



# Adenosine A<sub>2A</sub> receptors and A<sub>2A</sub> receptor heteromers as key players in striatal function

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A very significant density of adenosine A<sub>2A</sub> receptors (A<sub>2A</sub>Rs) is present in the striatum, where they are preferentially localized postsynaptically in striatopallidal medium spiny neurons (MSNs). In this localization A<sub>2A</sub>Rs establish reciprocal antagonistic interactions with dopamine D<sub>2</sub> receptors (D<sub>2</sub>Rs). In one type of interaction, A<sub>2A</sub>R and D<sub>2</sub>R are forming heteromers and, by means of an allosteric interaction, A<sub>2A</sub>R counteracts D<sub>2</sub>R-mediated inhibitory modulation of the effects of NMDA receptor stimulation in the striatopallidal neuron. This interaction is probably mostly responsible for the locomotor depressant and activating effects of A<sub>2A</sub>R agonist and antagonists, respectively. The second type of interaction involves A<sub>2A</sub>R and D<sub>2</sub>R that do not form heteromers and takes place at the level of adenylyl cyclase (AC). Due to a strong tonic effect of endogenous dopamine on striatal D<sub>2</sub>R, this interaction keeps A<sub>2A</sub>R from signaling through AC. However, under conditions of dopamine depletion or with blockade of D<sub>2</sub>R, A<sub>2A</sub>R-mediated AC activation is unleashed with an increased gene expression and activity of the striatopallidal neuron and with a consequent motor depression. This interaction is probably the main mechanism responsible for the locomotor depression induced by D<sub>2</sub>R antagonists. Finally, striatal A<sub>2A</sub>Rs are also localized presynaptically, in cortico-striatal glutamatergic terminals that contact the striato-nigral MSN. These presynaptic A<sub>2A</sub>Rs heteromerize with A<sub>1</sub> receptors (A<sub>1</sub>Rs) and their activation facilitates glutamate release. These three different types of A<sub>2A</sub>Rs can be pharmacologically dissected by their ability to bind ligands with different affinity and can therefore provide selective targets for drug development in different basal ganglia disorders.

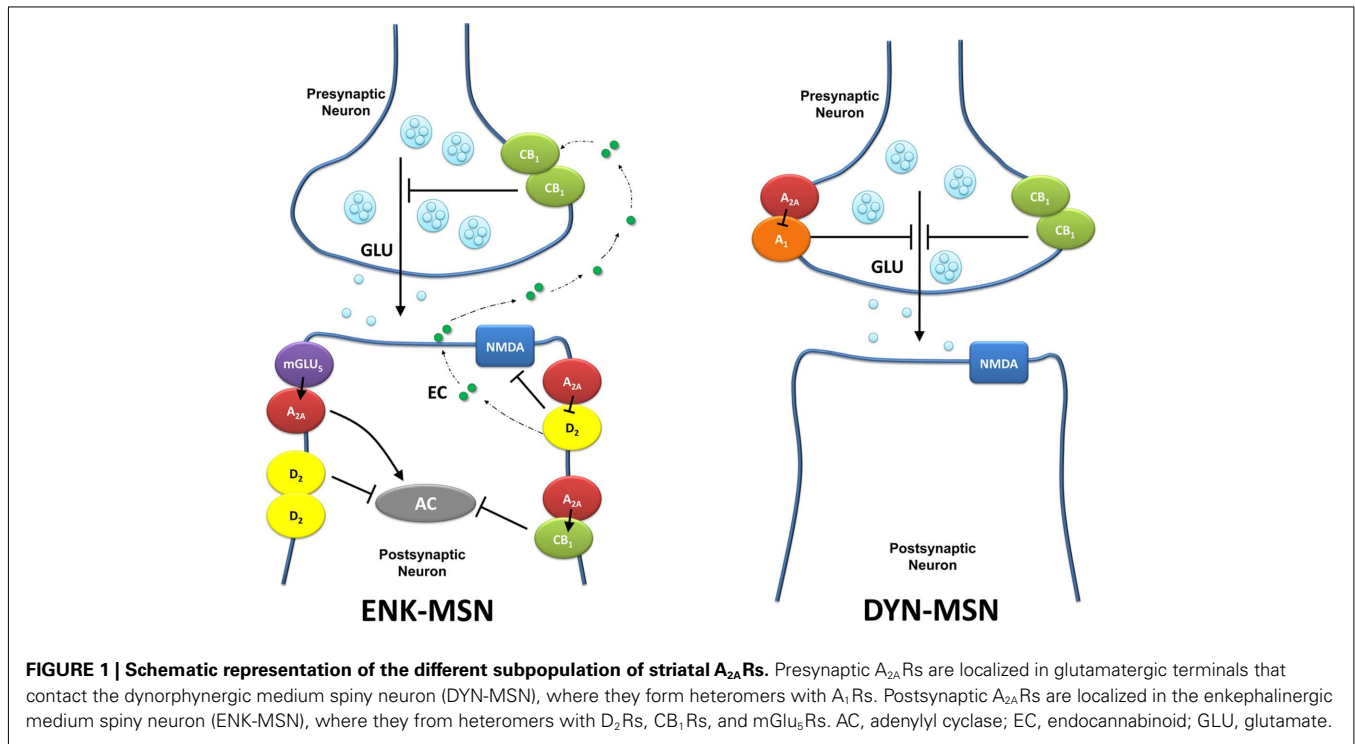
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## POSTSYNAPTIC STRIATAL ADENOSINE A<sub>2A</sub> RECEPTORS

