G-PROTEIN-COUPLED RECEPTOR OLIGOMERIZATION AND ITS POTENTIAL FOR DRUG DISCOVERY

Susan R. George‡§, Brian F. O'Dowd*§ and Samuel P. Lee**

G-protein-coupled receptors (GPCRs) represent by far the largest class of targets for modern drugs. Virtually all therapeutics that are directed towards GPCRs have been designed using assays that presume that these receptors are monomeric. The recent realization that these receptors form homo-oligomeric and hetero-oligomeric complexes has added a new dimension to rational drug design. However, this important aspect of GPCR biology remains largely unincorporated into schemes to search for new therapeutics. This review provides a synopsis of the current thinking surrounding GPCR homo-oligomerization and hetero-oligomerization and shows how new models point towards unexplored avenues in the development of new therapies.

POLYMORPHISM
The occurrence in a population of two or more variant alleles of a gene, for which the frequency of the rarer alleles is greater than can be explained by recurrent mutation alone.

RADIATION INACTIVATION
A technique in which proteins are inactivated with high-energy particles to determine the molecular mass of functional oligomers.

*Departments of *Pharmacology and ‡Medicine, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8. §Centre for Addiction and Mental Health, 250 College Street, Toronto, Ontario, Canada M5T 1A8. Correspondence to S.R.G. e-mail: s.george@utoronto.ca*
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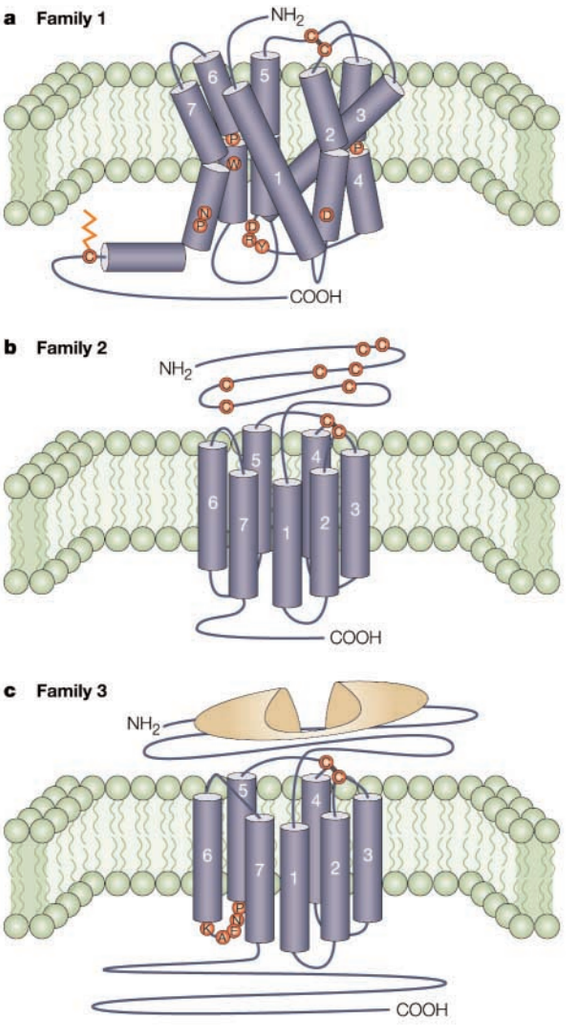
The G-protein-coupled receptors (GPCRs) are the largest class of cell-surface receptors and are encoded by >1,000 genes in the human genome[1]. GPCRs are activated by a diverse array of ligands, including hormones, peptides, amino acids, ions and photons of light, and transduce signals through a wide range of effectors. Not surprisingly, these receptors carry out a multitude of tasks in the central nervous system (CNS) and the periphery. Numerous diseases and disorders have been linked to mutations and POLYMORPHISMS in GPCRs[2,3], and they are the targets of an increasingly large number of therapeutic agents. It has been estimated that 50% of all modern drugs[1] and almost one-quarter of the top 200 best-selling drugs in 2000 modulate GPCR activity[4]. GPCRs can be grouped on the basis of sequence homology into several distinct families[5,6]. Although all GPCRs share a similar architecture of seven membrane-spanning α-helices, the GPCR families show no sequence homology to one another (BOX 1), indicating that they might be unrelated phylogenetically and that the similarity of their transmembrane (TM)-domain structure might be only to fulfil common functional requirements.

Traditionally, mechanisms of ligand binding and signal transduction by GPCRs were modelled on the assumption that monomeric receptors participate in the processes. Despite the fact that, for many other classes of receptors, such as the tyrosine-kinase receptors, constitutive or ligand-induced oligomerization has long been known to be essential for signalling[7], only a monomeric model for GPCRs was generally accepted. In retrospect, early studies using radioligand binding, cross-linking and RADIATION INACTIVATION (for a review, see REF. 8) had predicted homo-oligomeric receptors even before the structure of GPCRs became known, but the significance of these findings was not clear at the time. Nevertheless, since the mid-1990s, numerous reports have successively shown oligomerization of all the GPCRs for which it has been examined (TABLE 1), and it is now widely accepted that oligomerization is a universal aspect of GPCR biology.

The idea of receptor–receptor interactions has not only resulted in an important revision of the traditional models of GPCR structure and function, it has also created interesting new ones. Soon after the first reports of GPCR homo-oligomers, it was shown that some receptor subtypes formed hetero-oligomers (TABLE 2), and that these 'heteromeric' receptors had functional characteristics that differed from homogeneous populations

Box 1 | **Classification of G-protein-coupled receptors**

G-protein-coupled receptors (GPCRs) can be divided phylogentically into six families (see the GPCR database online). Schematic representations of receptor monomers showing some key structural aspects of the three main families are shown. Family 1 (panel **a**; also referred to as family A or the rhodopsin-like family) is by far the largest subgroup and contains receptors for odorants, small molecules such as the catecholamines and amines, some peptides and glycoprotein hormones. Receptors of family 1 are characterized by several highly conserved amino acids (some of which are indicated in the diagram by red circles) and a disulphide bridge that connects the first and second extracellular loops (ECLs). Most of these receptors also have a PALMITOYLATED cysteine in the carboxy-terminal tail. The recent determination of the crystal structure of rhodopsin has indicated that the transmembrane (TM) domains of family 1 receptors are 'tilted' and 'kinked' as shown. Family 2 or family B GPCRs (panel **b**) are characterized by a relatively long amino terminus that contains several cysteines, which presumably form a network of disulphide bridges. Their morphology is similar to some family 1 receptors, but they do not share any sequence homology. For example, the family 2 receptors also contain a disulphide bridge that connects ECL1 and ECL2, but the palmitoylation site is missing, the conserved prolines are different from the conserved prolines in the family 1 receptors and the DRY (aspartic acid, arginine, tyrosine) motif adjacent to TM3 is absent. Little is known about the orientation of the TM domains, but — given the divergence in amino-acid sequence — it is probably quite dissimilar from that of rhodopsin. Ligands for family 2 GPCRs include hormones, such as glucagon, gonadotropin-releasing hormone and parathyroid hormone. Family 3 (panel **c**) contains the metabotropic glutamate, the Ca^{2+}-sensing and the γ-aminobutyric acid $(GABA)_B$ receptors. These receptors are characterized by a long amino terminus and carboxyl tail. The ligand-binding domain is located in the amino terminus, which is often described as being like a 'Venus fly trap'. Except for two cysteines in ECL1 and ECL2 that form a putative disulphide bridge, the family 3 receptors do not have any of the key features that characterize family 1 and 2 receptors. A unique characteristic of the family 3 receptors is that the third intracellular loop is short and highly conserved. Although the structure of the amino terminus is well characterized, similar to the family 2 receptors, little is known about the orientation of the TM domains.



of their constituent receptors. The generation of new properties through hetero-oligomerization indicated a possible mechanism for generating diversity of function among GPCRs that had not previously been anticipated.

