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Allosteric mechanisms within the adenosine A_{2A}-dopamine D₂ receptor heterotetramer

Sergi Ferré^{a,*}, Jordi Bonaventura^a, Dardo Tomasi^b, Gemma Navarro^c, Estefanía Moreno^c, Antonio Cortés^c, Carme Lluís^c, Vicent Casadó^c, and Nora D. Volkow^b

^aIntegrative Neurobiology Section, National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Baltimore, MD 21224, USA

^bNational Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda MD 2092, USA

^cCentro de Investigacion Biomedica en Red sobre Enfermedades Neurodegenerativas and Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain

Abstract

The structure constituted by a G protein coupled receptor (GPCR) homodimer and a 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 its preferred G protein. GPCR homomers and heteromers can act as the conduit of allosteric interactions between orthosteric ligands. The well-known agonist/agonist allosteric interaction in the adenosine A_{2A} receptor (A_{2A}R)-dopamine D₂ receptor (D₂R) heteromer, by which A_{2A}R agonists decrease the affinity of D₂R agonists, gave the first rationale for the use of A_{2A}R antagonists in Parkinson's disease. We review new pharmacological findings that can be explained in the frame of a tetrameric structure of the A_{2A}R-D₂R heteromer: first, ligand-independent allosteric modulations by the D₂R that result in changes of the binding properties of A_{2A}R ligands; second, differential modulation of the intrinsic efficacy of D₂R ligands for G protein-dependent and independent signaling; third, the canonical antagonistic G_s-G_i interaction within the frame of the heteromer; and fourth, the ability of A_{2A}R antagonists, including caffeine, to also exert the same allosteric modulations of D₂R ligands than A_{2A}R agonists, while A_{2A}R agonists and antagonists counteract each other's effects. These findings can have important clinical implications when evaluating the use of A_{2A}R antagonists. They also call for the need of monitoring caffeine intake when evaluating the effect of D₂R ligands, when used as therapeutic agents in neuropsychiatric disorders or as probes in imaging studies.

*Corresponding author: Integrative Neurobiology Section, National Institute on Drug Abuse, IRP, Triad Technology Building, 333 Cassell Drive, Baltimore MD 2122. Tel: 443 7402647. sferre@intra.nida.nih.gov.

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Keywords

Adenosine A2A receptor; dopamine D2 receptor; heteromer; striatum; caffeine; Parkinson's disease

1. Allosteric properties within GPCR oligomers: Allosteric interactions between orthosteric ligands

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 functional complexes that include other receptors, forming 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 (Milligan and Bouvier, 2005, Pin et al., 2007, Ferré et al., 2009, Ferré et al., 2014). 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 of the bound agonist to induce a functional response (which is independent of its affinity for the receptor)). 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 (with two orthosteric binding sites) and one heterotrimeric G protein provides a main functional unit and oligomeric entities can be viewed as multiples of dimers (Ferré et al., 2014; Ferré, 2015). 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 binding to one of the protomers, to the same or another orthosteric ligand, which binds to the second protomer. The realization of these *allosteric interactions between orthosteric ligands* is leading to a profound modification of classical pharmacology.

It has long been recognized in radioligand binding experiments in membrane preparations from transfected mammalian cell lines or from native tissues that ligands, mostly agonists, show ‘binding heterogeneity’, different affinities for the same GPCR. This is seen as

