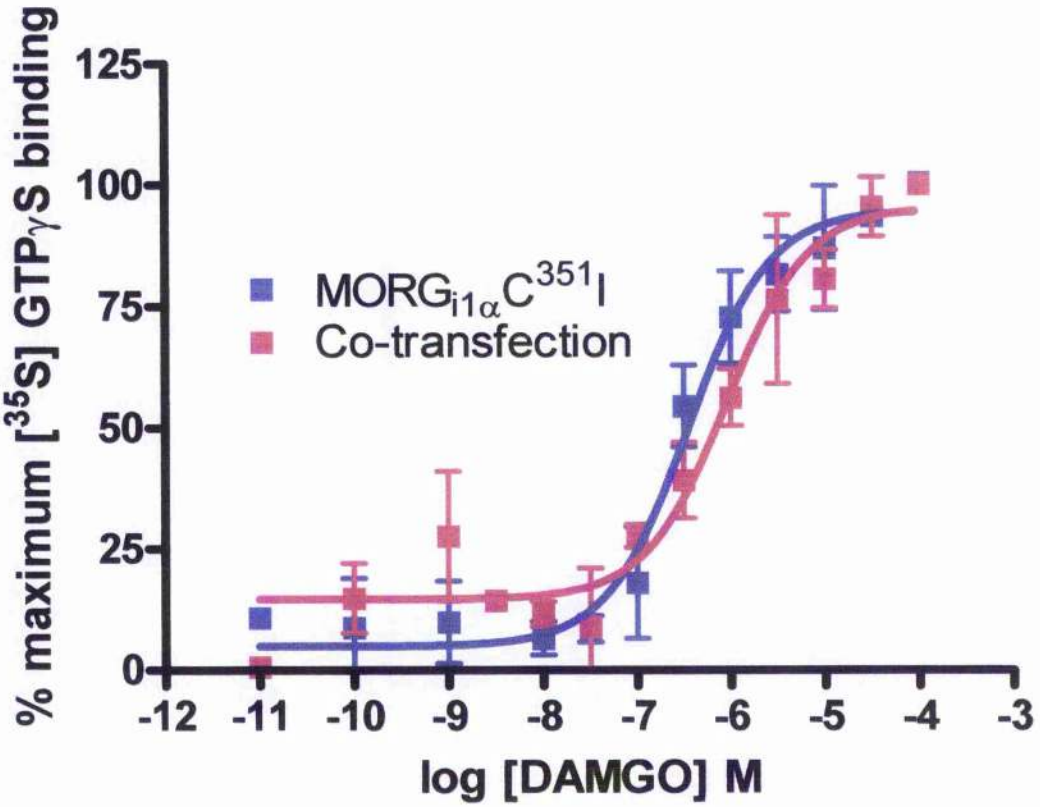

% maximum [35S] GTPγS binding
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
log [DAMGO] M
MORGi1αC351I
Co-transfection

Figure 3.17: Graphic representation of rKOR-$G_{i1\alpha}C^{351}I$, rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$

A. rKOR-$G_{i1\alpha}C^{351}I$ fusion protein

This fusion protein consisted of KOR fused to $G_{i1\alpha}C^{351}I$. The $G_{i1\alpha}$ contained a cysteine mutated into isoleucine to prevent ADP ribosylation by PTX.

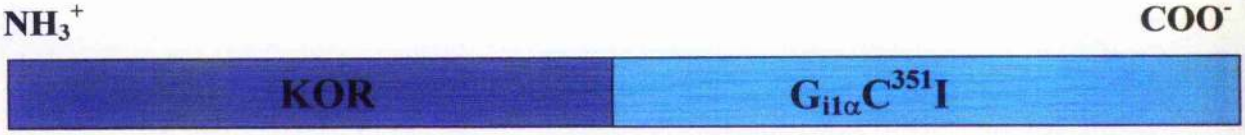
B. rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ fusion protein

This fusion protein consisted of KOR fused to the $G_{i1\alpha}C^{351}I$ subunit. The glycine at position 202 of $G_{i1\alpha}C^{351}I$ was mutated into an alanine.

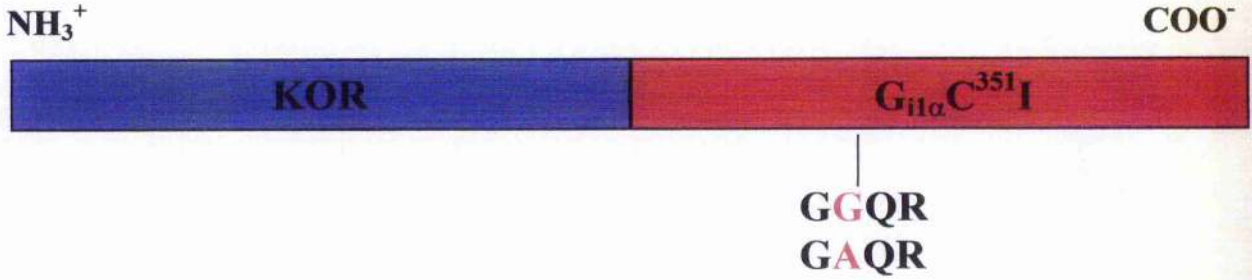
C. rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ fusion protein

This fusion protein consisted of KOR mutated in the 2nd intracellular loop of the receptor. Two valines in positions 160 and 164 were mutated into glutamic acid and aspartic acid respectively.

A


NH3+
COO-
KOR
Gi1αC351I

B


NH3+
COO-
KOR
Gi1αC351I
GGQR
GAQR

C

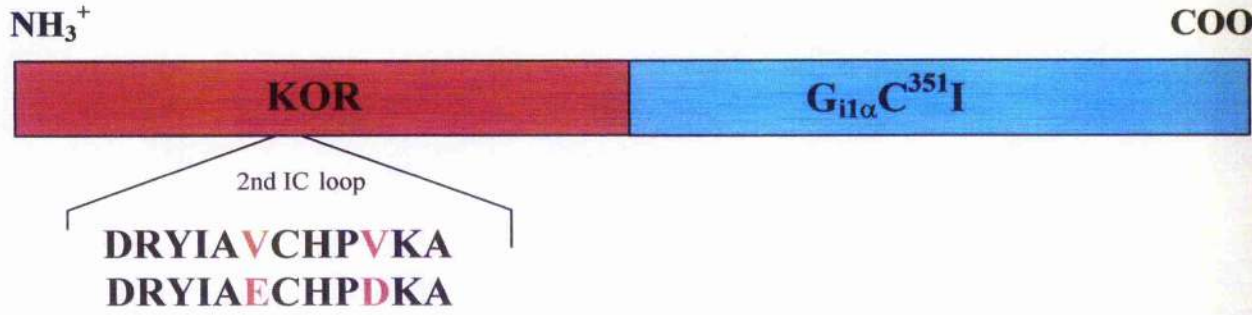

NH3+
COO
KOR
Gi1αC351I
2nd IC loop
DRYIAVCHPVKA
DRYIAECHPDKA

Figure 3.18: [^{3}H]-Diprenorphine saturation binding assays following expression of rKOR-$G_{i1\alpha}C^{351}I$ fusion proteins

Membranes expressing rKOR-$G_{i1\alpha}C^{351}I$ (dark blue squares), rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green dots), rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ (light blue dots) and co-expressing rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the specific binding of different concentrations of [^{3}H]-diprenorphine. Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM.

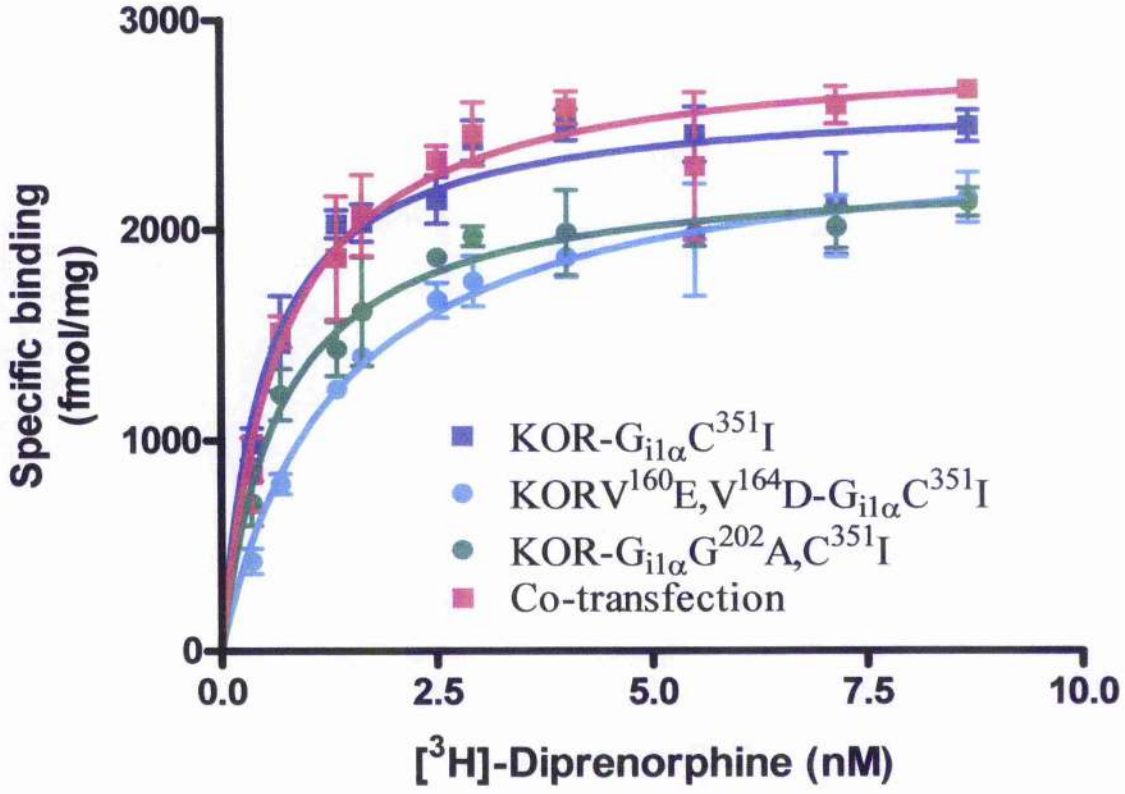

3000
2000
1000
0
Specific binding (fmol/mg)
0.0
2.5
5.0
7.5
10.0
[3H]-Diprenorphine (nM)
KOR-Gi1αC351I
KORV160E,V164D-Gi1αC351I
KOR-Gi1αG202A,C351I
Co-transfection

Figure 3.19: Co-expression of a pair of distinct non-functional mutants of rKOR-$G_{i1\alpha}C^{351}I$ fusion proteins reconstitute function

Membranes of HEK 293 cells expressing 15 fmol of rKOR-$G_{i1\alpha}C^{351}I$, rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$, rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or 30 or 45 fmol in the case of rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$+ rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ co-transfection were used to measure the [^{35}S]-GTPγS binding in the absence (open bars) or presence (filled bars) of 10 μM or (checkered bars) 100 nM U69593. Membranes expressing 15 fmol of rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ and 15 fmol of rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were also mixed and stimulated with U69593. Data represent n=4 experiments performed in triplicate. Data points represent means ± SEM.

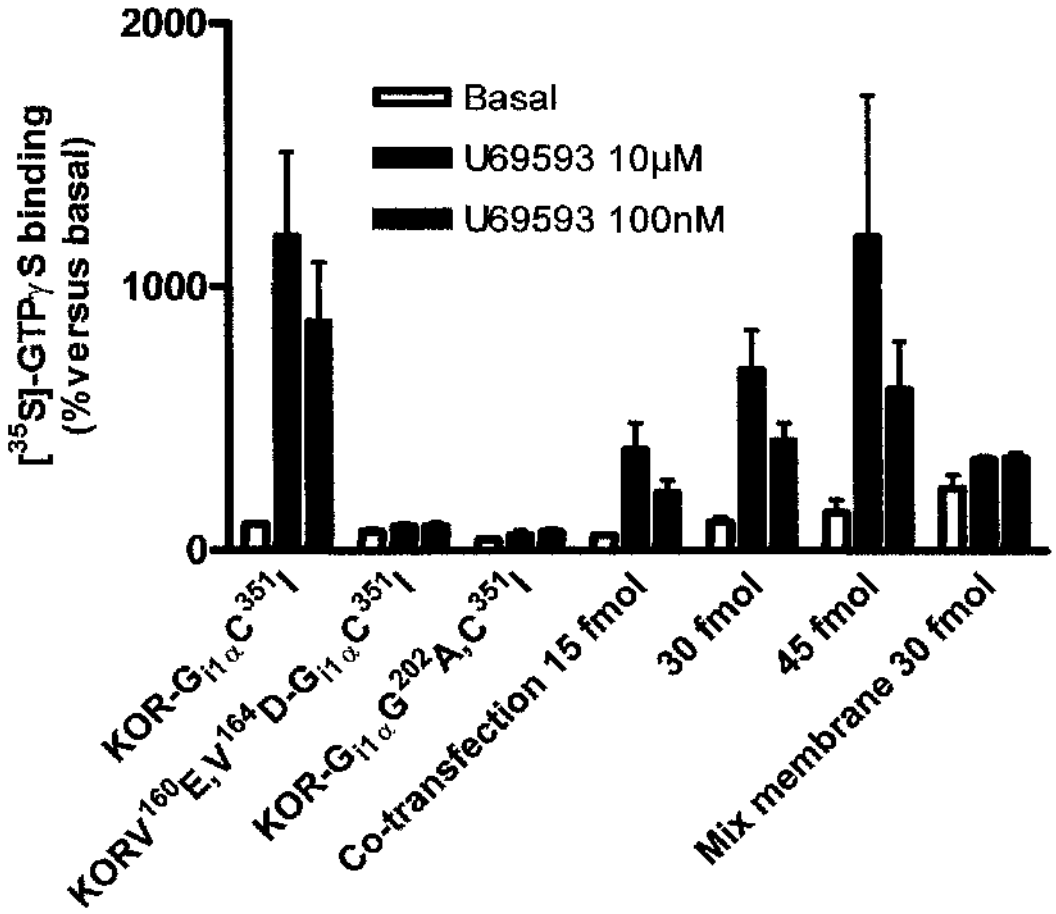

2000
1000
0
[35S]-GTPγS binding
(% versus basal)
Basal
U69593 10μM
U69593 100nM
KOR-Gi1α C351I
KORV160E,V164D-Gi1α C351I
KOR-Gi1α G202A,C351I
Co-transfection 15 fmol
30 fmol
45 fmol
Mix membrane 30 fmol

Figure 3.20: Schematic model of the complementation technique

The pair of distinct but complementary fusion proteins expressed alone are non-functional, no guanine nucleotide exchange was observed.

The reconstitution produced by the co-expression of two complementary mutant result from a transactivation in the dimer in which the wild-type receptor activates the wild type G protein even thought it is link to the inactive receptor.

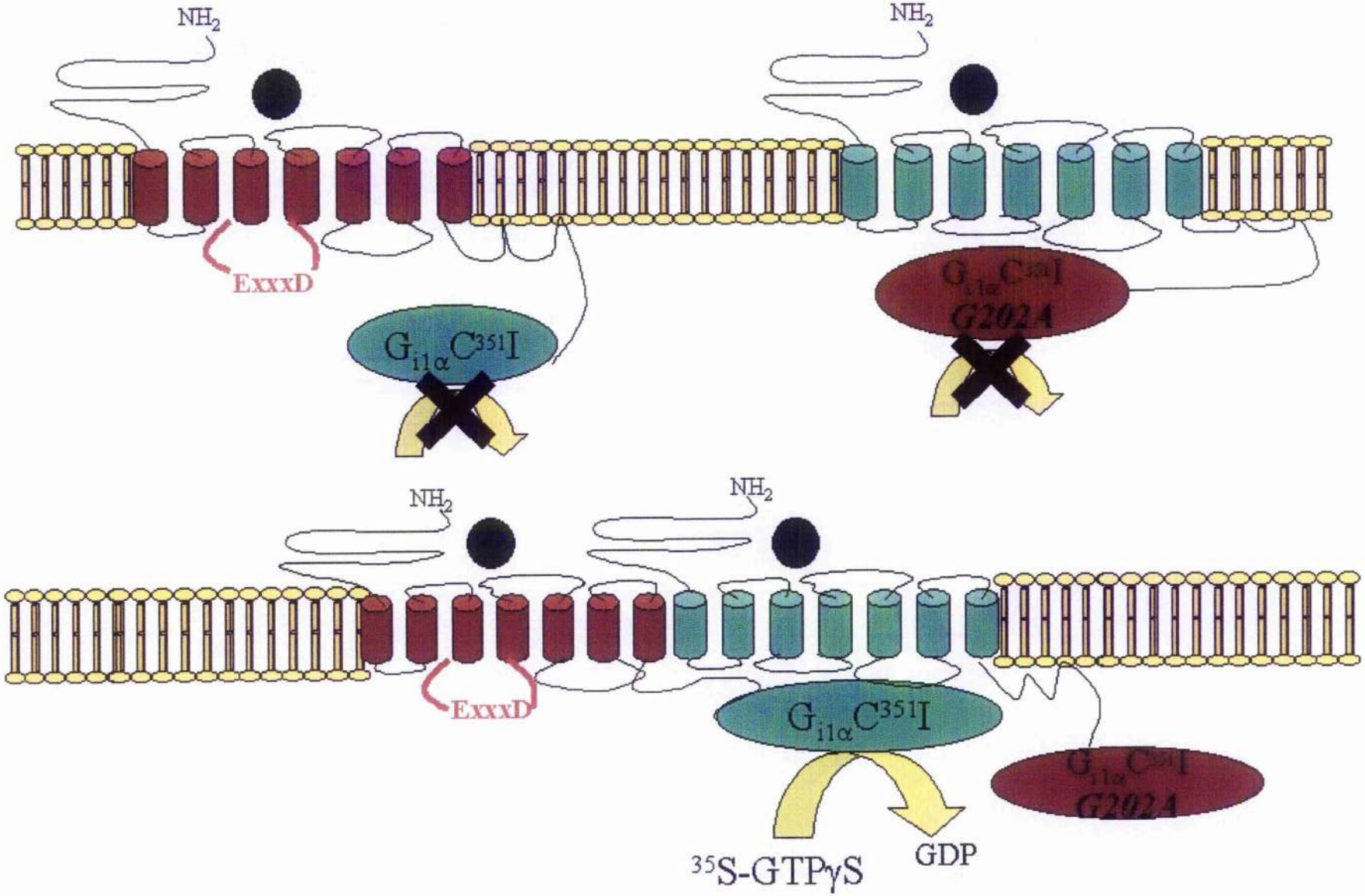

NH2
NH2
ExxxD
Gi1αC351I
Gi1αC351I
G202A
NH2
NH2
ExxxD
Gi1αC351I
Gi1αC351I
G202A
35S-GTPγS
GDP

Figure 3.21: Effect of mutation and co-expression of rKOR-$G_{i1\alpha}C^{351}I$ fusion proteins on competition between [^{3}H]-Diprenorphine and U69593

Membranes expressing rKOR-$G_{i1\alpha}C^{351}I$ (dark blue squares), rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green dots), rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ (light blue dots) and both rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ (pink squares) were used to measure the ability of varying concentrations of U69593 to compete with 1nM [^{3}H]-diprenorphine. Data are representative of n=4 experiments performed in triplicate. Data points represent means ± SEM.

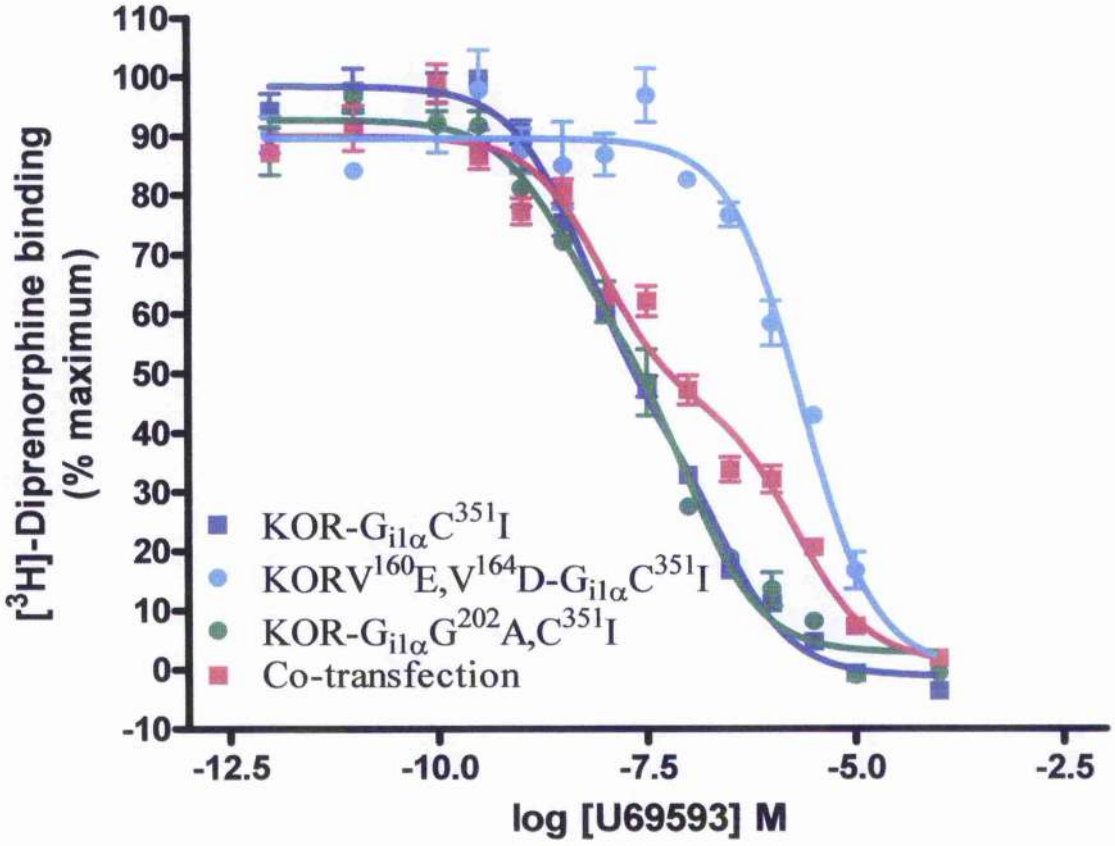

[3H]-Diprenorphine binding
(% maximum)
110
100
90
80
70
60
50
40
30
20
10
0
-10
-12.5
-10.0
-7.5
-5.0
-2.5
log [U69593] M
KOR-Gi1αC351I
KORV160E,V164D-Gi1αC351I
KOR-Gi1αG202A,C351I
Co-transfection

Figure 3.22: Effects of the mutations introduced in the 2nd intracellular loop of the receptor fused to the functional G protein on competition between [^{3}H]-Diprenorphine and U69593 in the presence or absence of GppNHp

Membranes expressing rKOR-$G_{i1\alpha}C^{351}I$ or $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ were used to compete 1nM [^{3}H]-diprenorphine by U69593 in presence (open squares) or absence (filled squares) of a 100μM of GppNHp. Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM.

