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# Class A G-Protein-Coupled Receptor (GPCR) Dimers and Bivalent Ligands

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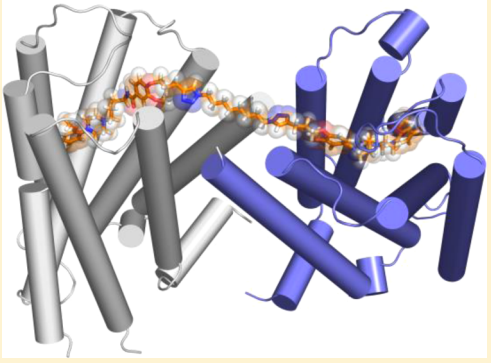
**ABSTRACT:** G-protein-coupled receptors (GPCRs) represent the largest family of membrane proteins involved in cellular signal transduction and are activated by various different ligand types including photons, peptides, proteins, but also small molecules like biogenic amines. Therefore, GPCRs are involved in diverse physiological processes and provide valuable drug targets for numerous diseases. Emerging body of evidence suggests that GPCRs exist as monomers or cross-react forming dimers and higher-ordered oligomers. In this Perspective we will review current biochemical and biophysical techniques to visualize GPCR dimerization, functional consequences of homo- and heterodimers, and approaches of medicinal chemists to target these receptor complexes with homo- and heterobivalent ligands.



## INTRODUCTION

G-protein-coupled receptors (GPCRs) represent the largest family of cell surface proteins encoded by the human genome. Structurally, these membrane proteins consist of seven transmembrane-spanning $\alpha$-helices linked by three altering intracellular and extracellular loops. GPCRs bind various ligands, including photons, ions, hormones, peptides, nucleotides, and also small molecules like biogenic amines. Therefore, GPCRs are involved in numerous physiological processes and display an attractive drug target for diverse diseases.

For many years, GPCRs have been considered to exist and function as monomers within the plasma membrane. However, in the past decades an expanding number of studies demonstrated that GPCRs are able to cross-react, forming dimers or higher-ordered oligomers, which are often essential for modulation of receptor function.[1−8] The first widely accepted evidence of functionally significant GPCR dimers was given by $GABA_B$ receptors that belong to class C GPCRs and form obligate heterodimers. Whereas the $GABA_{B1}$ subtype binds various $GABA_B$ receptor ligands, it is not functional when expressed alone. Only coexpression with $GABA_{B2}$, which is incapable of $GABA_B$ receptor ligand binding but promotes efficient cell surface trafficking and G protein coupling, allows the formation of a functional $GABA_B$ receptor.[9,10] Accumulating data indicate that receptors also belonging to the class A GPCR family are able to form dimers. Besides receptor function, dimerization can affect ligand pharmacology, signal transduction, and cellular trafficking. Hence, targeting GPCR dimers with bivalent or with monovalent dimer-specific ligands may result in more potent and selective compounds with reduced side effects. In this Perspective, we will focus on biological evaluation and strategies for targeting class A GPCR dimers with small molecules.

## EVIDENCE OF DIMERIZATION: BIOCHEMICAL AND BIOPHYSICAL TECHNIQUES TO STUDY GPCR DIMERS

**Coimmunoprecipitation.** Various publications reported that GPCRs often migrate on SDS−PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis) in a way that indicates approximately twice the expected molecular mass. Although the higher molecular weight of immunoreactive bands might represent specific receptor−receptor complexes, they could also be referred to any other receptor−protein aggregate. To investigate direct physicochemical interactions between two GPCRs, coimmunoprecipitation of differentially epitope-tagged receptors was established, which displays one of the most common techniques used to investigate GPCR dimerization.[11−14] This biochemical approach uses differentially epitope-tagged (cMyc-, hemagglutinin-, 6-histidine-, or FLAG-tagged) receptors, which are coexpressed in heterologous cells, and two specific antibodies. The potentially formed dimers are immunoprecipitated by a specific antibody against one epitope, followed by immunoblotting using a specific antibody against the other protomer. In a recent study a similar method was applied using a fluorescence-detected coimmunoprecipitation approach to investigate cross-interactions between dopamine $D_{2L}$ and neurotensin $NTS_1$ receptors. Fusion proteins of the $D_{2L}$ receptor with enhanced yellow fluorescent protein (eYFP) and of the $NTS_1$ receptor with enhanced cyan fluorescent protein (eCFP) were employed to examine colocalization of both receptors in the plasma membrane by confocal microscopy. The formation of physically interacting $D_{2L}$−$NTS_1$ heterodimers was investigated in a coexpression system

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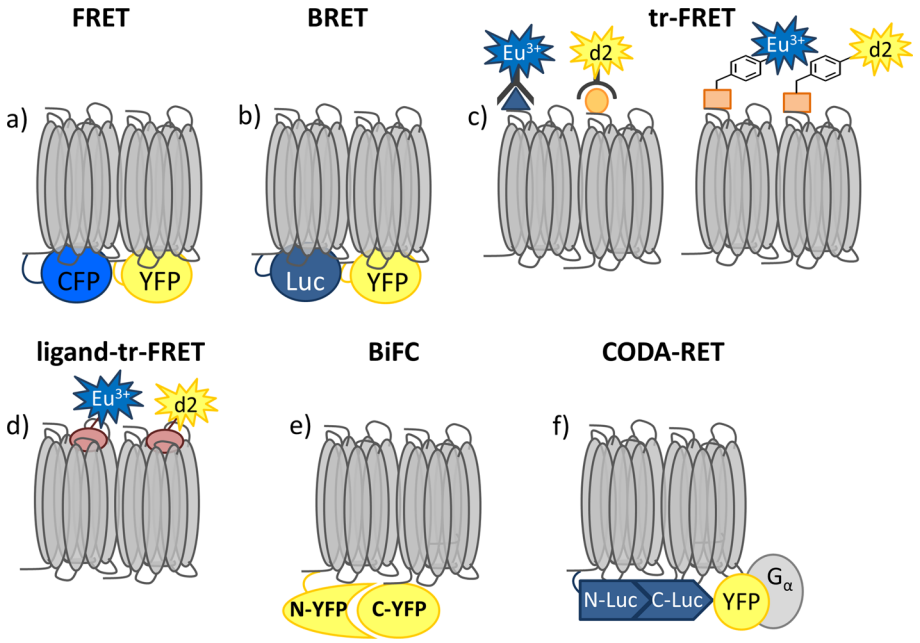


**Figure 1.** Fluorescence-based techniques.[25]

of C-terminally eCFP-tagged $NTS_1$ and wild-type $D_{2L}$ receptors. After cell lysis, $D_{2L}$ receptors were immunoprecipitated using a $D_{2L}$-specific antibody and heterodimer formation was evaluated by detection as well as quantification of the emission spectrum of the fluorescently labeled $NTS_1$ receptor. Comparison with the fluorescence intensity of the eCFP-labeled $D_{2L}$ receptor revealed a stable physical interaction between the two receptor protomers and the formation of stable $D_{2L}$–$NTS_1$ heterodimers.[15]

Because GPCRs are hydrophobic membrane proteins, formation of nonspecific aggregates is possible when the receptors are removed from the lipid environment of the plasma membrane because of insufficient concentrations of detergents used during the solubilization process. Therefore, control experiments with a mixture of cells, expressing only one of the two epitope-tagged receptors, are essential to exclude false positive results.[16,12,17] Because increasing the concentration of the detergents may result in the disruption of existing receptor–receptor interactions, cross-linking agents have been applied prior to the solubilization process to stabilize the preformed dimers during the subsequent steps.[18−22] Coimmunoprecipitation reveals the participation of both receptors to the same complex but does not provide evidence of physical interaction between the protomers. Hence, coimmunoprecipitation is frequently accompanied by fluorescence-based methodology in order to further validate the obtained results.

**Fluorescence-Based Techniques.** Resonance energy transfer (RET) based techniques have facilitated the visualization of GPCR dimers in intact, living cells. The most widely used approaches involve either fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET), which are based on the nonradiative transfer of energy from a donor to an acceptor molecule. By exploitation of FRET (Figure 1a), energy transfer-competent pairs of fluorescent proteins have been attached to the C-terminus of the GPCR (CFP as donor and YFP as acceptor). Taking advantage of BRET (Figure 1b), a bioluminescent protein, commonly luciferase from *Renilla reniformis* (Rluc), has been used as an energy donor and a fluorescent protein (YFP, GFP2, or mOrange) as an acceptor.[16] By use of time-resolved FRET (tr-FRET, Figure 1c), the inability to define the cellular location of the signal was overcome by fluorescence markers that are not able to penetrate the cell membrane. tr-FRET is based on the energy transfer between a lanthanide (terbium, dysprosium, samarium, or europium) and a compatible fluorophore (Alexa Fluor 647, DY-647, or d2). Because of the long-lasting light emission of the lanthanides, tr-FRET provides a significantly improved signal-to-noise ratio.[23] tr-FRET strategies use lanthanide-labeled antibodies against N-terminal-tagged GPCRs to monitor receptor dimerization at the cell surface. As an alternative to antibodies, the SNAP-tag technology allows covalent labeling of GPCRs with tr-FRET compatible fluorophores.[24] SNAP-tags that can be engineered into the N-terminus are derivatives of the DNA repairing enzyme $O^6$-guanine nucleotide alkyltransferase and covalently react with fluorophore-containing benzylguanine derivatives.[25]

All mentioned RET methods require receptor chimeras expressed at nonphysiological concentrations in heterologous cell lines. Alternatively, fluorescence-labeled ligands instead of labeled proteins can be applied to investigate GPCR oligomerization, especially heteromerization, with the advantage of a smaller size compared to antibodies and fluorescent proteins. Using ligands covalently linked to tr-FRET-compatible fluorophores (ligand-tr-FRET, Figure 1d), Albizu et al. have observed GPCR dimerization in native tissue for the first time. The authors recorded tr-FRET after treatment of isolated membrane fractions from mammary gland, expressing high levels of oxytocin receptors, with fluorescent oxytocin receptor antagonists.[26] Limitations of FRET-based techniques include the inability to differentiate dimers from higher-order oligomers and to investigate the stability of receptor complexes. An approach based on fluorescence recovery after photobleaching (FRAP) addresses these problems. FRAP measures the membrane diffusion of nonbleached fluorescent receptors into small, irreversibly bleached areas,[27] when the speed and extent of FRAP is dependent on the protein's mobility. Dorsch et al.


