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The role of dimerisation in the cellular trafficking of G-protein-coupled receptors

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The concept that G-protein-coupled receptors can exist as homomeric and/or heteromeric complexes is now well established. Despite this, how dynamic such interactions are and if this may be modulated during receptor trafficking remain topics of debate. Use of endoplasmic reticulum trapping strategies and the generation of asymmetric homomers have started to provide information on the contribution of protein–protein interactions to receptor maturation, cell surface delivery and ligand-mediated endocytosis. Although dimer/oligomer formation appears to be essential for cell surface delivery of class A and class C GPCRs, this may not be the case for class B receptors.

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Introduction

Following reconstitution of single, purified monomeric class A G-protein-coupled receptors (GPCRs) into high-density lipoprotein phospholipid bilayer particles agonist ligands are able to activate added G protein [1•,2]. Although such observations demonstrate clearly that monomeric GPCRs are able to initiate canonical, G-protein-mediated signal transduction, and there are suggestions that certain observations consistent with GPCR quaternary structure have been over-interpreted [3], substantial evidence indicates that GPCRs are able to form and exist as dimers and/or higher order oligomers [4,5]. The class C, metabotropic glutamate receptor-like $GABA_B$ receptor was the first GPCR recognised to require quaternary structure for cell surface delivery and function (see [6] for review). The functional $GABA_B$ receptor is a constitutive heterodimer (or possibly a hetero-tetramer [7••]) formed by direct interactions between the polypeptide products of two distinct, but highly related genes. When expressed alone, the $GABA_BR1$ polypeptide is retained in the endoplasmic reticulum (ER) because of the presence of an ER-retention sequence in the intracellular C-terminal tail. Co-expression of the $GABA_BR2$ polypeptide generates protein–protein interactions that result in the retention motif being masked and allows cell surface delivery of the hetero-complex. Despite there being no substantial sequence conservation between class C GPCRs and members of other GPCR families, models that explore the importance of successful pre-assembly of dimers/oligomers in the ER have been explored recently for other classes of GPCRs. The proportion of GPCR populations that exist as dimeric/oligomeric complexes has also been a topic of recent debate, as has whether GPCRs internalise as dimers/oligomers following exposure to agonist ligands. These topics will form the basis of the current review.

Where do GPCR dimers/oligomers form?

On the basis of understanding the ER assembly of the heteromeric $GABA_B$ receptor complex Salahpour *et al.* [8] replaced the C-terminal tail of the rhodopsin-like, class A β_2-adrenoceptor with the equivalent region of the $GABA_BR1$. This generated a form of the β_2-adrenoceptor that was trapped inside transfected cells. This construct was also able to prevent cell surface delivery of co-expressed wild type β_2-adrenoceptor and retained the wild type receptor in the ER [8]. These observations were consistent with the C-terminally modified receptor interacting with the wild type receptor but with an inability of wild type β_2-adrenoceptor to mask the $GABA_BR1$ ER-retention motif introduced into the modified β_2-adrenoceptor. Furthermore, these studies also indicated that homodimerisation of the β_2-adrenoceptor was unlikely to be governed largely or exclusively by interactions involving the C-terminal tail. Although other GPCRs closely related to the β_2-adrenoceptor were not tested, cell surface delivery of an N-terminally epitope-tagged form of the β_2-adrenoceptor was substantially higher when co-expressed with the full length $GABA_BR1$ polypeptide than with the β_2-adrenoceptor containing the $GABA_BR1$ C-terminal tail and ER-retention motif [8]. Importantly, bioluminescence resonance energy transfer (BRET) studies indicated little interaction between the β_2-adrenoceptor and $GABA_BR1$ [8], consistent with the concept that physical interactions between the wild type and the ER-retained, modified form of the β_2-adrenoceptor, rather than simply the presence of an ER-retained protein, was the basis for poor cell surface delivery of the wild type receptor. This general approach has the