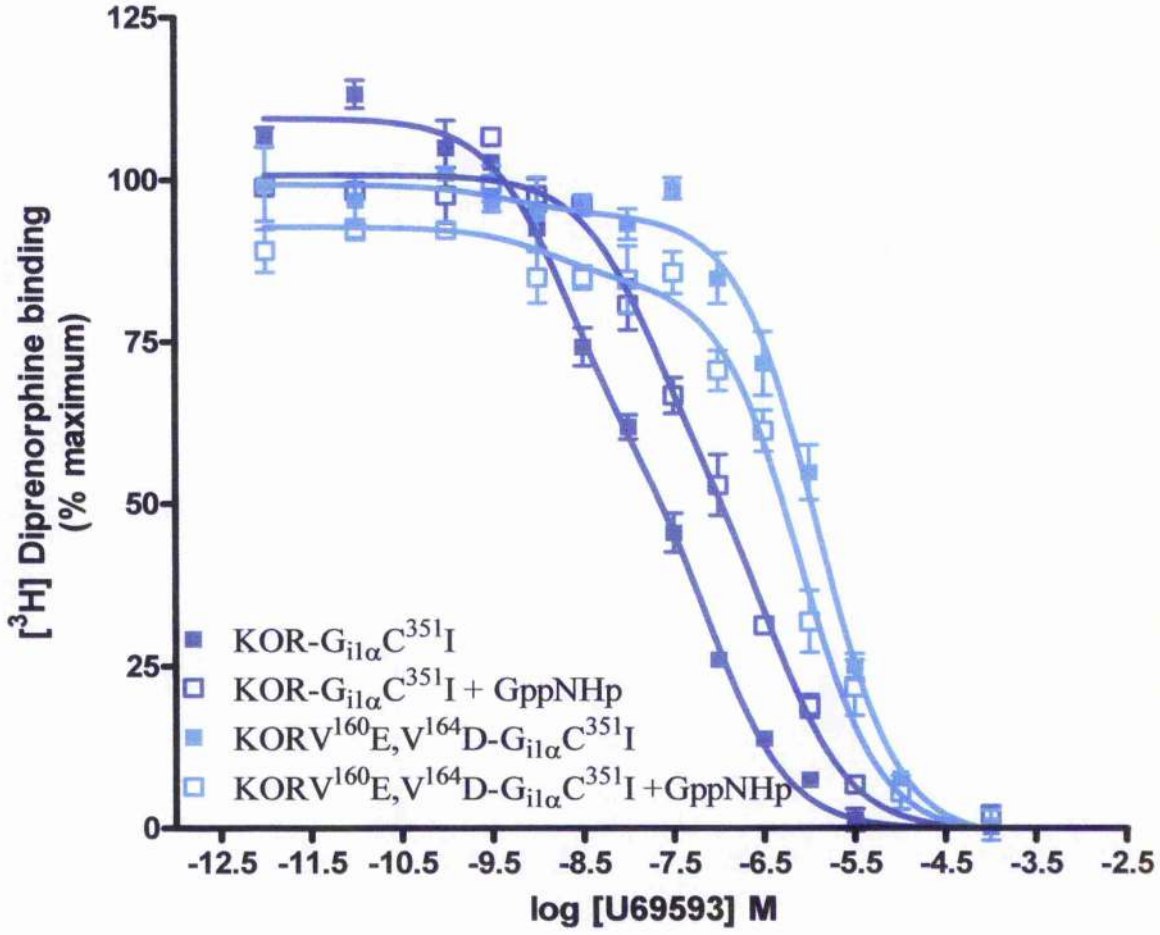
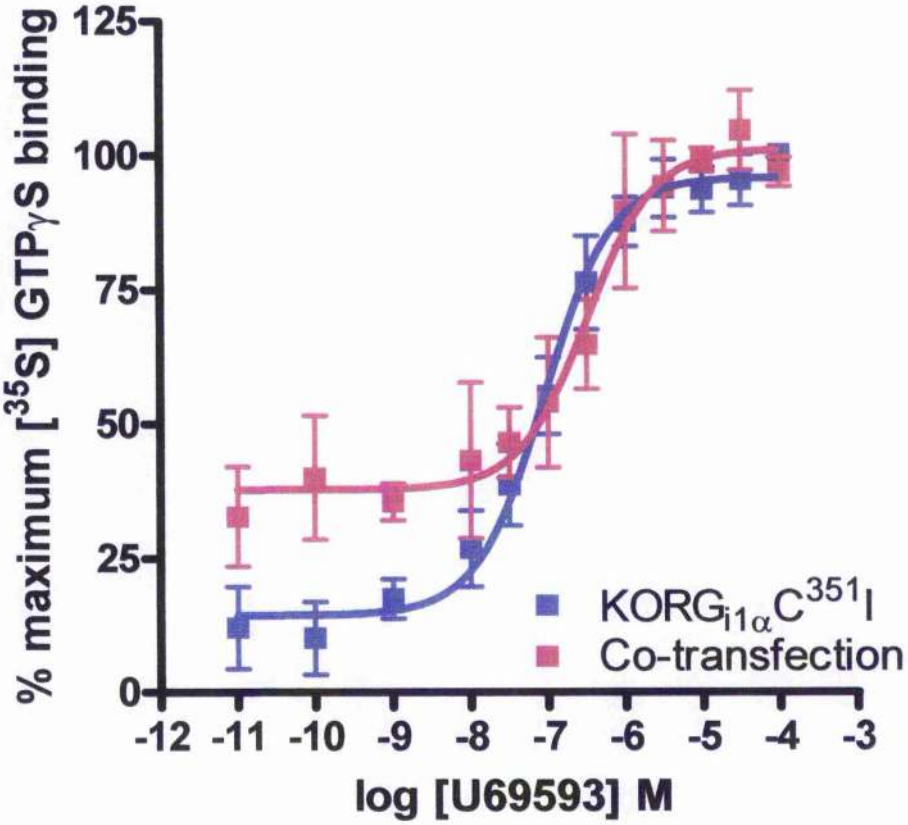

125
100
75
50
25
0
[3H] Diprenorphine binding
(% maximum)
KOR-Gilα C351I
KOR-Gilα C351I + GppNHp
KORV160E,V164D-Gilα C351I
KORV160E,V164D-Gilα C351I +GppNHp
-12.5 -11.5 -10.5 -9.5 -8.5 -7.5 -6.5 -5.5 -4.5 -3.5 -2.5
log [U69593] M

Figure 3.23: Comparison of agonist-stimulated [^{35}S]-GTPγS binding of rKOR-$G_{i1\alpha}C^{351}I$ and rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ reconstituted dimer

Membranes expressing rKOR-$G_{i1\alpha}C^{351}I$ or co-transfected with rKOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ were used to measure the ability of increasing concentrations of U69593 to activate [^{35}S]-GTPγS binding. Data represents n=3 experiments performed in triplicate. Data points represent means ± SEM.


% maximum [35S] GTPγS binding
125
100
75
50
25
0
-12 -11 -10 -9 -8 -7 -6 -5 -4 -3
log [U69593] M
KORGi1αC351I
Co-transfection

Chapter 4

Opioid receptor heterodimerisation studied using a complementation technique

4.1 Introduction:

GPCRs have been reported to form homo as well as heterodimers/oligomers that contain at least two distinct gene products (in the text heterodimer will be used in referring to either heterodimer or oligomer). A variety of GPCRs from classes A (George *et al.*, 2002) and C (Gama *et al.*, 2001) have been reported to heterodimerise. Different groups demonstrated that GPCR family subtypes such as dopamine D2 and D3 receptors (Maggio *et al.*, 1999) and serotonin $5HT_{1B}$ and $5HT_{1D}$ receptors (Xie *et al.*, 1999) were able to interact. The simplest hypothesis would be that closely related GPCRs are more likely to form heterodimers. However, this first hypothesis appears to be an over-simplification as the number of studies on heterodimerisation increased. This is exemplified by the detection of somatostatin receptor subtypes heterodimerisation. SSTr5 and SSTr1 interaction was detected whilst no association was noted between somatostatin SSTr5 and SSTr4 (Rocheville *et al.*, 2000a). Equally, for α_1-adrenoceptor subtype, heterodimerisation appears to be specific within the sub-group. The α_{1B} receptor was reported to dimerise with α_{1A} and α_{1D} whereas no experimental evidence suggested α_{1A} and α_{1D} interactions (Uberti *et al.*, 2003). Moreover, less-related receptors were showed to form dimer such as MOR with SSTr2 (Pfeiffer *et al.*, 2002). Furthermore, the class A adenosine A2 and class C glutamate mGlu5 receptors, which share no inherent homology beyond the seven transmembrane spanning domains, have been reported to interact (Ferré *et al.*, 2002). These observations suggest that GPCRs have a natural tendency to dimerise, however, the physiological relevance of these interactions is less clear and is dependent on *in-vivo* co-expression of the two receptors involved. Such studies are qualitative and do not take the affinity of these receptors to form dimers into consideration. Ramsay et al, (2002) evaluated dimer formation by use of a $BRET^2$ assay and observed that DOR and KOR were as efficient in forming heterodimers as homodimers but that less-related receptors such as the β_2-adrenoceptor or thyrotropin-releasing hormone receptor-1 required a higher level of expression to form detectable heterodimers with KOR. To further investigate the ability of different GPCRs to associate saturation BRET techniques were used. Mercier et al, (2002) described that β_2 and β_1-adrenoceptors have equal affinity to form homo and heterodimers. Similar observations were reported on the ability of β_2 and β_3 adrenoceptors to homo- or heterodimerise (Breit *et al.*, 2004).

The importance of heterodimerisation is based on the description of new pharmacology and functions due to these interactions. These new properties are different from those displayed by

the individual receptors and from what might be expected by simple combination of the two independent GPCRs. An example of this phenomenon is the greater number of high affinity binding sites for tripitramine and pirenzepine observed when dopamine D2 and D3 receptors are co-expressed (Maggio *et al.*, 1999). Effects on receptor internalisation have been described for SSTr2A and SSTr3, where dimerisation leads to a complex with a greater resistance to desensitisation compared to when the receptors are expressed individually (Pfeiffer *et al.*, 2001). The formation of this dimer also results in a dominant negative effect as the SSTr3 function is inactivated when co-expressed with SSTr2A. Enhanced functionality and changes in G protein coupling have also been demonstrated upon hetero-oligomer formation. Mellado et al., (2001) described that chemokine CXCR2 and CXCR5 receptor oligomers displayed an enhanced calcium response upon combination of chemokines that could be due to recruitment of $G_{q/11}$ protein by the heterodimer.

For opioid receptors the appearance of new ligand binding properties, alterations in receptor trafficking, new G protein coupling and synergy in signalling have been reported upon heterodimerisation. DOR/KOR, MOR/DOR and recently MOR/KOR heterodimer formation have been demonstrated (Jordan and Devi, 1999, George *et al.*, 2000, Wang *et al.*, 2005). The DOR/MOR complex has been described to be present in live cells and endogenous tissue such as spinal cord (Gomes *et al.*, 2004). For the KOR and DOR heterodimers, an attenuated affinity for selective agonists of both receptors as well as an increase in affinity for partially selective ligands was observed. KOR and DOR heterodimers also displayed a synergistic inhibition of adenylate cyclase activity along with an increase of MAPK phosphorylation. Moreover, internalisation induced by etorphine, which normally causes DOR but not KOR to internalise, did not occur upon heterodimer formation (Jordan and Devi, 1999). In the case of the MOR/DOR heterodimer, formation of a novel binding pocket was suggested with an altered rank order and potency for highly selective synthetic agonists and enhanced affinity for endomorphin and Leu-enkephalin (George *et al.*, 2000). Co-expression of MOR and DOR was also described as activating Ca^{2+} release in GH3 cells upon DAMGO treatment whereas cells only expressing MOR exhibited an inhibition of voltage-gated Ca^{2+} channels (Charles *et al.*, 2003). Evidence for a change in G protein coupling properties when DOR and MOR were co-expressed was observed by George *et al.*, (2000). The heterodimer was reported to be insensitive to PTX treatment implying coupling to G proteins other than $G_{i/o}$. However, this result is different to Law et al., (2005) whose recent observations on MOR and DOR heterodimerisation suggest no change in G protein coupling. Moreover, the authors described that MOR and DOR internalised independently and not as heterodimers.

Heterodimer formation can increase the repertoire of signalling mechanisms available for a specific ligand and therefore represents a potential avenue for new drug design. Bivalent ligands (an agonist and an antagonist or two agonists from different opioid receptors) can be designed in order to avoid side effects of opioids but keep its analgesic property but also to obtain molecules with higher affinity or potency. All the above studies were carried out on samples expressing heterodimers as well as homodimers, which raise the issue that observed changes of pharmacology or function of the receptors are simply a combination of signals in a mixture of co-expressed homo and heterodimers. In this second Result chapter, the protein complementation technique was used to study opioid receptor heterodimerisation as it allows monitoring of the heterodimer function alone, even if homodimers are also present. Thus heterodimer function and properties can be studied in the absence of confounding and extraneous signals originating from the co-expressed homodimers.

4.2 Results:

4.2.1 Study of membrane targeted $G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ co-expression

A/ Construction of a Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ fusion protein

To ensure that the complementation observed does indeed originate from dimer formation rather than from the fact that $G_{i\alpha}$ subunit is anchored to the membrane thus enabling its interaction with a nearby receptor, a Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ fusion protein was constructed (Figure 4.1A). In this construct, DOR N-terminus, first TM region and 1st intracellular loop were fused with $G_{i1\alpha}C^{351}I$. The N-terminus of the fusion protein was Flag-tagged to allow its detection in membrane preparations by immunoblot. As in the previous chapter, in all the experiments transiently tranfected cells were PTX treated for 16h prior to harvesting. This treatment should prevent any signal due to the activation of endogenous $G_{i/o}$ in HEK 293 cells.

B/ Analysis of Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ expression and determination of the dissociation constant for [^{3}H]-diprenorphine

Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ fusion proteins were expressed individually or co-expressed in HEK 293 cells and hDOR-$G_{i1\alpha}$ $C^{351}I$ was transfected as a reference. Membrane preparations were used to measure expression of the fusion proteins by [^{3}H]-diprenorphine saturation ligand binding (Figure 4.2). As expected Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ did not bind [^{3}H]-diprenorphine as one transmembrane domain is not able to form a binding pocket. The other fusion proteins and membranes co-expressing Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ displayed an expression level of approximately 1800 fmol/mg (Table 4.1).

The K_d values observed for the different membrane preparations were not significantly different from those expressing the wild type fusion protein hDOR-$G_{i1\alpha},C^{351}I$ ($P>0.05$, 1 way ANOVA).

Table 4.1: B_{max} and K_d of hDOR-$G_{i1\alpha}$,$C^{351}I$ full or truncated fusion proteins

Construct	B_{max}	K_d	pK_d
hDOR-$G_{i1\alpha}C^{351}I$	1816 ± 209	0.65 ± 0.05	9.2 ± 0.03
hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1777 ± 285	0.67 ± 0.07	9.19 ± 0.05
Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ +hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1839 ± 372	0.60 ± 0.04	9.23 ± 0.03

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

Statistics were performed using 1 way ANOVA on B_{max} and pK_d numbers.

C/ Co-expression of Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ with hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$: [^{35}S]-GTPγS binding

Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ and DOR-$G_{i1\alpha}G^{202}A,C^{351}I$ fusion proteins were expressed individually or co-transfected into HEK 293 cells. Expression of the fusion proteins was assessed by the binding of a single, near saturating concentration of [^{3}H]-diprenorphine in membrane preparations. An equal amount of [^{3}H]-diprenorphine binding sites (15 fmol) or 10μg of Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ membranes (protein) were used to measure [^{35}S]-GTPγS binding in either the presence or absence of 10μM or 100nM of DADLE (Figure 4.3). In each experiment [^{35}S]-GTPγS binding was monitored following an immunoprecipitation step with anti-$G_{i1/2}$ serum. As previously observed in Figure 3.6 no significant agonist-induced [^{35}S]-GTPγS binding was detected for the hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ fusion protein. Similarly, Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ did not bind [^{35}S]-GTPγS in the presence of 10μM or 100nM of DADLE. This result was anticipated as the construct was known to consist only of DOR N-terminal, transmembrane domain 1 and intracellular loop 1 fused to $G_{i1\alpha}C^{351}I$. Co-expression of Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ with hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ did not result in significant stimulation of [^{35}S]-GTPγS binding after DADLE exposure. Thus, simple membrane

expression of $G_{i1\alpha}$ did not result in its activation by the functional receptor fused to the non-functional G protein, excluding any transactivation due simply to physical proximity.

D/ Expression of Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$ fusion protein in samples used for [^{35}S]-GTPγS binding

As Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$ did not bind [^{3}H]-diprenorphine, it was important to check that this construct was expressed in membranes used in [^{35}S]-GTPγS binding experiments. These membranes were resolved by SDS-PAGE, and anti-$G_{i1/2}$ antiserum was used for protein detection (Figure 4.4). The membranes were compared with those from mock-transfected cells and a specific band at approximately 40 kDa was detected in all the samples. This band corresponds to the endogenous G_i protein. In addition to the band at 40 kDa, a specific band at 50 kDa could also be visualised in samples transfected with Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$ cDNA. The 50 kDa band is in agreement with the molecular size expected for DOR N-terminal plus TM 1 fused to G_i protein. This result demonstrated that Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$ was expressed in the samples used for [^{35}S]-GTPγS binding experiments. Thus, the absence of signal observed after agonist treatment in [^{35}S]-GTPγS binding experiments was not due to the absence of expression of Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$ but to the absence of activation of the membrane-anchored G protein by the receptor fused to a non-functional G protein i.e. hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$.

E/ Interaction of Flag-Nt-TM1$_{DOR}$ with hDOR (fused or not to $G_{i1\alpha}C^{351}I$) monitored by co-immunoprecipitation

In these studies Flag and c-Myc versions of Nt-TM1$_{DOR}$ and hDOR fused or not to $G_{i1\alpha}C^{351}I$ were used. c-Myc-Nt-TM1$_{DOR}$ and Flag-hDOR were previously generated in the laboratory (Feng *et al.*, unpublished). HEK 293 cells were mock transfected or transiently transfected with c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$, Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$, c-Myc-Nt-TM1$_{DOR}$, Flag-hDOR or co-transfected with c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$, c-Myc-Nt-TM1$_{DOR}$ + Flag-hDOR and c-Myc-Nt-TM1$_{DOR}$+ Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$ then immunopreciptated using an anti-Flag antibody and detected with anti-c-Myc antibody after being resolved by SDS-PAGE (Figure 4.5). Specific bands could only be detected where Flag

and c-Myc versions of the constructs were co-expressed. In lane 4, where c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ were co-transfected bands at 65 and 80 kDa were observed corresponding to the bands previously visualised in chapter 3 when the c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ monomer was detected (Figure 3.7A). This result showed that Flag-Nt-$TM1_{DOR}$ fused to $G_{i1\alpha}C^{351}I$ interacts with c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ demonstrating that the absence of signal observed in the [^{35}S]-GTPγS binding studies did not result from an absence of protein-protein contacts but that a single transmembrane domain associated with a functional receptor is not enough to activate a G protein.

In lane 7 corresponding to the co-expression of c-Myc-Nt-$TM1_{DOR}$ with Flag-hDOR two bands at 15 and 20 kDa were detected. These bands are in agreement with the molecular mass expected for c-Myc-Nt-$TM1_{DOR}$ and may correspond to differentially glycosylated forms of the c-Myc-Nt-$TM1_{DOR}$ construct. This hypothesis was not, however, examined directly. The upper band was not detected in lane 8 as c-Myc-Nt-$TM1_{DOR}$ expression was not as strong as in lane 7. This result demonstrated that Nt-$TM1_{DOR}$ can associate with the full lengh hDOR and may provide an important interface for DOR dimerisation. They also confirmed that the dimerisation observed was not forced by the presence of the G protein in these constructs.

To further investigate the importance of transmembrane 1 in the dimerisation process, c-Myc-Nt-$TM1_{DOR}$ was co-transfected with Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$. Following immunoprecipitation with anti-Flag antibody, a band at 15 kDa was immunodetected corresponding to c-Myc-Nt-$TM1_{DOR}$. This indicates that it is possible for two transmembrane 1 domains to interact and suggests a symmetrical TM1-TM1 interface, which may play an important role in DOR dimerisation.

Re-blotting of this gel with anti-Flag antibody revealed bands where the different Flag-tagged constructs were expressed. In lanes 3, 4 and 8 a band at 50 kDa was detected, corresponding to Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ expression. In lanes 5 and 7, expressing Flag-hDOR, bands at 40 and 80 kDa were revealed likely to correspond to the Flag-hDOR monomer and dimer forms.

4.2.2 DOR and β_2-adrenoceptor heterodimerisation studied using the protein complementation technique

A/ Construction of a β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ fusion protein

To study possible DOR and β_2-adrenoceptor heterodimerisation a fusion protein made previously in the laboratory, β_2-adrenoceptor-$G_{i1\alpha}$ (Feng *et al.*, unpublished) was used. The $C^{351}I$ mutation was introduced in the $G_{i1\alpha}$ protein of this fusion protein using a Stratagene point mutation kit (Figure 4.1B). The mutation of the cysteine residue into isoleucine confers resistance of the corresponding G protein to the ADP ribosylation activity of PTX. PTX treatment of β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ transiently transfected cells should eliminate any signal due to endogenous $G_{i/o}$ protein.

