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G protein-coupled receptor-receptor interactions give integrative dynamics to intercellular communication

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Abstract: The proposal of receptor-receptor interactions (RRIs) in the early 1980s broadened the view on the role of G protein-coupled receptors (GPCR) in the dynamics of the intercellular communication. RRIs, indeed, allow GPCR to operate not only as monomers but also as receptor complexes, in which the integration of the incoming signals depends on the number, spatial arrangement, and order of activation of the protomers forming the complex. The main biochemical mechanisms controlling the functional interplay of GPCR in the receptor complexes are direct allosteric interactions between protomer domains. The formation of these macromolecular assemblies has several physiologic implications in terms of the modulation of the signaling pathways and interaction with other membrane proteins. It also impacts on the emerging field of connectomics, as it contributes to set and tune the synaptic strength. Furthermore, recent evidence suggests that the transfer of GPCR and GPCR complexes between cells via the exosome pathway could enable the target cells to recognize/decode transmitters and/or modulators for which they did not express the pertinent receptors. Thus, this process may also open the possibility of a new type of redeployment of neural circuits. The fundamental aspects of GPCR complex formation and function are the focus of the present review article.

Keywords: allosteric interaction; exosomes; G protein-coupled receptors; oligomerization; receptor-receptor interactions.

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Introduction

In his essay 'Evolution and Tinkering' published 40 years ago, Jacob (1977) proposed to describe the process of evolution with the concept of 'tinkering', stating that the natural selection's creative force is evident in its ability to recombine old material into novelties. In other words, new biological structures emerge from previously unseen associations of already available material. Modern neuroscience provided several examples of this concept. Anderson (2007, 2010), for instance, has put forward the interesting proposal of the creative reuse of existing neural components, a process that likely played a significant role in the evolutionary development of cognition. A particularly interesting example of tinkering, however, may be found in nerve cells at the molecular level.

The G protein-coupled receptor (GPCR) superfamily represents the largest family of integral membrane receptors, contributing to all physiologic processes in mammals and representing the most common target of drugs (Wise et al., 2002; Lefkowitz, 2007). The GPCR family involves about 800 human receptors, organized into five subfamilies, namely classes A (the largest group), B, C, frizzled, and adhesion (Foord et al., 2002). It is well known from *in vitro* and *in vivo* experiments that GPCR monomers can recognize/decode signals. In this respect, worth mentioning are studies in which the monomeric entities of three class A GPCRs (namely rhodopsin, β_2-adrenergic, and μ-opioid receptors) trapped into nanodiscs were able to signal as monomers (Bayburt et al., 2007; Whorton et al., 2007; Kuszak et al., 2009). Furthermore, signaling from GPCR monomers is characterized by an intrinsic plasticity, as GPCR activation can result in different patterns of signal transduction, such as G protein and/or arrestin pathways (Zidar et al., 2009). The concept of biased GPCR agonism, meaning functional selectivity, was developed by Kenakin (2011). The agonist stabilization of distinct active states in receptor conformation was suggested to be the mechanism involved in producing the activation of discrete signal transduction pathways by GPCR.

In the 1980s, however, by means of *in vitro* and *in vivo* experiments, Agnati, Fuxe, and their coworkers gave

indirect biochemical and functional evidence that GPCRs could also establish structural receptor-receptor interactions (RRIs; Agnati et al., 1980, 1983; Fuxe et al., 1983). The term RRI emphasized the existence of an interaction requiring a direct physical contact between the involved receptor proteins leading to the formation of multimeric assemblies of receptors (dimers or high-order oligomers) at the cell membrane, operating as integrative input units of membrane-associated molecular circuits (see Kenakin et al., 2010). The concept of GPCR oligomerization was later confirmed in 1998–1999 by studies reporting that two nonfunctional class C GPCR monomers, $GABA_{B1}$ and $GABA_{B2}$, assembled in a signaling heterodimer (Marshall et al., 1999a). In the years that followed, several groups provided direct evidence for the existence of receptor complexes formed by GPCR (Fuxe et al., 1998; Bockaert and Pin, 1999; Marshall et al., 1999b; Xie et al., 1999; Franco et al., 2000; Lee et al., 2000; Overton and Blumer, 2000; Zeng and Wess, 2000; Angers et al., 2001; Dean et al., 2001; Kenakin, 2002; Waldhoer et al., 2005).

The amount of data supporting the existence of GPCR heteromers showed a huge increase as far as biophysical techniques capable of detecting the spatial proximity of protein molecules were developed and became widespread (Bai, 2004; Guidolin et al., 2015). Biological methods for identifying GPCR oligomers in cells and tissues or in recombinant mammalian expression systems presently include energy transfer-based methods [fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET); Fernandez-Dueñas et al., 2012], bimolecular luminescence or fluorescence complementation (Gandia et al., 2008), total internal reflection fluorescence microscopy (Hern et al., 2010), fluorescence correlation spectroscopy (Chen et al., 2003), analysis of colocalization in immunohistochemical preparations (Agnati et al., 2005a), coimmunoprecipitation (Skieterska et al., 2013), assays based on bivalent ligands (Yekkirala et al., 2013), and *in situ* proximity ligation assays (Trifilieff et al., 2011).

It is now well accepted that class C GPCRs form constitutive homomers or heteromers (Kniazeff et al., 2011) and some evidence exists suggesting that also class B GPCRs could oligomerize (see Ng and Chow, 2015).

The oligomerization process in class A GPCRs is a debated question (see Milligan, 2009), especially for what it concerns its occurrence in living tissues, as no single presently available experimental approach can lead to a conclusive demonstration of GPCR complexes *in vivo* (Lambert and Javitch, 2014). However, the overall available evidence (obtained through multiple approaches with consistent results) strongly supports the possibility of class A GPCR complexes in native systems (Bouvier and Hebert, 2014). Furthermore, in view of the fact that the three above-mentioned class A GPCRs shown to be functional as monomers also exist as dimers or higher-order oligomers (see below), the existence of class A GPCR functional oligomers cannot be excluded (see Franco et al., 2016, for a recent discussion of the topic). In this respect, of interest are studies showing that class A receptors appear to exist in a monomer-dimer equilibrium, where class A GPCR dimers are often transient as seen from their half-lives determined from the rate of association and dissociation (Gurevich and Gurevich, 2008). This may help explain opposing views on the role of class A GPCR monomers versus dimers (Chabre and le Maire, 2005).

The amount of RRIs identified so far is very high and their number is continuously increasing (see Farran, 2017, for a recent review). They are mostly stored in the GPCR Oligomerization Knowledge Base (http://www.gpcr-okb.org; Khelashvili et al., 2010) and, for what it concerns the heteromers, in the GPCR-HetNet (http://www.iiia.csic.es/~ismel/GPCR-Nets/index.html; Borroto-Escuela et al., 2014) containing more than 500 entries.

The basic molecular mechanism leading to the formation of these receptor assemblies are allosteric interactions (see Changeux, 2013), and as recently outlined by Changeux and Christopoulos (2016), the cooperativity that emerges in the actions of orthosteric and allosteric ligands of the GPCR forming the complex provides the cell decoding apparatus with sophisticated dynamics in terms of modulation of recognition and signaling. Thus, for an assembly of multiple receptors, the term 'receptor mosaic' (RM) was also introduced (Agnati et al., 1982) to better indicate the 'integrated output' of such an input unit, stressing the concept that the emergent properties of the receptor assemblage depend on the location and the order of activation of the participating receptors (Agnati et al., 2007) as well as on the type of allosteric interactions (entropic and/or enthalpic) within such an integrative complex (Fuxe et al., 2009; Agnati et al., 2010a). The assessment of RRI, therefore, provided a broadened view on the role of GPCR in the dynamics of synaptic function, indicating that they can operate not only as monomers but also as integrated units.

This finding led to the suggestion (Fuxe et al., 2013, 2014a; Gomes et al., 2013) that RRI could open new targets for drug development and allow new strategies of treatment. This aspect is presently the subject of intense research (see Guidolin et al., 2015; Borroto-Escuela et al., 2017; Farran, 2017, for recent reviews). In recent years, such an effort allowed the characterization of a panel of receptor complexes representing possible targets for the

treatment of pathologic conditions, such as Parkinson's disease (Fuxe et al., 2015), schizophrenia and depression (Fuxe et al., 2013; Sahlholm et al., 2017), neuropathic pain (Bushlin et al., 2012), addiction (Gomes et al., 2013), and food intake disorders (Kern et al., 2012). On this basis, novel strategies for drug treatment have also been proposed. Interestingly, such protocols when compared to the traditional ones often appear able to reduce collateral effects (Le Naour et al., 2014). Of particular interest were recent advances leading to the development of receptor complex-specific ligands (Bhushan et al., 2004; Daniels et al., 2005) that could lead to the identification of new tools for pharmacologic intervention.

