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Oligomerization of G protein-coupled receptors: A reality

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Abstract

As reviewed in the present issue, we have now an important amount of experimental evidence that indicates that G protein-coupled receptor (GPCR) oligomerization, including homo- and heteromerization, is a general phenomenon. Receptor heteromers possess unique biochemical characteristics that are demonstrably different from those of its individual components (protomers). Those properties include allosteric modulations between protomers, changes in ligand recognition, G protein-coupling and trafficking. The discovery of GPCR oligomers have been related to the parallel discovery and application of a variety of resonance energy transfer (RET) techniques, such as bioluminescence, fluorescence and sequential RET (BRET, FRET and SRET, respectively), time resolved FRET (T-FRET) and fluorescence recovery after photobleaching (FRAP) microscopy. However, RET techniques are difficult to implement in native tissues. For receptor heteromers, indirect approaches, such as the determination of a unique biochemical characteristic ('biochemical fingerprint'), are allowing their identification in native tissues and their use as targets for drug development. Dopamine and opioid receptor heteromers are being the focus of intense research, which is related to the possible multiple applications of their putative ligands in pathological conditions, which include basal ganglia disorders, schizophrenia, drug addiction and pain.

General concepts

G protein-coupled receptors (GPCRs) have been classically perceived as receptors that do not "need" to oligomerize to be functional, to execute their basic function of transducing a signal from ligand binding to G protein activation (see Lohse in this issue [1]). In fact, recent studies have shown that monomers of class A GPCRs (adrenergic β_2 , rhodopsin and opioid μ receptors) reconstituted in lipid vesicles couple and activate their respective G proteins upon agonist binding [2–4]. Also monomeric rhodopsin in solution can activate its G protein transducin [5]. Nevertheless, we have now an important amount of experimental evidence that indicates that GPCR oligomerization, including homo- and heteromerization, is a general phenomenon (see all articles in this issue) and it still needs to be determined if GPCR monomers are functionally present in the cellular plasma membrane.

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There has been some confusion about the terms used to describe GPCR oligomers. One of the main reasons has been the lack of knowledge about what constitutes a receptor unit. It is redundant to talk about a “functional receptor” if we consider that receptor is currently defined as ‘a signal transducing unit, a cellular macromolecule or an assembly of macromolecules that is concerned directly and specifically with chemical signaling between and within cells’ [6]. Recommendations for definitions and criteria for identification of receptor heteromers have recently been proposed [6]. First we should make a distinction between ‘heteromeric receptor’ or ‘homomeric receptor’ and ‘receptor heteromer’ or ‘receptor homomer’. Heteromeric receptor is a dimeric or oligomeric receptor for which the minimal functional unit is composed of two or more different subunits that are not functional on their own. Homomeric receptor is the same as heteromeric receptor but composed of two or more identical subunits that are not functional on their own. On the other hand, receptor heteromer is a macromolecular complex composed of at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components [6]. Then, a receptor homomer is the same as receptor heteromer but combining two or more identical (functional) receptor units.

Most ligand-gated ion channels (ionotropic receptors) are well-known heteromeric receptors, but there are also heteromeric GPCRs, such as the GABA_B receptor, which is composed of at least two seven transmembrane (7TM) protein units, GABA_{B1} and GABA_{B2}, to constitute a (functional) receptor. Thus, the GABA_{B1} subunit is responsible for ligand binding and the GABA_{B2} subunit is responsible for G protein activation and signaling (described in this issue by Prezeau et al. [7]). In this case we have a complete asymmetry in the function of both 7TM units in the heteromeric receptor. Functional asymmetry of the two receptors in a receptor homodimer has also been shown for several GPCRs [1]. In a recent study, using a functional complementation assay, D₂ receptor homodimers with a single G protein was found to be a minimal signaling unit, which is maximally activated by agonist binding to one of the promoters, whereas additional agonist or inverse agonist binding to the second promoter blunts or enhances signaling, respectively [8]. This allosteric modulation of signaling results from a direct interaction of the receptor homodimer with the G protein, rather than from a downstream effect. A similar situation, two receptor units and one G protein, is most probably found in a receptor heterodimer, but in this case two different ligands interact in the heteromer, and allosteric interactions in the receptor heteromer have also been described (see Fuxe et al., this issue [9]). It is currently believed that an allosteric interaction in a receptor heteromer involves an intermolecular interaction by which binding of a ligand to one of the protomers changes the binding properties of another promoter [6,9,10]. The same is believed to occur in receptor homomers, which is translated into a positive or negative cooperativity of ligand-receptor binding [10]. It is important to realize that some observed receptor-receptor interactions postulated to involve and intermolecular crosstalk in a receptor heteromer might still correspond to an oligomerization-independent intracellular crosstalk (see below the comments about ‘biochemical fingerprint’ and Prezeau et al., this issue [7]).