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applied dual-color FRAP to study $\beta_1$- and $\beta_2$-adrenoreceptor ($\beta_1$AR and $\beta_2$AR) oligomerization by using extracellularly YFP-tagged and intracellularly CFP-tagged $\beta_1$AR and $\beta_2$AR, expressed in equivalent amounts. Subsequent immobilization via a polyclonal anti-YFP antibody and membrane photobleaching resulted in FRAP of $\beta_1$AR-CFP, whereas only a small fraction of YFP-$\beta_1$AR diffused in the bleached region. Immobilization of YFP-$\beta_1$AR reduced the $\beta_1$AR-CFP mobility by approximately 15%, indicating a specific but transient interaction.[28]

Bimolecular fluorescence complementation (BiFC, Figure 1e) exhibits another approach for studying GPCR dimerization. The BiFC assay is based on the formation of a fluorescent complex by interaction of two GPCRs fused to inactive fragments of a fluorescent protein (Venus,[29] Cerulean,[30] mCherry[31]). Dimerization brings the fragments in proximity, leading to protein reconstitution and recovery of function.[32] On the basis of the above-mentioned methodology, Urizar et al. developed the complemented donor–acceptor RET (CODA-RET, Figure 1f) technology that combines features of protein complementation and resonance energy transfer.[33] In order to investigate $D_1$–$D_2$ receptor dimerization, luciferase was split into two nonfunctional protein fragments and fused to the C-termini of the two receptors, respectively. Receptor dimerization upon agonist binding reconstituted Rluc function, resulting in a BRET signal due to energy transfer to the acceptor protein mVenus, which was inserted into the G$\alpha$ subunit.

Fluorescence-based methods excite fluorophores in the entire sample, resulting in high background fluorescence. This intracellular, out-of-focus fluorescence can be minimized by application of total internal reflection fluorescence (TIRF) microscopy, a valuable tool for visualization and tracking of proteins at the plasma membrane of living cells. TIRF selectively excites fluorophores at the cell surface, which reduces cellular photodamage and increases the signal-to-noise ratio.[34] This approach was used to demonstrate reversible dimer formation of $M_1$ muscarinic receptors, which were labeled with Cy3B- or Alexa488-telenzepine.[35]

Recently, TIRF microscopy combined with direct labeling of the receptors with small organic fluorophores via SNAP-tag technology was applied to dynamically quantify $\beta_1$- and $\beta_2$-adrenergic receptor mobility and dimerization. Data obtained on $\beta_1$- and $\beta_2$-adrenergic receptors indicate that GPCRs are in a dynamic equilibrium on the cell surface. Both $\beta_1$AR and $\beta_2$AR undergo transient interactions that are not altered upon agonist stimulation.[36] These results are in accordance with those previously reported by Dorsch et al. using dual-color FRAP measurements.[28]

**Functional Complementation Studies.** The biochemical and biophysical experiments described above demonstrate that GPCRs can exist in proximity to each other. To investigate physical interactions across a GPCR dimer, functional complementation studies have been used successfully. These studies are based on the concept that two distinct nonfunctional GPCR–G protein fusion constructs restore signaling when coexpressed (Figure 2a). Such fusion proteins consist of a G protein $\alpha$ subunit linked via its N terminus to the C terminus of a GPCR. Hence, contrasting pairs of nonfunctional mutants can be generated, which contain either a mutated GPCR unable to activate the fused G protein or a mutated G protein unable to be activated by the fused GPCR. GPCR mutants are obtained by replacing hydrophobic residues in the second intracellular loop with acidic residues. This modification

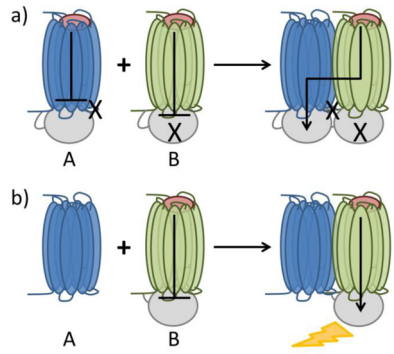


**Figure 2.** Complementation of receptor function by heterodimerization. (a) The GPCR–G protein fusion construct A contains a mutation within the second intracellular loop that prevents G protein activation, whereas protomer B is fused to a mutated G protein that is unable to exchange the guanine nucleotide.[39] (b) Functional reconstitution of a nonfunctional $D_2$–$G_{qi5}$ fusion construct B by coexpression with $D_2$ wild-type receptor A. Detection was by luminescence readout.[38]

eliminates G protein activation upon agonist binding without altering the orthosteric binding site. For the generation of the nonfunctional G protein, a conserved glycine unit of the G protein $\alpha$ subunit was mutated to an alanine residue, which prevents guanine nucleotide exchange and thus G protein activation. Coexpression of the complementary, nonfunctional fusion protein pairs resulted in functional dimers, which were quantified by determining increased [$^{35}$S]GTP$\gamma$S binding or enhanced intracellular $Ca^{2+}$ levels.[16,37] By use of this method, functional interactions between homo- and heterodimers could be detected. Because the G protein has been fused to the GPCR via, in some cases, its long cytoplasmatic C-terminal tail and a flexible peptide-based linker, GPCR–G protein fusion constructs might provide a G protein for the activation of another receptor without directly interacting in a dimeric receptor–receptor signaling complex. To circumvent this problem, Han et al. used a modified approach to prove functional complementation of dopamine $D_2$ receptor dimers (Figure 2b).[38] The authors developed a protomer that prevents G protein activation by using a $D_2$R–$G_{qi5}$ construct having the $G_{qi5}$ protein (a pertussis toxin resistant $G_q$ protein capable of signaling from $G_i$-coupled receptors) directly linked to the C terminus of the $D_2$ receptor. The consequence is a lack of signaling due to the inability of the short-tethered G$\alpha$ to couple to its linked GPCR, presumably as a result of the steric constraints. By use of the $G_{qi5}$-protein-coupled response as readout, coexpression with the wild type $D_2$R, endogenously deficient in $G_q$ coupling, resulted in a robust restoration of the $G_q$-dependent calcium signal, thus proving direct interactions between the two receptor protomers.

**Dimerization Interface: Hints from Crystal Structures.** Structural insights into GPCR dimerization were provided by recent crystal structures of the CXCR4 chemokine receptor[40] and the opioid receptor subtypes $\mu$OR[41] and $\kappa$OR.[42] Five independent crystal structures were obtained for CXCR4, each consisting of a parallel, symmetric dimer and showing identical dimeric interfaces. Interactions between the two protomers were observed at the extracellular side of the transmembrane domain (TM) 5 and TM6 burying 850 Å$^2$ of surface area.[40] The dimer interface of the $\kappa$-opioid receptor is formed through contacts of TM1, TM2, and helix 8, covering ~1100 Å$^2$ buried surface area.[42] Manglik et al. observed associated receptor pairs


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**Table 1. Dimers of G-Protein-Coupled Receptors, Class A[a]**

| dimer | detection method | pharmacological effect | ref |
|---|---|---|---|
| 5-$HT_{2A}$–$D_2$ | co-IP, FRET | B↑ and S↓ (by $D_2$ agonist) | 61, 62 |
| 5-$HT_4$–5-$HT_4$ | co-IP, BRET | nd | 63 |
| $\alpha_{1A}$–$\alpha_{1B}$ | co-IP, tr-FRET | I, S | 59, 64 |
| $\alpha_{1A}$–$H_1$ | FR | S↑ | 65 |
| $\alpha_{1B}/\beta_2$–$\alpha_{1D}$ | co-IP, LCM | E↑, S↑ (of $\alpha_{1D}$) | 66, 67 |
| $\alpha_{2A}$–$\beta_1$ | co-IP, LCM | I | 68 |
| $\alpha_{2A}$–$\mu$OR | FRET | S↓ (of $\alpha_{2A}$, by morphine) | 46 |
| $A_1$–$A_{2A}$ | co-IP, BRET, FRET | B↓ (agonist), S | 69 |
| $A_1$–$\beta_2$ | nd | S↓ (of $\beta_2$ upon coactivation) | 70, 71 |
| $A_1$–$D_1$ | co-IP | S↓ (of $D_1$ upon coactivation) | 72 |
| $A_{2A}$–$CB_1$ | BRET, IP | S ($CB_1$ activation requires $A_{2A}$ coactivation) | 73 |
| $A_{2A}$–$D_{2L}$ | BiFC, BRET, co-IP | B↓ (agonist), I | 13, 74–78 |
| $AT_1$–$B_2$ | co-IP, BRET | B↑ and S↑ (of angiotensin II) | 47, 79, 80 |
| $AT_1$–$\beta_1/\beta_2$ | co-IP, LCM | S↓ (antagonist) | 81 |
| $AT_1$–$CB_1$ | BRET, co-IP | S↑ (of angiotensin II), G (heterodimer coupled to $G_{\alpha q}$ and $G_{\alpha i}$, controlled by $CB_1$ activity) | 82 |
| $\beta_1$–$\beta_1$ | BRET, FRAP | nd | 28, 83 |
| $\beta_1$–$\beta_2$ | co-IP | S↑ (agonist), B↓ | 84 |
| $\beta_2$–$\beta_2$ | co-IP, BRET, FRAP | nd | 11, 28, 83 |
| $\beta_2$–$\delta$OR | co-IP | I | 85 |
| $\beta_2$–OT | co-IP, BRET | S↓ (agonist) | 86 |
| $CB_1$-$D_2$ | BiFC, FRET | B↓ (agonist), G ($G_{i/o}$ to $G_s$) | 49, 87, 88 |
| $CB_1$–$\delta$OR | BRET, co-IP | E (of $CB_1$)↑, S↓ (of $CB_1$ agonist), B↑ (of $\delta$OR by $CB_1$ agonist) | 89, 90 |
| $CB_1$–$\mu$OR | BRET | S↓ (agonist) | 90 |
| $CCK_2$–$\mu$OR | BRET | | 91 |
| $CCR_{2b}$–$CCR_5$ | BRET, co-IP | B↓ (agonist) | 45, 92 |
| $CCR_5$–$\mu$OR | co-IP | S↓ | 93 |
| $CXCR_1$–$CXCR_1$ | co-IP, BRET, FRET, tr-FRET | nd | 57 |
| $CXCR_2$–$CXCR_2$ | co-IP, BRET, FRET, tr-FRET | nd | 57 |
| $CXCR_2$–$\delta$OR | BRET, co-IP, FR, tr-FRET | S↑ (of $\delta$OR by $CXCR_2$ antagonist) | 94 |
| $\delta$OR–$\kappa$OR | co-IP | B↓ (selective agonist, antagonist), B↑ (partially selective ligand) | 12 |
| $\delta$OR–$\mu$OR | co-IP, BRET | B↑ and S↑ (by combination of $\mu$-agonist and $\delta$-antagonist), G ($G_i$ to $G_z$ and $G_i$ to $\beta$-arrestin), I↓ | 44, 50, 95–97 |
| $\delta$OR–$D_1$ | FR | G (preference for $G_i$ over $G_s$) | 98 |
| $D_1$–$D_2$ | co-IP, CODA-RET | G ($G_{i/o}$ to $G_{q/11}$), I | 33, 48, 99 |
| $D_1$–$D_3$ | BRET, co-IP, FRET | B↑ (agonist), S↑ (of $D_1$) | 100, 101 |
| $D_1/D_2$–$H_3$ | BRET | S↓ (by $H_3$ agonists) | 102 |
| $D_2$–$D_2$ | FR | S (modification depends on ligand) | 38 |
| $D_2$–$D_3$ | FRET, BRET | B↑ (of $D_3$ sel agonists) | 103, 104 |
| $D_2$–$SSTR_5$ | pb-FRET | B↑ (agonist), S↑ | 105 |
| $D_{2L}$–$NTS_1$ | fluorescence-detected co-IP | B↓ (agonist, NTS1 coactivation) | 15 |
| $\kappa$OR–$\mu$OR | BRET, co-IP | nd | 106 |
| $\mu$OR–$SSTR_{2A}$ | co-IP, LCM | I | 107 |
| $M_1$–$M_1$ | TIRF | nd | 35 |
| $M_3$–$M_3$ | co-IP + cross-linking | nd | 108 |
| $SSTR_1$–$SSTR_5$ | pb-FRET | I | 109 |
| $SSTR_{2A}$–$SSTR_3$ | co-IP | S↑ (of $SSTR_{2A}$), S↓ (of $SSTR_3$), greater resistance against agonist-induced internalization | 110 |
| $V_{1A}$–$V_2$ | BRET, co-IP | I, S (binding to $\beta$-arrestin↑) | 111 |
| $V_{1A}/V_2$–OT | BRET, co-IP | no effect on B | 112 |

