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J Recept Signal Transduct Res. Author manuscript; available in PMC 2013 March 13.

Published in final edited form as:

J Recept Signal Transduct Res. 2010 October ; 30(5): 287–303. doi:10.3109/10799891003786226.

On the expanding terminology in the GPCR field: the meaning of receptor mosaics and receptor heteromers

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Abstract

The oligomerization of G protein-coupled receptors (GPCRs) is a fact that deserves further attention as increases both the complexity and diversity of the receptor-mediated signal transduction, thus enriching the cell signaling. Consequently, in the present review we tackle among others the problems concerning the terminology used to describe aspects surrounding the GPCRs oligomerization phenomenon. Therefore, the theoretical implications of the GPCR oligomerization will be briefly discussed together with possible implications of this phenomenon especially for new strategies in drug development.

Keywords

Oligomerization; receptor–receptor interactions; stoichiometry; spatial arrangement

Introduction

In the eighties, our group gave strong experimental indications that G protein-coupled receptors (GPCRs) could interact, directly or via accessory proteins, at the plasma membrane level (1–3). The hypothesis was also put forward that GPCRs could form high-order oligomers (4–6). A fundamental consequence of this view was that not only the recognition/decoding processes at GPCRs, and hence the information handling at neural network level, but also the conventional design of drugs acting on GPCRs had to be revisited. Thus, it was no longer possible to describe signal transduction as the result of the binding of the chemical signal to its receptor, but rather as the result of a filtering/integration

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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of chemical signals in receptor assemblies. In addition, the drug design could no longer be built mainly on the activation or blockade of a single receptor at a usually well-defined ligand-binding site (orthosteric binding site).

GPCRs are currently thought to form and signal as monomers and/or dimers; whether they can form high-order structures (now called higher-order oligomers) is a topic of an intense debate (7). It is interesting that the existence of such higher-order complexes has been demonstrated for some GPCRs (8–10) but not for others (11); thus, the controversy is still alive (12). Collectively, these findings suggest, however, that our original proposal of high-order receptor oligomers is correct. In particular, by means of recent fluorescence-based approaches it has been possible to demonstrate the existence of these structures. Thus, by combining bimolecular luminescence complementation/bimolecular fluorescence complementation, respectively and resonance energy transfer (RET)-based approaches (e.g., bioluminescence-RET and fluorescence-RET) it has been demonstrated that at least three adenosine A_{2A} receptors (A_{2A}Rs) (13) and four dopamine D_2 receptors (D_2Rs) (14) are located in close molecular proximity in living mammalian cells, consistent with their organization as higher-order oligomers in the plasma membrane. On the other hand, by using sequential RET approaches it has been possible to demonstrate the existence of trivalent GPCRs complexes in living cells, for instance the existence of higher-order A_{2A}R-CB_1R-D_2R (15) and A_{2A}R-D_2R-mGlu$_5$R (16) heteromers has been shown. It is important to mention here that although this highly ordered organization has been supported by means of atomic force microscopy observations of native rhodopsin (17–19) the exact stoichiometry and the specific subcellular localization of these oligomeric structures has not been assessed yet.

Recently, other set of studies support the existence of higher-order receptor oligomers. In the case of the β_2-adrenergic receptor (β_2-AR), the group of Kobilka has demonstrated that the receptor is predominantly tetrameric following reconstitution into phospholipid vesicles (20). These authors made also the interesting observation that binding of inverse agonists leads to significant increases in fluorescence resonance energy transfer (FRET) efficiencies for most labeling pairs, suggesting that this class of ligand promotes tighter packing of protomers and/or the formation of more complex oligomers by reducing conformational fluctuations in individual protomers (20). Overall, our original hypothesis on the existence of high-order GPCR oligomers is now gaining new important experimental support.

It is obvious that the original hypothesis of high-order GPCR oligomers implies the existence of multiple receptor interfaces and accordingly it has been experimentally shown that in the case of D_2Rs as well as of other GPCRs (see, e.g., Table 1 in Filozola's article (21)) transmembrane domains (TMs) I, IV, and/or V play a role in dimerization/oligomerization (14,21). As mentioned above, the recent discovery of GPCR dimers/higher-order oligomers suggests that the selective targeting of these oligomers by specific drugs or by small molecules that interfere with the receptor–receptor interfaces may provide new opportunities for novel drug discoveries.

In the present review findings on the A_{2A}R–D_2R dimers/higher-order oligomers will be presented in the context of a careful discussion on the terminology to describe the phenomena. Finally, comments on possible new strategies on drug development based on these phenomena will be presented.

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Terminology aspects

Basic definitions

It is worthwhile to introduce by means of operational definitions some basic terms largely used in the study of protein networks at the plasma membrane level, and hence, also of the receptor function and the assembly of GPCRs into an integrative center:

Microprocessor is a term introduced by Kenakin (22) to indicate that 7TM receptors are not just "on-off" switches; rather, they have a high capability to elaborate information. In fact, by being allosteric proteins and having several intrinsically disordered domains (23) GPCRs are able to adopt many isoenergetic conformations. This idea is consistent with molecular dynamics data, which suggest that regions of intrinsic protein disorder are particularly high in cellular signaling proteins capable of assuming multiple conformations and of taking part in several protein–protein interactions. In agreement with this capability GPCRs also exist as clusters of interchanging conformations referred to as ensembles, and ligands produce changes in these ensembles by selectively binding to preferred conformations. Thus, stimulation of GPCRs by an agonist leads to a conformational change and to a transition of the receptor as it switches from an inactive to an active conformation, which can then couple to its cognate G protein. Conformational changes upon agonist binding have been well established to proceed through a rearrangement involving TMIII and TMVI (24–28). These changes are believed to be transmitted into the third intracellular loop. This loop seems to contain the key domains for coupling to G proteins, particularly in its C terminus (adjacent to TMVI) but also in its N terminus (adjacent to TMV) regions (29). Although classic theory assumed that receptors simply switch between "off" and "on" states, more recent data indicate that agonists of different efficacy might induce different changes in receptor conformations (30–32). Partial agonists do not induce a smaller proportion of the same active receptor conformation compared with full agonists but instead evoke ligand-selective conformations. Thus, a complex picture of receptor activation emerges in which distinct changes in different regions of a receptor may induce the ability to interact with different downstream proteins (33).

Colocalization means the simultaneous presence of the proteins under scrutiny in the same location, usually, within the same subcellular compartment. As far as GPCRs are concerned, they are colocalized when they are present in one and the same microdomain of the cell membrane, as, for instance, a membrane lipid-raft. Interestingly, colocalization can be assessed by means of computer-assisted image analysis of dual-channel fluorescence laser microscopy images (34) or by applying methods derived from spatial statistics to electron (35) or to atomic force microscopy images (see, e.g., ref. 36).

Receptor homodimers/heterodimers and higher-order receptor oligomers

This term describes the pairings of receptor in the cell surface forming a functional centre possibly possessing unique signaling and binding properties. Homodimers are pairs of the same receptor, whereas heterodimers are formed by different receptors. Each receptor in the dimer is referred to as a protomer (37). Usually, more than two receptors (or protomers) associated in an assembly of multimers are considered to form a higher-order receptor oligomer, which could either be a higher-order homomer or a higher-order heteromer.