potential to be used to explore the selectivity and location of GPCR dimerisation but is not restricted to the use of the $GABA_BR1$ C-terminal ER-trapping sequence. A number of other GPCRs, for example the α_{2C}-adrenoceptor [9], contain what appear to be arginine-based ER-retention motifs. Indeed, transient expression of the α_{2C}-adrenoceptor in HEK293 cells results in a pattern of expression consistent with predominant ER-retention [9]. After addition of this ER-retention motif to the C-terminal tail of the chemokine CXCR1 receptor, Wilson *et al.* [9] noted both that the modified chemokine receptor was unable to reach the cell surface and that the ER-trapped CXCR1 receptor was able to limit cell surface delivery of co-expressed, wild type CXCR1. Furthermore, the ER-retained CXCR1 receptor also prevented cell surface delivery of the closely related chemokine receptor CXCR2 [9], providing a clear example of the generation within the ER of a heteromer between closely related class A receptors. By contrast, co-expression of the ER-trapped CXCR1 receptor did not modulate cell surface delivery of the α_{1A}-adrenoceptor [9]. This was an important control because there are often concerns about the specificity of GPCR dimerisation, particularly in transient transfection studies where it is difficult to define expression levels in individual cells. Because parallel saturation BRET studies [10] indicated that CXCR1 generated homomers and CXCR1–CXCR2 heteromers with equal efficiency, and also confirmed that the CXCR1 receptor did not interact with the α_{1A}-adrenoceptor [9], such studies also suggested that the 'dominant-negative' effects of the ER-retained receptor to prevent surface delivery were correlated with, and might reflect, dimerisation potential. Since these studies a number of other reports have generated data consistent with physical interactions between GPCR monomers being initiated within the ER. For example, an ER-retained mutant of the α_{2B}-adrenoceptor is able to cause ER-retention of wild type α_{2B}-adrenoceptor [11] as well as of the α_{2A}-adrenoceptor and α_{2C}-adrenoceptor [11]. Furthermore, introduction of the ER-retained α_{2B}-adrenoceptor mutant into cells that express various α_2-adrenoceptor subtypes endogenously resulted in reduced cell surface delivery of the endogenous α_2-adrenoceptor [11]. Fluorescence resonance energy transfer (FRET) measurements performed in cells co-expressing C-terminally cyan fluorescent protein (CFP)-tagged and yellow fluorescent protein (YFP)-tagged serotonin $5\text{-}HT_{2C}$ receptors also identified interactions within the ER and, via time-lapse microscopy, such complexes were followed through the Golgi apparatus and to the plasma membrane [12]. As FRET efficiencies measured in the ER, Golgi and plasma membrane compartments were similar, this suggested that the $5\text{-}HT_{2C}$ receptor remained a dimeric or oligomeric complex as it trafficked to the cell surface after synthesis [12]. The ability to measure GPCR–GPCR interactions in cell fractions isolated from sucrose density and other gradient systems, initially employing time-resolved FRET measurements [13] and, more recently BRET studies [14], has also allowed demonstration of receptor homomers in distinct cellular compartments [13,14]. Although not providing direct evidence on the location of biogenesis of GPCR dimers, such studies have confirmed populations of receptor homomers to co-migrate with ER markers, such as the protein-folding chaperone calnexin, as well as plasma membrane markers such as the Na^+/K^+ ATPase [13,14]. Co-localisation with calnexin and related chaperone proteins is of particular relevance as direct interactions of a number of GPCRs with calnexin have been shown [15,16] and relate to the progress of receptors from the ER if they are correctly folded.

It is unclear how, subsequently, variation in identity of the final transport vesicle populations for different GPCRs is determined, but a series of elements, often in different regions of the receptor sequence, can modulate export from the ER [17,18], and in many GPCRs there may be multiple such elements. However, recently, a single, highly conserved Leu residue in the first intracellular loop has been suggested to play an important role in ER export for a large number of GPCRs, including a range of adrenoceptors and the angiotensin AT_1 receptor [19••] and mutation of this residue may offer a general means to generate ER-retained mutants that could be used in the types of studies described above.

Pathways of cell surface delivery of GPCRs are less well studied than pathways of receptor internalisation from the cell surface, but despite clear evidence of distinct vesicular pools being favoured by individual receptors [18,20], the early stages of ER-quality control involving interactions with ER-resident chaperone proteins, and the subsequent processing of N-linked glycosylation, as GPCRs move from the ER to the Trans-Golgi Network are similar to other proteins that are destined to be trafficked via the secretory pathway. These ensure that only properly folded and assembled proteins proceed. In this regard it is important to note that it is well established that the quaternary structure of many other classes of cell surface receptors, such as ion channels and transforming growth factor receptors, as well as certain ATP binding cassette transporters, is pre-fabricated into the correct quaternary organisation before cell surface delivery [21,22]. It is, therefore, hardly surprising that this general rule should also apply to GPCRs, and there is growing evidence that such quaternary complexes may contain G-protein subunits as well the GPCR(s) [23]. There have been sporadic reports of the inability to replicate specific examples of GPCR heteromerisation and, clearly, it is important to examine potential reasons for these discrepancies. For example, although the proclivity of the angiotensin AT_1 receptor to form heteromers with the bradykinin B2 receptor has recently been questioned [24], it has also recently been suggested that the expression

level of the chaperone protein calreticulin defines the effectiveness of these interactions [25•] as only the properly folded and fully mature bradykinin B2 receptor is reported to interact with the angiotensin AT_1 receptor [25•]. If this is a general feature, it may be helpful in defining the earliest stages of GPCR heteromer generation and, potentially, also homomer formation. In this context it is also interesting to note that cell surface delivery of a DOP–MOP opioid receptor heteromer can be controlled by a Golgi chaperone named RTP4 [26], potentially via RTP4 protecting the DOP–MOP heteromer from ubiquitination and degradation [26]. This can lead to an increase in surface heteromer levels [26].

