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**MINIREVIEW**

# The prevalence, maintenance and relevance of GPCR oligomerization

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**Running Title Page**

**Running title: GPCR oligomerization**

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**Non-standard abbreviations**

BRET, bioluminescence resonance energy transfer; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GPCR**,** G protein-coupled receptor; HTRF, homogeneous time-resolved fluorescence;

**Chemical names**

DAMGO, ([D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin; DOI, 2,5-dimethoxy-4-iodoamphetamine, O-2050, (6aR,10aR)-3-(1-methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran; rimonabant/ SR141716, 5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide

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**Abstract**

Over the past decade ideas and experimental support for the hypothesis that G protein-coupled receptors may exist as dimeric or oligomeric complexes moved initially from heresy to orthodoxy, to the current situation in which the capacity of such receptors to interact is generally accepted but the prevalence, maintenance and relevance of such interactions to both pharmacology and function remain unclear. A vast body of data obtained following transfection of cultured cells is still to be translated to native systems and, even where this has been attempted, results often remain controversial and contradictory. This review will consider approaches that are currently being applied, why these might be challenging to interpret and suggest means to overcome these limitations.

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## Introduction

In the last 10 years the question of whether G protein-coupled receptors (GPCRs) exist as monomers, dimers or oligomers has been a substantial component of many studies on members of this family of transmembrane signalling proteins (Milligan, 2004, 2008, Gurevich and Gurevich, 2008, Pétrin and Hébert, 2012). Until recently these were often considered to be mutually exclusive scenarios but the co-existence and potential interchange between such forms, based in part simply upon Mass-Action, has resulted in a more textured view (Calebiro et al., 2013, Patowary et al., 2013). It is clear that stable hetero-complexes formed by interactions between polypeptide products of distinct genes encoding members of the class C, metabotropic glutamate-related GPCRs define the pharmacology and function of certain receptors (Maurel et al., 2008, Pin et al. 2009, Kniazeff et al., 2011). These include interactions between the $GABA_{B1}$ and $GABA_{B2}$ polypeptides to generate the $GABA_B$ receptor (Kniazeff et al., 2011). Similarly, co-expression and interactions between the TAS1R1 and TAS1R3 polypeptides result in perception of savoury or umami flavors whilst similar co-expression and interactions between TAS1R2 and TAS1R3 polypeptides are required for identification of sweet tastes (Chandrashekar et al., 2006, Palmer, 2007). Furthermore, although members of distinct subgroups of metabotropic glutamate receptors appear unable to generate heteromeric interactions with each other (Doumazane et al., 2011) this does occur between more closely related polypeptides within the same subgroup and each individual member of the metabotropic glutamate receptor family is able to generate homomers, an organizational structure, that is integral to function (Doumazane et al., 2011).

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Despite these clear examples, all of which meet the broad guidelines proposed by the International Union of Basic and Clinical Pharmacology (IUPHAR) for acceptance of GPCR complexes as homomers and/or heteromers (Pin et al., 2007), the situation for the numerically much larger class A of rhodopsin-like receptors is far more complex. This article will attempt to appraise why this is so.

## Monomeric class A GPCRs are functional

In recent times a series of studies have purified class A GPCRs and, following insertion as monomers into various forms of phospholipid bilayers, have shown capacity of these to interact productively with appropriate heterotrimeric G proteins. For example Kuszak et al., (2009) took this strategy and employed a form of the μ opioid receptor with Yellow Fluorescent Protein and a series of epitope tags linked to the N-terminus. Addition of heterotrimeric $G_{i2}$ to the system resulted in both the opioid alkaloid morphine and the μ opioid receptor-selective synthetic enkephalin peptide [D-Ala$^2$, N-MePhe$^4$, Gly-ol]-enkephalin (DAMGO) displaying biphasic competition binding curves with the antagonist [$^3$H]diprenorphine, including a high affinity component that was not present in the additional presence of a poorly hydrolysed analog of GTP. Furthermore, in these studies DAMGO promoted binding of [$^{35}$S]GTPγS to the G protein in a concentration-dependent manner (Kuszak et al., 2009). Along with similar experiments using the $\beta_2$-adrenoceptor (Whorton et al., 2007) and rhodopsin (Whorton et al., 2008), these studies provided firm evidence that a number of class A GPCRs can function effectively as monomers. Furthermore, although there have also been suggestions that dimeric forms of GPCRs might be required for interactions with non-G protein effectors and adaptors, at least in the case

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of rhodopsin and interactions with arrestin-1 (Bayburt et al., 2011) again this seems to be accommodated by a monomeric GPCR.

**Even GPCRs that are capable of acting as monomers may exist as dimers or higher oligomers**

Interestingly, the three class A GPCRs noted above as being functional when forced to be strict monomers have each been shown to have the capacity to exist as dimers or higher oligomers. Indeed, in the case of the organization of rhodopsin, observation via atomic force microscopy of the receptor as paracrystalline arrays of dimers in mouse disc membranes (Fodiatis et al., 2003) was a key observation in providing evidence of physiologically relevant receptor-receptor interactions. Although there were immediate suggestions of concerns about technical details of these studies and that other evidence is consistent with rhodopsin acting as a monomer (Chabre et al., 2003), this remains a landmark study. In the case of the $\beta_2$-adrenoceptor, alongside rhodopsin the most extensively studied GPCR, it was one of the first GPCRs in which potential 'dimerization' was explored. Building on a series of co-immunoprecipitation studies (see Milligan and Bouvier 2005 for review), Angers et al., (2000) were instrumental in promoting the use of resonance energy transfer techniques to probe potential oligomeric organization of receptors in intact cells and showed such interactions for the $\beta_2$-adrenoceptor. These studies, and the rapid adoption of both bioluminescence- (BRET) and fluorescence-resonance energy transfer (FRET)-based approaches by many groups (Alvarez-Curto et al., 2010a) have generated a mini-industry on assessing GPCR-GPCR interactions, despite concerns that limited understanding of the limitations and caveats of such biophysical methodologies may compromise

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analytical analysis and result in over-interpretation of the observations (James et al., 2006). Although specific aspects of these criticisms were rebutted rapidly (Salahpour and Masri, 2007), the seemingly basic question of the size of GPCR complexes remains a core question. Importantly, each of fluorescence recovery after photobleaching (FRAP) studies (Dorsch et al., 2009) and co-internalization in response to addition of the β-adrenoceptor agonist isoprenaline of co-expressed wild type and chemically-engineered variants of the $\beta_2$-adrenoceptor that are unable to bind this ligand (Sartania et al., 2007) have provided evidence for oligomerization of this receptor via distinct methodologies. Indeed, quantitative analysis of the FRAP studies suggested that the $\beta_2$-adrenoceptor may exist as a stable oligomeric, rather than strictly dimeric, complex (Dorsch et al., 2009). In support of this Fung et al., (2009) employed FRET on purified $\beta_2$-adrenoceptors reconstituted into model lipid bilayers and obtained data consistent with the receptor existing predominantly in a tetrameric form.

