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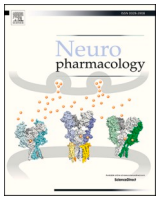

Presynaptic adenosine receptor heteromers as key modulators of glutamatergic and dopaminergic neurotransmission in the striatum

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ABSTRACT

Adenosine plays a very significant role in modulating striatal glutamatergic and dopaminergic neurotransmission. In the present essay we first review the extensive evidence that indicates this modulation is mediated by adenosine A_1 and A_{2A} receptors (A_1Rs and A_{2A}Rs) differentially expressed by the components of the striatal microcircuit that include cortico-striatal glutamatergic and mesencephalic dopaminergic terminals, and the cholinergic interneuron. This microcircuit mediates the ability of striatal glutamate release to locally promote dopamine release through the intermediate activation of cholinergic interneurons. A_1Rs and A_{2A}Rs are colocalized in the cortico-striatal glutamatergic terminals, where they form A_1R-A_{2A}R and A_{2A}R-cannabinoid CB_1 receptor (CB_1R) heteromers. We then evaluate recent findings on the unique properties of A_1R-A_{2A}R and A_{2A}R-CB_1R heteromers, which depend on their different quaternary tetrameric structure. These properties involve different allosteric mechanisms in the two receptor heteromers that provide fine-tune modulation of adenosine and endocannabinoid-mediated striatal glutamate release. Finally, we evaluate the evidence supporting the use of different heteromers containing striatal adenosine receptors as targets for drug development for neuropsychiatric disorders, such as Parkinson's disease and restless legs syndrome, based on the ability or inability of the A_{2A}R to demonstrate constitutive activity in the different heteromers, and the ability of some A_{2A}R ligands to act preferentially as neutral antagonists or inverse agonists, or to have preferential affinity for a specific A_{2A}R heteromer.

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1. Introduction

The role of adenosine and adenosine receptors in striatal function was initially focused on dopaminergic transmission, with the discovery of specific pharmacological interactions between ligands with preferential affinity for adenosine A_{2A} receptors (A_{2A}Rs) and dopamine D_2 receptors (D_2Rs). These interactions were related to the ability of A_{2A}R agonists to decrease locomotor activity induced by D_2R agonists in reserpinized mice (Ferré et al., 1991a) and the affinity of dopamine for D_2R in preparations of rat striatal membranes (Ferré et al., 1991b). Simultaneously, Schiffmann et al. (1991) reported a specific colocalization of A_{2A}Rs and D_2Rs in one of the two subtypes of striatal efferent neurons, the GABAergic enkephalinergic striato-pallidal neuron.

In 1992, we proposed the hypothesis that the striato-pallidal neuron is a main locus of interaction of adenosine and dopamine in the brain (Ferré et al., 1992). We also postulated that their interacting A_{2A}Rs and D_2Rs were involved in the behavioral activating effects of the non-selective adenosine receptor antagonist caffeine and that they could provide a new therapeutic approach for Parkinson's disease, with the application of A_{2A}R antagonists, which would increase the therapeutic effect of L-DOPA or selective D_2R agonists (Ferré et al., 1992). This was later confirmed, and the selective A_{2A}R antagonist KW-6002, also known as istradefylline, has been shown to be effective in Parkinson's disease (Jenner et al., 2021), and has recently been approved by the FDA as an

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antiparkinsonian agent (Chen and Cunha, 2020).

A series of studies would also confirm the initial hypothesis of a significant role of interacting A_{2A}Rs and D_2Rs in modulating the striato-pallidal neuronal function (for review, see Ferré, 2016; Ferré et al., 2016, 2018a), while another specific pharmacological interaction between adenosine and dopamine receptor ligands was found. This was related to the ability of adenosine A_1 receptor (A_1R) agonists to specifically decrease the locomotor activating effect of dopamine D_1 receptor (D_1R) agonists in reserpinized mice and the affinity of dopamine for D_1Rs in preparations of rat striatal membranes (Ferré et al., 1994). The significant role of the interacting A_1Rs and D_1Rs could then be demonstrated in the modulation of another subtype of striatal efferent neurons, the dynorphinergic striato-nigral, striato-entopeduncular neuron (Ferré et al., 1996, 1997, 1999).

This initial work on interactions between specific adenosine and dopamine receptors was followed by many studies by different research groups, using different biophysical techniques in mammalian transfected cells and parallel functional *in situ* and *in vivo* studies that promoted the development of the concept of the G protein-coupled receptor heteromer (GPCR) (Ferré et al., 2014; Gomes et al., 2016; Gaitonde and González-Maeso, 2017). Those included the striatal postsynaptic A_{2A}R-D_2R and A_1R-D_1R heteromers, localized in striato-pallidal and striato-nigral, striato-entopeduncular neurons, respectively. A long list of newly discovered putative GPCR heteromers would follow, although very few have been given a significant functional role, based on consensus guidelines for their true identification and function in native tissues (Ferré et al., 2009, 2014; Gomes et al., 2016). In the present article, we will focus on other striatal adenosine receptor heteromers, the presynaptic A_1R-A_{2A}R and A_{2A}R-cannabinoid CB_1 receptor (CB_1R) heteromers, which are localized in the cortico-striatal neuronal terminals and provide a pivotal role in the adenosine-mediated local striatal modulation of not only glutamate, but also acetylcholine and dopamine release. We also show that part of our knowledge about the functional and pharmacological properties of these striatal presynaptic adenosine receptor heteromers derived from their study in an animal model of restless legs syndrome (RLS). Their putative involvement in the pathogenesis of this prevalent disorder will also be discussed.

2. The GPCR heteromer and the GEMMA concept

A GPCR heteromer is defined as a macromolecular complex composed of at least two different GPCR units with biochemical properties that are demonstrably different from those of its individual components (Ferré et al., 2009). In the field of GPCR oligomerization, it is believed that a main, but not exclusive, functional unit is made up of a GPCR homodimer and one G protein, with its α, β and γ subunits, and GPCR heteromers would then be often constituted by heterotetramers, with two different homodimers coupled to their preferential G protein (Ferré et al., 2014). A common GPCR heterotetramer seems to be composed of two different GPCR homodimers, with one homodimer coupled to a stimulatory Gs/olf (Gs for short) protein and the other coupled to an inhibitory Gi/o (Gi for short) protein, providing the framework for the canonical Gs-Gi antagonistic interaction at the adenyl cyclase (AC) level, by which the activation of a Gi-coupled receptor inhibits the activation of AC by a Gs-coupled receptor (Ferré, 2015). This would include the A_{2A}R-D_2R and A_1R-D_1R heterotetramers, where A_{2A}Rs and D_1Rs are the corresponding Gs-coupled receptors and D_2Rs and A_1Rs are the corresponding Gi-coupled receptors (Bonaventura et al., 2015; Navarro et al., 2018a; Rivera-Oliver et al., 2019).

Four main concepts on GPCR heteromers should be emphasized: First, that different intracellular domains and transmembrane domains (TMs) are involved in GPCR oligomerization; second, that GPCR heteromers provide the framework for emergent allosteric interactions; third, that the same GPCR can display different oligomeric interfaces when forming heteromers with different GPCR, which determines different quaternary structures and therefore different properties; and fourth, that GPCR heteromers can constitute significant targets for drug development.

The TMs involved in GPCR oligomerization can be explored by analyzing the ability of synthetic peptides with the amino acid sequence of the different TMs to disrupt the GPCR oligomer. The peptides are usually attached to a TAT sequence (TAT stands for HIV transactivator of transcription), which determines the orientation of the peptide once integrated into the plasma membrane (He et al., 2011). TM peptides are then often used in bimolecular fluorescence complementation (BiFC) experiments, where the two putative oligomerizing proteins are fused separately to two complementary halves of a fluorescence protein (Ciruela et al., 2010). The TM-peptides that specifically disrupt BiFC, by potentially disclosing the TMs involved in the oligomeric interface, can also allow determining the properties of the oligomer, when those properties are selectively disrupted by the peptides that specifically disrupt BiFC (Ciruela et al., 2010).

With this strategy, we could establish the specific TMs that form the homomeric and ~~the~~ heteromeric interfaces of the A_{2A}R-D_2R heterotetramer (Bonaventura et al., 2015; Navarro et al., 2018a). In the same study, we could also demonstrate that TMs of AC (AC5 isoform) can establish intermolecular interactions with TMs of GPCRs (A_{2A}R and D_2R), promoting oligomerization of AC with GPCRs (Navarro et al., 2018a). Furthermore, it could be shown that the canonical Gs-Gi antagonistic interaction at the AC level mediated by activation of A_{2A}Rs and D_2Rs depends on the integrity of a macromolecular complex that includes the A_{2A}R-D_2R heterotetramer and AC5, since TM peptides that disrupt the A_{2A}R-D_2R heteromer or its oligomerization with AC5 disrupted the canonical interaction (Navarro et al., 2018a). Importantly, the results of these experiments predicted the right sequence of the first transmembrane domain TM1 of AC and the right orientation of the following TMs, which were wrongly predicted by currently used algorithms (Uniprot and TOPCONS), as was shown later with the first reported molecular structure of an AC (AC9) that included its TMs (Qi et al., 2019). This provided a strong validation of the TAT-TM peptide/BiFC approach.

The discovery of the functional A_{2A}R-D_2R heterotetramer-AC5 complex led to the development of the GPCR-effector macromolecular membrane assembly concept or **GEMMA**, which is defined as an assembly of directly interacting specific GPCRs, G proteins and effectors located in the plasma membrane with emergent functional and pharmacological characteristics (Ferré et al., 2022a). The GEMMA concept offers an alternative and complementary model to the canonical collision coupling model, allowing more efficient interactions between specific signaling components, as well as the integration of the concept of GPCR oligomerization and GPCR interactions with orphan receptors, truncated GPCRs, and other membrane-localized GPCR-associated proteins (Ferré et al., 2022a).