[a] B, binding affinity; co-IP, coimmunoprecipitation; E, cell surface expression; G, G protein switch; FR, functional reconstitution; I, cointernalization after agonist stimulation; LCM, laser confocal microscopy; nd, not determined; pb-FRET, photobleaching FRET; S, receptor signaling; ↑, enhanced; ↓, reduced; 5-HT, serotonin receptor; $\alpha$, $\alpha$-adrenergic receptor; A, adenosine receptor; AT, angiotensin receptor; $\beta$, $\beta$-adrenergic receptor; B, bradykinin receptor; CB, cannabinoid receptor; CCK, cholecystokinin; CCR, CXCR, chemokine receptor; D, dopamine receptor; $\delta$OR, $\delta$-opioid receptor; H, histamine receptor; $\kappa$OR, $\kappa$-opioid receptor; M, muscarinic receptor; $\mu$OR, $\mu$-opioid receptor; NTS, neurotensin receptor; OT, oxytocin receptor; SSTR, somatostatin receptor; V, vasopressin receptor.

with two different interfaces for the crystal structure of the $\mu$-opioid receptor. The smaller one with a buried surface area of 615 Å$^2$ involves TM1, TM2, and helix 8 analogous to the $\kappa$OR structure. The second interface covers 1492 Å$^2$ of buried surface area by interaction between TM5 and TM6.[41] These two distinct dimerization interfaces provide a structural basis for the existence of higher-order oligomers. Interestingly, some of the 28 amino acid residues of TM5 and TM6 that contribute to the dimer interface are interacting with amino acids of the ligand binding pocket. Thus, changes within TM5 and TM6


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upon GPCR dimerization may affect ligand binding or signaling properties.

## FUNCTIONAL CONSEQUENCES OF G-PROTEIN-COUPLED RECEPTOR DIMERIZATION

Even though accumulating biophysical and biochemical data demonstrate the interaction of different GPCR subtypes with each other, the question of the biological significance of these receptor complexes remains. In order to define the physiological relevance of GPCR dimers, the International Union of Basic and Clinical Pharmacology (IUPHAR) released three criteria of which at least two have to be fulfilled. First, the physical interaction of GPCRs has to be verified in native tissues or primary cells. Second, evidence of dimer-specific properties, like specific signaling or binding properties or the existence of dimer-selective ligands, must be given. The third criterion recommends the in vivo validation of GPCR dimerization by kockout animals or RNAi technology.[43] Recent literature addresses these issues and confirms altered pharmacological properties of GPCR oligomers resulting from allosteric communication between the protomers (Table 1).

**Dimerization Resulting in Distinct Ligand Binding Properties.** GPCR dimerization can result in altered binding properties that are not compatible with the model that one single, independent GPCR binds one ligand. Evidence for a possible cross-talk between protomers originates from radioligand binding studies measuring alterations in ligand binding affinity or dissociation kinetics and variations in the maximal binding capacity depending on the radioligand used. Conformational changes induced by the binding of an orthosteric ligand can be transmitted from the binding site of one protomer to the other, resulting in an increased or decreased propensity of the second molecule to bind. This phenomenon is known as positive or negative cooperativity, respectively. A study on $\delta$-opioid receptor/$\mu$-opioid receptor ($\delta$OR/$\mu$OR) interactions demonstrated the existence of $\delta$OR−$\mu$OR heterodimers in living cells and native membranes. Additionally, a substantial increase in the binding of $\mu$OR ligands (morphine and DAMGO) by various $\delta$OR antagonists was observed. This positive cooperativity was only discovered in $\delta$OR and $\mu$OR coexpressing cells.[44] Recently, a trans-inhibitory effect on agonist binding affinity was observed in cells coexpressing dopamine $D_{2L}$ and neurotensin $NTS_1$ receptors. In the presence of neurotensin, the $D_2$ agonist *N*-[(*N*′-indan-2-yl-*N*′-propyl)-4-aminobutyl]pyrazolo[1,5-*a*]pyridine-3-carboxamide (FAUC 326)[15] displayed a 34-fold decreased binding affinity at the $D_{2L}$ receptor subtype. Springael et al. discussed negative binding cooperativity between dimers of chemokine receptors. The influence of $CCR_2$-specific monocyte chemotactic protein 1 (MCP-1)[45] and $CCR_5$-specific macrophage inflammatory protein1$\beta$ (MIP-1$\beta$)[45] ligands on dimerization and ligand binding was tested in living cells, indicating that MIP-1$\beta$ inhibited the binding of MCP-1 in $CCR_2$−$CCR_5$ coexpression systems and vice versa.

In addition to ligand affinity, selectivity profiles can also be altered by GPCR dimerization. As an example, both protomers lost affinity for their highly selective agonists and antagonists in $\kappa$OR/$\delta$OR coexpression systems when the heterodimer showed high affinity for partially selective ligands instead.[12]

**Altered Signaling Properties of GPCR Dimers.** Besides alterations in the binding properties, conformational changes caused by GPCR dimerization were shown to result in various functional consequences. These include the modulation of existing functions of one or two protomers, such as enhanced or impaired signaling, and can lead to the generation of novel pharmacological effects through a switch in signaling. Vilardaga et al. investigated the influence of morphine on $\alpha_{2A}$-AR-mediated signaling in cells coexpressing $\mu$OR and $\alpha_{2A}$-AR. This study revealed the ability of morphine to inhibit $\alpha_{2A}$-AR activation by norepinephrine, which is probably due to stabilization of a $\alpha_{2A}$-AR inactive state. Morphine reduced the level of $G_i$ activation and ERK1/2 phosphorylation in the presence of norepinephrine resulting from a direct interaction between $\mu$OR and $\alpha_{2A}$-AR, since no effect was observed when the receptors were expressed singly.[46]

Communication between two receptor protomers can also enhance GPCR signaling. Heterodimerization between the vasoactive angiotensin receptor $AT_1$ and the bradykinin receptor $B_2$, which is coexpressed on smooth muscle cells, was shown to elicit an increased efficacy and potency of angiotensin II. This enhanced $AT_1$R-mediated signaling was not influenced by the binding of bradykinin but required an intact receptor/G protein interface of the $B_2$ receptor, which was demonstrated by coexpression of a $B_2$R mutant ($B_2$ Y157A) that is uncoupled from its G protein together with the $AT_1$ wild-type receptor. The $AT_1$/$B_2$Y157A heterodimer did not enhance $AT_1$R-mediated signaling.[47]

Functional studies indicate that dimerization can trigger a switch in G protein coupling. Whereas $D_1$R couples to $G_{\alpha s}$ and $D_2$R can activate $G_{\alpha i}$, $G_{q/11}$ coupling could be observed for the $D_1$−$D_2$ receptor heterodimer after coactivation of both receptor protomers.[48] An increase in intracellular calcium release occurred in consequence of phospholipase C (PLC) activation which was independent from adenylyl cyclase (AC) modulation. Distinct G protein coupling by dimerization was also shown for the $CB_1$−$D_2$ receptor dimer. Whereas the cannabinoid $CB_1$ receptor is known to couple $G_{\alpha i}$ and thus to inhibit cAMP accumulation, coactivation of the dopamine $D_2$ receptor provokes a switch in $CB_1$R coupling to $G_{\alpha s}$, resulting in elevated intracellular cAMP levels.[49]

Moreover, dimerization can cause a switch from G-protein- to $\beta$-arrestin-mediated signaling as recently demonstrated for the $\delta$OR−$\mu$OR heterodimer by Rozenfeld et al.[50] In contrast to cells singly expressing $\delta$OR or $\mu$OR, $\delta$OR−$\mu$OR heterodimers constitutively recruit $\beta$-arrestin. Subsequent stimulation of the heterodimer coexpressing cell lines by cotreatment with $\delta$OR and $\mu$OR ligands resulted in conformational changes leading to dissociation of $\beta$-arrestin followed by rapid ERK phosphorylation, whereas depletion of $\beta$-arrestin resulted in an ERK phosphorylation pattern similar to that of cells only expressing the $\mu$OR.