The widespread interest in efforts to develop non-addictive analgesics that function via the μ opioid receptor have also extended to studies on potential homomeric interactions in this receptor. Thus, the recent crystal structure of this receptor showing a clear dimeric interface based on a four-helix bundle with contributions from at least 28 residues along the length of transmembrane helices V and VI provides clear support for a dimeric model (Manglik et al., 2012). The involvement of so many residues, providing a buried footprint of nearly $1500A^2$ on each monomer, also suggests that it is likely to be a high affinity interaction that may be difficult to disassemble in a physiological setting, although this was obviously achieved by detergent solubilization to produce monomeric μ opioid receptor for the G protein-

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interaction studies of Kuszak et al., (2009) discussed above. Furthermore, the crystal structure also demonstrated the potential for a second dimeric interface involving residues from transmembrane helices I and II as well as the intracellular sequence usually referred to as 'helix VIII' (Manglik et al., 2012). This interface was not nearly as extensive as the one involving residues from helices V and VI and is at least compatible with the idea that this might provide a lower affinity interface that would allow dynamic interchange between dimeric and tetrameric forms of this receptor. Experimentally observed co-existence and the potential for interchange between dimeric and tetrameric forms of a GPCR at the plasma membrane (Patowary et al., 2013) support this concept. Furthermore, the proportions of different oligomeric states might vary for the same receptor in cells and tissues that express a GPCR to markedly different levels. The implications of this potential for the analysis of ligand binding studies have also been considered in a theoretical context (Rovira et al., 2009). The structures of the μ opioid receptor were obtained in the presence of an irreversibly bound antagonist and, therefore, although the general significance is currently unclear, the fact that Fung et al., (2009) observed that addition of an inverse agonist to lipid bilayer-reconstituted $\beta_2$-adrenoceptors seemed to enhance the organization of a tetrameric structure is certainly fascinating. Interestingly, although displaying a more limited interface, a number of individual crystal structures of the chemokine CXCR4 receptor bound by a small molecule antagonist also display a dimeric form with the interface again defined by residues from transmembrane helices V and VI (Wu et al., 2010), whilst crystals of the κ opioid receptor also show a parallel dimeric interface involving residues from transmembrane domains I, II and VIII (Wu et al., 2012).

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**Is a monomer more effective than a dimer for G protein activation?**

If certain GPCR monomers can couple effectively to heterotrimeric G proteins to initiate signal transduction upon binding of an agonist ligand, and the same receptors can exist as dimers or higher-order complexes, what are the implications for signalling and is one form more efficient than the other? Results on this topic are interesting but do not yet provide clarity. For example, for the leukotriene BLT2 receptor, expression in *E.Coli* followed by refolding and purification of dimers arranged with parallel organization suggested that the dimer activated purified G protein less effectively than receptor monomers (Arcemisbéhère et al., 2010). Although this is a technically exacting and precise study, it is difficult to be sure that the dimer interface in such studies equates fully to what might be found in a cell expression system. By contrast, when using expression of various forms of the serotonin 5-$HT_4$ receptor in COS7 cells activation of both elements of the dimeric complex was shown to result in greater activation of G protein than of a single protomer of such a complex (Pellissier et al., 2011).

An extension of this question and studies is whether each element of a dimer or higher-order complex of a class A GPCR is able to bind a molecule of agonist (at least with similar affinity) and if so does this influence the function of the partner protomer(s). Based on the studies of Pellissier et al. (2011), this is clear for the 5-$HT_4$ receptor. Moreover, studies such as those of Herrick-Davis et al. (2005) on the serotonin 5-$HT_{2C}$ receptor that employed combinations of a wild type and a variant receptor unable to bind serotonin are also at least consistent with a need to bind agonist to both protomers of a dimer to generate maximal function. Moreover, kinetic analysis of how the rate of dissociation of a fluorescent agonist from the adenosine $A_3$

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receptor expressed in CHO-K1 cells is increased markedly in the presence of both agonists and antagonists known to also bind the same, orthosteric site on the receptor, is not consistent with ligand dissociation occurring from a monomer (where ligand dissociation rate should be unaffected by the presence of a second ligand) and has been interpreted as evidence for a dimeric receptor (May et al., 2011) in which ligand binding to one protomer generates a co-operative allosteric effect on ligand binding to the other protomer. The extent of such effects may vary substantially between even closely related receptors. In equivalent studies using the adenosine $A_1$ receptor much smaller effects on ligand dissociation rate were recorded, although other evidence also indicated the receptor to form dimeric complexes (May et al., 2011), indicating that allosteric effects within different receptor dimers may not be sensed or transmitted to the same extent. A further approach that has recently been applied is to examine the effect of unmodified receptors on the behavior of a co-expressed receptor capable of reporting conformational change and activation. Using the angiotensin $AT_1$ receptor as a model, Szalai et al., (2012) first used a form of this receptor which although still able to bind the endogenous peptide ligand was unable to bind the small molecule antagonist candesartan. This allowed selective activation of the antagonist-resistant form in the presence of a combination of the antagonist and angiotensin II. Employing BRET to examine either β-arrestin-2 binding to the receptor or the activation of an intramolecular BRET sensor form of the receptor that was not blocked by the presence of candesartan, allowed these authors to observe effects also consistent with allosteric effects within a dimer (Szalai et al., 2012). Furthermore, these effects were lacking when using a mutant in the receptor in which the highly conserved DRY domain at the bottom of transmembrane domain III was mutated. Such a concept of

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asymmetry of the individual protomers of a dimeric or oligomeric complex is not new and has been discussed previously in detail (e.g. Maurice et al., 2011) but does provide a basis to probe the presence of homodimers. Clear asymmetry of function is integral for the individual promoters within receptor heteromers (see later) and the class C $GABA_B$ receptor in which the $GABA_{B1}$ subunit binds the orthosteric agonist ligand whilst the partner $GABA_{B2}$ polypeptide communicates this to G protein activation is both the prototypic and most fully analyzed example of this to date (see Pin et al., 2009 for review).