B/ β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ [^{35}S]-GTPγS binding

HEK 293 cells were transiently transfected with β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ or hDOR-$G_{i1\alpha}$ $C^{351}I$ and membranes prepared. These membranes were used to study [^{35}S]-GTPγS binding after treatment with 10 μM of opioid or β_2-adrenoceptor agonists, DADLE and isoproterenol respectively (Figure 4.6). The addition of either DADLE or isoproterenol did not result in any significant stimulation of [^{35}S]-GTPγS binding to the β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ fusion protein over basal (Figure 4.6). Isoproterenol was therefore unable to activate $G_{i1\alpha}C^{351}I$ fused to the β_2-adrenoceptor and neither did DADLE. These results were expected as the β_2-adrenoceptor is coupled predominantly to G_s, so isopreterenol was not able to activate G_{i1} and DADLE subsequently has no significant affinity for the β_2-adrenoceptor. In parallel experiments membranes expressing hDOR-$G_{i1\alpha}$ $C^{351}I$ produced a large signal in the presence of DADLE but not isoproterenol as would be expected from the nature of these two agonists. These results demonstrated that the β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ fusion protein is non-functional.

C/ Co-expression of β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$

The β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ fusion proteins were co-expressed in HEK 293 cells. A fixed amount (2 μg) of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ DNA was transfected in

combination with varying quantities of β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ cDNA. Expression of the two fusion proteins was assessed by the binding of single, close to saturating concentrations of either [^{3}H]-diprenorphine or [^{3}H]-dihydroalprenolol for the DOR and β_2-adrenoceptor fusion proteins respectively. Equal expression of each fusion protein was observed when 2 µg of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ cDNA was co-expressed with 1µg of β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ cDNA (Figure 4.7). This cDNA ratio was used for cell transfection in the following experiments to assure a 1:1 expression level of each fusion protein when co-expressed.

Expression levels and K_d for antagonist ligands were measured in membranes transfected with hDOR-$G_{i1\alpha}C^{351}I$, hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$, β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ or co-expressing hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ by saturation ligand binding. Increasing concentrations of the opioid and β_2-adrenoceptor ligands [^{3}H]-diprenorphine and [^{3}H]-dihydroalprenolol were used to determine total binding of DOR and β_2-adrenoceptor respectively (Figure 4.8 A and B). The antagonists naloxone and alprenolol were employed to define non-specific binding. When co-transfected each of the fusion proteins was expressed at approximately 1100 fmol/mg (Table 4.2) as predicted from the previous experiment (Figure 4.7), which added together give a total expression of some 2000 fmol/mg.

Saturation binding also permitted determination of the fusion proteins K_d values for [^{3}H]-diprenorphine or [^{3}H]-dihydroalprenolol. No significant differences in [^{3}H]-diprenorphine K_d values were observed between the individual DOR fusion proteins and when β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were co-expressed (Table 4.2 A, $P>0.05$, 1 way ANOVA). Similarly the [^{3}H]-dihydroalprenolol K_d values for β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ in the presence of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were not significantly different compared to that of the β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ expressed alone (Table 4.2 B, $P>0.05$, T test).

Table 4.2: B_{max} and K_d of the different fusion protein

A. [^{3}H]-diprenorphine binding experiments

Construct	B_{max} (fmol/mg)	K_d	pK_d
hDOR-$G_{i1\alpha}C^{351}I$	1816 ± 209	0.65 ± 0.05	9.2 ± 0.03
hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1777 ± 285	0.67 ± 0.07	9.19 ± 0.05
β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ +hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1135 ± 74	0.73 ± 0.15	9.17 ± 0.04

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

One way ANOVA were performed using pK_d

B. [^{3}H]-dihydroalprenolol binding experiments

Construct	B_{max} (fmol/mg)	K_d (nM)	pK_d
$\beta_2$adrenoceptor-$G_{i1\alpha}C^{351}I$	4877 ± 712*	0.60 ± 0.18	9.28 ± 0.18
$\beta_2$adrenoceptor-$G_{i1\alpha}C^{351}I$ +hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1295 ± 170	0.32 ± 0.03	9.5 ± 0.07

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

T test were performed using pK_d

D/ Reconstitution of function following co-expression of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$

The non-functional proteins hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ were individually expressed or co-transfected into HEK 293 cells. hDOR-$G_{i1\alpha}C^{351}I$ was also individually transfected as a reference. Expression of the fusions proteins was assessed by the binding of a single, close to saturating concentration of [^{3}H]-diprenorphine or [^{3}H]-dihydroalprenolol depending on the receptor anticipated to be present in each membrane preparation. An equal amount of each construct (15 fmol) was used to measure [^{35}S]-GTPγS binding in the presence of 10μM DADLE. As previously observed in Figures 4.6 and 3.6 no significant stimulation was noticed when β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were treated with DADLE. However, when β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were co-expressed, DADLE did enhance [^{35}S]-GTPγS binding (Figure 4.9). However this was not highly effective. When membrane containing 30 fmol of [^{3}H]-diprenorphine and [^{3}H]-dihydroalprenolol (1:1 ratio) binding sites were used, only some 40 % of the signal from hDOR-$G_{i1\alpha}C^{351}I$ was reconstituted (Table 4.3). This result demonstrates

that the functional DOR was able to activate the G protein fused to β_2-adrenoceptor. However, when compared to the DOR reconstituted homodimer, the effect of DADLE in membranes potentially expressing the β_2-adrenoceptor/DOR heterodimer was substantially smaller. When membranes containing 15 fmol of β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ and 15 fmol of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were prepared and combined before the assay, no significant agonist-stimulated [^{35}S]-GTPγS binding was observed (Figure 4.9 and Table 4.3). This demonstrates that the reconstitution detected was specific and required co-expression of the two constructs. Basal, constitutive activity was observed in the absence of agonist stimulation. As previously noticed for the other mutated fusion proteins, constitutive activity was significantly lower compared to the wild type fusion protein ($P<0.05$, 1 way ANOVA) and increased with the amount of co-expressed mutants.

Table 4.3: [^{35}S]-GTPγS binding of β_2-adrenoceptor and DOR fusion proteins

Construct	Basal	DADLE 10μM
hDOR-$G_{i1\alpha}C^{351}I$	29.3 ± 7.2	100
β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$	5.4 ± 2.1*	7.7 ± 2.1
hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$	8.3 ± 2.4*	9.5 ± 2.6
Co-transfection 15 fmol	13.3 ± 3.2	20.9 ± 0.9
30 fmol	16.8 ± 3.6	39.7 ± 4.6
45 fmol	22.5 ± 4.7	55.1 ± 4.5***
Mix membrane 30 fmol	14.2 ± 2	10.6 ± 5.2

Data represent n=5 experiments performed in triplicate on different membrane preparations and numbers are means ± SEM expressed as a percentage of agonist-induced stimulation of hDOR-$G_{i1\alpha}C^{351}I$.

* Significantly different $P<0.05$, 1 way ANOVA from wild-type basal

*** Significantly different $P<0.001$, 1 way ANOVA from wild-type stimulated

E/ The ability of the reconstituted dimer to activate G protein function

The wild type hDOR-$G_{i1\alpha}C^{351}I$ or the pair of non-functional hDOR-$G_{i1\alpha}C^{351}I$ + β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ fusion proteins were transiently transfected into HEK 293 cells and membranes prepared. An equal amount of [^{3}H]-radioligand receptor binding sites (15 fmol) were used to study G protein activation of the wild type fusion protein or the reconstituted heterodimer by measuring [^{35}S]-GTPγS binding in the presence of increasing concentration of DADLE (Figure 4.10). No significant difference in the potency of DADLE to activate G protein was observed ($P>0.05$, T test).

Table 4.4: EC_{50} of DADLE for hDOR-$G_{i1\alpha}C^{351}I$ versus the reconstituted dimer hDOR-$G_{i1\alpha}C^{351}I$ + β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ analysis by [^{35}S]-GTPγS binding studies

	EC_{50} nM	pEC_{50}
hDOR-$G_{i1\alpha}C^{351}I$	247 ± 68	6.67 ± 0.14
Co-transfection	181 ± 76	6.85 ± 0.24

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

T test was performed using pEC_{50}

4.2.3 MOR and DOR heterodimerisation studied using the protein complementation technique

To study MOR/DOR heterodimerisation, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ fusion proteins were used.

A/ Analysis of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}$ $C^{351}I$ co-expression and determination of the dissociation constant for [^{3}H]-diprenorphine

Based on the expressions levels previously determined in Chapter 3 (Table 3.1 and 3.7) membranes co-expressing 2 µg of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ cDNA and 1 µg of $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ cDNA were used to measure the specific binding of increasing concentrations of [^{3}H]-diprenorphine (Figure 4.11). The level of expression observed was significantly different (3425 ± 377 fmol/mg) compared to the hMOR-$G_{i1\alpha}C^{351}I$ fusion protein (1217 ± 72 fmol/mg). Saturation binding also permitted determination of the co-expressed mutated fusion proteins K_d value (Table 4.5). This value was significantly different ($P<0.001$, 1 way ANOVA) compared to the hMOR-$G_{i1\alpha}C^{351}I$ fusion protein. However, no significant difference was observed between the K_d value for the co-expressed mutated proteins and $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (Table 4.5). This result might reflect $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ overexpression compared to hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$. This was not obvious from the level of expression observed (3425 ± 377 fmol/mg). Although, this value seems to reflect the addition of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}$ $C^{351}I$ individual expression level (1251 ± 20 and 2181 ± 228 fmol/mg) and would imply that $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}$ $C^{351}I$ expression was two times higher compared to hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$. This is not likely as only half the cDNA amount used in the earlier $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}$ $C^{351}I$ studies was used in the co-expressed samples in an attempt to achieve a 1:1 MOR/DOR expression ratio.

Table 4.5: B_{max} and K_d for [^{3}H]-diprenorphine in membranes expressing hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}$ fusion proteins

Construct	B_{max} (fmol/mg)	K_d (nM)	pK_d
hMOR-$G_{i1\alpha}$ $C^{351}I$	1217 ± 72	0.36 ± 0.07	9.47 ± 0.08
hMOR-$G_{i1\alpha}G^{202}A$,$C^{351}I$	1251 ± 20	0.32 ± 0.05	9.52 ± 0.08
hDORV^{150}E,V^{154}D-$G_{i1\alpha}$ $C^{351}I$	2181 ± 228	1.65 ± 0.06	8.78 ± 0.01
hMOR-$G_{i1\alpha}G^{202}A$,$C^{351}I$+ hDORV^{150}E,V^{154}D-$G_{i1\alpha}$ $C^{351}I$	3425 ± 377	1.8 ± 0.16***	8.75 ± 0.04***

Data represent n=3 experiments performed in triplicate on different membrane preparations and numbers are means ± SEM

Statistics were performed using pK_d

*** Significantly different $P<0.001$ from hMOR-$G_{i1\alpha}$ $C^{351}I$

B/ Reconstitution of function when co-expressing hMOR-$G_{i1\alpha}G^{202}A$,$C^{351}I$ and hDORV^{150}E,V^{154}D-$G_{i1\alpha}$ $C^{351}I$

The mutated fusion proteins hDORV^{150}E,V^{154}D-$G_{i1\alpha}$ $C^{351}I$ and hMOR-$G_{i1\alpha}G^{202}A$,$C^{351}I$ were individually or co-transfected in HEK 293 cells. hMOR-$G_{i1\alpha}C^{351}I$ was also transfected. Expression of the fusions proteins was assessed by the specific binding of a single, close to saturating concentration of [^{3}H]-diprenorphine in membrane preparations. An equal number of [^{3}H]-diprenorphine binding sites (15 fmol) was used to measure [^{35}S]-GTPγS binding in the absence or presence of 10μM DAMGO. As previously observed in Figure 3.14, hMOR-$G_{i1\alpha}G^{202}A$,$C^{351}I$ did not produce any agonist-induced [^{35}S]-GTPγS binding as it is non-functional. hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ also did not bind [^{35}S]-GTPγS in response to DAMGO treatment (Figure 4.12). This result was not surprising as it was previously demonstrated that the hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ fusion protein was non-functional (Figure 3.6), and that the agonist used in this experiment, DAMGO, is a highly selective MOR agonist. Nevertheless, a large stimulation of [^{35}S]-GTPγS binding was observed when the two mutated fusion proteins were co-expressed (Table 4.6, Figure 4.12). Membranes of co-transfected cells expressing 30 fmol of [^{3}H]-diprenorphine binding sites were employed, DAMGO stimulation of [^{35}S]-GTPγS binding was as great as in membranes expressing 15 fmol of only hMOR-$G_{i1\alpha}$ $C^{351}I$.

Basal, constitutive activity was observed for hMOR-$G_{i1\alpha}C^{351}I$ in the absence of agonist treatment as previously reported (Figure 3.14). hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ basal [^{35}S]-GTPγS binding was significantly lower compared to the wild type fusion protein, raising the possibility that the mutation introduced altered basal binding and that this difference reflects the level of constitutive activity of hMOR. This could be adressed by examining the effect of MOR inverse agonists but was beyond the scope of the current study. A rise in basal activity was observed when increasing amounts of co-expressed mutated fusion proteins were used. Interestingly, as in the case of the agonist-stimulated reconstituted signal, 30 fmol of co-expressed fusion proteins was found to be sufficient to produce a level of constitutive activity similar to that corresponding to 15 fmol the wild type fusion protein.

Table 4.6: [^{35}S]-GTPγS binding of the mutated fusion proteins

Construct	Basal	DAMGO 10μM
hMOR-$G_{i1\alpha}$ $C^{351}I$	100	451 ± 72
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	41 ± 8*	66 ± 13
$hDORV^{150}E,V^{154}D$-$G_{i1\alpha}$ $C^{351}I$	61 ± 21	71 ± 20
Co-transfection 15 fmol	49 ± 14*	286 ± 62
30 fmol	127 ± 33	541 ± 91
45 fmol	212 ± 54	863 ± 161

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

* Significantly different P<0.05, 1 way ANOVA from hMOR-$G_{i1\alpha}$ $C^{351}I$ basal

100% is binding of [^{35}S]-GTPγS to hMOR-$G_{i1\alpha}$ $C^{351}I$ in the absence of agonist

C/ The affinity of different opioid antagonists for co-expressed hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$

- **Competition for [^{3}H]-diprenorphine binding by CTOP**

Membranes from HEK 293 cells transfected with hMOR-$G_{i1\alpha}C^{351}I$, hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or co-expressing the mutated pair of distinct fusion proteins (hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ + hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$) were used for [^{3}H]-diprenorphine competition experiments with varying concentrations of the highly selective MOR antagonist, CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Trh-Pen-NH_2) (Figure 4.13). One site binding curves were best fitted in each case, with no significant difference in Hill coefficients between the different samples (Table 4.7). Introduction in the hMOR fusion of the $G^{202}A$ mutation in the G protein subunit did not alter CTOP binding properties as similar K_i values were obtained for the mutated as for the wild type fusion (Table 4.7). The K_i value observed for hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ was >10,000 nM. This low affinity value was expected as CTOP is reported to be a selective MOR antagonist and thus should not bind DOR with high affinity. However, when the pair of inactive MOR and DOR fusion proteins were co-expressed, a significantly different K_i value was observed when compared to the wild type hMOR-$G_{i1\alpha}C^{351}I$ fusion protein ($P<0.001$, 1 way ANOVA), but similar to the value obtained for hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ fusion protein ($P>0.05$, 1 way ANOVA). This result might suggest that hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ was overexpressed compared to hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and indeed that no significant amounts of the MOR fusion protein was present but this is not consistent with the high DAMGO-induced stimulation of [^{35}S]-GTPγS binding observed when these two mutated fusion proteins were co-transfected (Figure 4.12).

Table 4.7: Binding affinity of CTOP for the different fusion proteins when expressed individually or co-expressed

Construct	K_i (nM)	pK_i	Hill coefficient
hMOR-$G_{i1\alpha}C^{351}I$	25.7 ± 13.4	7.72 ± 0.24	-0.79 ± 0.11
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	25.1 ± 12.3	7.71 ± 0.23	-0.83 ± 0.07
hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$	> 10,000***	<5***	-1.16 ± 0.07
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$+ hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$	>10,000***	<5***	-0.75 ± 0.23

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

Statistics were performed using pK_i

*** Significantly different $P<0.001$ from hMOR-$G_{i1\alpha}$ $C^{351}I$ fusion protein

- **Competition for [^{3}H]-diprenorphine binding by ICI 174 864**

The same membranes as used for [^{35}S]-GTPγS binding experiments, expressing hMOR-$G_{i1\alpha}C^{351}I$, hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or co-expressing the pair of mutated fusion proteins were used for [^{3}H]-diprenorphine competition experiments using varying concentrations of the selective DOR antagonist ICI 174 864 (Figure 4.14). Competition curves with one binding site were observed for fusion proteins individually expressed. A K_i value >10,000 nM was detected for hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$, this low affinity value was not surprising as ICI 174 864 is a selective antagonist for DOR (Table 4.8). hDORV^{150}E,V^{154}D-$G_{i1\alpha}$ $C^{351}I$ exhibited a K_i value of 124 ± 20 nM. However, when the mutated fusion proteins were co-expressed, competition curves were best fitted to a two ligand binding sites model (Hill coefficient = - 0.58 ± 0.03), with a higher and a lower affinity binding site. K_h and K_l values of 84 ± 26 and >10,000 nM, respectively, were observed (Table 4.8). No significant difference in value was observed between hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ K_i and the K_h for co-transfected fusion proteins. A similar K_l value compared to that for hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ K_i was also noted ($P>0.05$, 1 way ANOVA) (Table 4.8). These results suggested that the K_h and K_l values correspond to the binding affinity of ICI 174 864 for hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ and hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$, respectively. Moreover 58 ± 5 % of the [^{3}H]-diprenorphine displayed high affinity for ICI 174 864, suggesting an equal expression of both mutated fusion proteins in these membranes.

Table 4.8: Binding affinity of ICI 174 864 for the different fusion proteins and co-expression of the distinct mutated pair of fusion proteins.

Construct	K_i (nM)	pK_i	% High affinity site	Hill coefficient
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	> 10,000	4.53 ± 0.03		–
hDORV^{150}E,V^{154}D-$G_{i1\alpha}$ $C^{351}I$	124 ± 20	6.92 ± 0.07		- 0.88 ± 0.14
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$+ hDORV^{150}E,V^{154}D-$G_{i1\alpha}$ $C^{351}I$	84 ± 26/ >10,000	7.17 ± 0.15/5.27 ± 0.28	58 ± 5	- 0.58 ± 0.03

Data represent n=3 experiments performed in triplicate on different membrane preparations
Numbers are means ± SEM
Statistics were performed using pK_i

D/ Ability of the hMOR/hDOR reconstituted heterodimer to activate G protein function

The wild type hMOR-$G_{i1\alpha}C^{351}I$ fusion protein or the pair of complementary hDOR or hMOR fusion proteins were transiently transfected into HEK 293 cells and membranes prepared. An equal number of receptor [^{3}H] diprenorphine binding sites (15 fmol) were used to study G protein activation by measuring [^{35}S]-GTPγS binding in the presence of increasing concentrations of DAMGO (Figure 4.15). No significant difference in the potency to activate G protein was observed between the wild type fusion protein and the reconstituted hMOR/hDOR heterodimer (P>0.05, T test)

Table 4.9: EC_{50} of DAMGO to activate hMOR-$G_{i1\alpha}C^{351}I$ and the reconstituted hMOR/hDOR dimer: [^{35}S]-GTPγS binding studies

Construct	EC_{50} (nM)	pEC_{50}
hMOR-$G_{i1\alpha}$ $C^{351}I$	214 ± 72	6.72 ± 0.16
Co-transfection	279 ± 134	6.67 ± 0.24

Data represent n=3 experiments performed in triplicate and numbers are means ± SEM

Statistics were performed using pEC_{50}

E/ The combination of MOR and DOR agonists on [^{35}S]-GTPγS binding to the reconstituted dimer

Membranes containing 15 fmol of [^{3}H]-diprenorphine binding sites of the hDOR/hMOR reconstituted heterodimer were used to study the activation of $G_{i1\alpha}$ by a combination of the MOR and DOR selective agonists, DAMGO and DPDPE, respectively. A concentration of 200 nM DAMGO, corresponding to the EC_{50} determined previously, (Table 4.9) was used to stimulate [^{35}S]-GTPγS binding and increasing concentrations of DPDPE were applied (Figure 4.16). Membranes were also stimulated with 10 μM DAMGO as maximum stimulation control. No alteration in [^{35}S]-GTPγS binding was noted when 200 nM DAMGO and varying concentrations of DPDPE were applied simultaneously therefore no synergistic or co-operative effects on the heterodimer were observed.

4.2.4 MOR and KOR heterodimerisation studied using the protein complementation technique

To study potential MOR/KOR heterodimerisation, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $rKORV^{150}E,V^{154}D$-$G_{i1\alpha}$ $C^{351}I$ fusion proteins were used.

A/ Analysis of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ co-expression

Membranes co-expressing hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ were used to measure specific binding of increasing concentrations of [^{3}H]-diprenorphine (Figure 4.17). An expression level of 2211 ± 267 fmol/mg was observed when 2 μg and 1 μg of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ cDNA respectively, were co-transfected. These two different amounts of cDNA were used for co-transfection as $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ B_{max} was two times higher than hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (Table 3.7 and 3.11) and an equal expression level of each fusion protein was desired. Determination of the expression level of each individual fusion protein was not possible as [^{3}H]-diprenorphine is an antagonist with similar affinity at both MOR and KOR. The expression level of the co-expressed mutated fusion proteins was significantly different to hMOR-$G_{i1\alpha}C^{351}I$ (Table 4.10). It was also possible to determine the K_d value for the co-expressed mutated fusion proteins using saturation binding. The value observed was significantly different ($P<0.01$, 1 way ANOVA) compared to hMOR-$G_{i1\alpha}C^{351}I$, but did not differ from the K_d value for $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$.