In addition to allosteric modulations, GPCR oligomerization involves changes in ligand recognition, G protein-coupling and trafficking. Opioid receptor heteromers constitute an example of changes in ligand recognition in the receptor heteromer (see van Rijn et al. in this issue [11]). The opioid receptor subfamily comprises μ , δ and κ receptors, but at least two pharmacological ‘subtypes’, with different affinities for different ligands, have been identified for each of the three cloned receptors. The δ receptor can form heteromers with the μ and also with the κ receptors [11]. It seems that the two pharmacological subtypes of δ receptors, δ_1 and δ_2 receptors, correspond to a δ - μ receptor heteromer and a δ receptor homomer, respectively [11]. About changes in G protein-coupling, taking into account that recent models support that only one G protein can bind to two receptor units (see above), this means that a receptor heteromer will at least have to “decide” which G protein should bind to, if those

receptors are usually coupled to different G proteins. In fact, in some instances, the receptor heteromer can “choose” a completely new G protein. Dopamine receptors are classified in D₁-like, with the D₁ and D₅ receptor subtypes, which usually couple to G_{s/olf} proteins, and D₂-like, with the D₂ and D₄ receptor subtypes, which couple to G_{i/o} proteins. As described by Fiorentini et al. in the present issue [12], the D₁-D₃ receptor heteromer couples to G_{s/olf}. As reviewed by Hasbi et al. in the present issue [13], the D₁-D₂ and D₅-D₂ heteromers couple preferentially to G_q proteins.

There is experimental evidence that indicates that most GPCR oligomers form in the endoplasmic reticulum (ER) and that their synthesis is a ligand-independent process. In fact, it has been shown that in some cases dimerization/oligomerization allows the seven trans-membrane (7TM) protein or GPCR (depending on if we are dealing with a potential subunit of a homomeric or heteromeric receptor or a unit of a receptor homomer or heteromer) to escape from its conditional retention in the ER, such as the adrenergic α_{1D} receptor, which needs to heteromerize with α_{1B} to be transported to the cell membrane (see ref. [14] and Milligan, present issue [15]). Most probably the “chaperone” receptor simply masks the ER-retention motif in the “unmarried” receptor. ER-retention motifs can in fact be used to demonstrate heteromerization. For instance, cell surface delivery of the chemokine CXCR₁ receptor was impaired when fused to an ER-retention motif of the adrenergic α_{2C} receptor, but ER retention was prevented by co-transfection with the close-related chemokine CXCR₂ receptor [15]. Once in the cell surface, many GPCRs internalize spontaneously or upon agonist binding. Indeed, the capacity of a selective ligand of one GPCR to cause co-internalization of a second co-expressed GPCR which does not bind the same ligand is used experimentally as evidence for heteromerization. For instance, in cells co-expressing chemokine CXCR₄ and CXCR₅ receptors, their respective ligands (CXCL12 and CCL5, respectively) induced co-internalization of both receptors (see Contento et al, [16] and Thelen et al. in this issue [17]).

Investigation of receptor oligomerization by energy transfer-based techniques

The use of the energy transfer-based techniques bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET) has been fundamental for taking the issue of GPCR oligomerization at the front of GPCR research, providing evidence for an increasing number of GPCR receptor heteromers in living cells. RET consists of a nonradioactive transfer of energy from a chromophore in an excited state, the ‘donor’, to a fluorescent ‘acceptor’ molecule. In FRET both molecules are fluorescent, whereas in BRET the donor molecule is an enzyme which becomes bioluminescent upon catalyzation of its substrate. Importantly, the efficacy of energy transfer is inversely proportional to the sixth power of the distance between the donor and acceptor chromophores. This implies that the range of energy transfer from the donor to the acceptor is beneath 10 nm (see Ayoub and Pflieger, this issue [18]). When using this technology to study GPCR oligomerization the donor and acceptor molecules are usually fused to the C-termini of GPCRs and co-transfected in mammalian cell lines. Since the diameter of the seven TM helical core is estimated to be about 5 nm, a positive energy transfer strongly suggests the existence of oligomerization [18].

