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REVIEW ARTICLE

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Brain Receptor Mosaics and Their Intramembrane Receptor-Receptor Interactions: Molecular Integration in Transmission and Novel Targets for Drug Development

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Abstract
The concept of intramembrane receptor-receptor interactions and evidence for their existence was introduced by Agnati and Fuxe in 1980/81 suggesting the existence of heteromerization of receptors. In 1982, they proposed the existence of aggregates of multiple receptors in the plasma membrane and coined the term receptor mosaics (RM). In this way, cell signaling becomes a branched process beginning at the level of receptor recognition at the plasma membrane where receptors can directly modify the ligand recognition and signaling capacity of the receptors within a RM. Receptor-receptor interactions in RM are classified as operating either with classical cooperativity, when consisting of homomers or heteromers of similar receptor subtypes having the same transmitter, or non-classical cooperativity, when consisting of heteromers. It has been shown that information processing within a RM depends not only on its receptor composition, but also on the topology and the order of receptor activation determined by the concentrations of the ligands and the receptor properties. The general function of RM has also been demonstrated to depend on allosteric regulators (e.g., homocysteine) of the receptor subtypes present. RM as integrative nodes for receptor-receptor interactions in conjunction with membrane associated proteins may form horizontal molecular networks in the plasma membrane coordinating the activity of multiple effector systems modulating the excitability and gene expression of the cells. The key role of electrostatic epitope-epitope interactions will be discussed for the formation of the RM. These interactions probably represent a general molecular mechanism for receptor-receptor interactions and, without a doubt, indicate a role for phosphorylation-dephosphorylation events in these interactions. The novel therapeutic aspects given by the RMs will be discussed in the frame of molecular neurology and psychiatry and combined drug therapy appears as the future way to go.

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1. Introduction

The concept of intramembrane receptor-receptor interactions between different types of GPCRs and evidence for their existence was introduced by Agnati and Fuxe in 1980/81 through analysis of the effects of neuropeptides on the binding characteristics of monoamine receptors in membrane preparations from discrete brain regions [1–3]. These results were in line with Lefkowitz, Limbird et al's previous findings [4] showing negative cooperativity in β adrenergic receptors, which could be explained by the existence of homodimers leading to site-site interactions. In 1982 the Agnati and Fuxe teams proposed the existence of assemblies of multiple receptors of various types in the plasma membrane and coined the term receptor mosaic (RM) for the proposed assembly, as a molecular basis for the engram [5]. As a logical consequence for the indications of direct physical interactions between neuropeptide and monoamine receptors the well-known terms heteromerization *vs.* homomerization was introduced to describe this interaction between different types of GPCRs [5–7]. Thus, heteromerization was postulated to be the molecular mechanism for the intramembrane receptor-receptor interactions witnessed in biochemical pharmacology.

Thus, cell signaling became a branched process beginning at the level of receptor recognition in the plasma membrane where receptors can directly modify the ligand recognition, G protein coupling and signaling of the other receptor in a heterodimer or of other receptors in a high order receptor heteromer (RM) [8–10]. RM may be defined as: *an assemblage of more than two receptors, which binds and decodes signals (transmitters, allosteric modulators,…) to give out an integrated input, via direct allosteric receptor-receptor interactions, to one or more than one intra-cellular cascade*. The term *RM* gives a better vision of the integrative actions and the role of the spatial organization (topology) for the structure of the molecular fingerprint, giving rise to the intramembrane receptor-receptor interactions. Different orders of activation of the participating receptors dependent inter alia on transmitter concentrations and receptor affinities together with stoichiometry will also determine the direct receptor-receptor interactions and thus the integrative and emergent properties of the RM and its functional outcome [8,10–22]. The RM does not include other proteins than receptor proteins and thus not indirect receptor-receptor interactions via e.g. adapter proteins.

The RMs can be located in special regions of the plasma membrane, called lipid rafts rich in sphingolipids and cholesterol [23,24], but they are not necessary for the formation of RMs [25]. The lipid rafts may be regarded as liquid ordered platforms providing a microenvironment that may modulate the operation of the RMs and their receptor interacting proteins in signal integration and transduction involving also caveolins forming caveolae [26,27].

Early evidence for the view that GPCRs may function as dimers was obtained by Maggio et al [28] in 1993 using chimeric α2-adrenergic/M3 muscarinic receptors containing the first five TM domains of one receptor and the last two TM domains of the other receptor and vice versa. Expression of the two chimeras alone did not lead to recognition of ligands or G protein coupling. However, coexpression of the two chimeras led to restoration of binding and signaling to both adrenergic and muscarinic agonists. Thus, it seems possible that the two chimeras may directly interact to rearrange their TM domains into two novel binding pockets within a dimeric complex. Such a domain swapping can also explain the reconstitution of a binding site after coexpression of two deficient AT1 receptor mutants [29]. A computational approach has also been used to evaluate domain swapping in the formation of dimers [30]. It should be noted that domain swapping has only been observed for receptors with loss of function probably related to technical problems in demonstrating the domain swapping in fully functioning receptors [17].

The first major technique used to investigate GPCR dimerization was coimmunoprecipitation of differentially epitope-tagged receptors. In 1996 Hebert et al [31] demonstrated with this technique that β2-adrenergic receptors of class A form SDS resistant homodimers with the transmembrane (TM) VI being part of the interface. In fact, a peptide from this domain inhibited both dimerization and activation of the receptors. The same year Romano et al [32] demonstrated by Western blots that mGluR5 belonging to the class C is a homodimeric receptor becoming a monomer under reducing conditions. Coimmunoprecipitation as outlined above showed that these results were caused by the existence of a receptor homodimer stabilized by a disulfide bond between the two very long extracellular domains. This year it was also observed that the class A D2 receptors in the caudate upon solubilization and immunoprecipitation exist as homodimers and monomers [33]. D2-TM peptides converted the dimer into a monomer suggesting that the dimer was formed via intermolecular noncovalent interactions between TM domains of the two D2 receptors in line with the findings on the β2-adrenergic receptors [31].

A major breakthrough came in 1998 when the first heterodimer was discovered with the known GABAB receptor of class C GPCR shown to be an obligate heterodimer composed of subunits GABAB1

and GABAB2 [34,35]. The GABAB1 enabled agonist/antagonist binding while the GABAB2 was responsible for the G protein coupling. Yeast two-hybrid screening was used to demonstrate the heterodimer which was formed via TM interactions and coiled-coil interactions in the C-terminal tails leading to the formation of a functional GABAB receptor [4,36–38]. The two subunits could be coimmunoprecipitated from native brain tissue and colocalization was demonstrated at the ultrastructural level [39].

The heterodimerization between two functional GPCRs was first demonstrated by Jordan and Devi [40] in 1999 using again coimmunoprecipitation with differentially tagged κ and δ opioid receptors to demonstrate the kappa-delta heterodimer. Finally in 2000 it became possible in coimmunoprecipitation experiments to detect heteromeric receptor complexes of A1 and D1 receptors and thus of GPCRs using different ligands, adenosine and DA [41]. This year it was also shown with immuno-affinity chromatography after agonist ligand affinity-labelling of the receptors that AT1 and B2 receptors formed heterodimers representing the first demonstration of heterodimers between two different types of vasoactive hormone receptors [42].

Coimmunoprecipitation of membrane receptors, however, has the drawback that it requires their solubilization with detergents. This is a problem, since highly hydrophobic proteins like GPCRs may form artifactual aggregates upon incomplete solubilization. Therefore, the field of dimeric and multimeric RMs demanded the direct demonstration that they also exist in the living cells. This was made possible with the development and use of biophysical methods based on light resonance energy transfer. Nevertheless the coimmunoprecipitation technique when used with appropriate controls remains a highly useful method in establishing the existence of receptor heteromers especially in native tissue and can be further developed into demonstrating RMs in the plasma membrane by use of cell-impermeant crosslinking substances [43]. It has even been possible to demonstrate the trimeric potential of the M2 muscarinic cholinergic receptor by coimmunoprecipitation of receptor complexes coexpressed in Sf9 cells with three different epitope tags [44].

In the present review the identification of RMs with BRET and FRET will be discussed. We will then focus on the current understanding of the heteromeric interface involving electrostatic epitope-epitope interactions [45] and the structural plasticity of the GPCRs transferring conformational changes from one receptor to the other receptors in the RMs leading to changes in cooperativity in the binding sites. The functional properties of the RMs in terms of recognition with altered pharmaoclogy, G protein coupling and switching, and trafficking will also be covered with focus on the novel neuropsychopharmacology created by the RMs.

In the end the role of allosteric modulators and receptor interacting proteins in the function of the RMs will also be dealt with and also the function of RMs built up of GPCRs and ion channel receptors or of GPCRs and RTKs representing integrative nodes for the volume and wiring transmission signals and for information and trophic signals, respectively.

2. Identification of RMs

2.1. FRET

Theodor Förster was the first to establish a parametric proportionality between the rate of transfer and the radiative rate constant which operates over distances of 1 to 20 nm, forming the basis of the extensive application of Förster resonance energy transfer (FRET). This technique is applied extensively in biological research as a general "nanoruler" with a dynamic range corresponding to intramolecular and intermolecular distances of molecules controlling cellular functions. The molecular networks operating under strict spatial-temporal conditions deal with binding, conformational transitions, covalent alterations and transport. FRET gives information on all these molecular interactions with high specificity and sensitivity.

FRET represents a photophysical phenomenon in which energy is transferred from the first excited electronic state of a fluorophor (donor) to another close by absorbing molecule (acceptor). There occurs a concerted quenching of the fluorescence of the donor and activation of the acceptor fluorescence [46]. Thus, light resonance energy transfer approaches are based on the non-radioactive transfer of excitation energy between electromagnetic dipoles of an energy donor and acceptor.