Table 4.10: B_{max} and K_d of co-expressed hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}$ fusion proteins

Construct	B_{max}	K_d	pK_d
hMOR-$G_{i1\alpha}C^{351}I$	1217 ± 72	0.36 ± 0.07	9.47 ± 0.08
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	1251 ± 20	0.32 ± 0.05	9.52 ± 0.08
$rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$	2391 ± 177	1.35 ± 0.13	8.88 ± 0.04
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}$ $C^{351}I$	2211 ± 267**	0.89 ± 0.09**	9.06 ± 0.05**

Data represent n=3 experiments performed in triplicate on different membrane preparations
Numbers are means ± SEM
Statistics were performed on pK_d
**Significantly different P<0.01 ANOVA

B/ Reconstitution of function using the non-functional fusion proteins hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKORV^{160}E,V^{164}D-$G_{i1\alpha}$ C^{351}I

The mutated fusion proteins rKORV^{160}E,V^{164}D-$G_{i1\alpha}$ C^{351}I and hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were individually or co-transfected into HEK 293 cells. hMOR-$G_{i1\alpha}C^{351}I$ was transfected as a reference. Expression of the fusions proteins was assessed by the binding of a single, close to saturating concentration of [^{3}H]-diprenorphine on membrane preparations. Membranes expressing equal numbers of [^{3}H]-diprenorphine binding sites (15 fmol) were used to measure [^{35}S]-GTPγS binding in the presence of 10 μM or 100 nM DAMGO. As previously observed in Figure 3.14 and 4.12, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ did not exert any agonist-induced [^{35}S]-GTPγS binding as it is non-functional. rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ also did not bind [^{35}S]-GTPγS after DAMGO treatment (Figure 4.18). This result was expected as it was previously observed that rKORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ is non-functional (Figure 3.19), and that the agonist used in this experiment, DAMGO, is a highly selective MOR agonist. Nevertheless, stimulation was observed when the two mutated fusion proteins were co-expressed (Table 4.11, Figure 4.18). When twice the number of [^{3}H]-diprenorphine binding sites in membranes co-expressing the mutated fusion proteins were used a similar extent of agonist stimulation as for hMOR-$G_{i1\alpha}$ C^{351}I was observed for both agonist concentrations (Table 4.11). The level of reconstitution observed was similar as previously noted for the MOR/DOR reconstituted heterodimer (Figure 4.12). When membranes containing 15 fmol of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and 15 fmol of rKORV^{160}E,V^{164}D-$G_{i1\alpha}$ C^{351}I were prepared individually and combined before the assay, no significant difference (P>0.05, 1 way ANOVA) in [^{35}S]-GTPγS binding was observed in the presence or absence of agonist (Table 4.11). The reconstitution detected therefore required co-expression and not simply the presence of both constructs in the assay. This result was surprising as others have suggested that KOR and MOR are not able to dimerise (Jordan and Devi, 1999, Filizola *et al.*, 2002).

The constitutive activity observed for the wild type fusion protein was reached when 30 fmol of co-expressed mutated fusion proteins [^{3}H]-diprenorphine binding sites was used as

hMOR/rKOR reconstituted heterodimer. Twice the number of [^{3}H]-diprenorphine binding sites of the reconstituted dimer were needed to reconstitute a full wild type signal as well.

Table 4.11: [^{35}S]-GTPγS binding of the hMOR and rKOR fusion proteins

Construct	Basal	DAMGO 10μM	DAMGO 100nM
hMOR-$G_{i1\alpha}$ $C^{351}I$	100	430 ± 89	250 ± 29
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	102 ± 40	134 ± 49	120 ± 41
rKOR$V^{160}E,V^{164}D$-$G_{i1\alpha}$ $C^{351}I$	55 ± 6	77 ± 11	82 ± 27
Co-transfection 15 fmol	56 ± 5	213 ± 40	98 ± 7
30 fmol	116 ± 31	405 ± 116	210 ± 38
45 fmol	208 ± 4	638 ± 82	303 ± 16
Mix membranes 30 fmol	122 ± 23	150 ± 20	139 ± 35

Data represent n=3 experiments performed in triplicate and numbers are means ± SEM. 100% is binding of [^{35}S]-GTPγS to hMOR-$G_{i1\alpha}$ $C^{351}I$ in the absence of agonist

C/ Interaction of hMOR with rKOR observed by co-immunoprecipitation

Flag-hMOR and rKOR-eYFP constructs made previously in the laboratory, were individually or co-transfected into HEK 293 cells. Samples were immunoprecipitated using an anti-GFP antiserum. These were resolved by SDS-PAGE, and anti-Flag antibody used for protein detection (Figure 4.19A). No specific bands were visualised in samples expressing either Flag-MOR or KOR-eYFP. A specific band at 40 kDa was present only when the Flag and eYFP versions of the proteins were co-expressed. The expected molecular mass for MOR is around 42 kDa and is consistent with the 40 kDa band immunodetected. An aliquot of each sample was reserved before immunoprecipitation and eYFP fluorescence emission (480nm) was measured. Fluorescence above mock transfected samples was only detected in the samples expressing rKOR-eYFP and was similar in extent in samples expressing KOR-eYFP alone or where rKOR-eYFP and Flag-hMOR were co-expressed (Figure 4.19B). These results

demonstrated that hMOR and rKOR can interact and are in agreement with the signal reconstitution observed by [^{35}S]-GTPγS binding (Figure 4.18).

D/ The affinity of the MOR antagonist CTOP for hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKORV^{160}E,V^{164}D-$G_{i1\alpha}$ $C^{351}I$ fusion proteins

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$, rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$, hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ or co-expressing the mutated pair of distinct but complementary fusion proteins (rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ + hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$) were used for [^{3}H]-diprenorphine competition experiments using varying concentrations of the highly selective MOR antagonist, CTOP (Figure 4.20). One site binding curves, were best fitted in each case, with no significant difference in Hill coefficient between the different samples ($P>0.05$, 1 way ANOVA, Table 4.12). As previously reported (Table 4.7), introduction of the $G^{202}A$ mutation in the G-protein subunit did not alter CTOP binding properties as similar K_i values as observed with hMOR-$G_{i1\alpha}$ $C^{351}I$ were obtained (Table 4.12). A low affinity K_i value (2760 ± 513 nM) was noted for rKORV^{160}E,V^{164}D-$G_{i1\alpha}$ $C^{351}I$, a result that was expected as CTOP is a MOR antagonist. When the distinct but complementary pair of fusion proteins were co-expressed, a significantly different K_i value was observed ($P<0.001$, 1 way ANOVA) compared to the hMOR-$G_{i1\alpha}$ $C^{351}I$, but similar to the rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ fusion protein ($P>0.05$, 1 way ANOVA). This result could suggest that rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ was overexpressed compared to hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ but this was not obvious from the high DAMGO-induced stimulation observed when these two mutated fusion proteins were co-expressed.

Table 4.12: Binding affinity of CTOP for the different fusion proteins and following co-expression of the mutated pair of fusion proteins

	K_i (nM)	pK_i	Hill number
hMOR-$G_{i1\alpha}$ $C^{351}I$	14.3 ± 4.5	7.89 ± 0.14	-0.78 ± 0.02
hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$	24 ± 3	7.63 ± 0.06	-0.81 ± 0.03
rKORV^{160}E,V^{164}D-$G_{i1\alpha}$ $C^{351}I$	2760 ± 513***	5.57 ± 0.07***	-1.04 ± 0.13
Co-transfection	1350 ± 363***	5.90 ± 0.15***	- 0.77 ± 0.09

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

Statistics were performed using pK_i

***Significantly different from hMOR-$G_{i1\alpha}$ $C^{351}I$ P<0.001 ANOVA

E/ The ability of the reconstituted dimer to activate G protein function

The wild type hMOR-$G_{i1\alpha}C^{351}I$ fusion protein or the pair of non-functional fusion proteins were transiently transfected in HEK 293 cells and membranes were prepared. Equal amounts of receptor binding sites (15 fmol) were used to study G protein activation of hMOR-$G_{i1\alpha}$ $C^{351}I$ or the reconstituted hMOR/rKOR heterodimer by measuring [^{35}S]-GTPγS binding in the presence of increasing concentrations of DAMGO (Figure 4.21). No significant difference in the potency to activate G protein was observed between the wild type fusion protein and the reconstituted heterodimer with EC_{50} values of 417 ± 200 and 310 ± 106 respectively (P>0.05, T test).

Table 4.13: EC_{50} for DAMGO stimulated [^{35}S]-GTPγS binding studies at hMOR-$G_{i1\alpha}C^{351}I$ and the reconstituted hMOR/rKOR heterodimer

	EC_{50} (nM)	pEC_{50}
hMOR-$G_{i1\alpha}$ $C^{351}I$	417 ± 200	6.44 ± 0.24
Co-transfection	310 ± 106	6.56 ± 0.16

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

Statistics were performed using pEC_{50}

F/ The combination of hMOR and rKOR agonists on [^{35}S]-GTPγS binding of the reconstituted dimer

Membranes containing 15 fmol of [^{3}H]-diprenorphine binding sites were used to study the activation of the $G_{i1\alpha}$ subunit of the rKOR/hMOR reconstituted heterodimer by a combination

of MOR and KOR agonists, DAMGO and U69593, respectively. A concentration of 200 nM DAMGO was used to stimulate [^{35}S]-GTPγS binding and increasing concentrations of U69593 were applied (Figure 4.22). Membranes were also stimulated with 10 μM DAMGO as a maximum stimulation control. No alteration in [^{35}S]-GTPγS binding was noticed when DAMGO and different concentrations of U69593 were applied simultaneously therefore no synergistic agonist effect of the heterodimer was observed.

4.3 Discussion:

Although the protein complementation technique used in this investigation was useful for studying opioid receptor homodimerisation and provided evidence that the dimer was the active signalling unit, this technique was of greatest interest to study opioid receptor heterodimerisation. The complementation between pairs of mutated fusion proteins allowed monitoring of heterodimer pharmacology and function without the presence of the confounding signal of the homodimers that might also be expected in the samples upon co-expression of pairs of receptor (Figure 4.23).

Molinari et al, (2003) co-transfected a construct consisting of the vasopressin V2-transmembrane region 1 fused to G_o along with DOR and described that a G protein subunit simply inserted in the membrane via a transmembrane anchor was sufficient to enhance GTPγS binding to a nearby receptor. In their study a very high expression level of opioid receptor was employed which entered into the range of order of expression levels where dimers can potentially be formed by random collision events (Mercier *et al.*, 2002). Nevertheless, it was important to demonstrate that the proximity between a membrane tethered G protein and a GPCR did not represent the basis for signals observed using the complementation technique. The hDOR N-terminal domain and TM1 region were fused to $G_{i1\alpha}C^{351}I$ then Flag-tagged at the N-terminus (Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$) and co-expressed with the non-functional fusion protein hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$. As expected, Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ did not bind [^{3}H]-diprenorphine as a single transmembrane domain is not sufficient to create a ligand binding pocket. The K_d and B_{max} values observed for [^{3}H]-diprenorphine following co-expression of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ were not significantly different compared to individually expressed hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (Table 4.1). Immunoblots of membrane fractions used for [^{35}S]-GTPγS binding experiments clearly showed a specific band at 50 kDa, corresponding to the expected molecular size of Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ (Figure 4.4). [^{35}S]-GTPγS binding assays in which the Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ construct was immunoprecipitated with anti-$G_{i1/2}$ antiserum confirmed that this construct did not bind [^{35}S]-GTPγS in response to DADLE (Figure 4.3). In a parallel experiment hDOR-$G_{i1\alpha}C^{351}I$ agonist-induced [^{35}S]-GTPγS binding was observed, confirming that the absence of signal for Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ was due to

the lack of functionality of the construct generated. hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ was also non-functional as shown in previous experiments (Figure 3.6). The co-expression of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ did not result in significant detection of agonist-induced [^{35}S]-GTPγS binding (Figure 4.3). Therefore co-expression of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ with Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ did not allow significant transactivation of the G protein linked to DOR TM1, demonstrating that simple anchorage of a G protein to the membrane was not sufficient to result in activation of the G protein via a co-expressed receptor. As it was possible that co-expression of c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ did not result in their physical proximity (which might account for the lack of activation observed), immunoprecipitation with anti-Flag antibody and detection with anti-c-Myc antibody of membranes individually or co-expressing Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ and c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ was carried out (Figure 4.5). A specific band at 80 kDa was only detected when the Flag and c-Myc version of the constructs were co-expressed demonstrating that co-expressed Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ and c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ are able to interact. In parallel, samples singly or co-expressing c-Myc-Nt-$TM1_{DOR}$ and Flag-hDOR were also immunoprecipitated with anti-Flag antibody and immunodetected with anti-c-Myc antibody. A band at 15 kDa was detected only when both constructs were co-expressed. This result confirmed that DOR TM1 and full length DOR can interact, even in the absence of fused G protein, and consequently demonstrated that the presence of the G protein did not force dimerisation. Thus, the entity formed of a DOR-TM1 and a full-lengh receptor cannot activate the G protein, confirming that the recovered function observed in chapters 3 and 4 is due to receptor association. This provides further evidence that two GPCRs, as a dimer, are the platform for G protein activation, as discussed in chapter 3.

Different transmembrane domains have been described as being involved in receptor dimerisation. For example TM6 was reported as being a β2-adrenoceptor dimer interface (Herbert *et al.*, 1996), whereas TM4 was shown to mediate homodimerisation of the dopamine D2 receptor (Guo *et al.*, 2003). Rhodopsin crystallisation and visualisation of the dimeric organisation of rhodopsin units by atomic force microscopy have suggested involvement of TM4 and TM5 as contact interfaces whereas TM1, TM2 and the 3rd intracellular loop were proposed to be involved in dimer row formation (Fotiadis *et al.*, 2003, Liang *et al.*, 2003). c-Myc-Nt-$TM1_{DOR}$ was also co-immunoprecipitated when co-expressed with Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}$I, suggesting that TM1 can self-associate and hence that this domain might be a contact interface for DOR homodimer formation. This result was not

predicted by a computational study, which suggested that the TM4 and TM5 domains maybe involved in DOR homodimerisation (Filizola and Weinstein, 2002). However, experimental studies on GPCRs such as the yeast α-factor receptor, the α_{1b}-adrenoceptor or complement C5a receptor have also proposed the involvement of TM1 in dimerisation of these GPCRs (Overton and Blumer, 2002, Overton *et al.*, 2003, Carrillo *et al.*, 2004, Kleo *et al.*, 2003).

Although the β_2-adrenoceptor and opioid receptors couple to different G protein classes, their potential for heterodimerisation has been studied. Jordan et al, (2001) provided evidence by co-immunoprecipitation that DOR and KOR could both interact with the β_2-adrenoceptor. DOR/β_2-adrenoceptor heterodimer formation modified receptor trafficking properties with endocytosis of both receptor noted upon treatment with agonist at the other receptor. Nevertheless, no change in ligand binding or coupling properties was observed. In the case of the KOR/β_2-adrenoceptor heterodimer, KOR receptor had a dominant negative effect on β_2-adrenoceptor as no agonist-induced internalisation of β_2-adrenoceptor was observed when the two receptors were co-expressed. Futhermore, signal transduction was altered, MAP kinase phosphorylation was decreased in response to a β_2-adrenoceptor agonist when both receptors were co-expressed. However, when β_2-adrenoceptor and KOR heterodimerisation was studied using a BRET^2 assay and compared to KOR homodimer and KOR/DOR heterodimer formation, a higher level of receptor expression was needed to detect a BRET^2 signal. This suggests that KOR and β_2-adrenoceptor have less affinity to form a heterodimer compared to KOR/DOR or the KOR/KOR homodimer (Ramsay *et al.*, 2002). A similar observation for α_{1a}-adrenoceptor homodimers compared to the α_{1a}-adrenoceptor/DOR heterodimer has recently been reported (Ramsay *et al.*, 2004). Moreover, McVey et al., (2001) showed a weak BRET and FRET signals for DOR/β_2-adrenoceptor heterodimer in comparison to the DOR homodimer. Thus, it was interesting to study the possible formation of a DOR/β_2-adrenoceptor heterodimer using the complementation technique. This technique could provide information about the affinity and the functionality of any dimer formed by these two receptors. In order to do so, a β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ fusion protein was used. The β_2-adrenoceptor is usually associated with G_s but some groups have reported that the β_2-adrenoceptor is able to switch coupling from G_s to G_i to initiate new signalling events (Daaka *et al.*, 1997, Zhu *et al.*, 2001). However, when agonist-induced [^{35}S]-GTPγS binding to β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ was measured, no significant activation was recorded (Figure 4.6). Thus, in my study I was unable to demonstrate β_2-adrenoceptor activation of fused G_{i1}. The

discrepancy observed in comparison with other groups might be explained by the use herein of a fusion protein. The β_2-adrenoceptor switching from G_s to G_i coupling has been associated with PKA-mediated receptor phosphorylation (Zamah *et al.*, 2002). In my study, the fusion of G_{i1} protein with the receptor might have interfered with PKA phosphorylation of the receptor. The B_{max} and K_d of the different fusion proteins were determined by saturation binding using [^{3}H]-labeled antagonists for either DOR or β_2-adrenoceptor. No significant difference was observed in K_d values for the co-expressed β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ compared to the wild type fusion proteins (Table 4.2 A and B). A close to 1:1 expression level was achieved when the fusion proteins were co-expressed and used for the different assays. When the distinct but potentially complementary fusion proteins were co-expressed, [^{35}S]-GTPγS binding was observed after DADLE treatment. The functional DOR receptor was therefore able to activate the functional G protein fused to the β_2-adrenoceptor. This result favours DOR/β_2-adrenoceptor forming a signalling unit, therefore a dimer. However, less than half of the reconstituted signal observed for the DOR homodimer was recorded when equal numbers of DOR/β_2-adrenoceptor fusion protein [^{3}H]-antagonist binding sites were used. This might indicate that the propensity of DOR and β_2-adrenoceptor to interact is lower in comparison to the homodimer formed by two DOR. This is in agreement with the conclusions of McVey et al., (2001) as well as Ramsay et al., (2002) that less closely related receptors appear to have lower affinity to form dimers. No change in the potency of DADLE to activate the G protein was observed for the reconstituted dimer compared to hDOR-$G_{i1\alpha}C^{351}I$ fusion protein alone (Figure 4.10). This result is in agreement with the absence of alteration in signalling reported by Jordan et al, (2001) upon DOR/β_2-adrenoceptor co-expression. These workers did not observe any change in adenylate cyclase inhibition by opioid agonists.

In some cases, closely related GPCRs have been reported to fail to interact e.g the α_{1A} and α_{1D} adrenoceptors (Uberti *et al.*, 2003), MOR and KOR have also been reported not to contact. Absence of MOR/KOR interaction was described in co-immunoprecipitation studies when differentially epitope-tagged MOR and KOR were co-expressed (Jordan and Devi, 1999). A computational approach, suggesting transmembrane domains involved in opioid heterodimerisation has provided support for this lack of interaction (Filizola *et al.*, 2002). However in my study, when hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ were co-expressed, DAMGO-induced [^{35}S]-GTPγS binding was recorded (Figure 4.18) indicating recovery of function upon fusion protein interaction. hMOR/rKOR interaction was confirmed

by detection of a specific band at 40kDa following Flag-hMOR and rKOR-eYFP co-immunoprecipitation with a GFP antiserum and immunodetection with an anti-Flag antibody (Figure 4.19). Although these results are different from the finding of others, they do not seem surprising as the three opioid receptor share high sequence homology. Equally CCR2 and CXCR4 were widely reported not to dimerise (Mellado *et al.*, 1999) but they have recently been described to interact using a BRET based approach (Percherancier *et al.*, 2005).

As expected, when the hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ fusion proteins were co-transfected, [^{35}S]-GTPγS binding was observed after DAMGO treatment reflecting rescue of receptor function by dimer formation (Figure 4.12). Competition for [^{3}H]-diprenorphine binding by ICI 174 864 verified that the DOR and MOR fusion proteins were co-expressed a 1:1 ratio (Table 4.8). For both the hMOR/rKOR and hMOR/hDOR recontituted heterodimers, signal as great as for the wild type fusion protein was recovered when twice the numbers of [^{3}H]–antagonist binding sites in the co-expressed samples were used (Tables 4.6 and 4.11). This percentage may be expected if the functional element is a dimer, as discussed in Chapter 3. The results obtained for opioid receptor heterodimerisation are different to the observations in Chapter 3 for opioid receptors homodimers (Figures 3.6, 3.14 and 3.19) and could indicate that the mutations introduced into the receptor may affect the ability of the opioid receptors to form homodimers but not heterodimers. If this is true it may suggest that different interfaces are involved in homo- and hetero-interactions. No change in potency to activate the G protein upon DAMGO stimulation was recorded for hMOR/hDOR and hMOR/rKOR heterodimers (Tables 4.9 and 4.13). This contrasts with results obtained by George et al, (2000) that described the MOR/DOR heterodimer becoming insensitive to PTX, indicative of a change in G protein coupling. As fusion proteins were used in my work a change in G protein coupling would be difficult to observe, the fusion protein favouring its two elements to interact. However, the switch in G protein coupling upon DOR/MOR interaction observed by George et al., (2000) was recently contradicted by Law et al, (2005) who did not witness any change G protein coupling when MOR and DOR were co-expressed. This recent observation would be more in agreement with the absence of G protein enhanced potency to stimulate G protein of the MOR/DOR reconstituted dimer observed in my study.

Signalling potentiation upon heterodimer formation has been witnessed for some GPCRs. For example a CCR2/CCR5 heterodimer was described to be more efficient at inducing Ca^{2+} mobilisation. The authors suggested that this response was due to a co-operative effect when

both receptors were occupied by their ligands simultaneously (Mellado *et al.*, 2001). A synergistic inhibition of adenylate cyclase upon co-exposure to DAMGO with DPDPE and TAN 67 was noted when the DOR/MOR heterodimer was formed (Martin and Prather, 2001). Gomes et al., (2003) also recorded alteration in DOR/MOR signalling properties using [^{35}S]-GTPγS binding and adenylate cyclase assays. MOR and DOR co-expression resulted in significant enhancement of MOR agonist-mediated signalling by DOR ligands, both in heterologous cells and in endogenous tissue. As only the heterodimer signal is recorded by the complementation technique, it was interesting to use a combination of DOR and MOR or MOR and KOR agonists and observe the stimulation obtained for the reconstituted heterodimers by these agonists. No synergistic effect was detected either on hMOR/hDOR or the hMOR/rKOR reconsituted heterodimer (Figures 4.16 and 4.22). Although Gomes et al, (2003) demonstrated ligand synergism when MOR and DOR were co-expressed, the only DOR agonist used in the different ligand mixes tested was deltorphine. The majority of the synergisms were observed using a combination of MOR agonists and DOR antagonists, consequently their observations do not contradict my results. However, the findings of Martin and Prather, (2001) are in opposition to my observations, as they noted an increased adenylate cyclase inhibition upon co-treatmant with DAMGO and DPDPE. A reason for this would be that the synergy they observed is mediated through G_o not G_i therefore in my study as only a G_i mediated signal was measured, a G_o mediated synergy would not be observed.
Taken together, these results are in favor of unchanged signalling properties of MOR/DOR and MOR/KOR heterodimers.