Oligomer is defined as any multimeric protein that contains a finite, relatively small, number of subunits that are not in covalent linkage, but in a state of reversible association with each other (38). Oligomerization can occur also between GPCRs where the assembly of two or more than two receptors forms a complex of receptor multimers, which can be detected, for example, by means of coimmunoprecipitation (39–45). More recently, FRET and bioluminescence resonance energy transfer (BRET) have been used to detect both receptor-

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receptor interactions (RRIs), and receptor's conformational changes (32,46–56). As mentioned above, our group has suggested a completely different way on the basis of a statistical spatial approach to evaluate the distribution of immunogold particles in images acquired by means of atomic force microscopy analyzed by means of computer assisted image analysis (36). This approach is illustrated in Figure 1. It should be noted that all these techniques are highly indicative of the existence of class A GPCR dimers/higher-order oligomers, but none demonstrates the existence of these oligomers in living animals (see, e.g., ref. 57). Thus, the early studies on the functional counterpart of the receptor oligomerization, namely, the strong indications from binding studies on intramembrane neuropeptide–monoamine receptor interactions at the level of receptor recognition based on the existence of allosteric RRIs fully maintain their importance (1,3,5,58).

Allosteric RRIs have been defined as the intramembrane phenomenon by which the affinity of the orthosteric binding site of receptor B is changed by the binding of a transmitter to the orthosteric site of receptor A. As already mentioned, indications for RRIs have been obtained in the 1980s by means of binding experiments of radioactive ligands in membrane preparations and in brain sections. Number of binding sites and affinities for receptor B (in the absence and in the presence of the ligand for receptor A) have been evaluated by means of liquid scintillation counting of radio-labeled receptors and computer-assisted image analysis of the autoradiographic plates, respectively (see ref. 5). These studies allowed the demonstration that the ligand for receptor A could modulate the biochemical characteristics of receptor B. Thus, an activated GPCR could trigger a conformational change of functional relevance for another receptor in membrane preparations, even in the absence of the intracellular machinery involving phosphorylation events (for reviews see refs. 59–61). In the 1980s, correlates of such a molecular mechanism have also been obtained by means of *in vivo* studies carried out in physiological and pathological animal models (see refs. 62–64). Recently, more direct evidence for the existence of a conformational cross talk in receptor heteromers have been obtained in live cells by using a FRET approach (32).

Cooperativity

The binding of more than one molecule of the same ligand to a multimeric protein. Hence, cooperativity is manifested when binding of a ligand alters the affinity for subsequent binding of the same ligand to the other protein subunits via allosteric changes in the contact zone. In the case of receptors, cooperative binding is the binding of more than one molecule of the same transmitter to a receptor multimer (65). According to these definitions, cooperativity is a special case of allosteric interaction (see ref. 66).

Horizontal molecular networks

Proteins embedded and/or associated with the membrane form the so-called horizontal molecular networks (HMNs) that can also operate as autonomous integrative modules carrying out specialized tasks (see Figure 2 and refs. 67,68). Furthermore, HMNs consist of membrane proteins, which can be assembled according to specific topologies, hence forming "mosaics." Such mosaics may last longer or for shorter period of times; therefore, it can be surmised that they undergo a reshuffling, including addition of new proteins ("tesserae" of the mosaic) or alteration in their topology. A functional and structural relationship exists between GPCRs and HMNs. As mentioned previously, these receptors, like all proteins, can adopt numerous tertiary conformations. Thus, they can also interact with cellular proteins, playing an important role in the formation of HMNs where they can function as sophisticated signaling-processing centers (69).

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Receptor mosaic

Also receptors, in particular GPCRs, can form mosaics, which may or may not be part of a HMN. In the first instance, they work as a specialized input unit not only to the vertical molecular networks (VMNs, i.e., to molecular networks that extend into the cytoplasm and often reach the nucleus) but also to the HMN itself, allowing a major increase in the intramembrane elaboration of information (67). The existence of receptor mosaics (RMs) of different kinds of GPCRs has recently been demonstrated in cell lines (15,16). It should be noted that a RM being localized at the membrane level may sometimes in itself represent an isolated HMN. The basic properties of an RM can be deduced from the RRI phenomenon. As a matter of fact, a RM has been defined as an assembly of receptors with direct physical interactions between them, which has the following features (23):

- A receptor assembly works as an RM if and only if at least one receptor modulates via allosteric RRIs the biochemical/functional features of at least another receptor of the assembly.
- The fluctuations of each receptor (of the RM) among its possible conformational states are conditioned by the conformations of the other receptors in the mosaic. Hence, each receptor will respond to its ligand in a way that depends not only on its own conformation but also on its allosteric interactions with the other receptors of the macromolecular assembly.
- Given a certain stoichiometry of a RM vs. the dimer condition, the RRIs that take place obviously depend on the topology of the RM, that is, on the spatial arrangement of the receptors forming the assembly and on the efficacy of the "allosteric pathways" interconnecting the receptors with each other (70–72).

As anticipated already in the early studies (4,58), RMs are 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 fact, the effects of oligomerization on the functional features of the monomers can be shown already at the level of dimers. It may be mentioned that opioid receptor subtypes acquire new biochemical and functional characteristics when forming heterodimers. Thus, k-δ and δ-μ opioid receptor heterodimers represent a new receptor entity endowed with new characteristics with regard to ligand binding and functional properties (e.g., G-protein coupling) that are distinct from those of the participating monomer receptors (43,73,74). The same phenomenon has been observed for the D_1R-D_2R heteromer with a shift from G_s (D_1R) and $G_{i/o}$ (D_2R) to a $G_{q/11}$ coupling (75). Thus, we have introduced the concept of "phenotype-plastic receptors" to indicate the phenomenon in which some monomers after heterodimerization show a fully different recognition/decoding mechanism when compared to the single monomers or homodimers (36). In an interesting critical article, the possibility has been raised that GPCR oligomers of class A are not truely formed but only transient "kiss-and-run" encounters between the two receptors take place (57) in contrast to class C GPCRs where covalent bonds develop. Even if several experimental approaches suggest that long-lasting oligomerization occurs between GPCRs of class A, we have put forward the hypothesis that the "kiss-and-run" phenomenon could also be of importance in the transient change of synaptic weight obtained via the assembly of special RMs. It may be possible that the highly transient assembly (that we called the Casanova-type of RRI) can play a role in some form of ultra-short memory processes (36). Among these emergent properties it may be important to mention the following:

- The appearance of new characteristics of the binding sites present in each protomer or the appearance of entirely new binding sites for extracellular or for intracellular ligands (e.g., for receptor-interacting proteins like G proteins and β-arrestin).

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- Different localization of the RM with respect to the isolated protomers (e.g., preferential localization in the lipid rafts).
- Different turnover rate and desensitization with respect to the isolated protomers.

The scheme of Figure 2 conveys some main features of the terms introduced. The protomers are single GPCRs that can be in most cases fully functional, that is, capable of recognizing and decoding an extracellular signal. These protomers can associate into dimers that can also be fully functional. Protomers and/or dimers can be aggregated to form a RM. It should be noted that a protomer or a dimer or an RM can also become physically linked to other extracellular or intracellular proteins forming a HMN. The following nomenclature may be suggested:

- A first-order microprocessor (for the term *microprocessor* see ref. 22) gives rise to an intracellular cascade of the first-order, as it involves a single protomer. In the case that it interacts with some membrane protein, a first-order HMN is formed.
- A second-order microprocessor gives rise to an intracellular cascade of the second order, as it involves a dimer. In the case that it interacts with some membrane protein, a second-order HMN is formed.
- Higher-order microprocessor gives an intracellular cascade of the higher order when we are dealing with at least a trimer (i.e., an RM). Thus, it should be noted that only for higher-order microprocessors does topology play a role. In the case that the higher-order microprocessor interacts with some membrane protein, we have a higher-order HMN.