The most commonly used FRET pairs for tagging GPCRs are cyan fluorescent protein (CFP) as an energy donor and yellow fluorescent protein (YFP) as an energy acceptor and likewise for GFP2 and YFP, respectively. These fluorophors are covalently fused to distinct GPCRs and FRET occurs when the distance between the fluorophors is less than 10 nm corresponding to the masses of heterodimers/heteromers. Linear unmixing between the GFP2 and YFP emission spectra must be made since they substantially overlap [25,47]. The FRET is dependent on the orientation of the two fluorophors and the distance between them [43,48]. In fact, the efficiency of FRET is inversely related to the 6^{th} power of this distance and conformational changes are maximally discovered at a distance giving 50% of the

maximal transfer efficiency. So far it has not been possible to safely distinguish heterodimers *vs.* heteromers nor to quantitate the proportion of monomers *vs.* heterodimers/heteromers with the FRET technique.

FRET experiments have mainly been made with fluorimetric analysis on suspensions of living cells distributed into wells of microplates read in a fluorimeter [25]. Acceptor bleaching FRET with confocal microscopy on fixed cells plated on coverslips adds the advantage of allowing studies on FRET in various domains of the cell like the plasma membrane [25,49]. The FRET is shown by the rise of the emitted donor fluorescence after photobleaching of the acceptor since energy transfer cannot take place and instead the saved energy is spent as increased fluorescence output from the donor. The FRET efficiency is calculated as 1 minus the ratio of donor fluorescence before and after photobleaching of the acceptor and normalized to a scale from 0 to 1. These experiments also represent important controls for FRET experiments with fluorimetry since exogenous light sources can result in background signals that may be believed to be FRET signals [43]. Photobleaching fluorescence resonance energy transfer microscopy can also be performed by bleaching of the donor involving determination of histograms of time constants obtained from single-exponential fits to pixel-based photobleaching decay curves [49]. The presence of acceptor leads to larger donor photobleaching time constants.

Time-resolved FRET can be used to establish FRET between receptors at the plasma membrane. N-terminally epitope-tagged GPCRs are employed and can be reached by the corresponding antibodies in intact cells labeled with suitable FRET pairs [43,48,50]. By using Europium as an energy donor time-resolved FRET can be used since shortlived autofluorescence can decay during a period of 50 μs after which measurements of the long lived FRET signals can take place in periods of 100 to 400 μs [50,51]. This method is of substantial help in clarifying that the GPCR heterodimer/heteromer in fact exists in the plasma membrane of living cells and not only in the cytoplasm. However, there exist concerns that the antibodies used in time-resolved FRET may promote the oligomerization and interfere with the ligand binding to the oligomer [43].

Measurements of FRET by fluorescence lifetime microscopy in living cells involving time correlated single-photon-counting with a picosecond diode pulse laser have given safe quantitative results on FRET signals. The measurements of fluorescence lifetime are in the nanosecond range, and are reduced by the existence of FRET [52]. It should be noted that the measurements are independent of changes in fluorophor concentration and in excitation intensity offering reliable information on the existence of heterodimers/heteromers.

A small, membrane–permeant fluorescein derivative with two arsen-(III) substituents (fluorescein hairpin binder, FLAsH) targeted to a short tetracysteine sequence has been inserted to GPCR and used in a FLAsH based FRET approach to follow G protein activation in living cells [53]. The CFP/FLAsH-tetracysteine system in contrast to the CFP/YFP tagged to the A2A receptors did not block the AC activation and gave higher FRET signals underlining the usefulness of this system to determine GPCR activation. Today intramolecular FRET has been developed by Lohse and his group as a first choice method to monitor receptor signaling in space and with time in intact cells [54,55].

2.2. BRET

Bioluminescence resonance energy transfer (BRET) is similar to FRET and is a process in which the donor is bioluminescent and the acceptor is fluorescent. It demands that the emission spectrum of the donor and the excitation spectrum of the acceptor overlap and the existence of a less than 10 nm distance between the donor and the acceptor. The luminescence is a phenomenon taking place naturally in various marine animals like the sea pancy *Renilla reniformis (Rluc)*. In BRET the luminescence obtained from the luciferase substrate coelenterazine H (BRET[1]) when bound to *Rluc* (emission peak at 470 nm) is transferred to a variant of green fluorescent protein, so called enhanced yellow fluorescent protein (eYFP), which then in the case of the existence of dimerization emits fluorescence in its well known spectrum (emission peak at 530 nm). Therefore, the ratio between emission at 530/emission at 470 is increased. The BRET signal is measured by dividing the amount of yellow light from YFP by the amount of blue light. The versatility of this technique has become enhanced by constructing fusion proteins where the two tags are added either to the N-terminal or the C-terminal of the two GPCR's analyzed for BRET. The strict requirement for molecular proximity makes it an important technique for detecting homo and heteromerization.

Unlike FRET, BRET is not sufficiently sensitive to be used for single cell signaling and thus not capable to give information on subcellular locations of the signals but has the advantage over FRET of lack of potential direct excitation of the acceptor reducing the disturbances from background signals [43,48]. To further reduce background signals the *Renilla luciferase* substrate DeepBlueC can be used with an oxidation spectrum having an emission peak at 400 nm (BRET[2]) which can effectively activate the acceptor GFP[2] giving a fluorescence emission

with a peak at 510 nm having little overlap with the *Rluc* emission.

The introduction of the "saturation" BRET was an important development in the BRET field [43,56] showing that a saturation curve could be obtained by increasing the acceptor/donor ratios similar to those observed in ligand binding experiments. In this way a BRETmax value could be obtained dependent on the number of heteromers and the orientation of the *Rluc* and the acceptor fluorophor and $BRET_{50}$ value giving the value of the half-maximal signal giving information on the relative affinity of the interactions between the two participating GPCRs tagged for BRET [25].

In 2006 a novel approach to BRET was introduced to better separate BRET signals arising from random interactions at the plasma membrane *vs.* those occurring in homo or heteromeric proteins like receptor homo or heteromers [57]. This was made possible by the use of two types of experiments (Type-1 and Type-2). Type-1 experiments involve the analysis of BRET efficiency at a fixed surface density and varying the acceptor/donor ratio. In this case molecules that oligomerize show an increased BRET efficiency as the ratio increases since the donors and acceptors compete in forming oligomeric complexes. Type-2 experiments involve varying the expression levels at a fixed acceptor/donor ratio [57]. In this case constitutive homo-heteromers produce a BRET efficiency that is unaltered by changes in surface density since folding and complex formation are not affected by this parameter in contrast to monomers that show an increase in the likelihood of BRET efficiency due to random interactions [57]. However, these experiments also have pitfalls as discussed by Bouvier et al [58] and Salahpour and Masri [59].

These groups [59,60] and others [61] have, however, shown that type-2 experiments are in fact useful for demonstrating oligomers of class A GPCRs like beta2 adrenergic homodimers and D1 or D2 homodimers. Thus, the slope between BRET signals and receptor expression levels is zero and therefore the BRET signal cannot be due to random molecular interactions but to the formation of homodimers [59]. This contrasts with results from the type-2 experiments performed by James et al [57] who implied that BRET signals for class-A GPCRs are a result of random interactions. The experimental design of their type-2 BRET experiments may, however, be incorrect which became apparent from the concerns raised by Salahpour and Masri [59].

Finally, it should be noted when performing BRET and FRET that the fluorescence or bioluminescence signal per GPCR binding site varies from one GPCR to the other, which may possibly be related to differences in folding rates between the catalytic site of the *Rluc* or the fluorophor and the binding site among different GPCRs. Therefore, the sensitivity of the BRET and FRET procedure may depend on the GPCRs involved [43].

2.3. Limitations of the BRET-FRET techniques

Recently, however, it has been pointed out that pitfalls still remain with the BRET and FRET techniques since energy transfer between the two fluorescent moieties can take place when a tight dimer exists or when the receptors are more than 50 nm apart [62]. Thus, the molecular proximities can be reported but it is difficult to absolutely prove the direct physical interactions between two GPCRs. Nevertheless, most researchers have accepted their existence in cotransfected cell lines based on these biophysical techniques including also bimolecular fluorescence complementation (BiFC) [63,64] especially when energy transfer can be demonstrated in physiologically relevant expression levels and the problem of "crowding" is avoided. In the case of class C receptors there is no doubt that they exist as homo and heterodimers since the covalent bonding (disulfide bridges) of non-heptahelical domains is sufficiently strong through its high affinity to give an energetically acceptable mechanism for stable dimer formation [65]. The case of class A receptors, however, is more controversial. It has recently been proposed that the bonding may not have the binding energy necessary for stable dimers but may only allow transient "kiss-and-run" encounters. Instead a dynamic equilibrium between monomers and dimers of varying stability may exist [65]. However, the high energy strength of the electrostatic arginin-phosphate salt bridges has not been considered in this proposal (see below).

In understanding the existence and size and stability of receptor dimers and RMs it becomes important for the future to use site directed spin labeling and distance measurements by pulsed electron paramagnetic resonance (double electron-electron resonance, DEER) [62].

The major problem in the field is, however, the identification of the heterdimers and RMs in native brain tissue since here it is more difficult to use the BRET and FRET techniques. There is a need to introduce the generation of transgenic mice expressing receptor pairs or trimers tagged with different variants of GFP in order to perform confocal FRET based techniques in brain tissue and in this way identify the heterodimers and RMs. This approach involves also the use of GFP tagged mutant receptors that cannot heteromerize in cell lines in this way obtaining negative controls. So far mainly indirect techniques have been used to obtain indications

for their existence in native brain tissue like coimmunoprecipitation. Furthermore, ever since the early 1980s the discovery of large numbers of intramembrane antagonistic and facilitatory receptor-receptor interactions, studied especially at the recognition level [8,10] have given indications for the existence of different types of receptor heterodimers and RMs in various regions of the brain. These receptor-receptor interactions have successfully been interpreted as reflecting the existence of allosteric mechanisms operating over the interfaces of receptor heterodimers and RMs leading to an integrated and coordinated signaling to various effectors like ion channels, kinases and phosphatases giving rise to a fine-tuned and dynamic regulation of neuronal excitability, firing and gene expression. Presently in the field experiments are being performed to obtain irrefutable evidence that the intramembrane receptor-receptor interactions demonstrated in native brain tissue are linked to direct physical receptor-receptor interactions in heterodimers and RMs and not represent indirect receptor-receptor interactions mediated via inter alia adapter proteins.