The pharmacological profile of co-expressed GPCRs have been described to differ compared to that of the same receptors individually expressed. For example SSTr2A and SSTr3 interaction resulted in a new entity with the pharmacological profile of SSTr2A (Pfeiffer *et al.*, 2001). In a similar way when muscarinic M2 and M3 receptors were co-expressed high affinity site for muscarinic ligands were identified (Maggio *et al.*, 1999). For opioid receptor heterodimers new ligand binding properties have been documented. DOR/KOR heterodimer displayed no significant alteration in affinity for either KOR or DOR selective agonists and antagonists but showed greater affinity for partial agonists (Jordan and Devi, 1999). For the MOR/DOR heterodimer, George et al, (2000) reported a reduction of affinity for DOR and MOR selective agonists but an increase in affinity for endogenous opioid peptides. A recent study reported alteration of MOR binding as a result of heterodimerisation with DOR (Law *et*

al., 2005). However, they reported that some of the ligand binding data contrasted with the observations of George et al., (2000). Competition binding performed on membranes co-expressing hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ using the DOR antagonist ICI 174864 were best fitted to a two site binding curve with both high and low affinity sites reflecting presumably hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ and hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ expression respectively (Figure 4.14). The percentage of high affinity sites was close to 50% providing evidence of an equal co-expression of each construct and no change in affinity for ICI 174 864 upon MOR/DOR heterodimer formation (Table 4.8). When the MOR antagonist CTOP was used, however, samples co-expressing hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ and hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ displayed only a low binding affinity for this ligand similar to when hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ was expressed alone (Figure 4.7). This could be due to overexpression of hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ compared to hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ but it is unlikely as the competitions with ICI 174 864 supported equal expression of each fusion protein when co-transfected. These observations argue in favor of the MOR/DOR heterodimer having a reduced affinity for the MOR selective antagonist CTOP. This is consistant with a selective change of pharmacology as previously observed by other groups. Equally, it was observed that samples co-expressing hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ displayed a lower binding affinity for CTOP and that was similar to when rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ was expressed alone. Again this result might reflect an overexpression of rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ compared to hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ but as a strong reconstituted signal was observed by [^{35}S]-GTPγS binding upon MOR selective agonist stimulation (Figure 4.12) this seem unlikely. This result could be the first indication of a change in ligand binding affinity upon KOR/MOR heterodimerisation, but further investigation is needed.

Figure 4.1: Graphic representation of Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$ and β2-adrenoceptor-$G_{i1\alpha}C^{351}I$ constructs

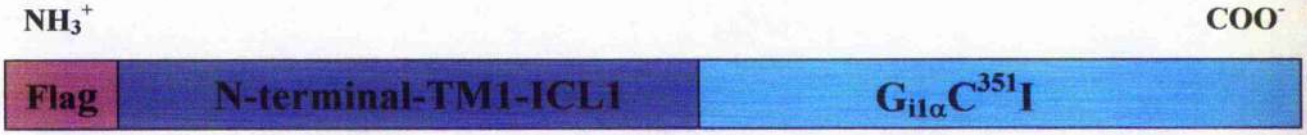
A. Flag-Nt-TM1$_{DOR}$-$G_{i1\alpha}C^{351}I$ fusion protein

This fusion protein consisted of DOR N-terminal, transmembrane domain 1 and 1st intracellular loop fused to $G_{i1\alpha}C^{351}I$. The $G_{i1\alpha}$ contained a cysteine mutated into isoleucine to prevent ADP ribosylation by PTX.

B. β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ fusion protein

This fusion protein consisted of β_2-adrenoceptor fused to $G_{i1\alpha}C^{351}I$.

A


NH3+
COO-
Flag
N-terminal-TM1-ICL1
Gi1αC351I

B


NH3+
COO-
β2-adrenoceptor
Gi1αC351I

Figure 4.2: [^{3}H]-Diprenorphine saturation binding assays following expression of hDOR-$G_{i1\alpha}C^{351}I$ and Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ fusion proteins

Membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ (blue squares), hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green circles) and co-expressing hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ (purple squares) were used to measure the specific binding of different concentrations of [^{3}H]-diprenorphine. Data are representative of n=5 experiments performed in triplicate. Data point represent means ± SEM of triplicates.

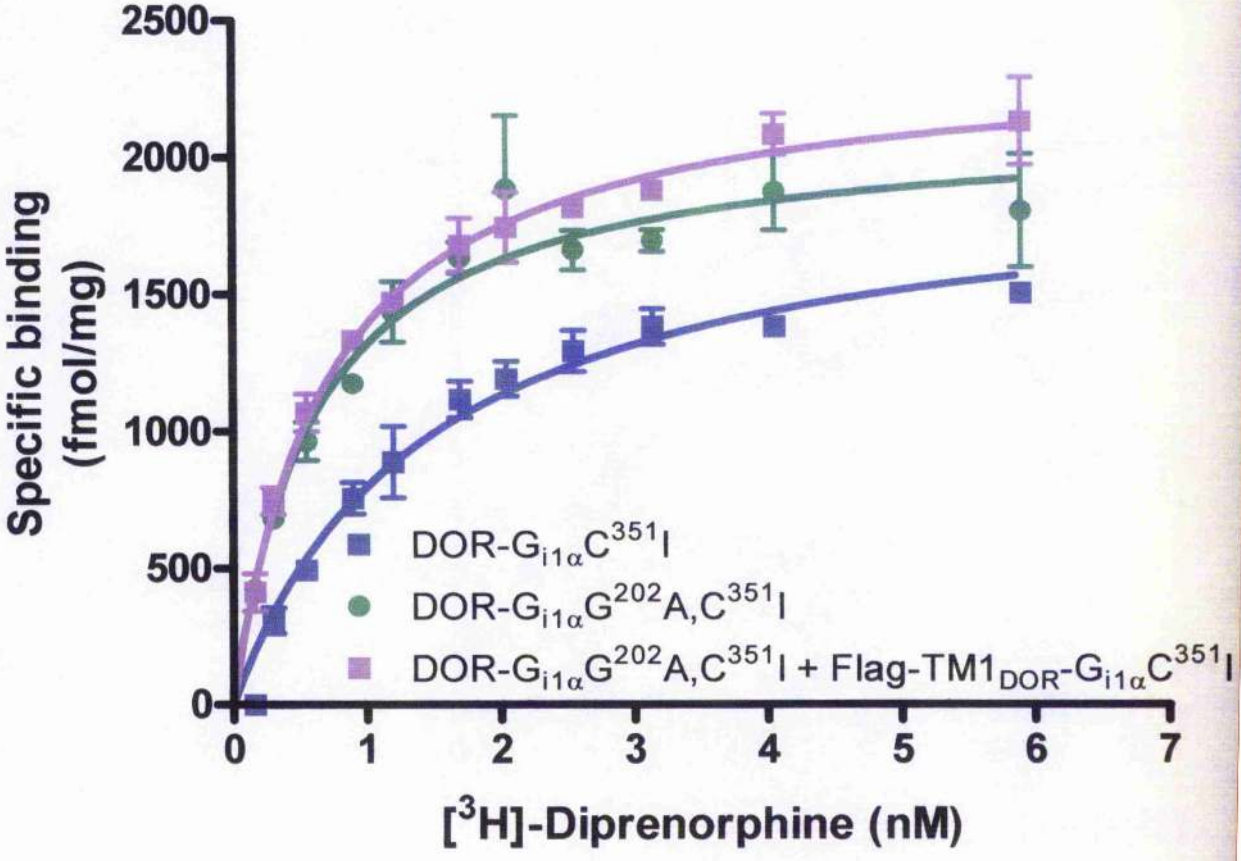

2500
2000
1500
1000
500
0
Specific binding
(fmol/mg)
0
1
2
3
4
5
6
7
[3H]-Diprenorphine (nM)
DOR-Gi1αC351I
DOR-Gi1αG202A,C351I
DOR-Gi1αG202A,C351I + Flag-TM1DOR-Gi1αC351I

Figure 4.3: Co-expression of Flag-Nt-TM1$_{DOR}$-G$_{i1\alpha}$C^{351}I with hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I does not induce receptor transactivation

Membranes of HEK 293 cells expressing 15 fmol of hDOR-G$_{i1\alpha}$C^{351}I, hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I or 30 or 45 fmol in the case of Flag-Nt-TM1$_{DOR}$-G$_{i1\alpha}$C^{351}I + hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I co-transfection or 10 μg of membrane containing Flag-Nt-TM1$_{DOR}$-G$_{i1\alpha}$C^{351}I were used to measure the binding of [^{35}S]-GTPγS in the absence (open bars) or presence of 10 μM (filled bars) and 100nM (checkered bars) DADLE. Membranes expressing 15 fmol hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I and 10μg of membrane expressing Nt-TM1$_{DOR}$-G$_{i1\alpha}$C^{351}I were also mixed and stimulated with DADLE. Data represent n=5 experiments performed in triplicate. Data point represent means ± SEM.

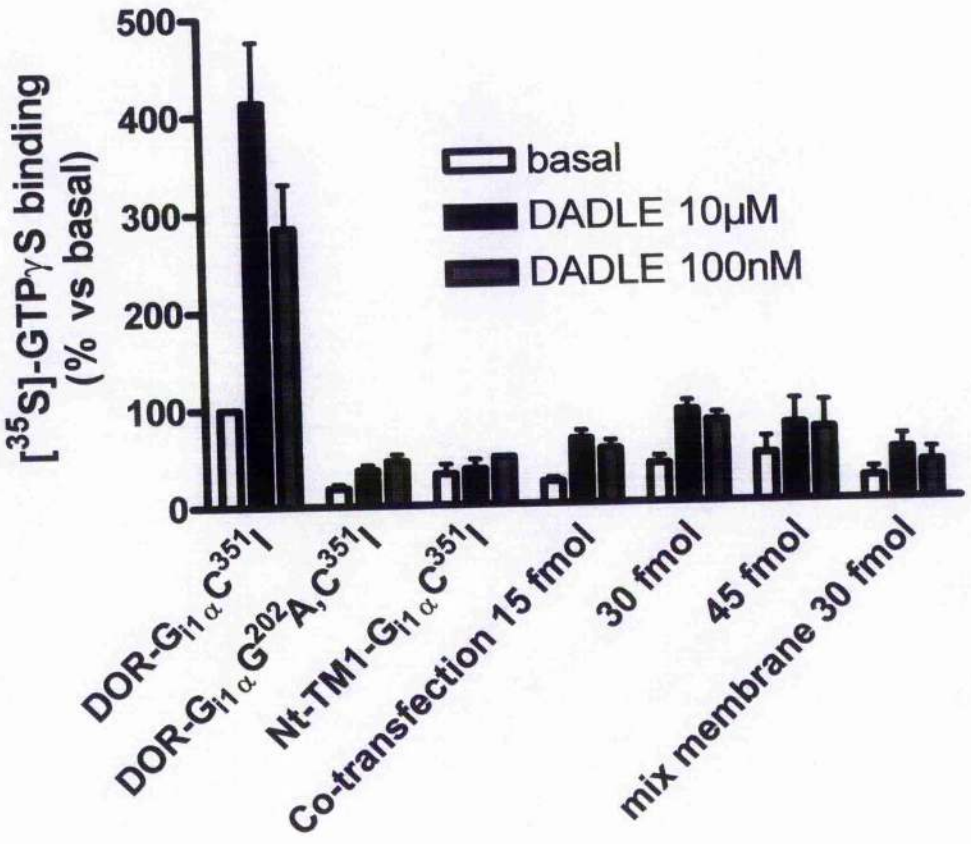

[35S]-GTPγS binding
(% vs basal)
500
400
300
200
100
0
basal
DADLE 10μM
DADLE 100nM
DOR-Gi1α C351I
DOR-Gi1α G202A,C351I
Nt-TM1-Gi1α C351I
Co-transfection 15 fmol
30 fmol
45 fmol
mix membrane 30 fmol

Figure 4.4: Immunodetection of Flag-Nt-TM1$_{DOR}$-G$_{i1\alpha}$C^{351}I in samples used for [^{35}S]-GTPγS binding

Membranes from control HEK 293 cells (1) and those used in reconstitution experiments expressing Flag-Nt-TM1$_{DOR}$-G$_{i1\alpha}$C^{351}I (2) or Flag-Nt-TM1$_{DOR}$-G$_{i1\alpha}$C^{351}I + hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I (3 and 4) were immunoblotted using anti-G$_{i1/2}$ antiserum after being resolved by SDS-PAGE.

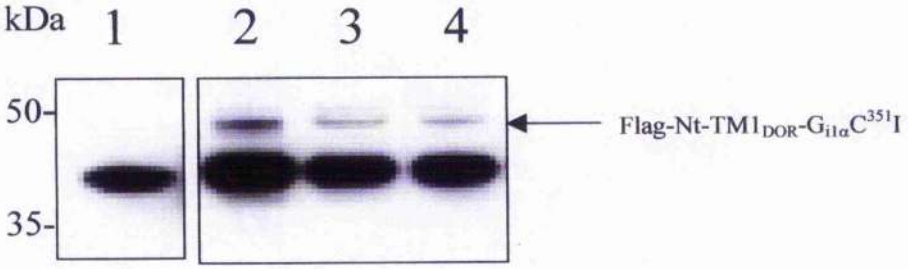

kDa
1
2
3
4
50-
35-
Flag-Nt-TM1DOR-Gi1αC351I

Figure 4.5: Co-immunoprecipitation of the different epitope tagged forms of Nt-TM1 and DOR fused or not to $G_{i1\alpha}C^{351}I$

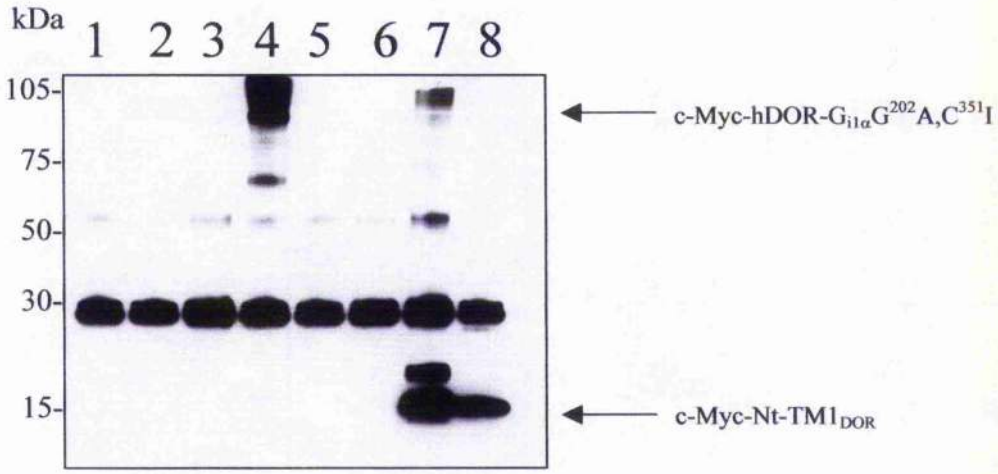
A. Co-immunoprecipitation

Membranes from HEK 293 cells (1) and cells transiently expressing c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (2), Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ (3), Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ + Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (4), Flag-hDOR (5), c-Myc-Nt-$TM1_{DOR}$ (6), Flag-hDOR + c-Myc-Nt-$TM1_{DOR}$ (7) and Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$ + c-Myc-Nt-$TM1_{DOR}$ were immunoprecipitated with anti-Flag antibody and detected with anti-c-Myc antibody after being resolved by SDS-PAGE. Data represents n=3 experiments.

B. Anti-Flag antibody was used to detect anti-Flag reactive proteins.

The same membranes as in panel A were reblotted using anti-Flag antibody.

A


kDa
1 2 3 4 5 6 7 8
105-
75-
50-
30-
15-
c-Myc-hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$
c-Myc-Nt-$TM1_{DOR}$

B

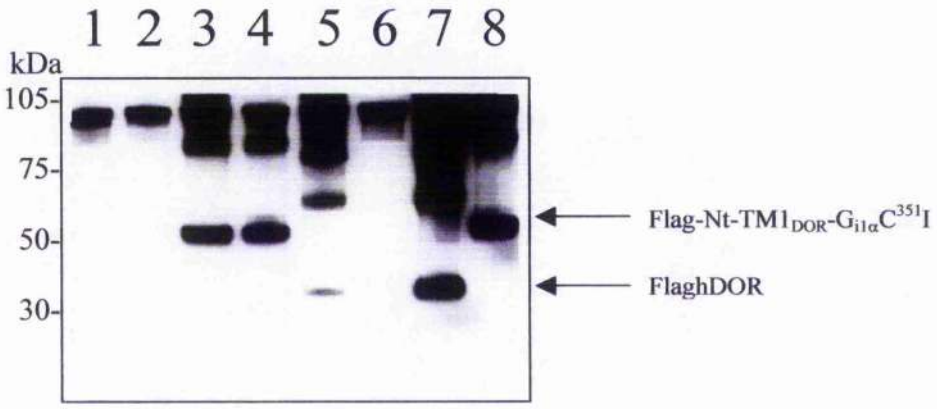

1 2 3 4 5 6 7 8
kDa
105-
75-
50-
30-
Flag-Nt-$TM1_{DOR}$-$G_{i1\alpha}C^{351}I$
FlaghDOR

Figure 4.6: β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ [^{35}S]-GTPγS binding

Membranes of HEK 293 cells expressing 15 fmol of β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ or hDOR-$G_{i1\alpha}C^{351}I$ were used to measure the binding of [^{35}S]-GTPγS in the absence (open bars) or presence of 10 μM isoproterenol (checkered bars) or DADLE (filled bars). Data represents n=3 experiments performed in triplicate. Data point represent means ± SEM of triplicates.

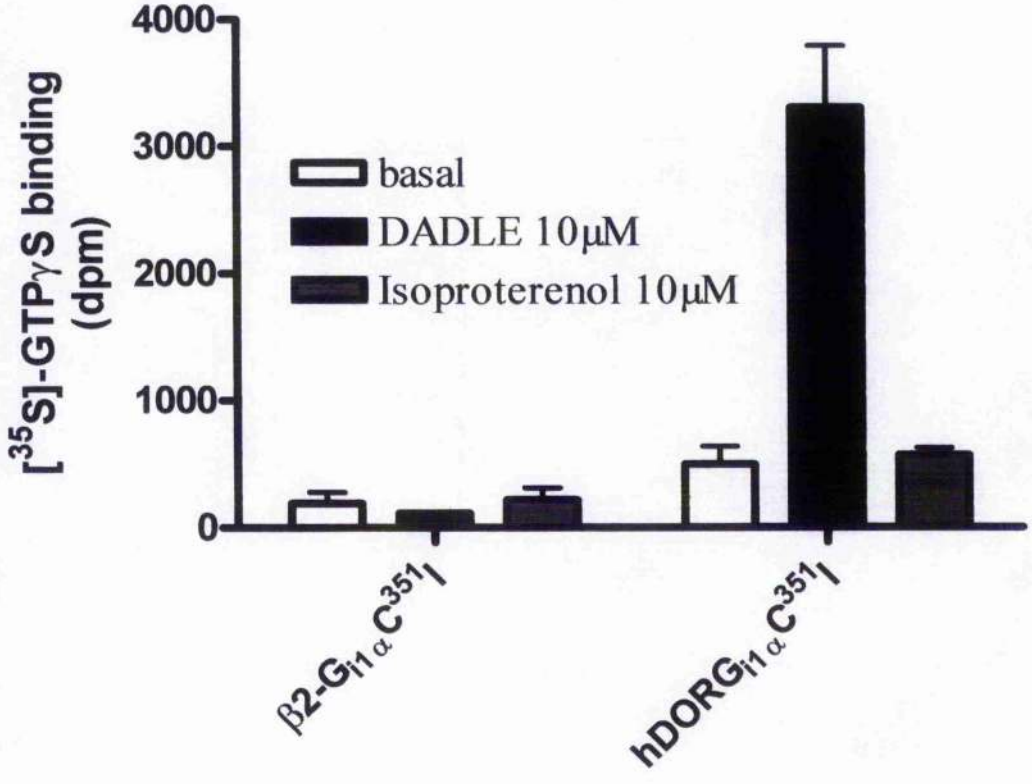

4000
3000
2000
1000
0
[35S]-GTPγS binding (dpm)
basal
DADLE 10μM
Isoproterenol 10μM
β2-Gi1α C351I
hDORGi1α C351I

Figure 4.7: Relative expression of DOR and β_2-adrenoceptor fusion proteins when co-expressed

Membranes co-expressing 2 μg of hDOR-$G_{i1\alpha}C^{351}I$ cDNA and varying amounts of β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ cDNA were used to measure specific binding of 2nM [^{3}H]-diprenorphine (open bars) and 2nM [^{3}H]-dihydroalprenolol (filled bars). Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

