J Mol Med (2001) 79:226–242
DOI 10.1007/s001090100219

REVIEW

Ivone Gomes · Bryen A. Jordan · Achla Gupta
Carl Rios · Nino Trapaidze · Lakshmi A. Devi

G protein coupled receptor dimerization: implications in modulating receptor function

Received: 22 May 2000 / Accepted: 26 February 2001 / Published online: 18 May 2001

Abstract Protein-protein interactions are involved in the regulation of a large number of biological processes. It is well established that a variety of cell surface receptors interact with each other to form dimers, and that this is essential for their activation. Although the existence of G protein coupled receptor dimers was predicted from early pharmacological and biochemical analysis, solid evidence supporting dimerization has come within the past few years following the cloning of G protein coupled receptor cDNAs. Using differential epitope tagging and selective immunoisolation of receptor complexes, dimerization of a number of G protein coupled receptors including members of the rhodopsin, secretin, and metabotropic glutamate receptor families have been reported. More recently fluorescence or bioluminescence resonance energy transfer techniques have been used to examine dimerization of these receptors in live cells. These studies have found that whereas in some cases there is an agonist induced increase in the level of dimers, in others there is a decrease or no change in dimer levels. Several recent studies have also reported the ability of related members of G protein coupled receptors to heterodimerize. These heterodimers exhibit distinct physical and functional properties. Examination of possible sites of interactions between receptors has implicated a role for extracellular, transmembrane and/or C-terminal region in dimerization. The functional consequences of dimerization, explored mainly using mutant receptors, have demonstrated a role in modulating agonist affinity, efficacy, and/or trafficking properties. Thus dimerization appears to be a universal phenomenon that provides an additional mechanism for modulation of receptor function as well as cross-talk between G protein coupled receptors.

Keywords Opioids · Oligomerization · Desensitization · Signal transduction · Downregulation

Abbreviations *BRET:* Bioluminescence resonance energy transfer · *cAMP:* Cyclic adenosine monophosphate · *CCR:* Chemokine receptor · *DAMGO:* [D-Ala^2,MePhe^4-Gly^5-ol]enkephalin · *Deltorphin II:* Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 · *DPDPE:* [D-Pen^2,D-Pen^5]enkephalin · *DTT:* Dithiothreitol · *FRAP:* Fluorescence resonance energy transfer after photobleaching · *FRET:* Fluorescence resonance energy transfer · *$GABA_BR$:* γ-Amino butyric acid receptor ·

IVONE GOMES received her Ph.D. degree in biochemistry from the All India Institute of Medical Sciences, New Delhi, India. She is presently in the Department of Pharmacology, New York University School of Medicine, New York. Her research interests are the study of G protein coupled receptors, particularly opioid receptors with emphasis on signal transduction pathways, receptor cross-talk, and mechanisms involved in G protein coupled receptor dimerization/oligomerization.

LAKSHMI DEVI received her Ph.D. in biology from University of Windsor, Ontario, Canada. She is presently Associate Professor at the Department of Pharmacology, New York University School of Medicine, New York. Her research interests are understanding the molecular mechanisms modulating G protein coupled receptor function by agonist-mediated endocytosis and receptor dimerization with particular emphasis on the heterodimerization of opioid receptors with other G protein coupled receptors.

I. Gomes · B.A. Jordan · A. Gupta · C. Rios · N. Trapaidze
L.A. Devi (✉)
Department of Pharmacology,
New York University School of Medicine, 550 First Avenue,
New York, NY 10016, USA
e-mail: Lakshmi.Devi@med.nyu.edu
Tel.: +1-212-2637119, Fax: +1-212-2637133

GIRK: G protein coupled inwardly rectifying K^+ channels · *GPCR:* G protein coupled receptors · *GST:* Glutathione-S-transferase · *HA:* Hemagglutinin · *MAPK:* Mitogen-activated protein kinase · *SDS-PAGE:* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis · *SSR:* Somatostatin receptor · *TIPPΨ:* H-Tyr-Tic[ψ,CH2NH]Phe-Phe-OH (Tic=1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid · *TM:* Transmembrane

Introduction

The activation of different classes of plasma membrane receptors regulates the activity of practically every cell of the body. The vast majority of these receptors belong to the superfamily of G protein coupled receptors (GPCRs) which at current estimates account for approx. 1% of the genes present in a mammalian genome. Agonists or antagonists of GPCRs as well as agents that interfere with cellular pathways regulated by these receptors are widely used in drug therapy.

All GPCRs share a common three-dimensional structure consisting of seven transmembrane (TM) helices (I–VII) linked by three alternating intracellular (i_1, i_2, and i_3) and extracellular (e_1, e_2, and e_3) loops. A disulfide bond formed by two cysteine residues conserved in most GPCRs (one in e_1 and the other in e_2) is probably important for the packaging and stabilization of a restricted number of conformations for these seven TM helices [1]. The extracellular and transmembrane regions of the receptor are involved in ligand binding [2, 3, 4] while the intracellular surface is important for G protein activation and function [5, 6, 7, 8]. Structurally GPCRs can be classified into three major receptor families [9]. Family A (rhodopsin/β_2-adrenergic receptor-like) receptors comprise by far the largest family of GPCRs and ligands acting on these receptors are remarkably diverse (photons, amino acids, proteins). Receptors belonging to this class are characterized by the presence of approx. 20 highly conserved amino acids localized mainly in the TM regions. A role for these residues in protein stability and/or in mediating agonist induced conformational changes has been proposed based on mutagenic studies [10]. Family B (secretin/glucagon receptor) receptors make up a relatively small group of receptors characterized by the presence of a large N-terminal extracellular domain that contains six well-conserved cysteine residues in addition to the some 20 highly conserved residues within the TM regions [11]. Family C (metabotropic glutamate and calcium-sensing receptor) receptors are characterized by a very long N-terminal extracellular domain that appears to be sufficient for ligand binding. These receptors share approx. 20 cysteine residues in the extracellular region and a TM receptor core that may be involved in ligand binding as well as other receptor functions [12].

Models describing the interaction of GPCRs with their G protein targets are generally based on the assumption that the receptors exist as monomers and couple to G proteins in a 1:1 stoichiometric ratio. However, recent studies suggest that these classical models of receptor/G protein coupling are oversimplified since a number of studies have reported not only the presence but also a role for GPCR multimers in modulating receptor function [13]. In addition, some GPCRs need to form heterodimers in order to be correctly folded, exported to the membrane and obtain their final identity [1, 14]. In this review we present several lines of evidence supporting dimerization of GPCRs as well as discuss possible mechanisms and functional implications of this phenomenon.

Homodimerization

The ability of GPCRs to dimerize or oligomerize was not recognized until recently despite a significant amount of circumstantial evidence derived from cross-linking experiments, target size analysis and hydrodynamic studies (Table 1). In recent years, with the availability of GPCR cDNAs and specific antibodies to the receptors, it has been possible to critically evaluate the hypothesis that "GPCRs physically interact with each other to form dimers/oligomers and this interaction modulates their function."

Early indirect evidence

Pharmacological evidence

Complex binding data from early pharmacological studies provided indirect evidence for the existence of GPCR dimers. Studies with adrenergic and muscarinic receptors showed homo- or heterotropic cooperativity in ligand binding analysis [15, 16, 17, 18, 19, 20, 21]. More recently receptor isolation using affinity chromatography followed by western blotting provided direct evidence for the ability of adrenergic and muscarinic m_3 receptor dimers/multimers to bind ligands [22, 23]. Radioligand binding studies with two different receptor antagonists suggested the existence of D_2 dopamine receptor dimers [24]. The receptor density was found to be approx. 1.5-fold greater with nemonapride than with spiperone. Photoaffinity labeling with radioiodinated ligands showed that spiperone labeled only the monomers while nemonapride labeled both the monomers and the dimers [24]. Radioiodinated ligand blotting studies with placental leteinizing hormone receptors also led to the detection of dimeric receptors (180 kDa) in solubilized preparations [25]. The apparent molecular weight of the receptor dimer is consistent with that determined by sucrose-density gradient centrifugation [26]. Finally, pharmacological evidence for the presence of opioid receptor dimers came from the use of dimeric enkephalin or morphine analogs, which exhibited higher affinity for δ or μ receptors [27]. Furthermore, these analogs exhibited sev-

Table 1 Homodimerization of G protein coupled receptors

Receptor	Technique	Year	References
Family A			
Monoamine			
β_2-Adrenergic	Binding assays	1975, 1976	15, 16
	Target size analysis	1982, 1983	28, 29
	Western blot analysis	1996	22
	Immunoprecipitation	1998	57[a]
	BRET	2000	68[a]
α_2-Adrenergic	Target size analysis	1983	29
Dopamine	Target size analysis	1987	39
	Photoaffinity labeling	1996	24
	Cross-linking studies	1996, 1998	24, 42
	Immunoprecipitation	1994, 1997	52, 53
Small molecule			
Muscarinic	Photoaffinity labeling	1983	43
	Binding assays	1985, 1987, 1988, 1989, 1991	17, 18, 19, 20, 21
	Target size analysis	1985, 1986	37, 38
	Western blot analysis	1995, 1999	63, 23
	Immunoprecipitation	1999	23[a]
Histamine	Immunoprecipitation	1997	60[a]
Platelet-activating factor	Immunoprecipitation	1994	61[a]
Peptide			
Opioid	Binding assays	1982	27
	Target size analysis	1983, 1986	30, 31, 33
	Hydrodynamic analysis	1986	32
	Immunoprecipitation	1997, 1999	58, 59[a]
Angiotensin II	Gel exclusion analysis	1980, 1983, 1987	50, 51, 47
	Cross-linking studies	1982, 1984, 1987, 1990, 1991	45, 46, 47, 48, 49
Vasopressin V_2	Immunoprecipitation	1996, 1998	22, 62[a]
Bradykinin	Cross-linking studies	1999	44
Somatostatin	FRAP	2000	69[a]
Protein			
Gonadotropin-releasing hormone	Target size analysis	1985	36
	FRET	2001	70[a]
Thyrotropin	Target size analysis	1987	35
	Cross-linking studies	1987	35
	Immunoprecipitation	1992, 1995	54, 55
Luteinizing hormone	Density gradient centrifugation	1990	26
	Ligand blotting	1991	25
	Immunoprecipitation	1992	56
Human chorionic gonadotropin	Immunoprecipitation	1992	56
Chemokine CCR2	Cross-linking studies	1999	41
	Immunoprecipitation	1999	41[a]
Family B			
Ig-Hepta	Immunoprecipitation	1999	64
Family C			
Calcium-sensing	Cross-linking studies	1998	40
	Density gradient centrifugation	1998	66
	Immunoprecipitation	1998, 1999	40, 65[a]
Metabotrophic glutamate	Western blot	1999	67
	Crystallography	2000	101[a]

[a] Indicates recent studies providing direct evidence for dimerization

eral-fold greater potency than their monomeric counterparts in the guinea pig ileum bioassay [27].