**Oligomeric organization of other class A GPCRs**

Although a potential dimeric form of rhodopsin was also identified via a crystal structure, for other GPCRs evidence for oligomeric organization has, to date, been derived from other approaches. Perhaps the most wide ranging group of studies have been performed on muscarinic acetylcholine receptor subtypes. Beginning with conventional pharmacological ligand binding (Park et al., 2002) and biochemical approaches (Ma et al., 2007), over a number of years Wells and colleagues have provided a portfolio of data on this topic for the muscarinic $M_2$ receptor that are consistent with the potential for tetrameric organization. Most recently this has derived from application of quantitative FRET (Pisterzi et al., 2010). Once again these studies provided evidence of tetrameric organization of the receptor, potentially with a rhombic or parallelogram shape, at least in transiently transfected CHO cells. A further refinement of these techniques has recently been employed to explore the oligomeric organization of the muscarinic $M_3$ receptor (Patowary et al., 2013). Herein, a HEK293-based cell line was established in which an energy acceptor-tagged

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form of the human muscarinic $M_3$ receptor was expressed stably and constitutively and in which a corresponding energy donor-tagged form of the receptor was harbored at an inducible locus (Alvarez-Curto et al., 2010b). This allowed varying amounts of the energy donor to be expressed in the presence of a constant level of energy acceptor. Quantitative FRET studies with spectral unmixing were then performed on these cells at the level of the plasma membrane. Mathematical analysis of the broad range of FRET efficiencies obtained from these complexes was compatible with the receptor existing within rhombus-shaped tetramers (Patowary et al., 2013) (**Figure 1A, 1B**). However, detailed analysis of the various FRET efficiency peak heights indicated the co-existence of a dimeric population of the $M_3$ receptor alongside the tetrameric form and co-immunoprecipitation studies indicated that there was dynamic interchange of units between these species (Patowary et al., 2013) (**Figure 1C, 1D).** **A**lthough clearly able to define, as anticipated, that the proportion of tetramers containing three energy donor-linked protomers and a single energy acceptor-linked one increased as the fraction of energy donor molecules was increased, the ability to vary the relative levels of energy donor and acceptor species in these studies was insufficient to define unambiguously if the proportion of tetramer to dimer increased as total expression levels increased, as predicted by Mass-Action. The capacity to control donor to acceptor ratios across a much broader scale, within such a regulated system rather than via transient transfection, will be required to clarify this question fully. It also remains uncertain if different tetrameric organizations of various receptors might exist, e.g. squares and parallelograms, but conclusions based on combinations of direct experimental and modelling studies on potential interfaces and stability of δ opioid receptor dimers suggest at least two possible, distinct orientations

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that might generate forms with different FRET efficiency signals (Johnston et al., 2011). The dopamine $D_2$ receptor has also been used widely to explore aspects of the organizational structure of class A GPCRs (Han et al., 2009). Using combinations of bioluminescence/fluorescence complementation and energy transfer to allow detection of complexes that are larger than dimers Guo et al., (2008) showed that this receptor could exist as a tetrameric complex based on symmetrical interfaces involving transmembrane domains I and IV. This expanded and built on a potential 'daisy-chain' model of the quaternary structure of the $\alpha_{1b}$-adrenoceptor developed by Lopez-Gimenez et al., (2007) in which symmetrical interfaces provided by residues in transmembrane domain I and transmembrane domain IV left the potential for further symmetrical interactions to extend the size of the complex. Indeed, in the analyses of muscarinic $M_3$ receptor organization of Patowary et al., (2013) a model based on a hexamer was also consistent with (but not required and therefore excluded on the principle of Occam's Razor) the experimental data. Despite these results a number of other studies are compatible with GPCR oligomers being restricted to dimers. These include studies in which the neurotensin 1 receptor behaved as a dimer when reconstituted in polar lipid bilayers (Harding et al., 2009) and fluorescence correlation spectroscopy studies on the brightness of complexes of the serotonin $5HT_{2C}$ receptor when tagged with a yellow fluorescent protein (Herrick-Davis et al., 2012). Although this review is centred on rhodopsin-like, class A GPCRs, information on this topic is currently more advanced for the class C receptors. Herein, directly comparable studies indicate that metabotropic glutamate receptors are restricted to dimeric pairs (Maurel et al., 2008) whilst the $GABA_B$ receptor, long appreciated as a heteromer containing both $GABA_{B1}$ and $GABA_{B2}$ subunits, is clearly able to exist as a tetramer (Maurel et

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al 2008) and potentially even as an octamer (Calebiro et al., 2013). Interestingly, efforts to assess subunit exchange within such heteromers have indicated the complex to be stable (Maurel et al., 2008), but this seems unlikely if dimers and tetramers as well as further higher order complexes can co-exist. FRET efficiency measurements using suitably labelled $GABA_{B1}$ and $GABA_{B2}$ subunits suggested that the $GABA_{B1}$ subunits are closer together in the tetramer than the $GABA_{B2}$ subunits (Maurel et al., 2008) suggesting direct interactions between $GABA_{B1}$ subunits but not between $GABA_{B2}$ subunits. These observations could be accommodated in either a 'linear' or a 'rhombic' model. However, despite the data discussed above, recent studies have shown the capacity of the large extracellular domain sections of $GABA_{B1}$ and $GABA_{B2}$ to interact directly with each other (Geng et al., 2012), and this may provide the additional binding energy to stabilise the tetrameric (or even larger) complex.

**Are dimers/oligomers of class A GPCRs stable complexes?**

A wide range of studies have provided evidence that class A GPCRs initially generate quaternary structure at an early stage of biosynthesis (Salahpour et al., 2004, Herrick-Davis et al., 2006, Wilson et al., 2005, Lopez-Gimenez et al., 2007, Kobayashi et al., 2009, Canals et al., 2009, Cunningham et al., 2012). This has implications for function as many GPCR mutants (Pidasheva et al., 2006) and non-synonomous single nucleotide polymorphisms (Leskela et al., 2012) may interact with the corresponding wild type receptor at this level and, by forming quaternary interactions and acting as dominant negatives, limit cell surface delivery. It has been suggested that early stage dimerization may encourage or be required for effective folding and maturation of the

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receptor. Studies of this type contributed to a view that GPCR dimers/oligomers would likely be stable complexes and might exist as such until turnover and destruction. Furthermore, a series of studies have suggested that such interactions can be measured across a wide range of expression levels (e.g. Guo et al., 2008), observations that are certainly consistent with such complexes being stable, long-lived entities. However, as well as a range of studies that indicate either the detailed organizational structure or indeed the extent of oligomerization can be modified upon ligand binding, a number of papers have suggested that oligomeric organization may alter over time or with cellular location. A key set of studies in this regard were performed by Dorsch et al., (2009). Although antibody-mediated immobilized of a defined proportion of cell surface $\beta_2$-adrenoceptors markedly restricted lateral mobility of other copies of this receptor, this was not observed in equivalent studies on the $\beta_1$-adrenoceptor. These results were interpreted to suggest that interactions between $\beta_1$-adrenoceptor monomers were limited and/or transitory whereas the $\beta_2$-adrenoceptor formed a stable oligomeric complex. However, in contrast to the studies of Sartania et al., (2007) which indicated that dimers/oligomers of the $\beta_2$-adrenoceptor internalized from the surface of cells as a maintained and presumably stable complex, recent studies from Lan et al., (2011), despite using the same mutational approach to ensure that only a fraction of the protomers were able to bind the agonist ligand isoprenaline, were unable to observe a similar co-internalization of agonist-binding competent and incompetent forms and concluded that $\beta_2$-adrenoceptors either associate transiently with each other in the plasma membrane, or that the complexes are actively disrupted during internalization. Similarly, by using an immobilization strategy conceptually similar to that of Dorsch et al., (2009), Foncesa and Lambert