complex data in saturation and competition experiments, such as concave Scatchard plots and biphasic or shallow-slope competitive inhibition curves. Classical pharmacology assumes that these complex radioligand data are due to the existence of two states of affinity of the receptor for the agonist that depend on the existence of two pools of receptors, coupled and not coupled to the G protein. Many data now indicate there is only one pool, always coupled to the G protein and that the biphasic nature of these data depends on the existence of cooperativity (usually negative) of the agonist within the homodimer, where the first molecule of the ligand binding to the dimer changes the affinity of the second molecule (Casadó et al., 2007; Ferré et al., 2014; Ferré, 2015). The new models of analysis of radioligand binding experiments that consider GPCR as dimers provide more robust results (less dependent on experimental conditions, such as the selected radioligand or its concentration) than the classical and still most used *two independent-site model* (that gives the classical $B_{\max}$ and K_D values from saturation experiments and K_H , K_L and R_H values from competition experiments). The *two-state dimer model* developed by Casadó et al. (2007) provides the most practical method to characterize ligand-receptor interactions when considering GPCR as homodimers. In saturation experiments, apart from providing affinity values and number of binding sites, it provides a dimer cooperativity index of the radioligand (D_C) that defines the ability of the radioligand binding to the first protomer to alter the affinity of the same radioligand binding to the second protomer (Casadó et al., 2007). From competition experiments, the two-state dimer model also provides a dimer cooperativity index of the competing ligand (D_{CB}) and indexes of allosteric modulations between the radioligand and the competing ligand. These two indexes measure the ability of the radioligand binding to the first protomer to alter the affinity of the competing ligand binding to the second protomer (D_{AB}) and the ability of the competing ligand binding to the first protomer to alter the affinity of the radioligand binding to the second protomer (D_{BA}) (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 (Figure 1A). This is probably the most quoted and reproduced allosteric modulation within a GPCR heteromer (Ferré et al., 1991; Dasgupta et al., 1996; Dixon et al., 1997; Diaz-Cabiale et al., 2001; Kudlacek et al., 2003; Bonaventura et al., 2015). The $A_{2A}R$ - D_2R heteromer is selectively localized in the GABAergic striato-pallidal neuron (also called indirect medium spiny neuron or iMSNs) (Ferré et al., 2007, Azdad et al., 2009, 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 et al., 2004, 2008; Ferré, 2008; Bonaventura et al., 2015).

In fact, the same mechanism provided the first rationale for the use of A_{2A}R antagonists in Parkinson's disease (Ferré et al., 1992; Muller and Ferré, 2007; Armentero et al., 2011).

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). There is a ligand-independent allosteric modulation where 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. Again, the A_{2A}R-D₂R 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 change in the binding properties of SCH 442416 for A_{2A}R when forming heteromers with D₂R, compared to when not forming heteromers or forming heteromers with adenosine A₁ receptor (A₁R) (Orrú et al., 2011a). Application of the two-state dimer model indicated that SCH 442416 binds with a strong negative cooperativity that appears when the D₂R interacts with the A_{2A}R in the heteromer (Orrú et al., 2011a, Ferré et al., 2014) (Figure 1B). This gave the first indication that the A_{2A}R-D₂R comprises at least two A_{2A}R protomers, supporting the existence of the A_{2A}R-D₂R heterotetramer. Being a weak ligand for the A_{2A}R-D₂R 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 A₁R. 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 A₁R-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 been 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 (Justinova 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 (Justinova 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.

2. Functional significance of allosteric interactions in the A_{2A}R-D₂R heterotetramer

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 pharmacological 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 physical 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 disrupting peptides, with the sequence of specific receptor domains putatively involved in receptor oligomerization (Hebert et al., 1996; Baneres and Parello, 2003; Azdad et al., 2009; Borroto-Escuela et al., 2010; He et al., 2011; Guitart et al., 2014; Bonaventura et al., 2015; Navarro et al., 2015). Studies with biophysical methods (such as Bioluminescence Resonance Energy Transfer or BRET) and mass spectrometry, led to the identification of intracellular epitopes of the D₂R (an arginine-rich epitope of the third intracellular loop) and the A_{2A}R (a distal Cterminal 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-D₂R heteromer (Ciruela et al., 2004; Woods and Ferré, 2005; Borroto-Escuela et al., 2010; Navaro et al., 2010; Bonaventura et al., 2015). 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 D₂R-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 D₂R-YFP, significantly reduce 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 D₂R agonists (Bonaventura et al., 2015). This indicates that the allosteric modulation between an A_{2A}R agonist and a D₂R agonist depends on the quaternary structure of the A_{2A}R-D₂R determined by the electrostatic interaction between intracellular domains of both receptors and, therefore, constitutes a biochemical property of the A_{2A}R-D₂R heteromer. Its demonstration in striatal tissue indicates the presence of the A_{2A}R-D₂R heteromer in the brain (Ferré et al., 1991; Bonaventura et al., 2015).