The formation of receptor complexes, however, has also an impact on neurophysiology (Farran, 2017), especially for what it concerns the emerging field of 'connectomics' (see Guidolin et al., 2017, for a recent review), as it allows an integration of the incoming signals already at the plasma membrane level and can significantly contribute to set and tune the efficiency of the connections between cells and, in particular, the synaptic strength.

The fundamentals of GPCR complex formation and the functional roles these structures can play at the synaptic level in terms of the modulation of signaling pathways [also called vertical molecular networks (VMNs); Agnati et al., 2005b] and interaction with other membrane proteins [i.e. in the so-called horizontal molecular networks (HMNs); Agnati et al., 2005b] will be the focus of the present review article. When available, molecular and bioinformatics models concerning the structure and function of GPCR complexes will be briefly summarized and discussed.

Structural biology of receptor complexes

GPCRs have a complex structure that occupies a volume of about 3–4 nm by side (Zoffmann et al., 2007) and ranges three different microenvironments (extracellular space, membrane lipid bilayer, and cytoplasm). From a global structural point of view, it is possible to distinguish seven α-helixes piercing the entire plasma membrane (transmembrane domains, TM), which are interconnected via an extracellular loop (ECL) and an intracellular loop (ICL). The extracellular region (comprising the N terminus of the protein) is characterized by a high structural diversity allowing the recognition of a wide spectrum of ligands. Interhelical bonds and hydrophobic interactions between highly conserved residues in GPCR provide the stability of the TM region (Palczewski et al., 2000; Katritch et al., 2012), which also harbors a number of kinks elicited by Pro residues, segregating the receptor into ligand binding and receptor signaling 'modules' (Latek et al., 2012). As shown by crystallographic studies, the overall structure of GPCR proteins is highly conserved, but significant diversities can be observed in the loop regions and in the pitch and orientation of individual TM in the helical bundle (Lu and Wu, 2016). Such a quite high plasticity of the GPCR structure is likely a consequence of the presence of intrinsically disordered segments that do not fold into a stable secondary structure (Agnati et al., 2008; Venkatakrishnan et al., 2014). Computational and structural studies have revealed that GPCRs harbor disordered segments in the extracellular N-terminus and large disordered areas in the cytosolic region, mainly in the intracellular C-terminus and in the ICLs, particularly ICL3 (Agnati et al., 2008; Guidolin et al., 2011a; Venkatakrishnan et al., 2014). These results have been recently supported by Tovo-Rodrigues et al. (2014) who provided a detailed analysis of disordered domains in 75 GPCRs involved in synaptic transmission using computational tools for the sequence-based prediction of intrinsically disordered regions within a protein. As, in many cases, disordered segments assume a stable folding following the binding with some partner, these unstructured sequences are particularly suited for interaction. Interestingly, some common interaction partners of GPCRs, such as GPCR kinases (GRK), have the possibility to couple to disordered regions of the receptor comprising ICL3 and the C-terminal tail (Boguth et al., 2010; Elgeti et al., 2013). The presence of these highly flexible linkers also facilitates conformational changes allowing large movements of the TM domains (Rasmussen et al., 2011), making possible a diversity of TM interactions.

Structural plasticity and malleability, however, are crucial not only for conformational fluctuations and intrareceptor interactions, but they are also of paramount importance to establish allosteric RRIs, allowing the formation of receptor complexes.

RRI as allosteric interactions

It has been known for a quite long time that receptors can functionally interact by sharing signaling pathways or by mechanisms of transactivation (Luttrel et al., 1999; Köse, 2017). This formally fits the definition of RRI in a functional sense, although the involved proteins may never physically come into contact with each other (see Prezeau et al., 2010, for a detailed analysis). What we are here discussing, on the contrary, are RRIs requiring a direct

physical contact between the involved receptors leading to the formation of receptor complexes at the cell membrane. The definition of 'physical contact', however, can be debated as proteins have varying degrees of association. A specific international consensus workshop in 2010 (see Kenakin et al., 2010) provided a definition that will be adopted here: 'Receptor-receptor interactions: when the binding of a ligand to the orthosteric or allosteric sites of one receptor causes, via direct allosteric interactions, a change in the ligand recognition, decoding and trafficking processes of another receptor'.

Allostery (Tsai et al., 2009; Tsai and Nussinov, 2014; Liu and Nussinov, 2016) is a mode of long-distance communication between distal sites in proteins, in which the energy released as a consequence of conformational or dynamic changes at one site can travel along specific pathways within the protein structure to other sites, changing their conformational or dynamic properties (Liu and Nussinov, 2017). Computational methods directly relating protein structural dynamics to information exchange between functional sites have also been devised (Lenaerts et al., 2008). Because allostery involves changes in protein conformation, the ability of a protein to take on new conformations is related to the ability of the protein to be allosterically modulated. Therefore, a protein with an already rigid structure is less inclined to be allosterically modulated than a protein with a high degree of intrinsic disorder. In this respect, molecular dynamics studies suggested that signaling proteins, such as GPCR, are ideal candidates to be allosterically modulated (Liu et al., 2006a; Hilser and Thompson, 2007). Thus, when two protomers establish direct RRI, the energy released following a perturbation event at one site of a protomer can pass over the receptor interface into the other protomer (Agnati et al., 2010b; Fuxe et al., 2012) to change its conformation and functional features. Extensive reviews on allostery at GPCR homomers and heteromers, with a clear discussion of the topic, have been provided by Kenakin and Miller (2010) and by Smith and Milligan (2010).

As far as the modes of association of GPCR monomers into oligomers are concerned, two modes have been proposed (Gouldson et al., 2000). One of them is called 'domain contact' dimerization, corresponding to the interaction of the molecular surfaces at specific binding interfaces, without largely changing the conformation of the monomer structure. The other one, termed 'domain swapping' dimerization, is a mechanism in which a substructure (or domain) of a monomer is exchanged with the corresponding substructure (or domain) of the other monomer. Thus, a large conformational change of a monomer structure is required for this mechanism. In the light of biophysical investigations (Fotiadis et al., 2003; Tateyama et al., 2004; Hern et al., 2010), the 'domain contact' is presently considered as the main mechanism of GPCR association. Regardless of the type of geometry assumed for the association, the specific interacting residues that form the interaction interface represent a significant target of current research on GPCR oligomerization (Skrabanek et al., 2007). In fact, the nature of the interaction interface not only specifies which GPCR can exhibit significant interactions but also influences the models for potential allosteric interactions between partners.

Interaction interfaces

The research in this field benefited of a combined use of bioinformatics methods to predict the amino acid sequences involved in the interaction interfaces and experimental work.

Several bioinformatics methods have been devised to predict the interfaces available to a given GPCR for RRI (Filizola and Weinstein, 2005; Guidolin et al., 2011a). They can, in principle, be categorized into three broad classes according to the type of strategy followed to perform the analysis (Simpson et al., 2010):

– The first type of approach is based on the identification of protein regions exhibiting some property (potentially relevant for the interaction with other proteins) that can be deduced simply by the analysis of the primary structure (i.e. the amino acid sequence). Using sequence features, for instance, several methods have been developed to classify whether any given residue belongs to protein segments potentially relevant for protein-protein interaction, such as intrinsically disordered regions (Ferron et al., 2006). Agnati et al. (2008) introduced a 'disorder index' as the weighted average of the results provided by 10 predictors, covering a wide spectrum of the strategies to identify disordered regions in proteins using their primary structure. The results suggested ICL3 and C-terminal domains as potential sites of interaction interfaces for A_1 and A_{2A} adenosine receptors, whereas, in A_{2B} and A_3 subtypes, only the C-terminal domain exhibited a significant score. The analysis of disordered domains, however, should be integrated with other methods, as it does not help for the identification of putative interaction sites located in the TM helices. Thus, an approach based on the evaluation of the aggregation propensity of natural amino acids (Sànchez de Groot et al., 2005) was also proposed (Agnati et al., 2009a), allowing the identification of

protein regions that are especially relevant for protein aggregation ('hot spots') by simply analyzing the amino acid sequence. When applied to the analysis of GPCRs, such as adenosine A_{2A}, dopamine D_2, cannabinoid CB_1, and glutamate $mGlu_5$, this approach predicted 'hot spots' in specific regions of TM4–TM6. Other sequence-based computational methods that have been used include variants of the 'evolutionary trace' method, 'level entropy' and 'sequence space automation' methods, and 'correlated mutation analysis' (see Filizola and Weinstein, 2005; Vohra et al., 2007; Guidolin et al., 2011a, for reviews). According to a meta-analysis reported by Filizola and Weinstein (2005), most of the identified residues are within TM4–TM6, further suggesting a specific role for these three helices in the dimerization/oligomerization interfaces of GPCR.