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(2009) concluded that the dopamine $D_2$ receptor also produced complexes that were transitory in nature, again in marked contrast to the observations and conclusions of Guo et al., (2008). Similar conclusions as to the transitory nature of at least certain family A GPCRs oligomers were reached for the $M_1$ muscarinic receptor using a combination of imaging and potential single molecule tracking via total internal reflection fluorescence microscopy (Hern et al., 2010). These studies concluded, at least when expressed in CHO cells, that at steady state-some 30% of the receptor was present as a dimer with no detectable presence of higher-order oligomers. However, these conclusions rest entirely on the view that the ligand used to the label the receptor was monitoring monomer-dimer transitions rather than, as suggested by Patowary et al., (2013) for the closely related $M_3$ receptor, potential dimer-tetramer transitions. Equally, single molecule imaging has been used to conclude that monomers and dimers of the chemoattractant N-formyl peptide receptor undergo rapid, sub-second interconversions (Kasai et al., 2011) and further, very recent, single molecule studies also favor rapid transitions of between monomers and dimers of β-adrenoceptor subtypes (Calebiro et al., 2013). This appears to contrast with recent studies on the serotonin $5HT_{2C}$ receptor where, using combinations of fluorescence correlation spectroscopy and photon counting histogram analysis, it was concluded that all of the cell surface receptor was dimeric (Herrick-Davis et al., 2012). A similar conclusion was recently reached for the orexin $OX_1$ receptor based on combinations of energy transfer and biochemical studies (Xu et al., 2011) and evidence based on the use of fluorescence lifetime measurements indicate that the serotonin $5HT_{1A}$ receptor exists as an oligomer (Ganguly et al., 2011). However, it may be that a number of the approaches used are unable to resolve fluctuations in receptor interactions occurring

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on a time scale of seconds. It will be interesting, therefore, to begin to see results that provide interaction affinities for different GPCR protomers and the results of single molecule tracking after GPCR protomers are linked together via cleavable cross-linkers. Modelling approaches have also been applied to efforts to predict if such interactions might be transitory (Provasi et al., 2010). If GPCRs routinely alternate between monomers and dimers/oligomers it should be possible to detect evidence of this biochemically. Indeed, for the muscarinic $M_3$ receptor analysis of interactions between recently synthesized, non-glycosylated, and fully N-glycosylated forms of the receptor that had been synthesized previously showed the presence of these two forms in the same molecular complex, arguing that there must be dynamic exchange between complexes (Patowary et al., 2013). The studies of Calebiro et al. (2013) are of particular interest for the range and selection of controls used. These included the use of a previously well characterized monomeric, single transmembrane domain polypeptide CD86, to which either a single or two copies of the 'SNAP' tag (Maurel et al., 2008, Alvarez-Curto et al., 2010a, Ward et al., 2011) was attached to the extracellular domain. This allowed labelling of the monomer with either one or two molecules of fluorophore for calibration purposes. Based on these measurements studies using transiently transfected SNAP-tagged forms of the $\beta_1$- and $\beta_2$-adrenoceptors indicated both were able to form dimers but that the proportion of such complexes was greater for the $\beta_2$-adrenoceptor at similar receptor density and that, in both cases, this increased with receptor density (Calebiro et al., 2013).

**What are the dimer/oligomer interfaces?**

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As noted earlier, both for the μ opioid receptor (Manglik et al., 2012) and the chemokine CXCR4 receptor (Wu et al., 2010) crystals have been obtained in which dimeric interfaces provided by residues from transmembrane domains V and VI have been highlighted. Such an interface is consistent with some previous biochemical studies, but less so with other datasets. These crystal structures were all obtained with the receptor occupied by small molecule antagonists/inverse agonists. One feature of class A GPCR activation that is consistent across approaches, ranging from atomic level structures (Rasmussen et al., 2011), via biophysical analyses on purified proteins following insertion of conformational sensors into the sequence of various receptors (Rosenbaum et al., 2009), to studies employing GPCRs engineered to act as intramolecular FRET sensors (Ambrosio et al., 2011, Xu et al., 2012) is that agonist-occupation and activation is associated with substantial movement of transmembrane domain VI. Assuming such a movement occurs within a dimer, then this might, at least in part, account for the agonist-induced intra-dimer communication reported in a wide range of studies (e.g. May et al., 2011). However, the potentially high affinity interaction produced by the extensive transmembrane domain V/VI interactions observed in the μ opioid receptor crystal structure (Manglik et al., 2012) might be anticipated to limit helix movement in this region. It is, therefore, interesting to note, although only a conceptual model at this point, that Manglik et al., (2012) have suggested a model in which a tetrameric form of the μ opioid receptor could interact with two heterotrimeric G proteins. Clearly, many more examples will be required before patterns emerge but it is interesting to note that May et al., (2011) observed a much smaller effect of othosteric ligands on dissociation of a fluorescent ligand from a potential adenosine $A_1$ receptor dimer than at the closely related adenosine $A_3$

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receptor. The other obvious feature of the μ opioid receptor crystal structure was the additional presence of a second set of contacts provided by residues from transmembrane domains I and II as well as from helix VIII. This second interface allows for both the presence of higher-order oligomers and, perhaps, may indicate why a number of efforts to define the structural basis of GPCR 'dimers' have resulted in evidence for contributions from a number of distinct elements. Although it is clearly not impossible to envisage that different GPCRs dimerize in very different ways, the overall structural conservation of the transmembrane helix bundle and the restricted number of ways in which the large family of class A GPCRs are likely to engage a limited population of heterotrimeric G proteins make this an intellectually unappealing hypothesis and suggests that there are likely to be general features that define or favor the stability of GPCR quaternary structure.

Although certain studies, not least a very early study on the $\beta_2$-adrenoceptor (Hebert et al., 1996), provided support for a role of transmembrane VI in dimer interactions and for the dimer as the functional signal transducer, a wide range of more recent studies have attributed key roles in dimer contacts to residues within other transmembrane domains including I (Wang and Konopka, 2009), IV (Lopez-Gimenez et al., 2007, de la Fuente et al. 2012) and V (Hu et al., 2012) in different receptors. Furthermore, despite the bulk of studies concentrating on the contribution of transmembrane domains to dimeric interfaces, a number of studies have also suggested key roles of intracellular loops (Navarro et al., 2010) with particular attention being given to potential electrostatic interactions between groups of positively and negatively charged residues and, also, of the extracellular N-terminal domain (Uddin et al., 2012). Modelling approaches have also contributed

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suggestions to the ways in which GPCRs may organize (Casciari et al., 2008, Johnston et al., 2012) but, although intrinsically of interest such studies need to be linked to direct experimental analysis to maximise their impact. There are, of course, a number of potential limitations in studies that rely extensively on mutagenesis to attempt to define protein-protein interaction interfaces, not least, as has also been an issue for mutagenic approaches to define ligand binding sites, that loss of function may reflect protein misfolding rather than reflecting the anticipated endpoint, and such potential effects must be considered as a realistic scenario.