Although GPCRs are the targets of numerous therapeutics, and it has been established that these receptors are oligomers, the link has not yet been made between oligomeric receptors and drug discovery. For example, the immense effort that continues in the search for new drugs has focused on identifying new GPCR targets by 'de-orphanizing' ORPHAN GPCRs[9]; however, the possibility that a substantial number of 'novel' drug targets could be generated from hetero-oligomerization of two known receptors has been neglected. In this review, an overview of what is known about the functional importance and structure of GPCR oligomers is presented, along with examples of how these concepts could be used in the drug discovery process.

PALMITOYLATION
A post-translationlational modification in which palmitic acid, a fatty carbon chain, is attached to a cysteine residue by a thio-ester bond.

ORPHAN RECEPTOR
A receptor for which no endogenous ligand has been identified.

COOPERATIVITY
A property of receptors that have interacting binding sites, for which the binding of a ligand to one site modulates the binding of a second ligand to another site.

How big is a GPCR oligomer?

Many studies of GPCR oligomerization do not make a clear distinction between dimers and larger receptor complexes, in part because it is easier to conceptualize a dimer, which is the smallest and therefore least complex oligomer. The term dimer is frequently used interchangeably with the terms oligomer and multimer. However, there are no conclusive data at present to indicate how large the oligomers of functional GPCRs are. In fact, it is not clear if there is one particular oligomeric state that all GPCRs attain. Tetramers of GPCRs have been reported in immunoblot analyses[10–12], and mathematical analyses of COOPERATIVITY in ligand binding indicate the existence of oligomers that are more complex than dimers and as large as octamers[13]. An intermolecular disulphide bond has been shown to link the amino termini of at least some receptors in family 3 (REFS 14–16), indicating that this family of receptors exists as dimers. However, as there seem to be several sites of intermolecular interaction,

higher-order oligomers might be possible, and dimeric receptors might simply represent the 'building blocks' of these oligomers.

In the light of numerous reports of GPCR oligomers, a question that has not been well addressed is whether or not these receptors exist functionally as monomers. Studies using the D_2 dopamine receptor indicate that GPCRs are only oligomeric, and that the receptor monomers and dimers that are observed after gel electrophoresis might be the result of dissociation from a larger array of receptors[12]. CO-IMMUNOPRECIPITATION studies using DIFFERENTIALLY TAGGED β_2-adrenoceptors[17] resulted in the detection of only heterodimers; that is no receptor monomers as the co-precipitate, indicating that gel electrophoresis does not disrupt receptor dimers. However, co-immunoprecipitation of differentially tagged receptors in other studies resulted in the detection of monomers, homodimers and, in some cases, higher-order homo-oligomers as the co-precipitate[10,18–24]. These results indicate that monomers observed after SDS–PAGE might have been associated as part of a larger oligomeric complex. It has been shown previously that oligomeric membrane proteins can be dissociated when solubilized in detergents such as SDS[25]. Therefore, how large GPCR oligomers actually are is unclear at present.

Homo-oligomerization and GPCR function

For other receptors, such as the tyrosine-kinase and the steroid-hormone receptors, the functional importance of oligomerization is much better defined[7,26] than it is for GPCRs. However, some potential roles of homo-oligomerization in receptor function have been identified in several GPCRs.

Cooperativity and signal amplification. Although the model that the ligand, receptor and G protein operate with a 1:1:1 stoichiometry is accepted by many as being too simplistic, it is still frequently used to describe GPCR signal transduction. However, this model is slowly undergoing revision for several reasons. In addition to the realization that GPCRs form oligomers, it has been postulated that G proteins also form complexes[27,28]. Furthermore, there have been many demonstrations of cooperativity between GPCR-binding sites[29–40]. The functional significance of this drug-concentration-dependent effect on binding kinetics is not well understood, but negative cooperativity might have a role in receptor DESENSITIZATION, as binding of the ligand to the receptor results in the accelerated dissociation of ligand in interacting binding sites[39]. Cooperativity in GPCR oligomers also allows multiple populations of binding sites in a single population of receptors, potentially creating an increased gradient of receptor-mediated signalling that is dependent on ligand concentration and receptor occupancy. Finally, understanding the stoichiometry of ligand, receptor and G protein has been complicated by observations that indicate that the binding of a single agonist to a single receptor might activate neighbouring receptors with which the agonist-bound receptor is oligomerized. In traditional models of GPCR signal transduction, signal amplification is generally thought to occur only at the level of the G protein or the effector and not at the receptor level. However, oligomerization could provide a means of signal amplification through the activation of many receptors by a single ligand (BOX 2).

Oligomerization in cellular transport of receptors. Oligomerization of many membrane proteins has been shown to be important for intracellular transport and

Table 1 | **GPCRs that form homo-oligomers**

Receptor	References
Family 1 receptors	
A_1 adenosine	96
β_2-adrenoceptor	17,71,73
AT_1 angiotensin II	40
B_2 bradykinin	67
CCR2 chemokine	24,89
CCR5 chemokine	24,97
CXCR4 chemokine	98
D_1 dopamine	11,99
D_2 dopamine	12,38,62
D_3 dopamine	10
H_2 histamine	100
H_4 histamine	101
Luteinizing hormone/hCG	102
MT_1 melatonin	103
MT_2 melatonin	103
M_2 muscarinic acetylcholine	36
M_3 muscarinic acetylcholine	60
μ-opioid	22
δ-opioid	18,71
κ-opioid	21
5-HT_{1B} serotonin	104
5-HT_{1D} serotonin	61
$SSTR_{1A}$ somatostatin	56
$SSTR_{1B}$ somatostatin	20
$SSTR_{1C}$ somatostatin	56
$SSTR_{2A}$ somatostatin	20
Thyrotropin	37
V_2 vasopressin	23
Family 2 receptors	
IgG hepta	105
Gonadotropin-releasing hormone	37
Family 3 receptors	
Metabotropic $mGlu_1$	16
Metabotropic $mGlu_5$	14,106
Ca^{2+}-sensing	15
$GABA_{B(1)}$	19
$GABA_{B(2)}$	19
Family 4 receptor	
Yeast α-factor receptor	107,108

GABA, γ-aminobutyric acid; GPCR, G-protein-coupled receptor.

CO-IMMUNOPRECIPITATION
A process that uses antibodies to isolate a protein that interacts with the protein of interest.

DIFFERENTIALLY TAGGED
Having two unlike epitope tags.