– A second type of approach is based on the analysis of the three-dimensional structure (as obtained by experimental investigation or by homology modeling) of the protein under scrutiny to identify the possible surfaces of interaction with other proteins. Of particular interest in this field are recent developments in protein-protein docking software that lead to the prediction of the possible binding sites between two molecules, allowing the formation of a stable complex (Simpson et al., 2010; Kaczor et al., 2015; Soni and Madhusudhan, 2017). Examples of this approach include the study of the lutropin receptor dimerization (Fanelli, 2007), studies aimed at characterizing the interaction interface in serotonin $(5\text{-HT})_4$ (Bestel, 2005; Soulier et al., 2007) and rhodopsin complexes (Han et al., 2009), and the generation of models for the heterodimeric $mGluR_2$-5-HT_{2A} complex (Bruno et al., 2009) and for the dopamine D_1-D_2 receptor dimer (Agnati et al., 2016).
– Molecular dynamics and coarse-grained simulations are one of the most versatile and widely applied computational techniques for the study of membrane proteins (Almeida et al., 2017), as they consider the tertiary structure of the studied proteins and can implement an energy landscape to estimate the molecular interactions, also accounting for the role of the lipid microenvironment. Such methods have also been used to study of GPCR dimerization and oligomerization (Fanelli et al., 2013; Jonas et al., 2015; Altwaijri et al., 2017). Examples include the study of rhodopsin dimer (Filizola et al., 2006) and simulations of vasopressin receptor oligomerization (Witt et al., 2008) and of the $mGluR_2$-5-HT_{2A} heterodimeric complex (Bruno et al., 2009). A structural characterization of the human A_{2A} adenosine receptor homodimer was very recently provided (Altwaijri et al., 2017) using a new coarse-grained approach to molecular dynamics simulations, specifically developed for identifying helix-helix interactions in GPCR.

Results obtained from computational predictions and concerning a panel of receptor complexes are summarized in Table 1. These analyses, however, only provide suggestions that should be confirmed by experimental data. Recent advances in experimental methods have equipped researchers with a repertoire of tools to get details about the interaction interfaces. The last years, for instance, have seen a significant advancement in crystallization techniques with important consequences for the analysis of GPCR and an increase of the number of experimentally assessed structures (Grisshammer, 2017). Additional experimental tools encompass atomic force microscopy (Liang et al., 2003; Agnati et al., 2010b) and novel super-resolution imaging approaches, such as photoactivated localization microscopy (PALM; Jonas et al., 2016), far-ultraviolet circular dichroism spectroscopy, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis using synthetic peptides corresponding to different TMs (Thevenin and Lazarova, 2008). Woods et al. (Ciruela et al., 2004; Woods et al., 2005) using mass spectrometry in combination with collision-induced dissociation experiments investigated intracellular domains (namely ICL3 and C terminus), demonstrating strong electrostatic interactions in heteromers. Results from experimental investigations are reported in Table 1. As shown, they were, in general, supportive of the predictions provided by bioinformatics. Both computational and experimental methods suggest that GPCR structures are capable of interacting via multiple interfaces, but some domains were observed more often than others. TM4–TM6 and ICL3, for instance, were reported as the main interfaces in a quite large number of GPCR complexes. For what it concerns the possible involvement of ECLs in the interaction, it was demonstrated for some class A GPCR (Huang et al., 2013), and evidence was provided for interaction by disulfide bridges between extracellular domains in some class C GPCR (Kniazeff et al., 2011).

An interesting finding from computational and experimental studies on GPCR oligomerization interfaces is the presence at the interface of specific motifs that appear of particular importance for the allosteric interaction. As demonstrated by Woods and colleagues (Woods, 2004; Woods and Ferré, 2005), the electrostatic interactions between intracellular domains occur between a negatively charged serine-phosphate-containing intracellular motif of one receptor and a positively charged arginine-rich

Table 1: Interaction interfaces: computational predictions and experimental findings.

GPCR complex	Predicted	References	Experimental	References
Homodimers				
Adenosine A_{2A}-A_{2A}	TM1,2,3–TM1,2,3 TM1–TM1	Fanelli and Felline, 2011	TM1,3–TM5,6	Liu et al., 2012
β_1-β_1-Adrenergic	TM1–TM1 TM5–TM5	Mondal et al., 2013	TM1,H8–TM1,H8 TM4,5–TM4,5	Huang et al., 2013 Cordomì et al., 2015
β_2-β_2-Adrenergic	TM6–TM6 TM4,5–TM4,5	Mondal et al., 2013	TM6–TM6	Hebert et al., 1996
	TM1–TM1	Prasanna et al., 2014		
	H8–H8	Ghosh et al., 2014		
Chemokine $CXCR_4$-$CXCR_4$	TM3–TM4,5	Rodriguez and Gutierrez-de-Teran, 2012	TM3–TM4	Wu et al., 2010
	TM5–TM5		TM5–TM6	
Dopamine D_2-D_2	TM4–TM4	Nemoto and Toh, 2005	TM4–TM4	Guo et al., 2003
δ-δ Opioid	TM4–TM4 TM4,5–TM4,5	Johnston et al., 2011	TM4–TM4	Johnston et al., 2011
κ-κ Opioid	TM1–TM2	Kaczor et al., 2013	TM1,2,H8–TM1,2,H8	Johnston and Filizola, 2014
μ-μ Opioid	TM1,2,H8–TM1,2,H8 TM5–TM5 TM1,2,H8–TM5	Marino et al., 2016	TM1,2,H8–TM1,2,H8 TM5,6–TM5,6	Manglik et al., 2012
Serotonin 5-HT_{1A}-5-HT_{1A}	TM4,5–TM4,5	Gorinski et al., 2012	TM4,5–TM4,5	Gorinski et al., 2012
Heterodimers				
A_{2A}-D_2	TM5,6,ICL3–TM3,4 TM4,5–TM3,4,5	Canals et al., 2003	TM5,6,ICL3–TM3,4	Canals et al., 2003
	TM4,5–TM4,5	Borroto-Escuela et al., 2010	TM4,5–TM4,5	Borroto-Escuela et al., 2010
	H8–ICL3	Agnati et al., 2008	H8–ICL3	Ciruela et al., 2004 Woods et al., 2005
D_1-D_2	TM4,5–TM1,2 H8–ICL3	Agnati et al., 2016	H8–ICL3	Hasbi et al., 2014
Trimers				
A_{2A}-CB_1-D_2	TM4–TM4 TM6–TM6 H8–ICL3	Agnati et al., 2010c	TM4–TM4 TM5–TM5 H8–ICL3	Navarro et al., 2010

H8, C-terminal amphipathic helix 8.

motif of a second receptor. Once established, they possess a covalent-like stability and likely represent the main mechanism for heteromer assemblage. More recently, it has been reported that a highly conserved *small-XXX-small* motif found in TM1 of the fungal GPCR Mam2 promotes TM1 self-association (Lock et al., 2014). *Small-XXX-small* motifs are motifs of two residues (typically glycine, but also alanine or serine) separated by three amino acids in the polypeptide chain, thus physically placing them on the same face of an α-helix. Colocation of these two small residues results in a 'groove' that allows two helices to interlock via many favorable van der Waals contacts, thereby promoting helix-helix interactions (Jenei et al., 2009). Based on a bioinformatics approach, Tarakanov and Fuxe (2010) have deduced a set of triplet homologies that may be responsible for RRIs. Such amino acid triplets resulted mainly located at the receptor-receptor interface. Most of them are leucine-rich motifs. Another minor type of triplets contains charged amino acids and the electrostatic interaction between triplets may guide-and-clasp protein-protein interactions (Borroto-Escuela et al., 2011; Fuxe et al., 2014a).