Biochemical evidence

Radiation inactivation studies. Radiation inactivation (target size analysis) is a technique based on the inverse relationship between the dose-dependent inactivation of a macromolecule by ionizing radiation and the size of that macromolecule. This technique was used to show that a number of GPCRs exist as multimeric arrays in the plasma membrane. For example, radiation inactivation showed that the molecular weight of the functional unit of the β_2-adrenergic receptor from mammalian lung membranes was approx. 109 kDa whereas the subunit molecular weight of the receptor was found to be 59 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Taken together these results suggested that the mammalian lung β_2-adrenergic receptor is a dimer of two identical subunits [28]. This technique was also used to demonstrate the functional size of the α_2-adrenergic receptor in human platelet membranes as approx. 160 kDa [29]. Radiation inactivation studies with the opioid receptor showed that the molecular mass of the membrane bound receptor ranges from 100 to 200 kDa depending on the assay conditions, and that it becomes 60 kDa in the presence of Na^+, Mg^{2+}, and guanosine triphosphate [30, 31]. Consistent with this, the hydrodynamic size of the solubilized opioid receptor complex was found to be 200 kDa [32]. These studies led to the suggestion that the native form of the opioid receptor is an oligomeric array of the receptor complexed with G proteins [33]. Analysis of the target size of thyrotropin receptor revealed the presence of a thyroid-stimulating hormone binding component of 71 kDa. This estimate is approximately twice that obtained by cross-linking studies (approx. 31 kDa), presenting the possibility that the functional receptor consists of a high molecular weight complex in its native state [34, 35]. Similar observations were also made for the gonadotropin-releasing hormone receptor [36] and for the muscarinic receptor [37, 38]. In the case of the D_1 dopamine receptor the functional molecular mass for the agonist binding site was found to be higher than the antagonist binding site, suggesting that agonists bound to the oligomeric receptor [39].

Cross-linking studies. Cross-linking studies have supported the existence of dimeric or oligomeric complexes of the dopamine D_2 receptor [24], calcium-sensing receptor [40], and chemokine receptor (CC^{R2}) [41]. In the case of the D_2 dopamine receptor, radioiodinated 4-azido-5-iodonemonapride detected both the monomeric (120 kDa) and the dimeric (approx. 250 kDa) forms of the D_2 receptor whereas radioiodinated azidophenethylspiperone detected only the monomeric form [42]. Photoaffinity labeling of muscarinic receptors present in partially purified membrane fractions from various brain regions indicated that the receptor is present in the brain as a dimer of 80 kDa and a tetramer of 160 kDa [43]. More recently dimerization of bradykinin B_2 receptors was demonstrated by cross-linking of agonists (but not antagonists) to endogenously expressed receptors in PC-12 cells [44]. Cross-linking of radioiodinated agonists to angiotensin II receptors in a variety of tissues showed that the receptor exists as a noncovalent dimer (approx. 116 kDa) with two subunits of similar molecular weight (approx. 60 kDa) [45, 46, 47, 48, 49]. The dimeric nature of this receptor was also supported by the detection of two peaks of radioactivity by gel exclusion chromatography [47, 50, 51]. Similar studies have indicated that the receptors for thyroid stimulating hormone are present in oligomeric complexes of a 31-kDa subunit in FRTL-5 thyroid cells [35]. In our laboratory we have seen that treatment of cells expressing either the β_2-adrenergic receptor or the D_2 dopamine receptor with a cross-linking agent such as dithiobis (succinimidyl propionate) leads to the detection of oligomeric complexes with a concomitant decrease in the monomeric receptor species (Fig. 1).

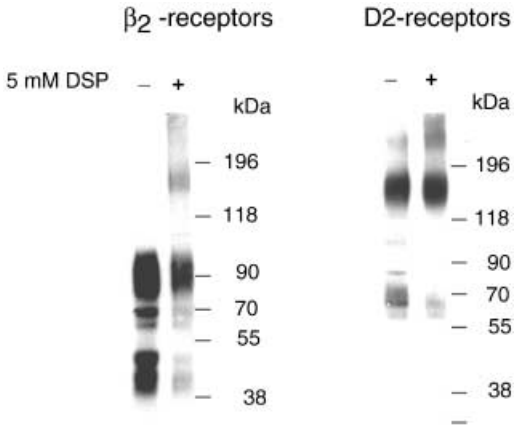

Fig. 1 Biochemical characterization of β_2-adrenergic receptor and D_2 dopamine receptors. Human embryonic kidney cells were transiently transfected with Flag-tagged human β_2-adrenergic receptor cDNA or D_2 dopamine receptor cDNA. After 72 h the cells were incubated without (–) or with (+) 5 mM Dithiobis-(succinimidyl-propionate) (*DSP*, Pierce) and subjected to Western blotting with anti-Flag (M1) antibody as previously described [58, 59]. Treatment with the cross-linking agent (DSP) results in a reduction in the level of both the approx. 45 kDa (monomer) and approx. 90 kDa (dimer) forms of the β_2-adrenergic receptor but stabilizes a higher molecular weight oligomeric form. Treatment with the cross-linking agent appears to decrease the level of the lower molecular weight (monomer) form of the D_2 dopamine receptor but does not affect the level of the major approx. 130 kDa (dimer) form

Immunoprecipitation studies using antibodies to endogenous receptors. Immunoprecipitation and Western blot analysis have been used to gain further support for the presence of dimers among several members of the rhodopsin family. Studies with dopamine receptors showed the presence of dimers in heterologous cells and in brain tissue. Immunoprecipitation of the D_2 long form of dopa-

mine receptors expressed in heterologous cells with a receptor specific antibody identified two major receptor species at approx. 44 kDa and at approx. 93 kDa suggesting the presence of receptor monomers and dimers [52]. Both receptor species could be immunoprecipitated from digitonin solubilized preparations of cells metabolically labeled with ^{32}P or [^{3}H]palmitate [52]. Immunoprecipitation studies with dopamine D_3 receptor specific antisera also showed two protein species of approx. 85 and 180 kDa (in addition to an approx. 45-kDa protein) in human, monkey, and rat brain membranes [53]. This suggests that the D_3 receptor exists as a dimer and a tetramer in both primate and rodent brain. In all three species examined the tetrameric form of the D_3 protein was found to be the most abundant species. These forms of the D_3 receptor were resistant to reducing agents and detergent treatment [53]. The presence of dimers was also observed with D_{3nf}, a naturally occurring truncated form of D_3 dopamine receptor lacking TM VI and VII [53]. Furthermore, when immunoblots of proteins extracted from rat prefrontal cortex or from cells cotransfected with both receptors were probed with the D_{3nf}-specific polyclonal antibody, both the tetrameric and the dimeric protein species gave immunoreactive signals [53]. In addition, the same receptor species was also identified in proteins from rat brain immunoprecipitated with anti-D_3 antibody [53] suggesting the dimerization between the D_3 and D_{3nf} proteins [53]. Confocal microscopy confirmed the presence of both proteins in the same pyramidal-like neurons with greatest colocalization in the proximal portion of the apical dendrites [53]; thus it is likely that the dimeric form of D_3-D_{3nf} plays a physiological role *in vivo*. Under nonreducing conditions membranes prepared from cells expressing thyroid-stimulating hormone were found to contain both glycosylated and unglycosylated dimeric and monomeric receptor forms [54]. Neither reducing agents nor high concentrations of a combination of detergents (SDS and Triton X-100) appeared to affect the level of dimers [54]. Immunoprecipitation with antisera raised against the thyroid-stimulating hormone receptor also identified the dimeric forms of the receptor in plasma membranes of human thyroids [55]. Similar experiments with membranes from ovaries of pseudopregnant rats revealed the presence of dimers of luteinizing hormone/human chorionic gonadotropin receptors [56].

Recent direct evidence

Immunoprecipitation studies using antibodies to epitope-tagged receptors

Differential epitope tagging of GPCR cDNA followed by selective immunoprecipitation of the dimer has been used to confirm the ability of GPCRs to dimerize in heterologous cells. Coexpression of differentially epitope tagged β_2-adrenergic receptor (with either c-*myc* or hemagglutinin (HA) epitopes) followed by immunoprecipitation and Western blotting with selective antisera resulted in the visualization of the dimeric (90 kDa) and monomeric (45 kDa) receptor forms [57]. Receptor dimers were also detected in digitonin-solubilized membrane extracts as well as in affinity purified receptor preparations [57]. The specificity of dimerization was demonstrated by the lack of coimmunoprecipitation of *c-myc* tagged muscarinic receptors when coexpressed with the HA tagged β_2-adrenergic receptors [57].

Direct demonstration of the existence of opioid receptor dimers came from similar immunoprecipitation studies of differentially tagged receptors [58, 59]. In these studies Flag and c-*myc* epitope tagged δ or κ receptor cDNAs were used. Immunoprecitipation with anti-*myc* antisera followed by Western blotting with anti-Flag antisera resulted in the visualization of δ [58] and κ [59] dimers only in cells expressing the receptors. These δ or κ opioid receptor dimers were seen in a variety of cell lines [58, 59]. The δ opioid receptor exhibits little or no dimeric forms in the absence of cross-linker; cross-linking agents are needed to stabilize the δ receptor dimers [58]. In contrast, κ receptors exist as SDS stable dimers regardless of the presence or absence of cross-linking agents [59]. Furthermore, treatment with reducing agents resulted in a decrease in the size of the immunoreactive material to one corresponding to that of κ receptor monomers suggesting the involvement of disulfide bonds in the dimerization of this receptor [59].

Western blot analysis of immunoprecipitates from cells expressing HA tagged-histamine H_2 receptors revealed four bands of molecular mass of approx. 30, 60, 80, and 120 kDa [60]. These bands were also detected when the same blots were probed with an antibody directed against the C-terminal 14 amino acid residues of the receptor, indicating that they correspond to full-length HA-tagged H_2 receptors. From the molecular mass as predicted from its amino acid sequence it was suggested that the 60-kDa band most likely represents the dimer, the 80-kDa band the trimer, and the 120-kDa band the tetramer of the histamine H_2 receptor [60].

Immunoprecipitation studies have also shown the presence of dimers in the case of the platelet activating factor receptor [61], the human V_2 vasopressin receptor [22, 62] and the chemokine CCR2 receptor [41]. Differential tagging of the m_3 muscarinic receptor at the C-terminal tail was used to confirm the presence of dimers. These dimers represented 45% of total receptors present on the cell surface and 30% of intracellular receptors [23]. Dimer and oligomer formation has also been observed in the case of the m_1 and m_2 muscarinic receptors [23, 63].

The members of the secretin family (family B of GPCRs) have also been examined for dimerization. Ig-Hepta is an unusual member in that its hepta-helical transmembrane region is similar to the secretin receptor family, but its large exodomain (1053 amino acid residues) is unique in having immunoglobulinlike repeats, a motif characteristic of the members of the Ig superfamily of cell surface receptors. Western blots, with Ig-Hepta

antisera, of detergent extracts from rat lung membranes detected a single band of approx. 160 kDa under reducing conditions [64]. Under nonreducing conditions significant amounts of Ig-Hepta migrated as an approx. 260-kDa species. Recombinant Ig-Hepta expressed in COS-7 cells behaved as an approx. 130 kDa and an approx. 260 kDa species under reducing and nonreducing conditions, respectively [64]. The presence of the dimeric form of Ig-Hepta receptor was confirmed by coimmunoprecipitation/Western blot analysis of the differentially tagged Ig-Hepta receptors [64].