Receptor co-expression can enhance cell surface delivery of GPCRs

In contrast to the 'dominant-negative' effects of certain GPCRs and mutants thereof, there are also a number of reports, apart from the $GABA_B$ receptor, in which co-expression of a second GPCR promotes cell surface delivery of the GPCR being studied [27,28]. For example, a number of adrenoceptor subtypes have been shown to both interact with the α_{1D}-adrenoceptor and to promote its cell surface delivery and function [28,29]. Although the physiological significance remains unclear, interaction with certain GPCRs has also been suggested as a means to promote cell surface delivery in heterologous systems of a number of olfactory receptors to allow their characterisation [30]. Although these examples do not require the co-presence of receptor ligands, the concept that cell permeant, small molecule ligands can promote cell surface delivery of a number of GPCR mutants by altering their conformation such that they can now (by)pass the quality control systems of the ER has been widely explored in recent times and such 'pharmacological chaperones' [27,31,32] have been discussed widely in terms of their clinical potential. Furthermore, extensions of the ability of such ligands to recover the structural organisation and trafficking of newly synthesised GPCRs have recently been used to provide novel insights into the location and relevance of GPCR dimerisation.

Do mutations and manipulations that interfere with GPCR dimerisation modulate ER release and cell surface trafficking?

Many GPCRs that are associated with disease lack function because they fail to pass ER export quality control and reach the cell surface [31,32,33•,34,35], rather than being inherently unable to bind ligands and generate signals. This lack of cell surface delivery results in the protein being routed for degradation. In many cases such mutants can also act as 'dominant-negatives' as they also prevent the cell surface delivery of a co-expressed wild type form of the GPCR and, in physiological settings, this can account for function being reduced by more than the anticipated 50% in individuals heterozygous for the mutation. For example, for the class C Ca^{2+} sensing receptor a series of mutants including Arg^{66}His and Arg^{66}Cys, that are associated with familial hypocalciuric hypercalcemia/neonatal severe hyperparathyroidism, lack mature glycosylation, and are localised within the ER but not within the Golgi apparatus [34]. Photo-bleaching FRET microscopy showed that these mutants, as well as the wild type receptor were dimerised in the ER [34]. Equally, a number of mutants of the melanocortin 1 receptor are retained intracellularly and have 'dominant-negative' effects on the function of the wild type receptor [35]. However, although it remains to be investigated fully, it is unlikely that many of these or related mutations are located directly at key GPCR dimeric interfaces (see next paragraph). Despite this, if GPCR mutants that are limited in their ability to dimerise are ER-retained they may be useful in understanding some aspects of the role of dimerisation in GPCR assembly.

Canals *et al.* [36••] took advantage of the disrupted oligomeric organisation of an α_{1B}-adrenoceptor that contains pairs of point mutations in transmembrane domains (TMDs) I and IV. This mutant was ER-retained when expressed either transiently [37] or stably [36••] in HEK293 cells and prevented cell surface delivery of co-expressed wild type α_{1B}-adrenoceptor [36••,37]. Although mutated at four positions the modified α_{1B}-adrenoceptor clearly retained core structure because it was able to bind α_1-adrenoceptor ligands, such as [^{3}H]prazosin, with affinity akin to the wild type receptor [36••,37] and, although ER-retained, did retain some capacity to generate oligomeric contacts because expression of pairs of this mutant that were C-terminally tagged with bimolecular fluorescence complementation-competent fragments of YFP [38], resulted in the generation of an ER-restricted fluorescent signal [37]. However, sequential three-colour FRET measurements indicated that the detailed organisational quaternary structure of the mutant was different from wild type α_{1B}-adrenoceptor [37]. The TMD mutant α_{1B}-adrenoceptor was transported to the surface in cells that were exposed to prazosin and other ligands with affinity for the α_{1B}-adrenoceptor [36••,37] and in the presence of such a 'pharmacological chaperone' the quaternary structure of the TMD mutant α_{1B}-adrenoceptor, monitored by three-colour FRET, was restored to something akin to that of wild type α_{1B}-adrenoceptor [37]. Interestingly, prazosin is not a highly cell-permeant ligand and the EC_{50} to promote cell surface delivery of the TMD mutant α_{1B}-adrenoceptor was some 50–100 fold higher than the K_d for binding in broken cell preparations [36••,37]. This was interpreted as indicating the poor access of prazosin to the ER-retained receptor as the ligand would be required to equilibrate across not only the plasma membrane but also the ER membrane. The studies indicated that binding of an appropriate ligand was able to either assist folding and/or engender a conformational alteration that enhanced dimerisation. To assess if the pharmacological chaperone caused cell