DESENSITIZATION
The mechanism by which a ligand becomes less effective at activating a receptor during prolonged application.

sorting[41]. Incorrectly oligomerized proteins can be retained and degraded in the cell, and many chaperone proteins are involved in ensuring that newly synthesized proteins assume the correct orientations and oligomerization before exiting the endoplasmic reticulum (ER)[42]. Oligomerization seems to be an early event during GPCR transport, as shown by the observation that the intracellular retention of receptor complexes occurs when truncated mutants of GPCRs are co-expressed with the wild-type receptor. Inhibition of cell-surface expression by receptor fragments has been shown for D_2 (REF. 12) and D_3 receptors[43], CCR5 chemokine receptors[44] and V_2 vasopressin receptors[45].

Numerous mutant GPCRs with diminished or no function have been described that are naturally occurring or the product of gene manipulation. It has largely been assumed that receptor mutants do not affect the function of the wild-type receptor. However, the occurrence of GPCR homo-oligomerization suggests the possibility that modulation of receptor function by intermolecular interactions can occur, indicating a potential physiological role for truncated receptor species that are generated by ALTERNATIVE SPLICING or mutation.

It has been shown that a prevalent truncation mutant of the human CCR5 receptor can inhibit CCR5-receptor-mediated human immunodeficiency virus (HIV) infection in individuals who are heterozygous for the mutant GPCR by forming heterodimers with the wild-type receptor and thereby preventing its transport[44]. Antagonism of the V_2 receptor by receptor fragments using a similar mechanism has been shown[45], and is a potential cause of some cases of nephrogenic diabetes insipidus in people who are heterozygous for the mutated V_2 receptor. Oligomerization of GPCRs with variant forms not only has been characterized as a potential disease mechanism, but also might have evolved as a cellular 'self-regulatory' mechanism. For example, a truncated splice variant of the D_3 receptor — D3nf — might attenuate D_3 receptor expression and binding[43,46]. Furthermore, a naturally occurring variant of the EP_1 prostanoid receptor suppressed wild-type EP_1 receptor signalling[47], and similar phenomena with potential clinical relevance have also been shown for the luteinizing-hormone[48] and gonadotropin-releasing-hormone receptors[49].

Functional role of GPCR hetero-oligomerization. GPCRs obviously interact and associate with a large number and wide range of proteins, including other types of receptor, ion channel and chaperone protein. A discussion of all the protein–protein interactions that involve GPCRs is beyond the scope of this review, and here, therefore, hetero-oligomerization refers strictly to interactions between different GPCRs. One of the most significant observations to indicate that GPCR dimerization might be important in receptor folding and transport to the cell surface came from studies of the metabotropic γ-aminobutyric acid $(GABA)_B$ receptor. Co-expression of two isoforms of the $GABA_B$ receptor, $GABA_{B(1)}$ and $GABA_{B(2)}$, was a prerequisite for the formation of a functional $GABA_B$ receptor at the cell surface[50–52]. Detailed analysis of this phenomenon showed that, when expressed alone, the $GABA_{B(1)}$ isoforms are retained intracellularly as immature glycoproteins[53]. By contrast, $GABA_{B(2)}$ is transported to the cell surface, even if expressed alone, but cannot bind GABA or promote intracellular signalling[52]. The idea has emerged from subsequent studies[54] that $GABA_{B(2)}$ serves as a chaperone that is essential for the proper folding and cell-surface transport of $GABA_{B(1)}$. $GABA_{B(1)}$–$GABA_{B(2)}$ dimerization, through a coiled– coil interaction of the carboxyl tails, masks an ER-retention signal, thereby allowing ER export and plasma-membrane targeting of the dimer.

Table 2 | **GPCRs that form hetero-oligomers**

Receptor	References
5-HT_{1B}–5-HT_{1D} serotonin	109
A_1 adenosine–D_1 dopamine	90
A_1 adenosine–$mGlu_1$	110
A_1 adenosine–$P2Y_1$ purinergic	111
A_2 adenosine–D_2 dopamine	83
AT_1–AT_2 angiotensin	112
AT_1 angiotensin–B_2 bradykinin	40,57
CCR2–CCR5 chemokine	24
D_2–D_3 dopamine	113
$GABA_{B(1)}$–$GABA_{B(2)}$	51,52,114
M_2–M_3 muscarinic acetylcholine	115
MT_1–MT_2 melatonin	98
$SSTR_{2A}$–$SSTR_{1B}$ somatostatin	20
$SSTR_{1A}$ somatostatin–μ-opioid	88
$SSTR_{1A}$–$SSTR_{1C}$ somatostatin	56
$SSTR_{1B}$ somatostatin–D_2 dopamine	75
T1R1–T1R3 amino-acid taste	116
T1R2–T1R3 amino-acid taste	116,117
δ- and κ-opioid	21
μ- and δ-opioid	22
δ-opioid–$β_2$-adrenoceptor	84
κ-opioid–$β_2$-adrenoceptor	84

GABA, γ-aminobutyric acid; GPCR, G-protein-coupled receptor.

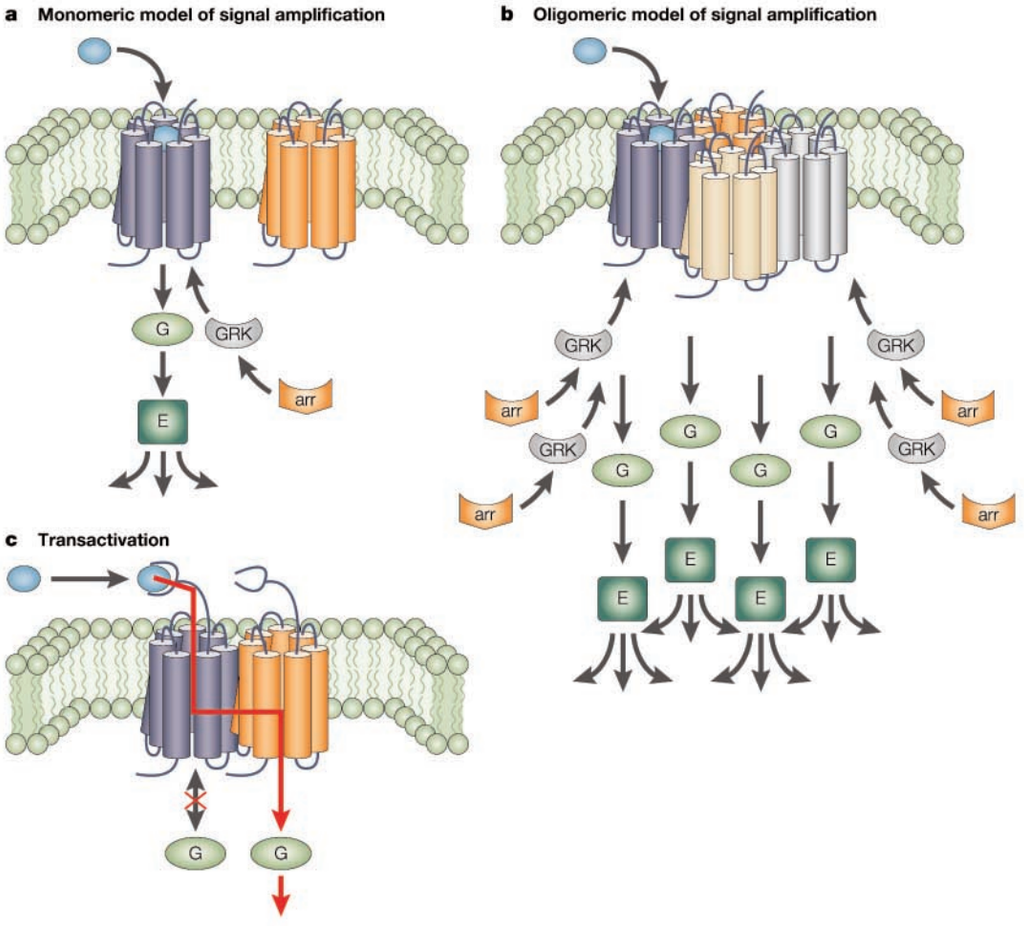
New properties from 'old' receptors. Arguably the most exciting aspect of GPCR oligomerization has been the observation that hetero-oligomerization can result in receptor complexes that have ligand-binding and signalling properties that are distinct from their constituent receptors. For example, it was shown that κ- and δ-opioid receptors formed a heteromeric complex, and that the heteromer showed no significant affinity for either κ- or δ-opioid receptor-selective agonists or antagonists, but showed high affinity for partially selective ligands[21]. Furthermore, these selective ligands were found to bind the heteromer synergistically when added simultaneously. Hetero-oligomers of μ- and δ-opioid receptors[22] also seemed to have distinct properties arising from hetero-oligomerization. Ligand binding in