Members of family C of GPCRs have also been shown to exist as dimers using immunoprecipitation and Western blot analysis. Surface labeled heterologous cells transiently expressing differentially tagged calcium-sensing receptors were found to exist as two major proteins of approx. 200–300 kDa and 300–500 kDa under nonreducing conditions and a major band of 160 kDa (mature monomeric receptor) under reducing conditions (in the presence of dithiothreitol, DTT) [40]. A dimeric band was also detected (under nonreducing conditions) with an anti-calcium-sensing receptor antiserum [40]. Iodoacetamide was included in the lysis buffer to prevent nonspecific disulfide bond formation between the free thiol groups of the native receptor. Use of a noncleavable membrane–impermeant cross-linker demonstrated that more than 85% of the calcium-sensing receptors are present on the cell surface in the form of dimers [40]. The presence of dimers under nonreducing conditions suggested that these receptors form dimers/oligomers via intermolecular disulfide bonds that are sensitive to reducing agents [40, 65]. In a related study, kidneys of anesthetized rats were perfused with *N*-ethylmaleimide to rule out any artifactual formation of disulfide bonds during tissue processing and endosome purification. Sucrose density gradient centrifugation of solubilized endosomal proteins detected the presence of the dimeric and monomeric forms as seen in Western blots [66].

Examination of metabotropic glutamate R1-α_2 receptors in baby hamster kidney cells or P2 membranes from rat cerebellum by Western blotting also detected dimers (approx. 300 kDa) in the absence of DTT and monomers (approx. 150 kDa) in the presence of DTT [67]. This strongly suggested a role for disulfide bonding in receptor dimerization. Treatment of BHK cells with brefeldin A, a drug that blocks the transit of proteins from the endoplasmic reticulum to the Golgi apparatus, did not prevent dimerization, suggesting that this process takes place in the endoplasmic reticulum [67].

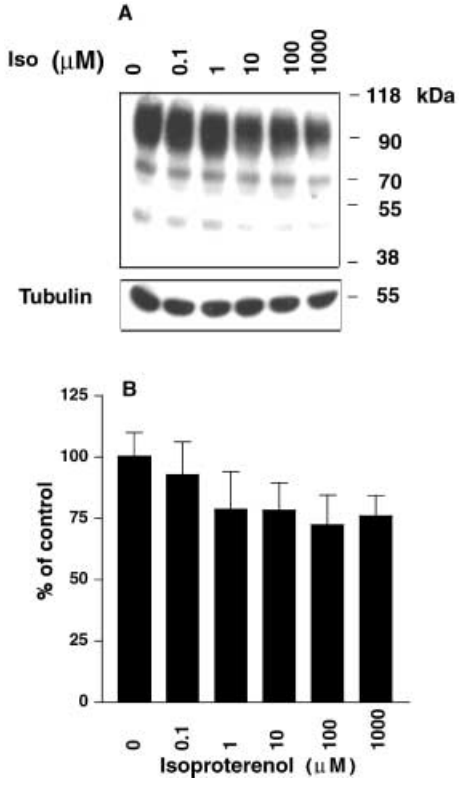

Fig. 2 Effect of agonist treatment on the level of the approx. 90 kDa (dimeric) form of Flag-tagged β_2-adrenergic receptor. Human embryonic kidney cells expressing β_2-adrenergic receptors were treated with increasing doses of isoproterenol for 30 min at 37°C. Following treatment the cells were washed and incubated with 5 mM DSP and subjected to Western blotting as described [58, 59]. The level of Flag-tagged β_2-adrenergic receptor was determined by Western blotting using Flag (M1) antibody and the levels of tubulin using anti-tubulin antibody (Sigma). **A** A representative autoradiogram showing the level of anti-Flag-tagged β_2-adrenergic receptors (*upper*) and tubulin (*lower*) following treatment with the agonist. **B** Autoradiograms from five independent experiments were densitized and the ratio of the β_2-adrenergic receptor to tubulin was determined as described [58]. The data present mean ±SEM (n=5). *Control* The ratio of β_2-adrenergic receptor levels to the tubulin levels; the level in untreated cells is taken as 100%. Statistical analyses did not reveal significant differences between treated and control groups

Effect of agonists on homodimerization

A number of studies have evaluated the effect of agonists on the level of receptor dimers and found that in some cases they stabilize and/or increase the level of dimers while in others they have no effect or decrease the level of dimers. A time-dependent increase in receptor dimerization, following agonist treatment, was observed in the case of β_2-adrenergic receptor as determined by agonist binding to membranes or affinity purified receptors [22]. Similarly, agonist treatment showed an increase in bioluminescence resonance energy transfer (BRET) signal [i.e., the emission from the yellow fluorescence protein (tagged to the receptor C-tail) as a consequence of the activation energy generated by the addition of the substrate to luciferase (also tagged to the C-tail)] [68]. This increase could be due to a change in the level of dimers and/or change in the conformation of the receptor upon agonist binding. We have recently found that treating whole cells expressing epitope-tagged β_2-adrenergic receptors with different doses of isoproterenol leads to a small decrease in the level of dimers, with no significant increase in the level of monomers (Fig. 2). Furthermore, treatment of these cells with isoproterenol

for a varied period of time did not significantly affect the level of receptor dimers (Nivarthi and Devi, unpublished). These findings are consistent with the possibility, that rather than a net increase in the number of dimers upon agonist treatment, there is a change in the conformation of the receptor that leads to the increase seen in BRET signal. Fluorescent resonance energy transfer after photobleaching (FRAP) was used to examine the effect of agonist treatment on the dimerization of the somatostatin receptor (SSR) in live cells [69]. Using fluorescein and rhodamine-conjugated monoclonal antibodies against the epitope tag on the SSR5 receptor, the authors showed that there was a significant slowing of the photobleaching process upon addition of the rhodamine-labeled cells to the fluorescein-labeled cells, and this was further decreased in the presence of the agonist [69]. These results suggest that there is an agonist-mediated increase in the level of dimers. However, it should be noted that these changes in energy transfer could be due to changes in receptor conformation leading to changes in the energy transfer. The fluorescence resonance energy transfer (FRET) method was used to demonstrate that only agonists and not antagonists promoted the microaggregation (dimerization/oligomerization) of the gonadotropin-releasing hormone receptor [70]. The doses of the agonists that promoted receptor microaggregation were within the physiological range and the time course indicated that these events occur immediately after agonist binding and persist up to 80 min [70]. These results led the authors to conclude that the gonadotropin-releasing hormone receptor undergoes agonist-mediated microaggregation.

In the case of the calcium sensing receptor, agonists induced a concentration-dependent increase in the dimeric (240–310 kDa) form which was accompanied by a decrease in the level of the monomeric (121 and 138–169 kDa) form in solubilized endosomes purified from rat kidney inner medullary collecting duct [66]. Dimers of differentially epitope tagged chemokine CCR2 receptors were detected on Western blots only after treatment of transfected cells with the agonist [41].

Stimulation of endogenously expressed bradykinin B_2 receptors in PC-12 cells with agonists also leads to the increased formation of receptor dimers and higher oligomerization states [44]. Chemical cross-linking with radioiodinated agonists showed an increase in receptor dimers with a concomitant decrease in monomers in cells transiently expressing the D_2 dopamine receptor [24] and the SSRs [69].

Agonist treatment does not affect the levels of some GPCRs dimers. In order to study the effect of agonists on α-mating factor receptor (STE2 in yeast) the receptor (lacking C-terminal tail regulatory domain) was C-terminally tagged with either cyan-fluorescent protein or yellow-fluorescent protein and subjected to FRET analysis [71]. Coexpression of the differentially tagged truncated receptors resulted in an increase in FRET efficiency, which was not affected by agonist treatment suggesting that agonists did not affect the levels of truncated receptor dimers [71]. Similarly, incubation of m_3 muscarinic receptor expressing cells with increasing concentrations of carbachol, followed by SDS-PAGE and Western blot analysis showed an absence of agonist effect on m_3 receptor dimer formation [23].

In the case of opioid receptors, agonist treatment had either no effect (κ receptors) or caused a decrease in the level of dimers (δ receptors) [58, 59]. Whereas several selective and nonselective agonists could decrease the level of dimers (with a concomitant increase in the level of monomers), morphine at concentrations that elicit functional responses did not decrease the level of δ dimers [58]. Taken together, these results propose differential effects of agonists in modulating the levels of GPCR dimers.

Functional implications of homodimerization

The function of GPCR dimerization/oligomerization has been addressed either through the use of peptides that block dimerization or mutant receptors that do not dimerize. Treatment of β_2-adrenergic receptors with a peptide that blocks dimerization (TM VI peptide) resulted in a decrease in agonist-mediated adenylyl cyclase activity [22]. Agonist treatment of membranes expressing the receptor induced a modest but reproducible increase in the amount of dimer in a time-dependent manner; agonist treatment also protected the dimer from the disruptive effect of TM VI peptide [22]. These observations suggest that agonist-induced dimerization is the mechanism by which the receptor activates Gs. Alternatively, dimerization may be the consequence of the interaction of the receptor with other membrane proteins. However, this appears less likely since an agonist-induced increase in the amount of receptor dimer was also observed with purified β_2-adrenergic receptors [22]. The role of dimerization in receptor activity was studied using a mutant β_2-adrenergic receptor (C341G) that exhibited lower levels of maximal agonist-stimulated adenylate cyclase activity i.e., a constitutively desensitized receptor [57]. If there was no functional interaction between the mutant and wild-type receptors, an intermediate phenotype for receptor activity would be expected upon coexpression of these two. In contrast to the expected results, full receptor activity was observed, suggesting an interaction between wild-type and mutant receptors [57]. Coexpression of the C314G mutant receptors with another β_2-adrenergic receptor mutant (S261, 262, 345, 346A), which has mutations in the two consensus protein kinase A sites and is somewhat resistant to agonist promoted desensitization, resulted in a receptor that was able to induce adenylate cyclase signaling with an efficacy similar to that of wild-type receptors [57]. This indicated that both the wild-type and mutant (S261, 262, 345, 346A) β_2-adrenergic receptors acted in a dominant positive fashion complementing the C314G phenotype [57].

A double cysteine mutant of the muscarinic m_3 receptor, which does not form disulfide bonded dimers (al-

though it forms noncovalent receptor dimers and multimers), displayed a greater than 50-fold reduction in binding affinities for agonists and antagonists than the wild-type receptor. Additionally, phosphoinositol assays showed that this mutant receptor exhibited substantially less agonist potency (>10,000-fold) than m_3 wild-type receptor [23].

Cotransfection of a mutant of the human platelet-activating factor receptor that did not exhibit coupling to G proteins (D63N) with the wild-type receptor resulted in the formation of a constitutively active receptor phenotype [72]. Agonist-induced inositol phosphate production was higher in cells transfected with a 1:1 ratio of wild-type:D63N mutant than with wild-type alone [72]. Controls showed that the mutant receptor alone could not induce inositol phosphate production in response to agonist stimulation. The same set of studies showed that coexpression of wild-type chemokine receptor CCR2B with a carboxyl-terminal deletion mutant resulted in a decreased affinity and responsiveness to agonists [72]. These studies suggest that dimerization plays an important role in the modulation of signal transduction in addition to influencing agonist affinity.