It is likely that GPCRs can be inserted in the membrane as protomers or as a dimers or even as oligomers. However, it is very well possible that oligomers are formed after the insertion of protomers or dimers at the membrane level and, in some cases, after the action of an extracellular signal (20).

It should also be noted that GPCRs may or may not belong to a HMN (see previous discussion). In the former case, they likely represent the hub proteins around which the HMN is organized. In agreement with this view is the case of the adenosine A_1 and A_{2A} receptors and their interacting proteins (see ref. 76). Crucial aspects not yet clarified in details concern the mechanisms that control the formation of dimers and higher-order oligomers, their insertion at membrane level, and in which membrane microdomain, as well as their participation in HMNs. It may be surmised that the endoplasmic reticulum and chaperones (i.e., quality control of protein folding and transfer), cytoskeleton, lipid composition of the membrane, all play a role, but how these mechanisms are interconnected in a complex control has not yet been clarified. Furthermore, it should also be considered that all the assemblies formed (dimers, RMs, and HMNs) are potentially plastic oligomeric structures. Hence, they cannot be internalized simply as such, but they can also be modified by the addition of new components and/or by disruption from some of the previously associated components. It is interesting to note that discrete conformations of GPCRs induced and/or stabilized by specific ligands, even in the absence of receptor phosphorylation, can promote distinct and functionally specific conformations in receptor-interacting proteins, for example, in the β-arrestins (77). Thus, β-arrestin can adopt multiple “active” conformations under the influence of 7TMRs operating as a hub in a VMN. As stressed by the Lefkowitz’s group their findings demonstrate that functionally specific receptor conformations can indeed be translated to altered direct coupling to downstream proteins, in particular to proteins working as effectors such as β-arrestins, thereby governing their functional specificity (77).

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Another important aspect that is under intense debate is whether the receptor-G protein complex is constitutively assembled in an inactive state and only becomes activated upon ligand binding to the receptor (precoupling model), or whether it only forms after agonist exposure, in which case the receptor-G protein interaction would be diffusion limited, that is, by free collision coupling (55). It has also been proposed that GPCR–G-protein signaling does not necessarily require G-protein dissociation. Thus, FRET and BRET studies in intact cells have led to the conclusion that at least some G proteins can signal in response to receptors or other stimuli without the need to dissociate (78). These studies can be important also for another interesting functional aspect, as it can be questioned whether G-proteins are organized in a chain as the original article by Rodbell (79) and some recent reports suggest (80). Nevertheless our group has proposed that such an organization could have some formal analogy with the Turing machine (36) even if it remains to demonstrate whether the G-protein chain can move in only one direction.

A theoretical comparison between the terms *RM* and *receptor heteromer*

The protein oligomerization is of fundamental importance for biochemical processes: it is sufficient to consider the cooperativity phenomenon, which can occur in multimeric proteins. To properly underline this aspect It should be mentioned that Monod pointed out the important biological role played by the “allostery” for protein functions by defining this phenomenon as “the second secret of life,” as allostery could be considered in importance second only to the genetic code (81,82). Thus, oligomerization of receptors and possible allostery including cooperativity in their assembly into oligomers can play a fundamental role in the recognition/decoding processes of extracellular and synaptic signals impinging on the cell membrane (36,83). Thus, it is worthwhile to point out some implications of the multi-facet meaning of the term *RM* by comparing it with the term *high-order heteromers*, introduced by our group (6) simply to indicate the receptor assembly by means of an already available biochemical term for protein assembly into multimers (see above “Basic Definitions”).

It is certainly possible to have receptor colocalization without receptor heteromerization and it is also possible to have receptor heteromerization without allosteric RRIs. Therefore, receptor assembly where RRIs take place is not simply heteromerization that is a structural clustering of monomers, but rather a functional construct capable of operating as an integrative centre. Thus, the term *RM* has been introduced, as it conveys a feature of the receptor assembly that can be of a basic importance for its function, namely, the spatial arrangement of the monomers, that is, the topology of the receptors in the oligomers (see below). As a matter of fact, the receptor topology, that is, receptor location with respect to each other, can affect:

- The order of progression of the allosteric RRIs, which can take place only between receptors that are in contact.
- The possible circulation of the allosteric RRIs within the RM is also dependent on the allosteric pathways, which are obviously determined by the spatial arrangement of the receptors and the biochemical characteristics of the receptor–receptor interfaces, in addition to the affinity and concentrations of the transmitters and allosteric modulators
- The existence of a “Hub Receptor” (23,67,84,85), that is of a receptor that in view of the number of its connections with the other receptors of the RM can play a special role. Obviously, the concept of hub receptor is strictly dependent on the location of that receptor with respect the other receptors of the mosaic and on the efficacy of the allosteric pathways of the hub receptor to transmit the allosteric modulatory signal.

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- The possibility of cooperativity in a RM of homomers (66,86,87) which can simply be a RM of homodimers coming together. It should be underlined that cooperativity markedly affects the behaviour of a RM and it has been suggested that positive cooperativity in a RM of homomers can be an important mechanism to enhance the capability of a RM located in an extrasynaptic position to detect low concentrations of the transmitter signal (see refs. 36,83). Negative cooperativity was the one first described and found to exist in β_2-ARs (88,89) and may represent a negative feedback mechanism at high transmitter concentrations found especially in the synapse. It should be noted that cooperativity is strongly affected not only by stoichiometry, but also by topology (see the Appendix).

Thus, due to topology and the rank order of activation it is possible to have markedly different integrations for RMs formed by the same set of receptors of different kinds (i.e., same stoichiometry) and certainly these features are not grasped by the term *higher-order heteromers*, a term conveying only the stoichiometry characteristics of the oligomer.

In an interesting recent commentary (7) it has been pointed out that ion channel receptors are oligomers formed by different nonfunctional subunits and only the complete assembly of protein subunits forms a functional complex by building up an ion channel. It has been proposed to use a dichotomy classification by naming "the ion-channel receptors "heteromeric receptors," whereas, in view of the fact that each GPCR protomer in a receptor oligomer is a functional receptor, the complex is called a receptor heteromer (7). This proposal is a timely and valuable effort to give a logical framework to a rapidly expanding field, but it has some pitfalls:

- The statement of the functional competence of each GPCR forming the oligomer is not true for the well-known GABA B R1 and GABA B R2 heterodimer, which is functionally competent only as a dimer, and, therefore, should be called, according to the proposed nomenclature, a heteromeric receptor (7). Furthermore, it should not be overlooked that some "orphan receptors" can operate to make functional other GPCRs (90) or, at least to modulate their recognition/decoding mechanisms as shown for the orphan 7TM protein GPR50 when forming a complex with the melatonin MT_1 receptor (91).
- In addition, the symmetrical definition of heteromeric receptors vs. receptor heteromers has also the fundamental defect of not taking into account the spatial design of the two types of assemblies. It is well known that the conformation of a protein determines its function and, similarly, the topological arrangement of monomers in an assembly determines the geometry of its quaternary structure and thus its function (92). Thus, in our opinion, it is the topology of receptor monomers in the oligomer that makes a clear cut dichotomy between the ion-channel receptors and the GPCR oligomers. As a matter of fact, whereas ion channel receptors have as topology an obligatory assembly of the subunits around a channel, the receptor GPCR heteromers can in principle be assembled according to several different topologies (93).