ALTERNATIVE SPLICING
Different products can be generated from a single gene by, for example, combining alternative forms of particular exons.

cells that co-expressed these opioid receptors had an altered rank order of potency, and selective synthetic agonists had a reduced affinity, whereas certain endogenous opioids had an increased affinity. Most notably, the hetero-oligomeric μ–δ complex had altered G-protein coupling. In contrast to individually expressed receptors, co-expressed μ- and δ-opioid receptors are insensitive to both GTPγS (a non-hydrolysable analogue of GTP) and PERTUSSIS TOXIN (PTX), and PTX-insensitive adenylyl cyclase inhibition was observed with co-expressed μ- and δ-opioid receptors. One of the great enigmas of opioid receptor pharmacology has been the cloning of relatively few opioid receptor genes (three), despite the existence of many pharmacologically defined opioid receptor subtypes (μ1, μ2, δ1, δ2, κ1, κ2 and κ3)[55]. It now seems likely that hetero-oligomerization of opioid receptors (between themselves and possibly with other GPCRs) and not other receptor genes might account for some of the complexity in opioid receptor pharmacology.

Box 2 | **Transactivation of GPCRs: signal amplification at the receptor level**

Traditionally, in models of G-protein-coupled receptor (GPCR) function, it has been assumed that a single agonist activates a single receptor and that signal amplification occurs at the level of the G protein or effector (panel **a**). However, investigations of the homo- and hetero-oligomerization of several GPCRs have shown that ligand binding to one receptor might activate neighbouring receptors in the oligomeric complex (panel **b**). For example, in one study, two partially active mutants of the $SSTR_{1B}$ somatostatin receptor were co-expressed[20]. One mutant could not bind the somatostatin peptides SST-14 and SST-28, and the other mutant showed a complete loss of adenylyl cyclase coupling. On co-expression and treatment with SST-14 or SST-28, agonist-induced adenylyl cyclase coupling was observed, indicating that agonist binding to signalling-deficient mutants resulted in the activation of associated agonist-binding-deficient mutants. Similar results have been observed for GPCR heteromers, for which agonist binding to one receptor partner in the heteromer results in activation of the other receptor[20,58,75,88]. Interestingly, in the native γ-aminobutyric acid $(GABA)_{B(1)}$–$GABA_{B(2)}$ heteromer, it seems that the $GABA_{B(1)}$ subunit is not capable of G-protein coupling, and the $GABA_{B(2)}$ subunit cannot bind ligand, indicating that heteromerization is required not only for proper transport in the $GABA_B$ receptor, but also for signalling, which seems to occur only through transactivation (panel **c**)[58]. arr, arrestin; E, effector; G, G protein; GRK, GPCR kinase.



PERTUSSIS TOXIN
A toxin that ADP ribosylates the inhibitory G protein G_i, thereby causing it to uncouple from G-protein-coupled receptors.

In another example of hetero-oligomerization that resulted in an altered ligand-binding pocket, chemokine agonists triggered calcium responses from CCR2 and CCR5 receptor hetero-oligomers at concentrations 10- to 100-fold lower than the threshold for either chemokine receptor alone[24]. Notably, the CCR2–CCR5 heterodimer also seemed to signal through a G protein that was different to that coupled to homogeneous populations of CCR2 or CCR5 receptors. In this case, the consequence of heterodimer formation is not only a new pharmacology, but also an increase in the sensitivity and dynamic range of the chemokine response of leukocytes *in vivo*.

Masking receptors by hetero-oligomerization. Hetero-oligomerization has been used to rationalize the CROSS-TALK that has been observed between two receptor systems, and is usually associated with increasing diversity of receptor pharmacology and function. However, hetero-oligomerization might also mask the individual properties of one of the constituent receptors. Heterodimerization of $SSTR_{1A}$ and $SSTR_{1C}$ somatostatin receptors seems to result in a new target, but with a pharmacological and functional profile that resembles that of the $SSTR_{1A}$ receptor[56]. $SSTR_{1A}$–$SSTR_{1C}$ heterodimers respond only to $SSTR_{1A}$ receptor ligands and do not bind $SSTR_{1C}$-receptor-selective ligands. Interestingly, $SSTR_{1A}$- and $SSTR_{1C}$-receptor homodimers underwent agonist-induced endocytosis, but, by contrast, the $SSTR_{1A}$–$SSTR_{1C}$ heterodimer separated at the plasma membrane, and only $SSTR_{1A}$ underwent agonist-induced endocytosis. So, inactivation of $SSTR_{1C}$ receptor function by heterodimerization with the $SSTR_{1A}$ receptor might explain some of the difficulties in detecting $SSTR_{1C}$-specific binding and signalling in mammalian tissues. More importantly, these observations illustrate the potential pitfalls of characterizing a receptor outside its endogenous environment, and how it is possible for a drug to work well *in vitro*, but have no efficacy *in vivo*.

Altered heterodimerization leading to clinical disorder. Although angiotensin II is a vasoconstrictor and bradykinin is a vasodilator, the angiotensin AT_1 and bradykinin B_2 receptors have been shown to form heterodimers[40]. In cells that heterologously express AT_1 and B_2 receptors, increases in the efficacy and potency of angiotensin II, but attenuation of the ability of bradykinin to stimulate the production of inositol phosphate were observed. An interesting relationship has been identified between AT_1–B_2 receptor heterodimerization and PRE-ECLAMPSIA[57]. In pre-eclamptic hypertensive women, there was found to be a significant increase in the amount of AT_1–B_2 receptor heterodimerization and in B_2 receptor density. Furthermore, evidence indicated that hypertension in pre-eclampsia might be related to this increase in AT_1–B_2 receptor heterodimerization. Therefore, it is possible that altered levels of GPCR hetero-oligomerization might represent the molecular basis of some physiological disorders.