In the case of the calcium-sensing receptor the functional consequences of cysteine to serine mutations on dimerization and signaling were examined by studying the effect of agonists on the accumulation of inositol phosphates in HEK293 cells [73]. The double point mutant receptor (C101S/C236S), which did not exhibit covalent dimerization, had a decreased affinity for extracellular Ca^{+2} and slower response kinetics with agonists [65]. Coexpression in HEK293 cells of specific pairs of mutant calcium-sensing receptors, each with a reduced or absent activity because of specific loss-of-function mutations, led to the partial reconstitution of extracellular calcium-dependent signaling [74], suggesting a role for dimerization in affecting signaling of this receptor.

The extent of involvement of dimerization in signaling was examined through the use of a number of deletion mutants of V_2 vasopressin receptor. Cotransfection of mutant V_2 vasopressin receptors, truncated by introduction of a translation stop codon into either the third intracellular loop, second intracellular loop, or second extracellular loop, with the wild-type V_2 receptor into COS-7 cells, led to an inhibition of maximum binding and of agonist-stimulated cyclic adenosine monophosphate (cAMP) levels. Little or no inhibition of agonist-induced cAMP responses was seen when receptors truncated at either the i_1 or e_1 loops were cotransfected with the wild-type receptor. The observed inhibition of agonist-stimulated cAMP levels by i_2, i_3, and e_2 mutant receptors, which do not have the intracellular domains predicted to be involved in G protein coupling, suggests that the observed effects did not occur via the sequestration of the stimulatory G protein [62]. In the case of the SSR coexpression of mutants that lack the second extracellular loop (and therefore lack ligand binding) with mutants lacking the C-terminal tail (and therefore lack signaling) led to a decrease in cAMP production in response to somatostatin 28 [75].

The role of dimers/oligomers of α-mating factor receptor (STE2) in signaling has been demonstrated by coexpressing a dominant interfering mutant of the receptor with the wild-type receptor; this led to the attenuation of signaling efficiency [69]. Signal attenuation was not due to G protein sequestration since the effect was observed even after overexpression of G proteins (α, β, and γ). Expression of the untagged mutant receptors with cyan fluorescent protein and yellow fluorescent protein tagged wild-type receptors led to the disruption of FRET efficiency. These results support a role for dimerization in modulation of receptor signaling. The α-mating factor receptors also undergo agonist-mediated internalization [71]. Interestingly, coexpression of an endocytosis defective mutant with the wild-type receptor led to efficient internalization of the mutant receptor, supporting a role for receptor dimerization in agonist-mediated endocytosis.

Dimerization appears to play a role in the endocytosis of opioid receptors [58, 76]. δ opioid receptors undergo agonist-induced monomerization [58]. Only agonists that induce receptor internalization are also able to induce receptor monomerization. Furthermore, a C-tail deletion mutant (ΔC15) that is dimerization deficient is unable to undergo rapid agonist-induced internalization. This suggests an active role for receptor monomerization in agonist-mediated endocytosis. It is interesting to note that κ receptors that do not undergo agonist-induced internalization are not able to undergo agonist induced monomerization [76] consistent with a role for receptor monomerization in agonist-mediated endocytosis.

In the case of bradykinin B_2 receptors dimerization does not appear to play a role in signal transduction since a mutant receptor truncated at the N-terminus, which did not exhibit agonist-induced receptor dimerization, behaved as the wild-type receptor with respect to agonist mediated increases in inositol phosphate levels [44]. Interestingly, these mutant receptors were not phosphorylated by G-protein coupled receptor kinase 2, did not undergo desensitization or significant agonist-mediated internalization or downregulation suggesting a role for dimerization in the trafficking of these receptors [44].

Heterodimerization

Pharmacological studies

Several lines of evidence have suggested the existence of GPCR heterodimers (Table 2). A phenomenon called "intramembrane receptor-receptor interaction" provided the first clues about the possible existence of GPCR heteromers. These studies showed that the binding of a neurotransmitter or modulator to its receptor modifies the characteristics of the receptor for another neurotransmitter or modulator [77, 78, 79, 80, 81]. Studies using chimeric receptors between the α_{2c}-adrenergic receptor and

Table 2 Heterodimerization of G protein coupled receptors

Receptor	Technique	Year	References
β_2-Adrenergic–α_2-adrenergic	Binding studies	1980	77
Cholecystokinin–dopamine	Binding studies	1981	78
Vasoactive intestinal peptide–serotonin	Binding assays	1983	79
α_{2c}-Adrenergic–m_3 muscarinic	Binding assays	1993	82
$GABA_BR1$–$GABA_BR2$	Immunoprecipitation	1998, 1999	89, 90, 91, 92
κ–δ Opioid	Immunoprecipitation	1999	59
M_2–M_3 muscarinic	Binding assays	1999	120
Serotonin 1B–1D	Immunoprecipitation	1999	93
Adenosine A_1–D_1 dopamine	Western blot	2000	99
Angiotensin AT_1–bradykinin B_2	Cross-linking/Western blots	2000	98
μ–δ Opioid	Immunoprecipitation	2000	95, 96
Somatostatin SSTR1–SSTR5	Binding assays, trafficking	2000	69
SSTR5–D_2 dopamine	Binding assays, pbFRET	2000	75
β_2-Adrenergic–δ opioid	Immunoprecipitation	2001	97
β_2-adrenergic–κ opioid	Immunoprecipitation	2001	97

m_3 muscarinic receptor, in which transmembrane domains VI and VII were exchanged (α_{2c}/m_3 and m_3/α_{2c}), also suggested the existence of GPCR heterodimers. When transfected separately the chimeric receptors were not able to bind to the specific radiolabeled antagonists [82]. However, when the receptors were coexpressed specific binding sites could be detected [82]. These sites displayed ligand binding properties similar to those of the two wild-type receptors providing support to the notion that the chimeric receptors heterodimerize to generate the ligand binding site [82]. Several pharmacological studies have suggested the possible heterodimerization between opioid receptor subtypes (for review see [83]). δ receptor ligands have been shown to modulate μ receptor mediated antinociception [84, 85]. Chronic treatment with morphine leads to the selective upregulation of a subpopulation of δ opioid receptors and treatment with β-funaltrexamine, an irreversible μ-antagonist, selectively modifies the binding of agonists for receptors [86, 87, 88]. These studies led Rothman et al. [86, 88] to divide the δ receptors into two subtypes, those that are associated with μ receptors (μ-δ complex) and those that are not. Recent studies with coexpression of cloned receptor cDNAs have allowed the critical evaluation of this issue.

Biochemical characterization

Direct evidence for GPCR heterodimerization between closely related members have come from studies with several members of family A GPCRs [e.g., γ-amino butyric acid B, opioid, serotonin, and SSRs) [59, 75, 89, 90, 91, 92, 93]. The γ-amino butyric acid B receptor ($GABA_BR$) 1 is a seven-transmembrane domain protein that has a high affinity for $GABA_BR$ antagonists [89, 90, 91, 92]. $GABA_BR1$ can account for some but not all of the functional properties of the native $GABA_BR$. Use of the yeast two-hybrid system (Y2H) and the COOH-terminal intracellular region of $GABA_BR1$, as a bait to screen a rat brain cDNA library as well as GenBank database searches for expressed sequence tags showing a high degree of homology to the $GABA_BR1$ sequence, led to the identification of a previously unidentified protein with seven internal hydrophobic segments characteristic for seven-TM GPCR proteins [94]. This protein exhibited a 36% amino acid sequence identity with $GABA_BR1$ and was denoted as "$GABA_BR2$" [94]. *In situ* hybridization of serial rat brain sections indicated that $GABA_BR1$ and $GABA_BR2$ show considerable overlap in distribution especially in the cerebellum, cortex, and medial habenula [94]. The pattern as well as the strength of expression of the mRNAs is largely consistent with the distribution and density of the $GABA_B$ receptor binding sites in the brain [94]. Differential epitope tagging of $GABA_BR1$ (RGS6His tag) and $GABA_BR2$ (HA tag) and immunoprecipitation of enriched plasma membrane fractions with anti-His antibody followed by Western blotting with anti-HA antibody detected the presence of heterodimers only in cells coexpressing both receptor proteins [89]. This indicates that both proteins are correctly targeted to the plasma membrane of HEK293 cells, and that they exist in a heteromeric complex, probably as dimers [89]. More importantly, antisera directed against endogenous $GABA_BR1$ and $GABA_BR2$ receptors were also able to immunoprecipitate endogenous receptor dimers from rat brain, supporting the presence of dimers in vivo [90, 92].

Recent studies have provided direct evidence for the heterodimerization of opioid receptors. The ability of κ opioid receptors to heterodimerize with δ opioid receptors was investigated by coexpressing differentially *myc*-tagged κ receptors with Flag-tagged δ receptors [59]. Flag-tagged δ opioid receptor was detected in material immunoprecipitated using antibodies specific to *myc*-tagged κ opioid receptor. The selectivity of heterodimerization was demonstrated using Flag-tagged μ opioid receptor; this receptor could not be immunoprecipitated with κ receptors under similar coprecipitation conditions [59]. This indicates that κ receptors can selectively dimerize with δ receptors. In order to ensure that the dimerization was not induced by the extraction and/or isolation procedure, cells individually expressing κ or δ receptors were mixed prior to extraction. Under identical extraction/immunoprecipitation conditions heterodimers

were not observed in the mixed cells, suggesting that the heterodimers were not artifacts of extraction [59]. κ-δ heterodimers are stable in a variety of detergents and extraction procedures and are destabilized by the treatment of cells with a reducing agent prior to extraction, suggesting a role for disulfide bonds in κ-δ heterodimerization. This study constituted the first direct evidence for the heterodimerization between opioid receptor types and also between two fully functional GPCRs [59]. Recently it has been shown that δ opioid receptors can dimerize with μ opioid receptors. These dimers are sensitive to reducing agents such as DTT and are not induced during solubilization/immunoprecipitation conditions because they are not seen in immunoprecipitates from a mixture of cells individually expressing μ and δ receptors [95, 96].

The serotonin (5-hydroxytryptamine) 1B and 1D receptor subtypes share a high amino acid sequence identity and have similar ligand binding properties [93]. A recent study has shown that both receptor subtypes exist as homodimers in addition to monomers when expressed alone and as heterodimers when coexpressed [93]. These results demonstrated the physical association of two GPCR subtypes. No heterodimers were observed when membranes expressing one receptor subtype were mixed with membranes expressing the other subtype, indicating that heterodimerization does not occur from the nonspecific aggregation of receptors. In addition only heterodimers and not homodimers were observed when both receptor subtypes were coexpressed on the same cells suggesting that the receptors favor the heterodimeric conformation [93].

A series of recent studies have shown that distantly related members of family A can heterodimerize with each other. These include δ and κ opioid receptors with β_2-adrenergic receptors [97], somatostatin receptors with D_2 dopamine receptors [75], angiotensin 2 receptors with bradykinin B_2 receptors [98], and A_1 adenosine receptors with D_1 dopamine receptors [99].