2.4. Determination of the receptor mass in protein-detergent complexes by size exclusion chromatography (SEC)

It is of substantial interest that the purified neurotensin receptor 1 NTS1 belonging to class-A GPCRs can dimerize in detergent solutions in a way which is concentration dependent and has a high affinity (low nanomolar concentrations) [66]. SEC was used with light scattering, refractive index and ultraviolet measurements to determine the molecular mass and homomeric state of the membrane proteins, which at the lowest concentrations of 1 nM and below show NTS1 existing as a monomer. This study together with the study on the leukotrien B4 receptor BLT1 [67] give further evidence that class-A GPCRs can dimerize and that the dimers formed develop allosteric interactions as shown in the case of the NTS1 dimer by the demonstration of positive cooperativity in the NT binding [66]. NTS1 dimerization was found not to be essential for G protein coupling with the NTS monomers activating the $G\alpha q\beta_1\gamma_2$ more efficiently and $\beta 2$ adrenergic receptor monomers in lipid discs also produce an effective activation of G proteins [68–70]. In fact, in solution the monomeric GPCR rhodopsin activates its G protein transducin at the diffusion limit giving evidence that an activated rhodopsin monomer is sufficient for a full G protein activation [71]. The reduced efficiency of the NTS1 dimer to activate the $G\alpha q$ was interpreted to reflect a result of an occlusion of one of the G protein subunit binding sites in the receptor dimer leading to internal competition for binding [66].

Using a novel flow cytometric FRET analysis combined with a 2 step approach it was possible to demonstrate serial transfer of energy from CFP to YFP to HcRed demonstrating that tumor necrosis factor receptor associated factor (TRAF) 3 can form heterotrimers with TRAF2 modulating its ability to produce NF-κB activation [72].

Recently it has been possible by means of a novel technique, the so-called sequential BRET-FRET technique, to obtain indications for the existence of trimeric RMs of A2A-CB1-D2 receptors in cotransfected cell lines [73]. Furthermore, a combined BRET-BiFC has been developed giving support for the existence of RMs built of at least three A2A receptors in living cells [63]. With bioluminescence/fluorescence complementation and energy transfer also at least four dopamine D2 receptors have been shown to be located in close molecular proximity in living cells likely forming RMs of four D2 receptors [64].

With atomic force microscopy Palczewski, Fotiadis and colleagues [74] could even obtain indications that rhodopsin exists as high order arrays of dimers in the native disc membrane in the retina having a paracrystalline arrangement. However, studies on rhodopsin diffusion in disc membranes are not compatible with such arrangements [75]. Also with this method in combination with immunogold technique indications have been obtained that in CHO cells stably transfected with human D2L receptors, RMs of D2L receptors may be formed with a ring structure [76]. The existence of a closed loop RM (ring model) of GPCRs had previously been postulated [77] based on interactions between the 5 and 6 TM domains and domain swapping [30].

3. The receptor interface among GPCR RMs

In the case of the GABAB receptor, which belongs to the family class C of GPCRs and is an obligate heterodimer, the interface is mainly formed by the TM domains and coiled-coil interactions in the C-terminal tails of the GABAB1 and GABAB2 receptor subunits [34]. This was in fact the first demonstration of a heterodimer among the GPCRs and took place in 1998/1999. The $mGluR_5$ and $mGluR_1$ receptor were instead shown in 1996 and 1998 to be disulfide-linked homodimers, respectively [32,78] with the interprotomer disulfide bridge existing in the large extracellular domains. The crystal structures of the ligand binding regions of the $mGluR_1$ show that glutamate binds to the crevice (venus flytrap domain; VFT) of the two binding domains [79]. The glutamate binding results in a rearrangement of the VFP which via an allosteric mechanism

is transferred to the heptahelical effector domain leading to a transduction of the glutamate induced conformational change in the binding pocket to the G protein as shown with FRET [80]. Thus, both intracellular and extracellular domains are involved in the dimeric interface [10,81]. Also the sweet and umami taste receptors belonging to the class-C of GPCRs have been shown to be functional only when forming heterodimers [82–84]. The sweet receptor is formed from T1R2 and T1R3 and the umami receptor from T1R1 and T1R3. Thus, the dimer is probably required in this class of GPCR for the transfer of the conformational change from the extracellular agonist binding pocket to the heptahelical TM domains [85].

It has been found, that it is more difficult to identify the TM domains that interact in various heteromers. The first studies in 1996 by Bouvier et al [31] on the β2 adrenergic receptors indicated that TM6 was involved since peptides from the TM6 domain, but not the mutated TM6 peptides blocked the dimerization. The first TM interaction to be demonstrated among the GPCRs was the one between the two TM4 domains in the interface of the D2 homodimer [86] and to-day most TM domains have been implicated in the dimeric interactions among class-A GPCRs [87,88].

In the beginning only contacts between TM helices (contact dimers) were considered but based on the functional rescue studies of Maggio et al [28] on chimeric (mutant) muscarinic-adrenergic receptors, Reynolds, Gouldson et al [30,87] introduced the model of domain swapping at the TM5 and TM6 interface based on molecular modeling and bioinformatics. It has also been possible to reconstitute the binding site of two deficient mutants of the type 1 angiotensin II receptor [29] and domain swapping has been demonstrated in the histamine H1 receptor but only from functionally impaired receptors [89]. It has also been proposed that both domain swapping and domain contact can take place (mixed–model dimer) allowing the possible assemblage of high order GPCR (RMs) into a ring structure (closed loop) [77]. Indications of the existence in cell lines of ring-like structures of D2 homomers have recently been obtained with atomic force microscopy [76]. In a classical paper by Palczewski, Fotiadis and colleagues [74] indications were obtained with atomic force microscopy of a paracrystalline arrangement of rhodopsin dimers in native disc membranes [90].

The pioneering work of Amina Woods [91] gave evidence of a fundamental role for electrostatic epitope-epitope interaction in the formation of the interfaces between proteins leading to the assemblage of the protein and RMs, which will be discussed below.

3.1. Electrostatic epitope-epitope interactions in RMs

Electrostatic interactions between an arginine rich, highly conserved epitope (basic motif), found in the third intracellular loop of Dopamine D2, D3 and D4 receptors, and a phosphorylated serine/threonine (acidic motif) on the C terminal tail of the Adenosine A2A receptor (Figure 1) are involved in the A2A/D2 receptor heteromerization [92]. This was demonstrated with mass spectrometry and confirmed in biochemical pulldown assays. The arginine-phosphate electrostatic interaction possesses "covalentlike" stability [93]. Hence, these bonds can withstand fragmentation by mass spectrometric collision-induced dissociation at energies similar to those that fragment covalent bonds and they show very slow dissociation of the noncovalent complex by plasmon resonance. The work also highlights the importance of phosphorylation-dephosphorylation events in the modulation of electrostatic attraction. Phosphorylation of the acidic motif by casein kinase on one receptor makes it available for interaction with the basic motif on the other. On the other hand, phosphorylation of serine and/or threonine residues downstream from the basic motif, by protein kinase A or C slows down the attraction between the epitopes. Although analyzed here in the frame of receptor

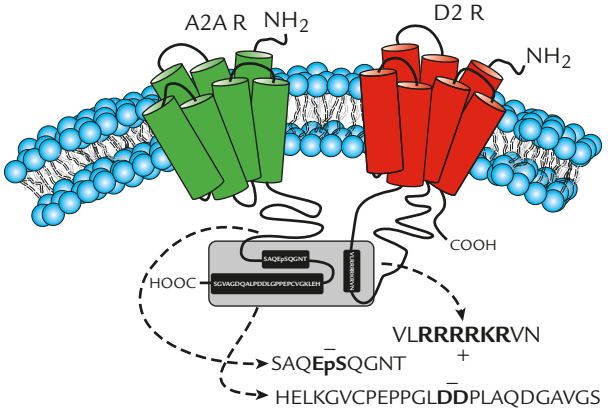

Figure 1 Illustration of the electrostatic epitope-epitope interactions in the intracellular parts of the A2A-D2 heteromers (seen in the box). Two epitopes with negative charges (in bold) exist in the C terminal tail of the A2A receptor, the one with the phosphorylated serine (SAQ**EpS**QGNT) being of special interest, since it underlines the role of phosphorylation processes in the electrostatic interactions. Positive charges (in bold) exist in the arginine rich epitope (VL**RRRRKR**VN) in the N terminal part of the intracellular loop 3 of the D2 receptor which interacts with the negatively charged epitopes seen in this figure. These non-covalent interactions in the intracellular part of the two receptors are strong and represent an important part of the A2A/D2 interface (see text for references).

heteromerization, the arginine-phosphate electrostatic interaction most likely represents a general mechanism in protein-protein interactions [45].

3.2. On the role of disordered domains

Based on the discovery of strong salt bridges between the guanidinium groups of arginines in the N terminal part of the IC loop 3 of the D2 receptor and the phosphorylated serine of the C-terminal tail domain of the A2A receptor [45,92] we became very interested in understanding the structural plasticity on the IC loop 3 and the C-terminal tail and also other domains of the GPCRs [94]. One form of receptor plasticity is the so called structural malleability related to the ability to interact with multiple protein partners [95]. Such properties are often dependent on the existence of disordered sequences of amino acids without a rigid secondary structure. The random coil is the typical disordered state. It should be considered that the fluctuations of the conformational states in the receptors of a RM are in fact conditioned by the conformations of the other participating receptors. The assemblage of RMs may inter alia take place via reciprocally induced-fit interactions between two malleable domains of two GPCRs [94]. It therefore becomes of substantial interest to detect the location of various disordered sequences in the domains of GPCRs since they are probably located in strategic positions for the development of receptor-receptor and receptor-protein interactions.

Computer-assisted analysis of amino acid sequences in 14 different GPCRs has been performed with 10 different computer-assisted programs to determine flexible and malleable domains [14,94,96]. A global index of disorder (DI) has been obtained by pooling the results from for the major GPCR domains including N-terminal, extra and intra cellular loops, TM helices and C-terminal of the 14 GPCRs examined. The DI of a domain was calculated on the basis of the number of disordered amino acids *vs.* the total number of amino acids in that domain.

The disorder index of the domain representing postgenomic plasticity was compared with coefficient of variation (CV) of the number of amino acids (length of chain) in that domain among the 14 GPCRs analyzed, representing genomic plasticity.