surface delivery of a quaternary homomeric complex rather than simply monomers of the α_{1B}-adrenoceptor, Canals *et al.* [36••] also generated a mutant α_{1B}-adrenoceptor that was unable to bind prazosin. This mutant Asp^{125}Ala α_{1B}-adrenoceptor, as for the wild type receptor, was both successfully delivered to the cell surface when expressed in isolation in HEK293 cells and became ER-trapped when co-expressed with the TMD mutant α_{1B}-adrenoceptor [36••]. Importantly, addition of prazosin to cells co-expressing Asp^{125}Ala α_{1B}-adrenoceptor and the TMD mutant α_{1B}-adrenoceptor resulted in their co-delivery to the cell surface [36••]. As a further control, addition of the Asp^{125}Ala mutation into the TMD mutant α_{1B}-adrenoceptor generated a form of the receptor that was retained in the ER when expressed but which could not be recovered and trafficked to the cell surface by prazosin or other ligands with α_{1B}-adrenoceptor affinity, demonstrating that interaction of the pharmacological chaperone at the orthosteric binding site was required. As the Asp^{125}Ala α_{1B}-adrenoceptor cannot bind ligands, the results of the co-expression studies indicated that it must have been carried to the cell surface in a homomeric complex with the TMD mutant α_{1B}-adrenoceptor [36••]. Previous studies had shown that the α_{1B}-adrenoceptor exists as an oligomer rather than a strict dimer [37]. However, these trafficking studies were not appropriate to usefully define whether the overall size of the complex changed in response to binding of the antagonist chaperone ligand, as has recently been suggested for the muscarinic M1 acetylcholine receptor upon binding of the antagonist pirenzepine [39].

In support of a model in which receptor homomers are pre-assembled in the ER and trafficked subsequently to the cell surface, Kobayashi *et al.* [40••] noted that mutation of residue Val 179 of the β_1-adrenoceptor (position 4.46 in the Ballesteros and Weinstein nomenclature, in which the most conserved amino acid in each transmembrane domain across the class A GPCR family is designated X.50 and other amino acids are then related to this position based on the primary amino acid sequence. Hence in TMD 4 of the β_1-adrenoceptor the Trp residue at position 183 in the primary sequence is residue 4.50 and residue 179 is four amino acids earlier in the primary sequence) or of Trp 183 (position 4.50) resulted in intracellular retention of the modified receptor. In both cases, cell surface delivery was enhanced by the treatment of cells with antagonists, such as alprenolol, that bind the β_1-adrenoceptor. The Val 179 and Trp 183 mutants also displayed increased $BRET_{50}$ values in 'saturation' BRET [10] studies designed to explore the avidity of protein–protein interactions, consistent with a reduced interaction affinity and propensity to dimerise. The $BRET_{50}$ value for Trp^{183} Alaβ_1-adrenoceptor was reduced to close to the value observed for the wild type β_1-adrenoceptor following treatment of cells with alprenolol, but whilst the EC_{50} for alprenolol was considerably greater than the anticipated K_d at the wild type β_1-adrenoceptor, direct estimates of the affinity of ligands for the mutated receptor were not reported. Despite this, these studies are entirely consistent with a model in which correct interactions to generate an appropriately folded receptor homomer are required for the receptor to be allowed to traffic to the cell surface. In a rather different, but conceptually related approach, Kong *et al.* [41] demonstrated that although when expressed individually, both the wild type dopamine D1 receptor and an Asp^{103} Ala mutant that is anticipated to be unable to bind catecholamine ligands, were delivered to the cell surface, their co-expression resulted in intracellular trapping of both. The asymmetric homomer so produced could be recovered to the cell surface only with the use of cell-permeant agonists and not antagonists [41], suggesting that a conformational change associated with receptor activation was required to generate trafficking-competent quaternary structure. Few studies have attempted to define whether the overall molecular organisation of GPCRs alters during trafficking from ER/Golgi to the cell surface but Vidi *et al.* [42] have used combinations of bi-molecular fluorescence complementation and FRET to explore this issue for the adenosine A2a receptor, concluding that dimers may be trafficked from the ER to the plasma membrane with subsequent higher order oligomerisation occurring at the plasma membrane. These are challenging experiments, not least because the essentially irreversible formation of the bi-molecular fluorescence complementation signal defines that interactions identified in this way are likely to remain at least in the dimeric state, and further work is required to explore this topic.

It is of considerable interest that in the studies of both Canals *et al.* [36••] and Kobayashi *et al.* [40••] mutation of amino acids within TMD IV resulted in ER-trapping, and inefficient, but not elimination of, homomerisation. Although other regions of GPCRs have been implicated as being dimer interfaces (see [4] for review), many recent studies have indicated elements of TMD IV to be important for homodimeric interactions [37,43,44]. It is further of interest that in the β_1-adrenoceptor one of these amino acids is the Trp residue that is the most highly conserved residue in TMD IV and, therefore, must play an important role because it is so highly conserved in class A GPCRs. However, at least in the adenosine A1 receptor, mutation of this residue to Ala does not alter homomer formation or cellular trafficking [45]. As such, a general role for this residue in dimerisation remains unproven and probably unlikely. Moreover, in a number of X-ray structures of GPCRs a molecule of cholesterol is bound at this location (see [46] for review) and the implications of this for GPCR quaternary structure are unclear. Furthermore, it is even more difficult to extrapolate predictions to GPCR heteromers. For example, although TMD IV mutations modulated homodimerisation of the β_1-adrenoceptor

they did not alter interactions between the β_1-adrenoceptor and the β_2-adrenoceptor [40••].