Functional implications of heterodimerization

Heterodimerization of GPCRs could modulate receptor function by regulating ligand binding properties, signaling as well as receptor trafficking properties. The first set of studies to address a role for heterodimerization was with receptor chimeras of the α_{2c}-adrenergic receptor and m_3 muscarinic receptor [82]. These chimeras were generated by exchanging the C-terminal region of the receptor, including transmembrane domains VI and VII (α_2/m_3 and m_3/α_2). Individually, the chimeras were able to signal only to a small extent. However, upon coexpression there was a pronounced increase in agonist-stimulated inositol monophosphate levels; a mutant m_3 muscarinic receptor generated by replacing a portion of the intracellular loop 3 with the corresponding m_2 receptor sequence showed a loss of stimulation of phosphoinositol hydrolysis. This receptor was cotransfected with either a truncated m_3 receptor (that was unable to mediate a functional response) or with a mutant m_3 receptor (containing a point mutation in transmembrane domain VII). This resulted in a greater level of signaling than with cells individually expressing these receptors [82]. These results provided evidence for the involvement of heterodimerization in modulation of receptor signaling.

Radioligand binding studies showed that in cells transiently transfected with $GABA_BR2$ there is no labeling with agonists. Also cells expressing recombinant $GABA_BR1$ exhibited 100- to 150-fold less binding potency for agonists than native receptors. However, in cells coexpressing $GABA_BR1$ with $GABA_BR2$ a tenfold increase in binding potency was observed with agonists and partial agonists [89, 90, 92]. A crucial physiological effect mediated by native $GABA_B$ receptors is the activation of outward potassium currents through the opening of G protein coupled inwardly rectifying K^+ channels (GIRKs). Reconstitution of $GABA_BR1$ or $GABA_BR2$ alone with GIRK1 and GIRK2 in HEK293 cells failed to mediate GIRK activation. However, coexpression of both receptor proteins mediated a robust increase in potassium conductance through GIRK activation in a pertussis toxin sensitive manner [89, 90, 92]. Thus, the physical interaction between $GABA_BR1$ and $GABA_BR2$ appears to be essential for the coupling of $GABA_B$ receptors to GIRKs [89, 90, 91, 92]. Also, coexpression of $GABA_BR1$ and $GABA_BR2$ (in combination with exogenous Gα in HEK293 cells resulted in a robust, agonist-dependent stimulation of [^{35}S]guanosine triphosphate-γ-S binding similar to that observed in rat brain membranes [92], supporting a role for heterodimerization in regulating the efficiency of coupling.

The ligand binding properties of cells expressing κ-δ heterodimers were compared with those of either κ or δ opioid receptors [59]. κ opioid receptors have a high affinity for the selective agonist (+)-(5a,7a,8b)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro [4,5]dec-8-yl]-benzeneacetamide (U69593) and antagonist norbinaltorphimine. Similarly δ opioid receptors have a high affinity for the selective agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE) and antagonist H-Tyr-Tic[ψ,CH2NH]Phe-Phe-OH (Tic=1,2,3,4-tetrahydroisoquinoline (TIPPψ). κ-δ heterodimers do not exhibit high affinity for either κ or δ opioid receptor selective agonists or antagonists [59]. However, the heterodimer binds to partially selective ligands with affinities that are virtually identical to those previously reported for κ_2 receptor subtype [59]. Furthermore, in the presence of a δ selective agonist (DPDPE) a κ agonist (U69593) binds the heterodimer with high affinity. Similarly, in the presence of the κ selective agonist (U69593) the δ agonist DPDPE binds the heterodimers with high affinity [59]. These results suggest that κ-δ heterodimers cooperatively bind to selective agonists. Interestingly, whereas a combination of two selective antagonists, norbinaltorphimine and TIPPψ, also binds with high affinity, a combination of a selective agonist, U69593, and a selective antagonist, TIPPψ, does not [59]. Also, synergistic binding is not observed in

membranes from cells individually expressing κ or δ opioid receptors. These results imply that heterodimerization of κ and δ receptors change the ligand binding properties of the receptor [59].

A significant alteration in ligand binding properties was also observed in cells coexpressing μ-δ receptors. These heteromers exhibited a substantial decrease in affinity for a number of selective ligands such as [D-Ala^2,MePhe^4-Gly^5-ol]enkephalin (DAMGO), DPDPE, and Deltorphin II. In these cells agonist binding to heteromers was insensitive to pertussis toxin treatment probably due to interaction with a different type of G protein [95]. Additional novel pharmacology of μ-δ heterodimers was found in whole cell binding assays by treatment of cells with a combination of μ and δ selective ligands. Extremely low doses of certain δ or μ selective ligands were able significantly to increase the number of binding sites for a μ or δ receptor agonist, respectively. These effects were observed only in cells coexpressing both receptor types and not in cells expressing each receptor individually [96].

The effect of heterodimerization on signal transduction was examined by determining opioid mediated decrease in the cAMP levels and phosphorylation of mitogen-activated protein kinase (MAPK) [59]. In cells coexpressing κ and δ receptors, selective agonists showed a dose-dependent decrease in the levels of cAMP and phosphorylation of MAPK [59]. Interestingly, treatment of the cells with a combination of ligands showed a 20-fold increase in potency for the decrease in cAMP levels and a 7-fold increase in potency for the level of increase in phosphorylated MAPK [59]. In membranes of cells coexpressing μ and δ opioid receptors DAMGO and DPDPE were able to inhibit forskolin-stimulated cAMP levels even after treatment with pertussis toxin suggesting the involvement of a pertussis toxin-insensitive G protein [95]. In addition treatment of cells coexpressing μ and δ opioid receptors with the TIPPΨ (δ antagonist) or Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (Deltorphin II, δ agonist) led to a potentiation in DAMGO (μ agonist) induced phosphorylation of MAPK. Also, the μ antagonist D-Phe-[Cys-Thr-D-Trp-Orn-Pen]-Thr-NH_2 CTOP was able to significantly increase the potency and efficacy of MAPK phosphorylation by Deltorphin II [96]. These results suggest that the heterodimerization of κ with δ receptors and of μ with δ receptors modulates the efficacy of agonists.

A role for heterodimerization of κ and δ receptors in agonist-mediated trafficking was examined using a universal opioid agonist, etorphine [59, 76]. κ opioid receptors do not internalize upon treatment with etorphine whereas δ receptors exhibit a robust rate of internalization. However, the extent of δ receptor internalization was found to be significantly lower when coexpressed with κ receptors [59]. These results support a role for opioid receptor heterodimerization in receptor trafficking.

Heterodimerization of differentially tagged SSR1 and SSR5 receptors was demonstrated using confocal microscopy [69]. In order to evaluate the role of these heterodimers, mutant receptors that are unable to signal were used in combination with ligands that bind selectively to these two receptors [69]. Coexpression of a C-tail deletion mutant of SSR1 (that binds somatostatin 28 but is unable to transduce signal) with a mutant SSR5 receptor (that is unable to bind to somatostatin 28) resulted in a significant reduction in cAMP levels supporting a role for heterodimerization in modulating signaling [69]. It appears that heterodimerization also plays a role in SSR trafficking [69]. Since SSR1 does not undergo agonist-mediated endocytosis and SSR5 does, the effect of SSR5 selective ligand on the trafficking of SSR1 was examined in cells coexpressing the receptors. It was found that SSR1 is internalized only as part of a heterodimer when coexpressed with SSR5 supporting a role for heterodimerization in receptor trafficking [69].

Heterodimerization of distantly related members of family A GPCRs alter their functional properties. D_2 dopamine receptors and SSTR5 are found to colocalize to the same neuronal subgroups in the striatum [75]. FRAP analysis was used to demonstrate agonist-mediated dimerization of these two receptors expressed in heterologous cells [75]. Studies examining functional interaction between these two receptors revealed that the heterodimer exhibits higher affinity for a combination of two selective agonists of dopamine receptor and SSTR5 receptor than for a single agonist alone [75].

A very recent study has demonstrated that heterodimerization of β_2-adrenergic receptor (stimulatory G proteins) with either the κ or δ opioid receptor (inhibitory G proteins) did not significantly alter the ligand binding properties and the ability to signal through adenylyl cyclase. However, it affected the trafficking properties since δ receptors associated with β_2-adrenergic receptors undergo isoproterenol-mediated endocytosis. The reciprocal is also true in that the β_2 adrenergic receptors undergo etorphine-induced endocytosis. However, β_2 receptors when coexpressed with κ opioid receptors do not undergo either isoproterenol or etorphine-mediated endocytosis [97]. These results suggest that heterodimerization of GPCRs that couple to different G proteins can lead to the modulation of receptor trafficking properties.

Domains involved in the dimerization/oligomerizaiton

Examination of the possible site(s) of receptor interactions implicated a role for extracellular, transmembrane and/or C-terminal region in GPCR dimerization. Receptor dimerization, brought about by the association of two monomers, could be mediated either by covalent (disulfide) and/or noncovalent (hydrophobic) interactions; these could be association of the extracellular domains, transmembrane domains and/or C-terminal tail. Several studies with a number of GPCRs have suggested that a combination of the above mentioned interactions occur during dimerization.

Studies carried out with m_3 muscarinic receptors have implicated the involvement of both covalent (disulfide)

as well as noncovalent interactions in dimerization [23]. Mutant receptors where specific cysteine residues were replaced with either alanine or serine indicated that Cys-140 and Cys-220 were involved in disulfide bond formation. Interestingly, cotransfection of the double mutant (C140A/C220A) receptors resulted in the formation of dimers, suggesting that m_3 muscarinic receptors are able to form noncovalent receptor dimers or multimers [23].

The involvement of the extracellular domain of the calcium-sensing receptor in disulfide bonded dimer formation was shown by the stable transfection of HEK293 cells with a mutant form of the receptor engineered to result in the secretion of the extracellular domain into the culture medium [100]. Western blot analysis of this protein showed that under nonreducing conditions it migrated as a band of greater than 200 kDa (glycosylated dimer) while under reducing conditions it migrated as a band of 78 kDa (glycosylated monomer) with a minor band of 48 kDa (proteolytic cleavage product). The extracellular domain protein is therefore able to dimerize using an intermolecular disulfide bond capable of being totally reduced by reducing agents [100]. The extracellular domain of the calcium-sensing receptor has four conserved cysteine residues, and although individual cysteine to serine mutations did not completely eliminate dimerization, mutations at C101S and C236S increased the amount of monomer observed under nonreducing conditions [65]. The double point mutant of the receptor (C101S/C236S)-GFP was present mostly in the monomeric form in the absence or presence of reducing agents, although noncovalent dimers could be detected in some cases [65]. Crystallographic studies have confirmed that the extracellular binding domain of metabotropic glutamate receptor is present in the form of disulfide-linked homodimers [101]

The involvement of the extracellular domain of bradykinin B_2 receptors in dimerization was investigated by incubating peptides corresponding to the amino acid sequence of the amino-terminus or connecting extracellular loops e_1, e_2, or e_3 with PC-12 membranes [44]. Receptor dimerization was examined following incubation with radioiodinated agonist followed by cross-linking of the receptor-ligand complex. Among these peptides, only addition of the peptide corresponding to the amino terminus reduced the levels of dimers, suggesting that this region is involved in triggering B_2 receptor dimerization [44].