Results suggest an involvement of $\delta$OR−$\mu$OR heterodimers in the regulation of pain and in opioid-mediated analgesia by heterodimer-specific $\beta$-arrestin signaling. Low doses of $\delta$OR antagonists enhanced morphine-mediated analgesia in vivo.[44,51] $\delta$OR knockout[52] and $\beta$-arrestin knockout[53] mice showed an enhanced effectiveness of morphine.

The results support the assumption that the formation of dimers is associated with unique signaling properties that are relevant for regulating physiological and pathophysiological processes.

**Altered Trafficking Properties.** Dimerization can trigger changes in receptor trafficking, including receptor maturation, cell surface delivery, and ligand-mediated endocytosis. Studies of a large number of potential dimers indicate that receptor oligomers are preassembled in the endoplasmatic reticulum


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**Table 2. Physiological Relevance of GPCR Dimerization**[a]

| receptor dimer | physiological relevance | ref |
|---|---|---|
| $D_1$–$D_2$ | Major depression: | 113 |
| | up-regulation of $D_1$–$D_2$ dimers in striatal regions; disruption of the dimeric $D_1$–$D_2$ complex exerts antidepressant-like effects. | |
| $\delta$OR–$\mu$OR | Analgesia, opioid-induced dependence, and tolerance: | 60, 115, 116 |
| | $\delta$OR–$\mu$OR heterodimers modulate $\mu$OR-mediated spinal analgesia negatively; disruption of the $\delta$OR–$\mu$OR interaction enhances morphine analgesia. | |
| $AT_1R$–$B_2R$ | Pre-eclampsia, hypertension: | 79 |
| | Elevated sensitivity to angiotensin II, increased levels of $AT_1R$–$B_2R$ dimers in pre-eclampsia | |
| $A_{2A}$–$D_2$ | Parkinson's disease: | 76–78 |
| | Possible synergistic effects between $A_{2A}$ antagonists and $D_2$ agonists in the treatment of Parkinson's disease | |
| $EP_1R$–$\beta_2$AR | Asthma: | 114 |
| | $PGE_2$ promotes dimerization of $EP_1R$ and $\beta_2$AR, leading to attenuated cAMP production and therefore to reduced bronchodilatation in response to $\beta_2$-agonists | |
| 5-$HT_{2A}$–5-$HT_{2C}$ | Drug addiction: | 117 |
| | Combination of 5-$HT_{2A}$ antagonist and 5-$HT_{2C}$ agonist synergistically suppressed cocain-induced hyperactivity and impulsive action | |

[a]Abbreviations: $EP_1R$, prostaglandin E receptor; $PGE_2$, prostaglandin $E_2$.

(ER) and are delivered together to the cell surface. Mutants of GPCRs have been constructed containing an ER-retention motif. Coexpression of these export-deficient mutants with wild-type receptors prevented the delivery of these receptors to the cell surface, indicating that GPCRs form dimers in the ER. ER trapping studies were performed for a variety of different GPCRs including $D_2R$,[54] $V_2R$,[55] $\beta_2R$,[11,56] and $CXCR_1$,[57] indicating that oligomerization during biosynthesis may be a general mechanism. Several reports also show that different, naturally occurring GPCR splice variants and mutants trap the wild-type counterparts in the ER likely because of receptor dimerization.

Dimerization can also enhance the cell surface expression of distinct receptors. For instance, the $\alpha_{1D}$ adrenoreceptor ($\alpha_{1D}$-AR) displays little or no functional activity when expressed alone because the receptor is mostly localized intracellularly and not delivered efficiently to the cell surface.[58] Uberti et al. discovered a 10-fold increase in cell surface expression of $\alpha_{1D}$-AR in coexpression systems together with $\alpha_{1B}$-AR, suggesting that $\alpha_{1B}$AR–$\alpha_{1D}$AR heterodimerization promotes transport of $\alpha_{1D}$-AR to the cell membrane.[59] Moreover, there is evidence that dimerization can alter ligand-induced internalization of either receptor.

Agonist-induced homologous desensitization is one of the major mechanisms involved in the regulation of GPCRs. Agonist binding leads to G protein activation and subsequent phosphorylation of intracellular serine/threonine residues by G-protein-coupled receptor kinases (GRKs). These phosphorylated GPCRs recruit $\beta$-arrestin, which then triggers receptor internalization. Besides monomers, there is growing evidence that GPCR dimers recruit $\beta$-arrestin too, whereas a single $\beta$-arrestin molecule might be sufficient to promote internalization. Cointernalization of distinct dimers was observed upon stimulation of only one protomer. In the case of the $\delta$OR–$\mu$OR heterodimer, treatment with $\delta$OR-selective agonists led to endocytosis of both types of opioid receptors.[60] This down-regulation of $\mu$OR cell surface expression by activation of $\delta$OR in combination with the above-mentioned observations suggests a negative modulation of morphine-mediated analgesia by heterodimerization of $\delta$- and $\mu$-opioid receptors.

These findings demonstrate that dimerization plays an essential role in the regulation of receptor processing and trafficking.

## STRATEGIES FOR TARGETING GPCR DIMERS

Dimerization and oligomerization of G-protein-coupled receptors can result in modified ligand binding and signaling properties (Table 2). Therefore, selective targeting of GPCR dimers by novel types of agonists or antagonists may provide significant therapeutic advantages in the treatment of distinct GPCR-sensitive diseases.

As an example of pathophysiological heterodimerization, Pei et al. reported on elevated levels of dopamine $D_1$–$D_2$ receptor dimers in striatal post-mortem brain samples of patients suffering from major depression.[113] Furthermore, heterodimerization of the prostaglandin $EP_1$ receptor and the $\beta_2$ adrenoreceptor in airway smooth muscle cells might play an important role in the pathogenesis of asthma. Prostaglandin $PGE_2$ promotes formation of a $EP_1R$–$\beta_2$AR heterodimer, leading to attenuation of cAMP production by uncoupling $\beta_2$AR from $G_s$ and thus reducing bronchodilatation in response to $\beta_2$ agonists.[114]

Tissue- or disease-specific formation of GPCR heterodimers implicates a great chance for the development of highly selective drug candidates with reduced side effects.

**Monovalent Heterodimer-Specific Ligands.** Accumulating evidence of GPCR heterodimers having distinct functional and pharmacological properties compared to their respective homodimers or monomers led to the hypothesis that there might be monovalent ligands that selectively target heterodimers. Heterodimer-selective ligands are considered to have higher affinity or induce higher activity by binding to a heterodimeric complex compared to the respective homodimer or monomer.

Waldhoer et al. demonstrated that compound **1**[118] (6′-guanidinonaltrindole, 6′-GNTI, Figure 3), originally designed as a $\kappa$OR agonist, preferentially activates the $\delta$OR–$\kappa$OR heterodimer.[118] When the agonist-mediated intracellular $Ca^{2+}$ release was measured, **1** proved to be the most potent and efficacious in cells expressing both $\delta$- and $\kappa$-opioid receptors, whereas a $\kappa$OR selective agonist was equally active in cells singly expressing $\kappa$OR and in coexpression systems. Signaling mediated by compound **1** was blocked by treatment with the $\kappa$OR-selective antagonist 17,17′-(dicyclopropylmethyl)-6,6′,7,7′-6,6′-imino-7,7′-bimorphinan-3,4′,14,14′-tetrol (**2**, NorBNI) and with the $\delta$OR-selective antagonist naltrindole. In vivo, **1** induced analgesia when administered intrathecally


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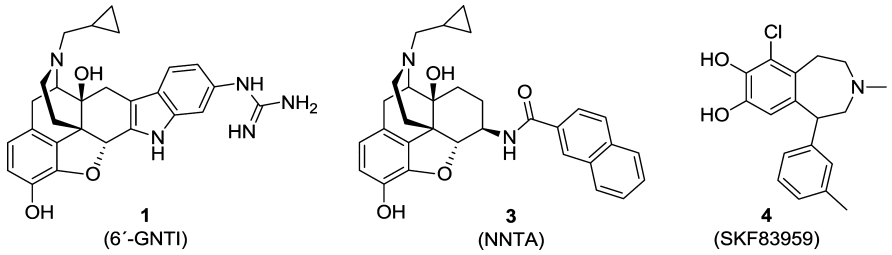


**Figure 3.** Heterodimer-specific GPCR ligands.

but not intracerebroventricularly. This analgesic effect was fully blocked by the antagonists **2** and naltrindole. The study suggests that opioid receptor heterodimers are expressed tissue-selectively and consequently represent potential targets for the development of tissue-selective analgesics that might display reduced side effects.