It is of substantial interest that the N-terminal, IC loop 3 and C-terminal domains have both the highest disorder index and highest coefficient of variation showing that postgenomic and genomic plasticity go together. In view of our special interest in the A2A/D2 heteromer, the A2A and the D2 receptors were included in the present computer assisted analysis [94] and thus we can state that the powerful electrostatic interactions demonstrated between the D2 IC loop 3 and the A2A C-terminal domains may be made possible by the high structural plasticity of these domains. In fact, the disorder index of D2 IC loop 3 was high (0.620) as was the disorder index of A2A C-terminal domains (0.613).

It may be stated that in the assembly of RMs, the receptors with many disordered domains may be the *hub* receptors, by interacting directly and at the same time with several participating receptors [97,98].

3.3. 3D model building of dimeric RMs

Early on in 2003 computational experiments were performed on the A2A/D2 heterodimeric RM by Fanelli and colleagues to generate a hypothesis on its interface [25]. It was based on docking simulations on theoretical models of A2A and D2 receptors. The whole sequences of the two receptors were modeled since the interface may also involve the intracellular and/or extracellular domains as suggested for rhodopsin [99]. Nine different average minimized structures of the A2A receptor were employed and each of them docked to the selected average minimized structure of the D2 receptor. Two different rigid-body docking programs ZDOCK and ESCHER were utilized resulting in a total of 32,000 and 9,400 filtered solutions which were markedly reduced upon additional filtering. Most realistic docking solutions revealed that the interface contained D2 portions including the C-terminal half of helix 5 and the N-terminal portion of IC3 with stretches of positively charged amino acids especially arginines [25]. Two sets of dimers with similar interfaces in each set were obtained. In the most populated dimer, helix 5 and/or helix 6 of the D2 receptor approach helix 4 of the A2A receptor and the N-terminal part of the IC3 domain of the D2 receptor approach the C-terminal portion of the C-tail of the A2A receptor validated by the results of the BRET experiments on the chimeric D2R-D1R and the A2A receptors.

This computational protocol has been further improved for the prediction of the supramolecular organization of the TM alpha helical proteins by rigid-body docking [25,100]. Such computational modeling including also ad hoc filtering, automatic cluster analysis and visual inspection of cluster centers have been employed to predict the interface of lutropin homodimers [101]. The results hypothesize the importance of helix 4, 5 and 6 and especially helix 4 in the inter-monomer interaction of this dimer along the lines of sequence based predictions [102] and may take place via weak Van der Waals interactions between hydrophobic amino acids. In this case the hydrophilic loops appear to play only a minor role and mutation induced lutropin receptor activation lead to increased contacts between helix 4-helix 4 of the lutropin dimer [101].

This computational approach has also recently been employed to hypothesize mechanisms of inter and intramolecular communication in GPCRs and G proteins [103].

4. Cooperativity in RMs as a molecular mechanism for the receptor-receptor interactions

It has been proposed that classical cooperativity plays an important role in the decoding of signals in RMs of GPCRs built up of homomers or of isoreceptors (receptor subtypes for the same transmitter) involving allosteric mechanisms with ligand induced conformational changes in one receptor passing via the interfaces to the other receptors [8,76,103]. This leads to alterations in the binding properties and G protein coupling of the adjacent receptors in the RM and is the major molecular mechanism for the intramembrane receptor-receptor interactions. The same principle molecular mechanism also mediates the intramembrane receptor-receptor interactions in RMs built up of heteromers but in view of the fact that the binding sites of the participating receptors are different the term for this type of cooperativity should be non-classical cooperativity [14,94,96]. The receptor monomers themselves in the absence of transmitters when binding to their receptor partners in the RM can be allosteric modulators and produce non-classical cooperativity in the RM involving not only the receptor recognition but also G protein coupling and the assemblage of the RM.

4.1. Dimer-based model of GPCR

The two state dimer receptor model can readily explain the nonlinear Scatchard plots for agonist binding to GPCRs since it predicts cooperativity in binding [104,105]. The model can explain both negative and positive cooperativity giving concave downward and upward Scatchard plots, respectively.

It is true that the fitting to the "two-state dimer model" was similar to that to the "two-independent site model" assuming two types of conformational states of the receptor. However, the two-state dimer model has less parameters and is therefore the simplest model to explain the binding results.

The ligand-binding process in the dimer-based model is strongly influenced by the binding cooperativity (μ) between the first and second A molecule to the A homodimer where A is the ligand. This is true also for the constant α reflecting the intrinsic efficacy of A and the constant θ reflecting the intrinsic efficacy of the second A molecule binding to the dimer. The appearance of the Scatchard plots is highly dependent on these values; high values of μ and low values of α as indicators for positive cooperativity, and low values for both μ and α as indicators for negative cooperativity (concave downward Scatchard plots). With a high value of θ positive cooperativity is mainly found. The two-state dimer model clearly demonstrated positive cooperativity for agonist binding to estrogen receptors known to show positive cooperativity [104]. Thus, this model may become a general model for receptor dimers and it also predicts the various responses of full and partial agonists, neutral antagonists and inverse agonists [106].

5. Altered recognition in receptor heterodimers and RMs gives unique pharmacology

In the case of class C GABAB receptors it became clear in 1998 that it is an obligate heterodimer formed from two GABAB receptor subtypes, GABAB1 and GABAB2 [34,107]. The GABAB1 gives the binding and GABAB2 the signaling. This was the first demonstration of a heterodimer and had a major impact on the field of receptor-receptor interactions. The same appears to be true for other class C GPCRs and dimerization in fact produces the receptor by enabling the signal to pass from the fly-trap domain of the GABAB1 receptor in the large extracellular region to the TM domain of the GABAB2 receptor via allosteric mechanisms. It has also in fact been shown that the GABAB2 receptor heptahelical domain expressed alone can signal after activation by a positive allosteric activator of the GABAB receptor, CGP7930 [108].

Recently the D1-D2 receptor heteromer has been discovered in brain by means of coimmmunoprecipitation and identified as a neuronal Gq/11 coupled heteromer with a unique pharmacology by the George group [109,110]. It was found that the D1 agonist SKF83959 was a selective agonist for this RM by being a full agonist at the D1 receptor partner and a partial agonist at the D2 receptor existing in a pertussis insensitive state leading to rapid activation of Gq/11 and phospholipase C with a robust intracellular release of calcium. It should be underlined that the D1 like agonist SKF83959 does not activate AC linked D1 or D2 receptors and may therefore be a unique agonist for the D1-D2 RM. Thus, this work represents an interesting example of how the RM field may generate new drugs in neuropsychopharmacology [14]. A diminished link between D1 and D2 receptors has been demonstrated in the striatum of schizophrenic patients [111] and the calcium signaling mediated increases in CaMKIIalpha is required for the sensitization to cocaine [112].

It is of substantial interest that the likely existence of α1B-α1D receptor heteromers may explain the disappearance of α1D receptors in native tissue based on the disappearance of high affinity binding sites for the selective α1D antagonist BMY 7378 [113]. Thus, the appearance of the α1B-α1D heteromer may result in a novel receptor in which the α1D binding pocket has become conformationally altered to obtain markedly different pharmacological properties. In line with these results α1B knockout mice demonstrate an increased affinity for the α1D receptor antagonist BMY7378 [114]. The existence of this heteromer may also help explain the discrepancy between α1D mRNA levels and expression of functional α1D receptors [117].

Very interesting changes in the A1 receptor pharmacology have been discovered by Nakata and his team in A1-P2Y1 receptor heteromers [115,116]. This heteromerization results in a conformational change in the A1 binding pocket leading to the appearance of an A1 receptor with P2Y like agonistic pharmacology. In fact, a P2Y1 agonist binds to the A1 receptor and produces an inhibition of AC which is blocked by an A1 antagonist. An ATP responsive A1 receptor has become generated that can be one mechanism for ATP induced inhibition of transmitter release as it is released from nerve terminal networks.

Altered recognition in the individual GPCR receptor of receptor heteromers almost always takes place after exposure to agonists for the other participating GPCR receptors as studied in membrane preparations and has been extensively reviewed [10]. This was the way the intramembrane receptor-receptor interactions were discovered in brain membrane preparations in 1980–1981 and indicated the existence of GPCR heteromers in which antagonistic or enhancing, receptor-receptor interactions at the recognition level could be observed and made possible via classical or non classical cooperativity with conformational changes passing via the receptor interfaces to the other receptor as discussed above. The receptor-receptor interaction gives a functional integrated outcome of the receptor heterodimer and the RM [5,8,14,76] the latter giving an improved nomenclature by underlining the importance not only of the stoichiometry but also of the topology of the different kinds of receptors involved.

6. Alterations of G protein coupling via receptor-receptor interactions in RMs

It has been suggested that dimerization is at least sometimes required for an efficient receptor-G protein coupling [19,22] and dimers are preferentially expressed on the plasma membrane *vs.* monomers as inter alia demonstrated for the A2A homodimers [50]. It should be considered, however that the expression of a GPCR monomer is sufficient for G protein coupling [63–65] and the GPCR rhodopsin monomer activates its G protein transducin at the diffusion limit [71]. Also some dimers have been found to show reduced ability to activate G proteins *vs.* their respective monomers [61,63]. Nevertheless in 1999 Jordan and Devi [40] demonstrated that combined activation of the two receptors in the δ-κ opioid receptor heteromer was necessary for an optimal activation of this RM and a proper MAP kinase cascade activation only took place upon combined agonist activation of the two receptors. Furthermore, George et al [15] found that in the D1-D2 receptor heteromer phospholipase C activation and thus signaling only developed if the two participating D1 and D2 receptors became activated [15,109,110].

On the other hand, a single agonist per mGluR homodimer gives signaling even if full activity demands two agonists per dimer [117]. Also Milligan's group has demonstrated transcomplementation between two class A GPCR defective in ligand binding or G protein coupling. Thus, agonist binding to one receptor of a dimer can give rise to substantial signaling [118,119]. Such a nonsymmetric activation of dimers is also in line with the demonstration of negative cooperativity in agonist binding as shown first by Lefkowitz and colleagues [4]. Negative cooperativity has now been shown to take place in heterodimers of chemokine CCR2 and CCR5 receptors and in glycoprotein hormone receptor dimers [120,121].