The involvement of disulfide bonds in the dimerization of the V_2 vasopressin receptor was suggested by the observed decrease in the levels of dimers with concomitant increase in the levels of monomer after treatment with DTT prior to SDS-PAGE [62]. In these studies a sulfhydryl alkylating agent, *N*-ethylmaleimide was used to rule out the artifactual formation of dimers due to disulfide bond exchange reactions [62]. Similar involvement of disulfide bonds in the formation of dimers has been suggested in the case of Ig-Hepta receptors [64] and κ opioid receptors [59] as well as heterodimers of κ and δ opioid receptors [59].

Transmembrane domains have also been implicated in GPCR dimerization. In the case of receptor dimers involving disulfide bonds transmembrane domains could provide the proper receptor conformation to facilitate the formation of these bonds. An involvement of transmembrane domains in receptor dimerization has been suggested for β_2-adrenergic and the dopamine receptors; disulfide bonds are not thought to be required for the dimerization of these receptors. β_2-adrenergic receptors have the dimerization motif, $^{75}LIXXGVXXG^{83}VXXT$, found also in glycophorin A [102] where a critical Gly^{83} appears to be essential for dimerization. This motif is present in the TM VI of β_2-adrenergic receptors [22]. This suggested the involvement of the dimerization motif in the formation of β_2-adrenergic receptor dimers. To test this hypothesis, membranes from cells expressing β_2-adrenergic receptors were incubated with a TM VI derived peptide. This led to a drastic reduction in the level of dimers within a short time after peptide treatment. A smaller reduction in the level of dimers was observed with a modified TM VI peptide where Gly-276, Gly-280, and Leu-284 were replaced with alanine [22]. This suggests that these three residues may be part of the interface between two receptor monomers. Studies with affinity purified β_2-adrenergic receptor incubated with increasing concentrations of TM VI peptide also showed a modest but reproducible increase in the apparent molecular weight of the monomer [22]. This suggests that the peptide forms a stable complex with the receptor monomer thus mimicking receptor-receptor interactions [22]. Taken together these results support the involvement of TM VI in β_2-adrenergic receptor dimerization.

Involvement of TM VI and TM VII in dimerization has been suggested for D_2 dopamine receptors since peptides corresponding to these regions dissociate dimer formation [24]. A small increase in the molecular mass of the receptor monomer was seen after peptide treatment; this suggested the formation of a peptide-D_2 receptor heterodimer confirming the involvement of these regions in dimerization [24]. These D_2 dimers are dissociated when the receptors are subjected to increasing temperature or acid pH (approx. 3). Taken together these results suggest that D_2 receptor dimerization is mediated at the protein level via specific intermolecular, noncovalent, electrostatic interaction of residues within transmembrane α-helices [24]. In the case of the D_1 dopamine receptor a peptide based on the sequence of TM VI did not affect receptor dimerization, although it affected ligand binding and receptor function [103]. This suggests that D_1 dopamine receptors may undergo dimerization by a mechanism independent of what has been proposed for the D_2 and β_2-adrenergic receptors [103].

The C-tail mediated dimerization has been well documented in the case of the $GABA_BR1$ and $GABA_BR2$ receptors. Glutathione S-transferase (GST) pull-down assays were used to confirm the involvement of the C-terminal region in the heterodimerization of $GABA_BR1$ and $GABA_BR2$ receptors. Western blot analysis indicated that only the GST fusion proteins containing the

$GABA_BR1$–C-terminus or the $GABA_BR1$ heterodimerization domain ($GABA_BR1\Delta7$), but not GST alone, were able to coprecipitate the $GABA_BR2$-C-terminus. No dimerization between $GABA_BR1$-C-termini was detected [91, 90] in these assays. Using this assay a critical role for coiled-coil domain (within the C-tail) in dimerization was reported [91, 92]. Similar studies have also suggested the involvement of the C-tail in the dimerization of metabotropic glutamate receptors [67].

A role for the C-terminal tail in dimerization of δ opioid receptors has been proposed [58] since a mutant receptor with a C-terminal 15 amino acid deletion does not exhibit significant level of dimers compared with the wild-type receptor [58]. A mutant receptor with a deletion of 7 amino acids is able to dimerize, suggesting that a portion of the receptor C-tail plays a role in the dimerization of this receptor (Cvejic and Devi, unpublished). However, the C-tail does not appear to be involved in the heterodimerization of μ and δ opioid receptors since a protein band representing the μ-δ heterodimer is seen when a mutant μ receptor lacking C-terminal 42 amino acids is cotransfected with wild-type δ receptors [96].

Mechanism of dimerization

Two mechanisms have been proposed for GPCR dimerization/oligomerization. One involves the association of 1:1 stoichiometric molecular complexes of receptors and the other involves the swapping of domains between two distinct receptor molecules resulting in a single binding domain [103].

Computer simulations of a model β_2-adrenergic receptor, built on the crystallographic data obtained from rhodopsin, were used to examine the mechanism of dimerization. This model was found to be consistent with known site-directed mutagenesis information on the receptor [104] as well as with biophysical data obtained from substituted cysteine accessibility studies [105], site-directed spin-labeling studies and zinc binding studies [106, 107, 108, 109, 110, 111]. Models of dimers involving interactions between TM V and VI suggested a high-energy structure for both the apo dimer and the antagonist-bound dimer; the energy of the dimer was significantly lowered in the presence of agonist [104]. This led to the suggestion that the agonist-induced conformational change optimizes the helix-helix interactions at the 5-6 interface. From these observations it was hypothesized that the agonist shifts the equilibrium so as to favor the TM V and TM VI domain swapped dimer [112]. Indirect evidence for the domain swapping hypothesis, was provided by the correlated mutations among the external residues that occur mainly at the 5-6 interface at precisely the locations predicted by the simulations [113, 114, 115, 116]. However, further evaluation of the data led the authors to suggest that a distinction between the domain-swapped dimer and a contact dimer could not be made. Using evolutionary trace analysis and correlated mutations, the authors propose that in addition to the 5,6 interacting dimer (either as a contact dimer or domain-swapped dimer) a secondary interaction occurs at the 2,3 interface [112]; this would result in a tetrameric receptor and/or higher order oligomeric complexes.

Another mechanism of dimerization that has been proposed involves disulfide bond exchange between two GPCR monomers. Support for this comes from modeling studies with the δ opioid receptor [117]. These studies have suggested that the interaction of protonated opioid agonists is able to catalyze the cleavage of the disulfide bond formed between the first and second extracellular loops (Cys-121–Cys-198). The accompanying cleavage of the disulfide bond may produce a conformational change in the extracellular loops such that the opening formed by the seven-helix bundle opens permitting the entry of the ligand, water, and ions into the cell. Results from biochemical studies in which a brief exposure to agonist results in an increase in cell surface thiol concentration are consistent with such a notion [117].

An alternative model is based on the discovery that mutations in the i_3 region of different adrenergic receptor subtypes dramatically increases their agonist independent activity. This led to the hypothesis that GPCRs can exist in equilibrium between two interconvertible allosteric states R and R* that could represent monomeric and dimeric forms. Also comparison between the structural dynamic features of the wild-type α_{1b}-adrenergic receptor versus a constitutively active structure suggested that the highly conserved arginine of the DRY sequence at the cytosolic end of helix 3 plays a fundamental role in promoting receptor isomerization into functionally different states [118, 119].

Future areas of reseach

Although several lines of evidence suggest that GPCRs can dimerize, it remains to be established whether this phenomenon is a general characteristic of these receptors, and whether it is essential for receptor function. Also, studies need to be carried out to understand whether these receptors dimerize prior to targeting to the plasma membrane and to what extent GPCRs dimerize *in vivo*. In addition, the possible involvement of other proteins such as anchoring proteins, caveolins, chaperones, etc., in facilitating homo/heterodimerization of GPCRs needs to be explored. The use of sensitive energy transfer techniques such as FRET, FRAP, and BRET has been helpful in answering some of these questions. Development of tools that will allow selective detection and/or activation of dimers is required to examine the role of GPCR dimers in vivo. These include the development of dimer-specific antisera that would permit the localization of dimers as well as the generation of ligands that would selectively bind to and activate receptor dimers.

A complete understanding of the mechanisms of heterodimerization and its functional implications has enormous clinical significance as well as a great impact on GPCR pharmacology since it represents another mecha-

nism that could modulate receptor function and thus provides a new strategy for the development of novel therapeutic drugs.

Acknowledgements We thank Dr. J.A. Javitch for the gift of Flag-tagged human β_2-adrenergic receptor cDNA and D_2 dopamine receptor cDNA. This work was supported in part by NIH grants DA 08863 and DA 00458 (to L.A.D.) and the postdoctoral training grant DA 07254 (to B.A.J).