**Physiology and pharmacology of class A GPCR homomers**

The equivalence of the protomers in class A GPCR homomers has meant that it is challenging to identify the presence of such complexes in native systems. By far the most enterprising, but also most challenging, route was taken by Rivero-Müller et al., (2010). Building on the concept that distinct non-functional forms of the glycoprotein hormone receptors can be generated by mutations that either prevent ligand binding or G protein activation they generated knock-in lines of mice in which either of two distinct, inactive forms of the luteinizing hormone (LH) receptor was introduced into LH receptor knock-out animals. Although neither of these was able to restore function, crossing of the lines to produce co-expression of the two individually inactive forms did result in rescue of function, implying a need for complementation between the two forms and, by extension, their dimerization. Nothing akin to this has been attempted *in vivo* for any other class A GPCR. Despite the apparent clarity of these results, even the basic premise of the mode of action of such complementation has recently been questioned and the interpretation challenged (Zhang et al., 2012).

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Within these studies Zhang et al., (2012) reported that the mutated variants of the receptor used by Rivero-Müller et al., (2010) may not be entirely lacking in function and that, in part, the reported lack of function might relate to limited cell surface delivery. Although certainly providing a series of challenging questions for previous interpretation, the studies of Zhang et al., (2012) were entirely limited to heterologous cell expression. This dichotomy clearly requires further study, but the commitment to production of transgenic animals to address these issues is not likely to be undertaken lightly.

Other approaches that might have value in either *ex vivo* or *in vivo* studies remain challenging. Conceptually, detailed analysis of the dissociation kinetics of receptor ligands may provide evidence in favour of models that are incompatible with the GPCR in question acting as single non-interacting species (Albizu et al., 2010, May et al., 2011) or if the behavior of certain ligands observed *in vitro* that can best be described in terms of receptor homomers (Brea et al., 2009) can be replicated *ex vivo*. Perhaps the most promising approach takes advantage of time-resolved FRET (Albizu et al., 2010, Cottet et al., 2011). Here, using FRET-competent ligands with affinity for the oxytocin receptor, FRET signals consistent with receptors with quaternary structure were detected in mammary gland patches known to express high levels of this receptor, but not in brain. Even here, however, the expression levels would probably have to be substantially higher than are known to be the case for many GPCRs to produce acceptable signal to background ratios.

There has also been interest in the idea that certain ligands or other receptor regulators may bind selectively to a receptor dimer. Perhaps the best example of this to date is

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not for a small molecule ligand, but for the snake toxin MT7 (Marquer et al., 2011). Here both extensive mutagenesis studies and a series of studies that indicated that the toxin favors either the formation or stability of such a complex of the $M_1$ muscarinic receptor have been produced. Even for this reagent, however, the suggestion that it favors the production of $M_1$ muscarinic receptor quaternary structure might result in an overestimate of the presence of such complexes in native tissues.

**GPCR heteromers**

Beyond the obvious examples of heteromeric GPCR complexes provided by the class C taste and $GABA_B$ receptors described earlier there is large literature on the ability of co-expressed class A GPCRs to form heteromers. Such interactions frequently result in markedly distinct receptor pharmacology and function, at least as defined in transfected cell systems

**GPCR quaternary structure: relevance to physiology, disease and drug design**

A major challenge has been and remains in defining the presence of GPCR heteromers in native tissues and either replicating or extending the functional and pharmacological sequelae noted following co-transfection of pairs of GPCRs into simple model cell systems. Despite these challenges significant progress is being made. One of the key examples has been to define the contribution of a potential 5-$HT_{2A}$-mGluR2 heteromer to both pharmacological and behavioral responses to a group of 5-$HT_{2A}$ agonists that generate hallucinogenic effects. Intriguingly, although drugs such 2,5-dimethoxy-4-iodoamphetamine (DOI) are able to induce a head-twitch response in mice that requires expression of the 5-$HT_{2A}$ receptor in cortical pyramidal

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neurons (Gonzalez-Maeso et al., 2007), this effect also requires expression of the mGluR2 receptor because it is also lacking in mGluR2 knock-out animals (Moreno JL et al., 2011). Moreover, this effect is restored in such animals by virally mediated re-introduction of the mGluR2 receptor (Moreno JL et al., 2012). A series of studies has shown the capacity of these two otherwise unrelated GPCRs to interact and form a functional complex both in cellular systems and in brain (Gonzalez-Maeso et al., 2008, Delille et al., 2012) whilst a range of other studies have also shown pharmacological interactions between these receptors (e.g. Molinaro et al., 2009). A key element of the studies of Gonzalez-Maeso et al., (2008), and which was extended by the further studies of Fribourg et al., (2011), was the alteration in signal transduction pathways produced via this heteromer. It must be noted that not all studies have been able to replicate the reported changes in ligand pharmacology and signalling (Delille et al., 2012) but further recent studies from Gonzalez-Maeso and colleagues (Moreno JL et al., 2012) utilized chimeric mGluR2/mGluR3 constructs and variation in sequence at the cytoplasmic end of transmembrane domain IV between these closely related receptors to define a small number of residues of mGluR2 that, when mutated to the equivalent sequence of mGluR3, greatly reduced interactions with the 5-$HT_{2A}$ receptor. When a mutant mGluR2 containing three such amino acid changes was introduced into mGluR2 knock-out mice, unlike wild type mGluR2, this construct was unable to restore the head-twitch response produced by DOI. It is well established that there is functional cross-talk between the glutamate and serotonergic systems in brain (e.g. Benneyworth et al., 2007), and the studies of Moreno et al., (2012) now appear to link the capacity of co-expressed $5HT_{2A}$ and mGluR2 receptors to form a heteromeric complex to a behavioral response to a

hallucinogenic drug. It remains possible, however, that observations consistent with altered function arising from a potential heteromer may instead reflect interactions between co-expressed but non interacting receptors via signalling pathway cross-talk. For example, although careful not to imply that previously reported heteromerization between the cannabinoid $CB_1$ and orexin $OX_1$ receptors (Ward et al., 2011) might not be the molecular explanation for previously reported allosteric effects between selective ligands at these two receptors, Jantti et al., (2013) recently highlighted that autocrine regulation of endocannabinoid generation can produce similar pharmacological effects. Moreover, although certain studies have indicated the capacity of co-expressed cannabinoid $CB_1$ and μ opioid receptors to interact directly (Rios et al., 2006), other studies suggest that pharmacological variation in the function of the opioid peptide DAMGO produced in the presence of the $CB_1$ receptor reflect constititive activity of this receptor. Evidence in favor of this model included that activity of DAMGO was restored by co-addition of the $CB_1$ receptor inverse agonist SR141716 (rimonabant) but not by the $CB_1$ receptor neutral antagonist O-2050 and that a mutationally modified form of the $CB_1$ receptor lacking constitutive activity was unable to mimic the effects of the wild type receptor (Canals and Milligan, 2008) A capacity of heteromers to alter the G protein coupling selectivity compared to those of the corresponding monomers/homomers has also been a central element in defining specific roles for the dopamine $D_1$-$D_2$ receptor heteromer (Ng et al., 2010). This pairing has been indicated to form a specific heteromer in parts of the striatum (Hasbi et al., 2011) and to mediate a group of distinct signals through engagement of $Ca^{2+}$-mediated signalling rather than the regulation of cAMP levels most normally anticipated to result from either dopamine $D_1$ or $D_2$ receptor activation. Recent work

