Neuropsychopharmacology REVIEWS (2014) 39, 131–155

Moonlighting Proteins and Protein–Protein Interactions as Neurotherapeutic Targets in the G Protein-Coupled Receptor Field

Kjell Fuxe*[,1], Dasiel O Borroto-Escuela[1], Wilber Romero-Fernandez[1], Miklós Palkovits[2], Alexander O Tarakanov[3], Francisco Ciruela[4] and Luigi F Agnati[5]

[1]Department of Neuroscience, Karolinska Institutet,, Stockholm, Sweden; [2]Department of Anatomy, Histology and Embryology, Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary; [3]Russian Academy of Sciences, St. Petersburg Institute for Informatics and Automation, Saint Petersburg, Russia; [4]Facultat de Medicina, Departament de Patologia i Terapèutica Experimental IDIBELL-Universitat de Barcelona, L'Hospitalet de Llobregat, Unitat de Farmacologia, Barcelona, Spain; [5]IRCCS, Lido Venice, Italy

There is serious interest in understanding the dynamics of the receptor–receptor and receptor–protein interactions in space and time and their integration in GPCR heteroreceptor complexes of the CNS. Moonlighting proteins are special multifunctional proteins because they perform multiple autonomous, often unrelated, functions without partitioning into different protein domains. Moonlighting through receptor oligomerization can be operationally defined as an allosteric receptor–receptor interaction, which leads to novel functions of at least one receptor protomer. GPCR-mediated signaling is a more complicated process than previously described as every GPCR and GPCR heteroreceptor complex requires a set of G protein interacting proteins, which interacts with the receptor in an orchestrated spatio-temporal fashion. GPCR heteroreceptor complexes with allosteric receptor–receptor interactions operating through the receptor interface have become major integrative centers at the molecular level and their receptor protomers act as moonlighting proteins. The GPCR heteroreceptor complexes in the CNS have become exciting new targets for neurotherapeutics in Parkinson's disease, schizophrenia, drug addiction, and anxiety and depression opening a new field in neuropsychopharmacology.
Neuropsychopharmacology Reviews (2014) **39,** 131–155; doi:10.1038/npp.2013.242; published online 9 October 2013

Keywords: receptor–receptor interactions; G protein-coupled receptors; moonlighting; heterodimerization; neurotherapeutic targets; neuropsychiatric disorders

INTRODUCTION

The focus of this review will be the special case of protein–protein interactions among receptors, especially GPCRs which results in the formation of heteroreceptor complexes in the CNS (see below). Lefkowitz has made the epoch making discoveries in the GPCR field. It started already in 1970 and the breakthrough came in 1986 when Lefkowitz and his group cloned the gene and cDNA for the beta2 adrenergic receptor and showed its homology with the rhodopsin sequence, which led to the discovery of the GPCR superfamily (Dixon *et al*, 1986; Lefkowitz, 2000).

Jeffery introduced the concept of moonlighting proteins in 1999 (Jeffery, 1999). Such proteins are special multifunctional proteins because they perform multiple autonomous, often unrelated, functions without partitioning into different protein domains. Thus, moonlighting proteins represent an interesting category of multifunctional proteins in which two or more functions can be found in a single-polypeptide chain. Jeffrey (2004) defined the term as: moonlighting proteins are proteins with more than one function, but do not include proteins where the multiple functions result from different RNA splice variants, gene fusions or several homologous proteins (isoforms) (Jeffery, 2004).

The existence of such proteins was reported roughly 20 years ago. It was found that certain structural proteins like crystallins, water-soluble structural proteins in the lens of the eye, are evolving and developed from metabolic enzymes (Huberts and van der Klei, 2010). A number of mechanisms have been proposed for the change of functions in a moonlighting protein (Jeffery, 1999), like different locations within a cell, intracellular versus extracellular location, presence in different cell types, binding of a substrate or cofactor including existence of different binding sites for different substrates, homomerization of the

*Correspondence: Professor K Fuxe, Department of Neuroscience, Karolinska Institutet, Retzius väg 8, 17177, Stockholm, Sweden, Tel: +46 8 52487077, Fax: +46 8 315721, E-mail: Kjell.Fuxe@ki.se
Received 18 February 2013; revised 4 July 2013; accepted 5 July 2013; accepted article preview online 6 September 2013

protein and formation of complexes with other proteins (heteroprotein complexes) (Jeffery, 1999).

It was recently proposed (Gancedo and Flores, 2008) that the moonlighting phenomenon is a consequence of the Jacob's principle, namely of the tinkerer's capability of performing evolution (Jacob, 1977), which means that novel functions can be carried out by adapting the existing devices. The concept of moonlighting proteins has now been introduced in the field of GPCR heteroreceptor complexes (Borroto-Escuela *et al*, 2011b). Moonlighting of receptors through heteromerization is in the current review operationally defined to take place when the allosteric receptor–receptor interactions lead to novel functions of at least one receptor protomer.

METHODS FOR STUDYING PROTEIN–PROTEIN INTERACTIONS

The uncovering of receptor–receptor interactions (that is, GPCR oligomerization) has been assessed through a plethora of methodologies which run from classical biochemical approaches to biophysical techniques. Indeed, each methodology used has provided precise and valuable information which has been considered with caution to avoid undesirable drawbacks. Thus, here we provide a historical and critical overview and description of the techniques used in the study of GPCR oligomerization paying special attention to common associated pitfalls. The first indication of GPCR oligomerization was obtained at the receptor-ligand recognition level by using classical radioligand-binding techniques (Fuxe and Agnati, 1985; Fuxe *et al*, 1983). Accordingly, distinct changes in the affinity and density of monoamine receptor binding sites have been observed after modulation by neuropeptide transmitters when analyzing saturation and competition radioligand-binding experiments in isolated brain membrane and cultured cell membrane preparations (Figure 1a). This phenomenon has been hypothesized to be due to physical intramembrane receptor–receptor interactions in heteromers. However, these experiments have been far from demonstrating a direct receptor–receptor interaction that takes place at the plasma membrane level. To tackle this last assumption, traditional biochemical techniques have been used including microscopy-based procedures

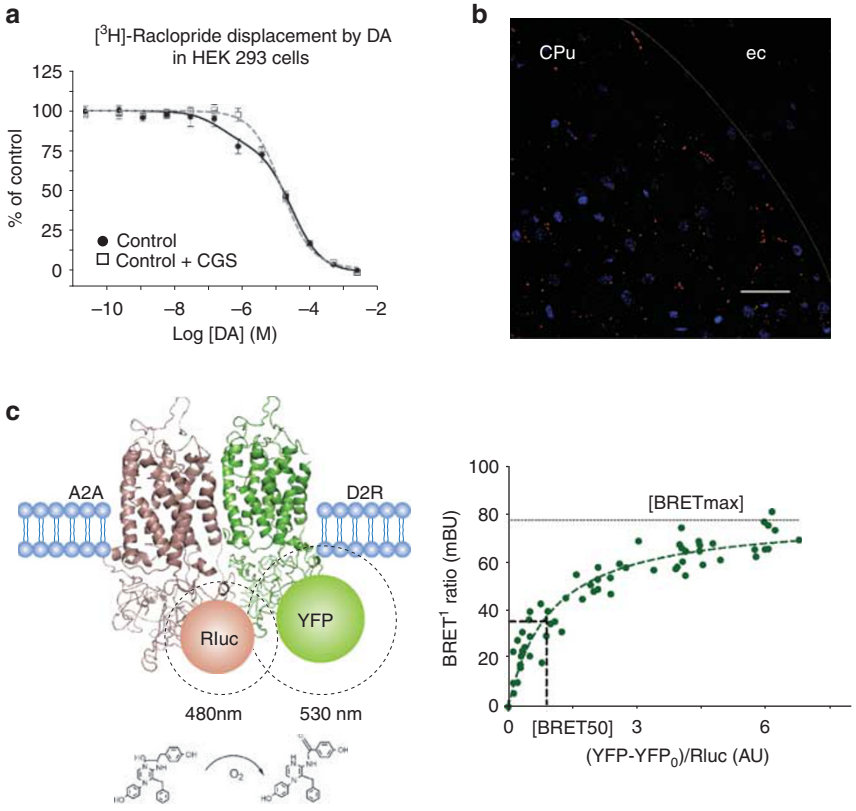

Figure 1. Different methods have been used to study GPCR homo and heteroreceptor complexes. As examples are shown methods used to study the existence of A2A–D2 heteroreceptor complexes in heterologous expression systems and in brain *ex vivo*. (a) Competition experiments using D_2-like receptor antagonist [^{3}H]-Raclopride (2 nM) versus increasing concentrations of dopamine in transiently cotransfected HEK293T cell membranes. Panel (a) illustrates the right shift of the competition curve after treatment with the A2A agonist CGS21680 in the high affinity range of the D2R agonist binding site, which demonstrates a reduction of D2R affinity in the high affinity state. (b) Detection of A2A–D2 heteroreceptor complexes in *ex vivo* brain sections can be found by *in situ* proximity ligation assay (PLA). PLA-positive A2A–D2 heteroreceptor complexes in striatal sections have been visualized as red clusters (blobs, dots) within the neuropil of caudate putamen (CPu), which were almost absent within the external capsule (ec). (c) In C the principle of the BRET^1 method is illustrated giving strong support for the existence of A2AR–D2R heteromers. In the presence of h-coelenterazine, an energy transfer between Rluc and YFP occurs when the distance between them is less than 100 Å. To the right a BRET^1 saturation curve for the A2A–D2 hetero receptor complex (D2LR^{Rluc} + A2AR^{YFP}, filled circles) is shown giving BRET max and BRET EC50 values.

(eg, coimmunolocalization) and immobilized protein–protein interaction assays (eg, coimmunoprecipitation). Again these approaches fail to provide unambiguous evidence for direct receptor–receptor interactions. In addition, their invasive nature still has disadvantages as false results were obtained due to the hydrophobic nature of receptors. Nevertheless, besides the inherent technical problems associated with these methods, they have proven to be very useful in initially confirming potential interactions between different GPCRs. Interestingly, to overcome the invasive nature of the immobilized protein–protein interaction assays, a set of fluorescence-based technologies have been developed. Some of these approaches are based on the resonance energy transfer (RET) principle (Figure 1c) and use receptor constructs which bear genetically fused 'donor' and 'acceptor' chromophores (Figure 1c). The bioluminescence-RET (BRET) approach involves an enzyme as a donor (eg, renilla luciferase, Rluc) which upon substrate oxidation generates bioluminescence engaging in a RET process with the appropriate acceptor molecule (eg, yellow fluorescent protein, YFP) provided they are in close proximity (<10 nm) (Figure 1c). Similarly, fluorescence-RET (FRET) techniques use a pair of fluorophores as donor and acceptor molecules (eg, cyan fluorescent protein, CFP and YFP). Thus, upon donor excitation with the matching monochromatic light the acceptor fluorophore can absorb resonant energy, if in close proximity. Overall, these experiments have favored the possibility of carrying out 'in cell' dynamic experiments. Therefore, the use of the previously mentioned RET-based techniques has emerged as a powerful tool in the study of GPCR oligomerization in living cells. However, it is important to keep in mind that there are several inherent problems associated with the use of these RET-based techniques. For instance, the attachment of such large chromophore proteins may preclude proper receptor function, which makes the interpretation of results difficult. In addition, the existence of false RET signals produced by photophysic cross-talks should be considered. Finally, an increase of non-specific RET can take place as a result of the random collision of intracellularly accumulated RET-tagged receptors when transiently overexpressed. As such, some controversy surrounds these approaches (Bouvier *et al*, 2007; James *et al*, 2006). Nevertheless, when RET-based results are properly assessed the elegant demonstration of dynamic oligomerization of ectopically expressed GPCRs and of cells from transgenic mice is possible (Audet *et al*, 2010; Marullo and Bouvier, 2007; Pin *et al*, 2007). There is consensus that RET methods strongly support the existence of receptor heteromers in living cells (Ferre *et al*, 2009). However, their existence in the CNS still remains a challenge but brain heteroreceptor complexes have been detected (see below) (Borroto-Escuela *et al*, 2013).

The demonstration of the existence of higher-order oligomers in living cells appears possible thanks to the combination of two fluorescence-based techniques. The detection of complexes containing metabotropic glutamate receptor 5 (mGlu5R), adenosine A2A receptor (A2AR) and dopame D2 receptor (D2R) may be achieved by combining the bimolecular fluorescence complementation (BiFC) technique with the BRET approach (Cabello *et al*, 2009). In brief, a fluorescence signal is created when two non-fluorescent fragments of a fluorescent protein (eg, YFP) when brought in close proximity by a receptor heterodimer (eg, mGlu5R/D2RYFP), allowing this reconstituted fluorescent protein to act as an acceptor fluorophore in a BRET process (eg, A2ARRluc-mGlu5R/D2RYFP). Indeed, by means of this BiFC/BRET approach it is possible to demonstrate the likely formation of A2AR-mGlu5R-D2R heteroreceptor complexes in cultured cells. Interestingly, by using triple-labeling post-embedding immunogold and detection at the electron microscopic level, the precise codistribution of these receptors in the extrasynapatic plasma membrane of glutamate synapses of the striatopallidal GABA neurons is achieved (Cabello *et al*, 2009). As such, it can be hypothesized that an A2AR-mGlu5R-D2R heteroreceptor complex exists in the striatum.

The sequential BRET–FRET technique is also developed specifically for the potential identification of higher-order heteroreceptor complexes in living cells, including the CB1-D2-A2A heteroreceptor complex (Carriba *et al*, 2008). Through a combination of BRET and FRET, results are obtained consistent with the existence of this higher-order heteroreceptor complex. This observation is in line with the indications for the existence of striatal CB1-D2 heteroreceptor complexes obtained in previous work on the brain (Fuxe *et al*, 2010; Marcellino *et al*, 2008). This higher-order heteroreceptor complex is an integrator of DA, adenosine, and endocannabinoid signals. Through a combined bioluminescence/fluorescence complementation and energy transfer, several dopamine D2 receptors have also been located in close molecular proximity in living mammalian cells, consistent with their organization as higher-order dimers at the plasma membrane (Guo *et al*, 2008).

