

Chapter 1

Historical Perspectives: From Monomers to Dimers and Beyond, an Exciting Journey in the World of G Protein-Coupled Receptors

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Abstract G protein-coupled receptors (GPCR)s are the largest family of proteins in the human genome, and for a long time they were thought to be monomeric in nature. Nowadays, this belief seems rather eccentric, and the concept of lonely GPCRs wandering around the cell membrane has been replaced by a different view in which GPCRs have instead a very active social life, with promiscuous coupling among, but not limited to, their family members. This short chapter summarizes the major steps that have led scientists to change their convictions, from strong supporters of GPCR individualism to enthusiastic appreciators of GPCR camaraderie. A fascinating journey started more than 40 years ago that keeps and will continue to fascinate and excite the scientific community for years to come.

Keywords G Protein-Coupled Receptor • Dimerization • Radioligand Binding • Resonance Energy Transfer • Bivalent Ligand

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Abbreviations

BRET	bioluminescence resonance energy transfer
ER	endoplasmic reticulum
FRET	resonance energy transfer
GPCR	G-protein-coupled-receptor
PALM	photoactivated localization microscopy
PLA	proximity ligation assay
RET	resonance energy transfer
SMT	single-molecule tracking
TM	transmembrane
TR-FRET	time-resolved resonance energy transfer

1.1 Introduction

Sherlock Holmes, the fictional detective, made famous by the pen of the Scottish writer and physician, Sir Arthur Conan Doyle, quoted: “when you have eliminated the impossible, whatever remains, however improbable, must be the truth”. This simple sentence holds also in science, especially when avant-garde ideas challenge pre-existing old concepts. In particular, because scientists are more likely to accept simpler ideas than complex-ground-breaking concepts, unconventional ideas have to go through a lot of scepticism, opposition and through intense validation processes before being fully accepted by the scientific community. The History of science offers numerous examples of this convoluted process [1] and one of the kind is certainly the concept of G protein-coupled receptor (GPCR) dimerization.

GPCRs, the largest family of cell surface proteins in eukaryotes, mediate the function of a remarkably large number of extra-cellular stimuli that range from light photons to large proteins. Importantly, about 4% of all human genes code for proteins of the GPCR family, and 30–40% of all the drugs currently in use target these receptors. Understanding the molecular mechanisms by which GPCR mediate signal transduction would therefore be crucial for the development of novel and more effective therapeutic drugs.

One of the earliest models of GPCR signal transduction, the “one ligand-one receptor interaction” model, identified receptor monomers as the “only” functional unit able to activate G-proteins. Indeed, experiments with GPCR single monomers incorporated into reconstituted lipid bilayers showed that single monomers of β_2 -adrenergic, rhodopsin and μ -opioid receptors were able to directly interact with G proteins [2–4] validating the concept of monomers as the “only” functional unit responsible for GPCR signalling. However, during the past two decades, an increasing number of experimental evidence has shown that GPCRs form dimers and oligomers and have suggested that the “one ligand-one receptor interaction” model is too simplistic and could not accurately explain the complexity of GPCR signalling [5].

Nevertheless, because GPCRs have been considered to exist exclusively as monomers for a long time, the dimerization concept had to go through a laborious and exhausting scientific analysis before being widely accepted.

This chapter summarizes the most significant steps, from the early pioneer works to the most recent and sophisticated studies that have led scientists to the validation of the well accepted ground-breaking theory of GPCR dimerization as a crucial mechanism in GPCR-mediated physiological processes.