It is therefore of substantial interest that asymmetric conformational changes have been observed in a leukotriene B4 receptor dimer controlled by G proteins [67]. The fluorescent properties of the dimer were measured based on a single 5-hydroxytryptophane labeled protomer and a single agonist per dimer caused full G protein activation. Also when the two receptors bind the agonist the conformational state of the two receptors differs in the presence but not in the absence of G protein. Thus, the G protein may mediate the asymmetric function of the dimer by altering the energy landscape of the second receptor. In the paper, an exciting theory is introduced inferring that the symmetric dimer instead is associated with arrestin and that stoichiometry of receptor activation in the RM may contribute to the selection among signal pathways available to the RM [67].

The D1-D2 receptor heteromer is an example of how the formation of a heteromer will switch the coupling of participating receptors to other G proteins [122]. In this case both the D1 and D2 receptors

become coupled to rapid activation of Gq/11 in the striatum instead of Gs and Gi/o respectively and the Gq/11 activation requires agonist binding to both the D1 and D2 receptors. In this way the PLC can become activated leading to intracellular calcium release and increased levels of calcium/calmodulin-dependent protein kinase IIα contributing to synaptic plasticity [109]. This represents an interesting example of how the formation of RMs increases the diversity of signaling pathways that can be used by receptor subtypes, in this case DA receptor subtypes and thus their functional repertoire in the brain. Its dysfunction may contribute to neuropsychiatric disease.

In the period 1968–1974 Anden, Corrodi and Fuxe and colleagues introduced the theory that a postjunctional 5-HT receptor in the brain may mediate the hallucinogenic effects of d-LSD, psilocybin and other hallucinogenic drugs of the indolalkylamine and phenylamine type based on studies on 5-HT turnover and 5-HT receptor activity [123–125]. This postjunctional 5-HT receptor has now been identified as the 5-HT2A receptor, enriched in layer IV of the cerebral cortex [126,127] since hallucinogens can recruit cortical 5-HT2A receptor mediated signaling pathways to affect behaviors [128].

Recently the exciting discovery has been made by Gonzalez-Maeso, Milligan and colleagues that 5-HT2A receptors form a RM with mGluR2 via TM helices 4 and 5 of mGluR2 in the brain cortex [129]. This RM may be the major target for hallucinogenic drugs since it allows the hallucinogenic 5-HT2A agonists to produce conformational changes in these 5-HT2A receptors in such a way that they become able to activate not only Gq/11 but also the Gi/o protein and its signaling pathways, necessary for their hallucinogenic effects [128]. This is another example of how unique conformational changes may develop in a receptor of a RM altering its pattern of G protein coupling in response to special agonists as a result of allosteric mechanisms operating via the receptor interfaces.

In this paper, it is also beautifully demonstrated that activation of mGluR2 antagonizes the 5-HT2A mediated hallucinogenic signaling in this RM via non-classical cooperative interactions at the recognition and G protein coupling level. In fact, there exist findings suggesting that they have an antischizophrenic potential [130,131]. This fine work of Gonzalez-Maeso, Sealfon, Milligan and colleagues [129] gives a novel target, the 5-HT2A-mGluR2 RM, for anti-schizophrenic drugs present in the cortical networks, especially in layer IV and involved in sensory processing. In this work they also report that in the schizophrenic brain the 5-HT2A receptors are increased and the mGluR2 reduced. It illustrates well the great impact the RMs and their receptor-receptor interactions will have for neuropsychopharmacology [14].

Alterations in G protein coupling have also been described for A2A-CB1 receptor heteromers [132] and for δ opioid receptor-μ opioid receptor heteromers compared the one observed with the individually expressed receptors like a switch from Gi to Gs [133].

7. Alterations in receptor trafficking and internalization via receptor-receptor interactions in RMs: relationship to desensitization

7.1. Receptor trafficking

One of the first observations that GPCR dimerization is important for receptor folding and transport to the plasma membrane came from the studies on the GABAB receptor in 1998 by several groups, the first heterodimer to be demonstrated [134]. The coexpression of the two isoforms of the GABAB receptor, GABABR1 and GABABR2, was found to be a prerequisite for the formation of a functional GABAB receptor at the cell surface. Thus, the GABABR1 is retained intracellularly as an immature protein if not expressed with GABABR2, while the latter isoform when expressed alone can be transported to the surface but cannot bind GABA. In fact, GABAR2 serves as a chaperone essential for the proper folding and cell-surface transport of GABAR1 [36]. The molecular mechanism responsible is the coiled-coil interaction of the carboxyl tails of the two GABABR isoforms leading to the heterodimerization which leads to the masking of the ER-retention signal thereby allowing ER transport and surface membrane targeting of the heterodimer [36]. Also for β2-adrenoceptors belonging to the group-A GPCRs dimerization is a prerequisite for delivery to the plasma membrane [134]. It appears that dimerization is an early event in receptor maturation and transport and intracellular retention of dimers occurs when truncated forms of V2 vasopressin and CCR chemokine receptors are expressed [135,136]. It has been demonstrated that oxytocin and vasopressin V1a and V2 form homo- and heterodimers during biosynthesis [22,137]. The need for dimers to reach the plasma membrane may stem from the indications that dimers can correspond to the major functional GPCR signaling unit engaging heterotrimeric G proteins. As an example, it should be mentioned that Canals et al [50] demonstrated that the A2A homodimers are preferentially expressed at the cell surface based on cell surface biotinylation of proteins followed by SDS-PAGE and immunoblotting with 90% of the cell surface receptor being in

the dimeric form. Furthermore, increasing evidence indicates that class A GPCR dimeric and high order RMs biogenesis occurs at an early time point during receptor biosynthesis and maturation in the ER and Golgi having a potentially important role in the quality control of newly synthesized receptors [138]. Also "non-obligatory" heterodimers in the plasma membrane display pharmacological and functional characteristics different from those of their constitutive monomers [139].

7.2. Internalization

A1-D1 RMs with antagonistic A1/D1 receptor interactions have been demonstrated in cell lines and in striatum [7,41,140–142]. It is of interest that in fibroblast cells and cortical neurons in culture A1 receptor agonists produced a coaggregation of A1 and D1 receptors suggesting movement and aggregation A1/D1 heteromers in the plasma membrane possibly associated with a certain degree of cointernalization [41]. This coaggregation was blocked by combined treatment with A1 and D1 agonists which was associated with a D1 receptor desensitization at the 2 hours time interval. The mechanism for the D1 desensitization may be that coactivation will make possible the full development of the antagonistic A1/D1 receptor interaction with uncoupling of the D1 receptor from the Gs protein and the disappearance of the high affinity D1 agonist binding site with maintained heteromerization and no signs of A1/D1 internalization. Thus, prolonged A1 activation can cause enduring conformational changes of the D1 receptor via the antagonistic A1/D1 interactions leading to reduced D1 signaling. D1 phosphorylation and the binding of β-arrestins to the D1 receptor may also contribute. However, in the case of the D1-D2 heteromer where no antagonistic receptor-receptor interaction exists cointernalization develops after coactivation [122]. D1 receptors have also been shown to diffuse in the surface membrane of spines where they can be trapped by activated NMDA receptors [143] contributing to the formation of the D1/NMDA RMs [144].

The failure of morphine to cause internalization of μ opioid receptors is regarded as one mechanism for morphine tolerance since dephosphorylation and reactivation of the μ opioid receptors may only develop upon internalization [145]. In view of the existence of μ opioid receptor homodimers it has been suggested that the μ agonist DAMGO by binding to one protomer may enhance the internalization of the morphine occupied protomer and counteract morphine tolerance due to the cotrafficking of the two protomers [146]. Cotreatment of animals with morphine and low amounts of DAMGO also resulted in the appearance of morphine analgesia without tolerance. Thus, the frequent existence of cotrafficking of homo and heteromers may be used in novel treatment strategies.

It should be noticed that A2A/D2 heteromers with antagonistic A2A/D2 receptor-receptor interactions has a different trafficking profile from the A1-D1 heteromers after coactivation of their receptors by longterm exposure to D2 and A2A agonists [25,147]. Thus, in D2 cotransfected neuroblastoma cells coactivation of A2A and D2 receptors led to coaggregation, cointernalization and codesensitization of A2A and D2 receptors. The A2A and D2 receptor functions are simultaneously altered which may help in understanding behavioral findings of cross-tolerance and cross-sensitization between D2 agonists on one hand and adenosine A2A agonists or A2A antagonists on the other hand. It seems likely therefore that the trafficking properties among the various types of heteromers may vary considerably and no general rules for their trafficking behavior can be introduced.

Recently evidence for the involvement of caveolin-1 in the internalization process of A2A-D2 RMs was studied by using computer-assisted image analysis [26]. In A2A-D2 cotransfected cells caveolin-1 colocalized with both A2A and D2 receptors. Interestingly, either the A2A agonist CGS21680 or the D2 like agonist quimpirole induced internalization of caveolin-1, A2A and D2 receptors with a preferential internalization of A2A and D2 receptors colocalized with caveolin-1. In contrast, the D2 antagonist stabilizes the D2 receptor and reduces the internalization of both the D2 and A2A receptors [148].

These results are indicative of the functional role of caveolin-1 in A2A-D2 cointernalization and thus in the permanence and plastic adjustments of A2A-D2 RMs in the plasma membrane and in the sensitization and desensitization of the participating receptors. The analysis in the above papers [26, 148] suggests that the A2A and D2 receptors internalize as a dimeric or high order RM in line with the early results obtained on the yeast alpha-factor pheromone receptor that homo-oligomers are the functional units of endocytosis [149].

8. Agonist regulation of receptor heteromerization

In 2000 Rocheville et al [150] demonstrated with FRET analysis that SSTR5 exists as a monomer in the basal state but becomes a dimer upon agonist activation. Also an agonist induced heteromerization of SSTR5 and SSTR1 was demonstrated and was subtype specific. Furthermore, with photobleaching FRET microscopy a SSTR5-D2 receptor heteromer

was observed but only after treatment with either agonist or coactivation of the two receptors had no further action [49]. Agonist induced enhancement of energy transfer for β2 adrenergic receptors was observed the same year by Angers et al [151] and for gonadotrophin-releasing hormone and thyrotropin-releasing hormone the following year [152,153]. An agonist promoted decrease of energy transfer has been reported for the CCK receptor related to an agonist dissociation of the CCK oligomers [154]. However, many of the RMs are constitutive and not agonist induced probably because they are formed in the ER and Golgi and then inserted in the surface membrane as discussed above. This may possibly not be true for the agonist induced heteromer like the SSTR5/D2 that may be fully formed only in the plasma membrane upon agonist activation.