Because single (and even the combination of multiple) point mutants have failed to result in the generation of GPCRs that are obvious monomers, other approaches have been used to try to interfere with GPCR dimerisation and hence to define its relevance. For example, addition of peptides that correspond to individual TMDs of GPCRs have been used both in intact cell and membrane-based studies. For the chemokine CXCR4 receptor a synthetic peptide corresponding to TMD IV reduced oligomerisation as reported by a reduction of FRET signals in cells co-expressing CFP-tagged and YFP-tagged forms of the receptor. It also inhibited ligand-induced actin polymerisation, and blocked chemotaxis of malignant cells [47]. These results have been interpreted as evidence for specific functional roles of CXCR4 dimers and build on other studies that have attempted to disrupt chemokine receptor homomers and heteromers (see [47,48] for details).

Although many fewer studies have examined homomer and heteromer formation for the class B receptor family, there is good evidence for such interactions (see [49] for review). Although there is no sequence similarity between class B and class A GPCRs, at least for the class B secretin receptor, TMD IV again appears to be a key element in allowing production of the homomer [50], but unlike many class A receptors, to date there is no evidence to support the formation of higher order multimers [51]. It remains to be established if this will be the pattern for other class B receptors and evidence suggests that this may vary between different class C GPCRs. Here the $GABA_B$ receptor can exist as at least a tetramer, whilst for metabotropic glutamate receptors only strict dimers have been detected [7]. Interestingly, by using a peptide corresponding to TMD IV of the secretin receptor Gao *et al.* [52••] were able to apparently monomerise this receptor and this was associated with the lack of high affinity, guanine nucleotide-sensitive binding of agonist ligand. These observations are at least consistent with the secretin receptor dimer being required for G-protein binding [52••], whilst monomeric class A GPCRs are sufficient to produce such pharmacology and function [1•,2,3]. A further element that may be different between class B and class A receptors is that following secretin receptor dimer disruption produced by the TMD IV peptide, the apparently monomeric receptor and various TMD IV point mutants were still able to traffic to the surface of cells [52••].

Do GPCRs internalise as dimers?

Once at the cell surface many GPCRs become internalised via endocytosis, either spontaneously or in response to the binding of agonist ligands. This topic has been reviewed extensively and is beyond the scope and capacity of this article. However, the physical organisation of internalised GPCRs, and if their oligomeric status is altered during this process, has been the subject of considerable debate. The capacity of a selective ligand of one GPCR to cause internalisation of both its cognate GPCR and a second co-expressed GPCR has been used as evidence to favour the presence and internalisation of intact GPCR heteromers (see [4] for review). This is more challenging to approach, however, for GPCR homomers. A developing strategy in this area is to generate asymmetric homomers following co-expression of a wild type GPCR and a variant of the GPCR that is unable to bind and respond to the same ligand(s) as the wild type receptor. For GPCRs with catecholamine ligands this can be achieved simply by alteration of the Asp residue at position 3.32 that is required to provide high affinity interaction with the amine headgroup of the ligand. Furthermore, in a limited number of cases ligands able to act as agonists at such mutated receptors but not at the corresponding wild type receptor have been synthesised. In the case of the β_2-adrenoceptor, regulated co-expression of a wild type and an Asp3.32Ser mutant resulted in co-internalisation of both forms of the receptor in response to isoprenaline, despite the mutant having no significant affinity for this ligand and not being internalised upon addition of isoprenaline when expressed in the absence of wild type β_2-adrenoceptor [53•]. Furthermore, a synthetic ligand that is a full agonist at the mutant receptor but has little affinity for the wild type also caused co-internalisation of both forms of the receptor [53•]. This basic approach requires to be extended to other GPCRs to test the generality of these observations and the expanding range of GPCRs for which variants that are activated solely by synthetic ligands are available offers a means to do so.

These range of approaches utilised in the studies discussed in this review are starting to unravel the basis and importance of GPCR–GPCR interactions in receptor synthesis and cell surface trafficking. As with other aspects of the importance of GPCR dimerisation, key studies need to be translated to physiologically relevant cells and tissues before clear understanding will be achieved.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. • Whorton MR, Bokoch MP, Rasmussen SG, Huang B, Zare RN, Kobilka B, Sunahara RK: **A monomeric G protein-coupled**

receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proc Natl Acad Sci U S A* 2007, **104**:7682-7687.
First demonstration that a purified, defined monomeric GPCR is able to activate provided G protein.