Overall, the RET and BiFC assays have inherent drawbacks mainly associated with the dependence of the ectopical expression of a fluorescent protein-fusion receptor, which may not necessarily behave exactly as their native counterparts. In addition, these assays include the risk of generating overexpression-mediated artificial receptor complexes. Therefore, the future of these techniques will rely on their ability to successfully detect native receptor oligomers, which has been accomplished with time-resolved FRET between GPCR ligands (Albizu *et al*, 2010), but high levels of receptor expression appears necessary.

The detection of native striatal A2AR–D2R heteroreceptor complexes has been achieved by means of another fluorescence-based approach, the protein ligation assay (Trifilieff *et al*, 2011; Borroto-Escuela *et al*, 2013). The PLA technique involves the use of two specific primary antibodies against the receptors being studied (ie, D2R and A2AR). The pair of secondary antibodies bears complementary oligonucleotides, which upon close proximity, usually a maximum of 16 nm between secondary antibodies, anneal into a circular dsDNA molecule. This

serves as a template for rolling circle amplification, resulting in a long single-stranded rolling circle product attached to one of the proximity probes. As the rolling circle product is linked to the proximity probe, it is attached at the site where the proximity probe bound and the location of the heteroreceptor complex is revealed (Borroto-Escuela *et al*, 2010a; Romero-Fernandez *et al*, 2012; Soderberg *et al*, 2006; Trifilieff *et al*, 2011; Borroto-Escuela *et al*, 2013). The rolling circle products can then be detected and quantified by hybridizing fluorescent oligonucleotides to the repeated sequences of the rolling circle products which render them visible by fluorescence microscopy. This makes it possible to study the localization and modulation of brain heteroreceptor complexes as fixed tissue is used. The PLA signal is influenced by the length of the oligonucleotides linked to the secondary antibodies. It is important to mention here that the main drawback of this technique lies in obtaining specific primary antibodies. Thus, appropriate controls should be used in each step. Another drawback is that the antibodies used can have a bivalent character and bind both receptors enhancing receptor cross-linking and the PLA reaction can give unspecific nuclear staining. The analysis shows that molecular proximity is necessary for the generation of the PLA signal eg, from A2A–D2 heteroreceptor complexes in striatum (Trifilieff *et al*, 2011; Borroto-Escuela *et al*, 2013). The findings indicate that PLA can be used to demonstrate GPCR heteroreceptor complexes *ex vivo* in brain tissue (Borroto-Escuela *et al*, 2013; Trifilieff *et al*, 2011).

Overall, besides the described weaknesses and drawbacks of the fluorescence-based assays, the strengths of these methodologies rely on their usefulness in the discovery and characterization of new GPCR-containing multimeric complexes in living cells and eventually in native tissue (Ferre *et al*, 2009; Kenakin *et al*, 2010; Pin *et al*, 2007). BRET has provided especially strong support for the existence of receptor heteromers in artificial cell systems (Marullo and Bouvier, 2007). It is therefore predicted that BRET will evolve into powerful biotechnological tools in several research areas, such as drug discovery, involving, eg, multiplexing of multicolor BRET (Breton *et al*, 2010). Another new approach is to measure BRET between a receptor heteromer and a subunit of the heterotrimeric G protein (Urizar *et al*, 2011). A complemented luminescence is obtained through the receptor–receptor interaction and communication can be measured between a defined heteromer and the G protein without interference from receptor homomers or cross-talk.

GPCRs AND THEIR RECEPTOR–RECEPTOR AND RECEPTOR–PROTEIN INTERACTIONS

Over the last 15 years large numbers of GPCR heteroreceptor complexes have been discovered characterized by allosteric receptor–receptor interactions, which indicates that such structures represent a general mechanism for molecular integration in the receptor field of high relevance for drug development (Agnati *et al*, 2003; Albizu *et al*, 2010; Ciruela *et al*, 2012; Ciruela *et al*, 2010; Fuxe *et al*, 2007b; Fuxe *et al*, 2009; George and O'Dowd, 2007; Lee *et al*, 2003; Milligan and White, 2001; Vilardaga *et al*, 2010). In contrast with the class C receptors, which exist as stable dimers (Gurevich and Gurevich, 2008a), the class A receptors appear to exist in a monomer–dimer equilibrium, which may vary at different stages of their life cycle (Gurevich and Gurevich, 2008b; Lambert, 2010). This may help explain opposing views on the role of GPCR monomers versus dimers (Chabre and le Maire, 2005; Fotiadis *et al*, 2006). The class A GPCR dimers are often transient as seen from their half-lives determined from the rate of association and dissociation (Gurevich and Gurevich, 2008b; Lambert, 2010). In the case of the neurotensin NTS1 receptor dimer, we observe a half life of 340 s at a Kd value of 20 nM for self-association (White *et al*, 2007). Furthermore, using total internal reflection fluorescence imaging of single molecules evidence has been found that M1 muscarinic acetylcholine receptors have an estimated half-life of 0.5 s (Hern *et al*, 2010). It has been proposed that transient kiss-and-run heteroreceptor complexes can be of relevance for some forms of ultra-short and short memory (less than 500 ms) (Agnati *et al*, 2010). One possible mechanism for encoding such memories may be the storage of information through on-going allosteric receptor–receptor interactions in higher-order heteroreceptor complexes like tetrameric complexes with a circular organization. In these receptor complexes the allosteric receptor–receptor interactions may result in reverberating receptor activity, which disappears when the transient tetrameric receptor complex falls apart into, eg, two heterodimers.

As to the possible existence of higher-order heteroreceptor complexes in the brain, functional A2AR–D2R-mGluR5 heteroreceptor complexes in the GABAergic striato-pallidal neurons have often been considered based on the high and selective co-expression of mGlu5R, D2R and A2AR in these particular nerve cells, on the existence of A2AR–D2R heteroreceptor complexes (Figure 2) (Canals *et al*, 2003) and A2AR–D2R-mGlu5R heteroreceptor complexes (Cabello *et al*, 2009) in living cells and on the existence of strong multiple interactions between the three receptors in striatum (Fuxe *et al*, 2008a; Popoli *et al*, 2001).

In cells nearly all functions are mediated by orchestrated multiprotein complexes. Interestingly, apart from interacting with and activating G proteins, GPCRs also bind a plethora of GPCR-interacting proteins (GIPs) (Figure 3). GIPs, either located intracellularly or associated to cell membranes, contain specific GPCR-interacting domains, which sustain, under some cellular conditions, the formation of functional multiprotein complexes necessary for both G protein-dependent and -independent signaling. On the other hand, some GIPs may just act as scaffold proteins which anchor GPCRs to specific plasma membrane domains and thus contribute to the targeting and subcellular distribution of GPCRs. Overall, by impinging on the GPCR trafficking, localization and/or pharmacological properties, GIPs have a prominent role in GPCR biology as they fine-tune the receptor functioning (Ritter and Hall, 2009).

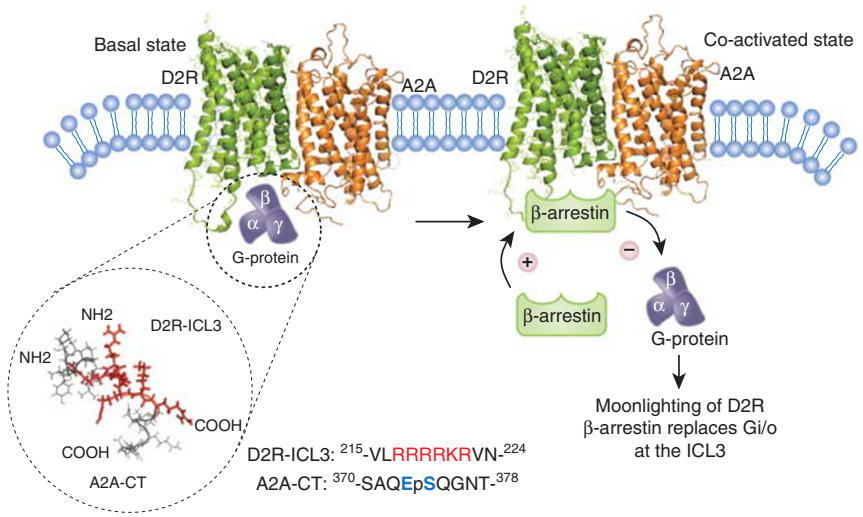

Figure 2. Illustration of receptor protomers in heteroreceptor complexes as moonlighting proteins.The A2A–D2 heteroreceptor complex example. The D2 protomer can moonlight under the influence of the allosteric antagonistic A2A–D2 receptor–receptor interaction (see broad arrow in right receptor complex from A2AR to D2R). It may especially involve the intracytoplasmatic part of the receptor interface with the positively charged arginine rich epitope in D2R-ICL3 (215-VLRRRRKRVN-224) interacting with the negatively charged phosphorylated serine epitope in the A2AR-CT (370-SAQEpSQGNT) seen in the left part. The A2A agonist activated receptor–receptor interaction is proposed to moonlight the D2 protomer through a conformational change in the ICL3 strand which switches the ICL3 from binding Gi/o to binding beta-arrestin (right part). Thus, Gi/o signaling of the D2R protomer is reduced and signaling over beta-arrestin dominates associated also with internalization of the receptor complex. A change of function takes place. ICL3, intracellular loop 3; CT, C terminal part.

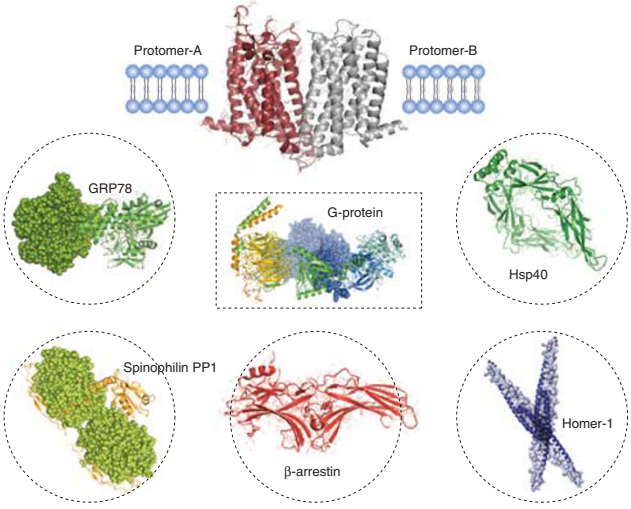

Figure 3. GPCR interacting proteins linked to GPCR heteromers. Apart from interacting with and activating G proteins, GPCR protomers also bind a plethora of GPCR interacting proteins (GIPs). GIPs, either located intracellularly or associated to cell membranes, contain specific GPCR interacting domains, which sustain, under some cellular conditions, the formation of functional multiprotein complexes necessary for both G protein-dependent and -independent signaling. On the other hand, some GIPs may only act as scaffolding proteins, which anchor GPCRs to specific plasma membrane domains (eg, lipid rafts, cell junctions, etc). In this way, they can contribute to the targeting and subcellular distribution of GPCRs. Hsp, heat shock protein; PP1, protein phosphatase 1; GRP, 78 kDa glucose-regulated protein.

RECEPTOR INTERFACE

Prediction of receptor interfaces may be made with intrinsic disorder analysis showing flexible and malleable regions (Agnati *et al*, 2008; Guidolin *et al*, 2010). Potential interfaces in the trans-membrane helices can be estimated using the G-protein-coupled receptor interaction partners method (Guidolin *et al*, 2010). Extracellular, intracellular, and transmembrane domains of 14 GPCRs have been considered and the propensity of each of these domains for a structured or unstructured conformation has been evaluated through *ad hoc* computer programs. The N- and C-terminals, as well as intracellular loop 3, have been shown to have a high propensity towards an unstructured conformation. Thus, they are potentially very plastic domains, which interact particularly with other protein domains. The disorder located especially in the intracellular loop 3 (ICL3) and in the C-terminus domain is strongly linked to receptor–receptor interactions.

As an example, we will show that three-dimensional molecular models of the seven TM regions of A2AR and D2LR can be built based on its crystal structure (PDB code 3EML, (Jaakola *et al*, 2008)) and the crystal structure of rhodopsin (PDB codes 2Z73, (Li *et al*, 2004)), respectively, as structural templates, by means of the homology modeling program Accelrys Discovery Studio 2.5 (San Diego, CA, USA). Dimerization of GPCR can result from either covalent (disulfide crosslink) or non-covalent unions (electrostatic and hydrophobic unions) between the receptor protomers. Helix–helix interactions are seen from a lateral view. Representation of the D2LR (TM-IV)-A2AR (TM-V) interaction in the A2AR–D2LR heterodimer is shown in Figure 4 (Borroto-Escuela *et al*, 2011a; Borroto-Escuela *et al*, 2010d).