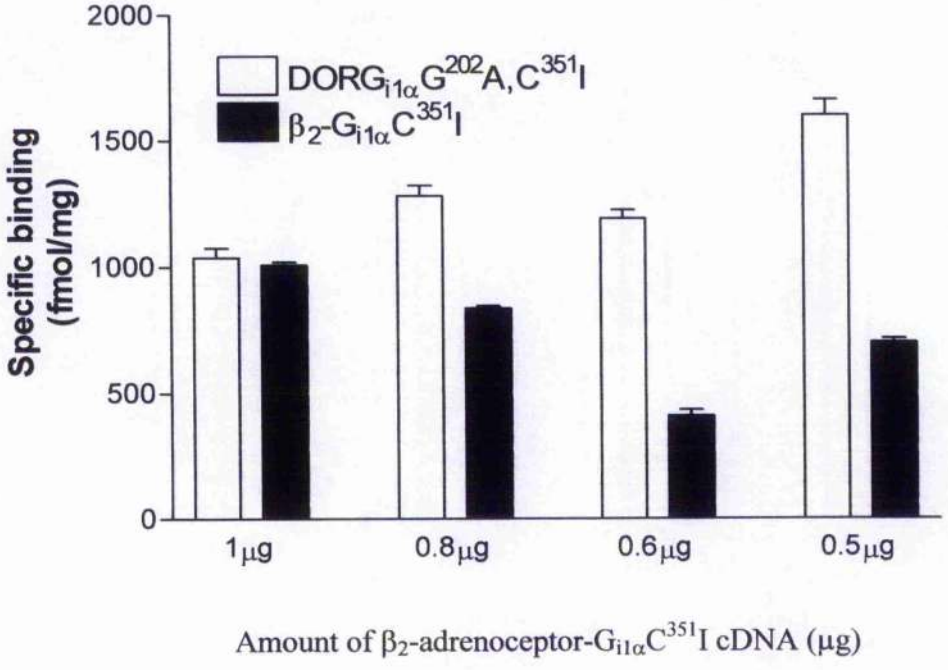

2000
1500
1000
500
0
Specific binding (fmol/mg)
DORG$_{i1\alpha}$G^{202}A,C^{351}I
β_2-G$_{i1\alpha}$C^{351}I
1μg
0.8μg
0.6μg
0.5μg
Amount of β_2-adrenoceptor-G$_{i1\alpha}$C^{351}I cDNA (μg)

Figure 4.8: [^{3}H]-diprenorphine and [^{3}H]-dihydroalprenolol saturation binding following expression of β_2-adrenoceptor and DOR fusion proteins

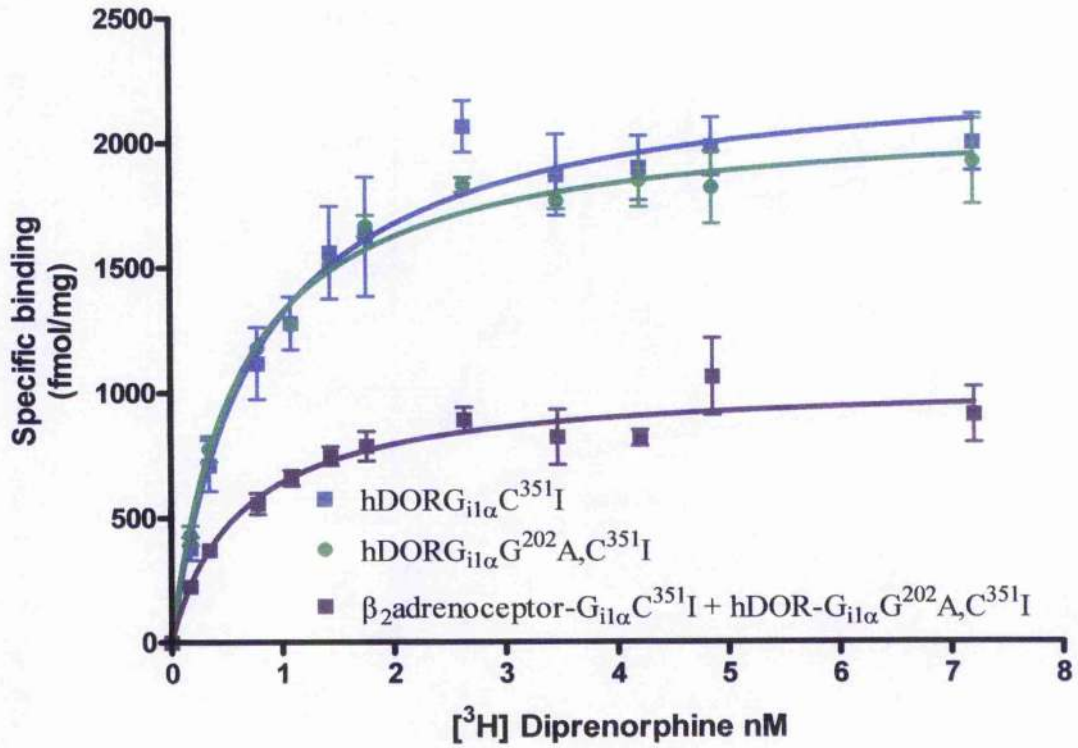
A

Membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ (blue squares), hDOR-$G_{i1\alpha}G^{22}A,C^{351}I$ (green circles), and both β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (purple squares) were used to measure the binding of different concentrations of [^{3}H]-diprenorphine. Data are representative of n=6 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

B

Membranes expressing β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ (blue squares) and β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (purple squares) were used to measure the binding of different concentrations of [^{3}H]-dihydroalprenolol. Data are representative of n=6 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

A


2500
2000
1500
1000
500
0
Specific binding (fmol/mg)
hDORG$_{i1\alpha}$C^{351}I
hDORG$_{i1\alpha}$G^{202}A,C^{351}I
$\beta_2$adrenoceptor-G$_{i1\alpha}$C^{351}I + hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I
0
1
2
3
4
5
6
7
8
[^{3}H] Diprenorphine nM

B

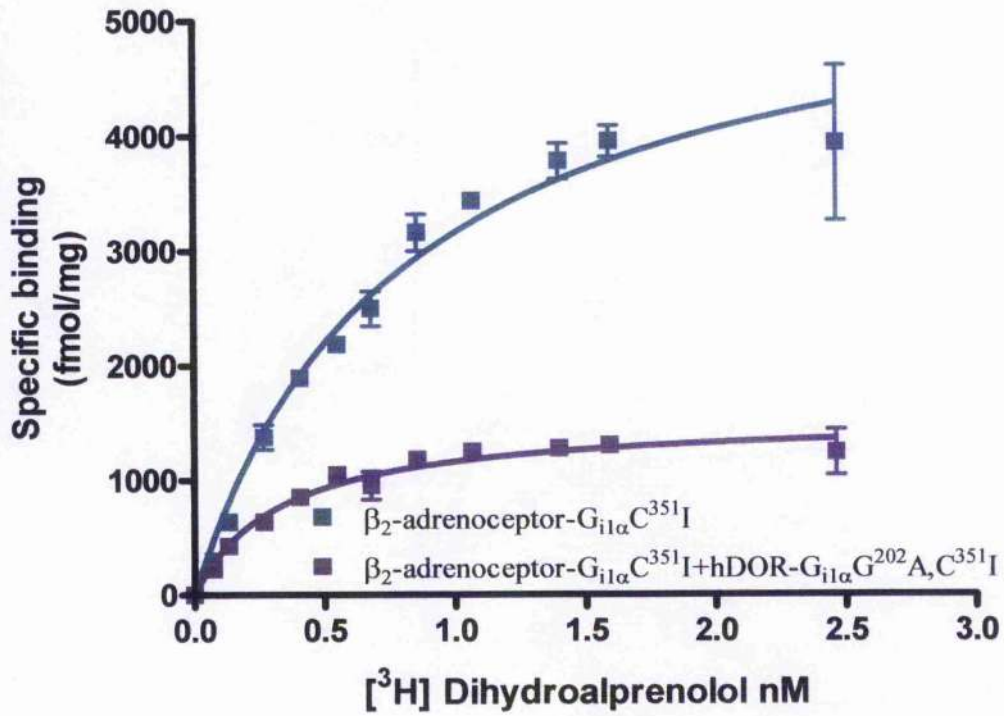

5000
4000
3000
2000
1000
0
Specific binding (fmol/mg)
β_2-adrenoceptor-G$_{i1\alpha}$C^{351}I
β_2-adrenoceptor-G$_{i1\alpha}$C^{351}I+hDOR-G$_{i1\alpha}$G^{202}A,C^{351}I
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[^{3}H] Dihydroalprenolol nM

Figure 4.9: Co-expression of non-functional fusion proteins, β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$, reconstituted function

Membranes of HEK 293 cells expressing 15fmol of hDOR-$G_{i1\alpha}C^{351}I$, hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$, β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ or 30 and 45 fmol β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ + hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ at a 1:1 ratio were used to measure [^{35}S]-GTPγS binding in the absence (open bars) or presence (filled bars) of 10 μM DADLE. Membranes expressing 15 fmol of β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ and 15 fmol of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ were also mixed and stimulated with DADLE. Data represent n=5 experiments performed in triplicate. Data points represent means ± SEM.

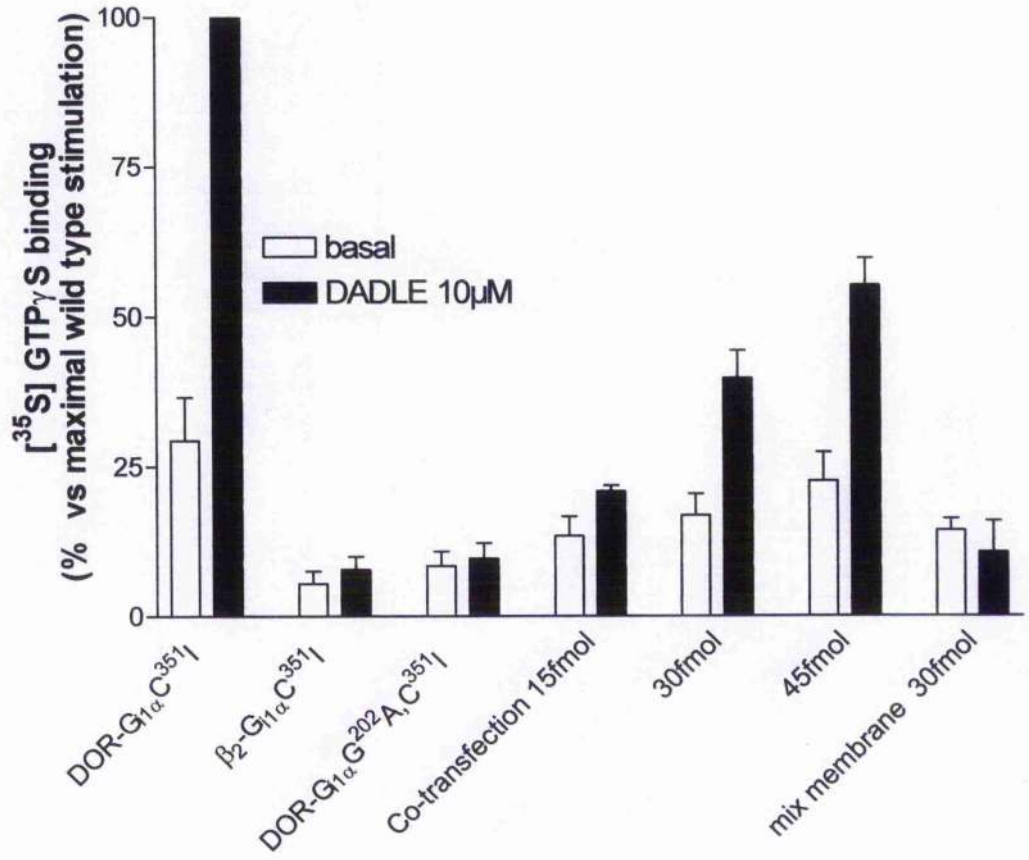

[35S] GTPγS binding
(% vs maximal wild type stimulation)
100
75
50
25
0
basal
DADLE 10μM
DOR-G1α C351
β2-G1α C351
DOR-G1α G202A, C351
Co-transfection 15fmol
30fmol
45fmol
mix membrane 30fmol

Figure 4.10: Comparison of agonist stimulated [^{35}S]-GTPγS binding to hDOR-$G_{i1\alpha}C^{351}I$ and hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ fusion proteins

Membranes expressing hDOR-$G_{i1\alpha}C^{351}I$ (blue squares) or co-transfected with 15 fmol of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ (purple squares) were used to measure the ability of increasing concentrations of DADLE to activate [^{35}S]-GTPγS binding. Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

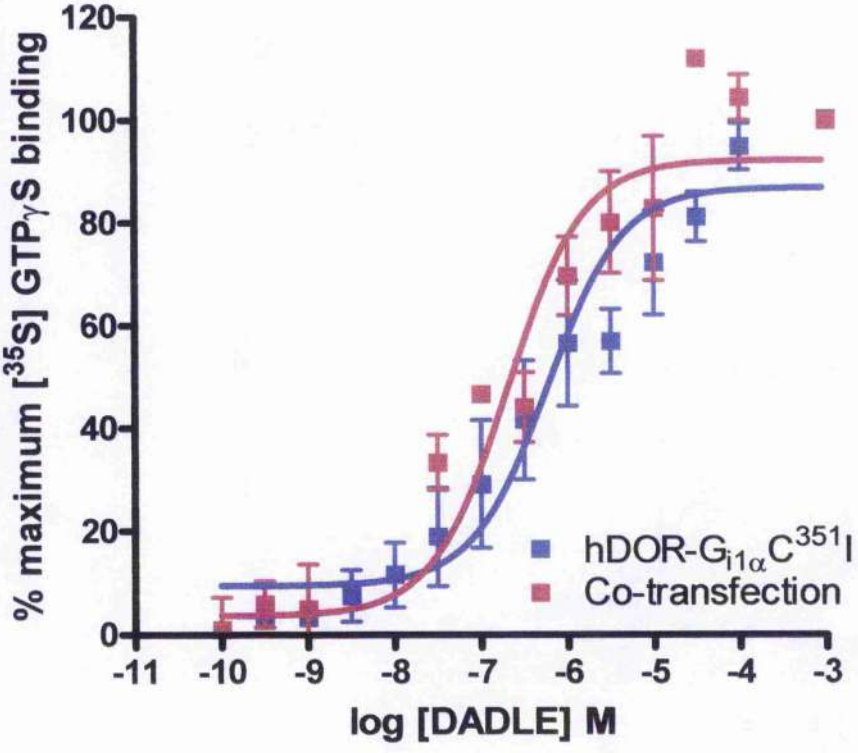

% maximum [35S] GTPγS binding
120
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
-3
log [DADLE] M
hDOR-Gi1αC351I
Co-transfection

Figure 4.11: [^{3}H]-diprenorphine saturation binding following co-expression of hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I and hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I fusion proteins

Membranes co-expressing hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I + hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I were used to measure the specific binding of different concentrations of [^{3}H]-diprenorphine. Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

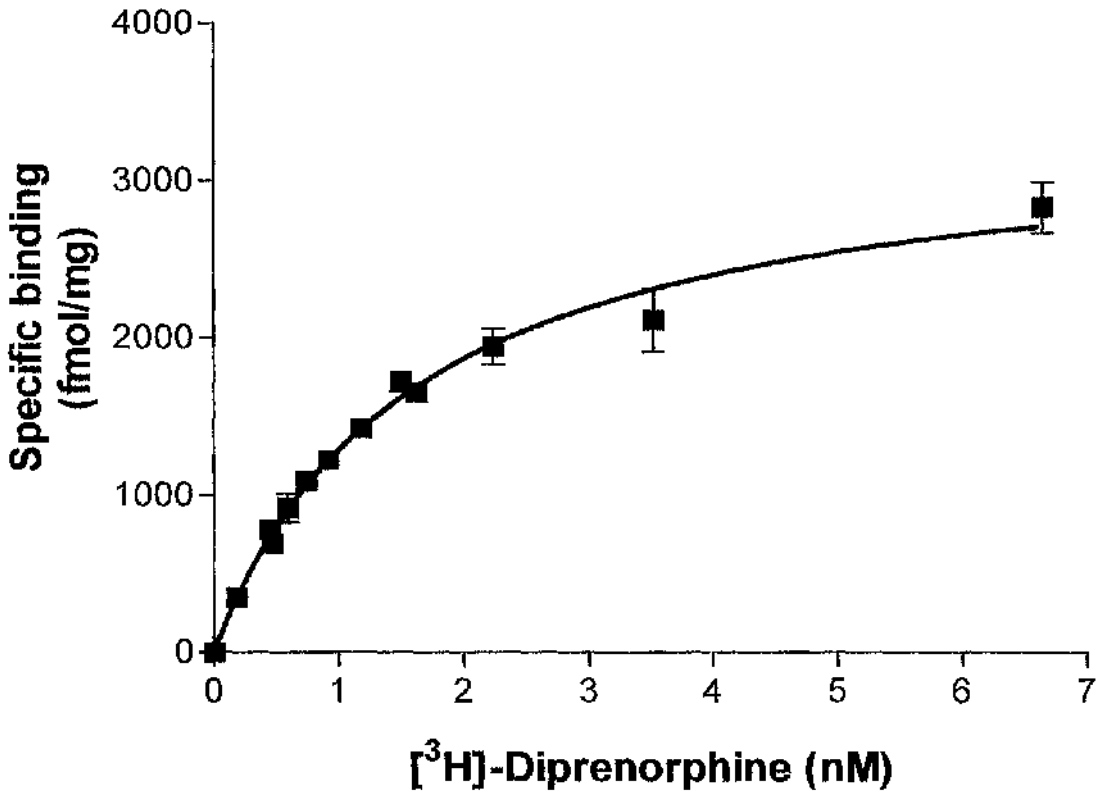

Specific binding
(fmol/mg)
4000
3000
2000
1000
0
0
1
2
3
4
5
6
7
[3H]-Diprenorphine (nM)

Figure 4.12: Co-expression of hDORV^{150}E,V^{154}D-G$_{i1\alpha}$C^{351}I and hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I reconstitutes function

Membranes of HEK 293 cells expressing 15 fmol of hMOR-G$_{i1\alpha}$C^{351}I, hDORV^{150}E,V^{154}D - G$_{i1\alpha}$C^{351}I, hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I or 30 and 45 fmol in the case of hDORV^{150}E,V^{154}D - G$_{i1\alpha}$C^{351}I + hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I co-transfection were used to measure [^{35}S]-GTPγS binding in the absence (open bars) or presence (filled bars) of 10 μM DAMGO. Data represent n=3 experiments performed in triplicate. Data points represent means ± SEM.

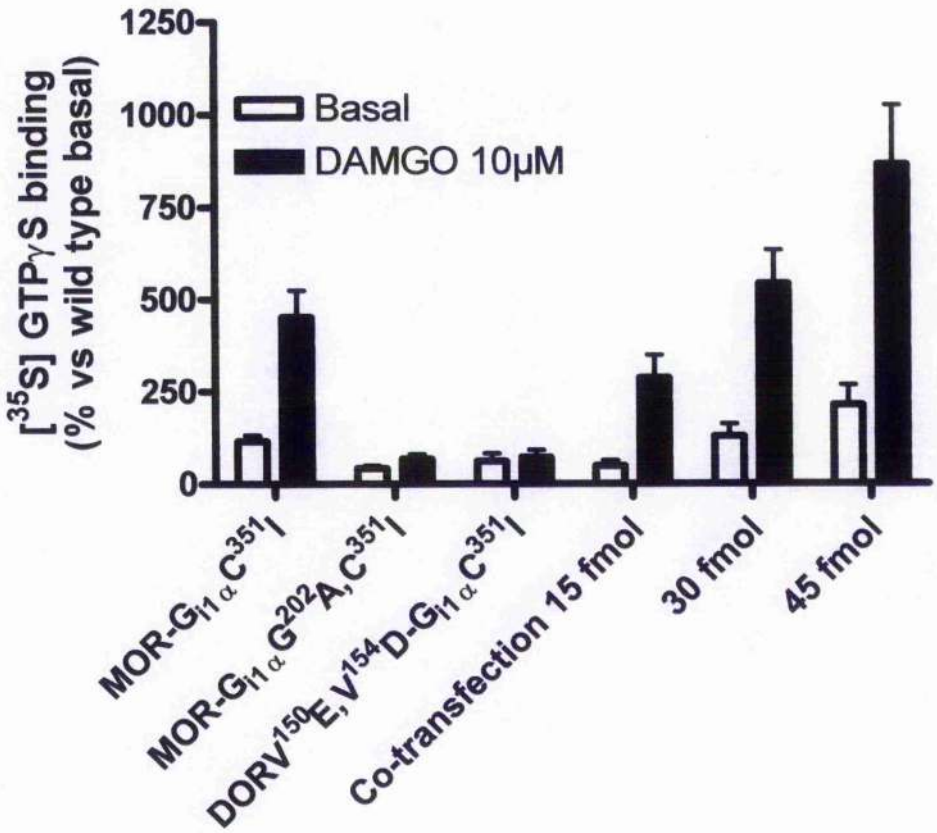

[35S] GTPγS binding
(% vs wild type basal)
1250
1000
750
500
250
0
Basal
DAMGO 10μM
MOR-Gi1α C351I
MOR-Gi1α G202A,C351I
DORV150E,V154D-Gi1α C351I
Co-transfection 15 fmol
30 fmol
45 fmol

Figure 4.13: Effect of mutations and co-expression of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ on competition between [^{3}H]-diprenorphine and CTOP

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$ (blue squares), hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green circles), hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ (light blue circles) and both hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ (red squares) were used to measure the ability of varying concentrations of CTOP to compete for binding with 1nM [^{3}H]-diprenorphine. Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