2. Kuszak AJ, Pitchiaya S, Anand JP, Mosberg HI, Walter NG, Sunahara RK: **Purification and functional reconstitution of monomeric mu-opioid receptors: allosteric modulation of agonist binding by Gi2**. *J Biol Chem* 2009. June 19 [Epub ahead of print] PMID: 19542234.

3. Chabre M, Deterre P, Antonny B: **The apparent cooperativity of some GPCRs does not necessarily imply dimerization**. *Trends Pharmacol Sci* 2009, **30**:182-187.

4. Milligan G: **G protein-coupled receptor dimerisation: molecular basis and relevance to function**. *Biochim Biophys Acta* 2007, **1768**:825-835.

5. Milligan G: **A day in the life of a G protein-coupled receptor: the contribution to function of G protein-coupled receptor dimerization**. *Br J Pharmacol* 2008, **153(Suppl 1)**:S216-S229.

6. Pin JP, Kniazeff J, Liu J, Binet V, Goudet C, Rondard P, Prézeau L: **Allosteric functioning of dimeric class C G-protein-coupled receptors**. *FEBS J* 2005, **272**:2947-2955.

7. •• Maurel D, Comps-Agrar L, Brock C, Rives ML, Bourrier E, Ayoub MA, Bazin H, Tinel N, Durroux T, Prézeau L, Trinquet E, Pin JP: **Cell-surface protein–protein interaction analysis with time-resolved FRET and snap-tag technologies: application to GPCR oligomerization**. *Nat Methods* 2008, **5**:561-567.
Novel approach, based on a combination of time-resolved FRET and CLIP/SNAP tagging, able to define the organisational structure of GPCR dimers and oligomers at the surface of intact cells.

8. Salahpour A, Angers S, Mercier JF, Lagacé M, Marullo S, Bouvier M: **Homodimerization of the beta2-adrenergic receptor as a prerequisite for cell surface targeting**. *J Biol Chem* 2004, **279**:33390-33397.

9. Wilson S, Wilkinson G, Milligan G: **The CXCR1 and CXCR2 receptors form constitutive homo- and heterodimers selectively and with equal apparent affinities**. *J Biol Chem* 2005, **280**:28663-28674.

10. Milligan G, Bouvier M: **Methods to monitor the quaternary structure of G protein-coupled receptors**. *FEBS J* 2005, **272**:2914-2925.

11. Zhou F, Filipeanu CM, Duvernay MT, Wu G: **Cell-surface targeting of alpha2-adrenergic receptors — inhibition by a transport deficient mutant through dimerization**. *Cell Signal* 2006, **18**:318-327.

12. Herrick-Davis K, Weaver BA, Grinde E, Mazurkiewicz JE: **Serotonin 5-HT2C receptor homodimer biogenesis in the endoplasmic reticulum: real-time visualization with confocal fluorescence resonance energy transfer**. *J Biol Chem* 2006, **281**:27109-27116.

13. Ramsay D, Carr IC, Pediani J, Lopez-Gimenez JF, Thurlow R, Fidock M, Milligan G: **High-affinity interactions between human alpha1A-adrenoceptor C-terminal splice variants produce homo- and heterodimers but do not generate the alpha1L-adrenoceptor**. *Mol Pharmacol* 2004, **66**:228-239.

14. Guan R, Feng X, Wu X, Zhang M, Zhang X, Hébert TE, Segaloff DL: **Bioluminescence resonance energy transfer studies reveal constitutive dimerization of the human lutropin receptor and a lack of correlation between receptor activation and the propensity for dimerization**. *J Biol Chem* 2009, **284**:7483-7494.

15. Free RB, Hazelwood LA, Cabrera DM, Spalding HN, Namkung Y, Rankin ML, Sibley DR: **D1 and D2 dopamine receptor expression is regulated by direct interaction with the chaperone protein calnexin**. *J Biol Chem* 2007, **282**:21285-21300.

16. Tarja T, Leskelä, Piia MH, Markkanen E, Pietilä M, Jussi T, Tuusa, Petäjä-Repo UE: **Opioid receptor pharmacological chaperones act by binding and stabilizing newly synthesized receptors in the endoplasmic reticulum**. *J Biol Chem* 2007, **282**:23171-23183.

17. Dong C, Filipeanu CM, Duvernay MT, Wu G: **Regulation of G protein-coupled receptor export trafficking**. *Biochim Biophys Acta* 2007, **1768**:853-870.

18. Duvernay MT, Dong C, Zhang X, Zhou F, Nichols CD, Wu G: **Anterograde trafficking of G protein-coupled receptors: function of the C-terminal F(X)6LL motif in export from the endoplasmic reticulum**. *Mol Pharmacol* 2009, **75**:751-761.

19. •• Duvernay MT, Dong C, Zhang X, Robitaille M, Hébert TE, Wu G: **A single conserved leucine residue on the first intracellular loop regulates ER export of G protein-coupled receptors**. *Traffic* 2009. February 8 [Epub ahead of print] PMID: 19220814.
Identification of a single Leu residue in the first intracellular loop and conserved in many class A GPCR that might provide a generic means to prevent ER to Golgi transport of GPCRs.