Regarding the second concept of GPCR heteromers, we have recently proposed a classification of allosteric mechanisms in receptor heteromers in types I, II, and III (Ferré et al., 2022a). Type I corresponds to interactions between orthosteric ligands of the different GPCRs, by which the ligand of one GPCR changes the properties (affinity or efficacy) of the ligand for the other GPCR or its constitutive activity. This type of allosteric interaction determines the ability of A_{2A}R ligands to modulate the potency and efficacy of D_2R ligands in the A_{2A}R-D_2R heteromer, and constitutes the main mechanism by which A_{2A}R antagonists potentiate the therapeutic effect of L-DOPA and selective D_2R agonists in Parkinson's disease (Ferré et al., 1991b; Bonaventura et al., 2015). Type II corresponds to a ligand-independent interaction, where one of the GPCRs, without interacting with any ligand, changes the properties of a ligand of the other GPCR or its constitutive activity. This type of allosteric interaction also occurs in the A_{2A}R-D_2R heteromer, with the disappearance of the constitutive activity of the A_{2A}R and, in the absence of D_2R ligands, with a decrease in the efficacy of A_{2A}R agonists (see below). Finally, the type III allosteric mechanism corresponds to an allosteric interaction through the GEMMA effector, such as the

canonical Gs-Gi antagonistic interaction at the AC level in the $A_{2A}R$-D_2R heterotetramer-AC5 GEMMA (Ferré et al., 2022a).

Regarding the third concept of GPCR heteromers, we have found that, in addition to the D_2R, the Gs-coupled $A_{2A}R$ can form heteromers with the Gi-coupled A_1R and CB_1R, with different homomeric and heteromeric TM interfaces (Köfalvi et al., 2020), providing them with different allosteric properties. We will expand on this later, when developing the functional, pharmacological and putative pathological role of the presynaptic striatal A_1R-$A_{2A}R$ heteromers and $A_{2A}R$-CB_1R, localized in the cortico-striatal glutamatergic terminals.

Regarding the fourth concept of GPCR heteromers and their potential use as therapeutic targets, this results from their specific allosteric mechanisms and their specific cellular localization. One approach is the simultaneous use of selective ligands for the two different protomers in the heteromer, such as the already used strategy of combining D_2R agonists and $A_{2A}R$ antagonists in Parkinson's disease, targeting the $A_{2A}R$-D_2R heteromer in the striato-pallidal neuron. A related approach is the development of heterobivalent ligands with two different pharmacophores linked by a spacer with a specific length, allowing the simultaneous binding of both pharmacophores to the respective orthosteric sites of the GPCR heteromer. This approach was pioneered by Philip Portoghese's group targeting opioid receptor heteromers (Daniels et al., 2005), and we recently obtained a heterobivalent ligand for the $A_{2A}R$-D_2R heteromer (Pulido et al., 2022). The indispensable simultaneous binding of this ligand to the $A_{2A}R$ and the D_2R orthosteric sites of the heteromer was demonstrated by radioligand competition binding assays in the absence and presence of specific TM peptides that disrupt the formation of the heteromer (Pulido et al., 2022). Another approach is the possibility of developing ligands that have specific properties, in terms of affinity, efficacy, or functional selectivity, for a GPCR when it is specifically forming heteromers with another GPCR. As discussed below, the proof of concept for this approach was obtained with the $A_{2A}R$ antagonist SCH-442416, which showed a specific decrease in affinity in the $A_{2A}R$-D_2R heteromer (Orrú et al., 2011a).

3. Local adenosine-mediated modulation of striatal glutamate release

Although initially the main pre- or postsynaptic neuronal localization of striatal $A_{2A}Rs$ was a contentious issue (Ferré et al., 1997; Richardson et al., 1997; Fredholm and Svenningsson, 1998), a large amount of evidence has accumulated to indicate that striatal $A_{2A}Rs$ are predominantly localized postsynaptically in the dendrites and spines of identified D_2R-expressing GABAergic striato-pallidal neurons, providing the frame for the formation of $A_{2A}R$-D_2R heteromers. Apart from the results of patch clamp electrophysiological studies in identified striato-pallidal neurons (Shen et al., 2008; Azdad et al., 2009; Higley and Sabatini, 2010), particularly compelling were the results of ultrastructural analysis obtained with well-validated $A_{2A}R$ antibodies (Hettinger et al., 2001; Cabello et al., 2009; Quiroz et al., 2009). These studies also demonstrated a lower but significant presynaptic expression of $A_{2A}Rs$, especially in terminals of asymmetrical synapses (Hettinger et al., 2001) and, more specifically, those colocalized with vesicular glutamate transported VGLUT1 (Quiroz et al., 2009), which correspond to cortico-striatal terminals, and not with VGLUT2, which correspond to thalamo-striatal terminals (Fujiyama et al., 2004). Presynaptic $A_{2A}Rs$ have also been suggested to predominate in the cortico-striatal terminals contacting D_1R-expressing GABAergic neurons. Nevertheless, blockade of presynaptic striatal $A_{2A}Rs$ leads to a complete counteraction of striatal glutamate release induced by cortical electrical activation or optogenetic activation of cortico-striatal terminal stimulation (see below). Finally, there is also evidence for lower expression of $A_{2A}Rs$ in astrocytes and for a lower or non-significant expression of functional $A_{2A}Rs$ in striatal interneurons (cholinergic or GABAergic) or dopaminergic terminals (Hettinger et al., 2001). On the other hand, A_1Rs have been consistently found to be expressed by most types of striatal neurons, including GABAergic efferent neurons and cholinergic interneurons (Ferré et al., 1996; Song et al., 2000), as well as by glutamatergic and dopaminergic terminals (see below).

In a series of *in vivo* microdialysis experiments, in Steve Goldberg's laboratory, we found evidence for a strong A_1R-mediated inhibitory tone of adenosine on striatal glutamate and dopamine release (Solinas et al., 2002; Quarta et al., 2004a,b; Borycz et al., 2007). Systemic or local perfusion of the non-selective antagonist caffeine or the selective A_1R antagonist CPT significantly increased extracellular glutamate and dopamine levels in the ventral striatum, in the shell region of the nucleus accumbens (NAc), suggesting that this mechanism also contributes to the psychostimulant effects of caffeine (Solinas et al., 2002; Quarta et al., 2004a,b). In addition to A_1R blockade, and as previously described (Popoli et al., 1995; Golembiowska and Zylewska, 1997), the local perfusion of the $A_{2A}R$ agonist CGS-21680 also produced glutamate and dopamine release (Quarta et al., 2004b). As expected, both effects of CGS-21680 were counteracted by the $A_{2A}R$ antagonist MSX-3, which did not produce a significant effect when administered alone, but unexpectedly, MSX-3 also counteracted the effect of CPT (Quarta et al., 2004b). This indicated that glutamate release induced by A_1R blockade was dependent on the activity of $A_{2A}Rs$. The mechanism involved would take almost two decades to discern (see below). An NMDA receptor antagonist, AP5, was also found to counteract the dopamine-releasing effect of the A_1R antagonist and the $A_{2A}R$ agonist (Quarta et al., 2004b), indicating a dependence on glutamate transmission.

From all these results, it was proposed that A_1Rs and $A_{2A}Rs$ are colocalized in striatal glutamatergic terminals, where they would mediate opposite effects of adenosine on glutamate release. The A_1R was known as the prototype of presynaptic GPCR which stimulation decreases the probability of neurotransmitter release by a Gi-βγ-dependent inhibition of voltage-dependent presynaptic Ca^{2+} channels (Wu and Saggau, 1997). On the other hand, presynaptic $A_{2A}R$ stimulation was known to facilitate the release of different neurotransmitters (Gubitz et al., 1996; Okada et al., 2001; Lopes et al., 2002), possibly involving a Gs-AC-PKA-dependent mechanism (Gubitz et al., 1996; Okada et al., 2001). Since adenosine has more affinity for the A_1R than for the $A_{2A}R$ (approximately three times according to IJzerman et al., 2022), we proposed that low concentrations would first activate A_1Rs and inhibit glutamate release, while high concentrations would also activate $A_{2A}Rs$, which would promote glutamate release by promoting Gs-dependent stimulatory signaling, overriding the inhibitory A_1R-mediated mechanism (Quarta et al., 2004b). Based on the demonstrated ultrastructural immunolocalization of NMDA receptors in dopaminergic terminals of the NAc (Gracy and Pickel, 1996), we also initially proposed that the glutamate-dependent release of dopamine was probably dependent on the activation of glutamate ionotropic receptors located in the dopaminergic terminals.

We then found evidence for the existence of A_1R-$A_{2A}R$ heteromers, first, in mammalian transfected cells, with coimmunoprecipitation and BRET techniques (Ciruela et al., 2006a). A_1Rs and $A_{2A}Rs$ were then also found to coimmunoprecipitate from rat striatal synaptosomal preparations and, using antibodies against glutamate vesicular transporters, we could demonstrate a substantial immunocolocalization of both receptors in striatal glutamatergic terminals (Ciruela et al., 2006a). A putative type I allosteric interaction in A_1R-$A_{2A}R$ heteromers could be observed in membrane preparations from cells cotransfected with A_1Rs and $A_{2A}Rs$ and from the rat striatum, by which the $A_{2A}R$ agonist CGS-21680 decreased the affinity of the A_1R agonist [^{3}H]R-PIA (Ciruela et al., 2006a), providing a mechanism by which $A_{2A}R$ ligands could counteract A_1R signaling. Finally, also in rat striatal synaptosomal preparations, we could observe the postulated concentration-dependent effect of adenosine, with low concentrations inhibiting and higher concentrations facilitating glutamate release (Ciruela et al., 2006a).