In the intracellular part of the receptor interface, a high energy strength double arginine-phosphate electrostatic interaction was found in the A2A–D2 heteromer by Ciruela *et al* (2004) and Woods *et al* (2008). It possesses a

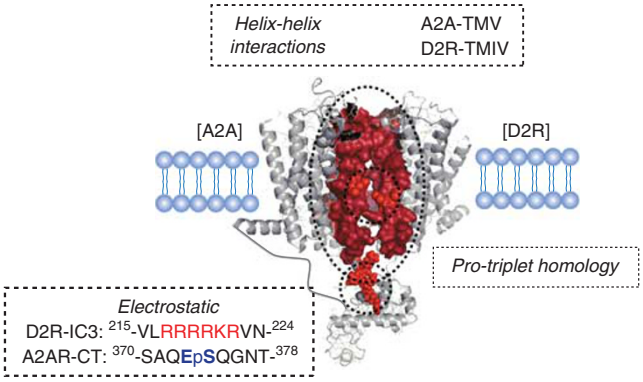

Figure 4. GPCR heteromer receptor interface illustrated in the A2AR–D2R heteromers. (Helix–helix interaction). Representations are shown from a lateral view of the D2R (TM-IV/V)-A2AR(TM-IV/V) model of the A2AR–D2R heterodimer. (Electrostatic interactions) Illustration of positively charged arginine-rich epitope 215VL**RRRRKR**VN224 in the N-terminal part of the third intracellular loop of D2R electrostatically interacting with the negatively charged C-terminal epitopes of the A2AR (370-SAQ**EpS**QGNT-378,388-H**E**LKGVCP**E**PPGL**DD**PLAQ**D**GAGVS-412). (Pro-triplet homology interactions) *In silico* analysis and sequence alignment of A2AR and D2R receptor homologies containing the triplet homologies AAR, AQE, VLS, and VYI (in red) in the receptor interface. The triplet homology VYI is located in the N-terminal of A2AR and in the fifth transmembrane α-helix (TM-V) of D2R, the triplet homology VLS is located in the TM-IV of both A2AR and D2R, the triplet homology AAR is located in the third intracellular loop (IL3, between TM-V and TM-VI) of both A2AR and D2R and the triplet homology AQE is located in the IL3 of D2R and in the C-tail of A2AR. Note that the triplet AQE is adjacent to S374, which seems to have a key role in the A2AR–D2R receptor interfaces. Also note that the triplet VYI cannot have a role as the homology is located in the N-terminal part of the A2AR and the fifth TM region of the D2R.

covalent-like stability as has been demonstrated with mass spectrometry in combination with collision-induced dissociation experiments and confirmed by pull-down techniques (Ciruela *et al*, 2004; Woods *et al*, 2008). A mass spectrum is produced from a mixture of A2AR and D2R epitopes. The mixture of the negatively charged A2A serine-phosphate containing motif from the C-terminal A2A and the positively charged D2 VLRRRRKRVN arginine rich motif from the IC loop 3 results in the formation of a noncovalent complex (Figure 4). These electrostatic interactions likely represent important hot spots also in the receptor interface (IC loop 3 of D3/D4-C-terminal of A2A) of the putative A2AR–D3R and A2AR–D4R heteromers (Fuxe *et al*, 2005; Torvinen *et al*, 2005). These electrostatic bonds in the intracellular part of the receptor interface may be dynamically regulated by kinases and phosphatases which increase and reduce the stability of the heterodimer formed.

The importance of these electrostatic interactions between A2AR and D2R has now been validated by a point mutation of serine 374 to alanine in the A2AR C-terminal tail which reduces the A2AR ability to interact with D2R. This is seen from the reduction of BRET signals and thus of heteromerization, which is enhanced in combination with mutation of the two aspartates (Borroto-Escuela *et al*, 2010b; Borroto-Escuela *et al*, 2010d). BRET1 studies have been performed on A2AR and D2LR heteromerization in HEK293T cells. BRET1 saturation curves have been obtained for the D2LR–A2AR heteroreceptor complex and compared with D2LR–A2AR mutant heteroreceptor complex. The comparison shows markedly reduced BRETmax values after serine 374 point mutation, especially in combination with the mutation of the two aspartates in the A2AR C-terminal. Moreover, this point mutation abolishes the A2AR-mediated inhibition of both the D2R high-affinity agonist binding and signaling as studied for example in a forskolin-induced cAMP-response element (CRE)-luciferase reporter gene assay. The forskolin-induced increase of luciferase activity by a direct activation of AC is significantly reduced by quinpirole, an action which is fully counteracted by the A2A agonist and by the D2R like antagonist raclopride. However, after the single point mutation of serine 374 in the A2AR, it is no longer possible for the A2A agonist (50 nM) to counteract the quinpirole-induced reduction of luciferase activity, while raclopride is still able to counteract it. These results validate a key role of serine 374 in the A2AR–D2R interface in making the allosteric modulation of the D2R possible. Serine 374 is also evolutionary conserved.

Electrostatic interactions have also been shown to be involved in the cytoplasmic receptor interface of the D1–D2R heteromer (O'Dowd *et al*, 2011). The two adjacent positively charged arginins in D2R IC loop3 are necessary for the D1–D2R heteroreceptor complex formation as were two adjacent negatively charged glutamate residues in the C-terminal tail of the D1. These results validate the impact of electrostatic interactions in the intracellular part of the receptor interface between IC3 and the C-terminal for the formation of GPCR heteroreceptor complexes.

THE ROLE OF THE TRIPLET PUZZLE IN THE RECEPTOR INTERFACE

Based on a mathematical approach, Tarakanov and Fuxe (2010, 2011a) deduced a set of triplet homologies ('triplet puzzle') that may be responsible for protein–protein interactions, including receptor heteromers, and human immunodeficiency virus (HIV) entry. For example, the triplet of amino-acid residues LLG (Leu-Leu-Gly) appears as a homology in six receptor heteromers (ie, in both receptors, see Table 1): 5-hydroxytryptamine 1A receptor (5HT1AR)–fibroblast growth factor receptor 1 (FGFR1), 5HT1AR–galanin receptor 1 (GalR1), FGFR1–platelet-derived growth factor receptor, alpha polypeptide (PDGFRA), 5-HT2A receptor (5HT2AR)–metabotropic glutamate receptor 2 (mGluR2), adrenergic receptor, alpha1B (ADRA1BR)–adrenergic receptor, alpha 1D (ADRA1DR), and cannabinoid receptor type 1(CB1R)–cannabinoid receptor type 2 (CB2R), but does not appear as a homology in any known non-heteromers (GABAB2 receptor subunit

TABLE 1 Example of Triplet Homologies in the Protomers of Human Receptor Heteromers and Marine Sponges

Receptor heteromer	Species	Reference	Function	LLG	KTV	TVS
5HT1A-FGFR1	Human	(Borroto-Escuela *et al*, 2012a)	MAPK signal and neuronal plasticity	+	+	+
D1-NMDA	Human	(Lee *et al*, 2002)	Modulation of NMDA signaling	−	−	+
5HT1A-GalR1	Human	(Borroto-Escuela *et al*, 2010c)	Modulation of serotonin signaling	#	−	+
FGFR1-PDGFRA	Human	(Faraone *et al*, 2006)	Receptor binding, signal and the control of endothelial cell function	+	#	−
5HT2A-mGluR2	Human	(Gonzalez-Maeso *et al*, 2008)	Activation of mGluR2 inhibits hallucinogen-specific signaling of 5HT2A	#	−	−
ADRA1B-ADRA1D	Human	(Hague *et al*, 2006)	Enhanced functional activity and receptor trafficking	#	−	−
CB1-CB2	Human	(Callen *et al*, 2012)		+	−	−
TLR1-TLR2	Human	(Jin *et al*, 2007)	Expression of pro- and anti-inflammatory cytokine genes and role in innate immune response	−	+	−
ITGA-ITGB	Sponge	(Pancer *et al*, 1997)	Cell-to-cell contacts and cell-to-extracellular matrix interactions	−	−	#

Abbreviations: ADRA1D, alpha-1D adrenergic receptor; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; D1, dopamine D1 receptor; FGFR1, fibroblast growth factor receptor 1; GalR1, galanin receptor type 1; 5-HT1A, 5-hydroxytryptamine receptor 1A; 5HT2A: 5-hydroxytryptamine receptor 2A; ITGA, metazoan adhesion receptor subunit integrin-α; ITGB, metazoan adhesion receptor subunit integrin-β; mGluR2, metabotropic glutamate receptor; 2PDGFRA, platelet-derived growth factor receptor alpha; TLR1, toll-like receptor 1; TLR2, Toll-like receptor 2.
Amino acid description one/three letter code: LLG (Leu-Leu-Gly), KTV (Lys-Thr-Val), and TVS (Thr-Val-Ser)
(+, yes in both receptors, #, may mediate their interaction, and −, not in any receptor).
The acronyms are listed according to the UniProtKG Accession number (in bold): 5-HT1A **(P08908)**; FGFR1 **(P11362)**; D1 **(P21728)**; GalR1 **(P47211)**; PDGFRA **(P16234)**; 5HT2A **(P28223)**; mGluR2 **(Q14416)**; Alpha-1D adrenergic receptor **(P25100)**; ADRA1D **(P25100)**; CB1 **(P21554)**; CB2 **(P34972)**; TLR1 **(Q15399)**; TLR2 **(O60603)**; ITGA **(CAA65943)**; ITGB **(CAA77071)**.

(GABAB2R)–A2AR, A2A-dopamine receptor 1(D1R), adenosine receptor 1(A1R)–D2R, neurotensin receptor 1(NTS1)–D1R, thyrotropin receptor (TSHR)–D2R, and cluster of differentiation 4 glycoprotein (CD4)–D2R; (Tarakanov and Fuxe, 2010). According to recent biochemical studies (Borroto-Escuela *et al*, 2012a; Romero-Fernandez *et al*, 2011), such triplets exist in the interacting domains forming the receptor interface. Furthermore, a 'guide-and-clasp' manner of receptor–receptor interactions has been proposed where the 'adhesive guides' may be the triplet homologies (Tarakanov and Fuxe, 2010). These interactions probably represent a general molecular mechanism for receptor–receptor interactions (Fuxe *et al*, 2012). This hypothesis is supported by the demonstration that the SVR triplet homology in IC loop3 of the mu-delta opioid receptor heteroreceptor complex (MOR-DOR) is crucial for the formation of this complex (O'Dowd *et al*, 2011). Based on this theory, for the first time it was indicated that several triplet homologies of the hetero-receptor complex 5HT1AR-FGFR1 as well as of other receptor heteromers of the human brain may be the same as in cell-adhesion receptors (built as heterodimers) of marine sponges (Tarakanov *et al*, 2012a, b), known to be highly conserved from the lowest metazoa to vertebrates (Buljan and Bateman, 2009; Gamulin *et al*, 1994; Pancer *et al*, 1997).

Materials and methods related to recent biochemical studies of the interacting domains of the receptor–receptor interface have been described thoroughly in our previous papers (for example, see Borroto-Escuela *et al* (2012a)). In brief, with *in situ* proximity ligation assay (PLA) and supported by coimmunoprecipitation and colocation of the 5HT1AR and FGFR1 immunoreactivities evidence for the existence of 5HT1AR–FGFR1 heteroreceptor complexes in the hippocampus and dorsal and median raphe nuclei of the rat has been obtained. Taken together, the 5HT1A receptors comprise part of a 5HT1AR–FGFR1 heteroreceptor complex. The triplet homologies and the existence of a basic set of triplets in the two participating protomers that may be responsible for their homo- or heterodimerization were computed using a modified version of our original software (Tarakanov and Fuxe, 2010).

The triplet homologies LLG (Leu-Leu-Gly), KTV (Lys-Thr-Val), and TVS (Thr-Val-Ser) exist in several categories of receptors in the human brain (Table 1) and also in marine sponges. Particularly, the triplet TVS (Thr-Val-Ser) exists in both integrin-α and integrin-β subunits of marine sponges. The triplet LLG (Leu-Leu-Gly) is only found in the integrin-β subunit, whereas the triplet KTV (Lys-Thr-Val) does not appear in any subunit (Table 1). The location of the KTV and TVS triplets in the different categories of receptor heteromers and proteins is displayed in Figure 5, whereas the location of the triplet LLG in these receptors and proteins (Table 1) is present and discussed in the paper by Tarakanov and colleagues (Tarakanov *et al*, 2012a). Among the GPCRs, such triplets can be located in several types of domains including their interface mediating the receptor–receptor interactions (see Figure 5 and the references in Table 1).

For example, the LLG triplet homology has been identified in six receptor heteromers: 5HT1AR–FGFR1, 5HT1AR–GalR1, FGFR1–PDGFRA, 5HT2AR–mGluR2, ADRA1BR–ADRA1DR, and CB1R–CB2R (Table 1). These LLG triplets are mainly located in the transmembrane interface of these heterodimers where hydrophobic leucine-rich motif residues enable the formation of a hydrophobic area at the helix–helix interface, facilitating receptor–receptor interactions (Tarakanov *et al*, 2012c). They may mediate

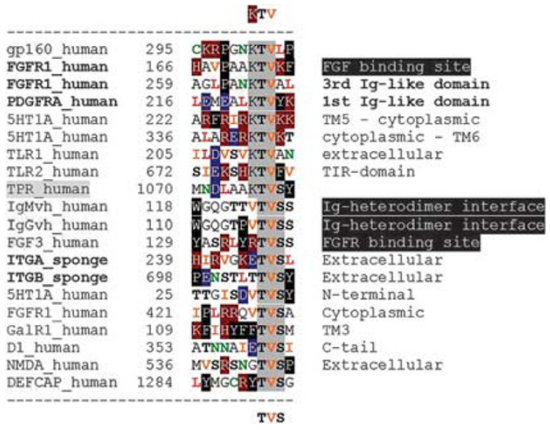

Figure 5. Examples of triplet homologies (dark shaded) KTV (Lys-Thr-Val) and TVS (Thr-Val-Ser) in marine sponges and different types of receptors and proteins: light shaded are six proteins (envelope HIV glycoprotein (gp160), tetratricopeptide repeat (TPR; scaffolds to mediate protein–protein interactions), IgG heavy chain variable region (IgGvh), IgM heavy chain variable region (IgMvh), FGF3, and death effector filament-forming CED4-like apoptosis protein (DEFCAP); bold text marks two receptor heteromers (human FGFR1-PDGFRA and marine sponge ITGA-ITGB), which may possess the corresponding triplet homologies KTV and TVS in their interfaces; TM is the transmembrane α-helix of GPCR. Three following amino acids are shown in white letters on the black background as the basic elements of protein binding: W (Trp), F (Phe), and P (Pro). The following amino-acid residues are marked by a color code as the basic elements of leucine-rich motifs. Red bold L is leucine (Leu). Orange bold I and V are isoleucine (Ile) and valine (Val) that may also occupy a position of Leu in leucine-rich motifs. Green N and C are asparagine (Asn) and cysteine (Cys). Black bold S and T are serine (Ser) and threonine (Thr) where agonist-regulated phosphorylation may occur. White letters are charged amino acids: negatively charged (dark blue background) E (Glu) and D (Asp) or positively charged (dark red background) R (Arg), K (Lys), and H (His).

interactions of 5HT1AR–GalR1 (via their TM1–TM4 interface), 5HT2AR–mGluR2 (TM2–TM2), and ADRA1BR–ADRA1DR (TM4–TM4). In line with this view, leucine-rich repeats are known to contribute to protein–protein interactions (Kobe and Kajava, 2001) and the leucine zipper functions as a structural motif in proteins where the hydrophobic leucine residues helps bonding of adjacent transmembrane helices (Landschulz *et al*, 1988). However, the same triplets will not interact in FGFR1–PDGFRA (as the triplet LLG is cytosolic in FGFR1 and extracellular in PDGFRA) and 5HT1AR–FGFR1 (transmembrane in 5HT1A and cytosolic in FGFR1).