A2A-D2 RM is a clear example of a constitutive RM. The BRET signal was not even modified by the agonist-induced activation of the A2A or the D2 receptor or by their coactivation in spite of being expressed on the plasma membrane [25]. These results also indicate that the coaggregation of A2A and D2 receptors described above after agonist treatment does not lead to a change in the distance between the two tags of the heterodimer in view of absence of a detectable change in the BRET signal. Failures of agonist induced alterations in FRET and BRET signals have also been observed for the yeast alpha factor receptor [155] and the human δ opioid receptor [51].

The agonist induced changes in BRET and FRET signals may sometimes be interpreted as increases, decreases or no changes in the amount of RMs. However, also other mechanisms may explain the observed changes in energy transfer. BRET and FRET efficacies vary with the 6^{th} power of the distance between the donor and acceptor. Also the receptor activation with agonists is associated with conformational changes within the TM core of the GPCR [156] with changes in G protein coupling, receptor phosphorylation and arrestin translocation. These events may affect the relative distance and orientation of the energy donor and acceptor depending on their position in the receptor and the structural features of each receptor. Therefore, it may often be difficult to interpret the agonist promoted changes in energy transfer as a result of a change in receptor oligomerization.

A study of melatonin receptor homomers shows that ligand-promoted BRET enhancement represent specific ligand induced conformational changes of preexisting receptor homomers linked to the activation state of the receptors [157]. For these homomers they deduced that the dimeric state was the constitutive one based on data fitting by adapting the dimer, trimer and tetramer model of energy transfer quenching in which the oligomeric state of a receptor could be deduced from an equation [157]. This work has continued and in 2004 Ayoub et al [158] demonstrated preferential formation of MT1-MT2 heterodimers with distinct receptor-receptor interactions when compared with MT2 homodimers, probably reflecting development of differential allosteric mechanisms between the binding sites in the heterodimers *vs.* the homodimers resulting in different alterations in cooperativity.

It is of substantial interest that the two chemokine receptors CCR2 and CCR5 have been shown to form homodimers and heterodimers with the heterodimers demonstrating receptor-receptor interactions characterized by the existence of negative cooperativity between their two binding sites [120,159,160]. Macrophage inflammatory protein 1β is a selective agonist at CCR5 and monocyte chemoattractant protein-1 a selective agonist at CCR2. Furthermore, the CXCR4 is the cognitive receptor for the stromal cell-derived factor 1α which induces a dimerization of its receptor necessary for signaling [161]. In view of the existence of these chemokine receptors in brain tissue including the substantia nigra as agonist dependent homomers and heteromers it has been postulated that in acute neuroinflammation the chemokines and also cytokines via their respective receptors can produce the formation of novel pathological RMs or dysfunctional RMs including also other types of receptors not belonging to the immune system [162–165]. This may be caused by the conformational changes induced in these immune receptors by the panorama of agonists present in the inflammatory brain regions which may lead to chronic neuroinflammation and neurodegenerative disease like Parkinson's disease.

The hypothesis has also been introduced that the viral-coded receptors not only operate as constitutively active monomers, but also can affect other receptor functions by interacting with receptors of the host cell forming novel pathological RMs with abnormal signaling [166]. Furthermore, it is suggested that viruses could insert not only single receptors (monomers), but also dimeric or high order RMs, altering the cell metabolism in a profound way. The prevention of the formation of viral receptor–host RMs with disease producing signaling may give rise to novel antiviral drugs that counteract virally-induced disease.

9. Role of allosteric modulators in the function of GPCR RMs

Previously it has been shown that homocysteine, a sulfur-containing amino acid formed by demethylation of methionine, can modulate NMDA receptor

function leading to increased NMDA receptor activity that may contribute to its neurotoxic effects [167]. Recently an allosteric modulation of D2 receptors has been demonstrated after homocysteine with lower concentration than those modulating NMDA receptor function [168]. The evidence obtained in A2A-D2 cotransfected CHO cell lines suggests that homocysteine acts as an allosteric D2 receptor antagonist. It reduces the affinity of the D2 agonist binding sites and decreases the ability of D2 receptor activation to produce an internalization of the A2A-D2 RM. Mass spectrometric analysis demonstrated that homocysteine forms noncovalent complexes with the two Arg-rich epitopes in the third intracellular loop of the D2 receptor one of them participating in the A2A-D2 receptor heteromerization. However, homocysteine could not prevent nor disrupt the A2A-D2 heteromerization as shown in FRET experiments. Therefore, the allosteric antagonistic action of D2 receptor recognition and signaling (evaluated in behavioral experiments) by homocysteine may depend on its binding to the Arg-rich region in the middle portion of the IC loop 3 of the D2 receptor leading to conformational changes causing reduction in recognition and G protein coupling [168,169]. The results indicate that this negative allosteric modulation may exist with maintained A2A-D2 heteromerization also inhibiting D2 receptor recognition and signaling via the antagonistic A2A/D2 receptor-receptor interaction [7,170]. Thus adenosine and homocysteine extracellular levels in brain may jointly act to silence D2 signaling and worsen the behavioral deficits in Parkinson's disease [168,171,172]. The L-dopa induced hyperhomocysteinemia may therefore contribute to the loss of therapeutic effects of L-dopa in this disease. Based on these observations allosteric intramolecular mechanisms within the receptor can operate together with allosteric intermolecular mechanisms passing from one receptor to the other one via the interface resulting in negative or positive cooperativity and establishing the receptor-receptor interactions.

10. Role of receptor interacting proteins in the modulation of GPCR RMs

It should be underlined that GPCRs contain sequence motifs known to have a theoretical capacity to interact with many other proteins. Such interactions will have an impact on the properties of the RMs formed through direct physical interactions between receptors like their compartmentalization. The integrated signaling of RMs are probably modulated by adaptor and scaffolding proteins [8,10, 140,173,174]. The long C-terminal tail and the long third intracellular loop have dominated these receptor-protein interactions which can be transitory or more stable. They have a clearcut impact on downstream signaling, trafficking, subcellular localization and cytoskeletal associations [175,176] and several GPCRs interact with cytoskeletal anchoring polypeptides like D2 receptors with alpha-filamin [177] and A2A receptors with α-actinin [178].

Interactions between GPCRs and PDZ-domain containing proteins play a special role by defining the molecular composition of signaling complexes within microcompartments and the precise placement of these complexes within the cell. Well-known proteins containing PDZ domains are PSD-95, Shank, and homers which function as multivalent scaffold proteins organizing receptor complexes and inter alia linking together NMDA, mGluR1α and mGluR5 in the glutamate synapses forming a high molecular signaling complex [175]. This receptor complex is however not a heterodimer or a RM since direct physical interactions between the receptors do not appear to exist. This complex becomes stabilized in the synapse by becoming linked to the actin cytoskeleton. Taken together, the interaction between NMDA and group-I mGluRs is mainly an indirect one via the PSD-95, GKAP, Shank, homer protein complexes with homer proteins directly interacting with the group-1 mGluRs. However, direct physical receptor interactions between these receptors can still exist in certain plasma membrane microdomains resulting in the formation of a heterodimer or a RM. The actin cytoskeleton may also assist in stabilizing RMs with their direct physical receptor interactions [175].

It is of substantial interest that calmodulin, an intracellular acidic calcium binding protein and a major transducer of calcium information [179] can directly interact with the third intracellular loop of the D2 receptor [180]. Exogenous calmodulin binds to the Arg-rich epitope domain in the amino-terminus in a calcium dependent manner to which also the C-terminal tail of the A2A receptor can bind and calcium induced activation of calmodulin inhibits Gi/o protein activation by the D2 receptor [180]. The functional relevance of this interaction has been further studied using a [^{35}S]-GTPγS binding assay in A2A-D2 cotransfected CHO cells [170,181]. Calcium ions were found to highly significantly and substantially increase basal and dopamine induced G protein coupling in A2A-D2 cotransfected cells but not in cells transfected only with D2 receptors. It was speculated that calcium/calmodulin complex could release the D2 receptor-Gi/o complex from the stronger inhibition caused by the A2A receptor. Furthermore, Neve's group has obtained indications that binding of endogenous calmodulin to the intracellular loop 3 of the D2 receptor enhances the

inhibitory D2 receptor signaling to the adenylate cyclase [182]. It may therefore be that the results of Terasmaa et al [181] can be explained by the existence of two types of dimers in the A2A/D2 cotransfected cells containing substantially higher densities of D2 receptor than of A2A receptors [183]. Thus, the existence of D2 homomers coupled to Gi/o and A2A-D2 heteromers with a reduced G protein coupling in the A2A/D2 cotransfected cells could help explain the additional increase in G protein activation found with combined treatment with DA and calcium ions through disruption by Ca2+/calmodulin of the antagonistic A2A/D2 interaction at the Arg-rich epitopes. Recently it has been suggested that the D2 receptor signaling in the A2A-D2 RM may preferentially involve the activation of Gq/11-PLC pathway [184] which however, remains to be established.

Thanks to the pioneering study of Woods et al [185] an increased understanding has been obtained of how calmodulin interacts with the A2A and D2 receptors. Mass spectrometric analysis revealed that electrostatic interactions involving the D2 receptor Arg rich epitope and several calmodulin acidic epitopes mediate the D2 receptor-calmodulin binding. The discovery was also made that calmodulin can form multiple non-covalent complexes involving electrostatic interactions with an epitope located in the proximal segment of the C-terminal of the A2A receptors. The dynamics of the A2A-D2-calmodulin interactions were also clarified by the ability of calmodulin to disrupt the electrostatic interaction between the D2 receptor epitope and the distal A2A receptor epitope in the C-terminal. Furthermore, calcium ions disrupted the binding of calmodulin to the D2 epitope but failed to do so at the A2A receptor epitope. These results give new models for A2A-D2-calmodulin interactions and illustrate the impact of receptor interacting proteins on the structure and function of RMs.