Signal amplification revisited. It has been shown that, in $GABA_B$ receptor heterodimers, agonist binding to $GABA_{B(1)}$ results in G-protein coupling/activation through the associated $GABA_{B(2)}$ receptor[58]. Interestingly, analysis of the CRYSTAL STRUCTURE of the dimeric amino-terminal domains of the $mGlu_1$ metabotropic glutamate receptor has shown that a 'closed–open' conformation of the ligand-binding regions is possible; that is, a conformation in which the ligand-binding domain of one receptor is occupied, whereas the ligand-binding domain of its partner is unoccupied[16].

$SSTR_{1A}$ receptor heterodimerization with the μ-opioid receptor has also been characterized. $SSTR_{1A}$–μ-opioid receptor heterodimerization did not substantially alter the ligand-binding or coupling properties of these receptors, although exposure of the $SSTR_{1A}$–μ-opioid receptor heterodimer to a $SSTR_{1A}$-selective ligand induced phosphorylation, internalization and desensitization of $SRIF_{1A}$, as well as the μ-opioid receptor[56]. Similarly, exposure of the heterodimer to a μ-opioid-receptor-selective ligand induced phosphorylation and desensitization of both partners. Heterodimerization might therefore represent a novel regulatory mechanism that could either restrict or enhance phosphorylation and desensitization of GPCRs.

Structural basis of GPCR homo-oligomerization

Although it is generally agreed that there are several mechanisms of intermolecular interaction in GPCR oligomerization, there is little consensus on precisely what these mechanisms are. Two specific types of intermolecular association have been identified in GPCR homo-oligomers: disulphide bonds and TM-domain interactions. However, no universal mechanism of oligomerization in GPCRs has been identified. One of the main difficulties associated with formulating such models has been the great diversity of receptor structure in the GPCR superfamily. Given the divergence in the different families of GPCRs, the idea that the mechanisms of oligomerization might be the same for all families of receptors is unlikely. For example, family 3 receptors have been more extensively studied in this regard than other GPCRs, in part owing to the crystallization of the extracellular ligand-binding region of $mGlu_1$ (REF. 16). In addition to other interactions, an intermolecular disulphide bond between the amino termini has been shown to be crucial for the dimerization of at least some family 3 receptors. However, the structure of the amino termini of the rhodopsin-like (family 1) receptors is very different from those of family 3, and the sites of intermolecular interaction are found elsewhere.

The determination of the crystal structure of rhodopsin[59] represented an exceptional advance in the understanding of rhodopsin-like GPCRs, but it provided little information about how these receptors form oligomers. In the rhodopsin crystal that was examined, the receptors were in a head-to-tail arrangement and the native quaternary structure was not analysed. Therefore, much of what is known about the oligomeric structure of family 1 GPCRs has been obtained through indirect biochemical means.

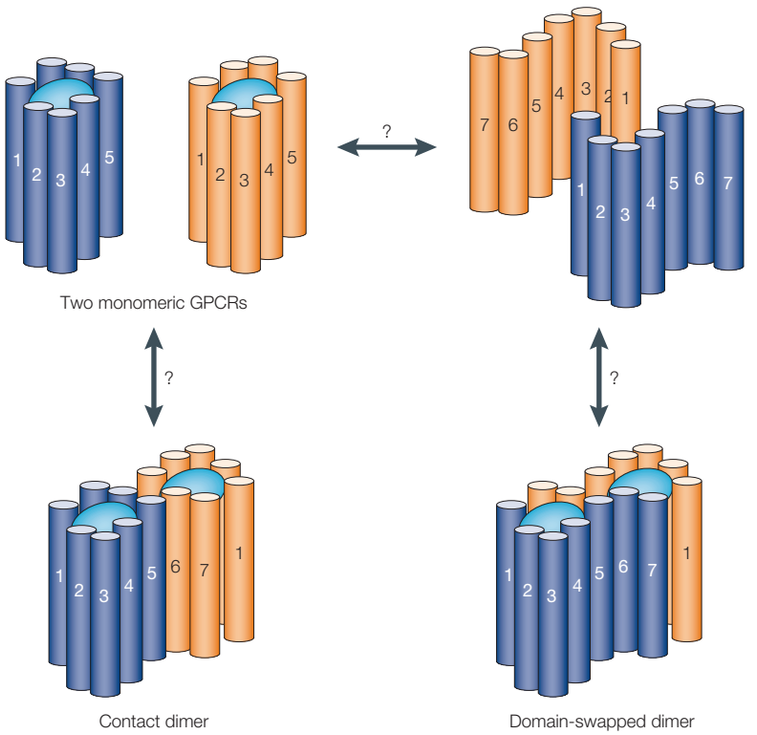
CROSS-TALK
An informal term that refers to the interaction or reciprocal modulation between two proteins.

PRE-ECLAMPSIA
A hypertensive disorder of pregnancy for which the cause is unknown.

CRYSTAL STRUCTURE
The three-dimensional arrangement of atoms in a protein that is determined by inducing the protein to form crystals.

Box 3 | **Domain-swapped dimers or contact dimers**

In the pursuit of determining how the transmembrane (TM) domains of family 1 G-protein-coupled receptors (GPCRs) interact to form oligomers, an interesting hypothesis has arisen. Computational modelling of adrenoceptors has indicated that GPCRs might participate in a process known as domain swapping[91]. Domain swapping has been shown to be a mechanism in the oligomerization of several other proteins and, if it occurred in GPCRs, it could provide a molecular model to explain the 'rescue-of-binding' experiments[92,93]. However, it might not be compatible with the accepted structure of rhodopsin-like receptors[59,94], in which the TM domains are arranged in a tightly packed hydrophobic bundle and are unlikely to undergo unfolding rearrangements. Furthermore, experiments using a photoaffinity label of the cholecystokinin receptor have shown that, although the cholecystokinin receptor might form oligomers, domain-swapped dimerization does not occur[95]. The photoaffinity label, a peptide agonist, had dual sites of covalent attachment, one on TM1 and the other on TM7, and it should have covalently linked two cholecystokinin receptors if dimerization was mediated by domain swapping. However, it was shown that the photoaffinity label bound to two regions of the same receptor. It is more likely that GPCR oligomers are 'contact' oligomers that are similar to those proposed by Schulz and co-workers[23], for which two binding pockets are formed from regions donated by both monomers instead of one binding site being formed by one 'subunit' receptor.



Disulphide bonds. Dissociation of receptor homo-oligomers by reducing agents has been shown for several rhodopsin-like GPCRs, including δ- and κ-opioid receptors[18,21], V_2 receptors [45], M_3 muscarinic-acetylcholine receptors[60], 5-hydroxytryptamine $(5\text{-HT})_{1B}$ and 5-HT_{1D} serotonin receptors[61] and D_1 receptors[61]. These observations indicate that a disulphide linkage is important in the formation of family 1 receptor oligomers, but it is not known whether the bond is intermolecular or intramolecular. Interestingly, the dissociation of homo-oligomers by a reducing agent is not detected in all receptors[61], and the complete dissociation by a reducing agent of all receptor dimers to receptor monomers is not always seen, even in receptors that are sensitive to this treatment. These observations indicate that other interactions might be involved in GPCR oligomerization in addition to disulphide bonds.