A very significant density of adenosine A<sub>2A</sub> receptors (A<sub>2A</sub>Rs) is present in the striatum (Rosin et al., 1998; Hettinger et al., 1998; Schiffmann et al., 2007; Quiroz et al., 2009), where they are preferentially localized postsynaptically in the soma and dendrites of GABAergic striatopallidal. These neurons also show a high density of dopamine D<sub>2</sub> receptors (D<sub>2</sub>Rs) and there is clear evidence for the existence of postsynaptic mechanisms in the control of glutamatergic neurotransmission to the enkephalinergic medium spiny neuron (MSN) by at least two reciprocal antagonistic interactions between A<sub>2A</sub>Rs and D<sub>2</sub>Rs (Ferré et al., 2008). In one type of interaction, stimulation of A<sub>2A</sub>R counteracts the D<sub>2</sub>R-mediated inhibitory modulation of NMDA receptor (NMDAR)-mediated effects, which include modulation of Ca<sup>2+</sup> influx, transition to the up-state and neuronal firing (Azdad et al., 2009; Higley and Sabatini, 2010; **Figure 1**). This interaction seems to be mostly responsible for the locomotor depressant and activating effects of A<sub>2A</sub>R agonists and antagonists, respectively (Ferré et al., 2008; Orru et al., 2011), which correlates with the results of behavioral experiments showing that A<sub>2A</sub>R activation or blockade decreases or

increases, respectively, the motor effects elicited by D<sub>2</sub>R activation (Ferré et al., 2008).

Initially, the main mechanism responsible for this A<sub>2A</sub>R–D<sub>2</sub>R interaction was attributed to what it was described as an “intramembrane interaction,” by which activation of A<sub>2A</sub>R could decrease the affinity of an adjacent D<sub>2</sub>R for agonists in striatal membrane preparations (Ferré et al., 1991). It was afterward hypothesized that this kind of intramembrane interaction was a biochemical property of receptor heteromers with important functional implications (Zoli et al., 1993). A receptor heteromer is now defined as a macromolecular complex composed of at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components (Ferré et al., 2009). The term “intramembrane interaction” is now known as “allosteric interaction in the receptor heteromer,” which is defined as an intermolecular interaction by which binding of a ligand to one of the receptor units in the receptor heteromer changes the binding properties of another receptor unit (Ferré et al., 2009). Another definition recently introduced in the field of receptor heteromers is “biochemical fingerprint,” which is a



biochemical characteristic of a receptor heteromer that can be used for its identification, even in a native tissue (Ferré et al., 2009). The introduction of this concept is important in view of the difficulty in demonstrating receptor heteromers in native tissues. Biophysical techniques, such as bioluminescence and fluorescence resonance energy transfer (BRET and FRET) techniques can be easily applied in artificial cell systems to demonstrate receptor heteromerization (Bouvier, 2001), but not in native tissues. Recent technological advances, using receptor labeling with selective fluorescent ligands, have allowed the demonstration of receptor homomers with time-resolved FRET in a native tissue (oxytocin receptor homomers in mammary glands; Albizu et al., 2010). However, this required the use of high quantities of a tissue with high expression of the receptor under study (Albizu et al., 2010).

The A<sub>2A</sub>R–D<sub>2</sub>R allosteric interaction, in fact, constitutes a biochemical fingerprint of the A<sub>2A</sub>R–D<sub>2</sub>R heteromer, since it depends on the proper quaternary structure of the heteromer. Thus, it has been recently shown that disruption of an electrostatic interaction between identified intracellular domains of the A<sub>2A</sub>R and D<sub>2</sub>R leads to a significant modification of the quaternary structure of the heteromer and to the disappearance of the A<sub>2A</sub>R–D<sub>2</sub>R allosteric interaction (Borroto-Escuela et al., 2010a; Navarro et al., 2010). The electrostatic interaction in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer involves an arginine-rich epitope of the third intracellular loop (3IL) of the D<sub>2</sub>R and a phosphorylated residue localized in the C terminus of the A<sub>2A</sub>R (Woods and Ferré, 2005; Navarro et al., 2010). It is important to point out that this electrostatic interaction is not directly involved in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer interface, which seems to be mostly determined by direct interactions between transmembrane domains (Borroto-Escuela et al., 2010b; Navarro et al., 2010).

A closer look to recent electrophysiological experiments (Azdad et al., 2009; Higley and Sabatini, 2010) indicates that, although useful as a biochemical fingerprint, the allosteric interaction in the receptor heteromer does not play a main role in the antagonistic A<sub>2A</sub>R–D<sub>2</sub>R-mediated functional interaction. In the study by Azdad et al. (2009), the D<sub>2</sub>R-mediated response consisted on the counteraction of NMDAR-mediated increase in firing rate by enkephalinergic MSNs (analyzed by patch-clamp experiments in identified striatal D<sub>2</sub>R-expressing MSNs). In this experimental setting, application of an A<sub>2A</sub>R agonist did not produce any significant effect on its own, but completely blocked the D<sub>2</sub>R-mediated response. Remarkably, this interaction was dependent on the integrity of the quaternary structure of the A<sub>2A</sub>R–D<sub>2</sub>R heteromer. Thus, the counteracting effect of the A<sub>2A</sub>R agonist disappeared after the application of peptides that selectively disrupted the intracellular electrostatic interaction (Azdad et al., 2009). Importantly, the counteracting effect of the A<sub>2A</sub>R agonist was detected in the presence of a high concentration of the D<sub>2</sub>R agonist, which should be able to surmount a decrease in the affinity of the D<sub>2</sub>R caused by A<sub>2A</sub>R occupation (Ferré et al., 1991). Therefore, although it might still be involved, the allosteric interaction, which leads to a lower affinity of D<sub>2</sub>R for dopamine when adenosine is activating A<sub>2A</sub>R, does not seem to be the main mechanism underlying the A<sub>2A</sub>R–D<sub>2</sub>R functional interaction in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer.