In 1990 we published the autoradiographic localization of *mas* proto-oncogene mRNA in rat brain using *in situ* hybridization [186]. The *mas* transcripts were linked to discrete nerve cell populations in the forebrain, especially in the limbic cortex, the olfactory tubercle and the olfactory bulb. There was no clear link to the distribution of the Angiotensin II high affinity agonist binding sites mainly found in the brain stem, especially the autonomic nuclei [187] in spite of the demonstration that the *mas* oncogene enhances the angiotensin induced calcium responses in cells with angiotensin receptors [188]. Also it was later on found based on studies in *mas* oncogene-deficient mice that there may exist direct interactions between mas and the angiotensin AT1 receptor in the amygdala with the heptahelical protein *mas* not being activated by angiotensin II [189]. Based on the mapping of *mas* mRNA levels discussed above it may be that the cortical limbic afferents to the amygdala contain *mas* protein in their nerve terminal networks on which AT1 receptors also may be located giving a structural correlate to a colocation.

It is therefore of substantial interest that a heterodimerization between *mas* and AT1 has been recently demonstrated in FRET and BRET experiments [190]. The heteromerization resulted in a reduction of AT1 signaling giving evidence that the role of this GPCR protein is to directly associate with the AT1 protein and via endogenous negative cooperativity bring down its AT1 signaling. There is no need to postulate that this heptahelical protein is an orphan receptor. In contrast, the receptor-receptor interactions in AT1-bradykinin B1 receptor heteromers in nuc tractus solitarius [191] and in smooth muscle cells [42] lead to enhanced AT1 receptor signaling and vasopressor responses. Increased presence of the AT1-B2 receptor heteromer contributes to the development of angiotensin II hypersensitivity in preeclampsia [192] and experimental hypertension [193].

Another interesting receptor interacting protein is adenosine deaminase (ADA) which metabolizes adenosine to inosine. This enzyme binds to cell surface A1 receptors as an ectoenzyme on the extracellular side [194] and is necessary for the high affinity agonist binding state of the A1 receptor [195]. An irreversible ADA inhibitor deoxycoformacin fully counteracted the antagonistic A1/D1 receptor interaction in the A1-D1 RM. This action was not due to blockade of enzymatic activity but to altered ADA-A1 protein interactions leading to altered allosteric mechanisms over the interface and loss of the A1 agonist high affinity state required for the A1/D1 receptor interaction [141]. A dysfunction of ADA may thus lead to exaggerated D1 signaling causing motor hyperactivity and EEG arousal.

11. Drug development based on targeting the RMs

Due to the existence of classical and non-classical cooperative interactions in RMs involving both extrinsic (induced by exogenous ligand) and intrinsic cooperativity (induced only via the receptor protein interfaces formed) taking place via allosteric mechanisms [8,10,14,76,141] novel receptor subtypes can appear as discussed above. Thus, the pharmacology of the binding pockets of the different receptors building up the RM can become markedly altered versus their pharmacology in the respective homomers. Through the operation of both exogenous and endogenous cooperativities,

an ample spectrum of pharmacological properties emerges in the RMs, which lead to the development of a novel neuropsychopharmacology.

This is the neuropsychopharmacology of the RMs, the hallmark being the use of combined agonist or antagonist treatment to optimally increase or reduce the signaling of one malfunctioning receptor in the RM. Thus, in addition to giving agonistic and antagonistic drugs acting on the malfunctioning receptor in the RM, other drugs are also given to increase or reduce the influence of surrounding receptors on the malfunctioning receptor in the RM. In this way also the doses of the combined drugs can be reduced leading to the appearance of fewer side effects but maintaining therapeutic effects [14]. The introduction of A2A antagonists combined with L-dopa in treatment of Parkinson's disease gives proof of concept that novel treatments can be introduced based on receptor-receptor interactions in RMs (Figure 2) [14,76,171]. Based on the putative existence of trimeric A2A/D2/CB1 RMs combined treatment with A2A and CB1 antagonists should also be considered in Parkinson's disease (Figure 2).

The conformational changes in the receptors of the RM caused by the receptor-receptor interactions through extrinsic and intrinsic cooperativity can also produce novel interactions among the participating receptors also including ion channels and RTK receptors. Thus, a rich spectrum of functions may emerge due to the formation of RMs. It should also be considered that in the RM novel interactions may develop through extrinsic and intrinsic cooperativity with receptor interacting and membrane associated proteins like scaffolding and adapter proteins. Thus, through the formation or disruption of RM novel cognition-decoding systems may appear or disappear in the brain membranes at synaptic and extrasynaptic locations and be a major molecular mechanism for learning and memory [14,76, 196,197]. Interactions between synaptic and extrasynaptic RMs with possible formation of new RMs may be a major mechanism for the integration of wiring and volume transmission [196,197].

The pharmacology of the RMs has also led to the introduction of bivalent ligands which can become useful in treatment of disease. On the basis of the existence of μ-δ opioid receptor heterodimers with δ ligands enhancing the efficacy of μ agonists, bivalent compounds have inter alia been developed built up of a μ agonist linked to a δ antagonist which show special analgesic properties [198]. The distance between the two components modulated the appearance of opioid tolerance and dependence giving indications that the bivalent ligands in fact targeted the μ-δ opioid receptor heterodimers.

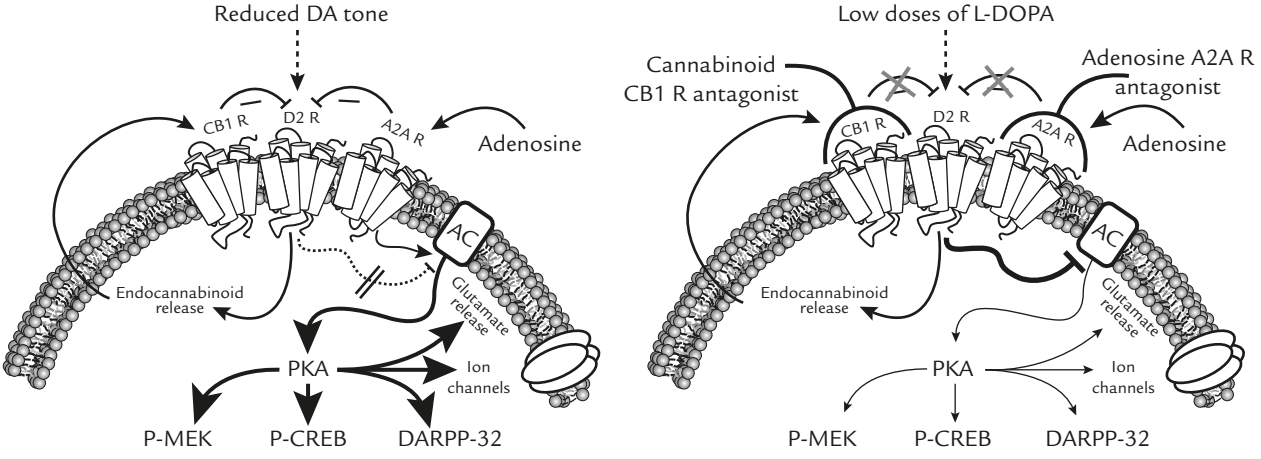

Figure 2 Scheme of receptor-receptor interactions in putative trimeric CB1/D2/A2A receptor mosaics in Parkinson's disease (PD) mainly located on dorsal striatal glutamate terminals with (right) or without (left) combined treatment with low doses of L-dopa and A2A and CB1 receptor antagonists. *Left panel:* Reduced tone of DA in PD leads to reduced signaling of D2 receptors to AC (inhibited by D2) and to ion channels (not shown). Instead dominance of A2A and CB1 receptor signaling will develop with increased inhibition of D2 receptor activity via antagonistic A2A/D2 and CB1/D2 receptor-receptor interactions and increased AC activity over the A2A activated AC. As a result PKA is strongly activated mediating increases in P-MEK (MAPK kinase), P-CREB and increased phosphorylation of Thr^{34} in DARPP-32 turning it into a protein phosphatase type-1 inhibitor. This will cause inter alia increases in striatal excitability with increased release of glutamate leading to inhibition of motor function. *Right panel:* By means of combined therapy with A2A and/or CB1 receptor antagonists and removal of the antagonistic A2A/D2 and CB1/D2 receptor interactions it will be possible to use very low doses of L-dopa with reduced side-effects like L-dopa induced dyskinesias. A proper tone of D2 receptor activity can therefore be restored to ion channels (not shown) and to Gi/o regulated AC with normalization of the activity of the A2A activated AC and reduction in the activity of PKA and thus in P-MEK, P-CREB and the activity of DARPP-32 and normalization of glutamate release.

Specific ligands have also been developed for the κ-δ opioid heterodimer, one being a compound with κ agonist and δ antagonist properties and it should be noted that its specific analgesic properties are different from the actions of combined treatment with δ antagonists and κ agonists [199]. Again bivalent compounds have been synthesized that target the heterodimer in this case the κ-δ opioid heterodimer [200,201].

12. RMs formed by GPCRs and ion channel receptors

The first one discovered was the one between GABAA and DA D5 receptors by Liu et al [202]. Coactivation of the two receptors is necessary for the formation of this RM with agonist-induced changes in the second intracellular loop of the γ subunit of the GABAA and in the C-terminal part of the D1 receptor being essential. One functional meaning of this RM appears to be the development of a mutually inhibitory cross-talk between the two receptors. In view of this agonist dependency it may be that this RM is formed in a transient way within the plasma membrane upon coactivation of the D5 and GABAA receptors in the surface membrane. GABAA receptor activation reduces D5/Gs coupling and D5 activation reduces the GABAA currents and thus synaptic strength at the GABA synapse [202]. The discovery of this GABAA and DA D5 RM gives new aspects on the role of these two receptors in schizophrenia [202,203].