It was therefore hypothesized that A_1R-$A_{2A}R$ heteromers located in the striatal glutamatergic terminals provide a molecular device that acts as an adenosine concentration-dependent switch (Ciruela et al., 2006a,

b). Low concentrations of adenosine would inhibit glutamate release by mostly binding and activating A_1Rs, which, by Gi-dependent effects, would directly inhibit voltage-dependent Ca^{2+} channels and indirectly counteract a weak A_{2A}R-mediated, Gs-dependent AC activation through a type III allosteric modulation (Fig. 1A). Higher concentrations of adenosine would promote A_{2A}R activation, which, through a type I allosteric modulation, would decrease the affinity of adenosine for the A_1R and promote A_{2A}R-Gs-mediated AC activation and facilitate glutamate release (Fig. 1B). However, although attractive, this initial hypothesis on the role of A_1R-A_{2A}R heteromers in the modulation of striatal glutamate release mediated by adenosine was recently challenged by new findings on the role of endogenous adenosine and the involvement of the constitutive activity of the A_{2A}R and presynaptic A_{2A}R-CB_1R heteromers. This also allowed a reevaluation of initial findings on the differential effects of different selective A_{2A}R ligands on the modulation of cortico-striatal glutamate release.

Thus, unexpected results were observed when comparing the ability of different selective A_{2A}R antagonists, when administered systemically in rats, to act postsynaptically in the A_{2A}R-D_2R heteromer, analyzing their ability to induce locomotor activity, and presynaptically in the A_1R-A_{2A}R heteromer, analyzing their ability to counteract jaw movements induced by cortical electrical stimulation in the orofacial area of the lateral agranular motor cortex, as an indirect measure of cortico-striatal transmission (Quiroz et al., 2009; Orrú et al., 2011a): MSX-3 produced both effects at similar doses; KW-6002 had a postsynaptic profile, with a strong locomotor activating effect and no effect at counteracting cortico-striatal transmission; and SCH-442416 showed a presynaptic profile, with no locomotor activating effects at doses with strong blockade of cortico-striatal transmission (Orrú et al., 2011a). The differential presynaptic-postsynaptic profile of SCH-442416 and KW-6002 was confirmed by microdialysis experiments, measuring glutamate release in the dorsal striatum induced by cortical electrical stimulation. SCH-442416 was very effective at counteracting glutamate release at an ineffective locomotor activating dose, while KW-6002 was ineffective at counteracting glutamate release at a very effective locomotor activating dose (Orrú et al., 2011a). In other studies, MSX-3, after systemic or local striatal administration, was also found to be effective at counteracting glutamate release induced by cortical electrical stimulation (Quiroz et al., 2009; Orrú et al., 2011b).

A mechanism for the presynaptic profile of SCH-442416 could then be demonstrated with radioligand biding experiments with the also selective A_{2A}R antagonist [^{3}H]ZM-241385 in mammalian transfected cells. In competitive inhibition experiments, significantly higher concentrations of SCH-442416 were necessary to displace [^{3}H]ZM-241385 binding in cells cotransfected with A_{2A}Rs and D_2Rs as compared to cells transfected only with A_{2A}Rs or cotransfected with A_{2A}Rs and A_1Rs (Orrú et al., 2011a). This was indicative of a type II allosteric interaction in the A_{2A}R-D_2R heteromer, by which heteromerization with the A_1R decreases the affinity of a selective ligand for the A_{2A}R. The results represent a proof of concept of GPCR heteromers as possible targets for drug development. KW-6002, istradefylline, can be used and is used as a postsynaptic striatal A_{2A}R antagonist in Parkinson's disease (Chen and Cunha, 2020; Jenner et al., 2021), while SCH-442416 could be used as a presynaptic A_{2A}R antagonist in other neuropsychiatric disorders, including substance use disorder (SUD) (Kravitz et al., 2015) and RLS (see below). In fact, an important qualitative difference in the effect of SCH-442416 and KW-6602 could be demonstrated in two different models of SUD. SCH-442416 significantly decreased while KW-6002 significantly increased THC self-administration in squirrel monkeys (Justinova et al., 2014) and cocaine self-administration in rats (Haynes et al., 2019).

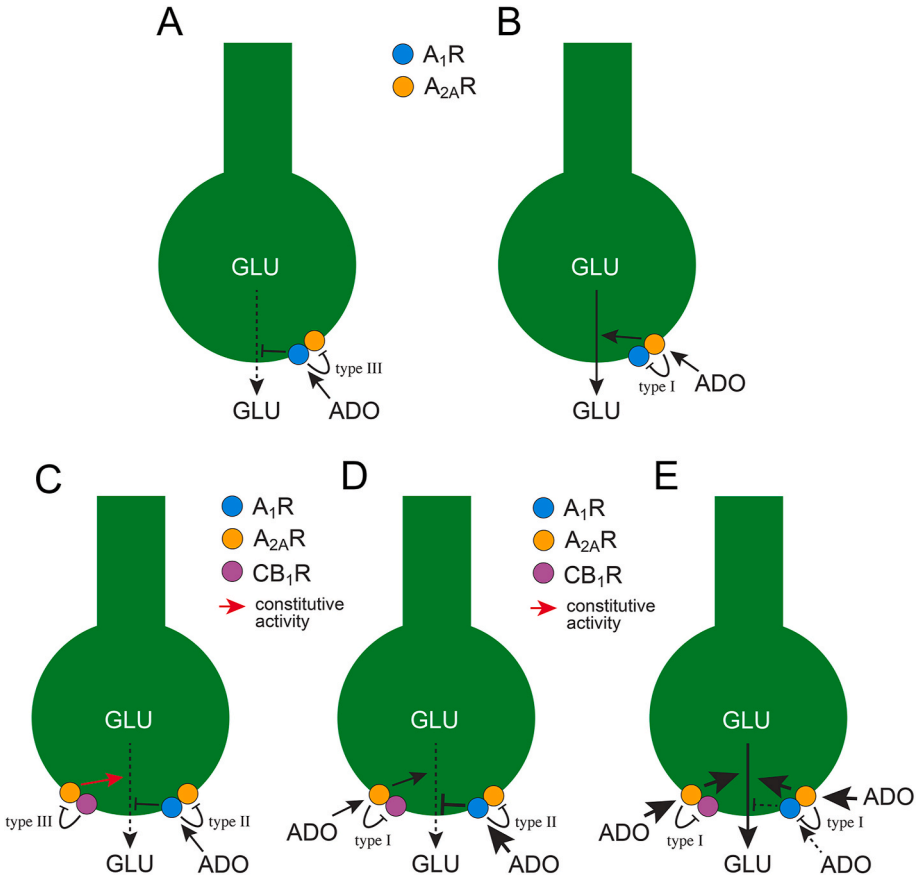

Fig. 1. Schematic representation of cortico-striatal glutamatergic terminals and their modulatory A_1R-A_{2A}R and A_{2A}R-CB_1R heteromers. Arrows represent receptor activation or facilitation of glutamate (GLU) release. Lines with perpendicular ending segments represent inhibitory allosteric modulation (types I, II or III; see text), or inhibition of glutamate release. Lower and higher degree of activation, facilitation, or inhibition is represented by broken and thicker arrows and lines, respectively. In **A** and **B**, initial hypothesis on the physiological role of the A_1R-A_{2A}R heteromer as an adenosine (ADO) concentration-dependent switch, where the A_{2A}R plays a role in the facilitation of GLU release at high physiological extracellular ADO concentrations (see text). In **C**, **D** and **E**, new hypothesis that involves the A_{2A}R-CB_1R heteromer, with the constitutive activity of the A_{2A}R (red arrow) playing a significant role in providing a basal sensitivity of the terminal to release GLU, while the A_{2A}R in the A_1R-A_{2A}R heteromer only plays a role with pathologically high concentrations of ADO. Although the adenosine receptor heteromers are proposed to be predominantly tetrameric, they are represented as dimers for the sake of simplicity.

4. Local adenosine-mediated modulation of striatal dopamine release

In view of the existence of conflicting results about the ability of caffeine to promote striatal dopamine release (Acquas et al., 2002; Solinas et al., 2002; Quarta et al., 2004a,b), we analyzed the effect of local infusion of the A_1R antagonist CPT in different striatal areas. In fact, significant qualitative results could be observed in the different striatal compartments. In most of the dorsal and ventral striatal areas analyzed, CPT promoted dopamine release, while only in the dorsomedial part of the shell of the NAc it also produced a significant glutamate release, and CPT-induced dopamine release was counteracted by an NMDA receptor antagonist (Borycz et al., 2007). Therefore, it could be concluded that, under *in vivo* basal conditions, adenosine exerts a local, tonic, A_1R-mediated inhibition of striatal dopamine release, which is glutamate independent in most striatal compartments and specifically glutamate dependent in the shell of the NAc (Borycz et al., 2007).

The most obvious mechanism for the glutamate-independent CPT-induced dopamine release was the blockade of A_1Rs located in the striatal dopaminergic terminals. In fact, using immunocytochemical techniques in rat striatal synaptosomal preparations, A_1Rs were found in a significant proportion of dopaminergic terminals, which were labeled with antibodies against tyrosine hydroxylase and the dopamine transporter (Borycz et al., 2007). Their functional significance could be demonstrated using the same striatal synaptosomal preparations, where an A_1R agonist (CPA) significantly inhibited KCl-induced [^{3}H]dopamine release, which was counteracted by an A_1R antagonist (DPCPX) (Borycz et al., 2007). These functional results represented a confirmation of previous studies using more intact preparations, such as rat striatal slices (Jin and Fredholm, 1997), which could not discard an indirect effect through A_1R-mediated modulation of glutamate release. In another study, a transient inhibitory effect of exogenously applied adenosine on electrically induced phasic dopamine release could also be demonstrated by fast-scan cyclic voltammetry in rat striatal slices, which was inhibited by the A_1R antagonist DPCPX, but not by the $A_{2A}R$ antagonist SCH-442416 (Ross and Venton, 2015). The fact that SCH-442416 did not modify the stimulated dopamine signal implied that the dopamine release was glutamate independent and it was generated by stimulation of the striatal dopaminergic axons (Ross and Venton, 2015).