In contrast, the triplet LLG is not present in the D1–NMDA receptor heteromer, toll-like receptor heteromers, and integrin heteromer of marine sponges (see Table 1). Although the triplet KTV is found in 5HT1AR (in the third intracellular loop between TM5 and TM6, see Figure 5), FGFR1 (in its ligand binding site and the third Ig-like domain), PDGFRA (in the first Ig-like domain), Toll-like receptor 1 (TLR1) (in the extracellular domain), and Toll-like receptor 2 (TLR2) (in the intracellular signaling domain TIR), an interaction may only be mediated in FGFR1-PDGFRA heteromers (by their Ig-like domains, see Figure 5 and Table 1). In addition, the triplet TVS may mediate an interaction between the subunits of the integrin receptor heteromer (integrin subunit, alpha (ITGA)- integrin subunit, beta (ITGB) of marine sponges (by their extracellular domains). These observations strengthen our theory that these triplet homologies ('triplet puzzle') participate in recognizing the other receptor protomer of the heteromer via postulated 'guide-and-clasp' interactions in the receptor interface (Fuxe *et al*, 2012; Tarakanov and Fuxe, 2010, 2011a; Tarakanov *et al*, 2012a). In fact, indications exist for their location in the receptor interface (Borroto-Escuela *et al*, 2012a; Romero-Fernandez *et al*, 2011), which can involve different categories of receptor heteromers.

It should also be noted that the set of integrin triplets of marine sponges does not exclude the presence of other sets of triplets, which seem to be typical for several kinds of receptor heteromers, including their possible origin from protein–protein interactions (Tarakanov *et al*, 2011b, 2012a). For example, the triplets SSS (Ser-Ser-Ser), SGS (Ser-Gly-Ser), and GGG (Gly-Gly-Gly) found in variable chains of IgG and IgM participating in antigen recognition also exist as triplet homologies in two GABAB1-containing heteromers: GABAB1–mGluR1 and GABAB1–C-X-C chemokine receptor type 4 (CXCR4) (Tarakanov *et al*, 2012c). Furthermore, substitution of any of the three GGG residues in the C-terminal tail of the delta opioid receptor has been found to disrupt the mu-delta opioid receptor heteromer (O'Dowd *et al*, 2012). Another example is the set of triplet homologies LLE (Leu-Leu-Glu), LEE (Leu-Glu-Glu), and NNL (Asn-Asn-Leu), which looks typical for a set of three receptor heteromers: GABAB1-GABAB2 (GABA B receptor), TLR1-TLR2, and TLR2-TLR6 (Tarakanov *et al*, 2012c). As for marine sponges, the triplet RAA (Arg-Ala-Ala) may mediate the heteromerization of the serotonin and the fibroblast growth factor receptors subtypes 5HT1AR and FGFR1 (Tarakanov *et al*, 2012b), whereas the triplets AVI (Ala-Val-Ile) and DLL (Asp-Leu-Leu) may help mediate the heteromerization of the dopamine receptors D1 and D2 (Tarakanov *et al*, 2012b). Thus, we can postulate that such sets of triplet homologies may determine the specificity of receptor–receptor interactions.

It appears that such triplets may have a role in neuroinflammation and other disorders (Tarakanov *et al*, 2011b). Such findings may also give a new molecular basis for the therapeutic actions of antidepressant drugs as their increase of extracellular 5-HT in the brain can activate the receptor heteromer 5HT1A–FGFR1 in the hippocampus restoring FGFR1 signaling and neuronal plasticity, which may potentially reduce the atrophy of the hippocampus found in depression (Borroto-Escuela *et al*, 2012a; Borroto-Escuela *et al*, 2011b).

Parts of the triplet puzzle of homologies in receptor heteromers appear to originate from the cell-adhesion receptor triplets of marine sponges, which are phylogenetically very old. Thus, the triplet puzzle may be part of a general mechanism for protein–protein interaction. For example, these triplets may 'guide-and-clasp' direct

protein–protein interactions by the involvement of leucin-rich motifs as well as a flexibility of the protein skeleton provided by LLG (Leu-Leu-Gly), phosphorylation of serine in TVS (Thr-Val-Ser) and electrostatic interactions of lysine in KTV (Lys-Thr-Val).

PROTOMERS IN HETERORECEPTOR COMPLEXES CAN ACT AS MOONLIGHTING PROTEINS VIA INTERMOLECULAR RECEPTOR–RECEPTOR INTERACTIONS

As mentioned in the discussion on moonlighting proteins, indications exist that changes in the allosteric receptor–receptor interactions among receptor protomers through the formation of different types of receptor heteromers can alter the function of the individual receptor protomer. Moonlighting can also develop in the receptor protomer via actions of receptor agonists and allosteric modulators. In this way, through conformational changes in these heteroreceptor complexes, moonlighting may develop in a single GPCR protomer by, for example, switching its coupling to other types of G proteins, to beta-arrestins, to other types of GPCR-interacting proteins, to receptor tyrosine kinases (RTKs) and to ion channel receptors and through changes in its orthosteric site altering its transmitter and agonist specificity (Bockaert *et al*, 2003; Borroto-Escuela *et al*, 2011a; Fuxe *et al*, 2007b; Hasbi *et al*, 2011).

A single GPCR protomer may undergo changes in cotrafficking with increased internalization through changes in allosteric receptor–receptor interactions due to its participating in altered heteroreceptor complex. The internalized receptor may then become linked to cytosolic proteins leading to an altered conformation and transport to the nuclei. The receptor may then moonlight as a transcription factor. As possible transcription factors, the GPCRs could directly modulate gene expression of high relevance for information handling, trophism, and neurodegeneration. A pathological change in the moonlighting properties of a receptor protomer may have major consequences for its function, especially of receptor protomers having a multitasking role with multiple interactions with other receptors and proteins, so-called hub receptors (Tarakanov *et al*, 2012b). A moonlighting dysfunction in a hub receptor protomer of key molecular networks may substantially contribute to the development of neurological and mental disorders.

One early example of moon-lighting is the GABAB receptor, which is a heterodimer formed from GABABR1 and GABABR2 subtypes that alone may only function as chaperone proteins and receptor interacting proteins. By their heteromerization mainly via their C-termini (coiled-coil interaction) they become the GABAB receptor, which bind GABA via GABABR1 and signal to the G protein via GABABR2 (Marshall *et al*, 1999). Thus, a novel function of acting as a receptor has developed through the protein–protein interaction.

The D1R–D2R heteroreceptor complex, discovered by the (George and O'Dowd (2007) and Rashid *et al* (2007b)), is a beautiful example of moonlighting of receptor function. The D1R–D2R heteroreceptor complex demonstrates how the formation of such a complex can switch the G protein coupling of participating protomers (D1R: Gs; D2R: Gi) to other G proteins (Gq) (Rashid *et al*, 2007a). Thus, upon coactivation of D1R and D2R in this heteromer, a selective Gq/11 activation occurs, which produces increases in phospholipase C (PLC) activity and a rapid rise in intracellular calcium levels without influencing adenylate cyclase activity regulated by Gs and Gi proteins. Thus, the PLC activation leads to intracellular calcium release and increased levels of calcium/calmodulin dependent protein kinase II alpha (CaMKII), which contributes to synaptic plasticity and behavioral sensitization to drug treatment (Hasbi *et al*, 2011; Rashid *et al*, 2007b). These results illustrate how allosteric receptor–receptor interactions between the D1R and D2R protomer can produce moonlighting of their functions by switching their coupling to Gq upon their coactivation. The dysfunction of the D1R–D2R heteroreceptor complex may contribute to neuropsychiatric diseases (Hasbi *et al*, 2011).

This heteroreceptor complex via the allosteric receptor–receptor interaction has also been shown to moonlight D1 and D2 protomer recognition giving them a unique pharmacology (Rashid *et al*, 2007a; Rashid *et al*, 2007b). The D1R agonist SKF83959 has been found to be a selective agonist for this receptor heteromer by being a full agonist at the D1R protomer and a partial agonist at the D2R protomer existing in a pertussis toxin-insensitive state leading to rapid activation of Gq/11 and PLC. On the other hand, the D1R-like agonist SKF83959 does not activate adenylyl cyclase (AC)-linked D1R and D2R and may, therefore, be a unique agonist for the D1R–D2R heteroreceptor complex. Thus, this work represents an interesting example of how moonlighting changes in the receptor-binding domains of the two protomers in the D1R–D2R heteroreceptor complex generate new recognition specificity and new drugs for neuropsychopharmacology. In fact, an increased proportion of the D1R–D2R heteroreceptor complex is in a high-affinity state in schizophrenia and after chronic amphetamine treatment.

Moonlighting in A2AR–D2R heteroreceptor complexes can also be seen from the A2AR agonist modulation of the D2R agonist induced beta-Arrestin2 recruitment (Borroto-Escuela *et al*, 2011a). The results indicate that the antagonistic allosteric receptor–receptor interaction in A2AR–D2R heteroreceptor complex favors beta-arrestin2 recruitment to the D2R protomer with subsequent cointernalization associated with a reduced time onset of Akt phosphorylation followed by a rapid dephosphorylation. Thus, beta-arrestin2 action becomes more rapid and short-lived and, in this way, mimics G protein-mediated signaling (Borroto-Escuela *et al*, 2011a). The moonlighting of the D2R protomer that takes place via the antagonistic allosteric A2AR–D2R interaction results in

a rapid switch from D2-Gi coupling towards beta-arrestin2-mediated D2R signaling linked to the formation of a possible A2AR–D2R-beta–arrestin2 complex (Figure1) (Borroto-Escuela *et al*, 2011a).

Another moonlighting phenomenon in the A2AR–D2R heteroreceptor complex is that the positively charged D2R N-Terminal ICL3 epitope (VLRRRRKRVN) may switch from binding to the negatively charged A2AR epitope (SAQEpSQGNT), located in the medium segment of the C-terminus of the A2AR (Ciruela *et al*, 2004), to several negative epitopes of calmodulin (Woods *et al*, 2008), which alters the function of the D2R protomer through the involvement of calmodulin mediated signaling. This moonlighting phenomenon is modulated by calcium ions since they disrupt the binding of calmodulin to the D2R but not the A2AR (Woods *et al*, 2008), which favors the binding of the D2R N-terminal ICL3 epitope to the A2AR epitope or to epitopes of Gi/o. It is likely that in the absence of A2AR agonist activation with reduced antagonistic allosteric A2AR–D2R interactions, the conformational state in the ICL3 of D2R may favor its link to Gi/o. This may involve inter alia electrostatic interaction between triplets AAR (in D2R ICL3) and AAE (Gi/alpha1) (and/or their symmetric variants), which contributes to guide-and-clasp D2R-Gi/o interactions with maintenance of its major physiological role as activator of Gi/o signaling.

Changes in A1R recognition have been discovered by Nakata *et al* (2005) in A1R-P2Y1 heteroreceptor complex which gives evidence for moonlighting in the A1 receptor protomer of this heteromer. This heteromerization results in a conformational change in the A1R binding pocket, which leads to the appearance of an A1R receptor with P2Y1-like agonist pharmacology. In fact, a P2Y1 agonist binds to the A1R and produces an inhibition of AC, which is blocked by an A1R antagonist. Therefore, this A1R-mediated ATP response can be one of the mechanisms that might account for the ATP-induced inhibition of transmitter release, as ATP is inter alia coreleased with glutamate from nerve terminal networks. The moonlighting achieved via the allosteric A1R–P2Y1 receptor–receptor interaction is the formation of an A1R protomer with a P2Y1-like pharmacology leading to ATP recognition.

It seems likely that moonlighting may develop in the intracellular loops and C terminal of the GPCRs, especially in the long ICL3, as a result of dynamic allosteric interactions between different types of G proteins and other receptor interacting proteins in these domains of the receptor. Because of these dynamic conformational changes, the same amino-acid strand in these domains may in one state bind to a certain G protein and in a different state to another signaling protein, which may be a result of changes in guide–clasp interactions of this motif in relation to the interaction domains of these two signaling proteins. In this way, the receptor may change its signaling function and dynamic multitasking actions could develop. In the case with receptor-interacting proteins, the moonlighting will mainly operate by having intracellular signals change the function of the GPCR as a result of intracellular demands. In the case of moonlighting in heteroreceptor complexes, it is instead the result of a demand from the environment to integrate two or multiple signals into novel functions via changes in single amino-acid strands of several receptor domains, which takes place through allosteric receptor–receptor interactions in the extracellular, transmembrane, and intracellular domains. The integration of signals in higher-order heteroreceptor complexes may result in a more complex moonlighting phenomenon.

The field of moonlighting proteins continues to expand in this case into the field of receptor proteins, especially in relation to their formation of GPCR–GPCR and GPCR–RTK heteroreceptor complexes. The allosteric receptor–receptor interactions lead to changes in the guide–clasp interactions of key receptor motives involved in agonist/antagonist binding and binding of signaling proteins so that moonlighting develops. Therefore, another type of recognition and pharmacology can be found and another signaling protein spectrum is selected (biased agonism). The moonlighting in the GPCR–RTK heteroreceptor complexes may have special relevance for counteracting neurodegenerative processes and depression induced atrophy of neurons in view of the impact of RTK signaling on nerve cell survival and differentiation. Moonlighting in GPCR homoreceptor complexes may likely also develop due to the binding of multiple GPCRs and signaling proteins including the G proteins to the intracellular domains of the protomers. This alters via allosteric mechanisms the guide–clasp interactions between the strands of amino acids belonging to the protomer motives and the domains of different signaling proteins, which changes the pattern of receptor–protomer signaling.