The same intracellular arginine-rich epitope of the D<sub>2</sub>R that is involved in the electrostatic interaction with A<sub>2A</sub>R in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer has been demonstrated to bind to calmodulin and also to be fundamental for the activation of G<sub>i/o</sub> proteins (Bofill-Cardona et al., 2000; Navarro et al., 2009). Since calmodulin binding to the same epitope of the D<sub>2</sub>R impairs its ability to signal

through G<sub>i/o</sub> proteins (Bofill-Cardona et al., 2000), it is likely that binding of the C terminus of the A<sub>2A</sub>R to the same epitope reduces the capacity of the D<sub>2</sub>R to bind calmodulin and to signal through G<sub>i/o</sub> proteins. In fact, it has recently been shown that the binding of calmodulin to the A<sub>2A</sub>R–D<sub>2</sub>R heteromer is occurring within the proximal portion of the A<sub>2A</sub>R but not with the D<sub>2</sub>R (Navarro et al., 2009). It is possible that agonist binding to the A<sub>2A</sub>R induces a conformational change in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer that causes an even further impairment in the coupling of the D<sub>2</sub>R to the G<sub>i/o</sub> protein. Thus, it seems that, in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer, D<sub>2</sub>R does not signal through G<sub>i/o</sub> proteins or that its main signaling is by a G-protein-independent mechanism. However, the recent study by Higley and Sabatini (2010) suggests that the D<sub>2</sub>R-mediated inhibitory modulation of NMDAR-mediated Ca<sup>2+</sup> signaling in the enkephalinergic MSN is mediated by PKA and, therefore, most probably related to the ability of D<sub>2</sub>R to couple to G<sub>i/o</sub> and to inhibit adenylyl cyclase (AC). Interestingly, in these experiments (and in agreement with the experiments by Azdad et al., 2009), an A<sub>2A</sub>R agonist did not produce any significant effect on its own, but counteracted the effect of a D<sub>2</sub>R agonist. Thus, although Higley and Sabatini (2010) suggested that this interaction between A<sub>2A</sub>R and D<sub>2</sub>R takes place at the AC level, it shows similar characteristics to the A<sub>2A</sub>R–D<sub>2</sub>R heteromer-dependent interaction. In summary, A<sub>2A</sub>R–D<sub>2</sub>R heteromers seem to play a key role in the modulation of NMDAR-mediated signaling in the enkephalinergic MSN, but the molecular mechanisms involved in these A<sub>2A</sub>R–D<sub>2</sub>R–NMDAR interactions are yet to be determined.

In addition to the antagonistic A<sub>2A</sub>R–D<sub>2</sub>R receptor interaction in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer, D<sub>2</sub>R stimulation impedes A<sub>2A</sub>R to signal through AC (Kull et al., 1999; Chen et al., 2001; Hillion et al., 2002; Håkansson et al., 2006; **Figure 1**). This D<sub>2</sub>R–A<sub>2A</sub>R interaction takes place at the second messenger level, and stimulation of G<sub>i/o</sub>-coupled D<sub>2</sub>R counteracts the effects of G<sub>s/olf</sub>-coupled A<sub>2A</sub>R (Ferré et al., 2007, 2008). Due to a strong tonic effect of endogenous dopamine on striatal D<sub>2</sub>R, this interaction keeps A<sub>2A</sub>R from signaling through AC. However, under conditions of dopamine depletion or with pharmacological D<sub>2</sub>R blockade, A<sub>2A</sub>R-mediated signaling through the cAMP–PKA cascade may be unleashed. Antagonism of D<sub>2</sub>R is biochemically associated with a significant increase in the phosphorylation of PKA-dependent substrates, which increases gene expression and the activity of the enkephalinergic MSN, producing locomotor depression (reviewed in Ferré et al., 2008). This appears to be the main mechanism responsible for the locomotor depression induced by D<sub>2</sub>R antagonists. Thus the motor depressant and most biochemical effects induced by pharmacologic blockade of D<sub>2</sub>R may be counteracted by pharmacological blockade of A<sub>2A</sub>R (Chen et al., 2001; Håkansson et al., 2006).

The two reciprocal antagonistic interactions, A<sub>2A</sub>R toward D<sub>2</sub>R (A<sub>2A</sub>R–D<sub>2</sub>R) and D<sub>2</sub>R toward A<sub>2A</sub>R (D<sub>2</sub>R–A<sub>2A</sub>R), take place simultaneously in the same cell, which suggest that are most likely mediated by the existence of at least two different populations of postsynaptic striatal A<sub>2A</sub>R in the enkephalinergic MSN (Ferré et al., 2008). One population would be forming heteromers with D<sub>2</sub>R and would determine that A<sub>2A</sub>R stimulation inhibits D<sub>2</sub>R-mediated signaling (A<sub>2A</sub>R–D<sub>2</sub>R interaction), while another population would not be forming heteromers with D<sub>2</sub>R and would