Yekkirala et al. discovered the monovalent ligand **3**[119] (NNTA, Figure 3) that selectively activates heterodimeric $\mu-\kappa$ opioid receptors in cultured cells and mice leading to potent antinociception without inducing physical dependence. **3** exhibited ~1000-fold enhanced agonist potency in cells coexpressing $\kappa$OR and $\mu$OR compared to $\kappa$OR monoexpression systems.[119]

Potent analgesics like morphine, fentanyl, and methadone are shown to produce antinociception via selective activation of $\mu$OR–$\delta$OR heterodimers. Whereas coadminstration of the $\delta$ antagonist naltrindole antagonized this activation in cells expressing both $\mu$- and $\delta$-opioid receptors, no effect was observed in $\mu$OR monoexpression systems. These data suggest on the one hand the involvement of $\mu$OR–$\delta$OR heterodimers in antinociception and on the other hand a strong allosteric modulation of the heterodimer by the $\delta$OR protomer. Besides the results of the in vitro studies, the antagonizing effect of naltrindole was observed in mice and rhesus monkeys after intrathecal administration.[120]

Selective targeting of GPCR heterodimers was also revealed for the dopamine $D_1$ receptor ligand **4**[48] (SKF83959, Figure 3). The compound binds to dopamine $D_1$ receptors with high affinity but fails to stimulate the production of cAMP. Nevertheless, it is able to induce a number of behavioral effects in animal models similar to those produced by standard dopamine $D_1$ receptor agonists. Further investigations revealed that **4** selectively acts at the heterodimeric $D_1$–$D_2$ signaling complex, resulting in increased intracellular calcium levels through activation of $G_{q/11}$. The adenylyl cyclase pathway, which is associated with the activation of $D_1$ or $D_2$ receptor homodimers or monomers, was not affected. This effect was abolished in cells expressing the $D_1$ receptor alone and could be blocked by $D_1$ or $D_2$ receptor antagonists. Radioligand binding studies using [$^3$H]raclopride, a $D_2$ receptor antagonist, in cells coexpressing $D_1$ and $D_2$ receptors revealed a high-affinity binding site for compound **4** on the $D_2$ receptor that was absent in cells expressing the $D_2$ receptor alone. Pretreatment of $D_1$–$D_2$ coexpressing cells with pertussis toxin (PTX), which uncouples $D_2$ receptors from $G_{i/o}$ proteins, only modestly reduced the proportion of high-affinity binding sites for **4**, demonstrating that **4** binds to PTX-resistant $G_{q/11}$-coupled $D_2$ receptors. These results indicate that **4** acts as a full $D_1$ agonist and a partial $D_2$ agonist with functional selectivity for the $G_{q/11}$ pathway. Subsequent radioligand binding studies using rat and mouse striatum verified the in vivo relevance of the functionally selective behavior of compound **4**, which was absent in $D_1$ knockout animals.[48]

**Bivalent Ligands as Molecular Tools To Investigate GPCR Dimerization.** In contrast to monovalent heterodimer-specific ligands, a bivalent ligand (Figure 4) consists of two pharmacophoric entities linked by an appropriate spacer. By selection of adequate, potent, and subtype-selective pharmacophores, it is feasible to target GPCR heterodimers or homodimers selectively, providing access to a rational design of bivalent ligands.

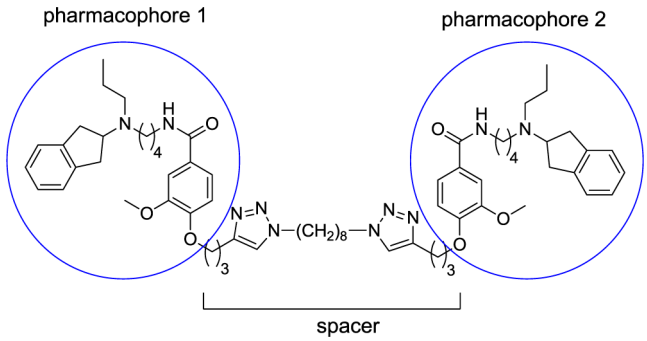


**Figure 4.** Bivalent dopamine $D_2$ receptor ligand.[121]

Whereas in a homobivalent ligand both pharmacophores are identical, a heterobivalent ligand comprises two different pharmacophores. On the basis of the pioneering work of Portoghese, who developed the first bivalent ligands as pharmacological tools for the investigation of opioid receptor dimerization,[122−125] a considerable number of compounds were synthesized to target various GPCRs including serotonin,[126−129] histamine,[130] dopamine,[121,131−136] adenosine,[137,138] chemokine,[139,140] and cannabinoid[141] as well as muscarinic[142,143] and adrenergic[144,145] receptors. Two possibilities have to be considered for the binding mode of bivalent ligands. The ligand can either target two orthosteric binding sites of two neighboring protomers simultaneously or interact with a primary, orthosteric, and an additional allosteric recognition site of the same receptor, which is frequently termed as a bitopic (or dualsteric) binding mode.[146−148] Bivalent ligands bridging the orthosteric binding sites of two physically interacting GPCRs provide valuable pharmacological tools to study the quaternary structure of receptor dimers and to gain insights into the functional relevance of GPCR dimerization. As a complement, bivalent ligands with a bitopic or dualsteric binding mode facilitate the development of subtype-selective compounds because allosteric regions are usually less conserved within a receptor family compared to the orthosteric binding pocket.


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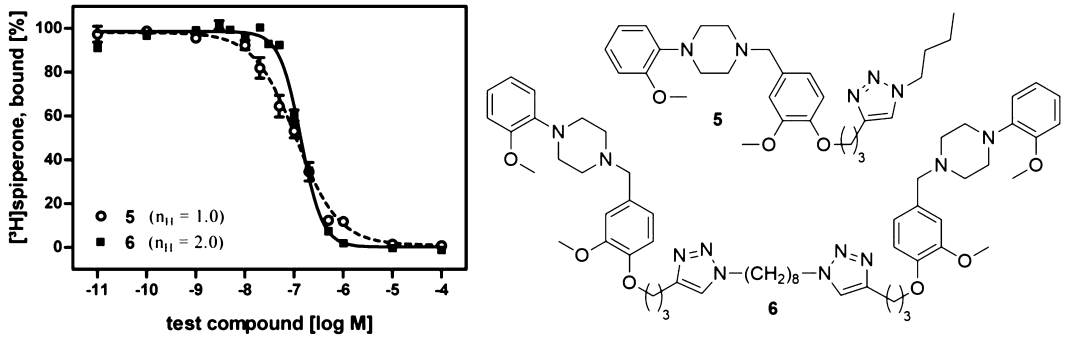


**Figure 5.** Bivalent and monovalent binding mode of **5** and **6** at the human dopamine $D_{2short}$ receptor.[134]

## BINDING PROPERTIES OF BIVALENT LIGANDS

Cross-talk between an orthosteric and an allosteric binding site of one receptor as well as conformational changes within the two protomers of a physically interacting GPCR dimer can evoke positive cooperativity. Also, bivalent ligands addressing two adjacent binding sites of a receptor dimer are able to induce such cooperativity because of the thermodynamic advantage of sequential ligand binding. Univalent binding of the first pharmacophore increases the local concentration of the second, unbound but tethered, recognition unit in the vicinity of the neighboring orthosteric binding site and thus significantly accelerates the second chelating binding event. By employment of radioligand competition experiments, bivalent binding leads to the liberation of 2 equiv of radioligand and thus to a substantial steepening of the competition curves compared to the monomeric controls (Figure 5).

This diagnostic effect was observed in recent studies of bivalent dopamine $D_2$ receptor ligands based on 1,4-disubstituted aromatic piperazine (1,4-DAP), aminoindane, and 5-OH-DPAT scaffolds. Competitive binding curves of monomeric antagonists such as the phenylpiperazine **5**[134] (Figure 5) usually display Hill slopes close to 1 ($n_H$ = 0.9−1.2), indicating that one receptor protomer binds one ligand. Binding studies of the bivalent dopamine $D_2$ receptors antagonist **6**[134] revealed significantly increased Hill slopes of 2.0.

Interestingly, bivalent agonists also showed steeper binding curves compared to their respective monovalent analogues. In contrast to antagonists, competition experiments between monomeric agonists, preferentially recognizing high affinity states of the respective receptor, and radiolabeled antagonists reveal shallow curves with Hill coefficients of 0.5−0.7, indicating a two binding site model. Binding studies of bivalent dopamine $D_2$ receptor agonists revealed significantly increased Hill slopes of 1.3−1.4.[121,134,136] Thus, careful analysis of Hill coefficients provides a valuable method to determine a bivalent binding mode.

## DESIGN OF BIVALENT LIGANDS

The design of bivalent ligands requires consideration of several aspects like the choice of an appropriate pharmacophore, an ideal attachment point of the linker, and the implementation of a spacer comprising optimal length and adequate physicochemical properties.

**Pharmacophores, Attachment Points.** Suitable pharmacophores for the synthesis of bivalent ligands should possess a low to medium molecular weight because incorporation of the linker and the spacer increases the molecular bulk significantly.[149] Additionally, lead compounds usually display high affinity in the low nanomolar range and subtype selectivity to ensure beneficial binding and selectivity profiles of the final bivalent ligands.

The identification of the most suitable attachment point of the spacer relies on two criteria: the feasibility of chemical modifications and the compatibility of these modifications with binding properties and intrinsic activity of the respective pharmacophore. Preferentially applied moieties for linking two pharmacological units are hydroxyl, amine, and carboxylic groups,[150,151] but also alkynes or azides are employed to create 1,2,3-triazole linkers via a copper-catalyzed 1,3-dipolar cycloaddition (Table 3).[131,152,153] The use of azides and terminal acetylenes requires additional reaction steps, as these functional groups are normally not present in common pharmacophores.

