Chapter 2
Allosteric Mechanisms in the Adenosine A_{2A}-Dopamine D_2 Receptor Heteromer

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Abstract The pentameric structure constituted by one G protein coupled receptor (GPCR) homodimer and one heterotrimeric G protein provides a main functional unit and oligomeric entities can be viewed as multiples of dimers. For GPCR heteromers, experimental evidence supports a tetrameric structure, comprised of two different homodimers, each able to signal with their preferred G protein. GPCR homomers and heteromers can act as the conduit of allosteric interactions of orthosteric ligands. One ligand binding to one of the receptor units (protomer) modulates the properties of the same or another orthosteric ligand binding to another protomer. The agonist/agonist interaction in the adenosine A_{2A} receptor ($A_{2A}R$)-dopamine D_2 receptor (D_2R) heteromer, by which $A_{2A}R$ agonists decrease the affinity of D_2R agonists, constitutes a well-known example and gave the first rationale for the use of $A_{2A}R$ antagonists in Parkinson's disease. We review most recent studies that extend those findings to, first, ligand-independent allosteric modulations of the D_2R protomer that result in changes of the binding properties of $A_{2A}R$ ligands in the $A_{2A}R$-D_2R heteromer; second, the differential modulation of the intrinsic efficacy of D_2R ligands for G protein-dependent and independent signaling; and third, the existence of the canonical antagonistic Gs-Gi interaction within the frame of the $A_{2A}R$-D_2R heteromer. These studies support the heterotetrameric structure of GPCR heteromers.

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The A_{2A}R- D_2R Heteromer as a Model to Understand Allosterism within GPCR Oligomers

John Newport Langley and Paul Ehrlich independently introduced the "receptor" concept in 1878. Since then receptors have mostly been considered as single functional units. But we know now that receptors form multimolecular aggregates that include other receptors with the formation of receptor oligomers (Ferré et al. 2009). Most evidence indicates that, as for family C G protein-coupled receptors (GPCRs), family A GPCRs form homo- and heteromers (Ferré et al. 2009, 2014; Milligan and Bouvier 2005; Pin et al. 2007). Receptor oligomer is defined as a macromolecular complex composed of at least two (functional) receptor units (protomers) with biochemical properties that are demonstrably different from those of its individual components (Ferré et al. 2009).

To understand the unique biochemical properties of receptor oligomers we need to understand the basis of allosterism, which is defined as the process by which the interaction of a chemical or protein at one location on a protein or macromolecular complex (the allosteric site) influences the binding or function of the same or another chemical or protein at a topographically distinct site (Smith and Milligan 2010). In this respect, it is useful to consider ligands as modulators and modulated entities, and the receptors or receptor oligomers as the conduits of the allosteric modulation (Kenakin and Miller 2010). An orthosteric agonist (which binds to the same receptor site as the endogenous transmitter) has two main properties: affinity (the avidity to bind to the receptor) and intrinsic efficacy (the power with which the agonist, once bound to the receptor, produces the functional response). In classical allosterism, the allosteric ligand, by binding to a non-orthosteric site, can modify either of these properties. In this frame, the GPCR is the conduit of the allosteric modulation and is usually considered as a monomeric entity.

A first important concept that arises from the new field of GPCR oligomerization is that the pentameric structure constituted by one GPCR homodimer and one heterotrimeric G protein provides a main functional unit, and oligomeric entities can be viewed as multiples of dimers (Ferré et al. 2014). Then, in the frame of GPCR homodimers, allosterism implies that the dimer can act as the conduit of the allosteric modulation by an orthosteric ligand, which binds to one of the protomers, to the same or another orthosteric ligand, which binds to the second protomer. The realization of these interactions is leading to a profound modification of classical pharmacology. For instance, application of new models of analysis of radioligand binding experiments that consider the homodimer as a fundamental functional unit is allowing a better understanding of complex binding saturation or competition curves. Particularly, the two-state dimer model (Casadó et al. 2007; Ferré et al. 2014) is a practical model to analyze allosteric modulations of one ligand molecule