determine that D<sub>2</sub>R stimulation inhibits A<sub>2A</sub>R-mediated signaling (D<sub>2</sub>R–A<sub>2A</sub>R interaction). This second population of postsynaptic A<sub>2A</sub>R would either not form heteromers or would form heteromers with other receptors, such as glutamate mGlu<sub>5</sub> receptors (mGlu<sub>5</sub>Rs; Ferré et al., 2002; **Figure 1**). Importantly, heteromerization of A<sub>2A</sub>R with mGlu<sub>5</sub>R is associated with a synergistic effect upon A<sub>2A</sub>R and mGlu<sub>5</sub>R co-activation at the level of AC and MAPK, providing a physiological mechanism by which A<sub>2A</sub>R can overcome the D<sub>2</sub>R–A<sub>2A</sub>R interaction (Ferré et al., 2002; Nishi et al., 2003). Co-stimulation of A<sub>2A</sub>R and mGlu<sub>5</sub>R *in vivo*, with the central administration of selective agonists, allowed A<sub>2A</sub>R to get rid of the inhibitory effect of the D<sub>2</sub>R and signal through the cAMP–PKA cascade (Ferré et al., 2002). Since this A<sub>2A</sub>R–D<sub>2</sub>R–mGlu<sub>5</sub>R interaction could be demonstrated in animal models of Parkinson's disease (Popoli et al., 2001; Kachroo et al., 2005), it was postulated that co-administration of A<sub>2A</sub>R and mGlu<sub>5</sub>R antagonists could be useful as a therapeutic strategy in this disease (Popoli et al., 2001).

Still a third population of postsynaptic A<sub>2A</sub>R would form heteromers with cannabinoid CB<sub>1</sub> receptors (CB<sub>1</sub>Rs; Carriba et al., 2007; **Figure 1**). In this heteromer, activation of A<sub>2A</sub>R is necessary to allow CB<sub>1</sub>R-mediated signaling. Thus, in a human neuroblastoma cell line, CB<sub>1</sub>R-mediated inhibition of AC activity was found to be completely dependent on A<sub>2A</sub>R co-activation (Carriba et al., 2007). Similarly, several biochemical effects of CB<sub>1</sub>R agonists in primary striatal cell cultures and striatal slices have been shown to depend on A<sub>2A</sub>R co-activation (Yao et al., 2003; Andersson et al., 2005). Accordingly, Tebano et al. (2009) reported that the depression of synaptic transmission induced by a CB<sub>1</sub>R agonist in cortico-striatal slices was prevented by A<sub>2A</sub>R antagonists and also by the conditional genetic blockade of striatal postsynaptic A<sub>2A</sub>R. The permissive effect of A<sub>2A</sub>R toward CB<sub>1</sub>R function did not seem to occur presynaptically, as the ability of the CB<sub>1</sub>R agonist to increase the R2/R1 ratio under a protocol of paired-pulse stimulation was not modified by an A<sub>2A</sub>R antagonist (Tebano et al., 2009). These results would predict that A<sub>2A</sub>R antagonists should produce similar behavioral effects than CB<sub>1</sub>R antagonists and, in fact, pharmacological or genetic inactivation of A<sub>2A</sub>Rs reduce the motor depressant, cataleptic, and rewarding effects of CB<sub>1</sub>R agonists (Soria et al., 2004; Andersson et al., 2005; Carriba et al., 2007; Justinova et al., 2011). Significantly, it has been recently reported that low doses of an A<sub>2A</sub>R antagonist (MSX-3) reduce in squirrel monkeys self-administration of THC and anandamide, but not cocaine (Justinova et al., 2011).

Although the studies just mentioned indicate that the motor (depressant) effects of CB<sub>1</sub>R agonists might depend on adenosine A<sub>2A</sub> receptor signaling, a recent study by Lerner et al. (2010) suggested quite the opposite, that CB<sub>1</sub>R signaling mediates the locomotor-activating effects of A<sub>2A</sub>R antagonists. Thus, pharmacological or genetic inactivation of CB<sub>1</sub>R reduced the locomotor activation induced by an A<sub>2A</sub>R antagonist in mice habituated to the testing environment (Lerner et al., 2010). The mechanistic explanation of this interaction is related to the previously reported D<sub>2</sub>R agonist-mediated endocannabinoid release by the enkephalinergic MSN, which by retrograde signaling would inhibit glutamate release by stimulating CB<sub>1</sub>R localized in glutamatergic terminals. This would lead to a decreased stimulation of the striatopallidal MSN, which would produce locomotor activation (Kreitzer and

Malenka, 2007). In fact, Kreitzer and Malenka (2007) advocated that, instead of direct postsynaptic effects, such as the previously mentioned D<sub>2</sub>R-mediated modulation of NMDAR-mediated signaling (Azdad et al., 2009; Higley and Sabatini, 2010), this indirect and endocannabinoid-mediated presynaptic effect is the main mechanism by which D<sub>2</sub>R stimulation produces inhibition of the enkephalinergic MSN function. According to Lerner et al. (2010), an A<sub>2A</sub>R antagonist would then produce locomotor activation by disinhibiting a tonic A<sub>2A</sub>R-mediated inhibition of D<sub>2</sub>R-mediated endocannabinoid release. However, this hypothesis would predict that CB<sub>1</sub>R agonists and antagonists should produce locomotor activation and depression, respectively, and that CB<sub>1</sub>R blockade should counteract the motor effects of D<sub>2</sub> receptor agonists. This is the opposite of what has been reported in previous studies (for a recent review, see Ferré et al., 2010). To reevaluate the findings by Lerner et al. (2010) we studied in detail the effects of pharmacological interactions between A<sub>2A</sub>R antagonists and CB<sub>1</sub>R antagonists on the locomotor activity in rats not habituated to the testing environment (Orru et al., submitted). Whereas we could indeed reproduce the results by Lerner et al. (2010) showing that a CB<sub>1</sub>R antagonist significantly decreases the locomotor effects induced by an A<sub>2A</sub>R antagonist, we found that the CB<sub>1</sub>R antagonist also produces a comparable decrease in locomotion in vehicle-treated animals (statistical analysis indicated that the locomotor effects of A<sub>2A</sub>R and CB<sub>1</sub>R antagonists were not interrelated). It was therefore the use of habituated animals (which display very low locomotor activity in the testing environment) what masked the depressant effect of CB<sub>1</sub>R antagonist in the vehicle-treated animals in the study by Lerner et al. (2010).