DRUG DEVELOPMENT BASED ON RECEPTOR–RECEPTOR INTERACTIONS IN GPCR HETERORECEPTOR COMPLEXES

The field of receptor–receptor interactions in GPCR containing heteroreceptor complexes and protein–protein interactions in general have opened up new targets for drug development (Agnati *et al*, 2003; Fuxe *et al*, 1989; George *et al*, 2002) and several strategies can be exploited to develop new drugs based on targeting the heteroreceptor complexes (Soriano *et al*, 2009). Bivalent and dimeric ligands were introduced early on and have proven to markedly increase receptor affinity (Halazy *et al*, 1996; Perez *et al*, 1998) but, paradoxically, this design was not based on the existence of heteroreceptor complexes (George *et al*, 2002). The potential value of this approach is illustrated by the demonstration that opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a dimeric ligand series (mu-delta agonist-antagonist (MDAN) (Daniels *et al*, 2005). These dimeric ligands likely target the mu-delta opioid heteroreceptor complexes (George *et al*, 2000; Gomes *et al*, 2004) in which the delta opioid receptor antagonist blocks

the antagonistic delta-mu opioid receptor–receptor interaction. This leads to enhanced mu opioid receptor activity and potentiated morphine analgesia. With MDAN spacer arms of 19 atoms or above morphine-induced dependence and tolerance have been counteracted giving indications that the antagonistic allosteric receptor–receptor interactions in the mu-delta opioid receptor heteroreceptor complex are involved in producing dependence and tolerance to morphine (Daniels *et al*, 2005).

An increased abundance of mu-delta opioid receptor heteroreceptor complexes has recently been found after chronic morphine administration (Gupta *et al*, 2010). Chronic, but not acute, morphine treatment causes an increase in the abundance of these heteroreceptor complexes in key areas of the CNS, which are implicated in pain processing. Because of its distinct signaling properties, the mu-delta opioid heteroreceptor complex may, as outlined above, counteract morphine action and be a therapeutic target in the treatment of chronic pain and addiction (Gupta *et al*, 2010). This work is possible through a subtractive immunization strategy, which generates antibodies that selectively recognize the endogenous mu-delta opioid heteroreceptor complex but do not recognize single mu or delta opioid receptors (Rozenfeld and Devi, 2010). All these data support the view that this heteroreceptor complex is a drug target for treatment of morphine tolerance and dependence, as this complex develops a reduced G protein-coupling with signaling mainly operating via β-arrestin2 upon chronic morphine treatment. Treatment with delta opioid receptor antagonists in animal models reduces the β-arrestin2 coupling in the mu-delta opioid heteroreceptor complex and enhances mu opioid-protomer binding, signaling, and morphine-induced antinociception, which may be associated with a disruption of the mu-delta opioid–receptor–heteroreceptor complex.

George and O'Dowd (2007) were the first to demonstrate that agonists selective for a discrete heteroreceptor complex can be developed, in this case the D1–D2 heteroreceptor complex (Lee *et al*, 2004; Rashid *et al*, 2007a; Rashid *et al*, 2007b). Thus, SKF 83959 is a specific D1–D2R heteroreceptor complex agonist at the orthosteric-binding sites. The search continues for specific heteroreceptor complex agonists and antagonists as tools and potential drugs for mental and neurological diseases treatment. Such drugs have the potential to offer therapeutic effects with substantially reduced side-effects.

One major strategy is to target heteroreceptor and other protein complexes to develop a molecule that antagonizes a receptor–receptor interaction and more generally a protein/protein interaction, which interferes with the complex formation. It is therefore of great importance to identify the molecular structure of at least part of the receptor interface. Transmembrane interface interfering peptides have successfully been introduced to target selected heteroreceptor complexes like, eg, the FGFR1–5HT1A heteroreceptor complex (Borroto-Escuela *et al*, 2012a) leading to a marked reduction of the receptor–receptor interaction. These transmembrane interfering peptides are also useful for determining if an observed change of a receptor protomer function is caused by an allosteric receptor–receptor interaction or by receptor cross-talk in the intracellular signaling cascades. In addition, with increased understanding of the receptor interface it may be possible to develop drugs, which by targeting the receptor interface enhances the allosteric receptor–receptor interaction and/or increases their formation. This will be beneficial in the case, eg, of the FGFR1–5HT1A heteroreceptor complex, as it may enhance the ability of the 5-HT1AR protomer to increase the activity of the FGFR1 protomer. Neuroplasticity and trophism should in this way be improved. It is also possible that positive and negative allosteric modulators of one protomer can have a major role in modulating the function of the other protomer in the heteroreceptor complex through similar mechanisms.

The receptor interface has become an important target for the development of neurotherapeutic drugs and for understanding the function of heteroreceptor complexes and other protein complexes. Disrupting heteroreceptor and protein complexes in the cytoplasmic part of the receptor interface is possible through the use of Tat-tagged peptide mimics (Li *et al*, 2011). Tat is an 11-amino acid protein transduction domain of the HIV-1 Tat protein, which allows the peptide that mimics part of the intracellular receptor interface to pass through the plasma membrane of live cells in brain tissue. In this way, it becomes possible to block, eg, the electrostatic interactions in the intracellular receptor interface of heteroreceptor complexes and to understand their role in heteroreceptor complex function. The identification of interacting hot spots of protein–protein interactions in the receptor interface like the electrostatic receptor–receptor interactions is of special interest. They likely represent key targets for novel neurotherapeutics acting as modulators of receptor–receptor interactions in the heteroreceptor complexes and of protein–protein interactions in general in protein complexes.

Based on RET methods alone or in combination with bimolecular fluorescence complementation, evidence shows that three GPCRs can exist in close proximity in living cells compatible with the existence of GPCR higher-order heteroreceptor complexes (see methodological section). It may therefore be speculated that in CNS higher-order heteroreceptor complexes (receptor mosaics; RM) may exist and offer several additional targets for drug development. Novel drugs may be developed to modify the composition of RM, their topography, order of activation as well as allosteric regulators modulating the functional state of the individual receptors in the RM. Drugs may affect, for example, (I) the synthesis and release of heteroreceptor complex building blocks from the endoplasmic reticulum, (II) the insertion of such building blocks into the plasma membrane, (III) the internalization of RMs, (IV) the **adapter** and scaffolding proteins organizing the RMs, and (V) ligand induced heteroreceptor complex assembly. The potential importance of developing allosteric modulators

has also been suggested (Milligan and Smith, 2007), as they may, inter alia, substantially affect the allosteric mechanisms within the RM, which leads to changes in its integrative function. Currently, it is relatively unknown how allosteric modulators at one receptor protomer influence the function of the other receptor protomers in a heteroreceptor complex via the allosteric receptor–receptor interactions. Understanding such actions of allosteric receptor antagonists and agonists may likely also offer opportunities for introduction of novel neurotherapeutic drugs.

Another approach in developing peptides that interfere with protein/protein interactions is that of replacing some natural amino acids with non-natural amino acids. This approach has been used to develop short peptides for the SH3 domains. These domains are very important for protein/protein interactions as they are small docking units present in many signal transduction proteins (Pawson *et al*, 2001; Pawson and Scott, 1997). It has therefore been possible to replace parts of the polyproline helix-recognition sequences with nonnatural, N-substituted glycine residues (Nguyen *et al*, 1998). These peptide-peptoid hybrids often have higher affinity than that of the natural peptide and improved specificity for SH3 domains (Cochran, 2000). By competing for binding to the SH3 domain, they may stop the signal along the wiring pathways in the cytoplasm such as the RTK–Ras–MAP kinase pathway.

A2A–D2 HETERORECEPTOR COMPLEXES AND THEIR RECEPTOR–RECEPTOR INTERACTIONS IN RELATION TO PARKINSON'S DISEASE AND AS TARGETS FOR NOVEL ANTIPARKINSON DRUGS

The striatal A2A–D2 heteroreceptor complexes (Borroto-Escuela *et al*, 2013; Canals *et al*, 2003; Trifilieff *et al*, 2011) have been proposed to be an important target for antiparkinsonian drugs based on the existence of antagonistic A2A–D2 receptor–receptor interactions within them (Fuxe *et al*, 1998). In the striato-pallidal GABAergic neurons, this heteroreceptor complex exists in equilibrium with A2A and D2 homoreceptor complexes (George *et al*, 2002) on the plasma membrane (Ciruela *et al*, 2012; Fuxe *et al*, 2007a; Fuxe *et al*, 2010; Fuxe *et al*, 2007c). A2A–D2 heteroreceptor complexes strongly modulate the excitability in the striato-pallidal GABAergic neurons via their ability to counteract the inhibitory D2R signaling to multiple effectors upon agonist activation of the A2AR protomer. The counteraction of the D2R-induced inhibition of the $Ca2+$ influx over the L-type voltage-dependent $Ca2+$ channels (Cav 3.1 channels) by A2AR activation leads to increased phosphorylation of this calcium channel, which causes an increased opening of the channel and to an upstate of the striato-pallidal GABAergic neurons leading to motor inhibition, see (Surmeier, 2007). Moreover, the D2R agonist-induced reduction of firing rates in the DA terminal denervated striatum is enhanced by A2AR antagonists and attenuated by A2AR agonists (Stromberg *et al*, 2000).

There also exist reciprocal antagonistic interactions within A2A–D2 heteroreceptor complexes as D2R can inhibit the A2AR-induced increase in cAMP accumulation via Gi/o at the level of the adenylate cyclase (AC), an interaction which can also take place as a cross-talk between A2AR and D2R mono-dimers. An antagonistic allosteric D2–A2A receptor–receptor also exists at the level of A2AR recognition in the heteroreceptor complex (Fernandez-Duenas *et al*, 2012). The negative allosteric modulation of the D2R on A2AR agonist binding has been visualized in real-time mode of FRET and D2R activation partially inhibits and also slows the binding association of the fluorescent A2AR agonist. Removal of the D2R brake on the A2AR signaling should therefore lead to an upstate of the striato-pallidal GABA neurons. In fact, it results in increased protein kinase A (PKA) activity causing increased phosphorylation of α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate and N-methyl-D-aspartic acid receptors (NMDA) receptors and of dopamine and cAMP-regulated neuronal phosphoprotein (DARPP-32) at the Thr34 position. The latter action leads to inhibition of protein phosphatase-1 further, which enhances the phosphorylation and the activity of these ion channel receptors.

Based on the use of D2 agonists and L-DOPA in treatment of Parkinson's disease, the discovered antagonistic A2AR–D2R interactions in A2A–D2 heteroreceptor complexes, even with these interactions increased in Parkinson's disease models (Ferre *et al*, 1991; Fuxe *et al*, 2003; Fuxe *et al*, 1993), leads to the proposal and use of A2AR antagonists as antiparkinsonian drugs (Bara-Jimenez *et al*, 2003; Fuxe *et al*, 2003; Fuxe *et al*, 1993; Hauser *et al*, 2003; Hauser and Schwarzschild, 2005). So far it has not been possible to develop A2AR antagonists, which selectively block the A2AR protomer of the A2A–D2 heteroreceptor complex. Different types of A2AR antagonists have consistently reversed the Parkinsonian deficits in non-human primates and rodents (Fuxe *et al*, 2007c; Hodgson *et al*, 2010; Morelli *et al*, 2007; Schwarzschild *et al*, 2006). A2AR antagonists, like SCH 58261, have been found to dose dependently increase locomotor activity in combination with sub-threshold doses of L-DOPA and D2R agonists in reserpinized mice (Morelli *et al*, 2007; Tanganelli *et al*, 2004). Such results can be elegantly explained by the hypothesis that A2AR antagonists can target the A2A–D2 heteroreceptor complex and increase D2R recognition, D2R–G protein coupling and D2R signaling in these heteromers.

In an early clinical trial (Bara-Jimenez *et al*, 2003), it was found that the addition of the A2A receptor antagonist istradefylline to a low dose L-DOPA infusion increases the therapeutic response to a level found with an optimal L-DOPA infusion dose. At the same time, the degree of dyskinesias is reduced. These results open up the possibility that with combined treatment with oral L-DOPA and istradefylline, a lower dose of L-DOPA can be used to obtain an optimal antiparkinson action associated with a

lowering of dyskinesias. The same year (Hauser *et al* (2003) found in a randomized trial in advanced Parkinson's disease that the combined treatment left the dyskinesias more or less unchanged in the presence of a reduction of OFF time. Today, the available clinical data in moderate to advanced Parkinson's disease with the A2A receptor antagonists istradefylline and preladenant do not show a reduction of L-DOPA induced dyskinesias (Hauser, 2011; Knebel *et al*, 2011; Morelli *et al*, 2012) despite evidence from animal models of Parkinson's disease that A2A antagonists can prevent the development of L-DOPA induced dyskinesias and attenuate apomorphine induced dyskinesias (Bibbiani *et al*, 2003).

Based on our hypothesis, treatment with an A2AR antagonist alone should in fact produce only weak to moderate effects in PD except during early stages of PD when DA is still being released from large numbers of remaining DA terminals. This hypothesis states that the major action of A2A antagonists may be to increase the D2R signaling in the A2A–D2 heteroreceptor complex. Therefore, the A2AR antagonist should ideally be given in early stages of Parkinsonin's disease in combination with close to threshold doses of L-DOPA and/or D2R agonists. Clinical results observed so far are in agreement with this view. Therefore, the A2AR antagonist may act mainly by targeting the A2AR protomer of the A2AR–D2R heteroreceptor complex to enhance D2R protomer signaling.