binding on the affinity of a second ligand molecule binding to a GPCR homodimer. From saturation experiments, the two-state dimer model provides an index of cooperativity of the radioligand (degree of modulation exerted by the first ligand molecule binding to the first protomer on the affinity of the second ligand molecule binding to the second protomer in the homodimer). From competition experiments, the two-state dimer model provides three more indexes: an index of cooperativity of the competing ligand; and index of the modulation of the affinity of the competing ligand binding to the second protomer by the radioligand binding to the first protomer; and an index of the modulation of the affinity of the radioligand binding to the second protomer by the competing ligand binding to the first protomer (Casadó et al. 2007; Ferré et al. 2014).

When considering receptor heteromers as conduits of allosteric interactions, two possible scenarios should be considered (Kenakin and Miller 2010). In the first scenario, a ligand binding to one of the receptors in the heteromer leads to changes in the properties (affinity or intrinsic efficacy) of a ligand binding to the second molecularly different receptor. The best example is the allosteric antagonistic interaction between adenosine A_{2A} receptor ($A_{2A}R$) agonists on dopamine D_2 receptor (D_2R) agonists in the $A_{2A}R$-D_2R heteromer, by which $A_{2A}R$ agonists decrease the affinity of D_2R agonists. This is probably the most quoted and reproduced allosteric modulation in a GPCR heteromer (Dixon et al. 1997; Ferré et al. 1991; Kudlacek et al. 2003). The $A_{2A}R$-D_2R heteromer is selectively localized in the GABAergic striato-pallidal neuron (also called indirect medium spiny neuron or iMSNs) (Azdad et al. 2009; Ferré et al. 2007; Trifilieff et al. 2011). It has been hypothesized that allosteric interactions between $A_{2A}R$ and D_2R agonists within the $A_{2A}R$-D_2R heteromer provide a mechanism responsible for the behavioral depressant effects of adenosine analogues and for the psychostimulant effects of selective adenosine $A_{2A}R$ antagonists and the non-selective adenosine receptor antagonist caffeine, with implications for several neuropsychiatric disorders (Ferré 2008; Ferré et al. 2004, 2008). In fact, the same mechanism provided the first rationale for the use of $A_{2A}R$ antagonists in Parkinson's disease (Armentero et al. 2011; Ferré et al. 1992; Muller and Ferré 2007). In the second scenario of allosteric modulation within GPCR heteromers, the modulator is not a ligand, but a protein (see the above-mentioned definition of allosterism): one of the receptors acts as modulator of a ligand binding to the other molecularly different receptor (Kenakin and Miller 2010). It is this allosteric modulation that can theoretically allow the selective targeting of different subpopulations of a particular receptor, like pre- *versus* postsynaptic receptors (see below). Again, the $A_{2A}R$-D_2R provides a valuable example. Screening with various *in vitro* and *in vivo* techniques led to the finding of very different qualitative properties of several selective $A_{2A}R$ antagonists. The most striking finding was a decrease in the affinity of SCH 442416 for $A_{2A}R$ when forming heteromers with D_2R, compared to when not forming heteromers or forming heteromers with adenosine A_1 receptor (A_1R) (Orru et al. 2011a). Application of the two-state dimer model indicated that SCH 442416 binds with low affinity due a strong negative cooperativity that appears when the D_2R binds to the $A_{2A}R$ in the heteromer (Ferré et al. 2014; Orru et al. 2011a), strongly suggesting that the $A_{2A}R$-D_2R comprises at least two $A_{2A}R$ protomers.