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from a number of teams has implicated charged residues in the 3rd intracellular loop of the $D_2$ receptor and in the carboxyl terminal tail of the $D_1$ receptor in defining this interaction, rather than the transmembrane domains (Pei et al., 2010, O'Dowd et al., 2012a) and in this regard has similarities to the studies of Navarro et al., (2010) described earlier. Furthermore, this had been predicted in earlier studies from Łukasiewicz et al., (2009), although these authors were careful to note mis-localization of mutants of the $D_2$ receptor as providing a possible contribution to lack of heteromer formation rather than a direct role for specific amino acids. Pei et al., (2010) have suggested a key role for this complex in major depression because higher levels of $D_1$ receptor were immunoprecipitated by an anti-$D_2$ receptor antibody from post-mortem human striatal tissue from individuals suffering major depression than from controls and, subsequent to demonstrating that a peptide corresponding to part of the third intracellular loop of the $D_2$ receptor was able to interfere with interactions with the $D_1$ receptor, infused such a peptide into the frontal cortex of rats. Remarkably this was as effective as treatment with the anti-depressant drug imipramine in reducing immobility in forced swim tests (Pei et al., 2010). It will be interesting to discover if other heteromers between dopamine receptor subtypes also are stabilized by interactions involving elements of the intracellular loops. Other heteromers between dopamine receptor subtypes have, indeed, been reported, including $D_1$-$D_3$ (Marcellino et al., 2008, Ferre et al., 2010, Moreno E et al., 2011), $D_2$-$D_3$ (Pou et al., 2012), and $D_2$-$D_4$ (González et al., 2011) complexes but the basis for their interactions and potential functional sequelae have not yet been explored in the level of detail of the $D_1$-$D_2$ heteromer. Interestingly, although it is widely accepted that the detection of receptor heteromers is likely to be associated with the concurrent presence of the

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corresponding homomers, this is difficult to assess in *ex vivo* situations, and even *in vitro* this has only recently been explored in cells co-expressing varying amounts of the dopamine $D_2$ and $D_3$ receptors to show that the expectation of a mixture of homomers and heteromers was likely to be correct (Pou et al., 2012).

Even at the level of dopamine receptor homomers, Wang et al., (2010) have suggested there might be a marked shift in the balance between monomers and dimers of the $D_2$ receptor in disease conditions highly linked to function of this receptor, including schizophrenia. Reviews on this topic have also highlighted the potential contribution of dopamine-containing heteromers (e.g. Maggio and Millan, 2010, Perreault et al., 2011). Although it is too early to draw general conclusions, the concept discussed earlier that inter-conversion between quaternary structure states of GPCRs may be rapid and extensive, linked to the idea that different organizational states of receptors might differentially affect the capacity for signal transduction, means that assessment of how the proportion of monomers, dimers and higher-order oligomers might be regulated and potentially vary with disease is an area ripe for re-investigation.

A number of other GPCR heteromers appear to play important roles in brain function. A recent study highlighted the potential importance of interactions between the dopamine $D_2$ receptor and the ghrelin receptor (Kern et al., 2012). These workers showed co-expression of the two receptors via a combination of staining brain sections from mice in which green fluorescent protein was driven from the ghrelin receptor promoter with an anti-$D_2$ receptor antibody and subsequently noted an alteration in signalling pathways for $D_2$ receptor agonists in cells engineered to co-express the two GPCRs that was re-capitulated in primary cultures of hypothalamic neurons. Time-resolved FRET studies employing SNAP- and CLIP-tagged (Maurel et

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al., 2008) forms of the two receptors were compatible with direct interactions between the two as were allosteric effects of receptor selective ligands (Kern et al., 2012). Such an interaction was also detected in native tissue using a ligand-based time-resolved FRET concept and approach. Herein, a red fluorophore-coupled form of ghrelin was used to label and bind to the ghrelin receptor and to act as energy acceptor, whilst an antibody against the $D_2$ receptor was labelled with an energy donor, cryptate-labelled fluorophore secondary antibody. Time-resolved FRET signals were detected in membranes from the hypothalamus of wild type but not ghrelin receptor knock-out animals (Kern et al., 2012). Furthermore, physiological relevance of the heteromer was assessed in a model of anorexia in wild-type animals. Here a selective ghrelin receptor antagonist was shown to block cabergoline (an ergot-related $D_2$ receptor agonist)-induced anorexia (Kern et al., 2012). The authors suggested that inhibiting dopamine $D_2$ receptor signaling in subsets of neurons with a ghrelin receptor antagonist would provide much greater pharmacological selectivity than the use of a direct dopamine $D_2$ receptor antagonist, as the effect would be restricted to cells expressing the heteromer. This concept of tissue selectivity provided by targeting GPCRs heteromers has been discussed extensively as a potential therapeutic approach (Milligan and Smith 20007, Casado et al., 2009, Saenz del Burgo and Milligan, 2010, Smith and Milligan, 2010). Observations of allosteric effects between highly selective ligands for pairs of receptors co-expressed in the same cell have become something of a mainstay in providing pharmacological support for the presence of receptor heteromers. Examples relating to opioid receptor subtypes are considered later but examples supporting interactions between chemokine receptor pairs have been perhaps the most numerous and best studied (e.g.

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Sohy et al., 2007). These can potentially involve more than two co-expressed receptor subtypes (Sohy et al., 2009), and can be observed in both native and transfected cells (Sohy et al., 2009). As a complex and wide ranging topic, this area has been reviewed extensively and readers are guided towards some recent viewpoints (Wang and Norcross, 2008, Appelbe and Milligan, 2009, Salanga et al., 2009, Thelen et al., 2010).

