Chapter 10
Adenosine Receptors as a Paradigm to Identify Dimer/Oligomers of G-Protein-Coupled Receptors and as Targets in Parkinson's Disease and Schizophrenia

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Abstract While adrenergic receptors were instrumental to start to understand the role of GPCRs, other receptors are taking the lead to understand why GPCR homo−/heteromers are needed and to address their physiological consequences in both healthy/homeostatic conditions and disease. Adenosine and dopamine receptors in the CNS are instrumental to understand pathogenic mechanisms in Parkinson's disease and to know the role of receptor heteromers. We here provide the account of the heteroreceptor complexes formed by adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3), and their potential as therapeutic targets. Both adenosine (A_1 or A_{2A})-dopamine (D_1 or D_2) and adenosine A_1A_{2A} heteroreceptor complexes are therapeutic targets in Parkinson's disease and may be altered after chronic levodopa treatment. A short account on the potential of adenosine receptors as targets in schizophrenia is also provided. Apart from potential in combating symptoms, adenosine receptors have potential as targets for neuroprotection. However, the design of neuroprotective drugs requires to understand how adenosine affects microglia and which adenosine-receptor-containing heteromers may be targeted.

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10.1 Introduction

Adenosine and dopamine receptors have been instrumental in identifying complexes with other members of the Class A G-protein-coupled receptor (GPCR) superfamily. For review on dopamine receptor homo−/heteromerization and its relevance, see Rashid et al. (2007), Fuxe et al. (2014a, b), George et al. (2014), Perreault et al. (2014), Borroto-Escuela et al. (2016), Borroto-Escuela and Fuxe (2017) and references therein. Dimers were first identified using coimmunoprecipitation and other biochemical approaches. Later, biophysical techniques were implemented to detect dimers (even trimers) in heterologous expression systems. The existence of receptor-receptor interactions between different GPCRs in the plasma membrane in brain tissue was first indicated in biochemical binding studies on neuropeptide modulation of the affinity and density of monoamine receptor subtypes using monoamine radioligands and membrane preparations from different brain regions (Fuxe et al. 1981, 1983, 1987; Agnati et al. 1982; Fuxe and Agnati 1985). The results gave rise to the concept of direct interactions in the plasma membrane of subtype-specific neuropeptide receptor and monoamine receptors. In 1993, it was proposed that the molecular mechanism for these GPCR receptor-receptor interactions was represented by the formation of a heterodimer in balance with the corresponding homodimers/monomers (Zoli et al. 1993).

Franco et al. (2016) reviewed the strategies that may lead to demonstrate that heteroreceptor complexes formed by GPCR are present in natural sources; in particular, the two that have provided more benefit in our experience are (i) the heteromer print (something that is particular to the complex and does not happen in individually expressed receptors) and (ii) in situ proximity ligation assays, a technique developed for assessing cancer types in samples from patients and that allows to detect GPCR clusters in cells, in samples from animal models, in samples from patients, or in samples from necropsies. The central nervous system (CNS) has been by far the substrate for identifying the complexes formed by adenosine receptors. Actually, the periphery lacks behind the CNS in identifying and addressing the physiological role of GPCRs. Exceptions do occur, and the most straightforward example in the periphery is likely provided by chemokine receptors, which may form homo- and heterodimers that provide pharmacological and signaling diversity to cells of the immunological system (see (Springael et al. 2005; Muñoz et al. 2009, 2011, 2012) and references therein). Indeed, there is consensus in that a receptor heteromer (Het) cannot be considered as such in the absence of any particular property, i.e., a given complex in a natural context should display a particular heteromer print (Het) (Ferré et al. 2009a).

10.2 Adenosine Receptors in the Formation of Heteromers with Non-purinergic GPCRs

Except for error, omission, or very recent discovery, the direct interactions reported for adenosine receptors with other members of the GPCR superfamily are those described below.