In addition to the three populations of postsynaptic striatal A<sub>2A</sub>R so far reported, there is also experimental evidence for a potentially more complex picture, which includes the possibility of receptor heteromultimers. Thus, using a new biophysical/based technology, sequential resonance energy transfer (SRET), and bimolecular fluorescence complementation plus BRET, evidence for A<sub>2A</sub>R–CB<sub>1</sub>R–D<sub>2</sub>R and A<sub>2A</sub>R–D<sub>2</sub>R–mGlu<sub>5</sub>R heteromers in transfected cells has been recently obtained (Carriba et al., 2008; Cabello et al., 2009; Navarro et al., 2010). Mutation experiments indicated that the interactions of the intracellular domains of the CB<sub>1</sub>R receptor with A<sub>2A</sub>R and D<sub>2</sub>R are fundamental for the correct formation of the quaternary structure needed for the function (MAPK signaling) of the A<sub>2A</sub>R–CB<sub>1</sub>R–D<sub>2</sub>R heteromers. It should be noted that the analysis of MAPK signaling in striatal slices of CB<sub>1</sub>R KO mice and wild-type littermates supports the existence of A<sub>2A</sub>R–CB<sub>1</sub>R–D<sub>2</sub>R receptor heteromers in the brain (Navarro et al., 2010). Despite the stoichiometry of the different populations of postsynaptic striatal A<sub>2A</sub>R heteromers (and homomers) is not known, taking into account the very high density of A<sub>2A</sub>R and D<sub>2</sub>Rs in the enkephalinergic MSM, we postulate that A<sub>2A</sub>R and D<sub>2</sub>R homomers and A<sub>2A</sub>R–D<sub>2</sub>R heteromers are the most common receptor populations, followed by combinations of those populations with CB<sub>1</sub>R and mGlu<sub>5</sub>R.

It is also of importance to mention that there is also evidence for the existence of A<sub>2A</sub>R receptors, also co-localized with D<sub>2</sub>Rs, in the somatodendritic and nerve terminal regions of the cholinergic striatal interneurons and that their interactions modulate acetylcholine release (James and Richardson, 1993; Jin et al., 1993;

Preston et al., 2000; Tozzi et al., 2011). The study by Jin et al. (1993) showed evidence for an antagonistic A<sub>2A</sub>R–D<sub>2</sub>R interaction in the modulation of striatal acetylcholine release. Thus, A<sub>2A</sub>R stimulation counteracted the ability of D<sub>2</sub>R activation to inhibit acetylcholine release. Similarly, a recent study showed that A<sub>2A</sub>R blockade potentiates D<sub>2</sub>R-mediated modulation of acetylcholine release (Tozzi et al., 2011), again indicating the existence of an antagonistic A<sub>2A</sub>R–D<sub>2</sub>R interaction and, probably, A<sub>2A</sub>R–A<sub>2A</sub>R heteromers in striatal cholinergic interneurons.

## PRESYNAPTIC STRIATAL ADENOSINE A<sub>2A</sub> RECEPTORS

Striatal A<sub>2A</sub>Rs are not only localized postsynaptically but also presynaptically, in glutamatergic terminals, where they heteromerize with A<sub>1</sub> receptors (A<sub>1</sub>Rs) and where they perform a fine-tuned modulation of glutamate release (Ciruela et al., 2006; Quiroz et al., 2009; **Figure 1**). Thus, A<sub>1</sub>R–A<sub>2A</sub>R heteromers seem to work as a concentration-dependent switch (Ferré et al., 2007), with adenosine acting primarily at A<sub>1</sub>Rs at low concentrations, and at both A<sub>1</sub>Rs and A<sub>2A</sub>Rs at higher concentrations. Activation of the A<sub>1</sub>R in the A<sub>1</sub>R–A<sub>2A</sub>R heteromer produces inhibition of glutamate release, while the additional activation of the A<sub>2A</sub>R produces the opposite effect, on a mechanism that seems to involve an allosteric modulation in the receptor heteromer and interactions at the G protein level (Ciruela et al., 2006; Ferré et al., 2007). Interestingly, presynaptic A<sub>2A</sub>Rs are preferentially localized in glutamatergic terminals of cortico-striatal afferents to the dynorphinergic MSN (Quiroz et al., 2009). Apart from morphological evidence provided by immunohistochemical and electron microscopy experiments, patch-clamp experiments in identified enkephalinergic and dynorphinergic MSNs provided a functional demonstration of the segregation of striatal presynaptic A<sub>2A</sub>Rs. Thus, an A<sub>2A</sub>R agonist and an A<sub>2A</sub>R receptor antagonist significantly increased and decreased, respectively, the amplitude of excitatory postsynaptic currents induced by the intrastriatal stimulation of glutamatergic afferents measured in identified enkephalinergic, but not dynorphinergic MSNs. Mean-variance analysis indicated a presynaptic locus for the A<sub>2A</sub>R-mediated modulation (Quiroz et al., 2009). Thus, there seems to be a selective A<sub>2A</sub>R-mediated modulation of glutamate release to the dynorphinergic MSN, which is in disagreement with the recently proposed role of postsynaptic A<sub>2A</sub>Rs in the modulation of glutamate release to the enkephalinergic MSN (Lerner et al., 2010).