The possibility of domain-swap models has been proposed, that is, models in which domains TM1–5 and TM6–7 could exchange between protomers (see ref. 23,94). Although there is limited direct evidence that supports such models (95), there are also enough data to support the view that GPCRs exist and function as oligomers. As a matter of fact, this is a reasonable assumption in view of data carefully reviewed by Filizola (21) supporting the view that GPCRs exist and function as contact dimers or higher-order oligomers with TM regions at the interfaces. In contact dimers/oligomers of GPCRs, the original TM helical-bundle topology of each individual protomer is preserved and interaction interfaces are formed by lipid-exposed surfaces.

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In any case, several authors have proposed on the basis of experimental evidence the simultaneous involvement of lipid-exposed surfaces of TM1, TM4 and/or TM5 at the interfaces of assembled GPCR complexes of oligomers (14,39,49). Thus, if more than one interaction surface exists in a GPCR it is reasonable to assume that several patterns of spatial arrangements (i.e., topologies) can take place when the GPCR takes part in an oligomer. Thus, the term *oligomerization* conveys only part of the information, as it does not take into account the spatial arrangement of the protomers. It could be stated that the relationships between oligomerization and topology for a RM are similar to those between “molecular formula” and “structural formula” of a compound in Chemistry. This analogy has the relevant consequence that as in Chemistry there can be more than one compound (i.e., structural formula) with the same molecular formula the same is true in a receptor oligomer where there can be more than one mosaic with the same oligomerization (see lower panel of Figure 3). Thus, the integrative action of a RM targeted by transmitters and allosteric modulators depends on both stoichiometry and topology. However, in the presence of multiple interacting surfaces leading to similar (or even identical) topologies the efficacy of the various interfaces in transmitting the allosteric signal may become especially relevant for the integrative action of the RM. As a matter of fact, we have pointed out in the past (refs. 87,96; see also refs. 97,98) that proteins can be assembled thanks to their *Lego property* and hence with the only restraints being the rules of protein-protein interactions giving rise to several different topologies (plans according to which monomers are assembled). Even for trimeric RM, for instance, two different plans of assembly, the linear vs. the triangular one (see ref. 93), could be hypothesized.

Another aspect that likely has a full meaning only with the GPCR heteromers (90) is the existence of *Hub Receptor*, which has been defined as the GPCR that can interact with multiple partners either receptors or proteins associated with the membrane (see also above and refs. 23,99).

Summing up, all these considerations underline the importance of the *RM* term, which conveys the concept of topology and may have a potentially great impact on the integrative functions of RMs and hence on the input signals to neurons (83,87,100–102). For a more penetrating analysis is left the problem of detecting the preferential allosteric pathways in the oligomer, which as discussed below may affect also cooperativity.

Some implications of the RM concept for the information handling

As pointed out by Milligan (103) a fundamental question is, why are receptors dimers? Or more generally why is the GPCR oligomerization phenomenon so widespread? Milligan considers different possibilities such as:

- *Facilitation of G-protein activation.* This does not seem to be the real reason of oligomerization, as the GPCR dimerization does not appear to be a general requirement for intracellular signal generation (see also ref. 57). As a matter of fact, the likely functional competence of single GPCR monomers has been demonstrated (14). Thus, both rhodopsin (104) and the β_2AR) (105) have been shown to signal efficiently to G proteins when reconstituted into lipid nanodiscs containing only a single receptor. Thus, after solubilization and reconstitution, these GPCRs can function on their own.
- *Control of membrane surface delivery.* Milligan and Bouvier discuss studies that appear to provide strong support for the concept that GPCRs traffic to the cell surface as dimers or higher-order oligomers and only after passing ER/Golgi export quality control. Thus, this can be an important regulatory mechanism of GPCR turnover involving oligomerization (103,106,107)

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Lohse has also made the interesting suggestion that among the possible functional effects of receptor dimerization a cross regulation in cell surface mobility should be considered (52). This aspect has been indicated in the scheme of Figure 2. where also the preferential targeting to membrane microdomains with a special composition of protomer vs. dimers and RMs is indicated.

Our group has put forward some other fundamental reasons (36,60,87,99) that can be summarized as follows:

- *Combinatorial property of high relevance for learning and memory* that is acquired by means of the different assembly of receptors (tesserae) in a mosaic not only taking advantage of different stoichiometry, but also of different topologies with the same stoichiometry. Thus, with the same set of receptors (tesserae of the mosaic) it is possible to obtain markedly different topologies and hence likely different integrative functions of the RM (Figure 3). This can be viewed as a "tinkering process" (108) that has high relevance in biochemistry as demonstrated by considering that, for example, the genome is basically built by only four nucleotides and proteins by 20 amino acids.
- *The possibility to have a preliminary elaboration of the information already at membrane level.* This elaboration could either filter the relevant signals that in fact reach the VMNs and/or give integrated signals to these molecular networks and to the HMNs. A very interesting case of such a filtering process is the one discovered by Vilargada, Lohse and Collaborators of a direct transinhibition by MOR of α_2A–AR that can also help explain previously described antagonistic NPYR-alpha2 RRIs at the membrane level (see ref. 99). Thus, morphine binding to the MOR triggers a conformational change in the norepinephrine-occupied α_2-AR that inhibits its signaling to Gi and the downstream MAP kinase cascade. It has been assessed that the cross-conformational switch that permits direct inhibition of one receptor by the other in the heteromer occurs with sub-second kinetics. In other words, the allosteric signal propagates over the receptor interface to induce its conformational changes, which are of high functional relevance (ref. 32; see also refs. 64,99).
- *To have negative or positive cooperativity in homomers with a great spectrum of different strengths* (83). Let us illustrate this point further by considering a special case, for example, of a tetramer formed by the same homodimer or two different types of homodimers. A tetramer can be surmised similar to that of hemoglobin (two α and two β subunits) binding the same ligand or a tetramer in which one dimer binds a ligand and the other dimer binds another ligand. This speculative example is only to give a hint of the extraordinary spectrum of possibilities of a biochemical high-order microprocessor formed by only four elements. In addition, it is also possible to surmise that a quadrangular topology has a completely different behaviour from a linear topology (see the Appendix).
- RRIs and more generally HMNs can play a role in enabling functional capabilities of certain membrane microdomains (109). In fact, it has been considered that particularly lipid rafts via protein–lipid interactions can play a role in favoring the localization of certain RMs and, more generally, the formation of specialized HMNs in these rafts but heteromerization can occur independently of lipid rafts (48,96). A similar proposal has been put forward by Maggio and Collaborators (110) by suggesting that protein–protein as well as protein–lipid interactions may provide the structural basis for organizing distinct cell compartments along the plasma membrane where different extracellular signals may be perceived and discriminated.

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- It has been also proposed that RMs especially in association with HMNs can play an important role in the modulation of the "synaptic weight" and thus in the process of learning and memory (100). Thus, a learning model that can take into account not only the connections between the cells, forming cellular networks able to adapt their function according to the stimuli they receive, but also molecular networks especially RMs and associated HMNs has been proposed. According to such a proposal, memory formation seems not only to be a distributed process, but also to follow a hierarchical morpho-functional organization (111).

Possible implications of the RM concept for new strategies in drug development

As discussed above, GPCR heterodimers can generate very distinct signals apart from the corresponding homodimers. The development of small-molecule ligands that are specific for these complexes is a potential new way to discover novel drugs with higher selectivity of action. Thus, one can surmise alternative approaches as schematically depicted in Figure 4. by developing the so called "bivalent ligands" and other drugs selective for a certain dimer or for a certain heterodimer (7, 21,102).