The first Het identified for receptors having different endogenous agonists was that constituted by adenosine A_1 (A_1R) and dopamine D_1 (Gines et al. 2000; Torvinen et al. 2002; Cao et al. 2006). In parallel, the Het for two different subtypes of receptors for the same endogenous agonist (mu and delta opioid receptors) was discovered by Gomes et al. (2000). In brain regions related to motor control, functional adenosine-dopamine receptor interactions were known. Also known was the segregation of striatal D_1 and D_2 receptors in, respectively, the so-called direct and indirect pathways of motor control. It turns out that whereas D_1 and A_1 colocalize in striatonigral GABAergic neurons, adenosine A_{2A} ($A_{2A}R$) and dopamine D_2 receptors colocalize in striatopallidal GABAergic neurons. Accordingly, we hypothesized, and later demonstrated, that A_{2A}-D_2 heteromerization in the indirect pathway paralleled the A_1-D_1 heteromerization in the direct pathway (Hillion et al. 2002; Canals et al. 2003, 2004; Fuxe et al. 2003, 2007; Ciruela et al. 2004). The highest $A_{2A}R$ expression in a mammalian body is found in the striatum, a fact whose extent is not fully known. Hence, interactions with other dopamine receptors, which are also expressed in motor control brain areas or with receptors widely distributed in the CNS, have been reported. On the one hand, the effect of activation of $A_{2A}R$ on in vivo actions mediated by dopamine D_3 (Hillefors et al. 1999) prompted us to investigate and identify A_{2A}-D_3 Hets (Torvinen et al. 2005). In vivo activation of A_{2A}Rs in the basal ganglia causes alterations in the pharmacological characteristics of dopamine D_3 receptors that may underlie the atypical neuroleptic-like effect of $A_{2A}R$ receptor agonists (Rimondini et al. 1997; Hillefors et al. 1999); as a matter of speculation, those in vivo effects may be a consequence of the particular pharmacological and functional properties of A_{2A}-D_3Hets. On the other hand, striatal adenosine A_{2A}Rs form functional heteromeric complexes with cannabinoid CB_1 receptors (Carriba et al. 2007) or with histamine H_3 (Márquez-Gómez et al. 2018) receptors; these Hets may, respectively, mediate the motor effects of cannabinoids and deserve attention on assessing the potential of antihistamines in the therapy of CNS diseases. Due to the intrinsic structural and conformational properties of the Class C GPCR subfamily, they can form a myriad of homo- and heteroreceptor complexes (Doumazane et al. 2011; Borroto-Escuela et al. 2014). Interestingly, the $A_{2A}R$ may form functional but also molecular complexes with Class C metabotropic $mGlu_5$ receptors (Ferré et al. 2002, 2003; Nishi et al. 2003; Kachroo 2005; Borroto-Escuela et al. 2017b). Ultrastructural studies have shown that the two receptors colocalize in the nonhuman primate striatum (Bogenpohl et al. 2012). Finally, the adenosine receptor is also able to interact with the orphan GPR37 receptor (Dunham et al. 2009). Pioneering evidence on functional interactions in rat caudate putamen

suggests that the adenosine receptors may also interact with some of the opioid receptor subtypes (Noble and Cox 1995; Borroto-Escuela et al. 2014).

$A_{2A}R$ may form homodimers (Canals et al. 2004) that likely interact with other GPCRs to form high-order heteroreceptor complexes. One example is the Het formed by A_{2A}, cannabinoid CB_1, and dopamine D_2 (Carriba et al. 2007; Navarro et al. 2008; Bonaventura et al. 2014; Pinna et al. 2014a, b). Another is the complex formed by A_{2A}, D_2, and $mGlu_5$ receptors (Cabello et al. 2009).

Consistent with the intense research on potential heteromerization of adenosine receptors, it has been shown that β_1- and β_2-adrenergic receptors may directly interact with the A_1R and that the resulting Het displays particular properties in terms of differential pharmacology and coupling to the signaling machinery (Chandrasekera et al. 2013). Finally, it has been confirmed that prostanoid receptors, namely, the thromboxane A_2 TP receptor, may form hetero-oligomers with the A_1R whose functional properties are conditioned by the presence and concentration of the endogenous agonist of the two receptors (Mizuno et al. 2012, 2013a). Heteromerization has been also reported for A_1R and class C metabotropic glutamate 1 alpha (Ciruela et al. 2001; Franco et al. 2001).

For reasons that are out of the scope of the present chapter, the two most studied adenosine receptors, in terms of receptor-receptor interaction research, are the A_1 and the A_{2A}. The other two types of adenosine receptors (A_3 and A_{2B}) are lacking behind, but, interestingly, the first identified Hets containing A_3 or A_{2B} are between adenosine receptors themselves (see next Sect. 10.3).