1.2 The Birth of the Dimerization Concept

The first indication that GPCRs could interact with each other goes back to 1975, when Lee E. Limbird, Pierre De Meyts and Robert J Lefkowitz, discovered a negative cooperative interaction among the β -adrenergic receptors [6]. The existence of such negative cooperativity was tested by a kinetic binding method, where the dissociation of receptor bound [^3H](–)alprenolol was studied either by the sole dilution of the ligand-receptor complex and by dilution in the presence of an excess of unlabeled (–)alprenolol. In particular, it was observed that the presence of the unlabelled (–)alprenolol increased the rate of [^3H](–)alprenolol dissociation, indicating that negatively cooperative interactions occurs among the β -adrenergic receptor binding sites. These data have become a milestone in the study of GPCR dimerization with radioligand binding assays [7]. However, in order to have more direct evidences of GPCR dimerization, we had to wait till the work of Claire M. Fraser [8] (1982), in which, analysis of mammalian lung membranes showed the existence, *in-vivo*, of β_2 -receptor dimers with an apparent molecular weight of $\sim 109,000$ Daltons. Moreover, in photoaffinity labelling experiments, Sofia Avissar et al. (1983) demonstrated that muscarinic receptors, as well, could dimerize [9]. Strikingly, Avissar found that muscarinic receptor monomers go through a different degree of dimerization depending on the tissue from which these receptors were isolated. Specifically, muscarinic receptors were found as dimers (86,000 Daltons) in the cortex and hippocampus whereas as tetramers (160,000 Daltons) in the medulla, pons, cerebellum, and cardiac atria of rats. Taken together these data suggested that muscarinic receptors form dimers and/or oligomers in a “tissue-specific” manner indicating that “dimerization” was important for muscarinic receptor activity, *in-vivo*.

Even more intriguing evidences of GPCR dimerization came from the studies of [10] Roberto Maggio, Zvi Vogel and Jurgen Wess, (1993) with α_2/M_3 and M_3/α_2 chimeras, in which, the transmembrane (TM) regions 6 and 7 were exchanged between the α_2 -adrenergic and M_3 -muscarinic receptors. Strikingly, even though these chimeras were no-longer functional, their responses were restored when co-expressed together. This clearly indicated that the chimeras regained their functionality through a dimerization process. In addition, data from epitope-tagged receptor techniques demonstrated that the TM regions of GPCRs were important for the interaction between GPCR monomers. In fact, a peptide corresponding to the TM6

region of the β_2 -receptor was able to disrupt β_2 -receptor dimerization and reduce β_2 -receptor function [11]. Moreover, in 1996 another independent study showed the importance of TM regions for the interaction between dopamine D_2 receptor monomers. In fact, in this study, a peptide corresponding to the TM regions 6 of the D_2 receptor was able to completely prevent dimerization of this receptor [12] strongly suggesting that GPCR-trans membrane domain interactions were a crucial step for GPCR dimer formation.

While at the beginning it was proposed that GPCR dimerization might occur by a mechanism of domain swapping, soon it became clear that the most plausible mechanism of dimerization was by lateral contacts among external surfaces of GPCRs [13]. This became even more evident with the finding that GPCRs not only could form homodimers, but they could also form heterodimers; in fact, it is important to notice that the receptor domains involved in the heterodimerization process are substantially different from each other and therefore the chances of domain swapping very unlikely.

One of the first clear evidence of heterodimerization between two distinct wild type GPCRs came from three works that appeared simultaneously in the same issue of Nature in 1998 [14–16]. Remarkably, these studies showed that GABA_{B1} and GABA_{B2} receptors had to assemble into heterodimeric complexes in order to proper function, given that the two monomers alone were inactive. In addition, a few years later, other studies have shown that the taste receptors go through heterodimerization processes in order to be functionally active [17, 18]. Strikingly, heteromerization was also demonstrated for other GPCR receptor subtypes, either within the same subfamily, such as between muscarinic M_2 and M_3 receptors [19], or across subfamilies such as between the distantly-related receptors dopamine D_2 and somatostatin SST₅ receptors [20]. Taken together these data began to raise the interest of the scientific community toward the concept of GPCR homo- and hetero-dimerization as important pharmaceutical targets for the development of new therapeutic strategies.