The powerful modulatory role of presynaptic A<sub>2A</sub>Rs on striatal glutamate release was first demonstrated with *in vivo* microdialysis experiments by Popoli et al. (1995), who showed that the striatal perfusion of an A<sub>2A</sub>R agonist produced a very pronounced increase in the basal striatal extracellular concentrations of glutamate. Also intrastriatal perfusion of an A<sub>2A</sub>R antagonist through a microdialysis probe could significantly counteract striatal glutamate release induced by cortical electrical stimulation in the orofacial premotor cortex (Quiroz et al., 2009). A striking unexpected finding was that the counteraction of glutamate release was also accompanied by a complete counteraction of the jaw movements induced by the cortical electrical stimulation, demonstrating the very important role of presynaptic A<sub>2A</sub>Rs in the control of cortico-striatal glutamatergic neurotransmission. By combining cortical electrical stimulation and recording of EMG activity of the mastication

muscles, a power correlation coefficient (PCC) was established as a quantitative *in vivo* measure of cortico-striatal neurotransmission (Quiroz et al., 2009). PCC was shown to be significantly and dose dependently decreased by the systemic administration of an A<sub>2A</sub>R receptor antagonist. PCC could therefore be used as a method to screen the presynaptic effect of A<sub>2A</sub>R antagonists (see below).

According to the widely accepted functional basal circuitry model (Obeso et al., 2002; DeLong and Wichmann, 2007), blockade of postsynaptic striatal A<sub>2A</sub>R in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer, localized in the enkephalinergic MSN should potentiate spontaneous or psychostimulant-induced motor activation. On the other hand, according to the same model, blockade of presynaptic striatal A<sub>2A</sub>R localized in the cortico-striatal glutamatergic terminals that make synaptic contact with the dynorphinergic MSN should decrease motor activity. The clear locomotor-activating effects of systemically administered A<sub>2A</sub>R antagonists could be explained by the significantly higher density of postsynaptic versus presynaptic striatal A<sub>2A</sub>R and to a stronger influence of a tonic adenosine and A<sub>2A</sub>R-mediated modulation of the enkephalinergic versus dynorphinergic MSNs under basal conditions. The results by Shen et al. (2008) about the differential effects of A<sub>2A</sub>R antagonists on psychostimulant-induced locomotor activation in WT versus conditional striatal postsynaptic A<sub>2A</sub>R KO mice (potentiation versus counteraction, respectively) support this hypothesis. As previously suggested (Ferré et al., 2007), activation of presynaptic A<sub>2A</sub>Rs seems to be highly dependent on the level of adenosine generated upon cortico-striatal glutamatergic input.

Striatal D<sub>2</sub>Rs are also localized presynaptically, in dopaminergic and glutamatergic terminals (Higley and Sabatini, 2010), giving the frame for the existence of interactions with A<sub>2A</sub>Rs at least in those terminals establishing contact with the dynorphinergic MSN. The experimental evidence suggest that there is also a presynaptic D<sub>2</sub>R–A<sub>2A</sub>R interaction by which D<sub>2</sub>R activation tonically inhibits the ability of endogenous adenosine to produce an A<sub>2A</sub>R-mediated increase in the basal extracellular levels of glutamate. Thus, dopamine denervation significantly potentiates A<sub>2A</sub>R agonist-mediated stimulation of glutamate release (Tanganelli et al., 2004). This has the biochemical characteristics of an interaction between A<sub>2A</sub>Rs and D<sub>2</sub>Rs at the AC level and not forming A<sub>2A</sub>R–D<sub>2</sub>R heteromers. Furthermore, results Rodrigues et al. (2005) have also demonstrated the existence of mGlu<sub>5</sub>Rs in striatal glutamatergic terminals co-localized with A<sub>2A</sub>Rs and which facilitate glutamate release in a synergistic manner. The interplay between adenosine- and dopamine-mediated actions at the presynaptic level is therefore affected by the occurrence of mGlu<sub>5</sub>Rs.

The presynaptic localization of CB<sub>1</sub>Rs in striatal glutamatergic terminals is well established, and therefore they can be co-localized with A<sub>2A</sub>R in terminals establishing contact with the dynorphinergic MSN (Ferré et al., 2010). The existence of A<sub>2A</sub>R–CB<sub>1</sub>R heteromers in striatal glutamatergic terminals which could mediate the reinforcing effects of cannabinoids has been recently postulated (Ferré et al., 2010; Justinova et al., 2011). However, a recent study by Martire et al. (2011) indicates that cannabinoid/adenosine functional interactions result from an interaction at the second messenger level. In the frame of heteromerization A<sub>2A</sub>R activation should facilitate the G<sub>i/o</sub>-mediated effect

of CB<sub>1</sub>R activation measured, as inhibition of glutamate release. Nevertheless, Martire et al. (2011), by studying extracellular field potentials recordings in cortico-striatal slices and superfused striatal nerve terminals, very convincingly showed that, instead, A<sub>2A</sub>R activation prevents CB<sub>1</sub>R-mediated inhibition of glutamate release. These results indicate that regulation of glutamate release by cannabinoids is not dependent on presynaptic A<sub>2A</sub>R–CB<sub>1</sub>R heteromers.

In summary, a great amount of available data indicates that, presynaptically, A<sub>2A</sub>Rs form heteromers mostly with A<sub>1</sub>Rs. In addition, there seems to be a population of A<sub>2A</sub>Rs not forming heteromers but establishing antagonistic interactions with D<sub>2</sub>Rs and CB<sub>1</sub>Rs and synergistic interactions with mGlu<sub>5</sub>Rs. Apart from co-expression, at this moment we do not know the variables that determine the ability of A<sub>2A</sub>Rs to bind to different receptors to form different pre and postsynaptic heteromers. Thus, D<sub>2</sub>Rs are also localized presynaptically, but yet they do not seem to form heteromers with A<sub>2A</sub>Rs. A<sub>2A</sub>Rs could bind with more affinity to A<sub>1</sub>Rs than to D<sub>2</sub>Rs or particular scaffolding proteins could favor a particular A<sub>2A</sub>R heteromer. All these are questions still need to be answered.