Being a weak ligand for the $A_{2A}R$-D_2R heteromer, SCH 442416 would not be useful in Parkinson's disease. Nevertheless, SCH 442416 acts preferentially on presynaptic striatal $A_{2A}R$ localized in cortico-striatal glutamatergic terminals that forming heteromers with A_1R. By blocking presynaptic $A_{2A}R$, SCH 442416 potently blocks cortico-striatal glutamatergic neurotransmission at doses that do not produce locomotor activation, that do not block postsynaptic $A_{2A}R$ (Orru et al. 2011a). The opposite pharmacological profile was obtained with KW 6002, which produced strong locomotor activity at doses that would be ineffective at blocking cortico-striatal glutamatergic neurotransmission (Orru et al. 2011a). KW 6002 would therefore be a promising antiparkinsonian agent. In fact, KW 6002 is already being successfully used in the treatment of Parkinson's disease (Jenner 2014; Pinna 2014).

The possibility of selectively targeting A_1R-$A_{2A}R$ heteromers with SCH 442416 was used to identify an important contributor to the reinforcing effects of cannabinoids: cortico-striatal glutamatergic neurotransmission. Initially, a paradoxical result had ben reported, by which the $A_{2A}R$ antagonist MSX-3 decreases THC and anandamide self-administration in squirrel monkeys at a relatively low dose, while a three-fold higher dose produced the opposite effect (Justinová et al. 2011). Based on results obtained in rats (Orru et al. 2011a), it was hypothesized that the different dose-dependent effects of MSX-3 could be related to a slightly selective presynaptic effect at lower doses with an overriding postsynaptic effect at larger doses. This hypothesis was confirmed by testing the effects of SCH-442416 and KW-6002 (Justinová et al. 2014). SCH-442416 produced a significant shift to the right of the THC self-administration dose-response curves, consistent with antagonism of the reinforcing effects of THC. On the other hand, KW-6002 produced a significant shift to the left, consistent with potentiation of the reinforcing effects of THC. These results show that selectively blocking presynaptic $A_{2A}R$ could provide a pharmacological approach to the treatment of marijuana dependence, and underscore cortico-striatal glutamatergic neurotransmission as a possible main mechanism involved in the rewarding effects of THC. At a more general level, these results also show that while the concept of using GPCR heteromers to target specific cell types is relatively new, it is a promising approach for targeting specific cell types to modulate specific symptoms of SUD.

Functional Significance and Regulation of Allosteric Interactions in the $A_{2A}R$-D_2R Heteromer

Demonstration of the functional significance of receptors heteromers is becoming an important goal in GPCR research. One main reason is their possible use as targets for drug development. The allosteric interactions in GPCR heteromers determine the specific biochemical properties of these heteromers, conferring their functional and pharmacologcical significance. In order to ascertain a biochemical property of the GPCR heteromer, which can then be used as a "biochemical fingerprint" for its identification in native tissues, the putative biochemical property should be

disrupted with molecular or chemical tools that destabilize the quaternary structure of the heteromer (Ferré et al. 2009, 2014). This can be achieved by introducing mutations that modify key determinant residues at the oligomerization interfaces or using competing peptides with the sequence of specific receptor domains putatively involved in receptor oligomerization (Azdad et al. 2009; Banères and Parello 2003; Guitart et al. 2014; He et al. 2011; Hebert et al. 1996; Pei et al. 2010). Studies of peptide-peptide interactions using biophysical methods (such as Bioluminescence Resonance Energy Transfer or BRET) and mass spectrometry, led to the identification of intracellular epitopes of the D_2R (an arginine-rich epitope of the third intracellular loop or 3IL) and the $A_{2A}R$ (a distal C-terminal epitope containing a phosphorylated serine, serine-374) that establish a strong electrostatic interaction and are important determinants of the quaternary structure of the $A_{2A}R$-D_2R heteromer (Borroto-Escuela et al. 2010; Ciruela et al. 2004; Navaro et al. 2010; Woods and Ferré 2005). In BRET, a bioluminescence donor molecule, *Renilla* luciferase (Rluc), emits light upon addition of its substrate coelenterazine H. If in very close proximity (less than 10 nm), this emission transfers energy to a fluorescence acceptor molecule, such as yellow fluorescence protein (YFP). When studying GPCR heteromerization, Rluc is fused to one of the receptors and YFP is fused to the other receptor unit. Heteromerization of $A_{2A}R$-Rluc and D_2R-YFP was then demonstrated in transfected cells (Canals et al. 2003). Subsequent studies showed that transfection with a mutant $A_{2A}R$ with substitution of serine-374 by alanine ($A_{2A}R^{A374}$-Rluc, instead of $A_{2A}R$-Rluc) and D_2R-YFP, significantly reduced BRET values (Borroto-Escuela et al. 2010; Navarro et al. 2010), and the potency of the $A_{2A}R$ agonist CGS 21680 to decrease the affinity of D_2R for dopamine agonists (Bonaventura et al. 2014; Borroto-Escuela et al. 2010). These results demonstrated that the agonist-agonist allosteric interaction constitutes a biochemical property of the $A_{2A}R$-D_2R heteromer. Therefore, its demonstration in striatal tissue indicates the presence of the $A_{2A}R$-D_2R heteromer in the brain (Ferré et al. 1991).