10.3 Adenosine Receptors May Interact with Other P1 (to Form Adenosine Isoreceptor Complexes) and with P2 Purinergic Receptors

Soon after the experimental confirmation of GPCR heteromerization and the extensive work made with A_1 and A_{2A} receptors, it was tempting to search for interaction between adenosine, i.e., P1 purinergic receptors, and "ATP" P2 purinergic receptors that are also GPCR members (metabotropic P2Y receptors). Pioneering studies to prove the hypothesis led to the discovery of interactions between A_1 and $P2Y_1$ receptors to form a functional unit with a particular pharmacological print (Yoshioka et al. 2001). Interestingly, A_1 and D_2 receptors were used as negative controls thus confirming previous results and the specificity of the interactions. Discovery of more P1-P2 receptor complexes (e.g., A_1-$P2Y_2$Hets), and/or their physiological roles (especially in the brain), were further reported (Yoshioka et al. 2001, 2002a, b; Suzuki et al. 2006; Tonazzini et al. 2007). The interplay between P1 and P2 receptors opens interesting avenues due, *inter alia,* to the fact that extracellular ATP acting on P2 receptors is degraded into adenosine, which activates P1 receptors (homoreceptors/monomers or forming Hets).

The interest of the P1/P2 receptor interplay prompted (Schicker et al. 2009) the performance of an ambitious project to discover mixed P1/P2 receptor-receptor interactions. The authors tested A_1, A_{2A}, $P2Y_1$, $P2Y_2$, $P2Y_{12}$, and $P2Y_{13}$ receptors and the $P2X_2$ (ligand-gated ion channel) ionotropic receptors. They provided evidence for the formation of *heterooligomers among each other*. $P2Y_1$, $P2Y_{12}$, $P2Y_{13}$, A_1, A_{2A}, and $P2X_2$ receptors are also able to exist as homomers (Schicker et al. 2009). Reviews on the role of P1/P2 receptor-receptor interactions may be found in Nakata et al. (2010) and Suzuki et al. (2013). Of further interest for the present article, these results confirmed the occurrence of A_1R homodimers (Ciruela et al. 1995), $A_{2A}R$ homodimers (Canals et al. 2004), and of A_1A_{2A}Hets for which a structural basis has recently been provided (see next Sect. 10.4).

After prediction by computational means of homodimerization of A_3 receptors (Kim and Jacobson 2006), Hill and colleagues detected both A_3homodimers and heterodimers with A_1 receptors (May et al. 2011; Hill et al. 2014). We also have evidence of A_1A_3 Het expression in the CNS (data in preparation). It is likely that more A_3-receptor-containing Hets exist, but they have not yet (to our knowledge) been identified.

Although some indirect evidence suggested that A_{2B} receptors ($A_{2B}R$) could be interacting with other GPCRs (Moriyama and Sitkovsky 2010), the direct proof is given in a recent publication (Hinz et al. 2018). As a matter of fact, the A_{2B} is an atypical receptor as the affinity for adenosine is very low, but its activation in lymphocytes may lead to calcium mobilization (Mirabet et al. 1997). In summary, it is assumed that A_{2B} receptors are activated is reservoirs with elevated adenosine levels or when hypoxic conditions lead to very high concentrations of the nucleoside. Also, there has been a lack of pharmacological tools that has been progressively solved. Intriguingly the A_{2B} protein has also been described in the CNS as a receptor for netrin-1, involved in axon guidance (Corset et al. 2000; Shewan et al. 2002). The discovery of heteromers formed by A_{2B} and A_{2A} receptors has led to a significant finding, namely, that the activation of the first alters the pharmacology and signaling of the latter. In a heteromeric context, the affinity of $A_{2A}R$ selective ligands is markedly reduced, i.e., the activation of the receptor demands higher concentrations of A_{2A} receptor agonists. Accordingly, the efficacy of ligands targeting the $A_{2A}R$ would be dependent on the heteromeric context, especially in the case of $A_{2A}A_{2B}$Het occurrence (Hinz et al. 2018).