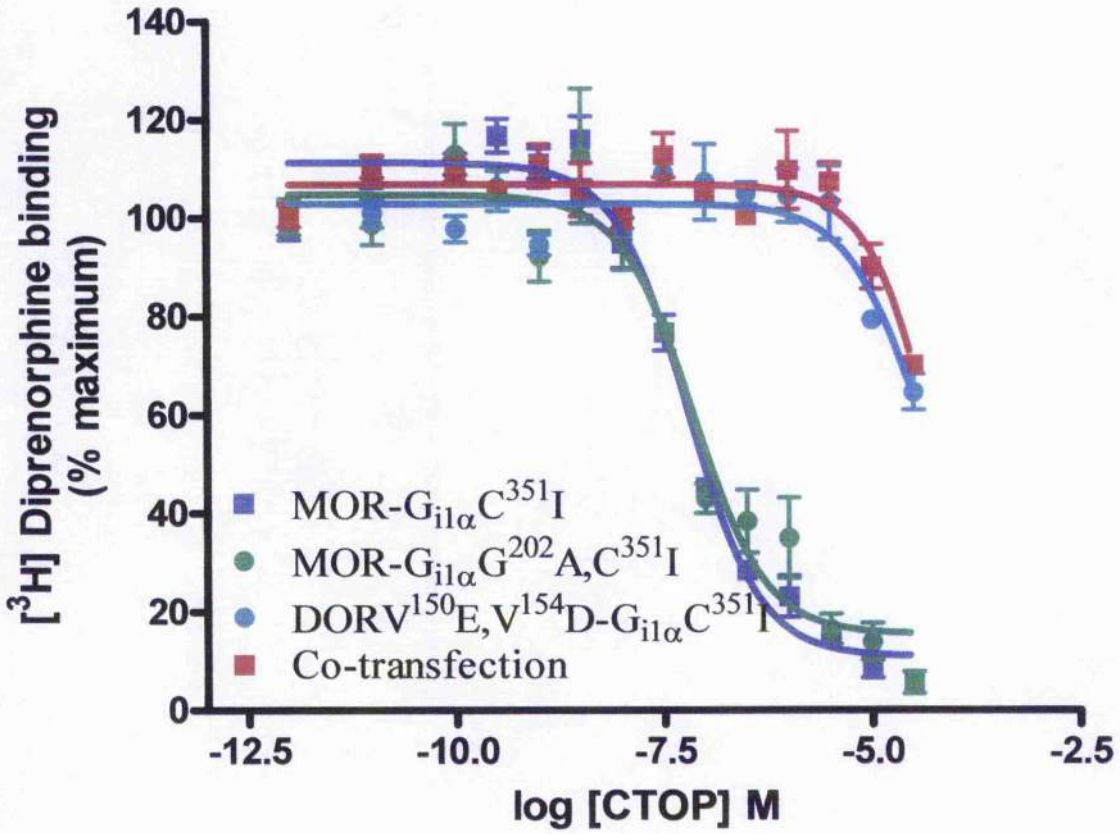

140
120
100
80
60
40
20
0
[3H] Diprenorphine binding (% maximum)
MOR-Gi1αC351I
MOR-Gi1αG202A,C351I
DORV150E,V154D-Gi1αC351I
Co-transfection
-12.5
-10.0
-7.5
-5.0
-2.5
log [CTOP] M

Figure 4.14: Effect of mutations and co-expression of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ on competition between [^{3}H]-diprenorphine and ICI 174 864

Membranes expressing hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green circles), hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ (blue circles) and both hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDORV^{150}E,V^{154}D-$G_{i1\alpha}C^{351}I$ (red squares) were used to measure the ability of varying concentrations of ICI 174 864 to compete with 1nM [^{3}H]-diprenorphine. Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

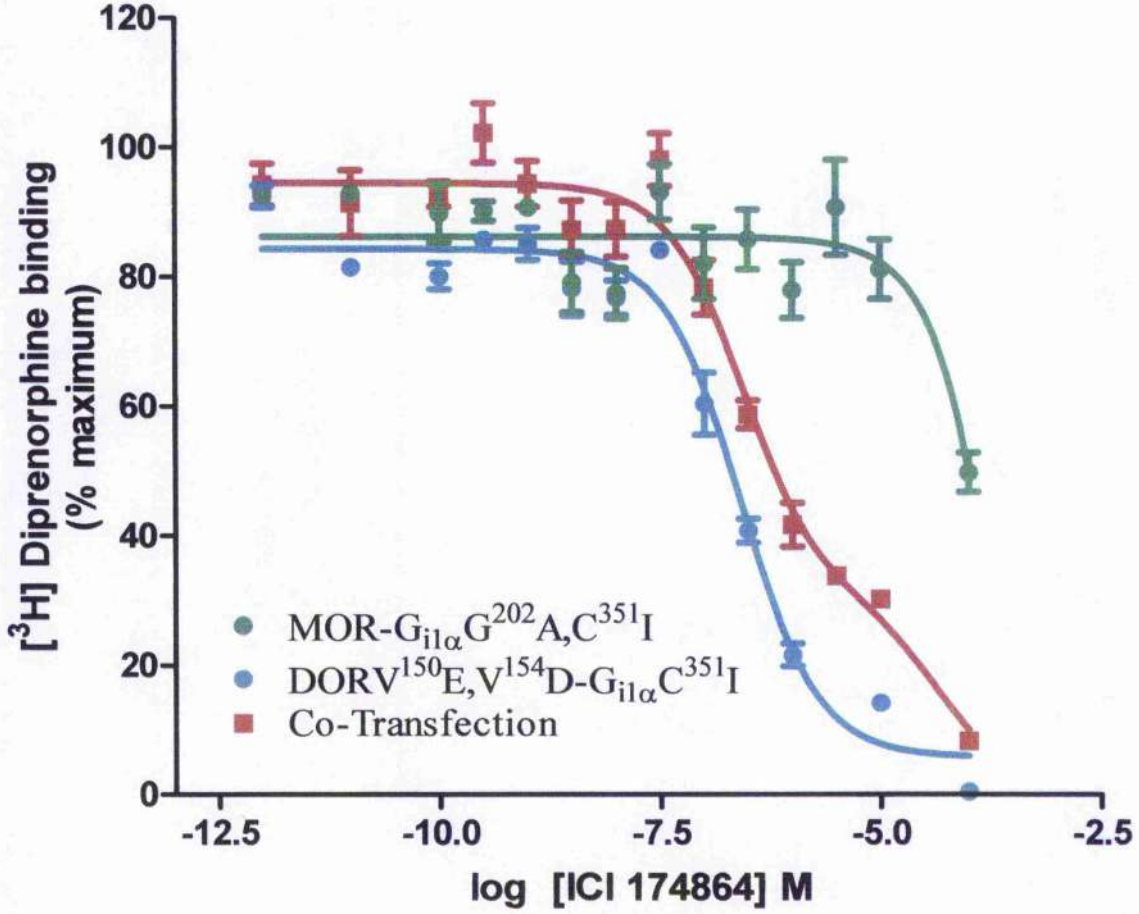

120
100
80
60
40
20
0
[3H] Diprenorphine binding (% maximum)
-12.5
-10.0
-7.5
-5.0
-2.5
log [ICI 174864] M
MOR-GilαG^{202}A,C^{351}I
DORV^{150}E,V^{154}D-GilαC^{351}I
Co-Transfection

Figure 4.15: Comparison of agonist stimulated [^{35}S]-GTPγS binding of hMOR-$G_{i1\alpha}C^{351}I$ and the hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ plus hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ reconstituted heterodimer

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$ (blue squares) or co-transfected with hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + hDOR$V^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ (red squares) were used to measure the ability of increasing concentrations of DAMGO to stimulate [^{35}S]-GTPγS binding. Data represents n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

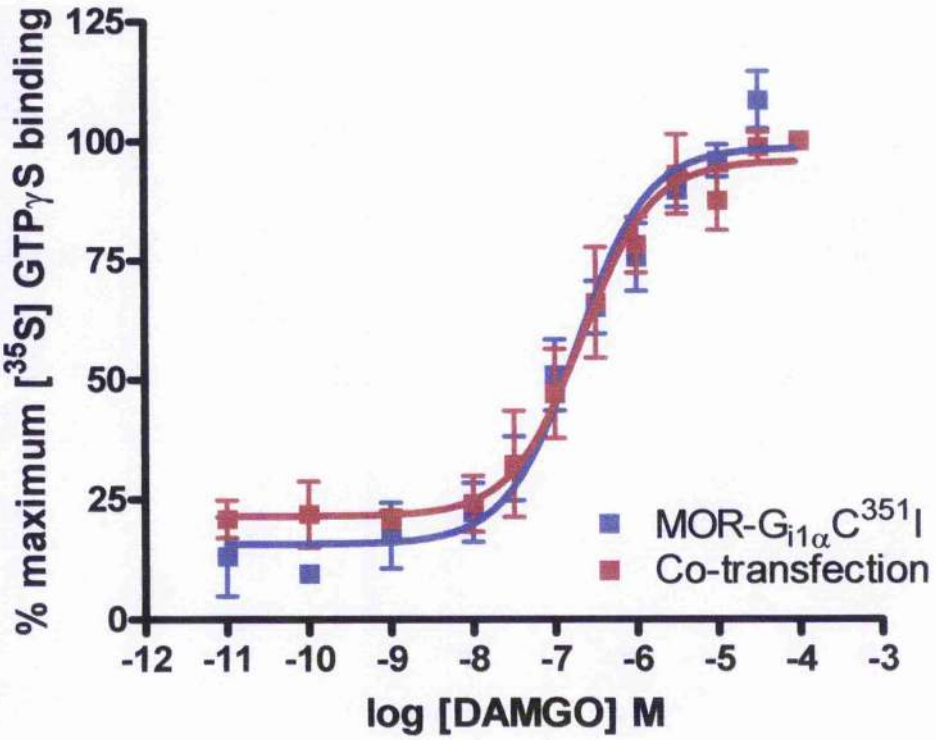

% maximum [35S] GTPγS binding
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
log [DAMGO] M
MOR-Gi1αC351I
Co-transfection

Figure 4.16: Effect of MOR and DOR selective agonists on [^{35}S]-GTPγS binding of the reconstituted heterodimer

Membranes co-transfected to express hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + $hDORV^{150}E,V^{154}D$-$G_{i1\alpha}C^{351}I$ were used to measure the ability of increasing concentrations of DPDPE in the presence of 200nM DAMGO to activate [^{35}S]-GTPγS binding. Data are representative of n=4 experiments performed in triplicate. Data points represent means ± SEM.

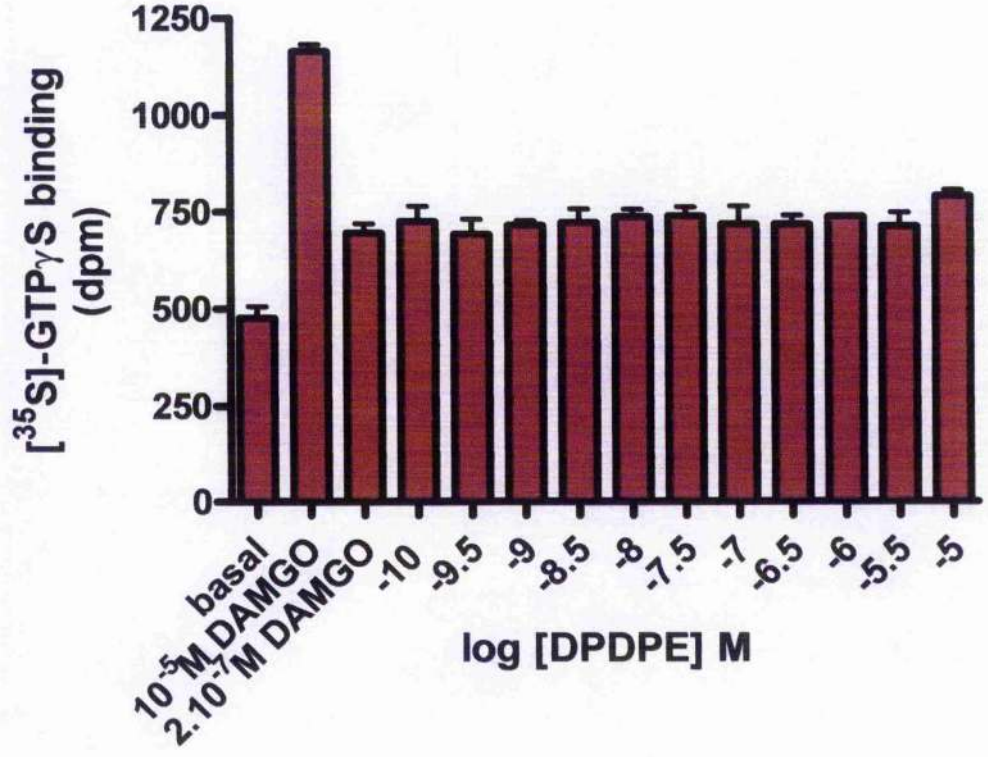

[35S]-GTPγS binding (dpm)
1250
1000
750
500
250
0
basal
10-5M DAMGO
2.10-7M DAMGO
-10
-9.5
-9
-8.5
-8
-7.5
-7
-6.5
-6
-5.5
-5
log [DPDPE] M

Figure 4.17: [^{3}H]-diprenorphine saturation binding following expression of of rKORV^{160}E,V^{164}D-G$_{i1\alpha}$C^{351}I and hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I fusion proteins

Membranes co-expressing hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I + rKORV^{160}E,V^{164}D-G$_{i1\alpha}$C^{351}I (red squares) were used to measure the specific binding of different concentrations of [^{3}H]-diprenorphine and this was compared to the individual expression of hMOR-G$_{i1\alpha}$C^{351}I (blue squares), hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I (green dotes) and rKORV^{160}E,V^{164}D-G$_{i1\alpha}$C^{351}I (light blue dots). Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

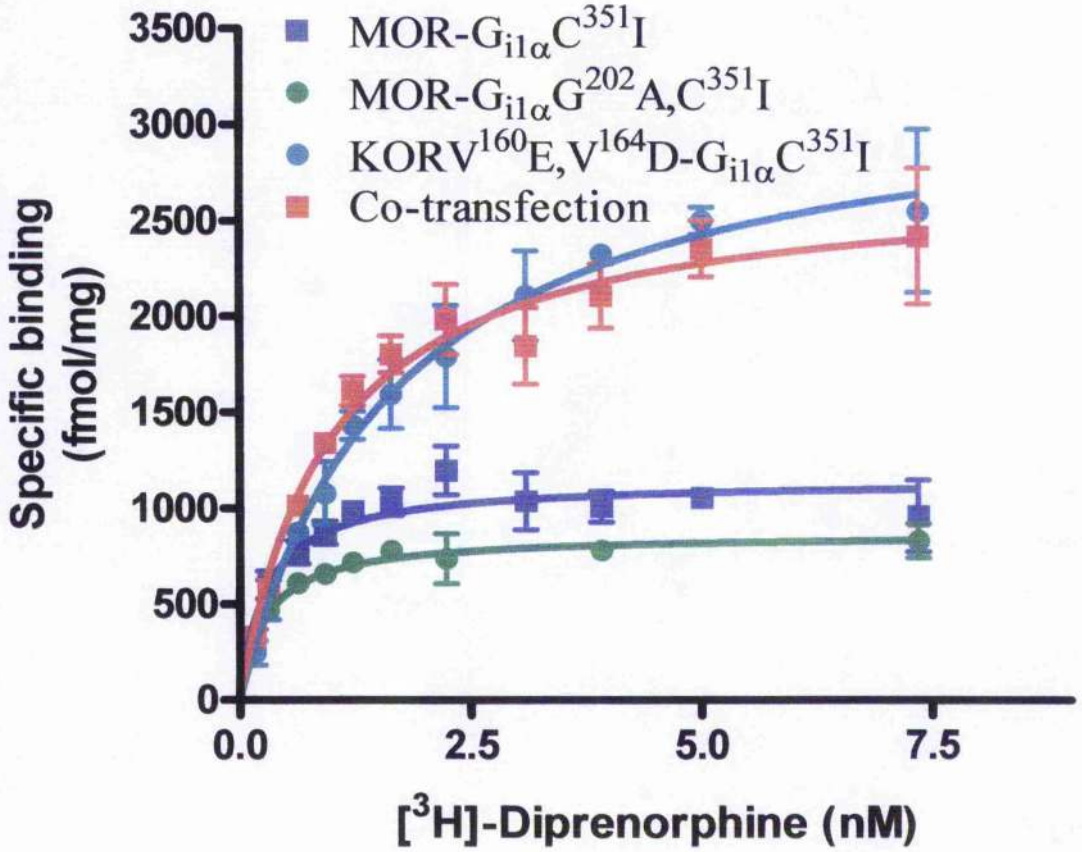

MOR-$G_{i1\alpha}C^{351}I$
MOR-$G_{i1\alpha}G^{202}A,C^{351}I$
KORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$
Co-transfection
Specific binding (fmol/mg)
3500
3000
2500
2000
1500
1000
500
0
0.0
2.5
5.0
7.5
[^{3}H]-Diprenorphine (nM)

Figure 4.18: Co-expression of rKORV^{160}E,V^{164}D-G$_{i1\alpha}$C^{351}I and hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I reconstitutes function

Membranes of HEK 293 cells expressing 15 fmol of hMOR-G$_{i1\alpha}$C^{351}I, rKORV^{160}E,V^{164}D - G$_{i1\alpha}$C^{351}I, hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I and 30 or 45 fmol in the case of the rKORV^{160}E,V^{164}D - G$_{i1\alpha}$C^{351}I + hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I co-transfection were used to measure the binding of [^{35}S]-GTPγS in the absence (open bars) or presence of 10 μM (filled bars) or 100 nM DAMGO (checkered bars). Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

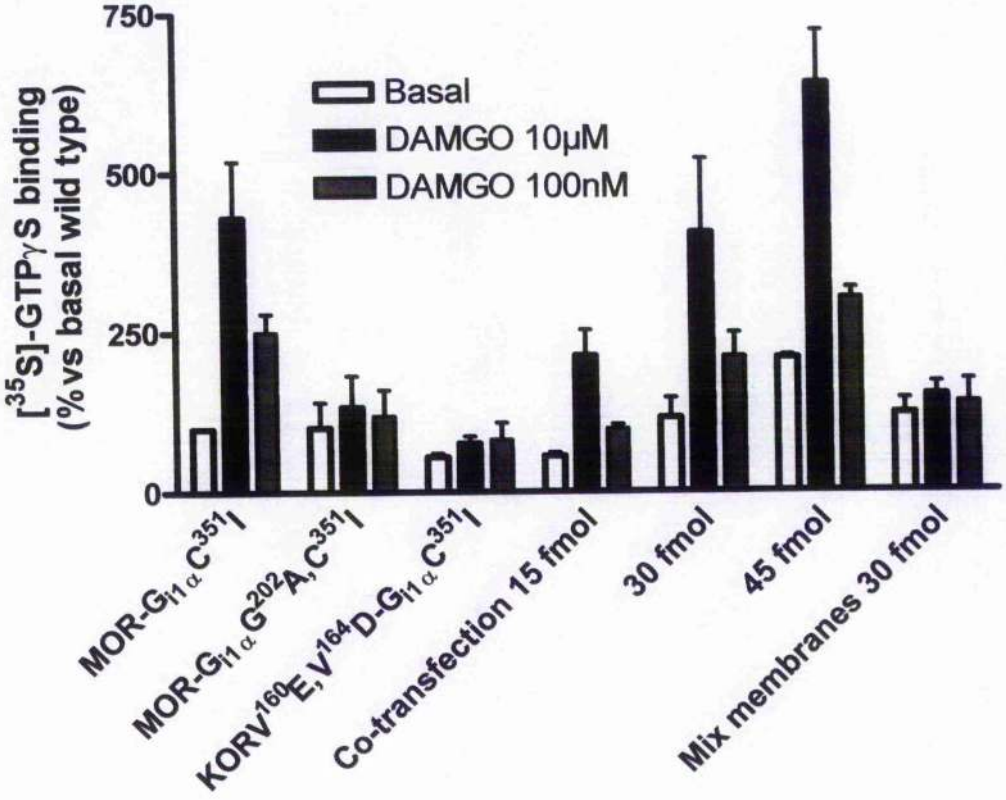

750
500
250
0
[35S]-GTPγS binding
(% vs basal wild type)
Basal
DAMGO 10μM
DAMGO 100nM
MOR-Gi1α C351I
MOR-Gi1α G202A,C351I
KORV160E,V164D-Gi1α C351I
Co-transfection 15 fmol
30 fmol
45 fmol
Mix membranes 30 fmol

Figure 4.19: Co-immunoprecipitation of rKOR-eYFP and Flag-hMOR constructs

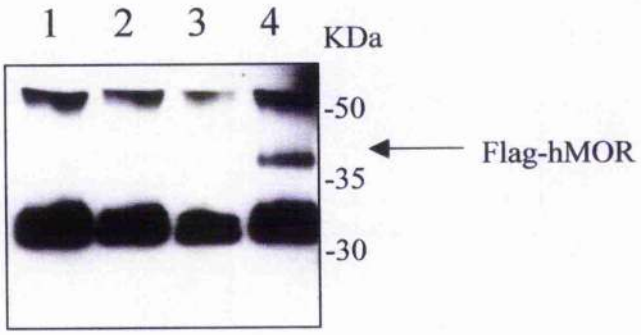
A. Co-immunoprecipitation of rKOR-eYFP and Flag-hMOR

Membranes from mock transfected HEK 293 cells (1) or those transiently expressing Flag-hMOR (2), rKOR-eYFP (3), rKOR-eYFP + Flag-hMOR (4) were immunoprecipitated with anti-GFP antiserum and detected with anti-Flag antibody after being resolved by SDS-PAGE.

B. Expression of KOR-eYFP in the membranes detected by measure of the luminescence.

The same membranes as in panel A: HEK 293 cells (white bar) or transiently expressing Flag-MOR (blue bar), KOR-eYFP (pink bar), KOR-eYFP + Flag-MOR (purple bar) were used to measure KOR-eYFP fluorescence using Victor machine at 480nm.