Transmembrane-domain interactions. In attempting to determine the sites of interaction of family 3 GPCRs, such as mGlu receptors, the Ca^{2+}-sensing receptor and $GABA_B$ receptors, the focus has primarily been on the amino and carboxyl termini, and potential hydrophobic interactions have not been investigated. However, in the family 1 receptors, intermolecular TM-domain interactions have received a great deal of attention in attempting to determine mechanisms of oligomerization, in part because the resistance of GPCR oligomers to dissociation by SDS[17,61,62] indicates a robust hydrophobic TM-domain interaction[63]. It is not clear precisely which TM domains interact with each other in family 1 GPCR oligomerization. Several theories have been proposed as to which TM domains are involved as sites of intermolecular contact, but there is no common theme (BOX 3). It is possible that different receptors might use different dimerization interfaces to associate, but it remains to be determined if the proposed mechanisms are simply variations of a common structural motif that applies to oligomerization in all rhodopsin-like GPCRs.

Intracellular- and extracellular-domain interactions. It has been established that dimerization of family 3 GPCRs is mediated, at least in part, by both covalent and non-covalent intermolecular interactions at the amino termini[16,64–66]. Nevertheless, little is known about the intermolecular interactions in the extracellular domains of the rhodopsin-like GPCR oligomers. The addition of synthetic peptides that correspond to the sequence of the amino terminus of the B_2 receptor has been reported to block B_2 receptor homodimer formation, indicating that it might be a site of interaction of rhodopsin-like GPCRs, but the mechanism that mediates this is not known[67]. Virtually nothing is known about potential intermolecular interactions between the intracellular domains of receptors that might mediate homo-oligomerization, although an interaction between the coiled-coil domains of the carboxyl termini of the $GABA_{B(1)}$ and $GABA_{B(2)}$ receptors is known to be a heterodimerization interface[68]. It has also been reported that the δ-opioid receptor is unable to form dimers when the terminal 15 amino acids are truncated, indicating that the carboxyl terminus might be a site of intermolecular interaction[18]. However, the basis of this possible interaction is not known, as there is no coiled-coil domain or other putative protein-interaction motif in this region of the δ-opioid receptor.

Structural basis of GPCR hetero-oligomerization

With the exception of the coiled-coil-domain interaction between the carboxyl termini of the $GABA_B$ receptors, little is known about specific mechanisms of intermolecular interaction in heteromer formation. Notably, $GABA_B$ heterodimers can still be formed by receptor mutants that lack coiled-coil

domains[69], indicating that there might be other interfaces between the $GABA_{B(1)}$ and $GABA_{B(2)}$ subtypes. It is possible that the mechanisms of heteromer formation are identical to those of homo-oligomerization, particularly in the cases of hetero-oligomerization of closely related receptors. However, there is no evidence to support or disagree with the possibility that an entirely different type of interaction might occur in heteromers.

Ligand-induced GPCR oligomerization

Little is known about the dynamics and regulation of GPCR oligomer formation. One of the most debated issues remains whether ligands promote association or dissociation of oligomers, or whether they bind to preformed oligomers and change the oligomeric receptor conformation. From crystallization studies, $mGlu_1$ receptors were shown to be constitutive dimers[16]; that is, dimeric receptors existed in the absence or presence of ligand. Nevertheless, the covalent nature of $mGlu_1$ dimers does not preclude the possibility of ligand-regulated formation of higher-order complexes in these receptors. For family 1 receptors, it is even less clear how ligand binding might affect oligomerization.

Studies that show that mutant GPCRs can inhibit the cell-surface expression of co-expressed wild-type receptors indicate that oligomer assembly occurs before receptor transport[12,44,45]. This is also supported by observations that some mutant GPCRs that are ineffectively transported to the cell surface can be rescued when co-expressed with the wild-type receptor, a fragment of the wild-type receptor or another related GPCR subtype[23,49]. However, it is unclear if dissociation and formation of oligomers can occur in the cell membrane.

Immunoblots of receptors that have been chemically crosslinked have indicated that GPCR oligomers are preformed in the cell membrane. Modulation of oligomerization by ligand binding has also been visualized in this way, although the presence of a CROSSLINKING AGENT is not always required to detect these changes. Immunoblot-detected, agonist-induced increases in homo-oligomer formation were seen for β_2-adrenoceptors[17], $SSTR_{1B}$ receptors[20] and CCR2 and CCR5 receptors[24]. When cells that express both CCR2 and CCR5 receptors were treated concurrently with agonists for both receptors, hetero-oligomers were detected by crosslinking and immunoblotting[24]. Interestingly, these chemokine receptor hetero-oligomers were not observed in the absence of agonist or in the presence of an agonist that was selective for just one of the subtypes, and the concentration of ligand that was required to induce hetero-oligomerization was ~100-fold lower than that required for homo-oligomerization. No ligand modulation of oligomerization could be seen in immunoblots of the D_2 receptor[70]. Immunoblot-detected, agonist-induced decreases in oligomer levels were observed for the δ-opioid receptor[18]; however, no effect on δ-opioid receptor oligomerization owing to agonist- or inverse-agonist occupancy was observed in a more recent study that used fluorescence resonance energy transfer (FRET) to measure changes in oligomerization[71].

CROSSLINKING AGENT
A chemical compound that forms a covalent link between two closely associated proteins.

Bivalent endogenous ligands. The existence of ET_A and ET_B endothelin receptor heterodimers has been proposed on the basis of binding experiments in the anterior pituitary gland (a tissue in which both receptors are expressed), which showed that the ET_B receptor can be detected only by radiolabelled endothelin-1 (ET-1)-peptide binding in the presence of an ET_A-selective antagonist[72]. The authors concluded that the ET-1 peptide is a bivalent ligand that has two distinct receptor recognition sites, one each for the ET_A and ET_B receptors, and that this ligand induced or stabilized the heterodimeric conformation. The binding of a monovalent ET_A-selective antagonist allowed for the detection of the ET_B subtype (as a non-heteromeric entity) by the ET-1 peptide. If this model is correct, it represents an interesting, although probably not unique, example of ligand-induced oligomerization (FIG. 1).