10.4 The A_1-A_{2A} Receptor Heteromer (A_1A_{2A} Het): A Unique Functional Unit

A_1A_{2A} Het is in itself a paradigm to understand a fact that was inscrutable for decades, namely, the co-expression of one receptor for adenosine coupled to G_s and another receptor for adenosine coupled to G_i. In such cells, adenosine would lead to a "contradictory" output as, on the one hand, it would increase adenylate cyclase

activity (via G_s), and, on the other hand, it would decrease adenylate cyclase activity (via G_i). Co-expression of different receptors for a given neurotransmitter in the same cell is quite common, for instance for serotonin receptors (Santana et al. 2004). One of the possibilities (especially in neurons) was to assume that one of the receptors was expressed in a specific location of the cell, whereas the second receptor was located in a different location (always in the cell membrane but far away in spatial terms). In the case of the A_1 and A_{2A} receptors, the explanation is totally different, and, furthermore, it constitutes a clear paradigm of the need of GPCR Hets. In brief, the A_1A_{2A}Het is a device to sense the adenosine concentration to act accordingly, i.e., decreasing cAMP levels when [adenosine] is low and to increase cAMP levels when [adenosine] is high. Adenosine not only increases in hypoxia but its level varies with the metabolic status. Again, this is especially important in regions where neurons are very active and the adenosine/ATP ratio is high. The A_1A_{2A}Het was discovered by Ciruela et al. (2006), and the results on heteromerization of those receptors were later validated by Schicker et al. (2009).

There are different cell types in which the two receptors are co-expressed and where they may likely form A_1A_{2A}Hets. The physiological role of the A_1A_{2A}Het has however shown in the CNS and in relationship to control of neurotransmitter transport by adenosine and, importantly, in both neurons and glial cells. Our results centered in the striatum showed that the levels of co-expression of the two receptors in glutamatergic terminals reaching the striatum were markedly high and that low or high concentrations of adenosine led to opposite effects on glutamate release (Ciruela et al. 2006). This finding in 2006 did not provide any molecular mechanism but suggested that the coupling was different, to either G_s or G_i, depending on the concentration of the nucleoside. Furthermore, it seemed that the heteromeric context was the substrate to block A_1R-mediated signaling when the A_{2A}R was activated. In summary at relatively low adenosine concentrations, the Het was providing A_1R-dependent signaling, whereas at relatively high concentrations it was providing A_{2A}R-dependent signaling.

Fairly similar results were obtained in astrocytes and the control of the transport of one of the main inhibitory neurotransmitters in the CNS, gamma-aminobutyric acid (GABA). First, colocalization of the two receptors and occurrence of A_1A_{2A}Het was demonstrated. Second, the regulation of GABA uptake by cultures of astroglia depended on the concentration of adenosine. Indeed, the regulation of GAT-1 and GAT-3 transporters was via G_i or via G_s depending on whether the receptor activated within the A_1A_{2A}Het was, respectively, A_1R or A_{2A}R (Cristóvão-Ferreira et al. 2013). The molecular basis of such a phenomenon was recently elucidated and described in the next Sect. 10.5.

10.5 The A_1-A_{2A} Receptor Heterotetramer: A Reliable Structural Model

On the one hand, the quaternary structure is crucial for Het function (Navarro et al. 2010). On the other hand, three-dimensional structures of GPCRs are difficult to decipher due to the technical difficulties in obtaining crystals of membrane proteins. Protein engineering and complementary technological advances have led to the elucidation of several GPCR structures and, also, to key structural elements of the GPCR-G protein interactions (see (Cordomí et al. 2015) and references therein). Those advances have served to understand that the most abundant G-protein-coupled signaling unit in the plasma membrane is a GPCR dimer. Exceptions may occur, i.e., a monomer GPCR may eventually couple to a G protein and be able to convey signal toward the inside of the cell. Indeed, this is not the case of the A_1A_{2A}Het whose minimal structure is likely constituted by one A_1R homodimer and one A_{2A}R homodimer, i.e., a heterotetramer. Identification of GPCR oligomers combined with structural data and with modeling and other in silico approaches has provided relevant information concerning the structure of heteroreceptor complexes and their coupled G proteins. Also relevant is the fact that a substantial movement occurs within a GPCR and a G protein when the receptor becomes activated by agonists. Overall, membrane-attached GPCRs, which contain seven transmembrane domains and a tightly coupled alpha G protein subunit, likely form homodimers in a head-to-head fashion. Even allowing to increase the size of the heteroreceptor complex by considering four GPCRs and two coupled G proteins, the number of possible structures for the macromolecular complex is very few, as reported in the quite revealing work by Cordomí et al. (2015)