Quaternary structure of GPCR complexes

The basic structure generated by the interaction of GPCR is the dimeric structure (Figure 1A), in which pairs of protein molecules (protomers) associate. Homodimers are pairs of the same protomer, whereas heterodimers are formed from distinct GPCR. The number of described homodimers and heterodimers in both cellular systems and native tissues is at present very high (see Fuxe et al., 2015; Guidolin et al., 2015; Borroto-Escuela et al., 2017;

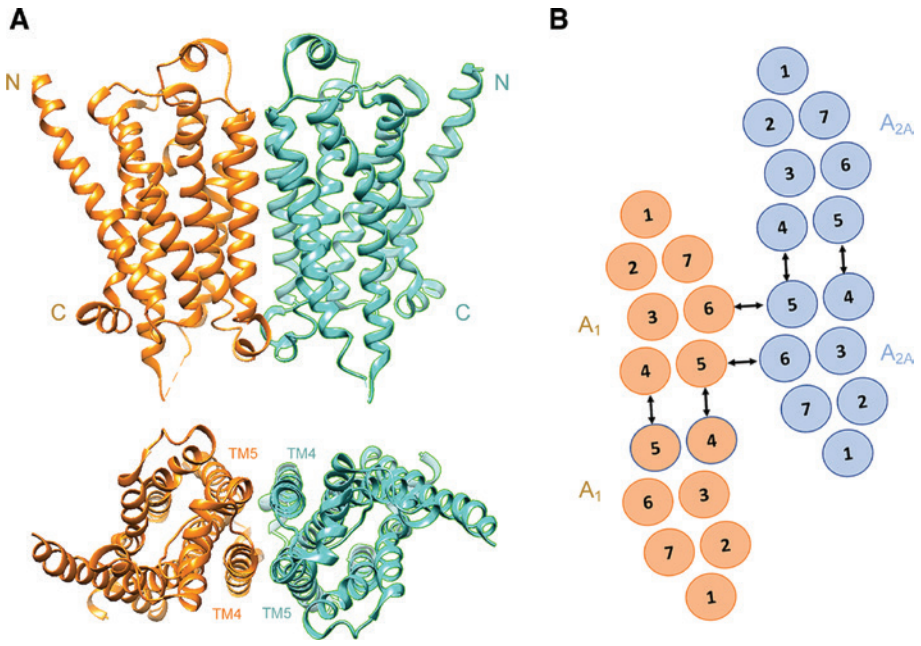

Figure 1: Examples of quaternary structures formed by GPCR.
(A) Monomers of the β_1-adrenergic receptor (PDB code: 4GPO) as obtained by X-ray crystallography (Huang et al., 2013), arranged to form a β_1-β_1 homodimer as suggested by Cordomì et al. (2015). Top, the N and C termini are indicated; bottom, a top view (from the extracellular surface) is provided with indication of the interacting TM4/TM5 domains. (B) Schematic representation of the heterotetramer formed by A_1 and A_{2A} adenosine receptors as proposed by Navarro et al. (2016). Arrows indicate the TM4–TM5 interface mediating homodimerization and the TM5–TM6 interface exploited for heterodimerization. These interfaces give a rhombus-shaped receptor complex organization.

Farran, 2017, for reviews), and Table 1 only provides some example. However, the evidence that a GPCR can exploit multiple interaction interfaces can significantly influence the architecture of the resulting receptor complexes in at least two aspects:

– The first concerns the stoichiometry of the complex (i.e. the number of component subunits) opening the possibility that oligomeric assemblies of different order could be formed (Agnati et al., 2010a). Navarro et al. (2010) were among the first to advance evidence for the role of interaction interfaces between protomers in orchestrating the quaternary structure of heteromers. This study was focused on adenosine A_{2A}, dopamine D_2, and cannabinoid CB_1 receptors. Each of them exhibited two intracellular domains that interacted in a specific manner with intracellular domains of the other two protomers through electrostatic interactions, leading to the assembly of dimers (A_{2A}-D_2, A_{2A}-CB_1, and CB_1-D_2) but also to the formation of a A_{2A}-D_2-CB_1 heterotrimer. Trimeric receptor complexes have been indeed identified (Gandia et al., 2008). They include the A_{2A}-D_2-$mGlu_5$ (Cabello et al., 2009) heteroreceptor complex, the muscarinic M_2 homotrimer (Park and Wells, 2004), the dynamic Gal_1-5-HT_{1A}-GPR_{39} heterotrimer (Tena-Campos et al., 2015), and the putative Gal_1-Gal_2-5-HT_{1A} heterotrimer (Millón et al., 2016). Tetrameric assemblies of β_2-adrenergic receptors were demonstrated by the group of Kobilka to occur spontaneously following reconstitution into phospholipid vesicles (Fung et al., 2009), suggesting that β_2-adrenergic receptor oligomerization is an intrinsic property of the receptor. A possible heterotetrameric structure has been recently proposed for the complexes formed by adenosine A_1 and A_{2A} receptors (Navarro et al., 2016), in which a TM4–TM5 interface mediates homodimerization and a TM5–TM6 interface is exploited for heterodimerization (Figure 1B). Some evidence also exists of higher-order GPCR oligomers. For instance, combined BRET/FRET and complementation studies have revealed that the assemblage of dopamine D_2 receptors by symmetrical interfaces at TM4 and TM1 can lead to a complex composed of at least four protomers in the plasma membrane of living mammalian cells (Guo et al., 2008). Moreover, based on an analysis of PALM data, it has been proposed that direct RRI could lead to the formation of high-order oligomers (tetramers, octamers, and larger-sized complexes) depending on the specific membrane microenvironment (Scarselli et al., 2016).

– The existence of multiple interaction interfaces opens the possibility that the assemblage of a given set of receptor molecules to form a complex could occur

in a number of different geometrical arrangements (Agnati et al., 2009a) depending on a number of conditions including not only the physical properties of the interacting proteins (surface charge, hydrophobicity, etc.) but also the microenvironment surrounding the interacting partners (i.e. the energy landscape; Frauenfelder et al., 1991). As better discussed in the next section, the topological arrangement of the receptor complex can influence its functional behavior. An interesting experimental evidence of this concept was recently provided by Jonas et al. (2015) using a super-resolution imaging approach. The study was focused on two functionally defined mutant luteinizing hormone receptors, which only function via intermolecular cooperation with favored oligomeric over dimeric formation. PD-PALM imaging of trimers and tetramers showed that monomers interconnected by complex helix interfaces can assume a variety of distinct spatial arrangements that also differ from each other in terms of signal sensitivity and strength.

A further aspect of substantial interest has been highlighted by studies using single-GPCR imaging (see Sungkaworn et al., 2013) in living cells. They revealed the kinetics of complex formation, indicating that GPCR can form either stable or transient complexes at the cell surface depending on the interaction energy (Gurevich and Gurevich, 2008). To exist as a stable dimer with a half-life comparable to that of even short-lived GPCR (2–20 h), a binding energy of at least ~60 kJ/mol is required. This condition is often fulfilled by class C GPCR, explaining why they often appear as stable dimers. For what it concerns family A GPCR dimers, they are often transient as seen from their half-lives. In the case of the neurotensin NTS_1 dimer, a half-life of 340 s has been observed (White et al., 2007) and evidence has been found that M_1 muscarinic receptor dimers have an estimated half-life of 0.5 s (Hern et al., 2010). In a study by Calebiro et al. (2013), β_1- and β_2-adrenoceptors were monitored on the surface of living cells and the kinetics of the interactions leading to the formation of oligomers was characterized. All these receptors dynamically formed dimers and high-order oligomers, with an apparent half-life in the order of 4–6 s. Thus, a dynamic equilibrium condition was established at the cell surface, with constant formation and dissociation of new receptor complexes. The relative amount of the different stoichiometries was dependent not only on the subtype of receptor but also on the receptor density.

A final relevant aspect of the receptor complex structure can be appreciated when the allosteric binding sites of the monomers are considered. In contrast to the orthosteric pockets, in receptor structures, allosteric binding sites can be located in various regions of the molecule (Bartuzi et al., 2017). For class A GPCR, in most cases, allosteric binding sites are located in the same region as the orthosteric one (i.e. within the seven TM), whereas, in class C GPCR, the two sites are usually well separated (see Wu et al., 2014). When a receptor complex forms, the allosteric binding sites on single monomers may undergo structural and functional changes (see Shivnaraine et al., 2016). Of significant interest, however, is the possibility that, when the complex forms, the quaternary structure could display novel specific allosteric sites suitable for the binding of some modulator. Thus, ligands could also exist specific to the receptor complex as such (see Fuxe et al., 2010). Studies on the effect of homocysteine (Agnati et al., 2006, 2008) on the A_{2A}-D_2 heterodimer provided a first example of the possible existence of allosteric modulators of a receptor complex. In Chinese hamster ovary cells stably cotransfected with adenosine A_{2A} and dopamine D_2 receptors, homocysteine selectively decreased the ability of D_2 receptor stimulation to internalize the receptor complexes. Mass spectrometric analysis showed that, by means of an arginine-thiol electrostatic interaction, homocysteine forms noncovalent complexes with the two arginine-rich epitopes of the third ICL of the D_2 receptor, one of them being involved in the receptor heteromerization interface. However, homocysteine was unable to prevent or disrupt A_{2A}-D_2 receptor heteromerization as demonstrated by FRET experiments. Thus, it likely acts as a modulator of the allosteric process of energy transmission between the two protomers.