A peptide approach was then used to evaluate the neuronal localization and functional significance of the $A_{2A}R$-D_2R heteromer. A very effective antagonistic interaction between $A_{2A}R$ and D_2R agonists was demonstrated with patch-clamp experiments (using knock-in mice expressing GFP) in D_2R-containing neurons in striatal slices (Azdad et al. 2009). CGS 21680 completely counteracted the ability of the D_2R agonist R(-)-propylnorapomorphine hydrochloride (NPA) to block NMDA-induced neuronal firing. This effect was selectively counteracted by the application of a small peptide with an amino acid sequence corresponding the epitope of the $A_{2A}R$ that includes serine-374 (Azdad et al. 2009). These results would suggest that this pharmacological interaction is determined by the agonist-agonist allosteric interaction in the $A_{2A}R$-D_2R heteromer, since both depend on the electrostatic interaction between intracellular domains of the $A_{2A}R$ and D_2R involved in the establishment of the quaternary structure of the $A_{2A}R$-D_2R heteromer. However, just a decrease in the affinity of NPA could not explain by itself the ability of CGS 21680 to abolish the decrease in excitability of D_2R-containing neurons induced by the high concentration of the D_2R agonist used, which should overcome the decrease in affinity. A decrease in the intrinsic efficacy of the D_2R agonist was therefore also involved

(Azdad et al. 2009). Importantly, we should not conclude from the peptide experiments that the electrostatic interactions between intracellular domains are the only ones determining the quaternary structure of GPCR heteromers, including $A_{2A}R$-D_2R heteromers. Also from experiments with peptides, it is becoming clear that interactions between specific transmembrane domains are also involved (as recently shown for the dopamine D_1R-D_3R heteromer; Guitart et al. 2014).