The interesting Filizola's perspective does not take into account an important feature of the GPCRs, namely, the possibility of a modulatory action on the orthosteric binding site via an allosteric modulator acting on the same receptor or on another receptor of the receptor heteromer (see refs. 112,113). In fact, as clearly stated by Kenakin (69,114) there is an increasing prevalence of allosteric ligands as investigational drugs. Although allosterism traditionally has been discussed in terms of affinity changes of receptors, the increasing use of functional pharmacological assays makes it mandatory to consider effects on both affinity and efficacy. Antagonism of agonist response can occur allosterically by reduction of affinity and/or efficacy but the antagonism will have different properties depending on which of these is primarily affected. It is clear that antagonism can be achieved by either blocking affinity but not efficacy, reducing efficacy but not affinity, or both (115).

Because an allosteric modulator allows agonist binding to the receptor, there can be differences in the resulting effect and allosterically-induced texture in antagonism can lead to other interesting properties. GPCRs are allosteric systems that have a modulator (ligand or protein) that interacts and transmits information through a conduit (receptor) to a guest (either other ligand, interacting protein or cytosolic protein). Thus, such receptor allostery can be discussed as vectorial transfers of information from ligand-binding domains ("classical" modulator allosterism) to the cytosol (functional selectivity) and along the plane of the membrane (receptor oligomerization) (37).

Thus, Kenakin notes that there are at least three advantages to use allosteric modulators for cell-surface receptors:

1. they are saturable and therefore there is a ceiling to the effects of a drug;
2. allosteric ligands have the ability to selectively fine-tune responses at receptors upon activation of the orthosteric sites in specific tissues;
3. allosteric drugs have the potential for greater receptor subtype selectivity, as the receptor subtypes are activated by the same transmitter.

It can be added that usually an allosteric modulator maintains the temporal code by which the endogenous orthosteric ligand acts on the target cell at any receptor illustrated in the three panels of Figure 4. It is possible to add the allosteric modulation of the orthosteric binding site with possible secondary effects on the orthosteric binding site of other receptors

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of the oligomer or on the heterodimer orthosteric binding sites as depicted in Figure 5. It should also be considered the development of drugs affecting not the orthosteric binding sites nor the formation and the maintenance of the oligomer (see ref. 21), but simply the transmission of allosteric signals along the amino acid pathways connecting binding sites of the oligomer. Thus, we have postulated the possible development of drugs acting on the "check points" localized along these pathways (36).

It is, therefore, clear that the RRI phenomenon has opened up a new field not only for a better understanding of the molecular mechanisms of recognition/decoding of the extracellular and synaptic signals and hence of the information handling by neural networks, but also of new strategies for drug development. A brief discussion of some aspects related to these new strategies for drug development has been previously presented by our group (102,116).

In conclusion, as we have already discussed, the early studies on RRIs carried out in the 1980s were fully aware of the potential importance of this molecular phenomenon and focused specifically on the molecular integration of signals, in order to achieve a better understanding of brain's functions. We and others have also suggested that an alteration in specific RRIs could play a role in pathogenic mechanisms leading to several diseases inter alia Parkinson's disease, hypertension, schizophrenia, addiction, and depression (102,117–122). Thus, it is only logical to propose that pharmacological targeting of RRIs could represent an important area to develop more selective drugs, including bivalent compounds and optimal types of combined treatments (see above and also refs. 7,73,123–126).

All the strategies listed in Figure 4 are important. However, it should be considered that there will always be a place for treatments based on the coadministration of drugs acting on different GPCRs even if these receptors form an heterodimer or a RM. Therefore, in our opinion, it could be important in many cases to either study each of them separately, on a case-by-case basis, or embark on a combined study, clubbing two or three cases together; thus, it would help protomers to take advantage of the different pharmacodynamics and/or pharmacokinetics of the coadministered drugs, which may more optimally target their respective receptor than bivalent drugs.

Acknowledgments

This work was supported by IRCCS San Camillo (L.F.A.), Swedish Research Council, Swedish Brain Fund and Torsten and Ragnar Söderberg's Foundation (K.F.) and by grants SAF2008-01462 and Consolider-Ingenio CSD2008-00005 from Ministerio de Ciencia e Innovación to F.C. who belong to the "Neuropharmacology and Pain" accredited research group (Generalitat de Catalunya, 2009 SGR 232).

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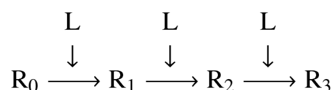
APPENDIX: Theoretical ligand binding curves

As illustrated in Figure 3, a cluster of receptors can in principle be arranged in different ways, depending on the physical constraints of the membrane environment in which the subunits are placed and on the interaction interfaces available to them.

To analyze the influence of the geometrical arrangement on the properties of the receptor clusters, theoretical ligand binding curves will be here derived for the different arrangements available to a trimeric receptor mosaic of identical subunits.

The analysis will be performed in the framework of the well-known Koshland–Nemethy–Filmer (KNF) model (127), based on a sequential scheme for the binding to a multisubunit protein:



For each binding step we have an equilibrium condition of the form (see (128)):

$$\frac{[R_i]}{[R_{i-1}][L]} = \frac{n_i}{n_{i-1}} \cdot e^{-\Delta G^0/RT} \qquad (1)$$

Where L denotes the ligand, R_i the protein complex with i occupied sites and ΔG^0 is the change in free energy involved in the transition. The multiplicity factor n_i accounts for the number of ways to achieve i occupied sites.

According to the KNF model, ligand binding at one subunit is assumed to cause a local conformational change (“induced fit”) leading to conformational changes at nearby subunits, affecting their affinity for the ligand. Thus, for each binding step the change in free energy at the equilibrium will depend on three energetic factors: the binding energy, the intrinsic

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energy difference associated with the conformational change of a subunit and the subunit-subunit interaction energy. To simplify things, however, it was also assumed that the conformational transition of each subunit is concomitant with ligand binding. As a consequence each subunit can be considered to have only two possible configurations (F or "free" and B or "bound") and the contributions due to ligand binding and conformational change can be lumped together taking the energy per binding event as the free energy difference per subunit between the completely free and completely occupied states. Considering as $G^0=0$ the free energy of the completely unoccupied receptor cluster, we can denote the free energy change associated with the binding of a ligand as:

$$\Delta G^0{}_L = -RT\ln K_L \quad (2a)$$

Where K_L is a binding constant including not only the energies directly relating to protein-ligand interactions, but also the energy of the obligatory conformational transition.

As far as the subunit-subunit interactions are concerned, they can be similarly denoted as:

$$\Delta G^0{}_{FB} = -RT\ln K_{FB} \quad (2b)$$

$$\Delta G^0{}_{BB} = -RT\ln K_{BB} \quad (2c)$$

Where K_{FB} and K_{BB} express the interaction energy between F-B and B-B pairs of interfaced subunits respectively.

Thus, for a state with i occupied sites, j F-B and z B-B interfaces between subunits, the free energy change relative to the completely unoccupied state is:

$$\Delta G^0 = -iRT\ln K_L - jRT\ln K_{FB} - zRT\ln K_{BB} \quad (3)$$

The different states available to a trimer arranged according to a linear or triangular topology are illustrated in Figure 6 together with the corresponding values of the parameters n, i, j, z.