1.3 Establishing the New Concept

A great improvement in the field of GPCR homo- and hetero-oligomerization occurred with the introduction of techniques such as the resonance energy transfer (RET) approach and the single-molecule microscopy detection system. In fact, fluorescent microscopy *per-se* would not be able to resolve densely packed fluorescent or bioluminescent probes due to the physical resolution limit imposed by diffraction; therefore, scientists had to wait for the development of these new methodologies in order to actually visualize GPCR-GPCR interactions. The RET methods, the fluorescent energy transfer (FRET) and the bioluminescence energy transfer (BRET), are based on the principle that energy can be transferred from one probe to another probe by resonance. In particular, energy would be transferred from a donor to a light sensitive acceptor only if donor and acceptor are sufficiently close to each

other, less than 10 nm. Thus, for instance, only when two GPCRs, one carrying the donor and the other carrying the acceptor, are in close proximity, and therefore interacting with each other, the energy can be transferred, detected and GPCR dimerization process studied [21, 22].

One of the first interesting observations researchers made using RET methods, was that GPCR dimerization occurred during the maturation process in the endoplasmic reticulum (ER), which suggested oligomerization as a quality control system for newly synthesized receptors [23]. This hypothesis was clearly demonstrated for some obligatory heterodimers, such as GABA_B receptors, and also proposed for other GPCRs. Therefore, GPCRs were thought to be organized in stable dimeric complexes before actually reaching the plasma membrane. However, this theory has recently been challenged by data obtained with the more sensitive single-molecule microscopy analysis. Nonetheless, improved RET methods such as the time-resolved FRET (TR-FRET) and the proximity ligation assay (PLA) indeed played a crucial role in validating the existence of GPCR complexes in primary cells and tissues [24, 25].

Today, the introduction of super resolution microscopy techniques such as the Photoactivated Localization Microscopy (PALM) has overcome the limit of resolution imposed by diffraction on fluorescence microscopy more effectively than RET approaches. In particular, PALM methods together with the single-molecule tracking (SMT) detection system have provided an extraordinary tool to visualize receptors at the single-molecule level. There is no doubt that these methods withhold the potential for understanding GPCR behaviors and for providing direct evidences of the existence of receptor dimers and oligomers in living cells. Importantly, PALM has permitted the visualization of single receptors highly expressed in fixed samples, while SMT has determined how GPCRs move and interact in living cells in the presence of different ligands [26, 27].

Strikingly, SMT has revealed the transient dynamic nature of dimer formation, where the GPCRs examined so far display a monomer-dimer equilibrium characterized by rapid association and dissociation [28]. At steady state, approximately 30–60% of receptors, depending from the subtype, are part of dimeric complexes. This is undeniably a breakthrough in the GPCR dimerization story; it proves that receptor monomers form transient dimers and at the same time it raises many questions about the molecular mechanisms involved in the dimerization processes and in the way dimer complexes function. In conclusion, the ability of GPCRs to form dimers *in-vivo*, has been supported by well-known direct visualization techniques, such as RET (as mentioned above), and more sensitive techniques like PALM and SMT.

1.4 Oligomers Make the Picture More Complicated

While evidences of GPCRs homo- and hetero-oligomerization were accumulating, it became clear that GPCRs could also assemble into higher-order oligomers. The first “direct” evidence of *in-vivo* GPCR oligomerization came from the seminal