The oligomeric state of receptors in living cells. Recently, studies using resonance energy transfer assays[71,73] have received a great deal of attention in the GPCR field, due in part to the ability of the assay to detect changes in the proximity of receptors in living cells. Agonist treatment has been shown to increase resonance energy transfer between some GPCRs, which has been interpreted as an increased formation of oligomers. Bioluminescence resonance energy transfer (BRET) and/or FRET studies on cells that express the β_2-adrenoceptor[71,73], the $SSTR_{2A}$ and $SSTR_{1B}$ receptors[20] and the thyrotropin-releasing-hormone receptor[74] have indicated that agonist-induced homo-oligomerization occurs in these receptors. Resonance energy transfer experiments have also reported that hetero-oligomerization of the $SSTR_{2A}$ and $SSTR_{1B}$ receptors[20], and of the $SSTR_{1B}$ and D_2 receptors[75], occurs owing to agonist binding. However, the data from these types of study cannot discern if the increase in energy transfer is due to a greater number of receptor associations or a conformational change in receptors that are already associated. For example, it is possible that increases in transfer energy might be the result of agonist-induced microaggregation of the receptors, such as occurs during internalization by clathrin-coated pits[76]. Furthermore, all BRET assays and some FRET assays that have been used to examine GPCR oligomerization have a significant drawback — they cannot distinguish between interactions at the cell surface and interactions that occur in intracellular compartments.

One study using resonance energy transfer assays has indicated that receptors in the inactivated state are monomeric and oligomerize only on ligand binding[20]. These authors have suggested that basal levels of resonance energy transfer that indicate constitutive oligomerization are observed only in heterologous expression systems in which the receptor density is significantly higher than physiological levels. However, this speculation is not supported by earlier findings[43–45], which indicate that receptor oligomerization occurs before transport to the cell membrane, and this study[20] is the only resonance energy transfer analysis of a GPCR in which constitutive oligomerization has not been observed.

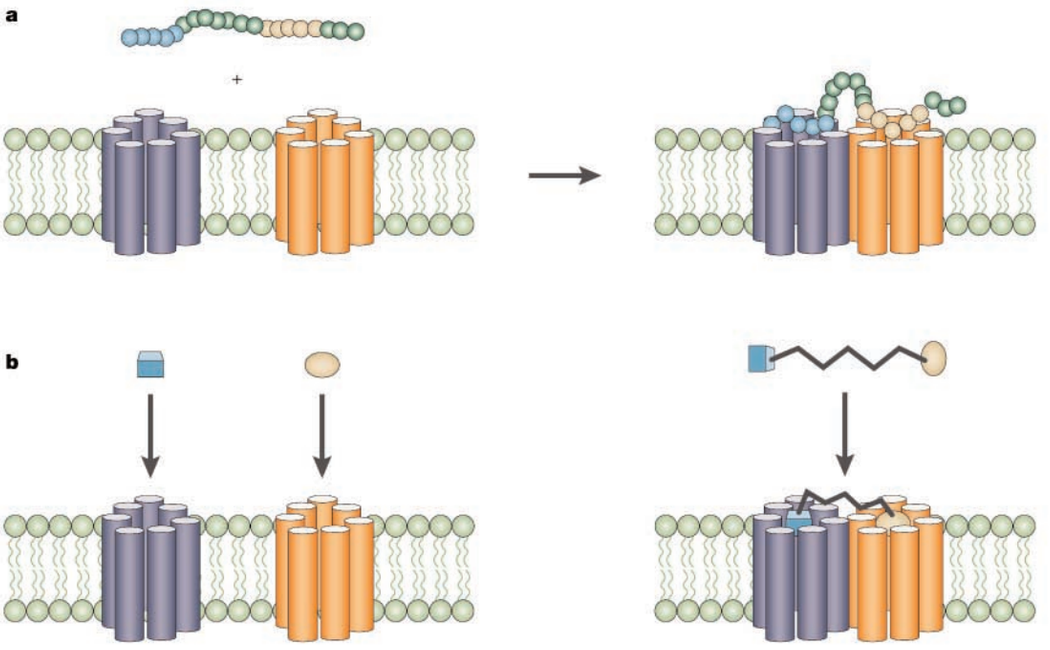


Figure 1 | **Bivalent and dimeric ligands. a** | Schematic representation of ligand-induced heterodimerization by a bivalent peptide ligand. A bivalent peptide with two distinct receptor-recognition sites might bind two different receptors simultaneously and induce receptor heterodimerization. If the two receptors pre-exist in an oligomeric state, the ligand might enhance the heteromeric conformation by crosslinking two receptors. **b** | Dimeric ligands generated from two monovalent ligands. Similar to a bivalent ligand, a dimeric ligand that has an appropriate linker might induce or enhance dimerization of two receptors. It is plausible that homodimeric and heterodimeric ligands might selectively bind homomeric and heteromeric receptors, respectively.

Novel therapies based on GPCR oligomerization

Dimeric ligands. It might be possible to exploit the oligomeric nature of GPCRs to improve drugs by developing dimeric ligands that act much like bivalent ligands. Before the recognition that GPCRs formed oligomers, several dimeric ligands were created by linking monovalent ligands with chemical 'spacers' or with bivalent antibodies (TABLE 3). The rationale for designing such ligands was not clear; however, it was postulated that these ligands would have increased affinity compared with their constituents because, theoretically, in dimeric ligands, the binding of one of the constituent pharmacophores proceeds through a univalently bound state and might therefore allow the unbound partner to be in closer proximity to neighbouring binding sites[77]. This would effectively increase the concentration of unbound pharmacophores in the vicinity of free receptors. Interestingly, these homodimeric and heterodimeric ligands had varying affinity, depending on the length of the spacer that linked the molecules, but, in general, tended to have increased affinity compared with their monovalent constituents. For example, dimers of 5-HT_1 receptor ligands had as much as a 700-fold increase in affinity for the 5-HT_{1B} receptor[78], and also became more selective for the 5-HT_{1B} and 5-HT_{1D} subtypes. Of particular note, when dimers of the 5-HT_{1B} agonist sumatriptan — a drug that is used clinically to treat migraines — were examined, it was found that its affinity for the 5-HT_{1B} receptor was increased by up to ~100-fold compared with monovalent sumatriptan[79].

In the light of the emergence of models of oligomeric GPCRs, an alternative theory for the mechanistic basis for increased affinity of dimeric ligands has been developed. As was proposed for the bivalent endothelin peptide, dimeric ligands might more readily induce or stabilize the dimeric conformation of the receptors[72,80] (or possibly 'crosslink' receptors), which could, in some manner, increase the affinity and/or potency of the ligand. This theory is supported by observations that the length and chemical properties of the spacer joining the dimeric ligands are crucial factors in its pharmacological characteristics.

Changes in selectivity have also been observed for bivalent opioid receptor ligands[81]. A non-selective, monovalent opioid receptor antagonist, when made bivalent with a short spacer, became selective for the κ-opioid receptor. However, when the spacer length was increased, the κ-opioid receptor selectivity was lost. Furthermore, changes in ligand potency as a result of ligand dimerization have also been observed. Dimerization of a gonadotropin-releasing-hormone receptor ligand altered it from an antagonist to an agonist[82], and the neurotensin–Leu-enkephalin bivalent ligand was shown to be a more potent stimulator of cyclic GMP production than neurotensin alone, indicating the possibility of oligomerization between neurotensin and opioid receptors[80]. In the case of the neurotensin–Leu-enkephalin dimer, as is the case with other dimeric ligands, the length of the spacer between the two ligands was crucial to its efficacy.