Typically, pharmacophores including the examples **7**[121,131,132,134]−**10**[127] (Table 3) possess more than one functional group and the linker has to be attached without impairing binding affinity or potency of the compounds. Therefore, a good understanding of structure−activity relationships is essential to identify an optimal anchor position. For instance, careful analysis of the potent and selective 5-$HT_4$ receptor partial agonist **9**[129] utilizing site-directed mutagenesis, molecular modeling studies, and the development of 5-$HT_4$-selective fluorescent probes revealed that bulky substituents in position 4 of the piperidine ring are well tolerated.[155] In contrast, modification of the aromatic amine moiety with fluorescent residues resulted in a complete loss of affinity.[156] On the basis of these results, Solier et al. successfully designed homobivalent serotonin 5-$HT_4$ receptor ligands incorporating two pharmacophores of the partial agonist **9** linked by different spacers in position 4 of the piperidine.[129]

**Spacer Length.** Critical parameters for the binding mode of bivalent ligands are both length and composition of the linking unit. Short spacer lengths favor targeting of a second allosteric binding pocket but inhibit the ability of the bivalent ligand to bridge two protomers of a GPCR dimer. Most successful studies on dimeric ligands demonstrated a correlation between spacer length and receptor affinity and determined an optimal distance range for spacer units connecting the two pharmacophores. In an early study on homobivalent ligands based on the scaffold of the $\mu$-OR agonist oxymorphamine, Portoghese et al. observed peak agonist potency at a spacer length of 18 atoms (~20 Å).[125] Further investigations on opioid receptors confirmed an optimal spacer length between 18 and 25 atoms.[91,115,154,157,158] This has been very indicative for these studies but not a general rule because the spacer length of a dimer spanning bivalent ligand depends on the


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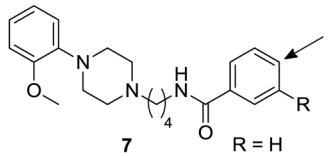
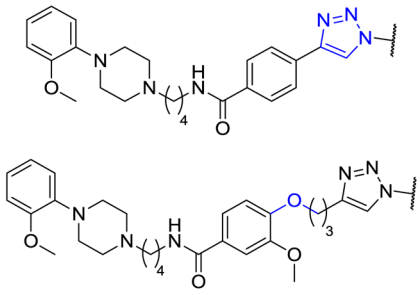
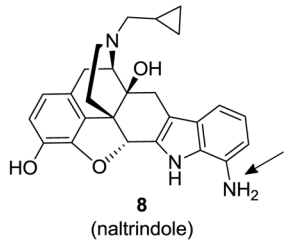
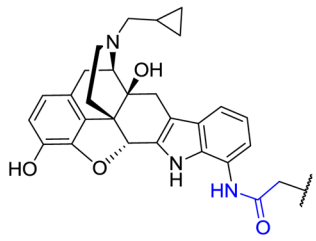
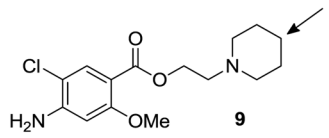
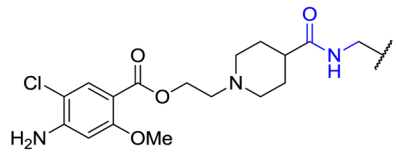
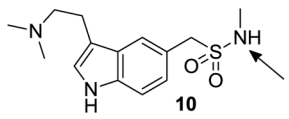
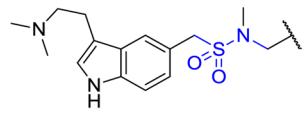
**Table 3. Pharmacophores, Attachment Points, and Linking Groups**[a]

| *Pharmacophore* | *Linking Group* | *Ref.* |
|---|---|---|
|  **7** R = H, R = OMe |  | 121, 131, 132, 134 |
|  **8** (naltrindole) |  | 154 |
|  **9** |  | 128, 129 |
|  **10** |  | 127 |

[a]Dopamine $D_2$ receptor partial agonists of type **7**,[121,131,132,134] $\delta$-selective antagonist **8**[154] (naltrindole), 5-$HT_4$-selective partial agonist **9**,[128,129] (ML10302), 5-$HT_{1B/1D}$ agonist **10**[127] (sumatriptan). Arrows indicate attachment points of linking groups.

dimer interface, the structure of the pharmacophores, and the topicity of the point of attachments.

Recently, McRobb et al. described homobivalent ligands to investigate dopamine receptor dimerization using the drug clozapine as pharmacophoric unit. Spacers of different length were attached at the N4′ position, and pharmacological evaluation of these compounds revealed significant gain in affinity and activity relative to clozapine for the compounds **11a**[135] and **11b**[135] (Figure 6) with spacer lengths of 16 and 18 atoms.

A library of bivalent ligands was synthesized to target the cannabinoid 1 ($CB_1$) receptor using two 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide (rimonabant, SR141716) pharmacophores.[141] This highly potent and selective $CB_1$ receptor antagonist was linked by spacers of various lengths, and the resulting bivalent ligands were investigated in radioligand binding studies and functional assays. The results of the pharmacological examination showed an initially increased and then decreased affinity and activity with elongation of the spacer. The highest gain in affinity compared to the monovalent control was determined for the bivalent ligand **12**[141] (Figure 6) composed of a 15-atom-containing spacer.

Interestingly, short spacer length can also improve binding or activity profiles compared to the monovalent control compounds. To determine the cross-talk between $\beta_2$-adrenergic ($\beta_2$AR) and adenosine $A_1$ ($A_1$AR) receptors, a series of bivalent

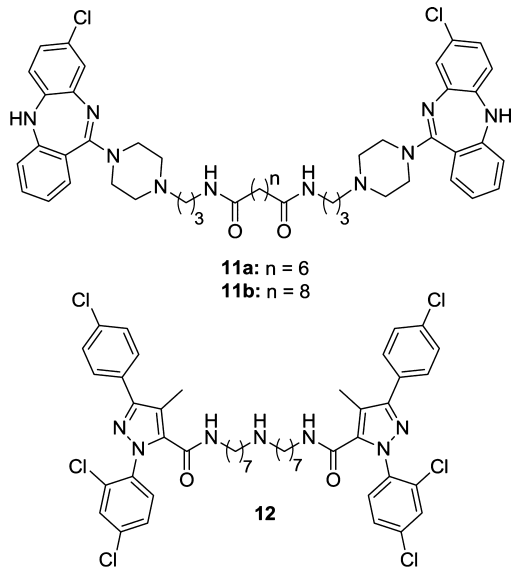



**Figure 6.** Homobivalent ligands based on clozapine and rimonabant.

agonists was synthesized incorporating the $\beta_2$AR agonist formoterol and the endogenous $A_1$AR agonist adenosine, whereby the side chain amine of formoterol and the $N^6$ position of adenosine proved to be suitable spacer connecting points. Best pharmacological properties were observed for the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hexyl-linked bivalent compound **13**[144] (Figure 7) that displayed the highest affinity for both receptor subtypes and was the most potent $\beta$-agonist.

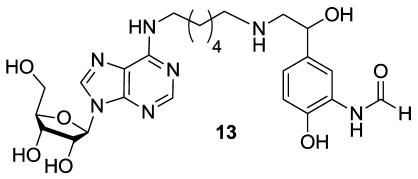


**Figure 7.** Bivalent ligand of the $\beta_2$ agonist formoterol and the $A_1$AR agonist adenosine.

Another study using the bivalent ligand approach focused on the development of highly potent and selective histamine $H_2$ receptor agonists based on acylguanidine scaffolds. The investigated bivalent compounds consisted of two pharmacophoric heteroarylpropylguanidine moieties acylated at the $N^G$ atom with dicarboxylic acids of different lengths.[130] Interestingly, compounds with sufficient spacer length (approximately 20 atoms or ~22−27 Å) for bridging two orthosteric recognition sites of a GPCR dimer displayed only weak agonism or a loss of agonistic activity and conversion to antagonism at the $H_2$ receptor, whereas incorporating shorter spacers resulted in potent $H_2$R agonists. The highest intrinsic activities were obtained by linking two heteroarylpropylguanidine entities by octandioyl or decanedioyl spacers (Figure 8).

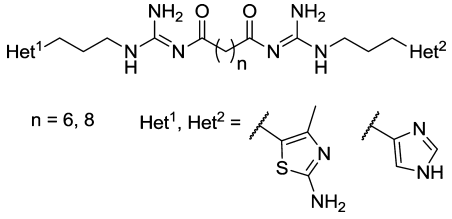


**Figure 8.** Homo- and heterobivalent $H_2$ receptor agonists.

Because these spacers are too short for simultaneously occupying recognition sites of two $H_2$R protomers, the remarkable increase in potency of the bivalent compounds relative to the monovalent controls is presumably caused by interaction with an accessory binding site at the same receptor.

These studies demonstrate that depending on the binding mode of the chosen pharmacophore and the respective relative position of the two linker attachment points as well as on the interface between the protomers, the optimal length of the connecting entity in a bivalent ligand may vary and needs to be determined empirically for each compound as well as for each receptor complex.

**Conformational Flexibility of the Spacer.** Besides spacer length, the chemical composition of the spacer plays an important role in modifying binding properties of the prospective bivalent ligands. In general, the spacer needs to be flexible enough to allow correct positioning of each pharmacophore in the binding pocket of each protomer. However, successful incorporation of a conformational restricted spacer into a bivalent ligand will provide valuable information of the required shape and nature of a linking entity and reduce the entropy penalty for the conformational restriction upon binding. Portoghese investigated the effect of conformational rigidization of the spacer on opioid receptor activity for the first time. The conformational mobility of the spacer was reduced by replacement of a succinyl group by a fumaryl moiety. Bivalent ligands consisting of two $\beta$-naltrexamine pharmacophores directly linked by a succinyl (**14a**, **14b**,[159] Figure 9) or fumaryl amide (**15a**, **15b**[159]) displayed

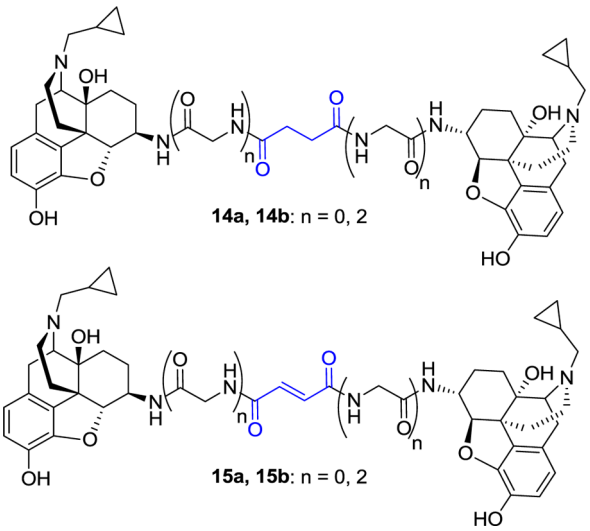


**Figure 9.** Homobivalent $\kappa$-opioid receptor antagonists.

significant differences in their selectivity profile. Whereas the succinyl bearing derivative **14a** was determined to be a potent $\kappa$OR antagonist, the corresponding fumaryl analogue **15a** displayed no $\kappa$-antagonistic properties. An increase in conformational flexibility by introduction of an additional four glycine units (**14b** and **15b**) led to a recovery of $\kappa$ antagonism.