work of the Palczewski' group [29], in which native membranes obtained from wild-type mouse retinal photoreceptors were analyzed by atomic force microscopy. In particular, this study gave direct experimental evidences that the prototypical member of class A GPCR rhodopsin receptor, formed paracrystalline arrays of dimers in rod outer-segment disc membranes of mouse retinal photoreceptors. Even though oligomerization could have been caused by the high density of rhodopsin receptors in the retina, the arrangement of dimers in rows strongly indicated that oligomer formations were the natural consequence of precise contacts between congruent interfaces of individual rhodopsin monomers. Afterward, Fotiadis showed that rhodopsin receptor oligomers, isolated with gentle detergents from native disk membranes, were able to activate G proteins more efficiently than rhodopsin-dimers or rhodopsin-single monomers [30, 31]. These data strongly suggested that tightly packed rows of rhodopsin-dimers were critical for proper light-mediated G-protein activation, in-vivo. The β_2 adrenergic receptor is another typical example of G-protein coupled receptor whose functions are finely regulated by oligomerization [32]. In particular, mutagenesis and co-expression experiments have shown that β_2 receptor oligomerization originates within the ER and is essential for the receptor maturation and trafficking to the plasma membrane [33]. The existence of β_2 receptor oligomers was also confirmed in cardiomyocytes by using super-resolution technique PALM [34]. An exciting hypothesis is that homo-oligomerization is a condition for GPCR compartmentalization, and therefore for confined increases of second messengers [35]. This hypothesis originates from a study with neonatal rat cardiomyocytes, in which β_1 adrenergic receptors were suggested to be co-localized with phospho-diesterase enzymes in specific areas of the cardiac myocyte plasma membranes. Specifically, Zaccolo and Pozzan [36] (2002) showed the formation of multiple micro-domains of stimulated β_1 receptors that produced cAMP accumulation in specific spots in cardiomyocytes, while co-localized phosphodiesterase prevented cAMP from diffusing. These cAMP "hot" spots would then activate a subset of protein kinase A molecules anchored in proximity to the T tubule and thus coordinate cardiomyocyte contractions [36].

However, in order to understand the role of oligomerization in GPCR functions, it is important to determine similarity and differences between oligomers and dimers in terms of GPCR-GPCR or GPCR- associated protein interactions that ultimately would affect GPCR signaling. For example, If we look at class C GPCRs, such as GABA_B receptors, it would appear clear that GABA_B heteromers are stable due to strong noncovalent interactions (same for GluR homomers), while GABA oligomeric complexes rely on weaker and transient interactions between heterodimers [27, 37]. Similar conclusions were also made for the class A GPCR M₃ muscarinic receptors, which might exist as either stable dimeric units or as tetramers [38]. Strikingly, another study demonstrated that the dopamine D₂ receptor formed tetramers and high-order oligomers using different TM domains [39]. These data further suggested that D₂ tetramer mediated signaling might dramatically differ from the signaling trigger by D₂ oligomer complexes [40]. Another interesting concept that would further emphasize the differences between dimer- and oligomer-mediated intracellular signaling is the spatial localization in specific "hot" spots of a cell type

that occurs for some higher-order oligomers (e.g. β_1 receptors co-localized with phospho-diesterase enzymes in cardiomyocytes). In fact, intracellular compartmentalization has been demonstrated to play a major role in generating physiologically-important cell-type specific signals” [40].

Other evidences of the importance of higher-order oligomers for GPCR function, in particular for hetero-oligomer complexes, came from the work of Bonaventura et al. (2015), with Adenosine A_{2A} and dopamine D_2 receptors [41]. They demonstrated that A_{2A} and D_2 receptors formed heterotetramers with unique pharmacological properties by showing a powerful allosteric type of interaction between A_{2A} and D_2 receptors, in which A_{2A} ligands decreased the affinity and intrinsic efficacy of agonists for D_2 receptors.

Moreover, another intriguing concept that must be mention about GPCR oligomer mediated signalling is the idea of “coincident-detection”, in which, different protomers within the same hetero-oligomer are activated simultaneously to generate a “synergic” increase in second messenger productions, and thus their downstream effects [35].

Even though, during these years, studies on GPCR dimerization and oligomerization have improved our knowledge on this fascinating field, many questions remain to be properly answered such as: What kind of interactions are responsible for the formation of dimers and eventually oligomers? What are the sizes of such oligomers? What functions do they serve? Which are the factors controlling their formation? Can we find new selective drugs active specifically on these receptor complexes? Notably, techniques like the single-molecule detection system described previously not only would play a crucial role in addressing these questions by deepening our understanding of the mechanisms involved in GPCR dimerization and oligomerization, but importantly, they would also help researchers to evaluate homo and hetero oligomer druggability.