Thus, with a ligand concentration of L, by using the equations (1) and (3) the equilibrium between a trimer with zero and one occupied site can be espressed as:

$$\frac{R_1}{R_0 L} = e^{\text{In}K_L + 2\text{In}K_{FB}} + 2e^{\text{In}K_L + 2\text{In}K_{FB}} = \left(K_{\text{FB}}^2 + 2K_{\text{FB}}\right) K_L \quad \text{for a linear trimer}$$

$$\frac{R_1}{R_0 L} = 3e^{\text{In}K_L + 2\text{In}K_{FB}} = 3K_{FB}^2 K_L \quad \text{for a triangular trimer}$$

And the concentration at equilibrium of single bound trimers (i.e., R_1) will be:

$$R_1 = R_0 \frac{R_1}{R_0 L} L = R_0 \left(K_{FB}^2 + 2K_{FB}\right) K_L L \quad \text{for a linear trimer} \quad (4a)$$

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$$R_1=R_0\frac{R_1}{R_0L}L=3R_0K_{FB}^2K_LL \text{ for a triangular trimer} \quad (4b)$$

R_2 and R_3 can be obtained following the same approach by using the corresponding data reported in Figure 6. We have:

$$R_2=R_0\left(K_{FB}^2+2K_{FB}K_{BB}\right)K_L^2L^2 \text{ for a linear trimer} \quad (5a)$$

$$R_2=3R_0K_{FB}^2K_{BB}K_L^2L^2 \text{ for a triangular trimer} \quad (5b)$$

$$R_3=R_0K_{BB}^2K_L^3L^3 \text{ for a linear trimer} \quad (6a)$$

$$R_3=R_0K_{BB}^3K_L^3L^3 \text{ for a triangular trimer} \quad (6b)$$

Being the fraction of occupied binding sites:

$$B=\frac{occupied}{total}=\frac{R_1+2R_2+3R_3}{3(R_0+R_1+R_2+R_3)}$$

by substituting R_1, R_2 and R_3 with the expressions provided by (4a–b), (5a–b) and (6a–b) we finally obtain:

$$B=\frac{\left(K_{FB}^2+2K_{FB}\right)K_LL+2\left(K_{FB}^2+2K_{FB}K_{BB}\right)K_L^2L^2+3K_{BB}^2K_L^3L^3}{3\left(1+\left(K_{FB}^2+2K_{FB}\right)K_LL+\left(K_{FB}^2+2K_{FB}K_{BB}\right)K_L^2L^2+K_{BB}^2K_L^3L^3\right)} \text{ for a linear trimer} \quad (7a)$$

$$B=\frac{K_{FB}^2K_LL+2K_{FB}^2K_{BB}K_L^2L^2+K_{BB}^3K_L^3L^3}{1+3K_{FB}^2K_LL+3K_{FB}^2K_{BB}K_L^2L^2+K_{BB}^3K_L^3L^3} \text{ for a triangular trimer} \quad (7b)$$

representing the saturation curves of the trimer in the two configurations.

It is noteworthy that with zero interaction energy, according to equations (2b) and (2c) we have $K_{FB}=K_{BB}=1$ and both (7a) and (7b) become:

$$B=\frac{K_LL+2K_L^2L^2+K_L^3L^3}{1+3K_LL+3K_L^2L^2+K_L^3L^3}=\frac{(1+K_LL)^2K_LL}{(1+K_LL)^3}=\frac{K_LL}{1+K_LL}$$

This is what one would expect for a cluster of completely independent subunits (Michaelis-Menten curve).

When subunit-subunit interactions are established, however, (7a) and (7b) define substantially different saturation curves as illustrated in Figure 7A, indicating that the existence of receptor–receptor interactions lead to a significant dependence of the receptor cluster response on the geometrical arrangement of the subunits within the cluster. In

particular, on comparing the curves obtained with identical values for the constants, it can be seen that the steepness of the curve increases and the mid-point shifts as the number of interactions increases.

The analysis of the tetramer further confirms this point. Three arrangements will be here considered for a cluster of four identical subunits: *linear* (in which the two interior subunits interact with each of the two neighbours, whereas the two terminal subunits interact with only one neighbour), *square* (in which the subunit are in a square pattern in which no interactions across the diagonal occur) and *tetrahedral* (in which each subunit interact with the other three). They are shown in Figure 7B together with their saturation curves, corresponding to the following equations as reported by Leskovac ((129); see also (130)):

$$B=\frac{2\left(K_{FB}+K_{FB}^{2}\right)K_{L}L+2\left(K_{FB}^{2}K_{BB}+2K_{FB}^{3}+K_{FB}^{2}+2K_{FB}K_{BB}\right)K_{L}^{2}L^{2}+6\left(K_{FB}^{2}K_{BB}+K_{FB}K_{BB}^{2}\right)K_{L}^{3}L^{3}+4K_{BB}^{3}K_{L}^{4}L^{4}}{4\left(1+2\left(K_{FB}+K_{FB}^{2}\right)K_{L}L+\left(K_{FB}^{2}K_{BB}+2K_{FB}^{3}+K_{FB}^{2}+2K_{FB}K_{BB}\right)K_{L}^{2}L^{2}+2\left(K_{FB}^{2}K_{BB}+K_{FB}K_{BB}^{2}\right)K_{L}^{3}L^{3}+K_{BB}^{3}K_{L}^{4}L^{4}\right)}$$

$$B=\frac{4K_{FB}^{2}K_{L}L+4\left(K_{FB}^{4}+2K_{FB}^{2}K_{BB}\right)K_{L}^{2}L^{2}+12K_{FB}^{2}K_{BB}^{2}K_{L}^{3}L^{3}+4K_{BB}^{4}K_{L}^{4}L^{4}}{4\left(1+4K_{FB}^{2}K_{L}L+\left(2K_{FB}^{4}+4K_{FB}^{2}K_{BB}\right)K_{L}^{2}L^{2}+4K_{FB}^{2}K_{BB}^{2}K_{L}^{3}L^{3}+K_{BB}^{4}K_{L}^{4}L^{4}\right)}$$

$$B=\frac{4K_{FB}^{3}K_{L}L+12K_{FB}^{4}K_{BB}K_{L}^{2}L^{2}+12K_{FB}^{3}K_{BB}^{3}K_{L}^{3}L^{3}+4K_{BB}^{6}K_{L}^{4}L^{4}}{4\left(1+4K_{FB}^{3}K_{L}L+6K_{FB}^{4}K_{BB}K_{L}^{2}L^{2}+4K_{FB}^{3}K_{BB}^{3}K_{L}^{3}L^{3}+K_{BB}^{6}K_{L}^{4}L^{4}\right)}$$

for the linear, square and tetrahedral arrangements respectively.

Thus, not only the stoichiometry of a receptor cluster (i.e., the number of component subunits), but also its topological arrangement (in particular the topological organization of the interactions between subunits) determines its behaviour. Stoichiometry and topology should, therefore, be considered as different and complementary characteristics of a receptor cluster, both significantly influencing its response to an incoming ligand.

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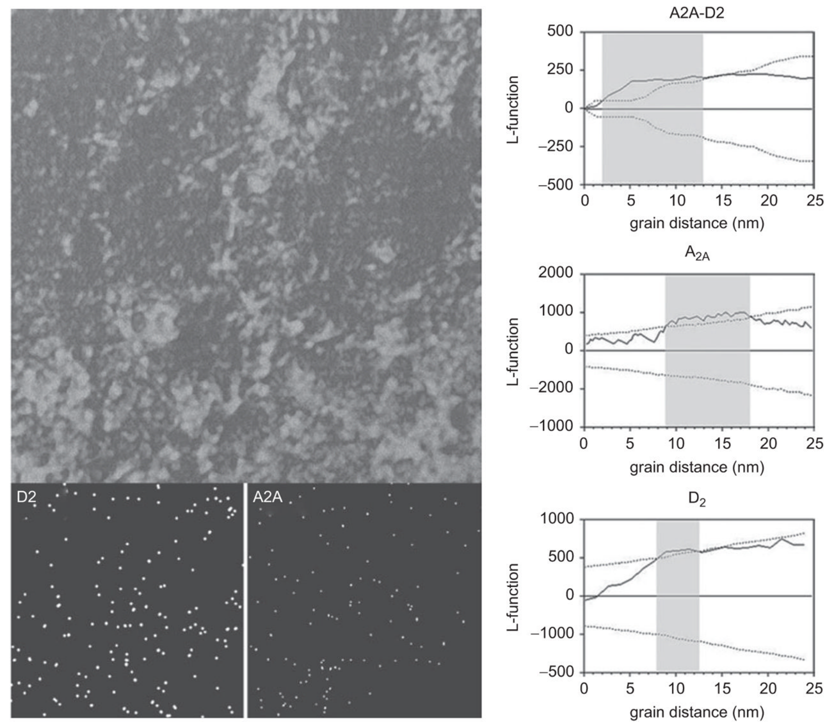

Figure 1.