This work was followed by the discovery of the constitutive D1/NMDA RM by Lee et al [144] where two regions exist in the C-terminal of the D1, one of them interacting with the NR1-1 and the other with the NR2A subunit of the NMDA receptor, respectively (Figure 3A) [203]. The D1/NR2A interaction reduced the NMDA currents upon D1 activation which may be caused at least in part by a reduced cell surface expression of the NMDA receptor [144]. There exist ER export signals in the C-terminal of the NR2 subunit that allows surface expression of assembled NMDA receptors. It was only in the presence of cotransfected NR2 subunits that the NR1-1 subunit-D1 receptor complex could be translocated to the surface membrane and D1 activation can reduce this process [204]. However, as pointed out earlier D1-NMDA RMs may also be formed in the spine surface membrane by diffusing D1 being trapped by NMDA receptors [143].

Instead the D1/NR1-1a interactions were involved in the rescue of NMDA receptor induced excitotoxicity by increasing the ability of the NR1-1a to form a complex with calmodulin and PI-3 kinase (Figure 3A) [144]. The two interacting regions of this interaction shows the presence of a phosphorylated serine and adjacent glutamates in the D1 receptor while NR1-1 contains three adjacent Arg residues which are all highly conserved [45]. Thus, similar electrostatic interactions exist as in the interface of the A2A/D2 RM [45,92]. These epitope-epitope interactions may represent general mechanism underlying receptor-receptor interactions as proposed by Woods and colleagues, where phosphorylation and dephosphorylation processes can play an important role since phosphate stabilizes these electrostatic interactions [93].

Finally a constitutive D2-NMDA RM has been demonstrated [205] taking place between the N-terminal part of the third intracellular loop of the D2 and C-terminal of the NR2B subunit located in microdomains of postsynaptic densities of glutamate synapses (Figure 3B). However, it was enhanced by D2 activation through cocaine treatment. This receptor-receptor interaction reduces the binding of Ca/calmodulin dependent protein kinase II to NR2B with diminished NR2B phosphorylation and reduction of NMDA signaling [205]. This RM may have a substantial role in Parkinson's disease, schizophrenia and cocaine addiction.

Thus, there exist highly specific RMs between GPCRs and key ion channel receptors that will fine-tune the excitability and firing of neural circuits in the brain involving modulation of phosphorylation and trafficking of the ion channel receptors to the surface membrane.

13. Putative RMs formed by GPCRs and tyrosine receptor kinases (RTKs): one possible mechanism for transactivation of RTKs

Receptor tyrosine kinases are a family of membrane-spanning receptors in mammals that mediate the TM signaling from ligands that include the majority of growth factor receptors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), neurotrophins (e.g., BDNF) and FGFs. In each case, dimerization and tyrosine phosphorylation of RTKs occur, and this phosphorylation serves as docking sites of adaptor proteins that lead to the activation of intracellular signaling pathways such as Ras-mitogen activated protein kinase (MAPK) cascade [206–208].

There is a new awareness that G protein-coupled receptor (GPCR) possesses the capacity for transactivation of RTK via GPCR induced release of neurotrophic factors [207] and via Gbetagamma subunits mediated Src-dependent phosphorylation [208] leading to increases in mitogen-activated protein kinase activity. This rise is rapid and transient

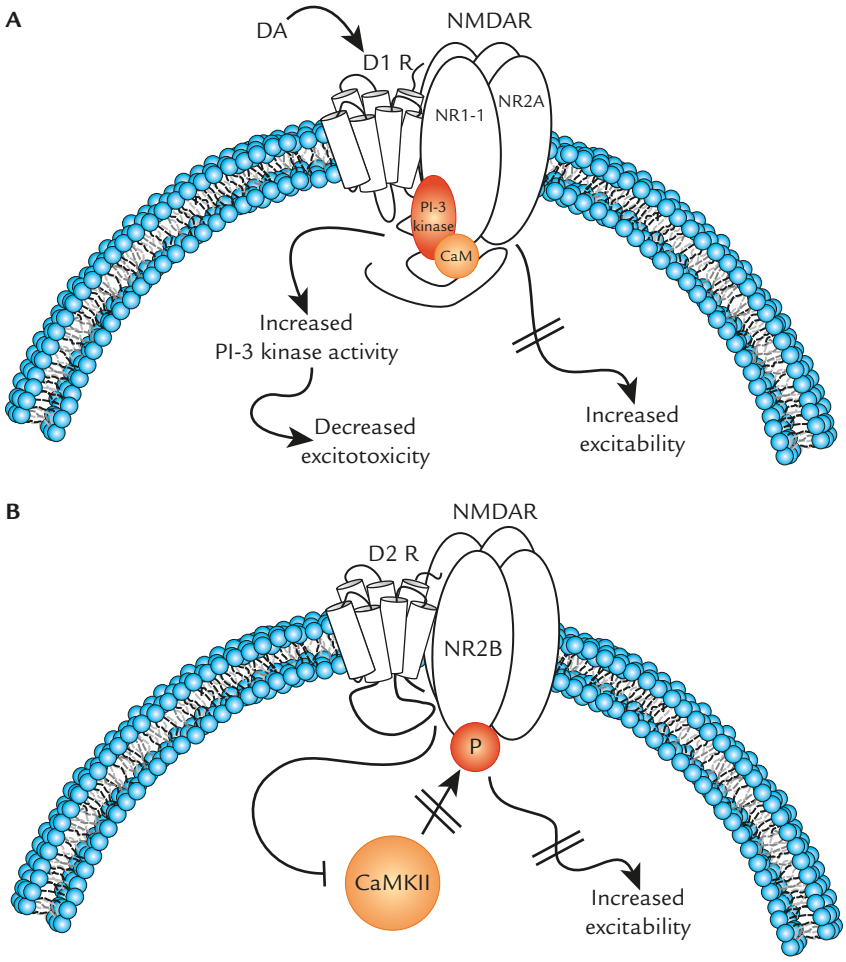

Figure 3 Scheme of the molecular mechanism for the receptor-receptor interactions in the D1/NMDA (A) and D2/NMDA (B) receptor mosaics. (A) One epitope of the C-terminal region of D1 can directly interact with the C-terminal of the NR2A and reduce its plasma membrane expression resulting in reduction of NMDA currents and in reduced excitability; another epitope of the C-terminal region of D1 can directly interact with the NR1-1a increasing the ability of the NR1-1 to form a complex with calmodulin and PI-3 kinase resulting in increased activity of the PI-3 kinase and increased cell survival (see text for reference). (B) The N terminal part of the third intracellular loop of D2 directly interacts with C terminal part of the NR2B subunit of synaptic NMDA receptors. This interaction is increased by D2 activation and reduces the binding of the calcium/calmodulin dependent protein kinase II to the NR2B which results in reduced phophorylation of the NR2B subunit, reduction of NMDA currents and thus reduced excitability (see text for reference).

and results in kinase translocation to the nucleus and transcriptional activation. It is of substantial interest that also beta-arrestins can bind to the GPCRs and transduce receptor signals causing a slow and sustained rise of MAPK activity mainly restricted to the cytoplasm [209]. Over the past decade many examples of transactivation of mitogenic growth factor receptors in response to GPCR signaling have been reported indicating that there are alternative modes of activating receptor tyrosine kinases in the absence of neurotrophic factor binding at the cell surface [207,210–215].

It has also been postulated that GPCRs and RTK can directly interact enabling allosteric receptor-receptor interactions within a GPCR-RTK heterodimer/RM like 5-HT1A-RTK fibroblast growth factor receptor 1 (FGFR1) [197]. Thus, direct GPCR-TRK receptor-receptor interactions in receptor heterodimer/RMs may develop in the cell surface membrane of substantial importance for neuronal trophism. It is therefore exciting to notice that Grengard and colleagues [216] in 2008 have discovered a direct physical interaction between the A2A receptor and FGFR1 and coactivation but not individual activation of these two types of receptors results in a marked activation of the MAPK/ERK pathway. This event is associated with differentiation, increased neurite extension, appearance of spines and striatal plasticity. It may play a crucial role in the induction of enduring change in the CNS trophism via VT signals operating through combined GPCR and RTK activation. The evidence [216]

suggests that FGF may act as a co-transmitter through A2A receptors to modulate neuronal plasticity.

The hypothesis has been introduced that the neurotrophic system FGF-2/FGFR1 may be a good candidate to mediate antidepressant induced improvement in 5-HT neuronal communication and neurotrophism with regeneration of connections lost during depression and recovery from depression [197]. It also states that FGFR1 transactivation mediates the antidepressant effects of 5-HT uptake blockers and that the molecular mechanism involves a receptor-receptor interaction between certain serotonin receptor subtypes like 5-HT1A and FGFR1 forming a RM [197].

14. Nomenclature and criteria for the existence of heteromers

Pin et al [107] in the IUPHAR report have proposed to name the GPCR heterodimer with the names of the two receptors separated by a hyphen in alphabetical and numerical order e.g. D1-D2 receptor "for the D1-D2 heteromer". In spite of the attractive simplicity we would agree with Ferre, Franco et al [217] to add "heteromer" after the word "receptor". This would be an improvement in view of the lack of knowledge on receptor stoichiometry. However, we would prefer to use the term "*receptor mosaic*" which we introduced already 1982 [5] when more that two receptors are involved instead of "receptor heteromer". The term "mosaic" implies the importance not only of stoichiometry but also of topology of the types of receptors present. Mosaic also gives a fine word for the structure of the molecular fingerprint from which the intramembrane receptor-receptor interactions in the receptor assembly arise via allosteric interactions. The RM can be built up of homomers or of heteromers and also be a mixture of homomers and heteromers. Finally we would recommend to use the word receptor and not subunit for the participating receptors in the RM in order to distinguish them from the subunits of the ion channel receptors which are not functional on their own.

We fully agree with the valuable criteria developed in the IUPHAR report for identification of receptor heteromers and appreciate their comment that as new criteria emerge they will become incorporated in the NC-IUPHAR recommendations.

In summary, the field of RM with their receptor-receptor interactions continues to expand and represents a new principle in molecular medicine[8] that continues to lead to new strategies in treatment of mental and neurogical diseases as well as of diseases in the immune, cardiovascular and endocrine systems. A2A antagonists have already been introduced in Parkinson's disease based on combined treatment with low doses of levodopa ad D2 agonists offered by the existence of antagonistic A2A/D2 interactions in A2A-D2 RMs [171].

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