Studies on GnRH and 5-$HT_4$ receptors analyzed the effect of rigid benzene diyne containing spacers on binding and functional activity of dimeric GnRHR antagonists or 5-$HT_4$ receptor agonists.[128,152] Comprising linking moieties as described in Figure 10, all dimeric compounds retained high

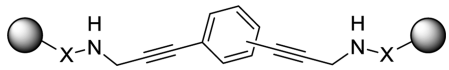


**Figure 10.** Conformationally constrained linking unit.

binding affinity, but their antagonistic or agonistic potency was significantly reduced compared to the monovalent analogues. In a BRET based assay ligand-induced conformational changes of the 5-$HT_4$ receptor by the 5-HT4-selective partial agonist **9** (Table 3) and the corresponding bivalent ligands were examined. In contrast to **9** and the control compound with a capped spacer, several bivalent ligands induced a significantly increased BRET signal, although no difference between flexible and rigidized linking entities was detected.

Forming well-defined helical structures that are maintained in aqueous solution, oligoprolines provide suitable rigidizing spacer elements and can be employed as molecular rulers to estimate distances, for instance, between two orthosteric binding sites of a GPCR dimer.[160] Poly(L-proline) helices are known to maintain a length of 0.9 nm per turn when one turn is formed by three proline residues in the case of a type II polyproline helix.[161] Tanaka et al. utilized oligoprolines as rigid linkers in the development of bivalent ligands targeting the


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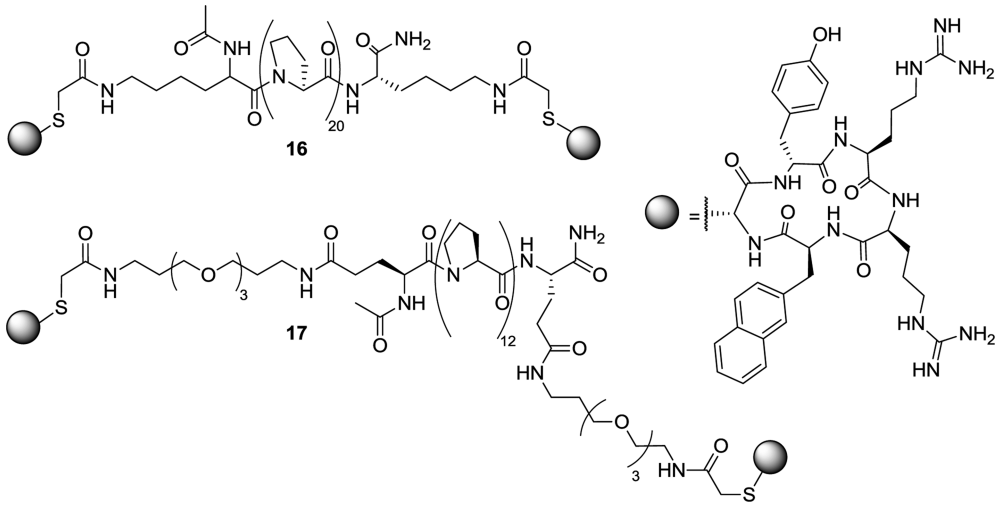


**Figure 11.** Oligoproline helices as molecular rulers.

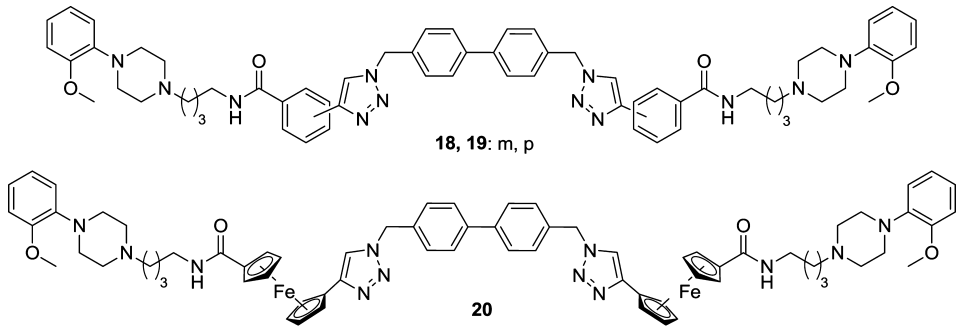


**Figure 12.** Ferrocene as a molecular hinge.

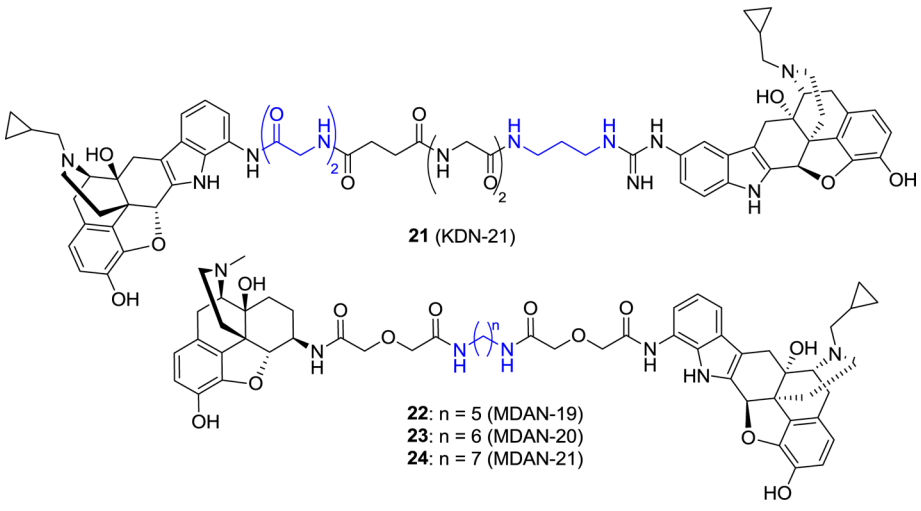


**Figure 13.** Linking units incorporating oligoglycine and diamine moieties.

chemokine CXCR4 receptor. Radioligand binding studies revealed that binding affinity of the bivalent ligands is dependent on linker length. The highest affinities were observed for compounds **16**[139] and **17**[139] (Figure 11) with a spacer length of 5.5−6.5 nm.

In addition to rigidizing elements, all above-mentioned spacers incorporated flexible entities that facilitate binding of the two pharmacophores. Bivalent ligands for the $D_2$-like receptors (Figure 12) were synthesized using less flexible bis-methyl-*p*-biphenyl spacers directly linked by 1,2,3-triazoles to 1,4-DAP pharmacophores. Characterization of the test compounds by radioligand displacement assays revealed poor binding affinities for the meta- and para-substituted benzamides **18**[131] and **19**[131] with $K_i$ values between 70 and 15 000 nM at the $D_2$-like subtypes. Interestingly, the ferrocenebenzamide **20**[131] showed excellent binding data ($K_i$ of 15, 14, 1.1, and 22 nM for $D_{2long}$, $D_{2short}$, $D_3$, and $D_4$, respectively).

These results clearly demonstrate the importance of conformational flexibility on potency of dimeric ligands. 1,1′-Disubstituted ferrocenyl subunits can serve as molecular hinges


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when rotation of the cyclopentadienyl moieties enables the two pharmacophores to adopt an optimal orientation.[131]

**Chemical Properties: Hydrophilicity, Polarity.** The polarity of the spacer is important for the solubility of the bivalent ligand, especially if the pharmacophoric unit is very hydrophobic. Implementation of hydrophilic groups improves aqueous solubility. Hence, many spacer groups involve polyamide or polyethylene glycol (PEG) units.[142,143,159] Compared to PEG, glycine oligomers are easier to synthesize but do not permit one-atom variations.[122,125] Therefore, polyamide spacers, most commonly oligoglycine moieties, are often combined with alkyldiamine or methylenediacyl cores (Figure 13).[154,158,162]

Besides water solubility, the spacer is also able to affect binding affinity and functional activity. Thus, it is essential to synthesize and biologically investigate monovalent controls containing the pharmacophore and a capped spacer in order to determine contributions of the linking unit to affinity and intrinsic activity.

## BIVALENT OPIOID RECEPTOR LIGANDS: PROOF OF CONCEPT

Opioid receptors are important targets for the treatment of pain. However, the administration of opioid analgesics is limited by several side effects, including respiratory suppression, obstipation, tolerance, and physical dependence. It has been demonstrated that the three opioid receptor subtypes $\delta$, $\kappa$, and $\mu$ are able to associate with each other, forming dimers with novel properties. Therefore, ligands specifically targeting these dimers provide a valuable approach to develop analgesics with improved side effect profiles.

In 2005, the group of Portoghese reported on bivalent ligands incorporating the pharmacophores of the highly potent $\mu$ agonist oxymorphone and the high affinity $\delta$ antagonist naltrindole tethered by spacers of different lengths in order to selectively target $\delta$OR−$\mu$OR heterodimers. This study addressed the question if physically interacting $\delta$OR−$\mu$OR heterodimers mediate opioid-induced tolerance and physical dependence. Chronic administration of the $\mu$ agonist−$\delta$ antagonist (MDAN) series in mice (**22**−**24**,[115] Figure 13) revealed that tolerance was clearly dependent on spacer length. Whereas ligands with short spacers containing 16−18 atoms exhibited tolerance comparable to the monovalent control (a $\mu$OR agonist containing a capped spacer) or morphine, ligands with linking units of 19 atoms or more displayed no significant tolerance. Furthermore, coadministration of a monovalent $\mu$OR agonist and a monovalent $\delta$OR antagonist produced a high degree of tolerance. Thus, the absence of tolerance is most likely due to the tethering of the pharmacophores and suggests the involvement of $\delta$OR−$\mu$OR heterodimers in opioid-induced tolerance. Additionally, bivalent ligands with spacers longer than 16 atoms were devoid of dependence. Altogether, ligands with spacer lengths longer than 22 Å (**22**−**24**) showed neither tolerance nor physical dependence. Moreover, ligand **24** (MDAN-21) exhibited high opioid agonist potency and was 50-fold more potent in vivo than morphine.[115] This study was a milestone, as it proved that rational designed bivalent ligands can be developed to confirm the existence and functional importance of GPCR heterodimers in vivo.