## TARGETING STRIATAL PRE AND POSTSYNAPTIC A<sub>2A</sub> RECEPTORS

A surprising yet fundamental finding of a recent study is that several A<sub>2A</sub>R antagonists previously thought as being pharmacologically similar present different striatal pre and postsynaptic profiles (Orru et al., 2011). Six compounds already known as selective A<sub>2A</sub>R antagonists were first screened for their ability to block striatal pre and postsynaptic A<sub>2A</sub>Rs with *in vivo* models. Locomotor activation was used to evaluate postsynaptic activity while PCC counteraction was used to determine presynaptic activity (see above). SCH-442416 and KW-6002, showed preferential pre and postsynaptic profiles, respectively, and four compounds, MSX-3, SCH-420814, SCH-58261, and ZM-241385, showed mixed pre–postsynaptic profiles. Combining *in vivo* microdialysis with cortical electrical stimulation was used as an additional *in vivo* evaluation of presynaptic activity of A<sub>2A</sub>R antagonists. In agreement with its preferential presynaptic profile, SCH-442416 significantly counteracted striatal glutamate release induced by cortical stimulation at a dose that strongly counteracted PCC but did not induce locomotor activation. On the other hand, according to its preferential postsynaptic profile, KW-6002 did not modify striatal glutamate release induced by cortical stimulation at a dose that produced a pronounced locomotor activation but did not counteract PCC.

Another important finding of the study by Orru et al. (2011) was that at least part of these pharmacological differences between A<sub>2A</sub>R antagonists could be explained by the ability of pre and postsynaptic A<sub>2A</sub>R to form different receptor heteromers, with A<sub>1</sub>R and D<sub>2</sub>R, respectively (see above). Radioligand-binding experiments were performed in cells stably expressing A<sub>2A</sub>R, A<sub>2A</sub>R–D<sub>2</sub>R heteromers, or A<sub>1</sub>R–A<sub>2A</sub>R heteromers to determine possible differences in the affinity of these different A<sub>2A</sub>Rs for A<sub>2A</sub>R antagonists. Co-expression with A<sub>1</sub>R did not significantly modify the affinity of A<sub>2A</sub>Rs for the different ligands, but co-expression with D<sub>2</sub>Rs decreased the affinity of all compounds, with the exception of

KW-6002 (Orru et al., 2011). The structural changes in the A<sub>2A</sub>R induced by heteromerization with the D<sub>2</sub>R could be detected not only by antagonists but also by agonist binding. Indeed, the affinity of the selective A<sub>2A</sub>R agonist CGS-21680 was reduced in cells co-transfected with D<sub>2</sub>Rs. When trying to explain the differential action of SCH-442416 observed *in vivo*, it is interesting to note that this compound in particular showed a much higher affinity for the A<sub>2A</sub>R in a presynaptic-like than in a postsynaptic-like context. In fact, the affinity of A<sub>2A</sub>R for SCH-442416 in cells expressing A<sub>2A</sub>R–D<sub>2</sub>R heteromers was markedly reduced (40 times higher B<sub>50</sub> values in competitive-inhibition experiments with [3H]ZM-241385 in cells expressing A<sub>2A</sub>R–D<sub>2</sub>R than A<sub>1</sub>R–A<sub>2A</sub>R heteromers).

The decrease in affinity upon co-expression with D<sub>2</sub>Rs was much less pronounced for ZM-241385, SCH-58261, MSX2, or SCH-420814, for which the affinity was reduced from two to about ninefold (Orru et al., 2011). Taking into account that these A<sub>2A</sub>R antagonists behaved qualitatively similar than the A<sub>2A</sub>R agonist CGS-21680 in terms of binding to A<sub>1</sub>R–A<sub>2A</sub>R and A<sub>2A</sub>R–D<sub>2</sub>R heteromers, it was expected that these four compounds compete equally for the binding of the endogenous agonist at pre and at postsynaptic sites. This would fit with the *in vivo* data, which showed that these compounds have a non-preferred pre-postsynaptic profile. Yet, KW-6002 was the only antagonist whose affinity was not significantly different in cells expressing A<sub>2A</sub>R, A<sub>1</sub>R–A<sub>2A</sub>R heteromers, or A<sub>2A</sub>R–D<sub>2</sub>R heteromers. Thus, KW-6002 showed the best relative affinity for A<sub>2A</sub>R–D<sub>2</sub>R heteromers of all compounds, which can at least partially explain its preferential postsynaptic profile. Experiments performed with the non-selective adenosine receptor antagonist caffeine also showed a correlation between the *in vivo* data and the *in vitro* preference for postsynaptic A<sub>2A</sub>R-containing heteromers. It was previously reported that in transfected mammalian cells the affinity of A<sub>2A</sub>R for the non-selective adenosine receptor antagonist caffeine did not change when co-transfected with D<sub>2</sub>R, but it was significantly decreased (about 10 times) when co-transfected with A<sub>1</sub>R (Ciruela et al., 2006). As predicted, caffeine did not significantly reduce PCC at doses that produce pronounced motor activation (Zanoveli et al., in preparation).