A2AR antagonists may therefore enhance D2R protomer signaling at the soma-dendritic level of the striato-pallidal GABA/enkephalin pathway, which leads to a reduction in its activity, reduced motor inhibition and increased motor drive. A combined treatment with L-DOPA, but not with D2R agonists, is optimal as L-DOPA restores D1R activity in the direct pathway that helps motor initiation (Fuxe *et al*, 2010; Morelli *et al*, 2012). The direct pathway becomes integrated with the indirect pathway in the globus pallidus interna and zona reticulata of the substantia nigra to optimally inhibit their GABA projections to the motor thalamus. In this way, the GABA inhibition of the excitatory glutamate thalamo-cortical pathway to the motor cortices will be removed and movements restored.

Another advantage of using combined treatment with L-DOPA and an A2A receptor antagonist is that DA formed from L-DOPA, unlike a D2R agonist, can probably activate all the DA receptor subtypes, which should all show deficits in DA receptor signaling in the forebrain of Parkinson's disease patients. Furthermore, also A2A–D3 and A2A–D4 heteroreceptor complexes probably exist in the human brain possessing antagonistic A2A–D3 and A2A–D4 receptor–receptor interactions, which can also be blocked by A2A receptor antagonists (Fuxe *et al*, 2005; Torvinen *et al*, 2005). It is therefore postulated that combined treatment with A2A antagonists and threshold doses of L-DOPA in early Parkinson's disease may substantially enhance the therapeutic benefits of L-DOPA treatment in this disease with the potential to also reduce dyskinesia development.

As discussed there is also a reciprocal interaction by which D2R inhibits A2A recognition and A2AR signaling at the level of adenylate cyclase either linked to the A2A–D2 heteroreceptor complex or located between A2AR homoreceptor and D2R homoreceptor complexes. Through such mechanisms we can understand why A2AR antagonists alone can counteract haloperidol-induced catalepsy, namely by blocking the excessive A2AR signaling that results from the removal of the inhibitory D2R signaling through the D2R-blocking action of haloperidol. This mechanism can also help explain some therapeutic effects of A2AR antagonists in advanced PD.

It may be of interest to discuss the possible role of adenosine mechanisms in the reduction of the therapeutic effects of L-DOPA and the appearance of L-DOPA-induced dyskinesias after long-term treatment. Our hypothesis is that upon chronic treatment a L-DOPA induced co-internalization of A2A–D2 heteroreceptor complexes and downregulation of D2R monomers and homoreceptor complexes leads to a dominance of A2AR signaling, which also involves an activation of *A2AR* gene expression via D2R-induced activation of the MAPK pathway (Fuxe *et al*, 2007c). In support of this hypothesis, increases of A2AR mRNA and A2AR immunoreactivity (IR) have also been demonstrated in animal models of L-DOPA-induced dyskinesias and in brains from dyskinetic PD patients, see Antonelli *et al* (2006). The resulting upregulation of A2AR leads to increases in PKA and phosphorylated DARPP-32 at Thr34 and increased inhibition of PP-1. This will result in an increase in protein phosphorylation including ion channels, which may help stabilize pathological RM formed under the influence of the transcriptional panorama caused by the L-DOPA-induced excessive D2R activation (Fuxe *et al*, 2007c). This may lead to a repeated appearance of an abnormal and fixed pattern of firing in the striato-pallidal GABA pathway, which could contribute to dyskinesia development through the indirect pathway to the motor cortex via the motor thalamus. This hypothesis can help explain the reported failure of A2AR antagonists to increase dyskinesias in spite of antiparkinson actions, which cannot be explained by the enhancement of D2-protomer signaling in the A2A–D2 heteroreceptor complex, as this would worsen dyskinesias. We have also postulated based on this hypothesis that A2AR antagonists may help counteract the disappearance of the therapeutic effects of L-DOPA after long-term treatment by counteracting the disbalance of A2A versus D2R signaling (Fuxe *et al*, 2007b; Fuxe *et al*, 2007c).

Taken together, the demonstrated anti-Parkinsonian effect of A2AR antagonists in clinical studies has given strength to the concept that modulation of allosteric receptor–receptor interactions in heteroreceptor complexes can lead to the development of novel therapies. The work of the Schwarzschild *et al* (2006) has also given indications for neuroprotective effects of A2AR antagonists in Parkinson's disease based on studies in animal models of Parkinson's disease. Epidemiological evidence also exists for an inverse association between intake of coffee and caffeine in Japanese–American men and the risk of development of Parkinson's disease.

A2A–D2 AND mGLu2–5-HT2A HETERORECEPTOR COMPLEXES AND THEIR RECEPTOR–RECEPTOR INTERACTIONS IN RELATION TO SCHIZOPHRENIA AND AS TARGETS FOR ANTIPSYCHOTIC DRUGS

The probable existence of A2A–D2 heteroreceptor complexes with antagonistic A2A–D2 receptor–receptor interactions in the ventral striato-pallidal GABA pathway (Borroto-Escuela *et al*, 2013; Diaz-Cabiale *et al*, 2001; Fuxe *et al*, 1993; Fuxe *et al*, 1998) introduced the strategy of using A2A agonists for schizophrenia treatment based on these antagonistic interactions, which leads to a reduction in the affinity of the high-affinity state of the D2R (Fuxe *et al*, 2005; Fuxe *et al*, 1998; Fuxe *et al*, 2010). The classical treatment for schizophrenia is the use of DA receptor antagonists like haloperidol and chlorpromazine (typical antipsychotic drugs) blocking the postjunctional DA receptors of the nigro-striatal and mesolimbic DA neurons as first indicated by Carlsson and Lindqvist (1963) and Carlsson (1988). This work has been further extended in functional and biochemical experiments (Anden *et al*, 1966). Fuxe (1970) a dysfunction of the meso-limbic DA neurons may be involved in mental disorders like schizophrenia and therefore DA receptors in meso-limbic DA transmission may be a major target for antipsychotic drugs most likely by improving the emotional state of the schizophrenic patients.

Later on the evidence obtained indicates that the D2R subtype is the key target for the typical antipsychotic drugs, its blockade leading to antipsychotic effects. D2R occupancy in the brains of schizophrenic patients also correlates with the antipsychotic effects (Farde *et al*, 1988). Indications for enhanced striatal DA responsitivity have been obtained in schizophrenic patients with PET imaging (Laruelle *et al*, 1996). According to the Seeman hypothesis of schizophrenia (Seeman, 2006), the major error in psychosis is an increased proportion of D2R in the high-affinity state resulting in an increase in D2R recognition and signaling. This makes our proposal on the antipsychotic potential of A2A agonist drugs of special interest, as A2A agonists targeting the A2A/D2R mosaic can preferentially reduce the affinity of the high-affinity agonist state of the D2R in the dorsal and ventral striatum (Fuxe *et al*, 1998).

The glutamate hypothesis of schizophrenia states that the meso-limbic DA neurons are hyperactive due to reduced NMDA receptor function (Jentsch and Roth, 1999; Svensson, 2000), which leads to reduced activity in the descending cortical glutamate projections to the ventral tegmental area. This results in an increased activity of the meso-limbic DA systems. The reason is that a reduced drive develops in the inhibitory ventral tegmental area GABA interneurons, which causes an increased firing in the meso-limbic DA neurons. The meso-limbic DA neurons therefore become hyperactive with increased inhibition of the ventral striato-pallidal GABA pathway (Fuxe *et al*, 2008b; Svensson, 2000). The ventral striato-pallidal GABA neurons integrate and transfer the emotional information from the limbic system via the mediodorsal thalamic nucleus to the prefrontal cortex. The increased activity of the D2R in the ventral striatum via this brain circuit reduces the glutamate drive to the prefrontal cortex, which worsens the hypoglutamatergic state in schizophrenia.

A2A agonists may be antipsychotic drugs mainly by antagonizing the D2R signaling in the A2A–D2 heteroreceptor complex in the soma-dendritic region and possibly also in the terminal region of the ventral striato-pallidal GABA pathway, which brings its GABA transmission back and leads to increased glutamate drive from the mediodorsal thalamic nucleus to the prefrontal cortex (Groenewegen, 1988). A2AR agonists strongly reduce the D2R agonist-binding affinity and probably also the D3 agonist-binding affinity in nucleus accumbens shell and core (Diaz-Cabiale *et al*, 2001; Torvinen *et al*, 2005). They diminish both D2R recognition and Gi/o coupling and potentially also the D3-mediated signaling in this region (Fuxe *et al*, 2008b). D3 antagonists may also have antipsychotic actions (Schwartz *et al*, 2000).

The A2A agonist also increases glutamate release in the nucleus accumbens in part via actions on the glutamate terminals (Fuxe *et al*, 2008b; Popoli *et al*, 1995), which will also contribute to increasing the excitability of the ventral striato-pallidal GABA pathway after A2A agonist treatment and thus to the antipsychotic activity of A2A agonists. It is important to point out that D2 autoreceptors are not directly modulated by A2AR agonists unlike D2R antagonists known to also block D2 autoreceptors and increase DA release. Instead A2A receptors do not exist in the DA terminal networks leaving the D2 autoreceptor function intact to reduce DA release (Fuxe *et al*, 2003).

A2A agonist treatment represents a new strategy for schizophrenia treatment especially in combination with low doses of atypical and/or typical D2R antagonists, which should produce antipsychotic actions in the presence of reduced extrapyramidal and other side effects provided the A2A–D2 heteroreceptor complex is the correct target. This may in fact be the case since the antagonistic A2AR–D2R interactions modulate the ventral striato-pallidal GABA pathway controling the glutamate projections to the prefrontal cortex via the ventral pallidum and the mediodorsal thalamic nucleus, a circuit with major disturbances in schizophrenia. Popken *et al* (2000) demonstrated that there is a subnucleus specific loss of nerve cells in the mediodorsal thalamus of schizophrenics. A 30% loss of nerve cells can be seen in the mediodorsal thalamic nucleus mainly confined to the parvocellular and densocellular subnuclei. The parvocellular part projects to the dorsolateral parts of the prefrontal cortex and other regions known to be compromised in schizophrenia (Popken *et al*, 2000). These results underline the relevance of the current antipsychotic strategy of targeting the A2A–D2 heteroreceptor complex in the nucleus accumbens.

A cortical mGlu2–5-HT2A heteroreceptor complex has been identified and implicated in psychosis (Gonzalez-Maeso *et al*, 2008). Previously, metabotropic glutamate 2/3 receptors were regarded as targets for antischizophrenic drugs (Aghajanian and Marek, 2000). In addition, a positive allosteric modulator of mGluR2, biphenyl-indanone A is effective in a hallucinogenic drug model of psychosis (Benneyworth *et al*, 2007). Furthermore, a randomized phase II clinical trial in schizophrenia using a mGluR2/3 agonist indicates their possible therapeutic potential for this disease (Patil *et al*, 2007).

It is of interest to note that the hallucinogenic 2,5-dimethoxy- 4-iodoamphetamine (DOI) stimulated [35S]GTPgammaS binding in primary cortical membranes, when linked to either Gai1, Gai2, or Gai3, is strongly reduced by the mGluR2/3 agonist LY379268 as is the hallucinogen-specific induction of egr-2 in mouse cortex (Gonzalez-Maeso *et al*, 2008). In contrast, the DOI-stimulated [35S] GTPgammaS binding when linked to Gaq/11 is only weakly affected by the mGluR2/3 agonist. Therefore, the mGluR2/3 agonist may in part produce their potential antipsychotic effects by targeting the mGlu2-5-HT2A heteroreceptor complex most likely located in distinct pyramidal and granular cells and reduce the hallucinogen-specific Gi/o signaling via allosteric interactions over the receptor interface (Gonzalez-Maeso *et al*, 2008).

An interesting consequence of restoring the glutamate drive to the prefrontal cortex by A2A agonists/D2R antagonists may in part be the increased activation of cortical mGluR2 possibly in part located in the mGlu2–5-HT2A heteroreceptor complex in cortical neurons. This may reduce the hallucinogenic-specific Gi/o signaling and behavior over the 5-HT2A receptor protomer with a reduction of primary disturbances in cortical sensory processing (Gonzalez-Maeso *et al*, 2008). Recently, this group identified three residues located at the intracellular end of transmembrane four of the mGlu2 receptor, which are necessary for this receptor heteromerization to take place (Moreno *et al*, 2012). Upon substitution of these residues, the mGlu2–5-HT2A heteroreceptor complex is no longer formed, which is associated with a reduction in the psychosis-like effects induced in mice by hallucinogenic 5-HT2A agonists. Furthermore, the active conformation of the 5-HT2A receptor is increased in the postmortum prefrontal cortex from schizophrenic subjects (Muguruza *et al*, 2012). It would be of interest to know if this dysregulation of 5-HT2A receptor recognition also involves the 5-HT2A protomer of a mGlu2–5-HT2A heteroreceptor complex of the human prefrontal cortex.

It may be speculated that in one group of schizophrenic patients, the heteroreceptor complex pathology could mainly exist, eg, in the cortical mGlu2–5-HT2A hetero-receptor complex. Therefore, these schizophrenic patients may have enhanced therapeutic responses to treatment with mGluR2 agonists and/or 5-HT2A antagonists with regard to antipsychotic activity. Other groups of schizophrenic patients may instead show improved therapeutic responses by targeting a postulated and malfunctioning D2–5-HT2A heteroreceptor complex in the ventral striatum. It has been shown that the D2R and the 5-HT2AR likely form stable and specific heteromers when expressed in HEK293T mammalian cells (Borroto-Escuela *et al*, 2010e; Lukasiewicz *et al*, 2010). Targeting both heteroreceptor complexes is also an interesting option which may already take place in the clinic, as many atypical antipsychotics like risperidone block inter alia both D2R and especially 5-HT2A receptors in clinically effective doses. Inverse 5-HT2A agonists also enhance the actions of atypical antipsychotic drugs (Meltzer and Massey, 2011).