A peptide approach was then used to evaluate the neuronal localization and functional significance of the A_{2A}R-D₂R heteromer. A very effective antagonistic interaction between A_{2A}R and D₂R agonists was demonstrated with patch-clamp experiments in D₂R-containing

neurons in striatal slices (Azdad et al., 2009). CGS 21680 completely counteracted the ability of the D₂R agonist R(–)-propylnorapomorphine hydrochloride (NPA) to completely block NMDA-induced neuronal firing. This effect was selectively counteracted by the application of a small peptide with an amino acid sequence corresponding to 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-D₂R heteromer, since both depend on the electrostatic interaction between intracellular domains of the A_{2A}R and D₂R involved in the establishment of the quaternary structure of the A_{2A}R-D₂R 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 D₂R-containing neurons induced by the high concentration of the D₂R agonist used, which should overcome the decrease in affinity. Therefore, a decrease in the intrinsic efficacy of the D₂R agonist had to be also involved (Azdad et al., 2009).

Also from experiments with peptides, it has been suggested that, in addition to intracellular domains, interactions between specific transmembrane domains (TMs) are also involved in GPCR oligomerization (He et al., 2011; Borroto et al., 2011; Guitart et al., 2014; Navarro et al., 2015). Borroto et al. (2010) reported the ability of peptides with the amino acid sequence of TM4 and TM5 from D₂R to disrupt BRET in cells transfected with D₂R-Rluc and A_{2A}R-YFP. In our hands, however, the application of TM peptides (at μ M concentrations) very strongly inhibited the enzymatic activity of Rluc, invalidating BRET as a method to evaluate modifications of receptor heteromer structure induced by synthetic hydrophobic peptides (Guitart et al., 2014). As an alternative method to BRET, we have successfully used bimolecular fluorescence complementation to demonstrate the involvement of specific TM domains in heteromerization of dopamine D₁ and D₃ receptors (Guitart et al., 2014) corticotropin-releasing factor CRF₁ and orexin OX₁ receptors (Navarro et al., 2015) and A_{2A}R and D₂R (Bonaventura et al., 2015). In our studies an HIV transactivator of transcription (TAT) peptide is fused to the corresponding TM peptide, which allows its effective insertion into the plasma membrane as a result of the penetration capacity of the TAT sequence and the hydrophobic property of the TM domain (He et al., 2010; Guitart et al., 2014; Bonaventura et al., 2015; Navarro et al., 2015). HIV TAT peptides fused to TM5 from both A_{2A}R or D₂R, but not TM6 or TM7, significantly reduced fluorescence values obtained with YFP reconstitution when HEK-293 cells were transfected with A_{2A}R fused to the YFP Venus N-terminal hemiprotein (A_{2A}R-nYFP) and D₂R fused to the YFP Venus C-terminal hemiprotein (D₂R-cYFP) (Bonaventura et al., 2015). The results strongly suggested that, in addition to intracellular domains, TM5 from both receptors form part of the heteromerization interface.

An enigma to be resolved about the function of A_{2A}R-D₂R 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 D₂R 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 D₂R activation to potently inhibit A_{2A}R adenylyl-cyclase signaling in transfected cells (Kull et al., 1999; Hillion et al., 2002) 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-D₂R heteromer, as