Dynamic behavior of receptor complexes and the concept of RM

The existence of these supramolecular assemblies is considered of particular importance because it allows the emergence of integrative functions (Agnati et al., 2010b) performed by a receptor complex as a whole. In fact, owing to allosteric RRI, a configuration change of a given protomer will change the probability of changing the configuration for the adjacent receptors in the complex and the effect will propagate throughout the cluster, leading to a complex collective behavior and to an integrated regulation of multiple effectors (Fuxe et al., 2012). These concepts were well illustrated by a simple mathematical approach to the cooperativity in complexes formed by dimers of identical receptors and/or by receptors binding to the same ligand (Agnati et al., 2005c). The model was

based on a 'symmetry rule', which has been proven for hemoglobin (Ackers et al., 1992), and this model maintains that a quaternary switching from tense form (the 'deoxy', low-affinity state) to relaxed form (the 'oxy', high-affinity state) occurs whenever heme-site binding creates a tetramer with at least one ligated subunit on each dimeric half-molecule. When the same basic rule is applied to assemblies formed by homodimers (as evidenced for dopamine receptors; Guo et al., 2008), the integrative cooperativity of the complex appeared to depend not only on the composition (number of dimers) but also on its spatial organization (respective location of the dimers) and order of activation (order according to which the single receptors are ligated). To investigate in more detail the potential complex cooperative behavior of the receptor assemblies, a number of computational models and computer simulations were proposed (see Guidolin et al., 2011a, for a review).

A first class of models aimed at describing the dynamics of receptor complexes was based on methods from discrete dynamics (Martelli, 1999). According to this approach, individual receptors can be assumed to have two broad classes of conformational states with respect to the macromolecular effectors: one active and one inactive. Owing to RRIs, however, a state change of a given receptor will change the probability of changing the state for the adjacent receptors in the RM and the effect will propagate throughout the cluster, leading to a complex cooperative behavior. On this basis, Boolean networks (BN) were proposed as a suitable abstract model to explore how complex properties may emerge from systems permeated by deterministic local interactions of many simple components operating in parallel (Agnati et al., 2007). Very common 'macroscopic' properties of a receptor system (such as a sigmoidal response curve to an extracellular ligand) were captured from a model of this type (Guidolin et al., 2011b). The response, however, was modulated by changes in the topology and/or in the local interactions between the receptor units forming the assembly. Furthermore, the system exhibited a limited number of equilibrium configurations or 'attractors', leading to the hypothesis that such a set of configurations could be interpreted as a form of information storage (engram) at the level of synapses and intercellular connections (Agnati et al., 1982, 2004; Guidolin et al., 2007). Thus, the suitable reorganization of receptor complexes in the postsynaptic membrane has been recently proposed as the molecular basis of learning and memory (Fuxe et al., 2014b; Borroto-Escuela et al., 2015).

Consistent with the just mentioned characteristics of a receptor complex suggested by BN models are also the results of thermodynamics-based approaches to modeling (Jackson, 2006). In this formulation, each protomer can exist in distinct conformational states and makes rapid stochastic transitions between these states, with Boltzmann-weighted probability. The energy of each protomer in the complex was in turn dependent on three energy terms: the energy associated with its configuration, the energy associated with external inputs, and the energy due to the coupling with adjacent protomers. In the model discussed by Duke et al. (2001), when coupling exists between neighboring proteins, a conformational spread occurs, driving the system to a switch-like, sigmoid response to changes in ligand concentration. A theoretical analysis of the role of the spatial arrangement of monomers within the complex based on thermodynamical considerations was provided by Agnati et al. (2010c), who showed that, for each given set of binding and interaction constants, the theoretical saturation curves of trimeric or tetrameric receptor complexes were dependent on the geometry of the receptor complex.

Thus, receptor complexes appear to be endowed with 'emergent properties', that is, with biochemical characteristics and functions that could not be fully anticipated by analyzing the characteristics of the single participating receptor monomers. In particular, due to differences in topology and rank order of activation, it is possible (at least from a theoretical standpoint) to have markedly different integrative functions for receptor complexes formed by the same set of monomers (i.e. same stoichiometry). To grasp this fundamental aspect of GPCR assemblies, the term RM was proposed (Agnati et al., 1982, 2010a) to identify them. The concept was inspired from the mosaic as defined in figurative art: namely the process of assembling images by inlaying small pieces of colored stones (tesserae) according to a structural plan (Agnati et al., 2009b). The term 'mosaic' thus describes how a limited set of building blocks could be arranged into different patterns according to distinct designs, resulting in sets of elements endowed with differential emergent properties depending on their respective interactions. According to a metaphor proposed by Kenakin (2009), RM would operate as a sort of molecular 'microprocessor', as they are not just 'on-off' switches but exhibit a high capability to elaborate information. A straightforward example is likely provided by RM formed by isoreceptors (i.e. receptors for the same ligand), such as, for instance, the D_1–D_2 (Lee et al., 2004) or the D_1–D_3 (Marcellino et al., 2008) isoreceptor complexes. It has been suggested that an analogy with an electronic apparatus like the 'demultiplexer' (a device taking one input signal and selecting one of many-data-output lines to send it) could be exploited to describe their functional

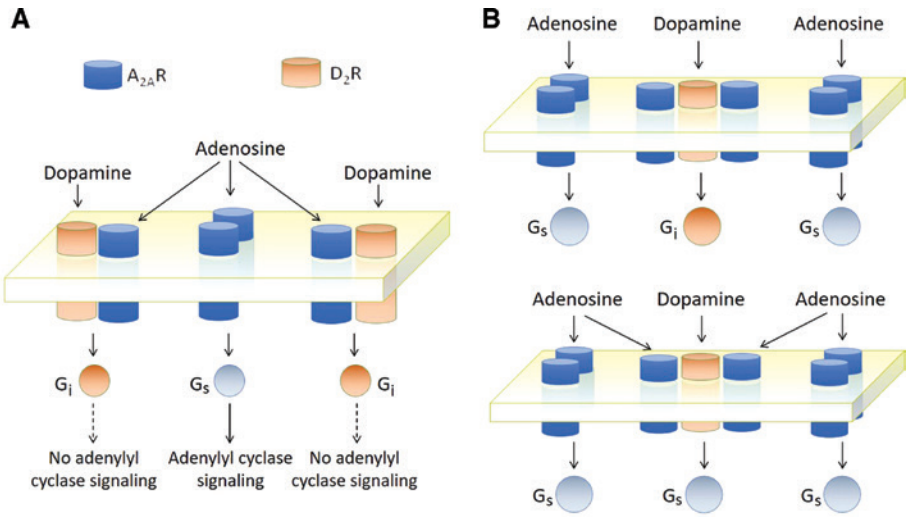

Figure 2: Basic operations described in Sherrington's studies that could be realized at the molecular level by receptor complexes (Guidolin et al., 2016).
(A) Possible "lateral inhibition" process at microscale level in a system of RM involving A_{2A} and D_2 receptors with reciprocal inhibitory activity (see Guidolin et al., 2015) and A_{2A} homodimers. The activation of D_2 receptors by dopamine leads to a reduction in the affinity of A_{2A} receptors in the heterodimers, hence in a sharpening of the G_s-mediated signaling. (B) Schematic representation of the possible implementation at molecular level of the concept of "fringe". A low concentration of adenosine can activate only G_s-mediated signaling from A_{2A} homodimers (top). However, high concentrations of adenosine can induce, via RRI, an inhibition of the D_2 receptor in the heterotrimer and its shift from G_i- to G_s-mediated signaling (bottom).

behavior (Agnati et al., 2016). Furthermore, it has been proposed (see Figure 2) that the interplay between RM may implement at membrane-level basic operations (e.g. 'lateral inhibition') that were described at the level of neuronal networks in Sherrington's studies (Guidolin et al., 2016).

As illustrated in Figure 3, RM operate as specialized devices in two complementary contexts (see Agnati et al., 2010a). From one side, they are part of the so-called VMNs, that is, the molecular pathways involved in signal recognition and transduction, extending from the extracellular space to the cytoplasm and nucleus. On the other side, they can also partake HMNs, i.e. networks formed by interacting membrane components, regulating informational exchange between the extracellular and the intracellular environments. This twin role will be the focus of the sections that follow.