An enigma to be resolved about the function of $A_{2A}R$-D_2R heteromers is the possibility of simultaneous antagonistic reciprocal interactions between the two different receptor units. As mentioned above, in the striatum, stimulation of $A_{2A}R$ counteracts a D_2R agonist-induced inhibitory modulation of NMDA receptor-mediated effects (Azdad et al. 2009, see also Higley and Sabatini 2010). But other studies have reported the ability of D_2R activation to potently inhibit $A_{2A}R$ adenylyl-cyclase signaling in transfected cells (Hillion et al. 2002; Kull et al. 1999) and it is not entirely clear if this canonical interaction between G_s- and G_i-mediated signaling pathways takes place in the frame of the $A_{2A}R$-D_2R heteromer, as recently suggested for other receptor heteromers (Cristóvão-Ferreira et al. 2013; Guitart et al. 2014). In the striatum, under normal conditions, the ability of $A_{2A}R$ to activate adenylyl-cyclase (and consequent expression of genes such as *c-fos* or *preproenkephalin* by the striato-pallidal neuron) seems to be restrained by a strong tonic inhibitory effect of endogenous dopamine on striatal D_2R, which efficiently inhibits $A_{2A}R$-mediated adenylyl-cyclase activation (Karcz-Kubicha et al. 2003; Svenningsson et al. 1999). Pharmacological or genetic blockade of D_2R produces a significant activation of the adenylyl-cyclase-cAMP-PKA cascade, and the consequent depressant motor effects and biochemical effects (such as increase in striatal *c-fos* or *preproenkephalin* expression) can be counteracted by genetic or pharmacologic blockade of $A_{2A}R$ (Bertran-Gonzalez et al. 2009; Chen et al. 2001; Håkansson et al. 2006). To explain the co-existence of these simultaneous reciprocal antagonistic interactions between striatal $A_{2A}R$ and D_2R, we previously postulated that they were mediated by two different subpopulations of $A_{2A}R$, forming and not forming heteromers with D_2R (Ferré et al. 2008; Orru et al. 2011b).

However, from recent experiments we could provide a heuristic model that allows understanding the possibility of different and simultaneous reciprocal interactions between $A_{2A}R$ and D_2R withion the $A_{2A}R$-D_2R heteromer. Depending on the intracellular Ca^{2+} levels, the neuronal Ca^{2+}-binding proteins NCS-1 and calneuron-1 exert a differential modulation of two different signaling pathways in the $A_{2A}R$-D_2R heteromer. Both Ca^{2+}-binding proteins were found to compete for the same binding sites in the $A_{2A}R$-D_2R heteromer. We first found that, in the absence of Ca^{2+}-binding proteins, an $A_{2A}R$ agonist decreases the intrinsic efficacy of a D_2R agonist-mediated G protein-dependent inhibition of adenylyl-cyclase and G protein-independent MAPK activation (Navarro et al. 2014). Thus, in transfected HEK-293 cells, the D_2R agonist quinpirole could not counteract the ability of the $A_{2A}R$ agonist CGS 21680 to induce cAMP accumulation, due to the allosteric modulation by which $A_{2A}R$ activation counteracts D_2R-mediated G protein-dependent signaling. However, this allosteric modulation was absent when cells were co-transfected with NCS-1 or calneuron-1 in the presence of low or high intracellular Ca^{2+} levels, respectively.

The same biochemical interactions were also found in striatal cells, where low or high intracellular Ca^{2+} levels determined if either NCS-1 or calneuron-1 bind to the $A_{2A}R$-D_2R heteromer. Knocking down the expression of NCS-1 or calneuron-1 led to the reappearance of the allosteric interaction under conditions of low or high intracellular Ca^{2+} levels, respectively, and quinpirole could not counteract the ability of CGS 21680 to stimulate adenylyl-cyclase (Navarro et al. 2014) (Fig. 2.1).

A different scenario was observed in relation to MAPK signaling. In transfected HEK-293 cells, MAPK activation (ERK1/2 phosphorylation) was similar under conditions of activation of either $A_{2A}R$ or D_2R or co-activation of both receptors.