recently suggested for other receptor heteromers (Cristovao-Ferreira et al 2013, Guitart et al 2014). In the striatum, under normal conditions, the ability of A_{2A}R to activate adenylylcyclase (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 D₂R, which efficiently inhibits A_{2A}R-mediated adenylylcyclase activation (Svenningsson et al., 1999; Karcz-Kubicha et al., 2003). Pharmacological or genetic blockade of D₂R 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 (Chen et al., 2001; Hakansson et al., 2006; Bertran-Gonzalez et al., 2009). To explain the co-existence of these simultaneous reciprocal antagonistic interactions between striatal A_{2A}R and D₂R, we previously postulated that they were mediated by two different subpopulations of A_{2A}R, forming and not forming heteromers with D₂R (Ferré et al., 2008, Orrú 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 D₂R considering only one predominant subpopulation of A_{2A}R, which forms A_{2A}R-D₂R heteromers (Figure 1C). Depending on the intracellular Ca²⁺ levels, the neuronal Ca²⁺-binding proteins NCS-1 and calneuron-1 exert a differential modulation of two different signaling pathways in the A_{2A}R-D₂R heteromer. Both Ca²⁺-binding proteins were found to compete for the same binding sites in the A_{2A}R-D₂R heteromer. We first found that, in the absence of Ca²⁺-binding proteins, an A_{2A}R agonist decreases the intrinsic efficacy of a D₂R 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 D₂R 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 D₂R-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 Ca²⁺ levels, respectively. The same biochemical interactions were also found in striatal cells, where low or high intracellular Ca²⁺ levels determined if either NCS-1 or calneuron-1 bind to the A_{2A}R-D₂R 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 Ca²⁺ levels, respectively, and quinpirole could not counteract the ability of CGS 21680 to stimulate adenylyl-cyclase (Navarro et al., 2014).

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 D₂R or co-activation of both receptors. The absence of at least an additive effect of A_{2A}R and D₂R agonists would indicate some degree of antagonistic interaction. But, under conditions of high intracellular Ca²⁺ levels and in the presence of calneuron-1, co-activation of A_{2A}R and D₂R did not produce a noticeable ERK1/2 phosphorylation (Navarro et al 2014). Since, as described previously (Klinger et al., 2002; Canals et al., 2005), we also found A_{2A}R-mediated MAPK activation be mostly dependent on G-protein-adenylyl-cyclase signaling, these results indicated that high intracellular Ca²⁺

levels allows calneuron-1 to selectively facilitate an allosteric interaction in the A_{2A}R-D₂R heteromer by which A_{2A}R agonists also blocks a G-protein-independent D₂R-mediated ERK1/2 phosphorylation. The same mechanisms were also found to operate in primary cultures of striatal cells and ERK1/2 phosphorylation was totally absent upon co-activation of A_{2A}R and D₂R under conditions of high intracellular Ca²⁺ levels (which induce binding of calneuron-1 to the A_{2A}R-D₂R heteromer). MAPK activation was nevertheless very significant under the same conditions but knocking down the expression of calneuron-1 (Navarro et al., 2004). Therefore, as recently found for the dopamine D₁R-D₃R heteromer (Guitart et al., 2014), we found functional selectivity of allosteric interactions within the A_{2A}R-D₂R heteromer, and this functional selectivity was found to be dependent on intracellular Ca²⁺ levels (Navarro et al., 2014) (Figure 1C). The functional inhibition by D₂R agonists of NMDA receptor-mediated Ca²⁺-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 D₂R-mediated signaling.

3. Pharmacological significance of allosteric interactions in the A_{2A}R-D₂R heterotetramer

Since it would be difficult for two GPCR protomers to simultaneously accommodate two trimeric G-protein molecules due to steric hindrance (Maurice et al., 2011), the results on allosteric interactions in the A_{2A}R-D₂R 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 (Guitart et al., 2014; Ferré et al., 2014; Ferré, 2015) (Figure 1c). Two more groups of experimental results support that the A_{2A}R-D₂R heteromer contains at least two A_{2A}R units: the abovementioned negative cooperativity of the A_{2A}R antagonist SCH 442416 (Orrú et al., 2011a) and our recent results on the allosteric properties of selective A_{2A}R antagonists and caffeine when binding to the A_{2A}R-D₂R heteromer (Bonaventura et al., 2015).