The significant functional role of A_1Rs localized in dopaminergic terminals in the establishment of a local, tonic, glutamate-independent, adenosine-mediated inhibitory modulation of dopamine release was recently validated by a recent *in vitro* study in mouse striatal slices by the research group of Stephanie Cragg, using fast-scan cyclic voltammetry, optogenetic and fiber-photometry techniques (Roberts et al., 2022). However, glutamate-dependent dopamine release could still be demonstrated in striatal areas other than the shell of the NAc, such as the dorsal striatum upon conditions of cortico-striatal stimulation (Quiroz et al., 2009).

To study in more detail the local *in vivo* modulation of glutamate and secondary dopamine release upon cortico-striatal glutamatergic stimulation, we designed a modified optogenetic-microdialysis probe, with an embedded optical fiber engineered to deliver light around the probe, to the area being sampled for glutamate and dopamine and being exposed to the compounds perfused by reverse dialysis (Quiroz et al., 2016a). Several weeks after injection of an adeno-associated virus expressing channel-rhodopsin into the infralimbic cortex of the rat, optogenetic stimulation in the shell of the NAc produced both glutamate and dopamine release, which were blocked by the local perfusion, by reverse dialysis, of MSX-3 (Quiroz et al., 2016a). Since the dopaminergic nerve terminals do not have a significant expression of functional $A_{2A}Rs$, these results supported that local dopamine release was glutamate dependent. With the same optogenetic microdialysis approach in mice, we could confirm the presynaptic profile of SCH-442416 and the postsynaptic profile of KW-6002. A low dose of SCH-442416, which did not produce locomotor activity, completely counteracted glutamate release, while a dose of KW-6002 that did produce locomotor activation was ineffective on glutamate release (Ferré et al., 2018a).

Previous studies had shown that selective activation of striatal cholinergic interneurons *in vitro* or *in vivo* induces dopamine release by activation of α_4-β_2* nicotinic acetylcholine receptors (nAChR; the asterisk indicates the possible presence of additional subunits) located in the striatal dopaminergic terminals (Cachope et al., 2012; Threlfell et al., 2012). Thus, ACh-induced dopamine release was antagonized by the α_4-β_2* nAChR antagonist dihydro-β-eritroidine (Cachope et al., 2012; Threlfell et al., 2012). Using the optogenetic microdialysis methodology in rats, in a recent study with the group of Veronica Alvarez, and in agreement with previous studies (Kosillo et al., 2016; Mateo et al., 2017), we found evidence for a key role of the cholinergic interneurons in the mediation of striatal dopamine release induced by activation of cortico-striatal glutamatergic terminals. Thus, in addition to dopamine release, optogenetic activation of cortico-striatal terminals promoted acetylcholine release and dopamine release was inhibited by dihydro-β-eritroidine, which did not counteract glutamate release (Adrover et al., 2020). In summary, cortico-striatal glutamate release promotes the activation of ionotropic glutamate receptors localized in cholinergic interneurons, and the consequent acetylcholine release promotes activation of α_4-β_2* nAChR localized in dopaminergic terminals, promoting dopamine release (Fig. 2). A similar mechanism appears to also be initiated by activation of the thalamo-striatal glutamatergic neurons (Threlfell et al., 2012; Kosillo et al., 2016).

A recent study, using a patch-clamp technique that allowed direct recordings of the neuronal axonal processes of striatal dopaminergic terminals in rodent and non-human primate striatal slices, demonstrated the existence of rapid spontaneous axonal excitatory postsynaptic potentials (axEPSPs) with properties characteristic of fast synapses (Kramer et al., 2022). These axEPSPs were mediated primarily by high-affinity α_4-β_2* nAChRs that also contain α_6 subunits, since they were abolished both by dihydro-β-eritroidine and by conotoxin-P1, a selective antagonist for α_6 subunit-containing nAChRs (Kramer et al., 2022). Interestingly, axEPSPs triggered spontaneous action potentials, suggesting that these axons can convert synaptic inputs into action potentials, revealing a synaptic-like neurotransmission that underlies cholinergic signaling onto dopaminergic axons independently of somatic firing (Kramer et al., 2022).

The α_4-β_2* nAChR localized in the striatal dopaminergic terminals appears to be part of a GEMMA that includes the short isoform of the D_2R ($D_{2S}R$) (Quarta et al., 2007), which is known to be the main isoform expressed by dopaminergic cells that acts as autoreceptor (Usiello et al., 2000). This autoreceptor complex depends on intermolecular interactions between the $D_{2S}R$ and the β_2 subunit of nAChR and allows $D_{2S}R$ activation to efficiently counteract dopamine release induced by activation of α_4-β_2* nAChRs, as demonstrated by both *in vitro* experiments in striatal synaptosomes (Grilli et al., 2009) and *in vivo* microdialysis in rats (Quarta et al., 2007). In addition to $D_{2S}R$, another subtype of D_2-like receptor, D_3R, is also expressed by dopaminergic cells (Diaz et al., 2000), which seem to also act as autoreceptor both in the mesencephalic somatodendritic area and in the striatal terminals (Tepper et al., 1997; Joseph et al., 2002; Zapata and Shippenberg, 2002; Maina and Mathews, 2010).

The contribution and mechanisms behind the putative autoreceptor function of D_3R are still a matter of debate (see for instance, Li et al., 2010), although a series of *in vitro* and *in vivo* studies strongly support a possible preferential inhibitory effect of D_3Rs on dopamine uptake and with less influence than D_2R on dopamine synthesis and release (Zapata et al., 2007; Castro-Hernandez et al., 2015; McGinnis et al., 2016; Manvich et al., 2019). Furthermore, using BRET and proximity ligation assays, Cristina Missale's group found evidence for the existence of α_4-β_2* nAChR-D_3R heteromers in mammalian transfected cells, in cultured dopaminergic cells, and in the mesencephalon, in the substantia nigra-ventral tegmental area (Bontempi et al., 2017). The heteromerization of α_4-β_2* nAChR and D_3R depended on an electrostatic