Very recently, a different approach was applied to characterize $\delta$OR−$\mu$OR heterodimers by using "tuned affinity" bivalent ligands. These ligands comprised a high affinity $\mu$OR pharmacophore linked to a low affinity $\delta$OR ligand. Binding of the highly potent $\mu$OR pharmacophore will increase the effective molarity of the tethered, low affinity $\delta$OR ligand, and thus, the bivalent ligand will display enhanced affinity at $\delta$OR−$\mu$OR heterodimers when compared to $\delta$OR monomers or homodimers. A bivalent ligand **25**[163] was synthesized containing the highly potent $\mu$OR antagonist naltrexone and the low affinity $\delta$OR agonist *N*,*N*-diethyl-4-(2,6-dimethylphenylpiperidin-4-ylidenemethyl)benzamide (DM-SNC80,[164] Figure 14). Radioligand competition binding assays were performed using monovalent controls comprising each pharmacophore and a methyl-capped spacer.

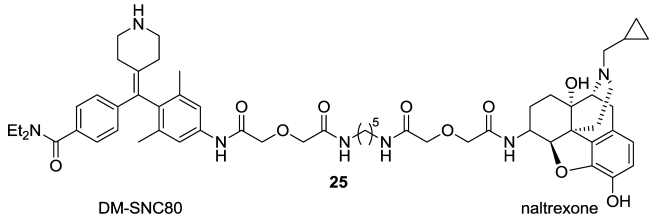


**Figure 14.** "Tuned-affinity" bivalent ligands.

Comparison of the binding data derived from cells expressing only $\delta$OR with data from cells expressing both $\delta$OR and $\mu$OR revealed a significantly (10-fold) increased affinity for the bivalent ligand **25** in the presence of both receptor subtypes. In contrast, the monovalent controls did not show a change in their binding profile. Hence, Harvey et al. were successful in designing a bivalent ligand that specifically targets the $\delta$OR−$\mu$OR heterodimer, and it provides a valuable tool to characterize this heterodimer as a novel therapeutic drug target.[163]

Another very important study demonstrating the value of bivalent ligands was published by Zheng et al.[91] Bivalent ligands were synthesized incorporating the $\mu$OR agonist oxymorphone and the $CCK_2$ antagonist *N*-[(3*R*)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-*N*′-(3-methylphenyl)urea (L-365,260) (Figure 15) to study the interaction of $\mu$OR with $CCK_2$ receptors, which are coexpressed in brain regions involved in the modulation of pain processing. BRET technology was used to measure the interaction of $\mu$-opioid and $CCK_2$ receptors. Under basal conditions, in the presence of monovalent ligands or of bivalent ligands with short spacers (nine atoms) no heterodimerization could be detected. However, the heterobivalent ligands **26a**−**c**[91] (Figure 15) with appropriate spacer length (16−22 atoms) did cause a BRET response, suggesting that they are capable of binding both receptors simultaneously and thus inducing the association of the receptors into a heterodimeric complex.

This was further investigated by the same group examining the effect of the bivalent ligands **26a**−**c** on heterodimeric receptor internalization.[165] Cells coexpressing the $CCK_2$ receptor and the $\mu$OR were treated with monovalent and bivalent ligands. While $CCK_2$ was not internalized upon treatment with the monovalent $CCK_2$ antagonist, agonist-induced endocytosis was observed for the $\mu$OR upon stimulation with the monovalent as well as with the bivalent ligand tethered by the short, nine-atom spacer. Interestingly, the bivalent ligands **26a**−**c** that are able to induce heterodimerization also led to cointernalization of the $\mu$OR together with the $CCK_2$ receptor, thus modulating the cell surface expression of the nonactivated $CCK_2$ receptor. Conclusively, these results demonstrate that bivalent ligands


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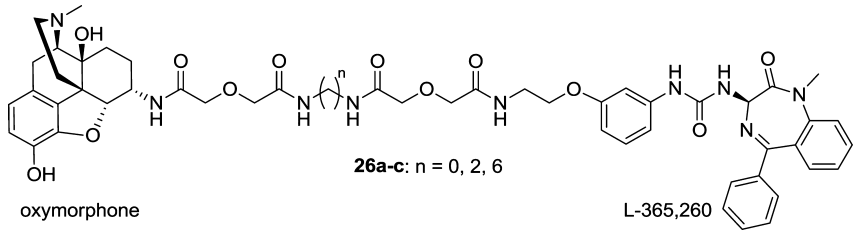


**Figure 15.** Bivalent ligand bridging $\mu$OR and $CCK_2$ receptors.

have the ability to induce association of receptors into heterodimeric complexes via a bridging mechanism even in the absence of native heterodimerization.

These inspiring examples highlight the feasibility and importance of the bivalent ligand approach to learn more about the dimeric properties of GPCRs as well as the physiological role of these complexes in vivo.

## CONCLUSION

Various studies employing different biochemical and biophysical techniques provide growing evidence/suggest that also class A GPCRs interact physically and form dimers or higher ordered oligomers. Besides coimmunoprecipitation of differentially epitope-tagged receptors, resonance energy transfer (RET)-based techniques have been utilized to visualize dimers in native tissues and in intact, living cells. Recent approaches using FRAP and TIRF microscopy indicate that GPCRs exist in a dynamic equilibrium between monomers and dimers. Allosteric communication between the protomers of a dimer can result in altered pharmacological properties compared to the respective monomers like altered ligand binding properties or modified receptor signaling and trafficking. Although the physiological significance of most GPCR homo- and heterodimers is not completely understood, targeting these complexes specifically may provide a great chance for the development of highly selective drug candidates with reduced side effects. Tethering two pharmacophoric entities by a spacer of specific length leads to bivalent ligands as valuable pharmacological tools for the investigation of both the quaternary structure and the functional consequences of GPCR dimerization. Several studies described that bivalent, two protomers bridging, ligands can be rationally designed if some important aspects are considered like the choice of an appropriate pharmacophore, an optimal attachment point of the connecting unit, and the composition of the spacer regarding length, hydrophilicity, and flexibility. Control experiments, including monovalent analogues and a combination of ligand binding studies with biochemical or biophysical techniques, are recommended to demonstrate a bivalent binding mode of the designed ligands. Recent crystal structures displaying parallel, symmetric dimers will facilitate the development and validation of bivalent ligands by structure-based design. The investigation of heterobivalent ligands is expected to give new insights into the physiological importance of GPCR heterodimerization in vivo and thus also in the understanding of potential heterodimer-specific diseases. The interplay between the development of new biological methods to test dimerization and the design of bivalent ligands will provide valuable insights into the function of GPCR dimers in vivo, their tissue-specific formation, and their contribution to the pathophysiology of diseases. The final goal is the development of more selective and efficacious drugs with fewer side effects.

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### Notes

The authors declare no competing financial interest.

### Biographies

**Christine Hiller** studied Pharmacy at the University of Erlangen-Nuremberg, Germany. Focusing on the synthesis and biological evaluations of enantiomerically pure dopamine $D_2/D_3$ receptor agonists, she received her Ph.D. under the guidance of Peter Gmeiner in 2012. Since then, she pursues research investigations in the GPCR field as a postdoctoral fellow at the University of Erlangen-Nuremberg.

**Julia Kühhorn** received a Bachelor's degree and a Master's degree in Molecular Life Sciences from the University of Erlangen-Nuremberg, Germany. She joined Peter Gmeiner's group as a Ph.D. student in 2007, where she worked on the design, synthesis, and biological evaluation of bivalent dopamine receptor ligands. She is now working as a postdoctoral researcher with Prof Guillermo Bazan at the University of California, Santa Barbara.

**Peter Gmeiner** received his Ph.D. in 1986 from the University of Munich, Germany. From 1987 to 1988 he was a postdoc at the University of California, Berkeley, U.S. Upon receiving his habilitation in 1992, he was appointed as a Professor of Pharmaceutical Chemistry at the University of Bonn, Germany. Since October 1996, he has held the Chair of Medicinal Chemistry at the University of Erlangen-Nürnberg, Germany. Peter Gmeiner's research spans the design, organic synthesis, and pharmacological investigation of bioactive molecules when class A G-protein-coupled receptors (GPCRs) are addressed. Within these studies, the development of novel types of GPCR ligands is of particular interest

## ABBREVIATIONS USED

5-HTR, serotonin receptor; 6′-GNTI, 6′-guanidinonaltrindole; $\alpha$AR, $\alpha$ adrenergic receptor; $A_1$R, $A_1$AR adenosine $A_1$ receptor; $AT_1$R, angiotensin $A_1$ receptor; AC, adenylyl cyclase; $\beta$AR, $\beta$ adrenergic receptor; $B_2$R, bradykinin $B_2$ receptor; BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; $CB_1$R, cannabinoid receptor 1; CFP, cyan fluorescent protein; co-IP, protein complex immunoprecipitation; CODA-RET, complemented donor−acceptor resonance energy transfer; CXCR, chemokine receptor; $D_{2L}$, dopamine $D_{2long}$ receptor; $\delta$OR, $\delta$-opioid receptor; DAMGO, [D-Ala$^2$,*N*-MePhe$^4$,Gly$^5$-ol]enkephalin; eCFP, enhanced cyan fluorescent protein; EP1R, prostaglandin E receptor; ER, endoplasmatic reticulum; eYFP, enhanced yellow fluorescent protein; FRAP, fluorescence recovery after photobleaching; GRK, G-protein-coupled receptor kinase; $H_2$R, histamine $H_2$ receptor; IUPHAR, International Union of Basic and Clinical Pharmacology; $\kappa$OR, $\kappa$-opioid receptor; M, muscarinic receptor; $\mu$OR, $\mu$-opioid receptor; MCP-1, mono-


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cyte chemotactic protein 1; MIP-1$\beta$, macrophage inflammatory protein 1$\beta$; NNTA, *N*-naphthoyl-$\beta$-naltrexamine; NorBNI, norbinaltorphimine; NTS, neurotensin receptor; OT, oxytocin receptor; PLC, phospholipase C; PTX, pertussis toxin; Rluc, luciferase from *Renilla reniformis*; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RNAi, ribonucleic acid interference; SSTR, somatostatin receptor; TIRF, total internal reflection fluorescence; tr-FRET, time-resolved fluorescence resonance energy transfer; TM, transmembrane; V, vasopressin; YFP, yellow fluorescent protein

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