1.5 Receptor Homo- and Heteromers as New Pharmacological Targets

One of the most intriguing consequences of receptor homo- and hetero-oligomerization is the possibility for the development of new pharmacological targets and new paradigms of allosteric regulations. In fact, receptor dimerization has open new ways to target GPCRs including the creation of bivalent ligands, molecules that consist of two pharmacophores linked by a spacer. A pioneer of this approach was Philip S. Portoghese who, even before the concept of receptor dimerization was born, constructed a bivalent ligand containing two beta-naltrexamine pharmacophores connected by oligoethylene glycol spanner that enhanced the ligand potency. Strikingly, these data laid the foundations for the concept of simultaneous occupation of proximal recognition sites for this type of ligands [42]. Such approach was then utilized in 2002 by Saveanu et al. [43] to target somatostatin SST_2 and

dopamine D₂ receptor heteromers. They demonstrated an enhanced potency of a chimeric somatostatin-dopamine molecule, BIM-23A387, in suppressing growth hormone and prolactin secretion from human pituitary somatotroph adenoma cells. Later on, the group of Portoghese developed an high affinity bivalent ligand for opioid DOR-MOR receptor heteromers [44] and for MOP-CB₁ receptor heteromers [45], both with potent analgesic effect but devoided of tolerance.

Interestingly, an important process that also characterized GPCR oligomers is the allosterism across homo- and heteromers that have been shown to play a crucial role in modulating receptor functions. Specifically, this mechanism has been shown to modify protomer conformations and eventually affinity for its ligands through conformational changes occurring in the other promoter of a dimer complex that bound a particular allosteric ligand [5]. One of the best characterized study of allosterism across dimers was by the group of Arthur Christopoulos [46]. They showed that the pure dopamine D₂ receptor allosteric modulator SB269652 [*trans*-1*H*-indole-2-carboxylic acid {4-[2-(cyano-3,4-dihydro-1*H*-isoquinolin-2-yl)-ethyl]-cyclohexyl}-amide] [47] mediated allosteric regulations on D₂ dopaminergic dimers by binding one of the promoter in the dimer complex and thus changing the binding properties of dopamine in the other associated protomer [46]. In particular, SB269652 is a bitopic compound able to engage both the orthosteric and allosteric sites of D₂ receptor promoters. This binding mode of SB269652, in fact, is typical of bifunctional compounds, that are called bitopic or dualsteric ligands [48]. Taken together, these data strongly suggest that the development of drugs targeting receptor homo- and heteromers would have tremendous implications for the development of new and more effective therapies.

1.6 Perspectives

The concept of GPCRs homo and hetero-oligomerization has now gone beyond the restricted circle of GPCR experts and it is accepted by the scientific community. Interestingly, studies on a relative large group of GPCRs have shown that the molecular mechanisms involved in the homo- and heterodimerization processes are not the same for all the GPCRs. In particular, these studies suggested that differences in the way GPCRs assemble together to form multimer complexes might withhold one of the key features responsible for tissue-specific, cell-specific, GPCR mediated signalling.

Indeed, there are many aspects of homo- and hetero-oligomerization that must be investigated thoroughly, and above all, their physiological and pathological implications.

One of the early studies that showed GPCR hetero-oligomerization as an important event for the pathogenesis of diseases came from the pioneering work by AbdAlla et al. (2001), in which preeclamptic hypertensive women showed a significantly clear positive correlation between the increase of angiotensin II AT₁/bradykinin B₂ receptor

heterodimerization and the angiotensin II hormone hypersensitivity associated with preeclampsia itself [49].

Therefore, taken together these data strongly suggest that in the near future GPCR dimers and oligomers will become extremely important pharmaceutical targets, given also the fact that GPCRs are virtually involved in every physiological process.

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