It is important to point out that BRET and FRET do not demonstrate the existence of physical contact between the fused proteins, but that there exists a very close proximity, which could of course depend on oligomerization. Nevertheless, when using BRET, physical contact can practically be shown by using several different experimental approaches. Those possibilities include BRET saturation assays, BRET competition assays and experiments that observe ligand-promoted changes in BRET [18]. In BRET saturation experiments, a constant amount of the donor fusion protein (usually a receptor fused to *Renilla Luciferase* (RLuc)) is co-expressed with increasing amounts of the acceptor fusion protein (usually a receptor fused to

yellow fluorescent protein (YFP)). If there is oligomerization, saturation is reached when all receptor-RLuc molecules are specifically associated with their receptor-YFP counterparts. By contrast, if the BRET signal results from random collision promoted by high receptor density, a quasi-linear curve is obtained. In BRET competition assays an untagged receptor A or B is co-expressed with the receptor A-RLuc and receptor B-YFP. Theoretically, if there is receptor A-receptor B oligomerization, the BRET signal is specific and decreases as a consequence of the untagged receptor interacting with one of the fusion proteins and competing for the complementary BRET fusion protein. Finally, in some cases, when receptor A-RLuc and receptor B-YFP are co-expressed, changes in BRET signal due to ligand binding to the heteromer can be detected, which can be indicative of ligand-induced conformational changes in the heteromer. This has been for instance observed in experiments with melatonin (MT) receptor heteromers [19]. FRET-based methods have also been introduced to monitor the signal transfer within a receptor heteromer upon ligand binding (described by Lohse in this issue [1]). These techniques detect movements (conformational changes) within a GPCR homo- or heteromer by inserting fluorophores, for instance cyan fluorescent protein (CFP) and YFP, in intracellular loops of both or just one of the protomers (intramolecular FRET and intermolecular FRET). A nice example was the recently reported application of this technique to the study of adrenergic α_2A -opioid μ receptor heteromer. In this case, when the double-inserted α_2A receptor was co-expressed with μ receptor decreased its response to noradrenaline in the presence of morphine [20]. This inhibitory effect was extremely fast (a half-life of less than 500 ms), strongly suggesting that it was due to direct protein-protein interaction rather than competition for G-protein subunits [20].

Sequential-BRET-FRET (SRET) has been introduced to identify oligomers formed by three different proteins [21]. In SRET, the oxidation of an RLuc substrate by an RLuc-fusion protein triggers the excitation of the acceptor GFP² by BRET² and subsequent energy transfer to the acceptor YFP by FRET [21]. BRET² differs from BRET¹ in the acceptor, CFP² instead of YFP, and the substrate for RLuc, DeepBlue C instead of coelenterazine. This technique has been used to demonstrate the heteromultimerization between adenosine A_{2A}, dopamine D₂ and cannabinoid CB₁ receptors [21] and between A_{2A}, D₂ and glutamate mGlu₅ receptors [22]. In the present issue Ferré et al. provide another example of the use of SRET, to demonstrate oligomerization of A_{2A}, D₂ and calmodulin [23]. A_{2A}-CB₁-D₂ and A_{2A}-D₂-mGlu₅ heteromers were also demonstrated by a combination of bimolecular fluorescence complementation (BiFC) and BRET techniques [22,24]. BiFC is based on the ability to produce a fluorescent complex from non-fluorescent constituents if a protein-protein interaction occurs. Two receptors are fused at their C termini with either N-terminal or C-terminal fragments of YFP, respectively, and receptor heterodimerization causes YFP reconstitution. Then, if there is heterotrimerization, BRET can be obtained when the cells also co-express the third receptor fused to RLuc [22,24].