20. Achour L, Labbé-Jullié C, Scott MG, Marullo S: **An escort for GPCRs: implications for regulation of receptor density at the cell surface**. *Trends Pharmacol Sci* 2008, **29**:528-535.

21. Greger IH, Ziff EB, Penn AC: **Molecular determinants of AMPA receptor subunit assembly**. *Trends Neurosci* 2007, **30**:407-416.

22. Robitaille M, Ramakrishnan N, Baragli A, Hébert TE: **Intracellular trafficking and assembly of specific Kir3 channel/G protein complexes**. *Cell Signal* 2009, **21**:488-501.

23. Dupré DJ, Robitaille M, Ethier N, Villeneuve LR, Mamarbachi AM, Hébert TE: **Seven transmembrane receptor core signaling complexes are assembled prior to plasma membrane trafficking**. *J Biol Chem* 2006, **281**:34561-34573.

24. Hansen JL, Hansen JT, Speerschneider T, Lyngsø C, Erikstrup N, Burstein ES, Weiner DM, Walther T, Makita N, Iiri T, Merten N, Kostenis E, Sheikh SP: **Lack of evidence for AT1R/B2R heterodimerization in COS-7, HEK293, and NIH3T3 cells: how common is the AT1R/B2R heterodimer?** *J Biol Chem* 2009, **284**:1831-18319.

25. • Abd Alla J, Reeck K, Langer A, Streichert T, Quitterer U: **Calreticulin enhances B2 bradykinin receptor maturation and heterodimerization**. *Biochem Biophys Res Commun* 2009, **387**:186-190.
A suggested explanation for literature reports for examples of GPCR heterodimerisation that have been difficult for other groups to replicate.

26. Décaillot FM, Rozenfeld R, Gupta A, Devi LA: **Cell surface targeting of mu-delta opioid receptor heterodimers by RTP4**. *Proc Natl Acad Sci U S A* 2008, **105**:16045-16050.

27. Dunham JH, Hall RA: **Enhancement of the surface expression of G protein-coupled receptors**. *Trends Biotechnol* 2009, **27**:541-545.

28. Minneman KP: **Heterodimerization and surface localization of G protein coupled receptors**. *Biochem Pharmacol* 2007, **73**:1043-1050.

29. Uberti MA, Hague C, Oller H, Minneman KP, Hall RA: **Heterodimerization with beta2-adrenergic receptors promotes surface expression and functional activity of alpha1D-adrenergic receptors**. *J Pharmacol Exp Ther* 2005, **313**:16-23.

30. Bush CF, Jones SV, Lyle AN, Minneman KP, Ressler KJ, Hall RA: **Specificity of olfactory receptor interactions with other G protein-coupled receptors**. *J Biol Chem* 2007, **282**:19042-19051.

31. Ulloa-Aguirre A, Conn PM: **Targeting of G protein-coupled receptors to the plasma membrane in health and disease**. *Front Biosci* 2009, **14**:973-994.

32. Conn PM, Janovick JA: **Drug development and the cellular quality control system**. *Trends Pharmacol Sci* 2009, **30**:228-233.

33. • Robben JH, Kortenoeven MLA, Sze M, Yae C, Milligan G, Oorchot VM, Klumperman J, Knoers NVAM, Deen PMT: **Intracellular activation of vasopressin V2 receptor mutants in nephrogenic diabetes insipidus by non-peptide agonists**. *Proc Natl Acad Sci USA* 2009, **106**:12195-12200.
Evidence to support the concept that receptors are linked to G proteins within the ER and before cell surface delivery and can generate second messengers at that location if ligands can cross membranes to interact with the complex.

34. Pidasheva S, Grant M, Canaff L, Ercan O, Kumar U, Hendy GN: **Calcium-sensing receptor dimerizes in the endoplasmic reticulum: biochemical and biophysical characterization of CASR mutants retained intracellularly**. *Hum Mol Genet* 2006, **15**:2200-2209.

35. Sánchez-Laorden BL, Sánchez-Más J, Martínez-Alonso E, Martínez-Menárguez JA, García-Borrón JC, Jiménez-Cervantes C: **Dimerization of the human melanocortin 1 receptor: functional consequences and dominant-negative effects**. *J Invest Dermatol* 2006, **126**:172-181.

36. •• Canals M, Lopez-Gimenez JF, Milligan G: **Cell surface delivery and structural re-organization by pharmacological chaperones of an oligomerization-defective alpha(1b)-adrenoceptor mutant demonstrates membrane targeting of GPCR oligomers**. *Biochem J* 2009, **417**:161-172.
Use of combinations of ER-retained and ligand-binding deficient mutants of a receptor to demonstrate that receptor traffic from the ER to the cell surface as a dimeric/oligomeric complex.