RM and VMNs

A wide variety of extracellular ligands (biogenic amines, amino acids, ions, lipids, peptides, proteins, sensory stimuli, etc.) can be detected by GPCR, and these events switch the receptor to an active conformational state that permits its coupling and activation of heterotrimeric GTP-binding proteins ($G_{\alpha\beta\gamma}$ proteins), leading to the regulation of multiple intracellular phosphorylation pathways involved in the regulation of gene expression and diverse biological responses, such as proliferation and differentiation. Activated GPCR, however, also interact with cytosolic ligands (see Premont and Gainetdinov, 2007; Magalhaes et al., 2012, for reviews), such as GRK (Premont et al., 1994) and arrestins (Lohse et al., 1990). GRK regulate GPCR desensitization by both phosphorylation-dependent and phosphorylation-independent mechanisms (Dhami and Ferguson, 2006). Arrestins turn off the GPCR response or adapt the system to a persistent stimulus by coordinating spatially and temporally the uncoupling of G protein from receptors and by mediating agonist-promoted receptor internalization (Lefkowitz and Shenoy, 2005). In addition, arrestins have been demonstrated to scaffold a wide variety of signaling complexes and there is now extensive evidence indicating that ligands that interact with GPCR can selectively activate G protein- versus arrestin-mediated signaling pathways (Reiter and Lefkowitz, 2006).

When RRI in the plane of the membrane occur, the formation of RM can lead to several changes in the chain of events linking ligand recognition to signal transduction from the single protomers. They can be briefly summarized as follows (see Guidolin et al., 2015; Borroto-Escuela et al., 2017; Farran, 2017, for more specific reviews):

– Modulation of the binding sites has been reported to occur in a variety of RM as a consequence of allosteric

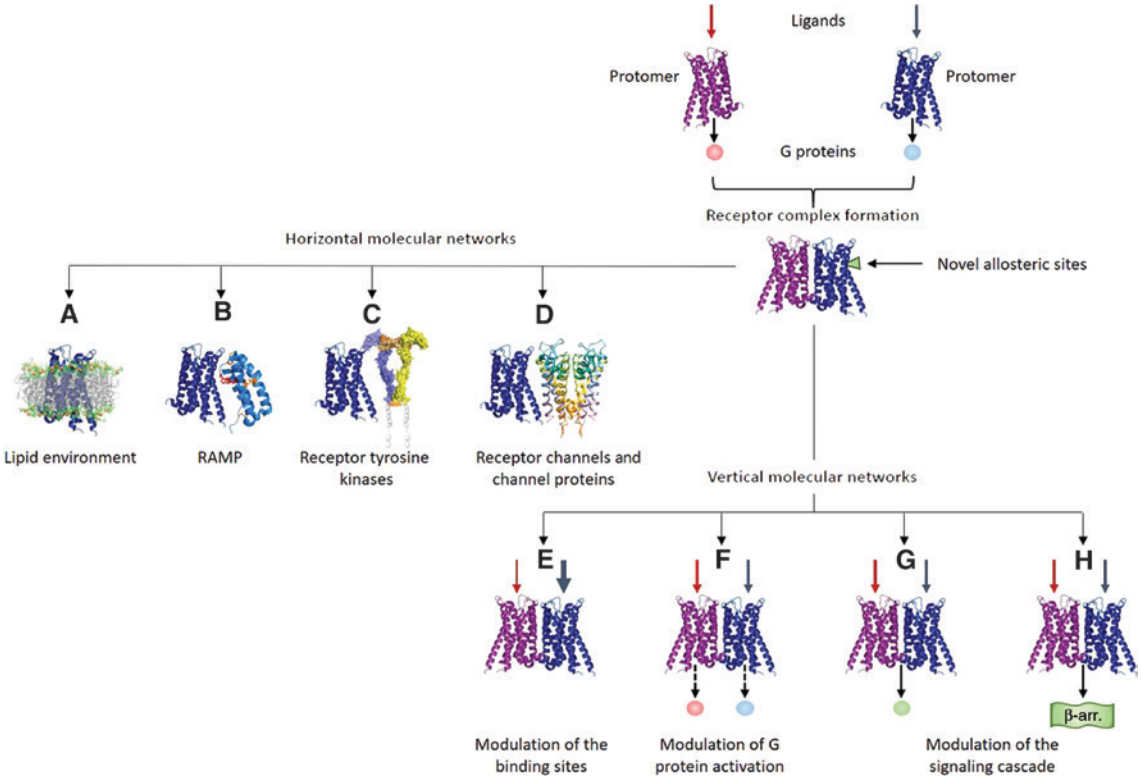

Figure 3: Receptor complexes appear to be endowed with characteristics and functions that could not be fully derived from the characteristics of the single participating receptor monomers.
When the complex forms, for instance, the quaternary structure could display novel specific allosteric sites (Agnati et al., 2008). Furthermore, RM are in the center of two complementary networks of interactions: HMN involving (A) the lipid environment (Gahbauer and Böckmann, 2016), (B) RAMP (Foord and Marshall, 1999), (C) RTK (Borroto-Escuela et al., 2012), and (D) membrane channels (Liu et al., 2006a; Gamo et al., 2015) and VMN leading to (E) modulation of the binding sites (Fuxe et al., 1998), (F) modulation of G protein activation (Ferrada et al., 2009), (G) modulation of the signaling cascade, among others, and (H) switching from G protein to β-arrestin signaling (Rashid et al., 2007; Rozenfeld et al., 2012).

RRI. One of the first examples was the A_{2A}-D_2 heterodimer, where the binding of the adenosine A_{2A} agonist CGS-21680 lead to a reduction of the affinity of the high-affinity dopamine D_2 agonist binding site (Fuxe et al., 1998). In this RM, a reciprocal interaction between D_2 and A_{2A} receptors also exists, as D_2 receptor can inhibit the A_{2A}-induced increase in cyclic AMP (cAMP) accumulation via $G_{i/o}$ at the level of the adenylate cyclase (Kull et al., 1999). As discussed by Woods and Jackson (2013), in this heterodimer, the first step driving heteromerization involves the phosphorylation of the serine/threonine in an epitope containing a casein kinase 1/2-*consensus site*, and dopaminergic neurotransmission, through cAMP-dependent protein kinase A (PKA), slows down heteromerization. In addition, the negative charge, acquired by phosphorylating a serine/threonine in a PKA consensus site in the arginine-rich epitope, affects the activity of the receptors involved in heteromerization by causing allosteric conformational changes, due to the repulsive effect generated by the negatively charged phosphate, thus modulating heteromerization and affecting the stability of heteromers' interactions and their binding affinity. In this instance, phosphorylation is not just an 'on-off switch'; instead, by weakening the noncovalent bond, heteromerization acts as the entity that controls the stability of the heteromer through the activation or inhibition of adenylate cyclase.

– Modulation of G protein activation causes changes in the decoding of signals impinging on protomers. An example is provided by the heterodimer formed by dopamine D_1 and histamine H_3 receptors. In this RM, there is a change in the D_1 receptor coupling from the G_s to the G_i protein, to which H_3 receptors are already coupled. In fact, in the presence of the H_3 receptor, D_1 receptors were no longer coupled to G_s and could not activate adenylyl cyclase but were coupled to G_i, which

transduced the signal toward the mitogen-activated protein kinase pathway (Ferrada et al., 2009).
– Modulation of the signaling cascade occurs when the RM recruits a G protein different from those usually associated to the monomers (as in the D_1-D_2 dimer; Rashid et al., 2007) or when the oligomerization process leads to a switch between G protein and β-arrestin signaling (Rozenfeld et al., 2012), such as, for instance, in the κ-μ and κ-δ opioid heteromers (Le Naour et al., 2014).

It has to be emphasized that the role played by RM in VMN is of particular importance from the pharmacologic point of view, as it discloses a marked rise of the repertoire of GPCR recognition and signaling (Guidolin et al., 2015; Borroto-Escuela et al., 2017).

RM and HMNs

The pattern of interactions between molecules embedded and/or associated with the cell membrane form the so-called HMNs that can operate as modules carrying out specialized tasks (Agnati et al., 2010a). A functional and structural relationship exists between GPCR and HMN. GPCR can interact with several membrane components (Gahbauer and Böckmann, 2016) and play an important role in the formation of HMN, where they can function as sophisticated signaling processing centers (Kenakin, 2007).