The second group of results originated from a PET study in humans, where we found that the acute administration of caffeine produces a significant increase in the binding of the D₂R antagonist [¹¹C]raclopride in putamen and ventral striatum (but not in caudate nucleus), when compared to placebo (Volkow et al., 2015) (Figure 2). In addition, caffeine-induced increases in D₂R availability in the ventral striatum were associated with caffeine-induced increases in alertness (Volkow et al., 2015). On the basis of the allosteric interaction between agonists in the A_{2A}R-D₂R heteromer, caffeine should have produced the opposite effect, by decreasing the ability of endogenous adenosine to inhibit the binding of endogenous dopamine to the D₂R. Furthermore, caffeine has also been shown to release dopamine in a subregion of the ventral striatum by blocking presynaptic A₁R localized in dopaminergic terminals (Ferré, 2008), which should also lead to a decrease in [¹¹C]raclopride binding. Therefore, we analyzed the possibility that caffeine binding to A_{2A}R could modulate D₂R agonist binding. In fact, CGS 21680 and caffeine significantly decreased [³H]quinpirole binding in membrane preparations from both sheep striatum and CHO cells transiently

transfected with A_{2A}R and D₂R (Bonaventura et al., 2015). In cells transfected with A_{2A}R^{A374} the potency of both CGS 21680 and caffeine at modulating [³H]quinpirole binding was significantly reduced, compared to cells transfected with A_{2A}R and D₂R (Bonaventura et al., 2015). This would indicate that the antagonistic allosteric modulation between an A_{2A}R antagonist and a D₂R agonist also constitutes a biochemical property of the A_{2A}R-D₂R heteromer.

Caffeine could therefore facilitate [¹¹C]raclopride binding by decreasing the binding of endogenous dopamine to D₂R and explain the results of the PET study. However, since both A_{2A}R agonists and antagonists produce a conformational change in the A_{2A}R-D₂R heteromer that leads to the same effect, a reduction in the affinity of agonists for the D₂R, this questions the validity of allosteric interactions between A_{2A}R and D₂R agonists within the A_{2A}R-D₂R heteromer as a main mechanism involved in the opposite behavioral effects of A_{2A}R agonists and caffeine. (Ferré, 1998). We then evaluated the combined effect of A_{2A}R agonists and caffeine or selective A_{2A}R antagonists on D₂R agonist binding. [³H]Quinpirole binding in membrane preparations from sheep striatum was measured in the presence of CGS 21680 and increasing concentrations of caffeine or the selective A_{2A}R antagonists SCH 58261 and KW 6002. The three compounds produced a biphasic effect on the ability of CGS 21680 to decrease [³H]quinpirole binding. Low concentrations counteracted the effect of CGS 21680, while high concentrations were associated with a significant decrease in [³H]quinpirole binding. These results showed that, although A_{2A}R agonists and antagonists who bind competitively to the orthosteric site (Lebon et al., 2011) produce the same allosteric modulation of D₂R agonist binding when individually administered, they can cancel each other's effect when co-administered. This would support that the A_{2A}R-D₂R heteromer contains two A_{2A}R protomers. A corollary of that assumption would be that simultaneous occupancy of the A_{2A}R homodimer in the A_{2A}R-D₂R heteromer by an agonist and an antagonist should not induce an allosteric modulation of D₂R agonist binding (Figure 1D). The dimeric nature of the A_{2A}R was confirmed with experiments of dissociation of [³H]ZM 241385 in sheep striatal preparations. The A_{2A}R agonist CGS 21680, but not caffeine or SCH 58261, significantly modified the dissociation rate of the labeled antagonist. Therefore, it is only the agonist that can exert an allosteric modulation of the labeled antagonist when both are occupying orthosteric sites in an A_{2A}R homodimer, since the four ligands, caffeine, ZM 241385, SCH 58261 and CGS 21680 all bind and compete for the same orthosteric site. This implies a different conformation of the A_{2A}R homodimer when occupied with an agonist and an antagonist compared to when occupied with two antagonists.