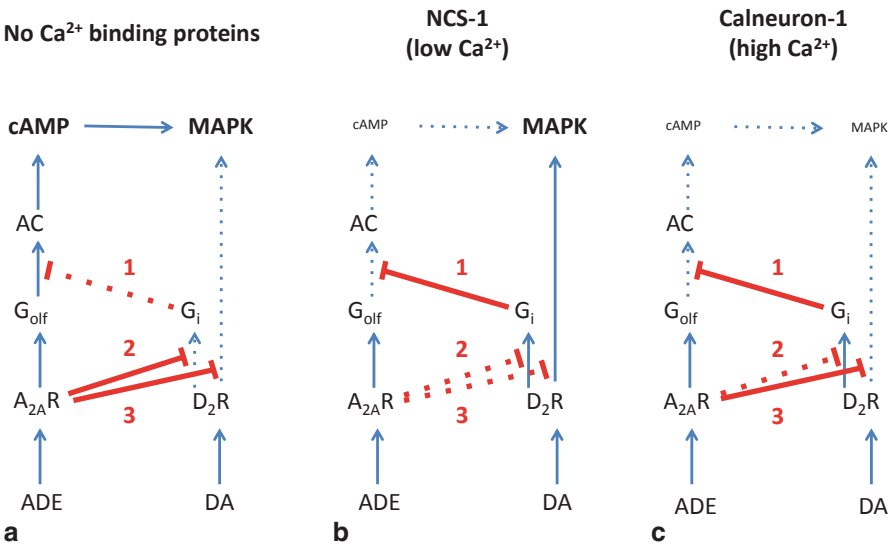

Fig. 2.1 Model representing the differential role of NCS-1 and calneuron-1 in $A_{2A}R$-D_2R heteromer signaling. Depending on the intracellular levels of Ca^{+2}, the neuronal Ca^{+2}-binding proteins NCS-1 and calneuron-1 exert a differential modulation of the $A_{2A}R$-D_2R heteromer signaling. In the absence of neuronal Ca^{+2}-binding proteins (a; non-transfected HEK-293 cells or knocking down protein expression in striatal cells in culture), the D_2R agonist cannot counteract the ability of the $A_{2A}R$ agonist to induce cAMP accumulation (*1*), due to an allosteric modulation by which $A_{2A}R$ activation inhibits D_2R-mediated G protein-dependent signaling (*2*). Under these conditions, $A_{2A}R$ activation also inhibits the D_2R agonist-mediated G protein-independent MAPK activation (*3*). These two allosteric modulations (*2* and *3*) are absent when NCS-1 binds to the receptor heteromer in the presence of low intracellular Ca^{+2} levels (b; transfected HEK-293 cells or striatal cells, where low intracellular Ca^{+2} levels determine the binding of NCS-1 to the $A_{2A}R$-D_2R heteromer). Under these conditions, co-activation of both receptors in the $A_{2A}R$-D_2R heteromer does not produce cAMP accumulation but still induces MAPK activation. When calneuron-1 binds to $A_{2A}R$-D_2R heteromer (c; transfected HEK-293 cells or striatal cells where high intracellular Ca^{+2} levels determine the binding of calneuron-1 to the $A_{2A}R$-D_2R heteromer), the allosteric modulation at the level of G protein-dependent signaling (*2*) is selectively disrupted, since the allosteric modulation at the level of G protein-independent signaling (*1*) is maintained. This results in very low activation of both MAPK signaling and cAMP production upon co-activation of both receptors in the $A_{2A}R$-D_2R heteromer, since $A_{2A}R$ agonist-mediated MAPK activation (*3*, which is dependent on adenylyl-cyclase signaling) is also inhibited.

The absence of at least an additive effect of $A_{2A}R$ and D_2R agonists would indicate some degree of antagonistic interaction. But, under conditions of high intracellular Ca^{2+} levels and in the presence of calcineuron-1, co-activation of $A_{2A}R$ and D_2R did not produce a noticeable ERK1/2 phosphorylation (Navarro et al. 2014). Since, as described previously (Canals et al. 2005; Klinger et al. 2002), we also found $A_{2A}R$-mediated MAPK activation be mostly dependent on G-protein-adenylyl-cyclase signaling (Fig. 2.1), these results indicated that high intracellular Ca^{2+} levels allows calcineuron-1 to selectively facilitate an allosteric interaction in the $A_{2A}R$-D_2R heteromer by which $A_{2A}R$ agonists also blocks a G-protein-independent D_2R-mediated ERK1/2 phosphorylation. The same mechanisms were also found to operate in striatal cells and no ERK1/2 phosphorylation was observed upon co-activation of $A_{2A}R$ and D_2R under conditions of high intracellular Ca^{2+} levels (which induce binding of calcineuron-1 to the $A_{2A}R$-D_2R heteromer). MAPK activation was nevertheless very significant under the same conditions but knocking down the expression of calcineuron-1 (Navarro et al. 2014). Therefore, as recently found for the dopamine D_1R-D_3R heteromer (Guitart et al. 2014), we found functional selectivity of allosteric interactions within the $A_{2A}R$-D_2R heteromer, and this functional selectivity was found to be dependent on intracellular Ca^{+2} levels (Navarro et al. 2014). The functional inhibition by D_2R agonists of NMDA receptor-mediated Ca^{2+}-dependent effects observed in striatal tissue preparations (Azdad et al. 2009; Higley and Sabatini 2010), which can be counteracted by $A_{2A}R$ activation, should depend largely on G-protein-independent D_2R-mediated signaling.