In this respect, the first aspect deserving consideration is the lipid environment. It was shown to influence GPCR function and several health disorders during aging were assigned to changes in the membrane composition that altered GPCR signaling (Alemany et al., 2007). The preferential localization of GPCR and other components involved in signal propagation in dynamic membrane nanodomains (lipid rafts and caveolae) has been reported in a vast number of studies (Insel et al., 2005; Lingwood and Simons, 2010; Simons and Sampaio, 2011). These nanodomains are densely packed, dynamic membrane areas with increased concentrations of glycosphingolipids and cholesterol. Caveolae ('little caves') show a similar lipid composition, but they additionally contain the protein caveolin on the inner leaflet of the bilayer (Insel et al., 2005). One of the most prominent membrane components, which shows enlarged concentrations in membrane nanodomains, is cholesterol. It was frequently reported to regulate GPCR signaling (Oates and Watts, 2011). The effect of cholesterol on the order and fluidity of the membrane may be an important parameter for receptor function (Mitchell et al., 1990), but direct cholesterol-receptor interactions have also been described (Albert et al., 1996). Remarkably, the effect of cholesterol on GPCR function is receptor dependent. For example, cholesterol modulates agonist binding to oxytocin receptors (Gimpl and Fahrenholz, 2002), 5-HT receptors (Pucadyil and Chattopadhyay, 2004) and μ-opioid receptors (Qiu et al., 2011), whereas other GPCRs are less influenced (Oates and Watts, 2011). Stability studies have demonstrated the binding of cholesterol molecules to a conserved motif located between helices 1–4 (Hanson et al., 2008). Recently, relevant phospholipids were found to affect GPCR function. In β_2-adrenergic receptors reconstituted in high-density lipoparticles, for instance, phosphatidylgycerol markedly favored agonist binding and facilitated receptor activation, whereas phosphatidylethanolamine favored antagonist binding and stabilized the inactive state of the receptor (Dawaliby et al., 2016). These data suggested that phospholipids could act as direct allosteric modulators of GPCR activity. Lipids, however, can also be covalently bound to GPCR (see Gahbauer and Böckmann, 2016). Due to a post-translational modification called palmitoylation, the saturated fatty acid palmitic acid (16 carbons) can be added to C-terminal cysteine residues via a thioester-type bond (Chini and Parenti, 2009). It was reported that GPCRs can be mono-, bis-, or even tris-palmitoylated and that this lipid modification is reversible as well as adjustable, thereby allowing the regulation of GPCR function (Qanbar and Bouvier, 2003).

As far as membrane proteins are concerned, the most interesting association with GPCR was identified in a set of three homologous transmembrane proteins that were named receptor activity-modifying membrane (RAMP) protein (Foord and Marshall, 1999). When associated to the calcitonin-like receptor (CLR), they significantly modify its function: the complex RAMP1-CLR behaves phenotypically as a calcitonin gene-related peptide receptor, whereas the association of RAMP2 or RAMP3 with CLR provides specificity for adrenomedullin (Poyner et al., 2002). Other family B GPCRs have also been shown to associate with RAMP. They include parathyroid hormone and glucagon receptors (see Kenakin and Miller, 2010).

Of special relevance for structural plasticity would be the recruitment of receptor tyrosine kinases (RTK) to the receptor complexes formed, which might result, for example, in synergistic increases in neurite densities and spines. Processes of transactivation of RTK by GPCR have been reported (Flajolet et al., 2008; Asimaki et al., 2011; Di Liberto et al., 2014), generating neuroplasticity in cultured neurons. Recently, however, the formation (by direct RRI)

of receptor complexes involving fibroblast growth factor receptor 1 and 5-HT_{1A} (Borroto-Escuela et al., 2012) or muscarinic M_1 receptor (Di Liberto et al., 2017) have been identified in the hippocampus with increased neurite densities after agonist coactivation.

It is likely that other transmembrane proteins (e.g. ion channel receptors, ion channels, and/or transmitter transporters) will also follow this theme and associate with GPCR within the lipid bilayer. A direct interaction between dopamine D_2 and N-methyl-D-aspartate (NMDA) receptors, for instance, has been identified in glutamate synapses (Liu et al., 2006b). D_2-NMDA heteroreceptor complexes form through the ICL3 of D_2 interacting with the NR2B subunit and lead to the inhibition of NMDA receptor signaling in the striatal glutamate synapses. Furthermore, recent data make possible to speculate that a direct interaction between D_1 dopamine receptor and hyperpolarization-activated nucleotide-gated (HCN) cation channels could exist in the prefrontal cortex. In fact, D_1 receptors and HCN colocalize in layer III of dorsolateral prefrontal cortex and the suppression of neuronal firing by D_1 signaling can be prevented by blocking HCN channels. Correspondently, working memory impairment induced by D_1 stimulation or pharmacologic stress can be prevented by blocking HCN channels in the rat prefrontal cortex (Gamo et al., 2015).

RM formation and trafficking

An important question can now be raised. It concerns where and how RM are formed, expressed, and modified at the cell surface. Based on available evidence, at least four aspects deserve consideration.

Influence of the lipid environment

Receptor complexes can arise in the plasma membrane (see Gahbauer and Böckmann, 2016). An evident mechanism how membrane properties may regulate GPCR assembly in membrane nanodomains is due to hydrophobic forces coupled to a hydrophobic mismatch. The hydrophobic mismatch is roughly defined as the difference between the hydrophobic membrane thickness and the height of the hydrophobic part of the protein. Consequently, if the protein's hydrophobic part exceeds the bilayer thickness, oligomerization might reduce the exposed hydrophobic area of the protein (Killian, 1998). Using computational methods, this process was analyzed with reference to the association of rhodopsin (Periole et al., 2007) and of the β_1- and β_2-adrenergic receptors (Mondal et al., 2013). Interestingly, it was observed that the protein domains most frequently involved in RRIs were also those showing the highest hydrophobic mismatch in monomers, which was substantially alleviated in oligomers. A few experimental studies addressed the relation between membrane thickness and oligomerization. Using FRET experiments, it was shown that the reduction of membrane thickness or the increase of the protein/lipid molar ratio promotes rhodopsin association (Brown, 1994; Botelho et al., 2006).

Specific membrane components can play a peculiar role in receptor assembly. For instance, the ligand-independent oligomerization of 5-HT_{1A} receptors in living cells was observed with FRET and appeared to be enhanced upon acute cholesterol depletion (Paila et al., 2011). This could be due to changes in membrane properties (e.g. larger hydrophobic mismatch caused by reduced membrane thickness) leading to the reorganization of the receptors or by disrupting specific cholesterol-protein interactions essential for the establishment of an interaction interface. Recently, the effect of polyunsaturated ω-3 fatty acid docosahexaenoic acid (DHA) chains on receptor oligomerization was studied with a combination of multiscale computer modeling and BRET experiments (Guixà-González et al., 2016). It was shown that DHA improves the oligomerization kinetics of adenosine A_{2A} and dopamine D_2 receptors.

Role of endoplasmic reticulum (ER) and chaperones

Accumulating evidence suggests that receptor dimerization and the assembly of GPCR with their signaling complex may occur before trafficking to the plasma membrane (Dupré et al., 2012). Indeed, receptor dimerization can occur in the ER (Smith and Milligan, 2010; Dupré et al., 2012). Mutagenesis studies targeting the putative interaction interface of β_2-adrenergic receptor showed that inhibiting β_2 dimerization caused its retention in the ER, thus supporting the hypothesis of receptor oligomerization in this cellular compartment (Salahpour et al., 2004). Other examples encompass the D_4 receptors (Van Craenenbroeck et al., 2011), $GABA_B$ receptors (Margeta-Mitrovic et al., 2000), and 5-HT_{2C} receptors (Herrick-Davis et al., 2006).

Newly synthesized GPCR at the ER interact with chaperones (e.g. Hsp-40, GRP78, and PDI) that aid the folding and maturation of receptors (see Fuxe et al., 2014b). Without the assistance of these chaperones, GPCR will be directed either to degradation (e.g. proteasomal

degradation) or to toxic ER accumulation prompting cell death. RTP4 is an example of chaperone implicated in receptor oligomerization. It has been reported to mediate the folding of the μ-δ opioid heteromer, thus masking the ER retention motif and permitting the export of the complex to the cell surface (Decaillot et al., 2008). Interestingly, RTP4 is thought to stay bound to the μ-δ opioid heteromer at the plasma membrane and participate in the regulation of heteromer-specific signaling. Another chaperone is calreticulin, which enhances the maturation and heterodimerization of the B_2 bradykinin receptor with the angiotensin II type 1 receptor (Abd Alla et al., 2009).

Thus, receptor oligomers and signaling complexes can be assembled in the ER and delivered to the cellular membrane as functional units. As emphasized by Farran (2017), this mechanism could contribute to the diversification and specificity of GPCR signaling complexes observed in mammalian cells.

GPCR interacting proteins (GIPs)

In 2003, already 50 or more GIPs had been demonstrated and Bockaert et al. (2003, 2010) in an interesting review described the C-terminal tail of the GPCRs as the 'magic tail' representing an important anchorage for functional protein networks. Thus, receptor complexes are in the center of multiple receptor-protein and protein-protein interactions that can influence their assemblage and stoichiometry (see Fuxe et al., 2014b).

Many of these GIPs serve as scaffolding or adapter proteins that modulate the physical RRIs in the receptor complexes (Franco et al., 2005). Adenosine deaminase provides an example. Apart from being able to deaminate extracellular adenosine, it has a nonenzymatical role through direct interaction with adenosine A_1 receptors at their extracellular side (Franco et al., 2005).