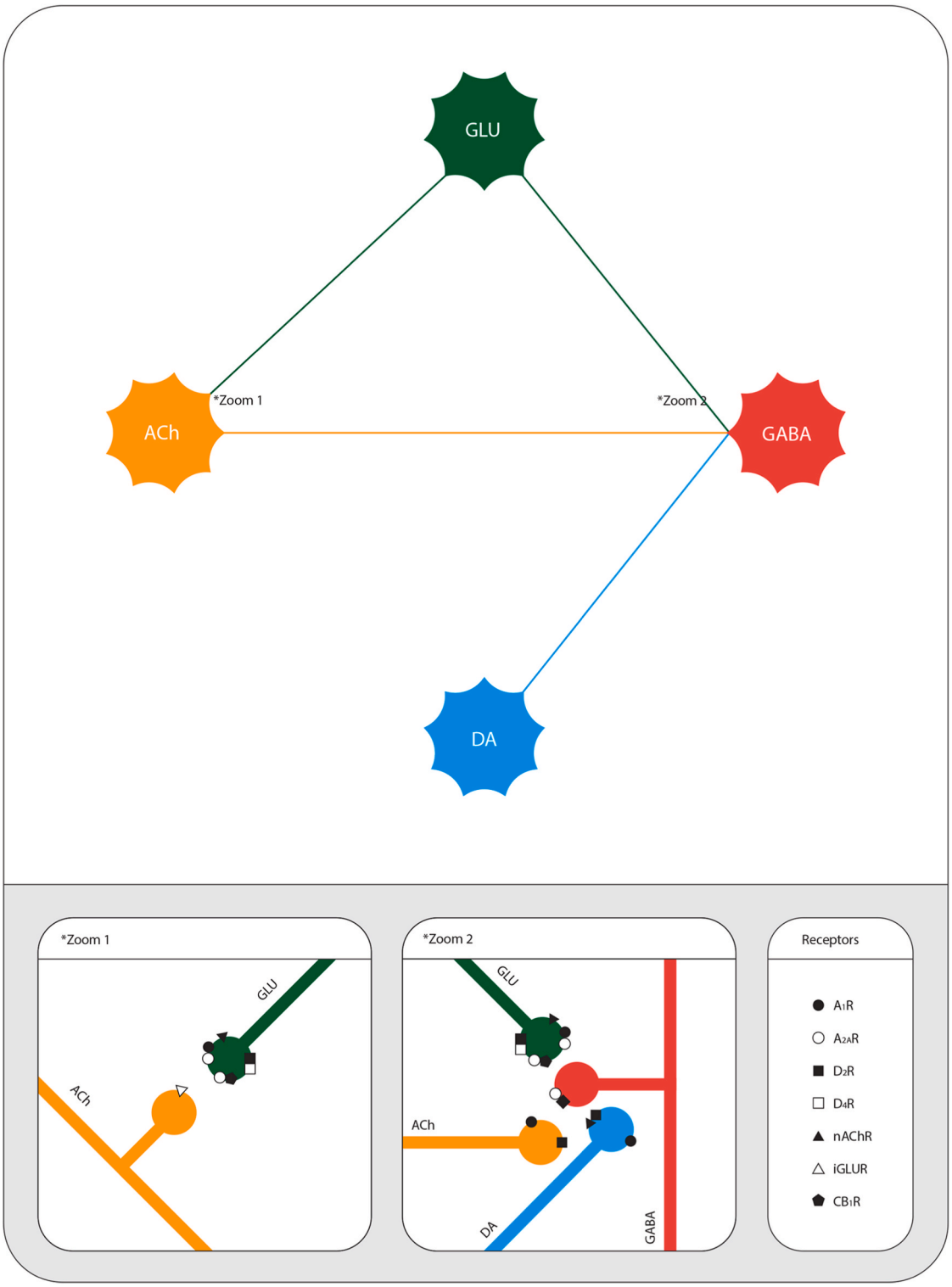

Fig. 2. Striatal microcircuit that includes the cortico-striatal glutamatergic terminal, the mesencephalic dopaminergic terminal, and the cholinergic interneuron. This striatal microcircuit mediates the ability of striatal glutamate (GLU) release to locally promote dopamine (DA) release through intermediate activation of cholinergic interneurons, by activating ionotropic glutamate receptors in the cholinergic neuron (iGLUR), promoting acetylcholine (ACh) release, which activates nicotinic ACh receptors (nAChRs; specifically, α_4-β_2* nAChRs) in the dopaminergic terminal, promoting dopamine release. Significantly functional A_{2A}Rs are only localized in the glutamatergic terminal, forming heteromers with A_1Rs and CB_1Rs. Dopamine directly inhibits the release of GLU, DA, and ACh by acting on D_2R-D_4R heteromers in the glutamatergic terminals, on D_2Rs (and possibly D_3Rs) that form heteromers with α_4-β_2* nAChRs in the dopaminergic terminals, and on D_2Rs in cholinergic neurons, respectively. ACh also directly activates GLU release by acting on α_7 nAChRs in glutamatergic terminals. Postsynaptic A_{2A}R-D_2R heteromers localized in the GABAergic striatal efferent (striato-pallidal) neuron are also shown. Although the adenosine receptor heteromers are proposed to be predominantly tetrameric, they are represented as dimers for the sake of simplicity.

intermolecular interaction between positively and negatively charged epitopes localized in the intracellular domains of D_3R and the β_2 subunit of nAChR, respectively (Bontempi et al., 2017). Targeting this electrostatic interaction with heteromer-disrupting peptides, the study disclosed a putative nAChR-D_3R heteromer-dependent mediation of the neurotrophic effects of nicotine and D_3R agonists on dopaminergic cells (Bontempi et al., 2017). However, a contribution of the nAChR-D_2R heteromer could not be discarded, since $D_{2S}R$ shares with D_3R the same type of positively charged epitope (Woods and Ferré, 2005), which could also determine the heteromerization of nAChR with $D_{2S}R$. More studies are needed to establish the proportion of nAChRs that form heteromers with $D_{2S}Rs$ and D_3Rs that locally mediate the ability of acetylcholine to release dopamine in different striatal compartments.

Finally, as mentioned above, there is also evidence for a significant expression of A_1Rs localized in cholinergic interneurons (Ferré et al., 1996; Song et al., 2000), whose activation inhibits the release of acetylcholine. Using single-cell reverse transcription–polymerase chain reaction, significant A_1R mRNA levels and very low $A_{2A}R$ levels were obtained from acutely isolated rat cholinergic interneurons, identified by the presence of choline acetyltransferase mRNA (Song et al., 2000). With whole-cell voltage clamping, it was then shown that activation of A_1Rs in cholinergic interneurons reduces currents mediated by Gi-dependent inhibition of the N-type voltage-dependent Ca^{2+} channel (Song et al., 2000). However, although this may be a mechanism by which adenosine reduces striatal acetylcholine release, the recent study by the Stephanie Cragg group showed that application of the α_4-β_2* nAChR antagonist dihydro-β-eritroidine did not counteract the inhibitory effect of an A_1R agonist on dopamine release (Roberts et al., 2022). This showed that activation of A_1Rs can suppress dopamine release independently of any indirect effects through cholinergic input to the nAChRs localized at the dopaminergic terminals.

5. Lessons from a rodent model of RLS

RLS is a common sensorimotor disorder, whose basic components include a primary sensory experience, akathisia (an urgent need to move) and, in about 80% of patients, a secondary motor component, periodic leg movements during sleep (PLMS), can be found (Allen et al., 2014; Manconi et al., 2021). It has been postulated that the overlying framework of the disease is a biological bias towards maintaining alertness even in the face of severe sleepiness (Ferré et al., 2019a). It is generally accepted that brain iron deficiency (BID) is one of the primary pathophysiological mechanisms of RLS (Earley et al., 2014; Ferré et al., 2019a). In fact, BID in rodents is used as one of the main animal models of RLS, with significant face and construct validity (Salminen et al., 2021, 2022). Therefore, BID in rodents recapitulates key neurochemical changes reported in patients with RLS and shows an RLS-like behavioral phenotype (Earley et al., 2022; Salminen et al., 2022), including a presynaptic hyperdopaminergic and hyperglutamatergic states. (Earley et al., 2014, 2022; Ferré et al., 2019a, 2021; Salminen et al., 2022).

Rodents with BID show an increase in the activity of the central dopaminergic system, with an increase in the synthesis and release of dopamine (Earley et al., 2014, 2022). In RLS patients, this presynaptic hyperdopaminergic state is revealed by an increase in tyrosine hydroxylase activity in the mesencephalon and striatum in postmortem tissue and an increase of tetrahydrobiopterin and 3-ortho-methyl-DOPA in the CSF (*in vivo* markers of increased L-DOPA synthesis in the brain), and a decrease in D_2R binding potential (as an indirect measure of an increase in dopamine release) (Salminen et al., 2022). Using the optogenetic microdialysis methodology mentioned above, it was demonstrated that a lower frequency of optogenetic stimulation was necessary to induce glutamate release from the cortico-striatal terminals of rats with BID, compared to controls, a BID-induced increase in the sensitivity of the cortico-striatal terminals to release glutamate (Yepes et al., 2017). This striatal hyperglutamatergic state is paralleled in RLS patients by an increased excitability of the motor cortex demonstrated by transcranial magnetic stimulation (TMS) studies (Salminen et al., 2022), which should imply an increased sensitivity of the pyramidal cortico-striatal neurons.

The importance of hyperexcitable cortico-striatal neurons and their glutamatergic terminals in the pathogenetic role of RLS was reinforced by demonstrating that the drugs commonly used in RLS could counteract the optogenetically-induced glutamate release by cortico-striatal terminals when locally perfused in the striatum through the optogenetic microdialysis probe, both in rats with BID and controls. Those drugs included the D_2-like receptor agonists pramipexole and ropinirole and the $\alpha_2\delta$ ligand gabapentin (Yepes et al., 2017). The mechanism of $\alpha_2\delta$ ligands depends on their ability to inhibit the function of voltage-dependent Ca^{2+} channels that contain $\alpha_2\delta$-accesory subunits and are localized in glutamatergic terminals (Dooley et al., 2007). The $\alpha_2\delta$ ligands are now the first recommended choice as pharmacological treatment for RLS instead of dopaminergic agonists, which have the risk of 'augmentation' (drug-induced worsening of symptoms) (Silber et al., 2021). To clarify the subtype of dopamine receptors involved in the effect of pramipexole, we analyzed the ability of different locally perfused dopamine receptor antagonists to block the effect of pramipexole. Those were the selective D_4R antagonist L745-870, the D_2R-D_3R antagonist raclopride, and the D_3R antagonist VK4-116. Since only VK4-116 was ineffective, we could conclude that D_2R and D_4R, but not D_3R, were involved. In a parallel study using the optogenetic-microdialysis technique in knock-in mice expressing a humanized D_4R with an intracellular loop corresponding to a common polymorphic variant and their wild-type littermates, we could demonstrate that, in fact, presynaptic striatal D_4Rs play a key role in the modulation of cortico-striatal transmission (Bonaventura et al., 2017). Furthermore, these D_4Rs seem to heteromerize with D_2Rs to a greater or lesser degree depending on the polymorphic variant of D_4R (reviewed in Ferré et al., 2022b).

Therefore, the same as for adenosine and A_1Rs, the striatal release of dopamine can be locally inhibited by activating those dopamine receptors localized in the dopaminergic terminals, the D_{2S} and D_3 autoreceptors, and those localized in the cortico-striatal terminals, which also appear to be predominantly $D_{2S}Rs$ (Centonze et al., 2004), and D_4Rs. The apparent conundrum of the initially very effective D_2-like receptor agonists, such as pramipexole and ropinirole, in alleviating the symptoms of RLS in the context of a hyperglutamatergic state, can then be explained by the targeting of these striatal presynaptic dopamine receptors (for recent discussion, see Ferré et al., 2021). Then it should be possible to obtain similar clinical results by targeting the striatal A_1Rs. In fact, a series of studies led to the discovery of alterations in the adenosinergic system in BID rodents. With radioligand binding and Western blot experiments in rodents, we found that BID induces down-regulation of A_1Rs in the cortex and striatum and up-regulation of $A_{2A}Rs$ in the striatum (Quiroz et al., 2010, 2016b). The results also showed that down-regulation of A_1Rs is a more sensitive phenomenon than up-regulation of $A_{2A}Rs$, since it was associated with a less severe iron-deficient diet (Quiroz et al., 2016b).

We then postulated that a hypoadenosinergic state, secondary to a BID-induced down-regulation of A_1Rs, could be a main mechanism responsible for the hyperglutamatergic and hyperdopaminergic states. The hypoadenosinergic state would represent a more proximal link in the chain of pathogenetic events that lead to the symptomatology of RLS (Ferré et al., 2019a). We also postulated that a change in the stoichiometry of both receptors in the cortico-striatal terminal in favor of $A_{2A}Rs$ could be main responsible for the increased sensitivity of those terminals with BID. This was recently demonstrated using a FACS-based synaptometric analysis in rat striatal synaptosomal preparations. BID was associated with a significant decrease in the ratio of A_1Rs versus $A_{2A}Rs$ in cortico-striatal glutamatergic terminals, labeled with the vesicular glutamate transporter VGLUT1 (Rodrigues et al., 2022). Consistent with this, we could also induce an increase in sensitivity of the cortico-striatal terminals in rats without BID by blocking A_1Rs, with striatal perfusion of

the A_1R antagonist CPT, which allowed the release of glutamate with a lower frequency of optogenetic stimulation (Ferré et al., 2019b).