The same allosteric modulation exerted by A_{2A}R agonists and antagonists on D₂R agonist affinity was also evident on D₂R agonist intrinsic efficacy. In HEK-293 cells transfected with A_{2A}R and D₂R, CGS 21680 significantly counteracted MAPK activation induced by a high concentration of quinpirole. Then, addition of increasing concentrations of either the A_{2A}R antagonist SCH 58261 or caffeine, which were ineffective when administered alone, produced the same biphasic effect than the one observed with radioligand binding experiments. Low concentrations counteracted the effect of CGS 21680 and this effect disappears with larger concentrations, which by themselves completely antagonized the effect of both CGS 21680 and quinpirole. These results predicted that, under specific

experimental conditions, A_{2A}R antagonists behaved as A_{2A}R agonists and decreased D₂R function in the brain and those effects were counteracted upon co-administration of A_{2A}R agonist and antagonist, as demonstrated with patch-clamp experiments in striatal slices and locomotor-activity experiments in rats (Bonaventura et al., 2015).

However, the results showing that A_{2A}R agonists and A_{2A}R antagonists can counteract each other's allosteric effects on D₂R agonist binding could not explain the ability of caffeine to increase striatal [¹¹C]raclopride binding (Volkow et al., 2015). They would again predict the opposite effect. We then explored the possibility of similar A_{2A}R ligand-mediated allosteric modulations of D₂R antagonist properties. Indeed, we could demonstrate that both CGS 21680 and caffeine significantly reduce [³H]raclopride binding in membrane preparations from sheep and human striatum and from CHO cells transfected with A_{2A}R and D₂R (Bonaventura et al., 2015). Again, the potency of both CGS 21680 and caffeine at modulating [³H]raclopride binding was significantly reduced in cells expressing the mutant A_{2A}R^{A374}, indicating dependence on A_{2A}R-D₂R heteromerization (Bonaventura et al., 2015). Importantly, the same peptides that destabilized the quaternary structure of the A_{2A}R-D₂R heteromer, HIV TAT peptides fused to TM5 from both A_{2A}R or D₂R, but not TM6 or TM7, selectively destabilized A_{2A}R-D₂R heteromerization in sheep striatal slices, as analyzed by proximity ligation assay, and caffeine-mediated decrease in [³H]raclopride binding in sheep striatal membrane preparations (Bonaventura et al., 2015). Therefore, any orthosteric A_{2A}R ligand, agonist or antagonist, can decrease the affinity and intrinsic efficacy of any orthosteric D₂R ligand, agonist or antagonist, and these constitute biochemical properties of the A_{2A}R-D₂R heteromer since they depend on the integrity of the right quaternary structure of the heteromer as demonstrated in transfected mammalian cells and striatal tissue, by using disrupting mutations and peptides, respectively.

Furthermore, the same biphasic effect observed with increasing concentrations of caffeine on the ability of the A_{2A}R agonist CGS 21680 to decrease [³H]quinpirole binding was also observed with [³H]raclopride binding in membrane preparations from sheep striatum. Thus, low concentrations antagonized the effect of CGS 21680, while high concentrations were also associated with a significant decrease in [³H]raclopride binding (Bonaventura et al., 2015). At last, these results provide a mechanistic explanation for the PET experiments in humans: Endogenous concentrations of adenosine would tonically decrease [¹¹C]raclopride binding, and caffeine would be counteracting this effect with the consequent increase in [¹¹C]raclopride binding. These results then call for the need of monitoring caffeine intake when evaluating the effect of D₂R ligands, when used as therapeutic agents in neuropsychiatric disorders or as probes in imaging studies.