It must be pointed out that to say that SCH-442416 is a selective presynaptic A<sub>2A</sub>R antagonist is an oversimplification. In fact, the *in vitro* data indicated that SCH-442416 binds equally well to the A<sub>2A</sub>R not forming heteromers than to the A<sub>2A</sub>R in the A<sub>1</sub>R–A<sub>2A</sub>R heteromer. Therefore, according to the previous description of the different populations of striatal A<sub>2A</sub>Rs, SCH-442416 should also be effective at counteracting D<sub>2</sub>R antagonist-induced motor depression. In fact, at doses that are not producing locomotor activation (but that reduce PCC), SCH-442416 significantly counteracts the locomotor depression induced by the D<sub>2</sub>R antagonist raclopride (Orru et al., submitted). On the other hand, KW-6002 produced the same locomotor activation with or without co-administration with raclopride, in agreement with its ability to block the three populations of A<sub>2A</sub>R studied so far *in vitro*, A<sub>2A</sub>R, A<sub>2A</sub>–D<sub>2</sub>R, and A<sub>1</sub>R–A<sub>2A</sub>R. Importantly, KW-6002 also produced the same locomotor activation when co-administered with the A<sub>2A</sub>R agonist CGS-21680, while SCH-442416, at the same dose that counteracted the depressant effect of raclopride, did not significantly counteract the depressant effect of CGS-21680. These

results, therefore agree with the hypothesis that the subpopulation of postsynaptic A<sub>2A</sub>R forming heteromers with D<sub>2</sub>R are mainly responsible for both the locomotor activation and depression induced by A<sub>2A</sub>R antagonists and agonists, respectively. In summary, SCH-442416 can be considered as a compound that at relatively low doses not only binds preferentially to presynaptic A<sub>2A</sub>Rs localized in cortico-striatal glutamatergic terminals (Orru et al., 2011), but also to a subpopulation of postsynaptic A<sub>2A</sub>Rs most probably not forming heteromers with D<sub>2</sub>Rs, but which function is tonically inhibited by D<sub>2</sub>Rs activated by endogenous dopamine. Interestingly, [<sup>11</sup>C]SCH-442416 has been used in rats, monkeys, and humans as a PET radioligand and shown to nicely label striatal A<sub>2A</sub>Rs (Moresco et al., 2005; Schiffmann et al., 2007; Brooks et al., 2010). The low doses used in PET experiments indicate that [<sup>11</sup>C]SCH-442416 is mostly labeling presynaptic A<sub>2A</sub>Rs and postsynaptic A<sub>2A</sub>Rs that do not form heteromers with D<sub>2</sub>Rs. The use of [<sup>11</sup>C]SCH-442416 and other less selective radioligand in combination with cold SCH-442416 could allow the identification of the different populations of A<sub>2A</sub>Rs in the human brain. The picture is still incomplete, and a further evaluation of the affinity of A<sub>2A</sub>R antagonists for A<sub>2A</sub>R–mGlu<sub>5</sub>R and A<sub>2A</sub>R–CB<sub>1</sub> heteromers (and of heterotrimers) is needed. Nevertheless, the information so far available is very valuable to attempt the design of more efficient A<sub>2A</sub>R antagonists to be used in basal ganglia disorders.

## A<sub>2A</sub> RECEPTOR HETEROMERS AS TARGETS FOR DRUG DEVELOPMENT

The results of the above mentioned studies support the notion that receptor heteromers may be used as selective targets for drug development. Main reasons are the very specific neuronal localization of receptor heteromers (even more specific than receptor subtypes themselves), and a differential ligand affinity of a receptor depending on its partner (or partners) in the receptor heteromer. Striatal A<sub>2A</sub>R-containing heteromers become particularly interesting targets, eventually useful for a variety of neuropsychiatric disorders. Blocking postsynaptic A<sub>2A</sub>Rs in the enkephalinergic MSN should be beneficial for Parkinson's disease because it would decrease the activity of the indirect striatal efferent pathway. On the one hand, one benefit would come from potentiating the effect of l-dopa or other dopamine receptor agonists on the D<sub>2</sub>R-mediated signaling in the A<sub>2A</sub>R–D<sub>2</sub>R heteromer. On the other hand, blockade of A<sub>2A</sub>Rs not forming heteromers with D<sub>2</sub>Rs (but antagonistically interacting with D<sub>2</sub>R at the AC level) should counteract the effects of the disinhibited A<sub>2A</sub>R signaling. However, blocking presynaptic A<sub>2A</sub>R in glutamatergic terminals contacting dynorphinergic MSN (either forming or not heteromers with A<sub>1</sub>R) should decrease glutamatergic transmission through the direct striatal efferent pathway, thus decreasing motor activity and, therefore, decreasing the antiparkinsonian efficacy of A<sub>2A</sub>R antagonists. The most convenient A<sub>2A</sub>R antagonist to treat Parkinson's disease patients would have more affinity for postsynaptic than for presynaptic receptors. Additionally, a selective blockade of presynaptic A<sub>2A</sub>Rs should be useful in dyskinesic disorders such as Huntington's disease and could also be useful in obsessive–compulsive disorders and drug addiction. Effective treatment of l-dopa-induced dyskinesia using “presynaptic” A<sub>2A</sub>R antagonists would be a possibility to explore.

The results by Orru et al. (2011) give a mechanistic explanation to the already reported antiparkinsonian activity of KW-6002 and suggest that SCH-442416 could be useful for the treatment of dyskinesic disorders, obsessive-compulsive disorders and in drug addiction. Medicinal chemistry and *in silico* modeling should help in elucidating the molecular properties that determine the particular pharmacological profile of SCH-442416 and

KW-6002, which may be used as lead compounds to obtain, respectively, more effective antidyskinetic and antiparkinsonian compounds.

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