References

1. Bockaert J, Pin JP (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J 18:1723–1729
2. Coughlin SR (1994) Expanding horizons for receptors coupled to G proteins: diversity and disease. Curr Opin Cell Biol 6: 191–197
3. Schwartz TW (1994) Locating ligand-binding sites in 7TM receptors by protein engineering. Curr Opin Biotechnol 5:434–444
4. Strader CD, Fong TM, Tota MR, Underwood D, Dixon RA (1994) Structure and function of G protein-coupled receptors. Annu Rev Biochem 63:101–132
5. Dohlman HG, Thorner J, Caron MG, Lefkowitz RJ (1991) Model systems for the study of seven-transmembrane-segment receptors. Annu Rev Biochem 60:653–688
6. Savarese TM, Fraser CM (1992) In vitro mutagenesis and the search for structure-function relationships among G-protein coupled receptors. Biochem J 283:1–19
7. Hedin KE, Duerson K, Clapham DE (1993) Specificity of receptor-G protein interactions: searching for the structure behind the signal. Cell Signal 5:505–518
8. Wess J (1997) G protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. FASEB J 11:346–354
9. Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 21:90–113
10. Wess J (1998) Molecular basis of receptor/G-protein-coupling selectivity. Pharmacol Ther 80:231–264
11. Laburthe M, Couvineau A, Gaudin P, Maoret J-J, Rouyer-Fessard C, Nicole P (1996) Receptors for VIP, PACAP, secretin, GRF, glucagon, GLP-1, and other members of their new family of G protein-linked receptors: structure-function relationship with special reference to the human VIP-1 receptor. Ann NY Acad Sci 805:94–111
12. Brown EM, Segre GV, Goldring SR (1996) Serpentine receptors for parathyroid hormone, calcitonin and extracellular calcium ions. Baillieres Clin Endocrinol Metab 10:123–161
13. Salahpour A, Angers S, Bouvier M (2000) Functional significance of oligomerization of G protein-coupled receptors. Trends Endocrinol Metab 11:163–168
14. McLachie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG, Foord SM (1998) RAMPs regulate the transport and ligand specificity of he calcitonin-receptor-like receptor. Nature 393:339
15. Limbird LE, Meyts PD, Lefkowitz RJ (1975) Beta-adrenergic receptors: evidence for negative cooperativity. Biochem Biophys Res Commun 64:1160–1168
16. Limbird LE, Lefkowtiz RJ (1976) Negative cooperativity among beta-adrenergic receptors in frog erythrocyte membranes. J Biol Chem 251:5007–5014
17. Mattera R, Pitts BJ, Entman ML, Birnbaumer L (1985) Guanine nucleotide regulation of mammalian myocardial muscarinic receptor system. Evidence for homo- and heterotropic cooperativity in ligand binding analyzed by computer-assisted curve fitting. J Biol Chem 260:7410–7421
18. Galper GB, Haigh LS, Hart AC, O'Hara DS, Livingston DS (1987) Muscarinic cholinergic receptors in embryonic chick heart: interaction of agonist, receptor, and guanine nucleotides studied by an improved assay for direct binding of the muscarinic agonist 3H-cis-methyldioxolane. Mol Pharmacol 32:230–240
19. Potter LT, Ferrendelli CA, Hanchett HE (1988) Two affinity states of M1 muscarinic receptors. Cell Mol Neurobiol 8: 181–191
20. Potter LT, Ferrendelli CA (1989) Affinities of different cholinergic agonists for the high and low affinity states of hippocampal M1 muscarinic receptors. J Pharmacol Exp Ther 248:974–978
21. Potter LT, Ballesteros LA, Bichajian LH, Ferrendelli CA, Fisher A, Hanchett HE, Zhang R (1991) Evidence of paired M_2 muscarinic receptors. Mol Pharmacol 39:211–221
22. Hebert TE, Moffett S, Morello J-P, Loisel TP, Bichet DG, Barret C, Bouvier M (1996) A peptide derived from a β_2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. J Biol Chem 271:16384–16392
23. Zeng F-Y, Wess J (1999) Identification and molecular characterization of m3 muscarinic receptor dimers. J Biol Chem 274:19487–19497
24. Ng GY, O'Dowd BF, Lee SP, Chung HT, Brann MR, Seeman P, George SR (1996) Dopamine D2 receptor dimers and receptor-blocking peptides. Biochem Biophys Res Commun 227: 200–204
25. Salesse R, Remy JJ, Levin JM, Jallal B, Garnier J (1991) Towards understanding the glycoprotein hormone receptors. Biochimie 73:109–120
26. Vuhai-Luuthi MT, Jolivet A, Jallal B, Salesse R, Bidart JM, Houiller A, Guiochon-Mantel A, Garnier J, Milgrom E (1990) Monoclonal antibodies against luteinizing hormone receptor. Immunochemical characterization of the receptor. Endocrinology 127:2090–2098
27. Hazum E, Chang K-J, Leighton HJ, Lever OW, Cuatrecasas OP (1982) Increased biological activity of dimers of oxymorphone and enkephalin: possible role of receptor-crosslinking. Biochem Biophys Res Commun 104:347–353
28. Fraser CM, Venter JC (1982) The size of the mammalian lung β_2-adrenergic receptor as determined by target size analysis and immunoaffinity chromatography. Biochem Biophys Res Commun 109:21–29
29. Venter JC, Schaber JS, U'Prichard DC, Fraser CM (1983) Molecular size of the human platelet a2-adrenergic receptor as determined by radiation inactivation. Biochem Biophys Res Commun 116:1070–1075
30. Ott S, Costa T, Wuster M, Hietel B, Herz A (1986) Target size analysis of opioid receptors. No difference between receptor types, but discrimination between two receptor states. Eur J Biochem 155:621–630
31. McLawhon RW, Ellory JC, Dawson G (1983) Molecular size of opiate (enkephalin) receptors in neuroblastoma-glioma hybrid cells as determined by radiation inactivation analysis. J Biol Chem 258:2102–2105
32. Simon J, Benyhe S, Borsodi A, Wollemann M (1986) Hydrodynamic parameters of opioid receptors from frog brain. Neuropeptides 7:23–26
33. Glasel JA (1987) Physical characterization of native opiate receptors. Additional information from detailed analysis of a radiation-inactivated receptor Biochim Biophys Acta 930:201–208
34. Nielsen TB, Totsuka Y, Kempner ES, Field JB (1984) Structure of the thyrotropin receptor and thyroid adenylate cyclase system as determined by target analysis, Biochemistry 23: 6009–6016
35. Gennick SE, Thomas CG Jr, Nayfeh SN (1987) Characterization of the subunit structure of the thyrotropin receptor in the FRTL-5 rat thyroid cell line. Endocrinology 121:2119–2130
36. Conn PM, Venter JC (1985) Radiation inactivation (target size analysis) of the gonadotropin-releasing hormone receptor: evidence for a high molecular weight complex. Endocrinology 116:1324–1326

37. Shirakawa O, Tanaka C (1985) Molecular characterization of muscarinic receptor subtypes in bovine cerebral cortex by radiation inactivation and molecular exclusion h.p.l.c. Br J Pharmacol 86:375–383
38. Collins SM, Jung CY, Grover AK (1986) Muscarinic receptor size on smooth muscle cells and membranes. Am J Physiol 251:G195–G200
39. Gredal O, Nielsen M (1987) Binding of [3H] SKF 38393 to dopamine D-1 receptors in rat striatum in vitro: estimation of receptor molecular size by radiation inactivation. J Neurochem 48:370–375
40. Bai M, Trivedi S, Brown EM (1998) Dimerization of the extracellular calcium-sensing receptor (CaR) on the cell surface of CaR-transfected HEK293 cells. J Biol Chem 273:23605–23610
41. Rodriguez-Frade JM, Vila-Coro AJ, de Ana AM, Albar JB, Martinez-A C, Mellado M (1999) The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. Proc Natl Acad Sci USA 96:3628–3633
42. Zawarynski P, Tallerico T, Seeman P, Lee SP, O'Dowd BF, George SR (1998) Dopamine D2 receptor dimers in human and rat brain. FEBS Lett 441:383–386
43. Avissar S, Amitai G, Sokolovski YM (1983) Oligomeric structure of muscarinic receptors is shown by photoaffinity labeling: subunit assembly may explain high- and low-affinity agonist states. Proc Natl Acad Sci USA 80:156–159
44. Abdalla S, Zaki E, Lother H, Quitterer U (1999) Involvement of the amino terminus of the B2 receptor in agonist-induced receptor dimerization. J Biol Chem 274:26079–26084
45. Paglin S, Jamieson JD (1982) Covalent crosslinking of angiotensin II to its binding sites in rat adrenal membranes. Proc Natl Acad Sci USA 79:3739–3743
46. Rogers TB (1984) High affinity angiotensin II receptors in myocardial sarcolemmal membranes. Characterization of receptors and covalent linkage of 125I-angiotensin II to a membrane component of 116:000 daltons. J Biol Chem 259:8106–8114
47. Carson MC, Harper CM, Baukal AJ, Aguilera G, Catt KJ (1987) Physicochemical characterization of photoaffinity-labeled angiotensin II receptors. Mol Endocrinol 1:147–153
48. Rondeau JJ, McNicoll N, Escher E, Meloche S, Ong H, De Lean A (1990) Hydrodynamic properties of the angiotensin II receptor from bovine adrenal zona glomerulosa. Biochem J 268:443–448
49. Siemens IR, Swanson GN, Fluharty SJ, Harding JW (1991) Solubilization and partial characterization of angiotensin II receptors from rat brain. J Neurochem 57:690–700
50. Capponi AM, Catt KJ (1980) Solubilization and characterization of adrenal and uterine angiotensin II receptors after photoaffinity labeling. J Biol Chem 255:12081–12086
51. Guillemette G, Escher E (1983) Analysis of the adrenal angiotensin II receptor with the photoaffinity labeling method. Biochemistry 22:5591–5596
52. Ng GY, O'Dowd BF, Caron M, Dennis M, Bran MR, George SR (1994) Phosphorylation and palmitoylation of human D2L dopamine receptor in Sf9 cells. J Neurochem 63:1589–1595
53. Nimchinsky EA, Hof PR, Janssen WG, Morrison JH, Schmauss C (1997) Expression of dopamine D3 receptor dimers and tetramers in brain and in transfected cells. J Biol Chem 272:29229–29237
54. Ban T, Kosugi S, Kohn LD (1992) Specific antibody to the thyrotropin receptor identifies multiple receptor forms in membranes of cells transfixed with wild-type receptor complementary deoxyribonucleic acid: characterization of their relevance to receptor synthesis, processing, structure, and function. Endocrinology 131:815–829
55. Grossman RF, Ban T, Duh QY, Tezelman S, Jossart G, Soh EY, Clark OH, Siperstein AE (1995) Immunoprecipitation isolates multiple TSH receptor forms from human thyroid tissue. Thyroid 5:101–105
56. Indrapichate K, Meehan D, Lane TA, Chu SY, Rao CV, Johnson D, Chen TT, Wimalasena J (1992) Biological actions of monoclonal luteinizing hormone/human chorionic gonadotropin receptor antibodies. Biol Reprod 46:265–278
57. Hebert TE, Loisel TP, Adam L, Ethier N, Onge SS, Bouvier M (1998) Functional rescue of a constitutively desensitized β_{2}-AR through receptor dimerization. Biochem J 330:287–293
58. Cvejic S, Devi LA (1997) Dimerization of the d opioid receptor: implication for a role in receptor internalization. J Biol Chem 272:26959–26964
59. Jordan BA, Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. Nature 399:697–700
60. Fukushima Y, Asano T, Saitoh T, Anai M, Funaki M, Ogihara T, Katagiri H, Matsuhashi N, Yazaki Y, Sugano K (1997) Oligomer formation of histamine H2 receptors expressed in Sf9 and COS7 cells. FEBS Lett 409:283–286
61. Ali H, Richardson RM, Tomhave ED, DuBose RA, Haribabu B, Snyderman R (1994) Regulation of stably transfected platelet activating factor receptor in RBL-2H3 cells. Role of multiple G proteins and receptor phosphorylation. J Biol Chem 269:24557–24563
62. Zhu X, Wess J (1998) Truncated V2 vasopressin receptors as negative regulators of wild-type v2 receptor function. Biochemistry 37:15773–15841
63. Wreggett KA, Wells JW (1995) Cooperativity manifest in the binding properties of the cardiac muscarinic receptors. J Biol Chem 270:22488–22499
64. Abe J, Suzuki H, Notoya M, Yamamoto T, Hirose S (1999) Ig-hepta, a novel member of the G protein-coupled hepta-helical receptor (GPCR) family that has immunoglobulin-like repeats in a long N-terminal extracellular domain and defines a new subfamily of GPCRs. J Biol Chem 274:19957–19964
65. Pace AJ, Gama L, Breitwieser GE (1999) Dimerization of the calcium-sensing receptor occurs within the extracellular domain and is eliminated by Cys→Ser mutations at Cys^{101} and Cys^{236}. J Biol Chem 274:11629–11634
66. Ward DT, Brown EM, Harris HW (1998) Disulfide bonds in the extracellular calcium-polyvalent cation-sensing receptor correlate with dimer formation and its response to divalent cations in vitro. J Biol Chem 273:14476–14483
67. Robbins MJ, Ciruela F, Rhodes A, MeIlhinney RA (1999) Characterization of the dimerization of metabotropic glutamate receptors using an N-terminal truncation of mGluR1α. J Neurochem 72:2539–2547
68. Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M (2000) Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci USA 97:3684–3689
69. Rocheville M, Lange DC, Kumar U, Sasi R, Patel RC, Patel YC (2000) Subtypes of the somatostatin receptor assemble as functional homo and heterodimers. J Biol Chem 275:7862–7869
70. Cornea A, Janovick JA, Maya-Nunez G, Conn PM (2001) Gonadotropin-releasing hormone receptor microaggregation. J Biol Chem 276:2153–2158
71. Overton MC, Blumer KJ (2000) G-protein-coupled receptors function as oligomers in vivo. Curr Biol 10:341–344
72. Le Gouill C, Parent J-L, Caron C-A, Gaudreau R, Volkov L, Rola-Pleszczynski M, Stankova J (1999) Selective modulation of wild type receptor functions by mutants of G-protein coupled receptors. J Biol Chem 274:12548–12554
73. Fan G-F, Ray K, Zhao X-M, Goldsmith PK, Spiegel AM (1998) Mutational analysis of the cysteines in the extracellular domain of the human Ca^{+2} receptor: effects on cell surface expression, dimerization and signal transduction. FEBS Lett 436: 353–356
74. Bai M, Trivedi S, Kifor O, Quinn SJ, Brown EM (1999) Intermolecular interactions between dimeric calcium-sensing receptor monomers are important for its normal function. Proc Natl Acad Sci USA 96:2834–2839
75. Rocheville M, Lange DC, Kumar U, Patel SC, Patel RC, Patel YC (2000) Receptors for dopamine and somatostatin: forma-