A direct evidence for the formation of heterotetramers, heteromers of A_{2A}R and D₂R homodimers, could be obtained with BRET with double bimolecular complementation of YFP and Rluc, as previously used to demonstrate D1R-D3R heterotetramers (Guitart et al., 2014). Rluc reconstitution after transfection of A_{2A}R fused to the Rluc N-terminal hemiprotein (A_{2A}R-nRluc) and D₂R fused to the Rluc C-terminal hemiprotein (D₂R-cRluc) was demonstrated by strong bioluminescence after the addition of the Rluc substrate coelenterazine H, indicating A_{2A}R(nRluc)-D₂R(cRluc) heteromerization. Significant BRET values were then obtained when co-transfecting A_{2A}R-nRluc, D₂R-cRluc, A_{2A}R-nYFP and

D₂R-cYFP (Bonaventura et al., 2014). The A_{2A}R-D₂R heterotetramer offers a model that explains the apparent contradiction of orthosteric A_{2A}R agonists and antagonists being able to produce the same modulatory effects on D₂R function and yet counteract each other's effects. The model assumes that occupancy of the A_{2A}R homodimer with either an agonist or an antagonist produces a conformational change that conduces the same allosteric modulation to the D₂R, while simultaneous occupancy of the A_{2A}R homodimer by an agonist and an antagonist would not allow this conformational change. The model has an important heuristic value. As the model predicted, in the brain, under specific experimental conditions, orthosteric A_{2A}R antagonists behave as A_{2A}R agonists and decrease D₂R function, effects that are counteracted upon co-administration of both A_{2A}R agonists and antagonists (electrophysiological and locomotor activity experiments). Nevertheless, motor depression by caffeine or A_{2A}R antagonists imply a significant displacement of endogenous adenosine and occupancy of the A_{2A}R homodimer in the A_{2A}R-D₂R heteromer, which can only be attained by large pharmacological doses.

4. 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-D₂R 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 old and new complex experimental results with pharmacological significance, such as: the selective negative cooperativity of the A_{2A}R antagonist SCH 442416 in the A_{2A}R-D₂R heterotetramer, which provides the proof of concept of the possibility that different GPCR heteromers can account for pharmacologically different subpopulations of receptors; the existence of multiple allosteric and reciprocal interactions between A_{2A}R and D₂R ligands, which are differentially modulated by different intracellular concentrations of Ca²⁺, making the A_{2A}R-D₂R heterotetramer a cellular device that integrates signals from the extracellular and extracellular compartments (dopamine, adenosine and Ca²⁺) to produce a specific functional response; the differential modulation of D₂R ligand properties by orthosteric A_{2A}R agonists and antagonists when administered alone or combined, giving new mechanisms for the motor stimulating effects of caffeine and selective A_{2A}R antagonists, with obvious implications for the treatment of Parkinson's disease and related basal ganglia disorders.

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Fig. 1. Scheme of different allosteric properties of ligands for the A_{2A}R-D₂ heterotetramer. **A:** Ability of A_{2A}R agonists (green circles; but also antagonists, see text) to decrease the affinity and intrinsic efficacy of D₂R agonists (orange circles; but also antagonists, see text). **B:** Ability of the D₂R to promote a negative cooperativity of a specific ligand, the A_{2A}R antagonist SCH 442416 (green squares). **C:** Ability of calneuron-1 (gray square with small red circles, representing Ca²⁺ ions) to promote functional selectivity of the allosteric modulations of A_{2A}R agonists (green circles) on D₂R agonists (orange circles) intrinsic efficacy (inhibiting D₂R-mediated MAPK activation but not G_i-mediated AC inhibition). **D:** Ability of caffeine or an A_{2A}R antagonist (green square) to block the A_{2A}R agonist (green circle)-mediated allosteric modulation of D₂R ligand (orange circles) functional properties.

Fig. 2. Brain maps showing significant differences in D_2R/D_3R availability (non-displaceable binding potential or BPND), between placebo and caffeine for the contrast Caffeine > Placebo. Threshold for significance corresponds to $p < 0.01$, clusters > 100 voxels. Images are in radiological coordinatea where right corresponds to left. Modified from Volkow et al. (2015).