A further heteromer involving the dopamine $D_2$ receptor that has been studied extensively is the potential adenosine $A_{2A}$-dopamine $D_2$ complex. Functional interactions between these two receptors have been studied for many years with the work of Fuxe and collaborators playing a central role. This team has written and speculated on the role of this and other potential heteromers so extensively (e.g. Fuxe et al., 2012, Filip et al., 2012) that it is almost impossible to summarize the available information and possibilities within a short review. However, as well as the usual range of transfected cell, co-immunoprecipitation and energy transfer approaches used to attempt to define the potential existence of such heteromers, it is noteworthy that recent proximity ligation studies that utilize immunohistochemical antibody detection of each partner followed by amplification of specific nucleotide sequences linked to secondary antibodies has provided evidence for proximity and, therefore, potential direct identification of an adenosine $A_{2A}$-dopamine $D_2$ heteromer in striatum (Trifilieff et al., 2011). Although technically challenging, not least because of wide ranging concerns over the specificity of many of the available GPCR antibodies (Beermann et al., 2012) and the modest expression levels of many GPCRs, approaches such as this offer the opportunity to begin to examine potential co-localization of GPCRs without resorting to more traditional methods based on e.g.

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immuno-electron microscopy (Moreno JL et al., 2012). Interestingly, each of large-scale GPCR protein production for crystallography trials, novel approaches to GPCR antigen presentation (Larsson et al., 2011) and indications that anti-GPCR directed 'biologicals' may have clinical utility (Harris et al., 2012) hint at ways forward in providing a wider range of more specific and high affinity immunological reagents. Furthermore, despite some of the concerns noted above about antibody specificity, in relation to the identification of heteromers, a small number of heteromer-specific antibodies have been described (Gupta et al., 2010, Rozenfield et al., 2011) that potentially identify such complexes directly. Rozenfield and colleagues (2011) employed an antibody reported to selectively detect angiotensin $AT_1$ receptor-cannabinoid $CB_1$ receptor heteromers to detect higher levels of such a complex in hepatic stellate cells from ethanol-administered rats, whilst Gupta et al., (2010) used a potentially δ-μ opioid receptor heteromer specific antibody to detect higher levels of this complex in chronic morphine-treated animals in areas of the central nervous system important for pain processing. It would be interesting to see the behaviour of this antibody in cells co-expressing forms of these two receptors reported to disrupt heteromer formation (O'Dowd et al., 2012b).

Interactions between opioid receptor subtypes and the implications of this for both pharmacology and function have been studied perhaps more widely than in any other group of class A GPCRs. As noted earlier, crystals of the μ opioid receptor formed with a strong potential dimeric interface involving many residues from transmembrane helices V and VI and a more limited second interface involving residues from helices I and II as well as the intracellular helix VIII (Manglik et al., 2012). Similarly, crystals of the κ opioid receptor containing a bound antagonist also

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display a parallel dimeric interface involving residues of helices I, II and VIII (Wu et al., 2012). By contrast, no parallel interaction interfaces were observed in crystals of the δ-opioid receptor bound by naltrindole (Granier et al., 2012). Despite this, reported heteromeric opioid receptors are not restricted to the κ-μ receptor pairing. Indeed, for the potential δ-μ receptor pairing it has been suggested that each individual receptor may exist as a homo-dimer that can combine to form a hetero-tetramer (Golebiewska et al., 2011). Interactions between δ and μ receptors have been widely implicated in efforts to understand specific features of the action of morphine and to consider if targetting such a heteromer might overcome aspects of the development of tolerance to this clinically important drug (Berger and Whistler, 2010, Costantino et al., 2012). Interestingly, given the potential role of transmembrane domains in such heteromers, it is noteworthy that alteration of a few key residues of the intracellular loops of these receptors is reported to interfere with heteromer formation (O'Dowd et al., 2012b). On this basis it should be possible to disrupt such interactions using either cell permeable peptides or peptidometic small molecules and hence assess functional significance as for dopamine $D_1$-$D_2$ heteromer (Pei et al., 2010). This, however, remains to be tested. The δ-μ opioid receptor heteromer is also reported to display unique ligand pharmacology (e.g. Kabli et al., 2010, Gomes et al., 2011). The opioid receptors have been particularly amenable to such studies because of the complex pharmacology of opioid receptors that has been described, which is not easily defined or replicated by studies performed on cells expressing only one of the cloned subtypes, as well as the vast array of both peptide- and alkaloid-based opioid ligands that have been generated to explore the function of these receptors. Given the vast literature around this topic only an expert on opioid receptor

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pharmacology could provide a balanced short overview. However, among key observations are those that have reported marked variation in, and often unique, ligand pharmacology upon co-expression of pairs of opioid receptors (e.g. Kabli et al., 2010, Gomes et al., 2011). A key question that remains to be established firmly is the extent and distribution of this heteromer. Detailed studies by Scherrer et al., (2009) indicated a limited overlap of δ- and μ opioid receptor expressing neurones and, if confirmed, this would indicate, at most, a limited distribution of the heteromer.

As well as heteromers incorporating only opioid receptor subtypes, reports of heteromers in which an opioid receptor interacts with a less related GPCR are widespread. Co-expression of such pairs, e.g. the cannabinoid $CB_1$ receptor with either the δ (Bushlin et al., 2012, Rozenfeld et al., 2012) or μ (Rios et al., 2006) opioid receptor also result in substantial alterations in ligand pharmacology and function. Furthermore, when the μ opioid receptor was co-expressed with a form of the $\alpha_{2A}$-adrenoceptor engineered to function as an intramolecular FRET sensor co-addition of opioid ligands was able to modify the FRET response to the presence of agonists of the $\alpha_{2A}$-adrenoceptor (Vilardaga et al., 2008). Although potentially a powerful approach other examples have not yet been reported as suitable FRET sensors are not generally available. A further example of a potentially heteromeric interaction involving an opioid receptor with implications for physiology and behavioral response was described by Liu et al., (2011). Herein interactions between the gastrin-releasing peptide receptor and different μ opioid receptor splice variants had distinct functional sequelae. Opioid-induced itch is linked to use of morphine in pain management and Liu et al. (2011) demonstrated that interaction of the gastrin-releasing peptide receptor specifically with the MOR1D splice isoform was required

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to cause morphine-induced scratching, whereas the MOR1 receptor was key for morphine-induced analgesia. These two splice variants of the μ opioid receptor displayed limited overlap of distribution but strong co-expression of the MOR1D and the gastrin-releasing peptide receptor was observed. Equally, and rather like the loss of the behavioural function of 5-$HT_{2A}$ receptor-directed hallucinogenic agonists in mGluR2 knock-out mice, morphine-induced scratching but not morphine-induced analgesia was almost lacking in gastrin-releasing peptide receptor knock-out mice (Liu et al. (2011) and a gastrin-releasing peptide receptor antagonist greatly reduced morphine-induced scratching but not analgesia in wild type mice. As these slice variants of the μ receptor differ only in the intracellular C-terminal domain a peptide containing the sequence variance between the two μ receptor forms was introduced into the spinal cord of mice. This both markedly reduced the ability of the gastrin-releasing peptide receptor and MOR1D isoform to be co-immunoprecipated and the extent of morphine-induced scratching (Liu et al. 2011).