Given that dimeric ligands seem to have altered properties, such as increased efficacy and potency, there seems to be great potential in developing new drugs by linking monovalent drugs to generate engineered bivalent ligands. It has even been speculated that heteromeric dimeric ligands might be selective for heteromeric GPCRs[55], indicating that it might be possible to stimulate or block the novel signalling of heteromers selectively and not markedly affect the 'traditional' signalling by homogeneous populations of the receptors.

New approaches with old drugs. By identifying receptors that directly interact with each other, it might be possible to develop new therapies simply by using available drugs in new ways. For example, a recent study on the heteromerization of the A_{2A} adenosine receptor and the D_2 receptor has shown that the functions of both receptors are simultaneously altered after long exposure to agonists, which potentially explains behavioural findings that show cross-tolerance and cross-sensitization between dopamine agonists and compounds that are active at adenosine receptors[83]. The authors of this study note that there is evidence to indicate that A_{2A} receptor function might be involved in the secondary effects, such as DYSKINESIA, that are observed after chronic treatment with levodopa, a drug that is used to treat PARKINSONISM. Evidence also indicates that the attenuation of the anti-Parkinsonian action of levodopa treatment might, in part, be caused by the simultaneous chronic activation of A_{2A} and D_2 receptors. Therefore, it was proposed that the co-administration of A_{2A} receptor antagonists, together with levodopa, could provide a new therapeutic approach that lacks the secondary effects of chronic levodopa treatment. If therapeutic strategies continue to be devised on the basis of the model that GPCRs function as monomers, it is possible that simple, effective therapies could be overlooked.

Enhancing and disrupting oligomerization. As noted earlier, there is evidence that GPCRs may undergo agonist-induced oligomerization, indicating that oligomeric receptors possibly represent activated signalling units. Therefore, drugs that can enhance or disrupt GPCR oligomer formation might also regulate oligomerization-dependent functions. Furthermore, as some GPCRs form both agonist-induced homo-oligomers and hetero-oligomers[24,84], and as receptor hetero-oligomers might have functional properties that are distinct from their constituent receptors, drugs that selectively induce hetero- over homo-oligomerization (or vice versa) could have enormous clinical value. At present, no drug development strategies to exploit receptor oligomerization specifically have been developed (with the possible exception of dimeric and/or bivalent ligands). Several studies have used synthetic peptides that mimic certain TM domains to attenuate the function of the GPCRs from which they were derived[11,17,85]. In one of these studies, a peptide based on TM6 of the β_2-adrenoceptor was shown to reduce the quantity of receptor dimer detected by immunoblot, and it was therefore postulated that TM6 was a dimerization interface and that the peptide blocked receptor function by preventing dimerization[17]. However, although an analogous peptide based on the D_1 receptor was shown to affect receptor function, it did not have any effect on the oligomeric state[11]. For the oestrogen receptor, a receptor from a family of proteins for which dimerization interfaces are better defined, it has been shown that a peptide that mimics the dimerization interface can block receptor function, but the mechanism for this effect is disruption of the monomeric receptor (by precipitating the protein from solution) and not the prevention of dimerization[86]. Nevertheless, compounds that disrupt the quaternary structure of GPCRs could have great potential as therapeutic agents and be worthy of some investigation.

Improving lead discovery and optimization processes. Contemporary drug discovery for GPCRs has largely been a process of high-throughput trial-and-error, often using a single GPCR of interest expressed in a recombinant cell line and with innumerable 'non-hits' for every breakthrough. There has been an increased awareness of the expanding number of GPCR targets and of the need for smarter combinatorial chemistry and compound library design. Nevertheless, these improved strategies generally still do not incorporate the possibilities added by GPCR homo- and hetero-oligomerization. For future lead-compound identification, the current understanding of GPCR oligomerization has mandated that hetero-oligomeric receptors must be considered as novel targets in the screening of compounds as drug candidates.

The realization that the function of GPCRs can be greatly influenced by neighbouring receptors also demands consideration in the lead development process. In the drug optimization process, data gathered from studies using 'isolated' receptors might be misleading, and the receptor in the physiological state needs to be analysed. Furthermore, on the basis of knowledge of the oligomeric state of the receptors that they target, novel regimens with 'old' drugs could be formulated as a means of enhancing some therapies. The use of dimeric ligands to modulate hetero-oligomers selectively or increase potency and selectivity of monovalent ligands for homomeric and heteromeric receptors also has great potential as a means to develop and improve candidate drug molecules.

DYSKINESIA
An impairment in the ability to control movements — characterized by spasmodic or repetitive motions or lack of coordination.

PARKINSONISM
Any of a group of nervous disorders that are similar to Parkinson's disease — characterized by muscular rigidity, tremor and impaired motor control.

Table 3 | **GPCRs targeted by dimeric ligands**

Receptors	References
5-HT_1 serotonin receptor	78,79,118
A_1 and A_3 adenosine receptors	119
Bombesin receptor	120
Gonadotropin-releasing-hormone receptor	82
Muscarinic receptors	121
Neurotensin and opioid receptors	80
Opioid receptors	81,122–124
$P2Y_1$ purinergic receptor	125
α-Melanocyte-stimulating-hormone receptor	120

GPCR, G-protein-coupled receptor.

A potential caveat. Given the trial-and-error processes of modern drug screening, it might be tempting to co-express all possible combinations of GPCRs in a search for new drugs. However, it is important to be aware that not all receptors that interact in heterologous cell systems are present together physiologically. A recent study has indicated that GPCRs, even receptors from different families, are promiscuous in their interactions and have a 'natural tendency' to co-immunoprecipitate when co-transfected into cells[87]. Although the conclusions of this study can be challenged by a multitude of evidence that shows specificity of interaction[24,56,83,88–90], the underlying caveat is still poignant.

Conclusion

The unprecedented advances of the past decade have presented a great new challenge for the field of GPCR drug discovery. Molecular biology and cloning techniques have vastly expanded the number of potential GPCR targets — far more than predicted by early physiological and pharmacological data — and great strides forward in understanding the secondary and tertiary structures of these receptors have been gained from biochemistry and protein crystallography. However, the realization of the significance of protein–protein interactions in GPCR function has added an immense degree of complexity to the attempts to understand this important class of receptors. For the future, perhaps the greatest challenge facing the pharmaceutical industry will be to integrate GPCR homo- and hetero-oligomerization (as well as GPCR interactions with other proteins) into the molecular models that are used in the development of novel and improved therapeutics. The consideration of GPCR quaternary structure has been slow to permeate into the thinking of the drug discovery mainstream, despite the potential to exploit it for improved therapies.

It is an exciting time for GPCR research. The realization that oligomerization is a pivotal aspect of the structure and function of GPCRs, which has implications for receptor transport, signalling and pharmacology, has provided better, albeit more intricate, models for understanding the physiological roles of these receptors. Not only have fascinating new possibilities been exposed, a re-evaluation of established ideas has been prompted. There will be new perspectives on the mechanisms of action of established drugs and on the molecular models of diseases that have been studied for many years. The increasing understanding of the implications of receptor–receptor interactions among GPCRs has yielded, and will undoubtedly continue to yield, great insights into their structure–function relationships and into behavioural and clinical disorders that are mediated by these receptors. Most importantly, the incorporation of oligomeric receptor models into strategies for GPCR drug discovery might result in better therapeutic agents that target these receptors.

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