We could then demonstrate that perfusion with dipyridamole, a non-selective inhibitor of the equilibrative nucleoside transporters ENT1 and ENT2, which increases extracellular levels of adenosine, decreases glutamate striatal levels and optogenetic-induced cortico-striatal glutamate release both in rats with BID and controls, and its effect was counteracted by CPT (Ferré et al., 2019b). This would indicate that the increase in endogenous adenosine induced by nucleoside transporter inhibition only promotes A_1R activation and does not significantly activate presynaptic $A_{2A}Rs$, which should have promoted glutamate release. The results of these experiments indicated a possible therapeutic effect of dipyridamole, which was recently demonstrated by Diego García-Borreguero's group in two clinical studies. First, an open trial and, second, a randomized, placebo-controlled crossover study (García-Borreguero et al., 2018, 2021), providing a new therapeutic approach for RLS and validating the adenosinergic hypothesis of RLS.

The experimental results with dipyridamole indicating that endogenous adenosine does not significantly activate presynaptic $A_{2A}Rs$ localized in cortico-striatal terminals made difficult to understand those previous results obtained with $A_{2A}R$ antagonists, which when administered systemically or locally counteracted electrical or optogenetically-induced glutamate release (Quiroz et al., 2009, 2016a; Orrú et al., 2011a,b; Ferré et al., 2018a). The main clue to resolve this enigma came from the lack of presynaptic effect of KW-6002 and its previously described unique property as a neutral antagonist, while most of the other selective $A_{2A}R$ antagonists (including ZM-241385, SCH-58261 and SCH-420814) are inverse agonists (Bennett et al., 2013). This could indicate that the previously described strong constitutive activity of $A_{2A}Rs$ (Klinger et al., 2002) plays a significant role in providing sensitivity to glutamatergic terminals. Consistent with this hypothesis, we could reproduce the finding of a very low inverse agonistic efficacy of KW-6002 and we could also demonstrate that SCH-442416 has a strong inverse agonistic efficacy (Fig. 3). We also analyzed the effect of caffeine, which, as previously described (Bennett et al., 2013), showed low inverse agonistic efficacy (Fig. 3). Now we can also understand our initial findings about the ability of MSX-3 to counteract glutamate release induced by the A_1R antagonist CPT (Quarta et al., 2004b). Furthermore, the lower $A_{2A}R$ inverse agonistic efficacy of the non-selective adenosine receptor caffeine would explain its predominant presynaptic A_1R antagonistic effect (Solinas et al., 2002; Quarta et al., 2004a,b).

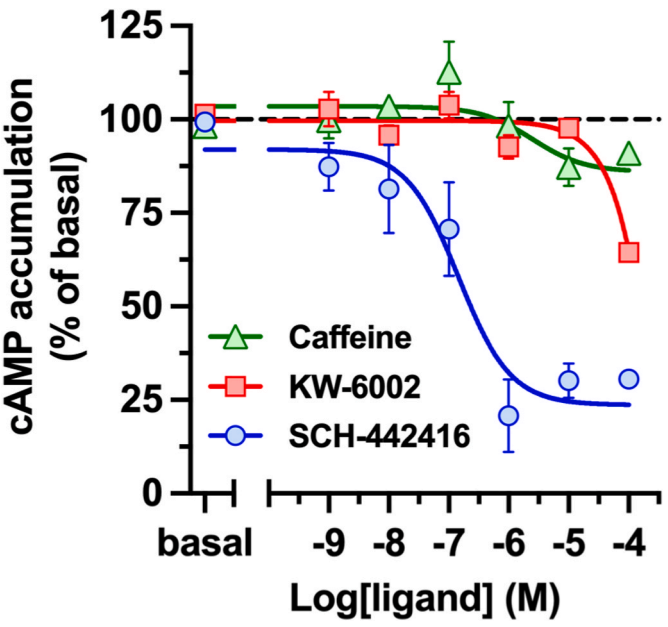

Fig. 3. Inverse agonisticefficacy of SCH-442416, KW-6002, and caffeine at the $A_{2A}R$. The inverse agonistic activity of SCH-442416, KW-6002 and caffeine was assessed by monitoring their effect on constitutive activation of AC in HEK-293 T cells permanently expressing $A_{2A}Rs$. Basal cAMP accumulation was determined using the LANCE Ultra cAMP detection kit, as described elsewhere (Sarasola et al., 2022). In summary, HEK-293 T-$A_{2A}R$ cells (2×10^5 cells/ml) were preincubated with stimulation buffer (DMEM, 0.1% BSA, 0.5 U/ml adenosine deaminase, 100 μM zardaverine) for 1 h at 37 °C in constant agitation (550 rpm) before being treated with increasing concentrations of SCH-442416 (blue circles), KW-6002 (red squares) and caffeine (green triangles) for 30 min at 37 °C in constant agitation (550 rpm). Subsequently, cells (10 μl) were seeded in a 384-well plate and the accumulation of cAMP was quantified following the indications of manufacture (Sarasola et al., submitted). The cAMP levels were normalized using vehicle-stimulated HEK-293 T-$A_{2A}R$ cells as 100%. Results are represented as means ± SEM of three independent experiments performed in triplicate. SCH-442416 induced a strong concentration-dependent reduction in the accumulation of basal cAMP (pIC_{50} = 6.8 (6.3–7.4, 95% CI); I_{max} = 77.7% (94.8–61.6, 95% CI)) which differs significantly from that induced by KW-6002 (non-adjustable fitting curve) and caffeine (pIC_{50} = 5.6 (4.1–6.9, 95% CI); I_{max} = 14.1% (2–36.2, 95% CI)) ($F_{(4, 47)} = 27.81$; $P < 0.0001$; extra sum of squares F test).

6. Heteromer-dependent modulation of the constitutive activation and ligand-mediated signaling of the $A_{2A}R$

The significance of the constitutive activity of striatal presynaptic $A_{2A}Rs$ is reinforced by the results of the studies on $A_{2A}R$-CB_1R heteromers, which were first identified in mammalian transfected cells with BRET techniques (Carriba et al., 2007). In these cells, the ability of the CB_1R agonist ACEA to inhibit AC, forskolin-induced cAMP accumulation, was also found to be counteracted by the $A_{2A}R$ inverse agonist ZM-241385, indicating that the CB_1R signaling depended on $A_{2A}R$ activation. In agreement, *in vivo,* locomotor depression induced by the intrastriatal administration of the CB_1R agonist WIN55,2121–2 was counteracted by the systemic administration of MSX-3 (Carriba et al., 2007). Immunohistochemical analysis in the rat striatum showed a significant colocalization of both receptors in the neuropil, compatible with their preferential colocalization in nerve terminals (Carriba et al., 2007). In another study, a FACS-based synaptometric analysis supported a significant colocalization of $A_{2A}Rs$ and CB_1Rs in striatal glutamatergic terminals (Ferreira et al., 2015). Subsequently, a study carried out by the research groups of Joseph Cheer and David Lovinger (Mateo et al., 2017) provided morphological and functional evidence for a significant role of CB_1R localized in the cortico-striatal terminals as the main mediators of the local inhibitory modulation of endocannabinoids in the release of striatal glutamate and, following the striatal microcircuit shown in Fig. 2, in the release of acetylcholine by the cholinergic interneurons and consequent release of dopamine by dopaminergic terminals. In fact, neither cholinergic interneurons nor dopaminergic neurons express CB_1R (Hohmann and Herkenham, 2000; Julian et al., 2003; Mateo et al., 2017).

We recently obtained results that strongly suggest that the ability of CB_1R agonists to inhibit striatal glutamate release is mainly mediated by the presynaptic $A_{2A}R$-CB_1R heteromer and, in addition, that this depends on a type III allosteric mechanism in the heteromer. More specifically, it depends on the ability of CB_1R activation to counteract the constitutive activation of the $A_{2A}R$ in the heteromer by a canonical Gs-Gi antagonistic interaction at the AC level (Köfalvi et al., 2020). This could be first demonstrated by the ability of a synthetic peptide that disrupts $A_{2A}R$-CB_1R heteromerization and the canonical Gs-Gi antagonistic interaction in transfected cells, to specifically disrupt the ability of the CB_1R agonist WIN55,2121–2 to inhibit the release of depolarization-induced glutamate release of rat striatal glutamatergic terminals. Second, in the same *in vitro* preparation, by showing that blocking the constitutive activity of $A_{2A}Rs$ with a high concentration of the inverse agonist SCH-58261 (Bennett et al., 2013) produces the same effect as WIN55,2121–2, significantly reducing depolarization-induced

glutamate release (Köfalvi et al., 2020). In summary, the results imply that the constitutive activity of A_{2A}Rs in the A_{2A}R-CB_1R heteromer exerts a tonic facilitation of glutamate release, which can be counteracted by an A_{2A}R inverse agonist or by activation of the CB_1R when forming heteromers with the A_{2A}R (Köfalvi et al., 2020).

In the same study, it was also demonstrated in mammalian transfected cells that the constitutive activity of the A_{2A}R is abrogated in cells cotransfected with A_1Rs or D_2Rs, but not with CB_1Rs (Köfalvi et al., 2020). A tetrameric structure of the A_{2A}R-CB_1R hetomer could be established using TM peptides from both receptors in BiFC experiments, which showed the same heteromeric interface than the A_1R-A_{2A}R heterotetramer, but different homomeric interfaces than those of the A_1R-A_{2A}R and A_{2A}R-D_2R heterotetramers (Navarro et al., 2018a, 2018b; Köfalvi et al., 2020). This provided a rationale for their differences in the constitutive activity of the A_{2A}R. The proof of concept for a significant functional role of the homomeric interfaces in a GPCR heterotetramer came from a recent study on the μ-opioid receptor (MOR)-galanin Gal_1 receptor (Gal_1R) heterotetramer, where it is shown that the change in the homomeric interface of Gal_1R determines a change in the preference for G protein coupling, allowing the Gal_1R homodimer to couple to Gs (De Oliveira et al., 2022).