tion of hetero-oligomers with enhanced functional activity. Science 288:154–157
76. Jordan BA, Cvejic S, Devi LA (2000) Kappa opioid receptor endocytosis by dynorphin peptides. DNA Cell Biol 19:19–27
77. Maggi A, U'Prichard DC, Enna SJ (1980) Beta-adrenergic regulation of alpha2-adrenergic receptors in the central nervous system. Science 207:645–647
78. Fuxe K, Agnati LF, Benfenati F, Cimmino M, Algeri S, Hokfelt T, Mutt V (1981) Modulation by cholecystokinins of 3H-spiroperidol binding in rat striatum: evidence for increased affinity and reduction in number of binding sites. Acta Physiol Scand 113:567–569
79. Rostene WH, Fischette CT, McEwen BS (1983) Modulation by vasoactive intestinal peptide (VIP) of serotonin receptors in membranes from rat hippocampus. J Neurosci 3:2414–2419
80. Fuxe K, Ferre S, Zoli M, Agnati LF (1998) Integrated events in central dopamine transmission as analyzed at multiple levels. Evidence for intramembrane adenosine A2/dopamine D2 and adenosine A1/dopamine D1 receptor interactions in the basal ganglia. Brain Res Rev 26:258–273
81. Zoli M, Agnati LF, Hedlund PB, Li XM, Ferre S, Fuxe K (1993) Receptor-receptor interactions as an integrative mechanism in nerve cells. Mol Neurobiol 7:293–334
82. Maggio R, Vogel Z, Wess J (1993) Co-expression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein linked receptors. Proc Natl Acad Sci USA 90:3103–3107
83. Traynor JR, Elliott J (1993) Delta-opioid receptor subtypes and cross-talk with mu-receptors. Trends Pharmacol Sci 14: 84–86
84. Jiang Q, Mosberg HI, Porreca F (1990) Selective modulation of morphine antinociception, but not development of tolerance, by delta receptor agonists. Eur J Pharmacol 186:137–141
85. Jiang Q, Mosberg HI, Porreca F (1990) Modulation of the analgesic efficacy and potency of morphine by [D-Pen2, D-Pen5]enkephalin in mice. Prog Clin Biol Res 328:449–452
86. Rothman RB, Danks JA, Jacobson AE, Burke TR Jr, Rice KC, Tortella FC, Holaday JW (1986) Morphine tolerance increases mu-noncompetitive delta binding sites. Eur J Pharmacol 124: 113–119
87. Rothman RB, Long JB, Bykov V, Jacobson AE, Rice KC, Holaday JW (1988) beta-FNA binds irreversibly to the opiate receptor complex: in vivo and in vitro evidence. J Pharmacol Exp Ther 247:405–416
88. Rothman RB, Bykov V, Long JB, Brady LS, Jacobson AE, Rice KC, Holaday JW (1989) Chronic administration of morphine and naltrexone up-regulate mu-opioid binding sites labeled by [3H][D-Ala2,MePhe4,Gly-ol5]enkephalin: further evidence for two mu-binding sites. Eur J Pharmacol 160:71–82
89. Jones KA, Borowski B, Tamm JA, Craig DA, Durkin MM, Dai M, Yao W-J, Johnson M, Gunwaldsen C, Huang L-Y, Tang C, Shen Q, Salon JA, Morse K, Laz T, Smith KE, Nagarathnam D, Noble SA, Branchek TA, Gerald C (1998) $GABA_B$ receptor function as a heterotrimeric assembly of the subunits $GABA_B$R1 and $GABA_B$R2. Nature 396:674–679
90. Kaupmann K, Malitschek B, Schuler V, Heid J, Froestl W, Beck P, Mosbscher J, Bischoff S, Kulik A, Shigemoto R, Karschin A, Bettler B (1998) $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. Nature 396: 683–686
91. Kuner R, Köhr G, Grünewald S, Eisenhardt G, Bach A, Kornau H.-C (1999) Role of heteromer formation in $GABA_B$ receptor function. Science 283:74–77
92. White JH, Wise A, Main MJ, Green A, Fraser NJ, Disney GH, Barnes AA, Emson P, Foord SM, Marshall FH (1998) Heterodimerization is required for the formation of a functional $GABA_B$ receptor. Nature 396:679–682
93. Xie Z, Lee SP, O'Dowd BF, George SR (1999) Serotonin 5-HT1B and 5-HT1D receptors form homodimers when expressed alone and heterodimers when co-expressed. FEBS Lett. 456:63–67
94. Marshall FH, Jones KA, Kaupmann K, Bettler B (1999) GABAB receptors-the first 7TM heterodimers. Trends Pharmacol Sci 20:396–399
95. George SR, Fan T, Xie Z, Tse R, Tam V, Varghese G, O'Dowd BF (2000) Oligomerization of mu and delta opioid receptors. J Biol Chem 275:26128–26135
96. Gomes I, Jordan BA, Gupta A, Trapaidze N, Nagy V, Devi LA (2000) Heterodimerization of mu and delta opioid receptors: a role in opioid synergy. J Neurosci 20:RC110(1–5)
97. Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA (2001) Oligomerization of opioid receptors with b2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. Proc Natl Acad Sci USA 98:343–348
98. AbdAlla S, Lother H, Quitterer U (2000) AT1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. Nature 407:94–98
99. Gines S, Hillion J, Torvinen M, Crom S, Casado V, Canela EI, Rondin S, Lew JY, Watson S, Zoli M, Agnati LF, Vernier P, Lluis C, Ferre S, Fuxe K, and Franco R (2000) Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes. Proc Natl Acad Sci USA 97:8606–8611
100. Goldsmith PK, Fan G-F, Ray K, Shiloach J, McPhie P, Rogers KV, Spiegel AM (1999) Expression, purification and biochemical characterization of the amino-terminal extracellular domain of the human calcium receptor. J Biol Chem 274:11303–11309
101. Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H, Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotrophic glutamate receptor. Nature 407:971–977
102. Lemmon MA, Flanagan JM, Treutlein HR, Zhang J, Engelman DM (1992) Sequence specificity in the dimerization of transmembrane α-helices Biochemistry 31:12719–12725
103. George SR, Lee SP, Varghese G, Zeman PR, Seeman P, Ng, GY, O'Dowd BF (1998) A transmembrane domain-derived peptide inhibits D1 dopamine receptor function without affecting receptor oligomerization. J Biol Chem 273:30244–30248
104. Gouldson PR, Snell CR, Reynolds CA (1997) A new approach to docking in the β_2-adrenergic receptor that exploits the domain structure of G-protein coupled receptors. J Med Chem 40:3871–3886
105. Javitch JA, Fu D, Chen J, Karlin A (1995) Mapping the binding-site crevice of the dopamine D2 receptor by the substituted-cysteine accessibility method. Neuron 14:825–831
106. Altenbach C, Yang K, Farrens DL, Farahbakhsh ZT, Khorana HG, Hubbell WL (1996) Structural features and light-dependent changes in the cytoplasmic interhelical E-F loop region of rhodopsin: a site-directed spin-labeling study. Biochemistry 35:12470–12478
107. Farahbakhsh ZT, Ridge KD, Khorana HG, Hubbell WL (1995) Mapping light-dependent structural changes in the cytoplasmic loop connecting helices C and D in rhodopsin: a site-directed spin labeling study. Biochemistry 34:8812–8819
108. Elling CE, Schwartz TW (1996) Connectivity and orientation of the seven helical bundle in the tachykinin NK-1 receptor probed by zinc site engineering. EMBO J 15:6213–6219
109. Elling CE, Nielsen SM, Schwartz TW (1995) Conversion of antagonist-binding site to metal-ion site in the tachykinin NK-1 receptor. Nature 374:74–77
110. Thirstrup K, Elling CE, Hjorth SA, Schwartz TW (1996) Construction of a high affinity zinc switch in the kappa-opioid receptor. J Biol Chem 271:7875–7878
111. Sheikh SP, Zvyaga TA, Lichtarge O, Sakmar TP, Bourne HR (1996) Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F. Nature 383:347–350

112. Gouldson PR, Snell CR, Bywater RP, Higgs C, Reynolds CA (1998) Domain swapping in G-protein coupled receptors. Protein Eng 11:1181–1193
113. Main BG (1990) β_2-adrenergic receptors. In: Emmett JG (ed) Comprehensive medicinal chemistry, vol 3. Pergamon, Oxford, pp 188–228
114. Timmermans PBMWN, Chui AT, Hoolen A, Main BG (1990) β_2-adrenergic receptors. In: Emmett JG (ed) Comprehensive medicinal chemistry, vol 3. Pergamon, Oxford, pp 133–187
115. Göbel U, Sander C, Schneider R, Valencia A (1994) Correlated mutations and residue contacts in proteins. Proteins 18:309–317
116. Pazos F, Helmer-Citterich M, Ausiello G, Valencia A (1997) Correlated mutations contain information about protein-protein interaction. J Mol Biol 271:511–523
117. Brandt W, Golbraikh A, Täger M, Lendeckel U (1999) A molecular mechanism for the cleavage of a disulfide bond as the primary function of agonist binding to G-protein-coupled receptors based on theoretical calculations supported by experiments. Eur J Biochem 261:89–97
118. Fanelli F, Menziani C, Scheer A, Cotecchia S, Benedetti PG (1998) Ab Initio modeling and molecular dynamic stimulation of the alpha1b-adrenergic receptor activation. Methods 14:302–317
119. Fanelli F, Menziani C, Scheer A, Cotecchia S, Benedetti PG (1999) Theoretical study of the electrostatically driven step of receptor-G protein activation. Proteins 37:145–156
120. Maggio R, Barbier P, Colelli A, Salvadori F, Demontis G, Corsini GU (1999) G protein linked receptors: pharmacological evidence for the formation of heterodimers. J Pharmacol Exp Ther 291:251–257