The use of acceptor and donor molecules that are genetically fused to GPCRs in classical FRET and BRET approaches can alter the functionality of the receptor heteromer under study. Furthermore, the fusion proteins can also be expressed in intracellular compartments, making difficult to demonstrate that the resonance energy transfer results from a direct interaction of proteins at the cell surface. Finally, an additional limitation of classical FRET is the low signal-to-noise ratio resulting from the intrinsic fluorescence of the cells and the overlap between the emission spectra of FRET donors and acceptors. Time resolved FRET (TR-FRET) is a FRET variant designed to circumvent these problems. TR-FRET is based on the prolonged fluorescence characteristics of certain rare earth tracers, lanthanide compounds, which enable the processes of excitation and detection to be separated temporally. In TR-FRET, the acceptor and donor fluorophore molecules are usually conjugated to antibodies against N-terminally epitope-tagged sequences and membrane-impermeant antibodies are used to exclusively assess cell surface GPCR oligomerization. This technology has been used to demonstrate

oligomerization of vasopressin and oxytocin receptors as reviewed in this issue by Cottet et al. [25]. A combination of TR-FRET with snap-tag technology has been recently introduced [26]. A snap tag, two-thirds the size of YFP, is introduced at the N-terminal end of a GPCR. The snap tag derives from O⁶-guanine alkyltransferase, which covalently reacts with benzyl guanine, and non-permeant benzyl guanine derivatives covalently labeled with a fluorophore compatible with TR-FRET measurements are applied. This technology has been used to demonstrate the existence of homomers of the heteromeric GABA_B receptor and to confirm the predominant arrangement of glutamate mGlu receptors as homodimers (see ref. [26] and Prezeau et al., in the present issue [7]).

Finally, another recently introduced new RET methodology to study GPCR oligomerization is called fluorescence recovery after photobleaching (FRAP) microscopy (described by Lohse in the present issue [1]). One receptor carries YFP at the extracellular N-terminus and another receptor carries CFP at the intracellular C-terminus. An area of the cell membrane is bleached and the exponential time-course of the fluorescence recovery is monitored. Binding of an antibody to the YFP-tag markedly impairs fluorescence recovery of receptor-YFP, while does not modify recovery of receptor-CFP, unless they are in close proximity. This technique can provide information about the dynamics of the interactions between protomers in GPCR oligomers [1]. Thus, a moderate delay or a lack of delay in fluorescence recovery upon immobilization of a partner receptor indicates a transient or a stable interaction, respectively. For instance, the results by Dorsch et al. indicate that adrenergic β_1 and β_2 receptors form transient and robust homomers, respectively [27].

Pharmacological implications of receptor oligomerization

Receptor heteromers, in particular, open up many implications for pharmacology, since they constitute new targets for drug development. An important step in this research field is the identification of receptor heteromers in native tissues. Thus, RET techniques are only straightforward when studying receptor heteromerization in artificial cell systems. Direct identification could be achieved by taking advantage of selective probes (for example, specific antibodies or labeled selective ligands) that could discriminate between the receptor heteromer and other configurations of the individual components. Bivalent ligands, with selective ligands for each protomers attached by a spacer with variable length can become a good strategy, as recently been reported for the A_{2A}-D₂ receptor heteromer [28]. No specific receptor heteromer antibodies have yet been reported. An indirect but valid strategy is to determine a 'biochemical fingerprint' of the receptor heteromer, i.e. a biochemical characteristic of a receptor heteromer, which can be used for its identification in a native tissue [6]. A strong suggestion that a biochemical fingerprint is specific for a receptor heteromer can be obtained by showing that it is abolished or altered when the heteromerization is disrupted, or alternatively when the quaternary structure of the heteromer is significantly modified without disrupting heteromerization. This of course could be shown with RET in the artificial cell system and requires identification of the domains or epitopes (of at least one of the receptors) that form the interaction surface in the heteromer. This is now an area of intense research, which uses computation techniques and bioinformatics (reviewed by Simpson et al, present issue [29]. These studies can guide experiments with mutated/chimeric receptors or the design of peptides that can selectively occupy and disrupt the receptor heteromer interface [6].