Blocking the constitutive activity of the A_{2A}R in the A_{2A}R-D_2R and A_1R-A_{2A}R heteromers, as mentioned before, represents a type II allosteric interaction. When comparing other allosteric mechanisms in the three heterotetramers, they all showed the same type I allosteric interaction, by which orthosteric ligands of A_{2A}Rs decrease the ability of orthosteric agonists to activate D_2Rs, A_1Rs or CB_1Rs in the corresponding heteromer (Ferré et al., 2018a; Navarro et al., 2018b; Köfalvi et al., 2020). This type I allosteric interaction provides the main explanatory mechanism for a series of results showing that A_{2A}R ligands decrease the pharmacological effects of A_1R, D_2R and CB_1R activation (Ferré et al., 1997, 2016, 2018a; Ciruela et al., 2006a; Azdad et al., 2009; Bonaventura et al., 2015; Ferreira et al., 2015; Navarro et al., 2018b; Köfalvi et al., 2020). Differences were also found with the type III allosteric interaction related to the possible Gi-coupled receptor-mediated inhibition of the Gs-coupled receptor-mediated activation of AC. D_2R and CB_1R agonists, but not A_1R agonists, inhibited A_{2A}R-mediated AC activation in the respective heterotetramers (Navarro et al., 2018a, 2018b; Köfalvi et al., 2020). The lack of a canonical Gs-Gi antagonistic interaction at the AC level in the A_1R-A_{2A}R heteromer was found to depend on interactions with the long C-terminus of the A_{2A}R, which deletion enabled the type III allosteric interaction (Köfalvi et al., 2020). Therefore, we could not support our initial hypothesis of a type III allosteric interaction in the A_1R-A_{2A}R heteromer localized in cortico-striatal terminals playing a significant role in the modulation of striatal glutamate release under conditions of low extracellular concentrations of adenosine (Fig. 1A). Finally, in the A_1R-A_{2A}R heteromer, in addition to the counteraction of the constitutive activity of the A_{2A}R, we recently found another type II ligand-independent allosteric interaction, by which heteromerization with A_1Rs significantly decreases the potency and efficacy of A_{2A}R agonists (Sarasola et al., 2022).

The new evidence on the functional significance of A_1R-A_{2A}R and A_{2A}R-CB_1R heteromers and their specific properties, with their ability to promote different allosteric interactions, as well as the evidence obtained from the experiments with dipyridamole that indicates a lack of activation of presynaptic A_{2A}Rs with endogenous adenosine, means that we need to reappraise our initially hypothesized role of A_1R-A_{2A}R heteromers in the modulation of glutamate release by cortico-striatal terminals mediated (Ciruela et al., 2006a,b). As mentioned above, this initial hypothesis assumed, first, that a predominant activation of A_1Rs or A_{2A}Rs in the A_1R-A_{2A}R heteromer promotes inhibition or facilitation of glutamate release, respectively; second, that low concentrations of adenosine preferentially activate A_1Rs, related to the higher affinity of adenosine for this receptor and its ability to inhibit A_{2A}R signaling through a type III allosteric interaction; and, third, that at higher concentrations adenosine also binds to the A_{2A}R, which counteract A_1R signaling by a type I allosteric interaction (Fig. 1A and B).

Regarding the A_1R-A_{2A}R heteromers, the A_1R seems to be the main adenosine receptor involved in the effects of physiologically low and high concentrations of adenosine, since, by type II allosteric mechanisms, the A_1R counteracts the constitutive activity of the A_{2A}R and further decreases its affinity for agonists (Fig. 1C and D). This seems to imply that the A_{2A}R in the A_1R-A_{2A}R heteromer can only signal at non-physiological concentrations of adenosine, such as under hypoxic conditions. High pathological concentrations of adenosine could then activate the A_{2A}R in the A_1R-A_{2A}R heteromer and overcome the decrease in affinity mediated by type II allosteric modulation and counteract A_1R-mediated signaling by type I allosteric modulation (Ciruela et al., 2006a; Navarro et al., 2018b) (Fig. 1D). The activation of the A_1R in the A_1R-A_{2A}R heteromer would then play an important role in providing a basal inhibitory effect of endogenous adenosine on cortico-striatal glutamate release (Fig. 1C and D).

A_{2A}Rs would also play an important role in modulating cortico-striatal glutamate release under basal conditions, with low extracellular concentrations of adenosine, exerting significant constitutive activity through the A_{2A}R-CB_1R heteromer. Under these conditions, the sensitivity of the cortico-striatal terminals to produce glutamate release would be mostly determined, on the one hand, by the degree of constitutive activation of the A_{2A}R in the A_{2A}R-CB_1R heteromer, which would be dependent on the level of endocannabinoids, that can counteract the constitutive activity of the A_{2A}R through a type III allosteric mechanism. On the other hand, the basal sensitivity of the cortico-striatal terminals to release glutamate would depend on the degree of activation of the A_1R in the A_1R-A_{2A}R heteromer by endogenous adenosine (Fig. 1C and D). These mechanisms operating at low extracellular concentrations of adenosine allow explaining the ability of A_1R antagonists to elicit striatal glutamate release and the ability of A_{2A}R inverse agonists to block this effect, as well as the A_{2A}R-dependent ability of CB_1R agonists to inhibit glutamate release from cortico-striatal terminals.

Physiologically higher concentration of adenosine would occupy A_{2A}Rs forming heteromers with CB_1Rs, which should counteract CB_1R activation through a type I allosteric interaction in the heteromer and potentially promote glutamate release (Ferreira et al., 2015; Köfalvi et al., 2020) (Fig. 1D). However, the increase in adenosine secondary to inhibition of ENT1/ENT2 did not induce an increase, but rather a decrease in basal and optogenetically-induced glutamate release. This could be explained by the concomitant significant A_1R occupation in A_1R-A_{2A}R heteromers, which would still be able to oppose the effects of A_{2A}R activation in A_{2A}R-CB_1R heteromers (Fig. 1D). Pathologically higher concentrations of adenosine should then be able to promote glutamate release by promoting higher occupancy and activation of A_{2A}Rs in both the A_{2A}R-CB_1R and A_1R-A_{2A}R heteromers, counteracting CB_1R and A_1R activation by type I allosteric interactions (Fig. 1E).

One more question remained to be answered after the results with dipyridamole in optogenetic-microdialysis experiments which indicated a lack of activation of presynaptic A_{2A}Rs. As mentioned in the Introduction, the systemic administration of A_1R and A_{2A}R agonists in reserpinized mice selectively antagonizes the locomotor activation induced by D_1R and D_2R agonists, respectively (Ferré et al., 1991a, 1994), which are pharmacological interactions mediated by post-synaptic striatal A_1R-D_1R and A_{2A}R-D_2R heteromers. The enigma was related to previous results obtained with the systemic administration of dipyridamole in reserpinized mice, which significantly decreased locomotor activation induced by D_1R or D_2R agonists and, in both cases, the effect of dipyridamole was reverted by caffeine (Ferré et al., 2018b). These results would indicate that, differently from presynaptic striatal A_{2A}Rs, the dipyridamole-induced increase in extracellular adenosine concentration can promote significant activation of postsynaptic striatal A_{2A}Rs, in addition to postsynaptic A_1Rs. An obvious explanation is the lack of functional opposition of inhibitory A_1Rs in the postsynaptic striato-pallidal neuron, which, as postulated above, it occurs

presynaptically under conditions of high physiological extracellular concentrations of adenosine (Fig. 1D). However, it should also depend on the specific affinity of adenosine for A_{2A}Rs and A_1Rs in their respective striatal postsynaptic heteromers.

Therefore, we analyzed the effect of CGS-21680 on the activation of the G protein with NanoBiT technology and, similarly to the type II allosteric interaction in the A_1R-A_{2A}R heteromer, we found a decrease in the efficacy of CGS-21680 in cells cotransfected with A_{2A}Rs and D_2Rs as compared with cells only transfected with A_{2A}Rs or with A_{2A}Rs and CB_1Rs (Fig. 4B). Nevertheless, different from the A_1R-A_{2A}R heteromer (Sarasola et al., 2022), there was no change in EC_{50} (Fig. 4B), adding a mechanistic explanation for a preferential affinity of endogenous adenosine for the striatal postsynaptic A_{2A}R-D_2R versus the presynaptic A_1R-A_{2A}R heteromer. With the same experimental preparation, we could also reproduce the significant reduction of constitutive activity of the A_{2A}R in cells cotransfected with D_2Rs as compared with cells only transfected with A_{2A}Rs or cotransfected with CB_1Rs (Fig. 4A).