Evaluations by means of spatial statistical analysis of atomic force microscopy images of HA-tagged A_{2A} and D_2 receptor distributions in CHO cell membranes. (A) Experimental procedure: CHO cells were cultured as described in previous articles (see, e.g., ref. 130). CHO cells were stably transfected with the human dopamine D_2L (long form) receptor cDNA (2600 kb cDNA fragment cloned into the Plxsn-vector, which confers resistance to geneticin), and the clones resistant to geneticin were selected (for further details, see ref. 131). As far as the immunogold staining is concerned, cells were grown on glass slides (Chamber Slide Culture, Labtek = Nunc, VWR International srl, Milano, Italy) coated with poly-L-lysine (Sigma, Milano, Italy). Cells were then rinsed in PBS, fixed in 4% paraformaldehyde and glutaraldehyde 2% for 20 min and washed with PBS containing 20 mM glycine and subsequently treated with PBS = 20 mM glycine = 1% BSA for 30 min at room temperature. Immunostaining was performed with the affinity purified mouse anti-HA antibody (Roche SpA, Milano, Italy) and rabbit anti-D_2 antibody in PBS, pH 7.4, supplemented with 1% normal serum at 48°C overnight. The cells were then rinsed three times for 10 min in Tris pH 7.4, three times for 5 min in Tris pH 7.4 BSA 0.2%, one time for 15 min in Tris pH 8.2 BSA 1% and incubated with 15 nm gold particles-conjugated anti-mouse (1:25) and 25 nm gold particles-conjugated anti-rabbit antibody in Tris pH 8.2, BSA 1% for 1 h at room temperature. Cells were then rinsed twice for 10 min in Tris pH 7.4. (B) Atomic force microscopy procedure: images were acquired in tapping-mode (radius of curvature of the probe tip: 10 nm; frequency: 120–130 kHz; AFM, PARK Autoprobe CP instrument) to obtain a phase image of a 2 × 2 μm area of the cell membrane (upper panel). Image analysis was performed according to Agnati et al. (36) and the binary images of the most probable 15 nm (labeling A_{2A} receptors) and 25 nm (labeling D_2 receptors) gold particles were obtained (lower panel). (C) Spatial statistical analysis: from the x, y-coordinates of the gravity centres of the 15 and 25 nm gold particles the cumulative distributions (K(d)) of the distances of each particle from its nearest neighbour of the same type or of the other type was obtained. Distances were normalized in order to account for the finite grain size. Thus, a distance of 0 means that two gold particles are adjacent. A number

of random (Poisson) bivariate point patterns of the same size was also generated in order to evaluate the distributions in conditions of complete spatial randomness (i.e., K0(d)) and the 95% confidence envelope around it. If $L(d) = K(d) - K0(d)$ is significantly greater than 0 for any range of d, then particles are colocalized, that is, they are closer to each other than could be expected by chance (35,132). As illustrated in a range around 10 nm significant A_{2A}–A_{2A}, D_2–D_2, and A_{2A}–D_2 associations can be detected.

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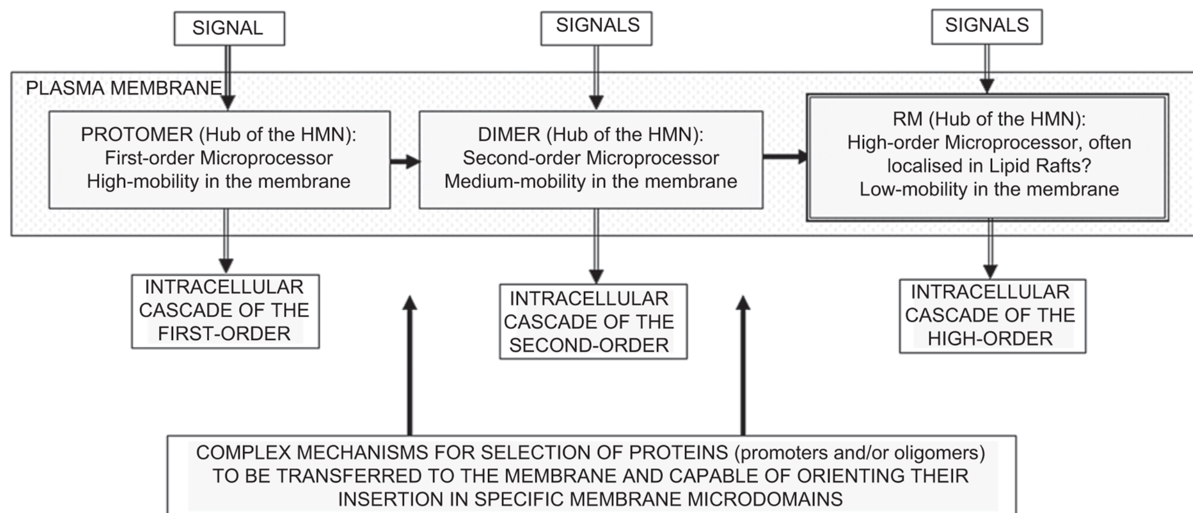

Figure 2.
Schematic representation of a horizontal molecular network (HMN), which by elaborating the extracellular fluid signals and the cytosolic signals can work as an “intelligent interface” between the two environments.

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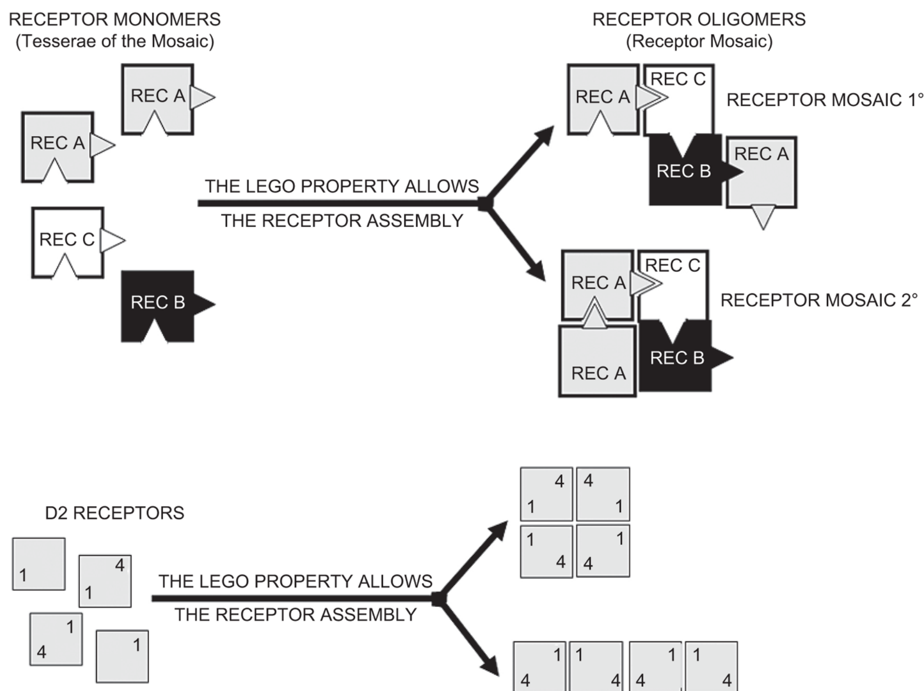

Figure 3.
Relevance of the topology for the possible assembly of different receptor mosaics, which have the same stoichiometry. It is likely that in several instances these stoichiometrically identical, but topologically different mosaics can be endowed with different functional characteristics. The lower panel shows two possible topological arrangements of D_2, which are based on the demonstration that both TM1 and TM4 can be involved in the oligomerization process via a symmetrical TM1-TM1 and TM4-TM4 interactions (14).