Opioid and dopamine receptor heteromers are being the focus of intense research, which is related to the possible multiple applications of their putative ligands in pathological conditions, which include basal ganglia disorders, schizophrenia, drug addiction and pain (in the present issue, see reviews by Fuxe et al. [9], van Rijn et al. [11], Fiorentini et al. [12], Hasbi et al. [13], Maggio and Millan [30] and Bushlin et al. [31]). In some cases, the allosteric interactions in the receptor heteromer can be used as a strategy to modify the effect of already existing

drugs. The best example is the addition of an A_{2A} receptor antagonist as a means to potentiate the antiparkinsonian effects of L-dopa [9]. In other cases targeting both promoters in the heteromer can modify some trafficking-related non-wanted effects. For instance, the opioid δ - μ receptor heteromer seems to provide a better target than either μ or δ receptors alone, since blockade of the δ receptor decreases tolerance to the analgesic effects of the most used μ receptor agonist, morphine. In the same line, bivalent ligands (with a μ receptor agonist and a δ receptor antagonist attached by a spacer with variable length) are being developed [11]. It is important to comment that δ - μ receptor heteromer is being the focus of debate, since a recent study questioned the co-localization of δ and μ receptors [32], an obvious prerequisite for receptor heteromerization. Using a recently generated δ receptor-eGFP knock-in mice [33], δ and μ receptor cellular co-localization in dorsal root ganglia was carried out using anti-GFP and anti- μ receptor antibodies, and was reported to be ~5% [30]. But given the increased level of δ receptor expression in these knock-in mice [33] and the high avidity of the anti-GFP antibody as compared to the anti- μ receptor antibody, it is most likely that the level of μ receptor co-expressed with δ receptor was underestimated in this study.

Furthermore, previous studies have found that the GFP tag at the C-terminus affects the maturation of δ receptors [34] and that the levels of δ receptor attenuate the maturation of μ receptors [35]. Finally, an important point to consider is that GPCR expression is altered during development and in pathology. Under these conditions, the level of receptor co-expression is likely to be significantly altered, making some particular GPCR heteromers attractive drug targets. The dopamine D₁-D₃ receptor heteromer constitutes a putative example of a pathology-involved receptor heteromer. It is thus possible that an increase in the expression of D₁-D₃ receptor heteromers be directly involved in L-dopa-induced dyskinesia in patients with Parkinson's disease (see Fiorentini et al. in this issue [12]).

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Biographies

Sergi Ferré is principal investigator at the National Institute on Drug Abuse, I.R.P., N.I.H., D.H.H.S. He is also guest professor from the Department of Biochemistry and Molecular Biology at the Faculty of Biology, University of Barcelona and adjunct associate professor from the Department of Pharmacology and Experimental Therapeutics at the University of Maryland School of Medicine. His general research interest is the understanding of the functional role of neurotransmitter receptor heteromers and their potential use as targets for drug development in neuropsychiatric disorders and drug addiction. He is particularly interested in the discovery of heteromers of receptors that are targets for addictive drugs (such as dopaminergic, cannabinoid, and nicotinic acetylcholine receptors) and with the analysis of their biochemical and pharmacological properties involving studies at the cellular level as well as at the in vivo level. At a more molecular level, he is also interested in finding the molecular determinants of the quaternary structure and function of receptor heteromers. He is principal editor of 'The Scientific World Journal' and member of the editorial board of 'Central Nervous System Agents in Medicinal Chemistry' and 'Recent Patent Reviews on CNS Drug Discovery'.

Rafael Franco is a professor of Biochemistry and Molecular Biology. He has been professor at the University of Barcelona, Spain, for 13 years. While keeping a link with this University he has recently moved to the Center for Applied Medical Research (www.cima.es), at the University of Navarra, Pamplona, Spain, where he heads the laboratory of Molecular Neurobiology and Neuropharmacology. His research interest focuses on the understanding of the physiological role of G-protein-coupled receptor heteromerization. He is particularly interested on the interactions established by adenosine, dopamine and cannabinoid receptors in the striatum with emphasis in the relationships between quaternary structure of the heteromers and signaling and function. He has developed the framework of a dimer-based pharmacology based on the idea that drug development should contemplate G-protein-coupled receptor heteromers instead of monomers. He has helped in developing a dimer-based model, which is a more robust model than the ternary-complex model, to better understand G-protein-

coupled receptor operation, improve data handling and optimize drug discovery strategies. He serves on a number of grant review panels and consults for Pharmaceutical and Investment companies.