A


1
2
3
4
KDa
-50
-35
-30
Flag-hMOR

B

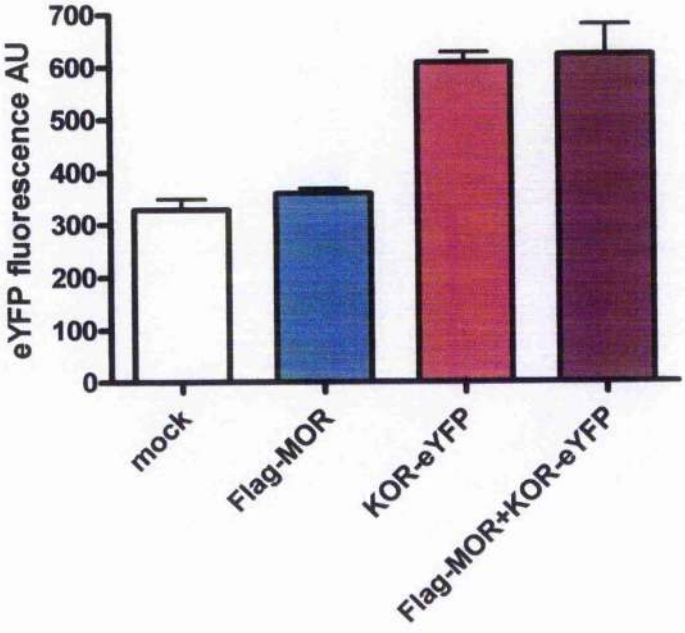

eYFP fluorescence AU
700
600
500
400
300
200
100
0
mock
Flag-MOR
KOR-eYFP
Flag-MOR+KOR-eYFP

Figure 4.20: Effect of mutations and co-expression of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ fusion proteins on competition between [^{3}H]-diprenorphine and CTOP

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$ (blue squares), hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ (green circles), rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ (blue circles) and co-transfected hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ (orange squares) were used to measure the ability of varying concentrations of CTOP to compete for binding with 1nM [^{3}H]-diprenorphine. Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

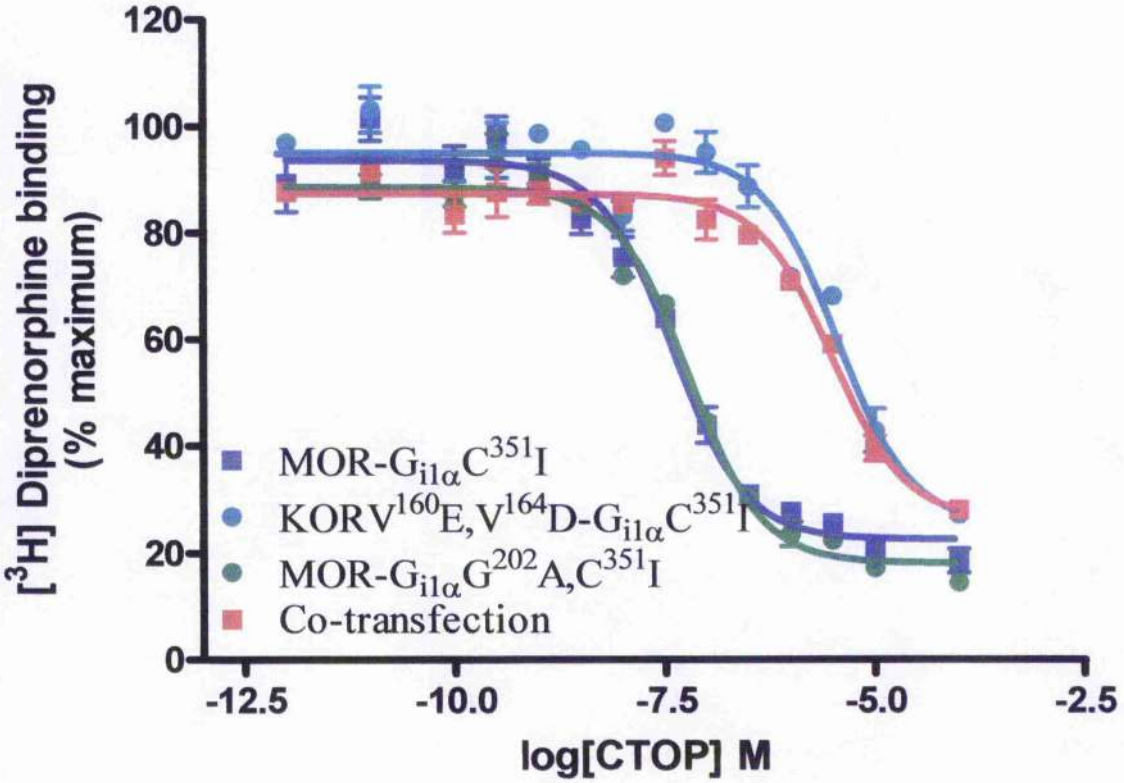

120
100
80
60
40
20
0
[3H] Diprenorphine binding (% maximum)
MOR-Gi1αC351I
KORV160E,V164D-Gi1αC351I
MOR-Gi1αG202A,C351I
Co-transfection
-12.5
-10.0
-7.5
-5.0
-2.5
log[CTOP] M

Figure 4.21: Comparison of agonist stimulated [^{35}S]-GTPγS binding to hMOR-$G_{i1\alpha}C^{351}I$ and the hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$/hKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ reconstituted heterodimer

Membranes expressing hMOR-$G_{i1\alpha}C^{351}I$ (blue squares) or the co-transfection of hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ (orange squares) were used to measure the ability of increasing concentrations of DAMGO to activate [^{35}S]-GTPγS binding. Data represents n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

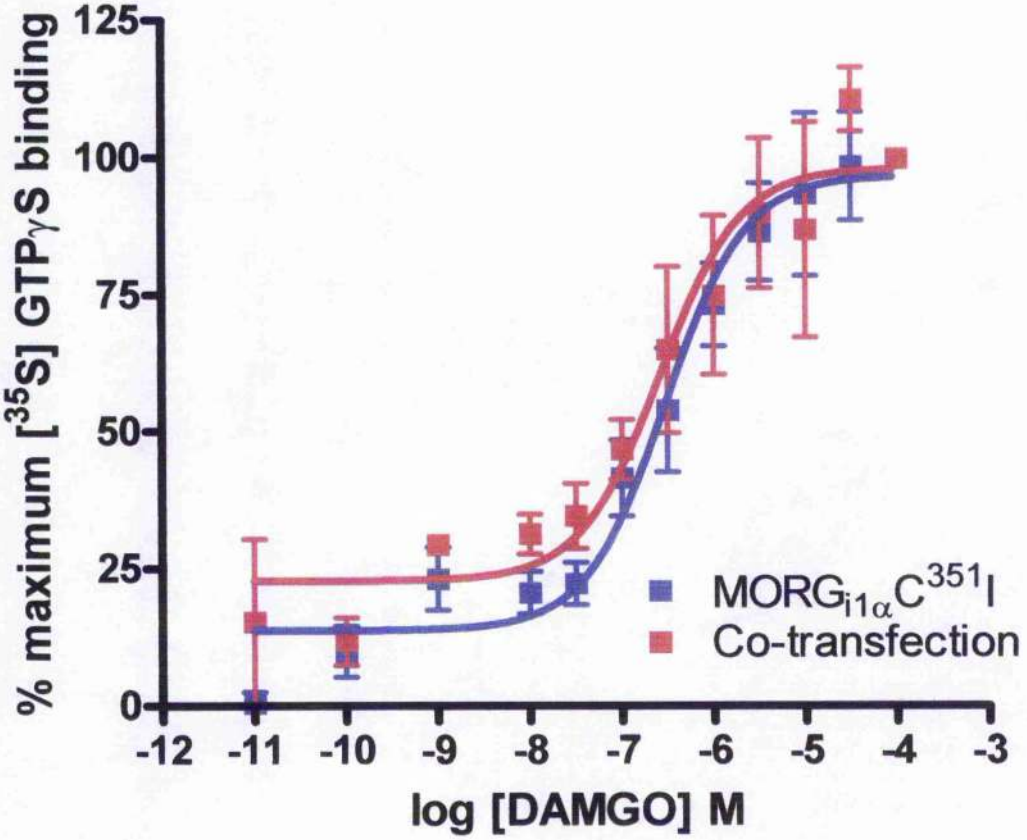

% maximum [35S] GTPγS binding
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
log [DAMGO] M
MORGi1αC351I
Co-transfection

Figure 4.22: Effect of MOR and KOR agonists on [^{35}S]-GTPγS binding of the reconstituted heterodimer

Membranes co-transfected with hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ + $rKORV^{160}E,V^{164}D$-$G_{i1\alpha}C^{351}I$ were used to measure the ability of increasing concentrations of U69593 in presence of 200nM DAMGO to activate [^{35}S]-GTPγS binding. Data are representative of n=3 experiments performed in triplicate. Data points represent means ± SEM of triplicates.

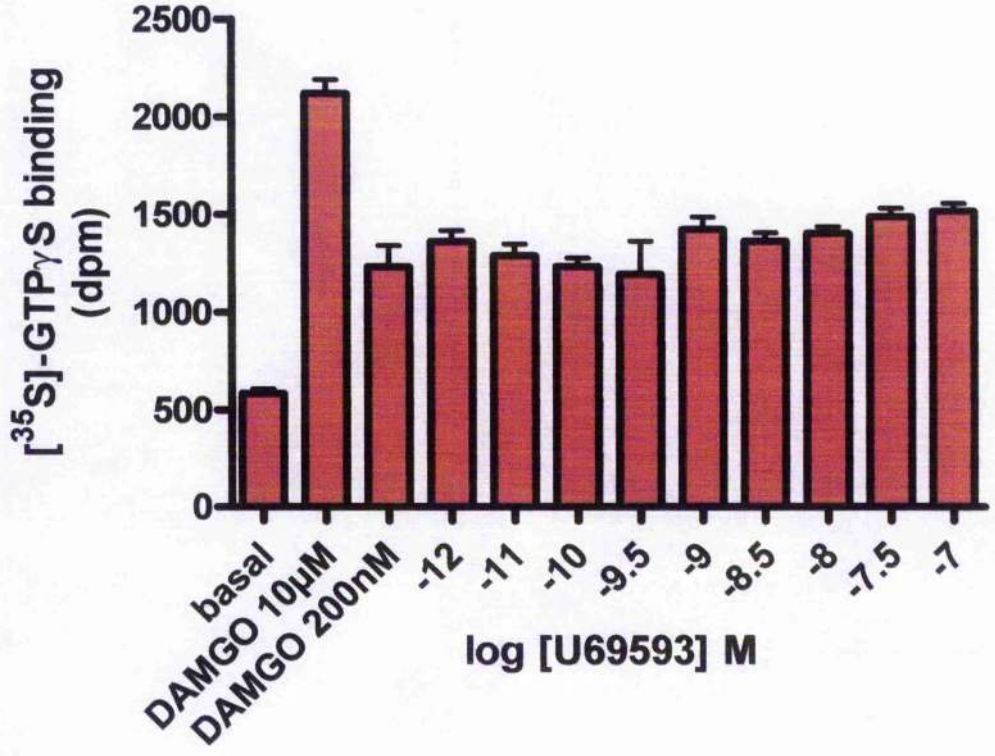
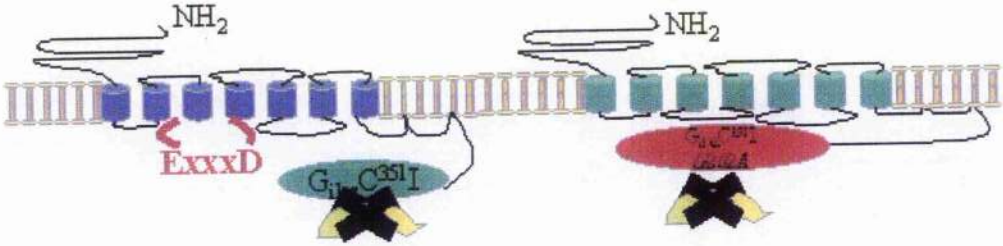
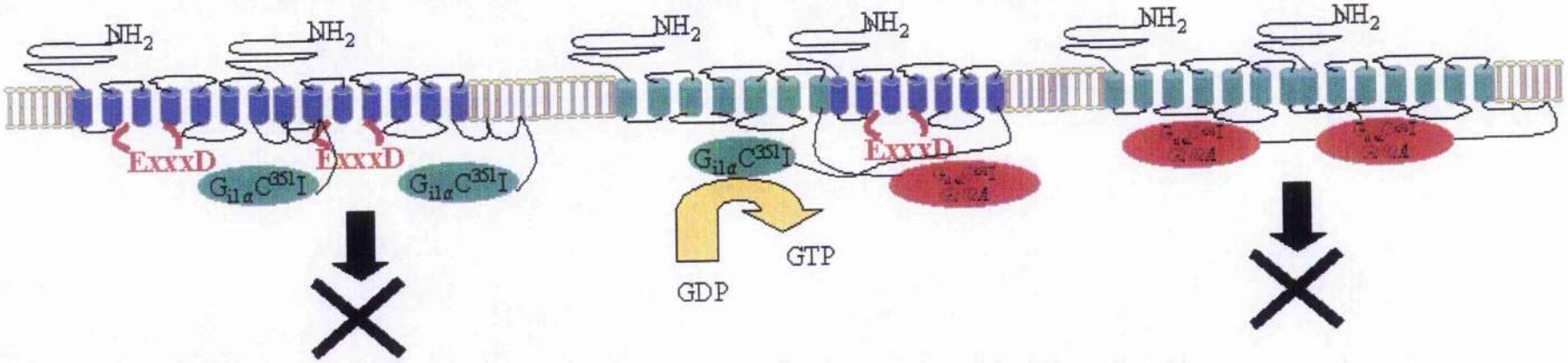

[35S]-GTPγS binding (dpm)
2500
2000
1500
1000
500
0
basal
DAMGO 10μM
DAMGO 200nM
-12
-11
-10
-9.5
-9
-8.5
-8
-7.5
-7
log [U69593] M

Figure 4.23: Only the heterodimers should signal when the complementation technique is used

When the wild type MOR fused (green) to the mutated $G_{i1\alpha}$ (red) and the KOR or DOR mutated opioid receptor (blue) linked to the wild type $G_{i1\alpha}$ (green) are co-expressed only the MOR/DOR or MOR/KOR heterodimer formed will be functional whereas the homodimers will not signal upon agonist stimulation.


NH2
NH2
ExxxD
Gilα C351I


NH2
NH2
NH2
NH2
NH2
NH2
ExxxD
ExxxD
ExxxD
Gilα C351I
Gilα C351I
Gilα C351I
GTP
GDP

CHAPTER 5

Final Discussion

GPCRs are now widely accepted as forming dimeric entities (Milligan *et al.*, 2003; Devi *et al.*, 2001). Biochemical and biophysical approaches as well as computational studies have contributed to this. Dimers formed by two identical GPCRs, homodimers, or composed of two different GPCRs, heterodimers, have been described (Herbert *et al.*, 1996; Ng *et al.*, 1996; Jones *et al.*, 1998; Abdalla *et al.*, 2001b). New pharmacological and signalling properties have been demonstrated upon GPCR heterodimer formation.

Opioid receptors belong to the GPCR family and have been demonstrated to form homo and heterodimers. Previous study failed to demonstrate MOR and KOR interaction (Jordan and Devi, 1999). It is only recently that Wang et al., (2005) showed their heterodimerisation. Changes in pharmacology and signalling properties have been reported for all three opioid receptor heterodimers (George *et al.*, 2000; Gomes *et al.*, 2004; Law *et al.*, 2005; Wang *et al.*, 2005). Differences in ligand binding, endocytosis patterns and G protein or adenylate cyclase coupling were observed. Nevertheless, many aspects of opioid receptor dimerisation still need to be investigated. Few data on the molecular basis of opioid receptor interaction or possible disparity in dimer formation with closely or less related GPCRs have been reported. The numbers of studies examining the proportion of dimers formed by GPCRs are also limited. Moreover, the signalling variation reported upon co-expression of distinct opioid receptors cannot be firmly established as reflecting heterodimerisation as a mixture of homo/heterodimers and monomers could be present in the samples analysed.

In this study I have taken advantage of a fusion protein strategy to build pairs of complementary constructs containing hDOR, hMOR or rKOR fused with the $G_{i1\alpha}$ subunit. Conserved residues have been mutated either in the 2nd intracellular loop of the opioid receptors or in the G_{α} protein to create non-functional fusion proteins. Two valines present in the 2nd intracellular loop of opioid receptors and highly conserved among most class A GPCRs were mutated into glutamic acid and aspartic acid. These mutations were shown to abolish G protein activation by the opioid receptors. Similarly, a highly conserved glycine residue in G_{α} protein subunit, the glycine 202 in $G_{i1\alpha}$, was mutated into alanine (Milligan *et al.*, 2005). Introduction of the G^{202}A mutation resulted in a form of the G protein that could not exchange GDP for GTP. However, when these mutated fusion proteins were co-expressed agonist-induced signalling was recovered for all three opioid receptors demonstrating cross-

talk between identical DOR, MOR and KOR subtypes consistent with their homodimerisation.

To verify the specificity of the interaction observed, DOR N-terminal and TM1 was fused to $G_{i1\alpha}$ and co-expressed with the fusion protein containing hDOR fused to the non-functional G protein. No significant [^{35}S]-GTPγS binding was observed after agonist treatment. Nevertheless, these fusion proteins were shown to interact as they could be co-immunoprecipitated following co-expression. These results provide evidence that two full length receptors might be necessary for G protein activation. However, contrasting data have been reported by Molinari et al, (2003). In their study the vasopressin V2 TM1 linked to G_o was activated by the full length DOR. The disparity observed could be due to the high receptor expression achieved and used in their study. Indeed, high level receptors overexpression has been reported to induce random collision events, thus unspecific physical contacts between proteins (Mercier *et al.*, 2002). These "bystanders" could have been misinterpreted as specific interactions by the authors.

In the present report DOR N-terminal and TM1 were observed to self-associate. Therefore, one or both of these domains contribute to hDOR dimer formation. Although TM1 was not proposed as a possible dimerisation interface for DOR by computational study (Filizola and Weinstein, 2002), this domain has been reported to play a key role in C5a receptor, yeast α factor receptor and α_{1a}-adrenoceptor dimerisation (Kelo *et al.*, 2003; Overton *et al.*, 2003, Carrillo *et al.*, 2004). Moreover, no experimental data have yet supported the informatic analysis, which suggested TM4 and TM5 as possible interfaces for DOR homodimerisation. Detailed analysis of the domains involved in opioid receptor interaction will be of interest. The putative glycophorin A dimerisation motif GXXXG was recently described as playing a key role in yeast α factor receptor and β2-adrenoceptor dimerisation (Overton et al., 2003; Salhapour *et al.*, 2004). Identification and mutation of this motif in opioid receptors would offer additional understanding of their dimerisation mechanism. Overall, identification of any residues responsible for opioid receptor interaction will be of prime importance to learn more about dimerisation and the role in opioid receptor function. Genetically modified animal: expressing opioid receptors mutated at these residues could be produced to bring insights into the role of dimerisation in opioid physiological responses such as analgesia, tolerance and dependence.

Pharmacological properties of the diverse fusion proteins were also investigated herein using a rage of synthetic agonists (DADLE, DAMGO, DPDPE, U69593). Loss of agonist binding affinity was observed when the two valines were mutated into glutamic acid and aspartic acid. This alteration in ligand binding could be due to a change in G protein coupling. A modification in receptor/G protein coupling is likely as no agonist-induced [^{35}S]-GTPγS binding was observed for the fusion proteins containing these mutations. Thus, the two mutated valines from the 2nd intracellular loop can be considered as key residues for opioid receptor/G protein activation for all three opioid receptors/G protein interactions. These data contrast with other studies, which described no involvement of the 2nd intracellular loop for effective G protein coupling with MOR and DOR (Georgoussi *et al.*, 1997; Merkouri *et al.*, 1996).

Opioid receptor/G protein uncoupling could be confirmed by comparing the wild type opioid receptors and the version of the receptor mutated in the 2nd intracellular loop competition binding in the presence and absence of GppNHp.

Investigation of opioid receptor heterodimerisation by the complementation technique has confirmed DOR/MOR heterodimerisation and revealed MOR/KOR interactions. MOR/KOR dimer formation was also observed by co-immunoprecipitation. Such observations were unexpected as two other groups reported an absence of interactions between KOR and MOR either using co-immunoprecipitation technique (Jordan and Devi, 1999) or by computational analysis (Filizola *et al.*, 2002). However, during the completion of my thesis Wang et al, 2005 have demonstrated MOR/KOR dimerisation using BRET approach, thus in agreement with my observations. Similar contradictions have been reported for other GPCRs. CCR4 and CCR2 were first documented as not interacting (Mellado *et al.*, 1999), however, recently this observation was refuted by Percherancier *et al.*, (2005). This may reflect the improving techniques and approaches as this field matures.

Consequently, MOR/KOR heterodimer pharmacology and signalling properties need to be further explored. A first attempt was made in this study by examining MOR/KOR binding affinity for the MOR selective antagonist CTOP. A loss of affinity for this ligand was observed in the sample co-expressing the fusion proteins. It could be argued that this observation is the result of rKORV^{160}E,V^{164}D-G$_{i1\alpha}$C^{351}I fusion protein overexpression compared to hMOR-G$_{i1\alpha}$G^{202}A,C^{351}I, however, this was not obvious from the DAMGO-

induced stimulation observed when hMOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and rKORV^{160}E,V^{164}D-$G_{i1\alpha}C^{351}I$ were co-expressed. Moreover, in their recent study Wang et al, (2005) have suggested that MOR/KOR heterodimer pharmacology may resemble that of KOR. They observed a lower affinity for the MOR agonists (DAMGO and endomorphin 1) when tested on the sample co-expressing MOR/KOR heterodimers compare to the MOR expressing cells. These results are in agreement with my data.

The complementation technique developed in our laboratory (Carrillo *et al.*, 2003) allows specific analysis of signalling of the reconstituted heterodimers. Even if a combination of monomers, homodimers and heterodimers exist in the samples analysed, the response observed will only originate from the heterodimer. This is the first approach allowing exclusive monitoring of opioid receptor heterodimerisation without non-specific background i.e. monomer and/or homodimer signalling. Taking advantage of this method, agonist combinations were tested on the reconstituted DOR/MOR and MOR/KOR heterodimers. No synergistic responses were observed with the agonist mixes used. However, only one ligand combination for each heterodimer was examined. To complete this study more ligand combinations should be examined and mixtures of agonists and/or antagonists should be tested. Modulation of 2nd messengers such as cAMP or Ca^{2+} could also be investigated in an effort to reveal distinct properties of opioid heterodimers.

Potential heterodimerisation between DOR and a less related receptor, the β_2-adrenoceptor, was also investigated. Co-expression of hDOR-$G_{i1\alpha}G^{202}A,C^{351}I$ and β_2-adrenoceptor-$G_{i1\alpha}C^{351}I$ resulted in agonist-induced signal reconstitution thus confirming previous reports which described their heterodimerisation (Jordan *et al.*, 2000; McVey *et al.*, 2001; Ramsay *et al.*, 2002). However, the extent of DOR/β_2-adrenoceptor reconstitution was two times lower compared to DOR/DOR suggesting better affinity for DOR to self-associate than to interact with the β_2-adrenoceptor. This difference did not reflect an alteration in G protein coupling as a similar potency to activate G protein was observed for the reconstituted dimer and the homodimer anticipated from expression of hDOR in isolation. Affinity for opioid receptors to interact with other GPCRs could be explored with this approach. Depending on the affinity of the receptors to interact new and original heterodimer properties observed can may be relevant to physiology.

In general, understanding of all aspects of GPCR dimerisation is essential if control of important functions such as pain relief or HIV infection are regulated by this mechanism.

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