## Conclusions

Although clearly requiring substantial physiological insight as well as high level technical skills, a number of recent examples of disruption of GPCR heteromers, based partly on phenotypes of GPCR knock-out mice, is both providing increasing support of the presence of such complexes in native cell and tissue settings and confirming their potential as distinct therapeutic targets. Despite the fascinating physiological effects noted to be associated with understanding the presence of such heteromers and targetting them either pharmacologically or via transgenic or other interventions, there remains an enormous lack of knowledge of even the basic

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principles of interactions between class A GPCRs, the overall size of such complexes, and their stability and dynamics. A clear understanding of all of these features will be required before a systematic means to target them therapeutically will become a reality.

**Authorship Contribution:** *Wrote the manuscript* Milligan

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**Footnotes**

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**Legends for Figures**

**Figure 1 Spectrally-resolved FRET and co-immunoprecipitation studies define the complex organization and dynamics of the muscarinic $M_3$ receptor quaternary complex at the surface of cells**

Spectrally resolved FRET was employed to investigate the organization of the muscarinic $M_3$ receptor at the surface of Flp-In™ T-REx™ 293 cells constitutively expressing a form of the muscarinic $M_3$ receptor that contains a N-terminal FLAG epitope tag and with the fluorescent protein Citrine linked in frame to the C-terminus (the energy acceptor) and also harbor at the inducible Flp-In™ T-REx™ locus a variant of this receptor containing a N-terminal MYC epitope and C-terminal Cerulean fluorescent protein (the energy donor). Expression from the Flp-In™ T-REx™ locus is controlled by the addition of various concentrations of doxycycline, **1A**. A broad range of FRET efficiencies were measured at the surface of cells induced to express varying amounts of the energy donor. This is not consistent with the receptor existing simply as a dimer because this scenario would be anticipated to result in a single FRET efficiency peak. Analysis of the experimental data based on the receptor existing , at least partially, as a rhombic tetramer with individual species containing varying numbers of acceptor (**yellow**) and donor (**blue**) species (**1B**) showed that the experimentally derived and simulated results were highly similar (**1A**).

**1C.** Lysates from cells as above were resolved by either Native-PAGE (**i**) or SDS-PAGE (**ii-iii**). Immunoblots of Native-PAGE resolved samples confirmed doxycycline-induced expression of the MYC-tagged protein and both constitutive and

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maintained expression of the FLAG-tagged form, whilst addition of SDS to samples prior to addition to the Native gel resulted in production of two, potentially differentially N-glycosylated, variant monomer forms of each (**i**). Treatment of cells with PNGaseF confirmed this (not shown).

Maintenance of the cells in the presence of the *de novo* N-glycosylation inhibitor tunicamycin (**TUN**) during the period of induction of the MYC-tagged energy donor resulted in all cell surface anti-MYC reactivity lacking carbohydrate (**90 kDa**) whilst only the proportion of the FLAG-tagged energy acceptor synthesized during this period lacked N-linked glycosylation (**ii**). Anti-MYC immunoprecipitates performed on lysates from cells treated with both doxycycline and tunicamycin resulted in the co-immunoprecipitation of both N-glycosylated (110 kDa) and non-glycosylated (90 kDa) FLAG-tagged energy acceptor (**iii**). This must reflect the presence of FLAG-tagged proteins both made before and after addition of tunicamycin in complex with the MYC-tagged variant and therefore dynamic interactions between the forms. Results are adapted from Patowary et al., (2013).

**1D**. A cartoon representation of the experiments illustrated in **1C**, based on a tetrameric organization of the $M_3$ receptor. For '**no treatment**', i.e. in the absence of doxycycline, only the energy acceptor species (yellow) is present and all copies are N-glycosylated. Following addition of '**doxycycline**' a mixture of energy donors (blue) and acceptors are present, all are N-glycosylated and exist as a mixture of homo- and hetero-tetramers. With co-treatment of the cells with '**doxycycline and tunicamycin**' a more complex pattern is predicted in which all energy donors lack N-glycosylation while, based upon their time of synthesis the energy acceptor species may be either N-glycosylated or non-glycosylated. As shown in **1C (iii)**, immunoprecipitation of the

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MYC-labelled energy donor results in the co-immunoprecipitation of both N-glycosylated and non-glycosylated versions of the energy acceptor and these are resolved in SDS-PAGE. CHO = carbohydrate.

# FIGURE 1

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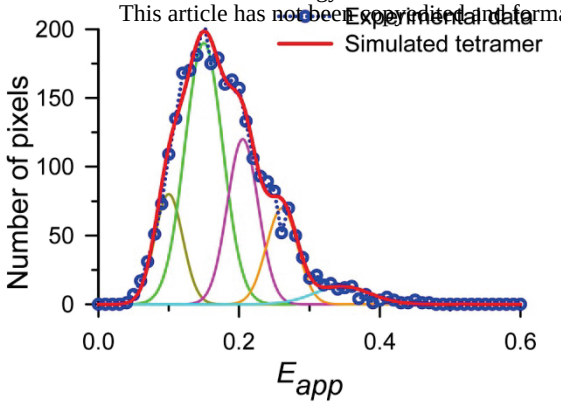
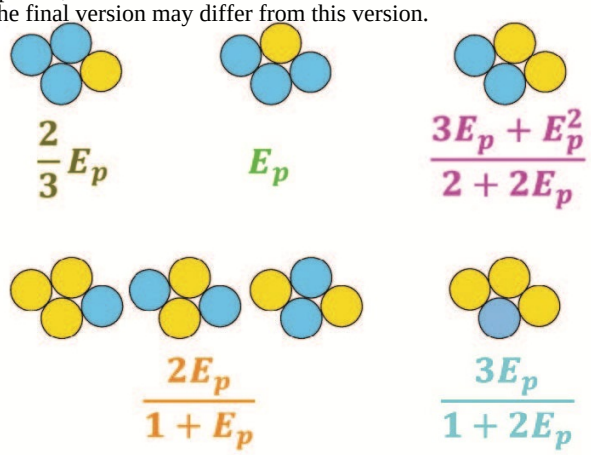



A

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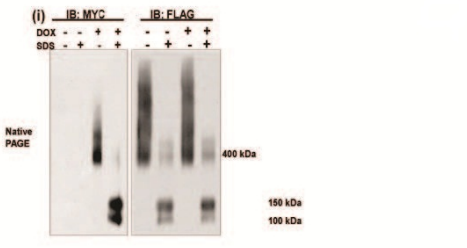


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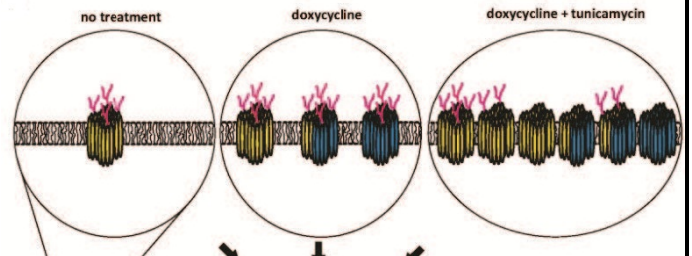


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