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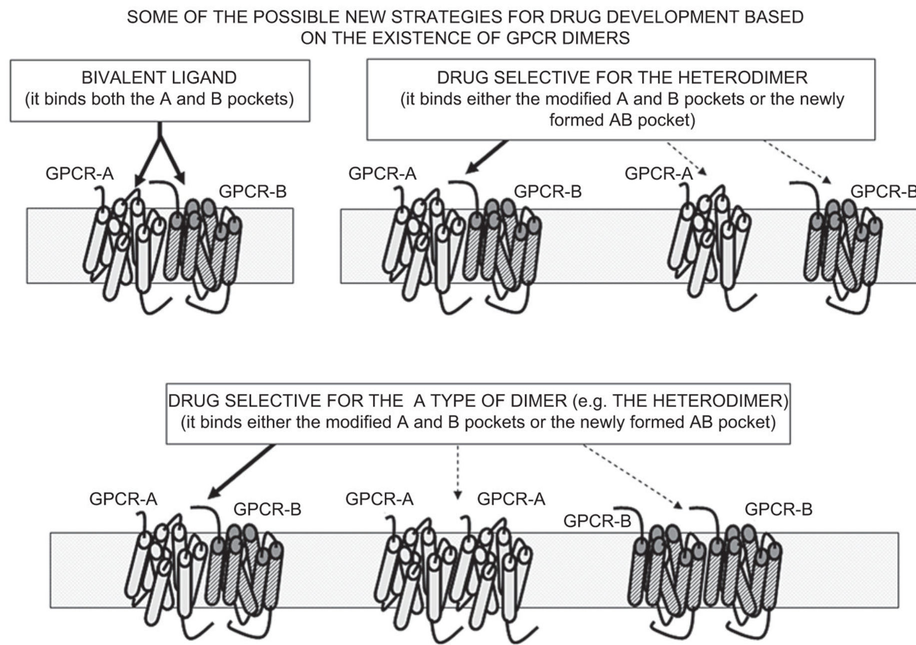

Figure 4.
Schematic representation of new strategies for drug development on the basis of the existence of GPCR oligomerization and especially receptor mosaics. For further details, see text.

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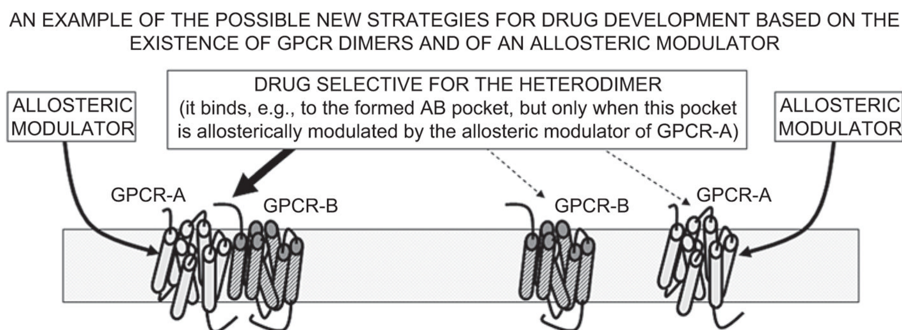

Figure 5.
Schematic representation of a possible new strategy for drug development on the basis of the existence of allosteric modulators of GPCRs. It should be considered to combine the allosteric modulation of a GPCR with some of the drugs indicated in Figure 4. In the scheme of the figure is illustrated an allosteric modulator that by binding to GPCR-A modulates the heterodimer in such a way to enhance the affinity for the heterodimer ligand. For further details, see text.

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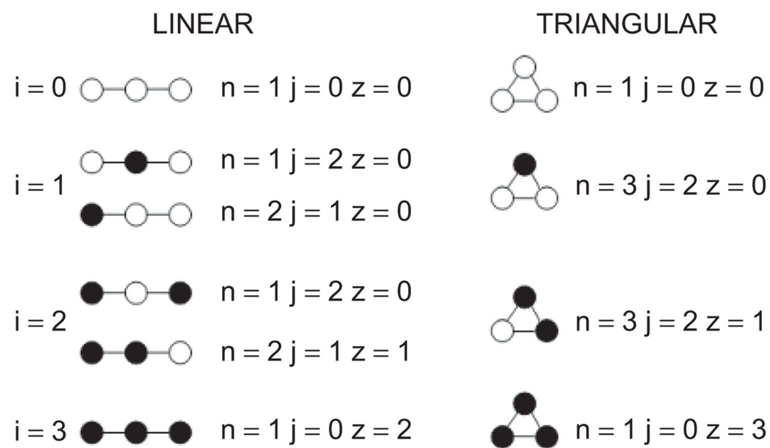

Figure 6.

Possible states available to a trimer arranged according to a linear or a triangular structure. Circles indicate subunits and pairs of interacting subunit are connected by a line segment. Black circles represent subunits in the “bound” configuration. The parameters (see text for a definition) i, n, j, and z characterizing each possible state are indicated.

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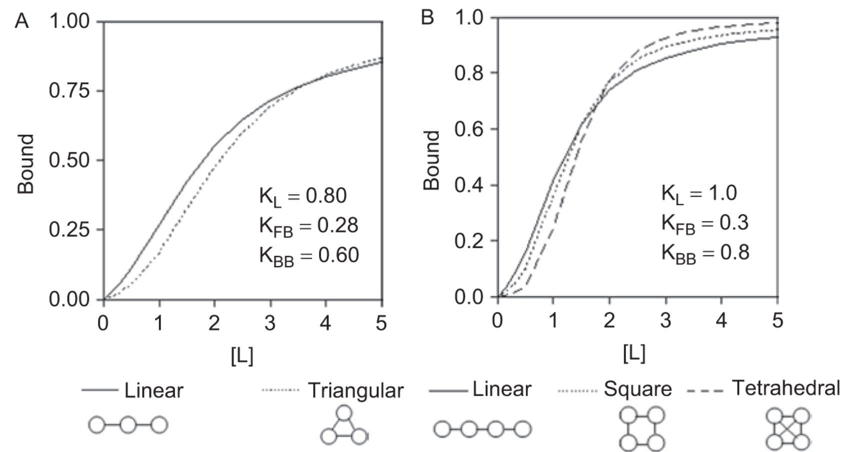

Figure 7.

Examples of saturation curves having the binding and interaction constants indicated and comparing the various interaction geometries (schematically shown at the bottom) of a trimer (A) and a tetramer (B). As illustrated, although the curves were characterized by the same set of constants, they showed a clear-cut dependence on the way the receptor cluster was arranged.