7. Conclusions

We have reviewed the significant role that adenosine and A_1Rs and A_{2A}Rs play in the local integration of glutamate, acetylcholine, dopamine and endocannabinoid release in the striatum, which depends on the striatal microcircuit that includes cortico-striatal glutamatergic terminals, dopaminergic nerve terminals and cholinergic interneurons, and the specific localization of heteromers of adenosine, dopamine, acetylcholine and cannabinoid receptors in these striatal neuronal elements (Fig. 2). The different properties of A_{2A}Rs when forming heteromers with adenosine, dopamine, and cannabinoid receptors, which depend on their different tetrameric quaternary structures, provide an example of the functional and pharmacological significance of GPCR heteromers. With the reappraisal of the role of A_1R and A_{2A}R heteromers in the cortico-striatal terminals, at the functional level, we still provide a mechanism that allows for a similar fine tuning of adenosine and endocannabinoids-mediated modulation of glutamate release as previously proposed within the frame of a putative differential heteromer-dependent G protein coupling of the A_{2A}R (Ferré et al., 2010). The strongest inhibition of glutamate release should be observed under conditions of high and low extracellular concentrations of endocannabinoids and adenosine, respectively, while high concentrations of adenosine and low concentrations of endocannabinoids should lead to significant glutamate release. As reviewed here, new experimental evidence supports the same coupling of the A_{2A}R to its preferred Gs protein in the tetrameric A_1R-A_{2A}R and A_{2A}R-CB_1R heteromers (Bonaventura et al., 2015; Navarro et al., 2018a,b; Köfalvi et al., 2020). And new experimental evidence indicates that fine-tuning the modulation of glutamate release could be conceptualized by functional competition between the mostly inhibitory A_1R-A_{2A}R and stimulatory A_{2A}R-CB_1R heteromers (Fig. 1).

Apart from adenosine receptor heteromers, we have also reviewed the functional significance of dopamine receptor heteromers within the striatal microcircuit that involves cortico-striatal glutamatergic and mesencephalic dopaminergic terminals, and the cholinergic interneuron (Fig. 2). A main functional output of this microcircuit is local glutamate-mediated and acetylcholine-dependent dopamine release, which involves autoreceptor complexes constituted by heteromers of α_4-β_2* nAChR and D_2R and possibly D_3R (Quarta et al., 2007; Bontempi et al., 2017). Through these heteromers localized in the dopaminergic terminals and, also, α_7 nAChR localized in the glutamatergic terminals (Kaiser and Wonnacott, 2000; Rassoulpour et al., 2005) (Fig. 2), acetylcholine predominantly exerts a facilitation of dopamine release, although muscarinic acetylcholine autoreceptors should moderate acetylcholine release (Muramatsu et al., 2022). Through the α_4-β_2*-D_2R/D_3R heteromers and the D_2R-D_4R heteromers localized in the glutamatergic terminals and possibly D_2R localized in cholinergic interneurons (Muramatsu et al., 2022) (Fig. 2), dopamine predominantly exerts an inhibition of dopamine release.

The analyzed microcircuit only included neuronal elements, but the role of astrocytes in the adenosine-mediated control of striatal glutamatergic and dopaminergic neurotransmission should not be ignored. In fact, astrocytes play a main role in the control of extracellular levels of adenosine, by their ability to produce vesicular release of ATP, which is immediately converted by plasma membrane ectonucleotidases to adenosine, and by their ability to promote the intracellular transport and

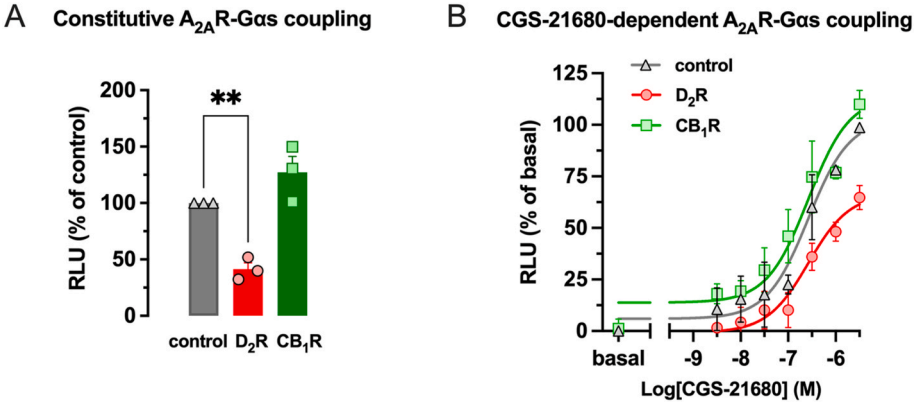

Fig. 4. Heteromer-mediated allosteric modulation of A_{2A}R-mediated Gs protein activation. A_{2A}R-mediated changes in the coupling to Gαs were monitored using a NanoBiT assay using $A_{2A}R^{SmBiT}$ and $G\alpha s^{LgBiT}$, as described elsewhere (Sarasola et al., 2022). In brief, HEK-293 T cells (5×10^5 cells/ml) transiently transfected with $A_{2A}R^{SmBiT}$ and $G\alpha s^{LgBiT}$ plus D_2R (red circles), CB_1R (green squares) or control pcDNA3.1 (gray triangles) were transferred (90 μL) into a white 96-well plate and incubated with 0.5 U/ml adenosine deaminase in OptiMEM for 1 h at 37 °C with constant agitation (550 rpm). Subsequently, 10 μl of a 10 μM coelenterazine 400a solution was added and after a 1-min incubation the end-point luminescence was determined using a CLARIOstar plate reader and the output luminescence reported as integrated relative light units (RLU). (**A**) Constitutive coupling of the A_{2A}R to Gαs-protein; the basal NanoBiT signal (RLU) of HEK-293 T cells transiently transfected with $A_{2A}R^{SmBiT}$ and $G\alpha s^{LgBiT}$ in the absence (control) or presence of D_2Rs or CB_1Rs was evaluated as described above; results were normalized as percentage of the constitutive activity in cells transfected with control pcDNA3.1 and are represented as means ± SEM of three independent experiments performed in triplicate; *$P < 0.01$, one-way ANOVA with Dunnett's *post-hoc* test when compared to control pcDNA3.1 transfected cells. (**B**) Concentration-response of CGS-21680-induced change in the A_{2A}R coupling to Gαs-protein; HEK-293 T cells transiently transfected with $A_{2A}R^{SmBiT}$ and $G\alpha s^{LgBiT}$ and D_2R, CB_1R or control pcDNA3.1, were incubated with increasing concentrations of CGS-21680 and the coupling of the A_{2A}R to Gαs-protein was monitored by NanoBiT; results show the RLU recorded at the NanoBiT signal peak (10 min after adding CGS-21680) normalized by the corresponding signal obtained without CGS-21680 (basal); results are expressed as means ± SEM of three independent experiments performed in quadruplicate; in cells transfected with $A_{2A}R^{SmBiT}$ and $G\alpha s^{LgBiT}$ and the control plasmid, CGS-21680 induced a concentration-dependent increase in the values of the A_{2A}R coupling to Gαs-protein (pEC_{50} = 6.5 (6–6.9, 95% CI); co-expression of CB_1Rs did not alter the CGS21680-induced A_{2A}R coupling to Gαs-protein (pEC_{50} = 6.8 (6.3–7.3, 95% CI; E_{max} = 107% (88–133, 95% CI), while co-expression of D_2Rs significantly reduced the maximum effect (E_{max} = 70.6% (55–94, 95% CI); $F_{(1, 38)} = 4.18$; $P = 0.047$) but not the potency of CGS-21680 (pEC_{50} = 6.5 (6–6.9, 95% CI); $F_{(1, 38)} = 0.01$; $P = 0.92$).

metabolization of extracellular adenosine by equilibrative nucleoside transporters and the enzyme adenosine kinase (Cunha, 2016). As part of the glutamatergic tripartite synapse, astrocytes provide an adenosine-mediated feedback inhibitory control of glutamate release upon activation of astrocytic metabotropic glutamate receptors, which promotes astrocytic ATP release and the subsequent preferential adenosine-mediated activation of presynaptic A_1Rs in the striatal glutamatergic terminals (Cavaccini et al., 2020). The same mechanism seems to operate upon dopamine release and activation of astrocytic D_1Rs (Corkrum et al., 2020) and, could also be mediated by acetylcholine and endocannabinoids, by acting on astrocytic α7 nAChRs and CB_1Rs, as recently demonstrated in cortical astrocytes in culture (Secci et al., 2019).

Furthermore, although with lower expression than their neuronal neighbors (Hettinger et al., 2001), striatal astrocytes seem to express functional A_{2A}Rs (Matos et al., 2012), which have been suggested to form striatal astrocytic A_{2A}R-D_2R heteromes (Cervetto et al., 2017). Similarly, there is also evidence for a significant functional expression of A_1Rs in striatal astrocytes in culture (El-Etr et al., 1992) and of functional A_1R-A_{2A}R heteromers in cultured cortical astrocytes (Cristóvão-Ferreira et al., 2013). More research is nevertheless needed to establish the functional and pharmacological significance that astrocytes play in the local integration of adenosine, glutamate, acetylcholine, dopamine, and endocannabinoid neurotransmission in the striatum, in the function of the striatal microcircuit that includes cortico-striatal glutamatergic terminals, dopaminergic nerve terminals and cholinergic interneurons, as well as in the function of basal ganglia circuitry.

We have also reviewed the evidence supporting the use of different striatal adenosine receptor heteromers as targets for drug development, depending on the differential ability of the A_{2A}R to demonstrate constitutive activity upon heteromerization with different GPCR (Köfalvi et al., 2020) and the different ability of A_{2A}R ligands to act as neutral antagonists or inverse agonists (present results and Bennett et al., 2013), and depending on the differential ability of A_{2A}R ligands to bind to different A_{2A}R heteromers (Orrú et al., 2011a). In general, this implies that the properties of ligands with selectivity for a specific GPCR should not just be evaluated in preparations only expressing the GPCR, but include other possibly interacting GPCRs and other plasma membrane-interacting proteins, i.e., considering the GEMMA concept (Ferré et al., 2022a). As a final conceptual consideration, the realization of a significant functional role of the constitutive activity of some GPCRs, as well as their potential ligand-dependent ability to allosterically influence the function of another GPCR when forming heteromers, implies that receptors should not only be considered as mediators of the effects of their neurotransmitters, but as direct key modulators of neurotransmission.

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Data availability

Data will be made available on request.

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