A2A–D2 AND D2–NMDA HETERORECEPTOR COMPLEXES AND THEIR RECEPTOR–RECEPTOR INTERACTIONS IN RELATION TO COCAINE ADDICTION AND AS TARGETS FOR DRUGS AGAINST COCAINE ADDICTION

Adenosine has an important modulatory role in accumbal/striatal function. Adenosine A2ARs are concentrated in these brain regions more than anywhere else in the brain and directly regulate the release of glutamate and the activity of the striato-pallidal GABA neurons (Fuxe *et al*, 2007b; Fuxe *et al*, 2010). In accordance with these neuroanatomical and neurochemical links, it has been demonstrated that central reward processes are modulated by A2AR function. As such, activation of A2ARs inhibits brain stimulation reward and cocaine withdrawal by elevating current reward thresholds without apparently affecting performance, whereas the blockade of A2ARs reverses the reward impairment produced by cocaine withdrawal (Baldo *et al*, 1999). The blockade of adenosine receptors by caffeine, its main mechanism of action is antagonizing A1R and A2AR, leads to a more rapid acquisition of cocaine self-administration in rats (Horger *et al*, 1991). Thus, specific antagonists acting at these receptors lead to high levels of cocaine-lever selection in substitution tests and produce a leftward shift in the cocaine dose–response curve, and the administration of a selective A2AR antagonist (MSX-3) enhances the expression of cocaine sensitization (Filip *et al*, 2006). In contrast, rats pre-exposed to A2AR agonists show a reduced initiation of cocaine self-administration (Knapp *et al*, 2001). We have found an increase in A2ARs in the nucleus accumbens after extended cocaine self-administration (Marcellino *et al*, 2007). This effect is strictly dependent on active self-administration since yoked controls that get passively infused with cocaine show different alterations in a state and brain region dependent way (Frankowska *et al*, 2012), which provides a rational for the aforementioned pharmacological interventions.

A2AR ligands have also been tested in the reinstatement paradigm, which provides a measure for drug-seeking and thus models the motivational aspects of craving. The A2AR

antagonist CGS 15943 reinstates cocaine-seeking behavior and maintains self-administration in baboons (Weerts and Griffiths, 2003), whereas pre-treatment with the A2AR agonist CGS 21680 dose-dependently blunts cocaine- and cue-induced reinstatement of drug-seeking behavior without affecting sucrose seeking (Bachtell and Self, 2009). These studies show that A2AR activation via A2AR agonists can reduce cocaine-seeking behavior. This conclusion is further supported by the fact that pharmacological stimulation of A2ARs protects against both the development and expression of cocaine sensitization (Filip *et al*, 2006).

Other studies utilizing genetic deletion of the A2ARs report findings that at first glance to conflict with pharmacological intervention studies. Mice lacking the A2AR display attenuated locomotor responses to cocaine and reduced reinforcing efficacy in cocaine self-administration (Chen *et al*, 2003; Soria *et al*, 2006). These opposing findings are most likely due to the lack of neuroanatomical specificity of knock-out of A2AR in neural circuits regulating cocaine-induced behaviors. In fact, conditional mouse models support this, as striatal-specific knock-out of A2ARs enhances the effects of cocaine, whereas more widespread forebrain knock-out of A2ARs reduces cocaine-induced locomotion (Shen *et al*, 2008). In conclusion, stimulation of A2ARs in accumbal/dorsal striatal sites may reduce cocaine-seeking behavior.

A large number of studies have dealt with the role of the mesocorticolimbic and nigrostriatal dopamine neurons and their D1, D2, and D3 receptors in mediating a variety of cocaine effects relevant for the development of addictive behavior (Everitt *et al*, 2008; Spanagel and Weiss, 1999; Wise, 2009). In particular, stimulation of D2Rs in the nucleus accumbens and the dorsal striatum is involved in mediating the behavioral effects of cocaine and these actions are antagonized by activation of A2ARs (Durieux *et al*, 2009; Filip *et al*, 2006; Soria *et al*, 2006). The higher the addicted cocaine state the higher the degree of D2like agonist induced relapse following chronic cocaine self-administration indicating a major role of D2like receptor sensitization in cocaine addiction (Self, 2010). It is further known that DA transmission can be reduced by A2AR agonists, while A2AR antagonists increase it. It is suggested that striatal A2A–D2 heteroreceptor complexes and their antagonistic receptor–receptor interactions contribute to these effects.

An increase in functional A2A receptors in the nucleus accumbens was observed after extended cocaine self-administration in Sprague–Dawley rats (Marcellino *et al*, 2007). This 10 day cocaine self-administration procedure produces increases in extracellular DA levels and the resulting increase in DA receptor signaling gives rise to an upregulation of functional A2A receptors likely mainly present in the ventral striato-pallidal GABA neurons of this region. The disappearance of the A2AR upregulation during the withdrawal period may help explain the increased reinforcing efficacy of cocaine in the 7-day withdrawal group (Morgan *et al*, 2002). The mechanism may involve increased signaling via nucleus accumbens D2R and D3 receptors by reducing the A2AR brake on D2R and D3R signaling (Marcellino *et al*, 2007). The results indicate a possible role of antagonistic receptor–receptor interactions in accumbens A2A–D2 and A2A–D3 heteroreceptor complexes and/or at the level of adenylate cyclase in the prevention and/or treatment of cocaine addiction.

In support of a role of antagonistic A2A/D3 interactions (Fuxe *et al*, 2005; Torvinen *et al*, 2005) in cocaine actions in the nucleus accumbens (Marcellino *et al*, 2007) D3 receptor antagonists also counteract cocaine seeking and cocaine enhanced reward and may be used in treatment of cocaine addiction (Vorel *et al*, 2002). An upregulation of D3 mRNA levels has been found in reward networks of human cocaine fatalities. Therefore, the reduction not only of the antagonistic A2A/D2R interaction but also of antagonistic A2A/D3 interaction in the 7-day cocaine withdrawal group (Marcellino *et al*, 2007) may contribute to the increased motivation to self-administer cocaine in the 7-day cocaine withdrawal group (Morgan *et al*, 2002). These results indicate that A2A agonists can represent cocaine antagonists to be used in the prevention and treatment of cocaine addiction. A2AR–D2R heteroreceptor complexes are hypothesized to be critically involved in cocaine addiction, and more specifically that the activation of the A2AR protomer of the heteroreceptor complex inhibits the development of addictive behavior.

The observed increase of A2A receptor density in nucleus accumbens after extended cocaine self-administration may depend on the existence of an atypical CRE in the core promotor of the A2A receptor gene (Chiang *et al*, 2005). CREB (CRE-binding protein) diminishes cocaine reward in this region (Carlezon *et al*, 1998) and is enabled by increased activation of the extracellular signal-related kinase (Mattson *et al*, 2005). Cocaine-induced activation of D2-like receptors can produce an increase in CREB phosphorylation via several intracellular mechanisms (Marcellino *et al*, 2007).

It should be considered that the A2A receptor upregulation reflects not only increases in, eg, A2A–D2 like heteroreceptor complexes and/or their antagonistic receptor–receptor interactions but also in increased presence of A2AR monomers and formation of A2A homoreceptor complexes. They may all increase the excitability of the striato-pallidal GABA neurons (Antonelli *et al*, 2006; Fuxe *et al*, 2007b) thus opposing D2like inhibition of these neurons. Another mechanism could be that the persistent D2R-like activation sensitizes A2A signaling at the level of the AC via release of $\beta\gamma$ dimers from the activated Gi proteins (Vortherms and Watts, 2004), leading to a dominance of A2AR signaling.

By comparing the cocaine self-administration group with the 'yoked' cocaine group in Wistar rats evidence has been obtained for the existence of motivational mechanisms that guide adaptive changes in the A2AR and D2R and in the D2R-Gi coupling, which differentially developed in the ventral and dorsal striatum during cocaine maintenance

and extinction (Frankowska *et al*, 2012). Differences observed in the adaptive changes occurring in A2A and D2R in response to cocaine self-administration of Sprague–Dawley and Wistar rats may, in addition to genetic differences, also result from differences in the cocaine self-administration schedule used, namely in the doses of cocaine administered, the total cocaine intake per animal and the schedule of cocaine reinforcement (Frankowska *et al*, 2012).

Filip *et al* (2012) have extensively discussed the importance of A2AR–D2R interactions in drug addiction in general. Behavioral studies indicate that A2A agonists may block drug abuse reinstatement in general as well as actions of withdrawal from drugs of abuse but the mechanisms still remain to be explored in the context A2A–D2 heteroreceptor complexes versus A2A receptor monomers and A2A homoreceptor complexes.

Our working hypothesis is that A2AR–D2R heteroreceptor complexes in the ventral striatum are critically involved in cocaine addiction, and more specifically that the activation of the A2AR protomer of this heteroreceptor complex inhibits the development of addictive behavior. A2A agonists are therefore a viable option for treatment of cocaine addiction, with special emphasis placed on the development of A2A agonists that preferentially target the A2A protomer in this heteroreceptor complex. A combined treatment with low doses of A2A agonists and D2 amtagonists will also be tested and should lead to synergistic effects as to counteract cocaine addiction development. The functional role of striatal A2A–D2 heteroreceptor complexes and their ratios with striatal A2AR or D2R populations remains to be characterized in the course of the addiction cycle involving the progression from the onset of cocaine taking behavior to the maintenance phase and sub-sequent addicted versus non-addicted stage.

The D2R and NMDA receptors form D2–NMDA heteroreceptor complexes (Figure6) through the IC3 loop of D2R interacting with the NR2B subunit (Liu *et al*, 2006) leading to inhibition of NMDA receptor signaling in the striatal glutamate synapses. This interaction is enhanced by cocaine and the D2R/NR2B interaction has been shown to be critical for the full behavioral response to acute cocaine treatment (Liu *et al*, 2006). It is therefore suggested that enhancers of signaling in NR2B containing NMDA receptors in combination with low doses of D2R antagonists may be of value cocaine addiction for treatment. Otherwise the cortical control of the striatum would be markedly reduced and the striato-pallidal GABA neurons will become silenced by cocaine-induced D2R activation involving the mesolimbic and nigrostriatal DA neurons unopposed by NMDA synapses and cocaine addiction may develop.

To stop the pathological control by enhanced D2R signaling of the ventral and dorsal striato-pallidal GABA pathways, both the extrasynaptic A2A–D2 and synaptic D2–NMDA heteroreceptor complexes should be targeted. This may, eg, be possible by low doses of D2R antagonists characterized to reduce D2R protomer signaling in both heteroreceptor complexes in combination with A2A agonists, which target and reduce the D2R protomer signaling in the A2A–D2 heteroreceptor complexes. This treatment should also reduce development of side effects as the combined treatment may allow low doses to be used to restore firing of the striato-pallidal GABA neurons through reduction of D2R-mediated inhibition.

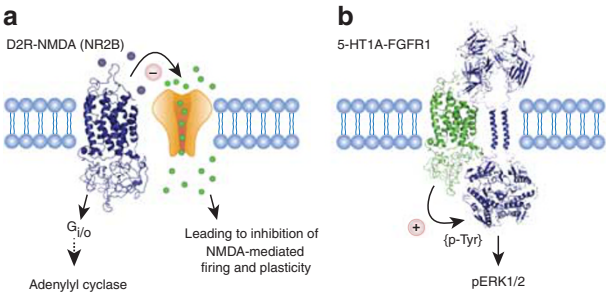

Figure 6. Receptor–receptor interactions in different types of heteroreceptor complexes in the CNS have now been obtained. The heteroreceptor complexes may allow direct physical interactions between the receptors with allosteric receptor–receptor interactions between them. The schematic representation depicts some of the principal, non-exclusive, molecular mechanisms by which GPCR heteromers produce novel functions or modulation. (a) Ion channel receptor-GPCR heteroreceptor complexes and their receptor–receptor interactions. Direct interaction of the dopamine D2R with the NMDA (NR2B unit) receptor leads to a D2-mediated inhibition of the NMDA receptor signaling with reduction of NMDA receptor-dependent firing and plasticity. (b) Receptor tyrosine kinase (RTK)-GPCR heteroreceptor complexes and their receptor–receptor interactions. Transactivation can occur during signal initiation and propagation, using shared signaling pathways. In the case of GPCRs, they can use RTK themselves as signaling platforms via direct receptor–receptor interactions. A heteroreceptor complex between 5HT1A receptor and FGFR1 has recently been discovered in the rat hippocampus and the midbrain raphe nuclei. *In vitro* assays on extracellular signal-regulated kinases 1 and 2 phosphorylation demonstrated synergistic increases in signaling and structural plasticity in the hippocampus and mesencephalic raphe through the allosteric receptor–receptor interactions.

FGFR1–5-HT1A HETERORECEPTOR COMPLEXES AND THEIR RECEPTOR–RECEPTOR INTERACTIONS IN DEPRESSION AND AS TARGETS FOR ANTIDEPRESSANT DRUGS

The focus of research into antidepressant drug action has shifted in recent years towards their gradually developing effects on intraneuronal signal transduction and cellular plasticity (Coyle and Duman, 2003; Manji and Duman, 2001). Several findings suggest that antidepressants facilitate activity-dependent selection of functional synaptic connections in the brain, and through their neurotrophic effects improve information processing within neuronal-glial networks compromised in mood disorders (D'Sa and Duman, 2002). According to such a

view, antidepressants induce processes of neuroplasticity that lead to a reorganization of central neural networks, which generates their therapeutic effects (Duman, 2002b; Nestler *et al*, 2002).

Increasing evidence suggests that antidepressant drugs via actions on the 5-HT and NA neurons may exert their therapeutic activity, at least in part, through the enhancement of neurotrophic factor expression and function (Duman, 2002a; Saarelainen *et al*, 2003). In this context, several lines of converging evidence suggest that brain-derived neurotrophic factor and its RTK trkB have a central role in the mechanism of antidepressant action, including electroconvulsive shock treatment (D'Sa and Duman, 2002; Nestler *et al*, 2002; Saarelainen *et al*, 2003; Shirayama *et al*, 2002).

Recently, an involvement of the fibroblast growth factors (FGFs) was proposed in mood disorders (Turner *et al*, 2006). Antidepressant drugs and chronic electroconvulsive shock treatment may increase the expression of fibroblast growth factor-2 (FGF-2) in frontal cortices and hippocampus (Mallei *et al*, 2002; Maragnoli *et al*, 2004), which suggests that potentially the FGF-2 expression could also mediate the antidepressant effects. In line with these results, chronic antidepressant treatment increases neurogenesis in adult rat hippocampus and depression is associated with reduced cortical thickness and neuronal size (Rajkowska *et al*, 1999).