Together with cytoskeletal proteins, some GIP also target and anchor the receptor protomers to the plasma membrane and may also participate in stabilizing direct interactions between receptors, necessary for the formation of receptor complexes (Ciruela et al., 2005). A family of proteins termed homers (Brakeman et al., 1997) provides a significant example. All homers contain a similar N-terminal domain, named Ena/VASP homology domain 1 (~150 amino acids), which has a characteristic Gly-Leu-Gly-Phe motif that is responsible for binding to metabotropic glutamate receptors of group I ($mGluR_{1\alpha}$ and $mGluR_5$). This allows the linkage of the receptor to the cytoskeleton via a homer/shank/actin interaction (Ciruela et al., 2005).

Intercellular transfer by microvesicles (MVs)

In the last decade, evidence was obtained that cells can exchange a set of chemical messages via extracellular vesicles (acting as protective containers; Simons and Raposo, 2009). Different types of MV have been described (see Agnati et al., 2014), but two types are of importance for our discussion. Exosomes are vesicles (40–100 nm in diameter) contained in the so-called early, late, or recycling endosomes, a type of multivesicular bodies. Endosomes usually transport newly synthesized material from the Golgi complex and endocytosed material from the plasma membrane to various intracellular destinations. Alternatively, however, they can fuse with the plasma membrane and release exosomes into the extracellular milieu both constitutively and in a regulated manner (Lakkaraju and Rodriguez-Boulan, 2008). Extracellular vesicles can also be formed from lipid raft domains of the plasma membrane and are then called shedding vesicles (Smalheiser, 2007). Thus, shedding vesicles show surface markers that are dependent on the composition of the membrane of origin and constitute a larger and more heterogeneous population of extracellular vesicles, ranging from 100 to 1000 nm in diameter.

Recent data were obtained demonstrating that GPCR can be transported by MV and in particular by exosomes (Agnati et al., 2011). In a study by Guescini et al. (2012), two populations of cells were created: the first transfected with a CFP-labeled dopamine D_2 receptor (D_2R-CFP) and the second with a YFP-labeled adenosine A_{2A} receptor (A_{2A}R-YFP). These two types of cells were cocultured, and acceptor photobleaching FRET analysis demonstrated cells positive to both D_2R-CFP and A_{2A}R-YFP. Treatment with two inhibitors of MV release (methyl-β-cyclodextrin or GW4869) abolished the effect. In a further test, cells not expressing the A_{2A} receptor were incubated for 24 h with MV carrying the receptor. When treated with the adenosine A_{2A} receptor agonist CGS-21680, the significant increase in cAMP accumulation clearly demonstrated that A_{2A} receptors were not only safely transferred via MV to target cells, but in the target cells they were also capable of recognizing and decoding their signal.

Concluding remarks

Communication, in particular intercellular communication, is a fundamental feature of living organisms, and in some apparatuses [e.g. the central nervous system (CNS)], it plays a particularly important role, determining virtually all aspects of their function. The interaction of

chemicals and/or energy forms released by a source with specific receptors expressed by the target cells likely represents the main mechanism of communication in biological organisms. In this respect, the GPCR superfamily of seven-TM proteins represents the largest family of integral membrane receptors. Members of this superfamily are encoded by 4% of all genes and contribute to a variety of physiologic processes in mammals (Lefkowitz, 2007). Their signaling as monomeric units has been well characterized in several different experimental contexts (Chabre and le Maire, 2005; Kenakin, 2011).

In the 1980s, RRI and their relevance for receptor diversity were proposed based on studies on neuropeptide/dopamine interactions (Agnati et al., 1980; Fuxe et al., 1983). The vast majority of subsequent investigations, although each characterized by points of strength and weakness, provided consistent evidence in support of this proposal (see Milligan, 2009; Guidolin et al., 2015; Farran, 2017, for reviews) and the need for a broader view on intercellular communication. Indeed, allosteric RRI made possible through receptor oligomerization may lead to novel receptor dynamics during which the receptor protomers change their recognition, pharmacology, signaling, and trafficking and novel allosteric binding sites can develop (Agnati et al., 2010b; Fuxe et al., 2012; Fuxe and Borroto-Escuela, 2016). Thus, the formation of the receptor complexes can allow an integration of the incoming signals already at the plasma membrane level.

The GPCR oligomerization process in cellular models is presently not questioned and increasing evidence (see Borroto-Escuela et al., 2013) supports its occurrence in behaving animals (i.e. *in vivo*). In this respect, a first example was the demonstration of A_{2A}-D_2 heteroreceptor complexes in the striatum of mice (Trifilieff et al., 2011) and rats (Fernandez-Dueñas et al., 2015), where the alteration of native A_{2A}-D_2 oligomers was also observed in experimental parkinsonism. Further examples (see Borroto-Escuela et al., 2017, for a detailed review) include the galanin Gal_1-Gal_2 heteroreceptor dimer in the midbrain, the FGF_1-5-HT_{1A} in the hippocampus, and the 5-HT_{1A}-5-HT_{2A} isoreceptor complex in cortical regions. Worth mentioning are the result of a recent study (Beggiato et al., 2017) supporting the existence of functional dimers between dopamine D_2 and σ_1 receptors in rat striatal dopaminergic and glutamatergic nerve terminals where nanomolar cocaine concentrations appear to alter the allosteric RRIs in such complexes leading to the enhancement of $G_{i/o}$-mediated D_2 signaling. Thus, the oligomerization process is of potential great importance for neuropsychopharmacology and the GPCR complexes have become exciting new targets for the development of novel therapeutic strategies in CNS diseases (see Guidolin et al., 2015; Fuxe and Borroto-Escuela, 2016; Borroto-Escuela et al., 2017; Farran 2017). The main focus of this research line is presently on the identification or development of new ligands specific for the receptor complexes (Bhushan et al., 2004; Daniels et al., 2005). However, the identification of receptor complexes that are typically expressed in human nerve tissues and the structural or biochemical changes they undergo in pathologic conditions (Zalewska et al., 2014) should represent further lines of future pharmacologic research.

Not only GPCR complexes are specialized regulators of signal detection and transduction but also are in the center of multiple interactions with membrane components and can significantly contribute to finely tune the efficiency of the connections between cells and, in particular, the synaptic strength (Agnati et al., 2010a). Recently identified direct interactions with RTK (Borroto-Escuela et al., 2012; Di Liberto et al., 2017), indeed, open the possibility that GPCR complexes could modulate structural changes at the level of dendrites and dendritic spines. Furthermore, the reorganization of GPCR complexes in the postjunctional membrane of synapses and their possible stabilization by the interaction with specific adapter proteins could represent a molecular mechanism for learning and memory (Guidolin et al., 2007; Fuxe et al., 2014b; Borroto-Escuela et al., 2015).

Based on these features, it has been proposed (Guidolin et al., 2017) that allosteric RRI and RM may be of relevance for the emerging field of 'connectomics' (see Sporns, 2013), that is, the comprehensive study of the structural connections in the brain, mediating the communication processes between brain regions and cells. A hierarchical or nested architecture has been suggested as a suitable model providing a unified view of the different spatial scales characterizing the brain network organization (Sporns et al., 2005; Sporns, 2013; Guidolin et al., 2016). In this respect, an almost general consensus exists (see Sporns et al., 2005; Sporns, 2013) in targeting at least three levels of organization: the macroscale of brain areas and regions, the mesoscale involving nerve cells networks, and the microscale where single cells and synaptic clusters (Cutsuridis et al., 2009) can be found. However, to capture properties concerning the strength and plasticity of synapses (and, more in general, of intercellular connections), a 'nanoscale' level should also be considered. This further level of miniaturization is exactly the level at which RM and related molecular networks operate as molecular devices regulating the intercellular communication. Interestingly, as briefly illustrated in the third section, methods from the graph and network theory appear appropriate to describe not only the higher levels

of brain organization but also the overall dynamic behavior of interacting receptors and of the molecular networks they partake (Guidolin et al., 2007).

In addition, it is interesting to cite Smalheiser's (2007) proposal that the exosomal transfer of proteins and RNA, especially from the postsynaptic dendrite to the presynaptic terminal, can play a role in synaptic plasticity. Consistent with this view is the available experimental evidence on the intercellular transfer of GPCR and GPCR complexes by MVs (Guescini et al., 2012). This process can lead to a transient acquisition of recognition/decoding apparatuses by target cells. Such a transient change of cell phenotype (Guidolin et al., 2016) could be a mechanism that modulates the intercellular connectivity and represents a new aspect of the extraordinary plasticity of the communication processes in the CNS.

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