These results allow understanding the co-existence of reciprocal antagonistic interactions between striatal $A_{2A}R$ and D_2R, considering only one predominant population of $A_{2A}R$, which forms heteromers with D_2R. This could account for different G protein-dependent or independent functional responses, which could be differentially modulated by intracellular Ca^{+2} levels. Apart from adenosine and dopamine, the Ca^{+2}-dependent modulation of $A_{2A}R$-D_2R heteromer function allows further integration of other neurotransmitter systems such as glutamate (through NMDA receptor activation) and acetylcholine (through G_q-coupled muscarinic receptors) (Tozzi et al. 2011).

As mentioned before the existence of negative cooperativity of the $A_{2A}R$ antagonist SCH 442416 (Orrú et al. 2011a) strongly suggested that the $A_{2A}R$-D_2R comprises at least two $A_{2A}R$ protomers. Also, it would be difficult for two GPCR protomers to simultaneously accommodate two trimeric G-protein molecules due to steric hindrance (Maurice et al. 2011). Therefore, the results on allosteric interactions in the $A_{2A}R$-D_2R heteromer at the level of adenylyl cyclase signaling supports a tetrameric structure, comprised of two different homodimers, each able to signal with their preferred G protein. This molecular arrangement would allow the canonical interaction between G_s- and G_i-mediated signaling to take place in the frame of the heteromer (Ferré et al. 2014; Guitart et al. 2014).

Conclusions

GPCR oligomerization is a reality and it is becoming obvious that GPCR homodimers constitute not only functional but also structural building blocks. In this way, receptor heteromers would be comprised of two different homodimers, each able to signal with their preferred G protein. We postulate that the canonical interaction between G_s- and G_i-mediated signaling is in fact a biochemical property of GPCR heteromer. Experiments are now in progress to validate this hypothesis. But what it is already obvious, and here exemplified from the studies on $A_{2A}R$-D_2R heteromers, is that allosteric mechanisms in the frame of GPCR heterotetramers provide them with multiple unique biochemical properties, including ligand and functional selectivity. These properties allow understanding complex experimental results with pharmacological significance, such as: the existence of reciprocal interactions between activated $A_{2A}R$ and D_2R, which are differentially modulated by intracellular Ca^{2+}, making the $A_{2A}R$-D_2R heterotetramer a cellular device that integrates signals from the extracellular and intracellular compartments (dopamine, adenosine and Ca^{2+}) to produce a specific functional response; the selective negative cooperativity of the $A_{2A}R$ antagonist SCH 442416, which provides the proof of concept of the possibility that different GPCR heteromers can account for pharmacologically different subpopulations of receptors. In fact, SCH 44416 has been successfully used to target selectively striatal presynaptic $A_{2A}R$ in a non-human primate model of addiction to cannabinoids (Justinová et al. 2014). Research is in progress to obtain molecules that selectively target striatal postsynaptic $A_{2A}R$, i.e. the $A_{2A}R$-D_2R heterotetramer.

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