37. Lopez-Gimenez JF, Canals M, Pediani JD, Milligan G: **The alpha1b-adrenoceptor exists as a higher-order oligomer: effective oligomerization is required for receptor maturation, surface delivery, and function**. *Mol Pharmacol* 2007, **71**:1015-1029.

38. Vidi PA, Watts VJ: **Fluorescent and bioluminescent protein-fragment complementation assays in the study of G protein-coupled receptor oligomerization and signaling**. *Mol Pharmacol* 2009, **75**:733-739.

39. Ilien B, Glasser N, Clamme JP, Didier P, Piemont E, Chinnappan R, Daval SB, Galzi JL, Mely Y: **Pirenzepine promotes the dimerization of muscarinic M1 receptors through a three-step binding process**. *J Biol Chem* 2009, **284**:19533-19543.

40. •• Kobayashi H, Ogawa K, Yao R, Lichtarge O, Bouvier M: **Functional rescue of beta-adrenoceptor dimerization and trafficking by pharmacological chaperones**. *Traffic* 2009, **10**:1019-1033.
Identification of residues in TMD IV of the β1-adrenoceptor that modulate the effectiveness of dimerisation, that this results in ER trapping and that recovery of organisational structure following binding of a pharmacological chaperone promotes receptor trafficking.

41. Kong MM, Fan T, Varghese G, O'dowd BF, George SR: **Agonist-induced cell surface trafficking of an intracellularly sequestered D1 dopamine receptor homo-oligomer**. *Mol Pharmacol* 2006, **70**:78-89.

42. Vidi PA, Chen J, Irudayaraj JM, Watts VJ: **Adenosine A(2A) receptors assemble into higher-order oligomers at the plasma membrane**. *FEBS Lett* 2008, **582**:3985-3990.

43. Wang HX, Konopka JB: **Identification of amino acids at two dimer interface regions of the alpha-factor receptor (Ste2)**. *Biochemistry* 2009. July 9 [Epub ahead of print] PMID: 19588927.

44. Han Y, Moreira IS, Urizar E, Weinstein H, Javitch JA: **Allosteric communication between protomers of dopamine class A GPCR dimers modulates activation**. *Nat Chem Biol* 2009, **5**:688-695.

45. Suzuki T, Namba K, Yamagishi R, Kaneko H, Haga T, Nakata H: **A highly conserved tryptophan residue in the fourth transmembrane domain of the A adenosine receptor is essential for ligand binding but not receptor homodimerization**. *J Neurochem* 2009, **110**:1352-1362.

46. Paila YD, Tiwari S, Chattopadhyay A: **Are specific nonannular cholesterol binding sites present in G-protein coupled receptors?** *Biochim Biophys Acta* 2009, **1788**:295-302.

47. Wang J, He L, Combs CA, Roderiquez G, Norcross MA: **Dimerization of CXCR4 in living malignant cells: control of cell migration by a synthetic peptide that reduces homologous CXCR4 interactions**. *Mol Cancer Ther* 2006, **5**:2474-2483.

48. Appelbe S, Milligan G: **Chapter 10. Hetero-oligomerization of chemokine receptors**. *Methods Enzymol* 2009, **461**:207-225.

49. Harikumar KG, Morfis MM, Sexton PM, Miller LJ: **Pattern of intra-family hetero-oligomerization involving the G-protein-coupled secretin receptor**. *J Mol Neurosci* 2008, **36**:279-285.

50. Harikumar KG, Pinon DI, Miller LJ: **Transmembrane segment IV contributes a functionally important interface for oligomerization of the Class II G protein-coupled secretin receptor**. *J Biol Chem* 2007, **282**:30363-30372.

51. Harikumar KG, Happs RM, Miller LJ: **Dimerization in the absence of higher-order oligomerization of the G protein-coupled secretin receptor**. *Biochim Biophys Acta* 2008, **1778**:2555-2563.

52. •• Gao F, Harikumar KG, Dong M, Lam PC, Sexton PM, Christopoulos A, Bordner A, Abagyan R, Miller LJ: **Functional importance of a structurally distinct homodimeric complex of the family B G protein-coupled secretin receptor**. *Mol Pharmacol* 2009, **76**:264-274.
Using combinations of mutagenesis and a peptide corresponding to TMD IV the authors suggest means to limit production of dimers of class B receptors in cells and that a dimer of the secretin receptor may be required to interact effectively with G protein.

53. • Sartania N, Appelbe S, Pediani JD, Milligan G: **Agonist occupancy of a single monomeric element is sufficient to cause internalization of the dimeric beta2-adrenoceptor**. *Cell Signal* 2007, **19**:1928-1938.
Generation of 'asymmetric' homodimers of the β2-adrenoceptor in cells by regulated expression of a receptor activated solely by synthetic ligand in the face of constitutive expression of the wild type β2-adrenoceptor allowed demonstration that the β2-adrenoceptor is internalised as an dimer and that this required binding of agonist to only one of the protomers.