The hippocampal atrophy found in major depression may be involved in the pathophysiology of this disease in view of the critical role of the hippocampus (Hip) in the emotional networks (Castren *et al*, 2007; Rajkowska *et al*, 1999; Schmidt and Duman, 2007). The atrophy may result from the downregulation of neurotrophic factors in the Hip. FGF-2 mRNA levels in the hippocampus are reduced in major depression (Charney and Eric, 2008) and this region appears to show an especially strong atrophy (Charney and Eric, 2008). Treatment with antidepressant drugs in rodents can increase Hip FGF-2 levels (Schmidt and Duman, 2007). The therapeutic effect of the most commonly prescribed antidepressants, like the selective serotonin reuptake inhibitors (SSRIs), eg, fluoxetine and the 5-HT1A partial agonist Buspirone (Carlsson *et al*, 1968; Wong *et al*, 1995), may in part be related to the enhancement of 5-HT transmission in the Hip mainly involving the postjunctional 5-HT1A receptor.

There is evidence for the existence of heteroreceptor complexes, which even in the absence of neurotrophic factor binding to the RTK, can lead to transactivation of RTKs with effects on neuronal plasticity (Flajolet *et al*, 2008; Fuxe *et al*, 2007b; Lee and Chao, 2001; Luttrell *et al*, 1999).

In our paper (Borroto-Escuela *et al*, 2012a) evidence is given for the existence of FGFR1–5-HT1A heteroreceptor complexes, using BRET, coimmunoprecipitation, and the PLA with a partial characterization of their interface (Figure 6). Evidence is given for their involvement in enhancement of hippocampal neuroplasticity in the rat (Borroto-Escuela *et al*, 2012a). *In vitro* and *in vivo* studies reveal a 5- HT1A agonist induced phosphorylation of FGFR1 and extracellular signal-regulated kinase (ERK) 1/2 in rat hippocampus without changing FGF2 levels. Co-activation of the heteroreceptor complex also results in synergistic increases in extensions of PC12 cells and neurite densities and protrusions in primary hippocampal cultures dependent on the receptor interface. The structural plasticity is linked to synergistic increases in ERK1/2 signaling upon coactivation with fibroblast growth factor 2 (FGF-2) and a 5-HT1A agonist, and dependent on the heteroreceptor interface. We have also found that acute and a 10 day i.c.v. treatment with FGF-2 and the 5-HT1A agonist 8-OHDPAT in the Sprague–Dawley rat can produce synergistic antidepressant effects in the forced swim test. Neurotrophic and antidepressant effects of 5-HT in brain may therefore, in part, be mediated by activation of the 5-HT1A receptor protomer in the hippocampal FGFR1–5-HT1A heteroreceptor complex, which enhances the FGFR1 signaling via receptor–receptor interactions. Therefore, this cotreatment may result in more rapid and stronger antidepressant actions than found with SSRIs.

Evidence is also presented for the existence of FGFR1–5-HT1A heteroreceptor complexes with facilitatory receptor–receptor interactions in the mesencephalic raphe 5-HT nerve cells with relevance for neuroplasticity. The raphe 5-HT1A autoreceptor, when being part of the FGFR1–5-HT1A heteroreceptor complex, may therefore have a beneficial role in depression, its activation leading to a FGFR1 transactivation through an allosteric receptor–receptor interaction. This can assist in the recovery of 5-HT nerve cell trophism including 5-HT synthesis and storage as well as increased outgrowth of dendritic, and terminal networks in the raphe-hippocampal 5-HT neuronal system. These results indicate that the 5-HT1A autoreceptors may have a trophic role in the midbrain raphe 5-HT neuronsystems by being part of a FGFR1–5-HT1A receptor heterocomplex in the midbrain raphe 5-HT nerve cells, in addition to having a key role in reducing the firing of these neurons. Future work will show whether this hypothesis is relevant for understanding mechanisms of major depression.

The receptor–receptor interaction in the FGFR1–5-HT1A heteroreceptor complex may change preferred signaling pathways of the individual receptors and lead to biased signaling and/or altered receptor trafficking and/or recognition, which may provide targets for the development of novel antidepressants.

The results obtained underline a role of RTK–GPCR heteroreceptor complexes in depression and its treatment and that transactivation of RTK by GPCRs (Lee and Chao, 2001; Luttrell *et al*, 1999) can occur via facilitatory receptor–receptor interactions in such complexes, namely in the FGFR1–5-HT1A heteroreceptor complex as postulated in 2007 (Fuxe *et al*, 2007a). It is certainly possible that in another RTK–GPCR complex antagonistic receptor–receptor with transinhibition of the RTK signaling develops.

In a brilliant paper from Flajolet *et al* (2008) demonstrate the existence of A2A–FGFR1 heteroreceptor complexes by

yeast two-hybrid analysis. Coactivation of the A2AR and FGFR1 results in a synergistic activation of the ERK/MAPK pathway, synergistic facilitation of corticostriatal LTP as well as structural signs of enhanced synaptic plasticity. FGF is proposed to act as a co-transmitter through adenosine A2A receptor to regulate synaptic plasticity in the striato-pallidal GABA neurons (Flajolet *et al*, 2008). It is of interest that in this case the GPCR A2A operates mainly via Gs/olf while the 5-HT1A receptor in the FGFR1–5-HT1A hetero-receptor complex operates mainly via Gi/o. Nevertheless, a similar strong enhancement of synaptic plasticity develops. One explanation may be that it is the allosteric receptor–receptor interactions in the heteroreceptor complex, which make the synergistic enhancement of synaptic plasticity possible and involve inter alia development of biased agonism.

In view of the presence of A2A–D2 heteroreceptor complexes in the striato-pallidal GABA neurons, the existence of FGFR1–A2A–D2 higher-order heteroreceptor complexes in these neurons may be postulated. It may be speculated in view of the existence of bidirectional antagonistic A2A–D2 receptor–receptor interactions (Fuxe *et al*, 2007a; Fuxe *et al*, 2010) that D2R activation can bring down the synergistic enhancement of synaptic plasticity found upon A2A and FGFR1 coactivation. Thus, a dynamic formation of such a heteroreceptor complex may allow a fine tuning of synaptic plasticity in the striato-pallidal GABA neurons of high relevance to their function in the basal ganglia circuits. A dysfunction of such a higher-order heteroreceptor complex may contribute to pathologies found in Parkinson's disease, schizophrenia, and drug addiction.

FUTURE RESEARCH DIRECTIONS

The D2Rs are regarded as hub receptors, as they participate in many different types of heteroreceptor complexes (of unknown stoichiometry and topology) (Tarakanov *et al*, 2012b). The standard treatment with L-dopa and/or D2R agonists in PD builds especially on their activation of these D2R in moderate to high doses and the accessory A2A, mGluR5 and CB1 receptors in the different heteroreceptor complexes are not targeted.

A novel principle may now be used to develop D2R agonists for treatment of Parkinson's disease based on the existence of various D2R heteroreceptor complexes. Thus, the conformational state of the D2R may differ from one heteroreceptor complex to the other one, which may also be due to different types of receptor–protein interactions. It is also influenced by the local molecular histology of the surface membrane of, eg, discrete striato-pallidal nerve cell populations and DA nerve terminal networks.

Thus, the agonist pharmacology of D2R in terms of potency and efficacy may show substantial differences among various types of heteroreceptor complexes like A2A–D2, A2A–D2–mGlu5, D2–NR2B containing NMDA hetero-receptor complexes and also versus D2R monomers and D2 homoreceptor complexes. The development of specific D2R agonist drugs for the D2R short autoreceptor may be especially hopeful, as the D2 autoreceptor isoform not only interacts with other types of receptors but also with special types of proteins like the DA transporter on the DA terminals. A disruption of the DAT–D2R interaction may exist in schizophrenia leading to enhancement of DA release (Lee *et al*, 2009).

This approach should give exciting possibilities to develop novel and more selective D2R agonist drugs for Parkinson's disease treatment by preferentially acting on certain postjunctional heteroreceptor complexes in, eg, the striato-pallidal GABA neurons and their glutamate inputs. In support of this approach, the striatal D1–D2R hetero-receptor complexes have been shown to possess a unique pharmacology and couples with Gq/11, as discovered by the group of Dr George and Dr O'Dowd (Rashid *et al*, 2007a; Rashid *et al*, 2007b). The DA agonist SKF-83959 is a specific agonist for this heteroreceptor complex by being a full agonist at the D1 receptor and a partial agonist at the pertussis-toxin resistant D2R protomer in this heteroreceptor complex.

The same principle may also be used to develop novel D2R-based antipsychotics for treatment of schizophrenia. Thus, the potency and efficacy of full and partial D2R antagonists and their inverse D2R agonist activity may vary among the different D2R heteroreceptor complexes due to differences in the conformational state of the participating postjunctional D2R and/or D2 autoreceptors. This can involve the allosteric receptor–receptor interactions in the heteroreceptor complexes giving them differences in D2R antagonist pharmacology.

The major target may be the postjunctional D2R in the ventral striato-pallidal GABA neurons inhibiting the glutamate drive to the prefrontal cortex in view of likely increases in meso-limbic DA activity in schizophrenia (Fuxe *et al*, 2008b; Svensson, 2000). It should be mentioned that the atypical antipsychotic-like drug remoxipride, unlike haloperidol, *in vivo* blocks only a subpopulation of D2R in nigro-striatal and meso-limbic/cortical regions as evaluated by the protection against the N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)-induced decreases in D2R binding due to its irreversible inactivation of D2R (Ogren *et al*, 1994). The blockade of this D2R subpopulation *in vivo*, which may belong to a certain type of D2R heteroreceptor complex, could be the basis for its atypical antipsychotic-like profile with reduced extrapyramidal side effects. Thus, the underlying mechanism may be that this selective D2R antagonist can only bind and block D2R protomers in distinct D2R heteroreceptor complexes present in these regions due to their unique D2R antagonist pharmacology. This may now be tested by studying how the potency and efficacy of remoxipride in blocking D2R could possibly vary in cell lines upon D2R co-transfection with other receptors known to form heteroreceptor complexes with the D2R (Fuxe *et al*, 2009). This approach could also be

developed for the treatment of cocaine addiction where novel and more selective D2R antagonists for certain D2R heteroreceptor complexes in the ventral striatum may also offer treatment improvements.

The other novel principal strategy based on the different D2R heteroreceptor complexes is the targeting of their accessory receptor protomers antagonistically interacting via receptor–receptor interactions with the D2R protomer (hub receptor), which involves, eg, the A2A and the mGlu5 receptors. A large number of observations suggest that A2AR and mGluR5 appear to synergize in counteracting D2R signaling in the postulated A2A–D2–mGluR5 higher-order heteroreceptor complexes in the striatum (Cabello *et al*, 2009; Ciruela *et al*, 2012; Fuxe *et al*, 2008a).

In PD and models of PD it is possible to test low doses of L-dopa and D2R agonists in combination with A2A and/or mGluR5 antagonists to reduce the inhibitory impact of these receptors on D2R recognition and signaling in A2A–D2, A2A–D2–mGlu5 and D2–mGlu5 heteroreceptor complexes. Reduced collateral effects of L-dopa and D2R agonists may be observed despite hypokinesia improvement, resting tremor, and rigidity (Fuxe *et al*, 2007c; Schwarzschild *et al*, 2006). In early PD, it may be possible to substantially delay the onset of L-dopa and D2R agonist treatment by introducing monotherapy or combined therapy with A2A and/or mGluR5 antagonists. Combined treatment with L-DOPA should also be tested since then a certain increase in DA signaling may also take place also at D1 and D5 receptors.

However, in schizophrenia and schizophrenia like models, only low doses of typical and atypical antipsychotics may be used in combined treatment with low doses of A2A agonists and/or mGluR5 agonists to obtain reduced collateral effects as these agonists will synergistically reduce D2like signaling by activating the A2A and/or mGluR5 protomers of the D2R heteroreceptor complexes. Therefore, with this combined treatment antipsychotic actions may be obtained with reduced extrapyramidal and other side effects and monotherapy with A2A agonists should also be considered in view of their atypical antipsychotic profile. A similar strategy may also be developed for treatment of cocaine addiction.

A major task for the future will be to characterize the receptor interface in the different types of existing heteroreceptor complexes in the CNS. In this way interface interfering peptides and non-peptide compounds (receptor–receptor interaction inhibitors) may be developed to target the receptor interface and block the dysfunctional receptor–receptor interactions. This will also unravel the function of the different heteroreceptor complexes and their role in disease development in the CNS. It is likely that targeting the receptor interface will be a fruitful avenue for drug development in the mental and neurological diseases. The GPCR-interacting protein field (GIP) faces similar challenges (Bockaert *et al*, 2010).

In the future research on GPCR heteroreceptor complexes and GIP research should join forces in understanding the full architecture of the molecular world of GPCRs and their interacting proteins and its dynamics in space and time. An increased understanding of the allosteric receptor–receptor interactions is equally important and may be assisted by bimolecular dynamics simulations to create atomic-resolution models of the various receptor interfaces. This type of research in cellular models and in brain tissue should provide exciting developments in novel drugs in neuropschopharmacology.

FUNDING AND DISCLOSURE

This work was funded by the Grants from the Swedish Research Council (04X-715), Torsten and Ragnar Soderberg Foundation, Hjarnfonden and Marianne and Marcus Wallenberg Foundation to KF, by a Grant from the Swedish Royal Academy of Sciences to DOB-E and by the Grants SAF2011-24779 and Consolider-Ingenio CSD2008-00005 from Ministry of Science and Innovation and ICREA Academia-2010 from the Catalan Institution for Research and Advanced Studies to FC. Dr Romero-Fernandez, Dr. Agnati, Dr. Palkovits and Dr. Tarakanov, declare no potential conflict of interest.

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