Using such in silico information, interfering peptides containing transmembrane sequences and, also, data from resonance energy transfer (using both receptors and G protein subunits as probes) and complementation assays, the first reliable structure for a GPCR Hets in complex with one G_s and one G_i protein was provided (Navarro et al. 2016b). The rhombus-shaped structure that contains alpha subunits of G_s or G_i bound to the outer protomers (in both A_1R and A_{2A}R homodimers) would allow signaling via A_1R and via A_{2A}R. We mean that such a symmetrical structure cannot provide an asymmetrical signaling as that involved in the control (by adenosine) of glutamate or GABA transport regulation. The clue that explains the uniqueness and the functional properties of the A_1A_{2A}Het is a recent finding involving the C-terminal tail of the A_{2A}R (Navarro et al. 2018). Different GPCRs display a wide range of lengths in their C-terminal domain. Whereas A_1R has a short C-terminal end, the tail of the A_{2A}R is quite long. Then we hypothesized that a long C-terminal tail would, upon activation of the receptor, block the G-protein-mediated signaling arising from a closely located receptor. Exhaustive experimental and in silico work has provided reliable data showing that removal of the C-terminal domain of the A_{2A}R leads to the disappearance of the Het fingerprint, i.e., activation of a truncated A_{2A}R does not result in impairment of A_1R activation and G_i-mediated signaling. The huge diversity

in the length and structure of C-terminal domains deserves a closer look and is a challenge in future work in the GPCR field.

10.6 Adenosine-Receptor-Containing Heteromers and Schizophrenia

Although the evidence is higher in Parkinson's disease and the success is already evident by the approval of an $A_{2A}R$ antagonist in the therapy of Parkinson's (see below), we would like to make a brief account of data showing that adenosine receptors have also potential in the therapy of schizophrenia. Fuxe et al. (2005) reviewed possibilities of the heteromer as target for schizophrenia. Moreover, the dopamine D_3 receptor is one of the proposed therapeutic targets for treatment of the disease and, accordingly, the discovery of the $A_{2A}D_3$Het receptor (Torvinen et al. 2005) place $A_{2A}R$ ligands as potential therapeutic drugs. Also along this line of reasoning is the above-described occurrence of occurrence of $A_{2A}mGlu_5$Hets. Reviews on the cumulative data that, based on adenosine-receptor-containing heteromers, open new perspectives in antischizophrenia therapy were provided by Fuxe et al. (2008, 2010) and Wardas (2008).

10.7 Adenosine-Receptor-Containing Heteromers and Parkinson's Disease (PD) and Levodopa-Induced Dyskinesia

In this section, we will first focus on the antiparkinsonian efficacy of adenosine receptor ligands to then take into consideration that any drug used by patients is – mostly – targeting Hets. Afterward, we will focus on the adenosine-receptor-containing heteromers that have been studied in both healthy and parkinsonian conditions. Hets constituted by adenosine receptor themselves and by adenosine and dopamine receptors fulfill these rules. The main objective in translational research is to identify suitable targets and efficacious drugs. To this respect dual adenosine-dopamine receptor ligands and bivalent compounds have been developed. The former (dual compounds) (Vendrell et al. 2007) may constitute the basis for the development of novel antiparkinsonian drugs. Instead, the latter (bivalent ligands), being unable to cross the blood-brain barrier and susceptible of being hydrolyzed soon after intake, have been instrumental to confirm the occurrence of adenosine-dopamine Hets in the striatum (A_{2A}-D_2 bivalents in Soriano et al. (2009) and A_1-D_1 bivalents in Shen et al. (2013). Hence, such heteromers are demonstrable targets of antiparkinsonian drugs.

10.7.1 Efficacious Antiparkinsonian $A_{2A}R$ Antagonists

Levodopa-based dopamine replacement therapy started decades ago and is still regarded as being of highest benefit for today's patients (Birkmayer and Hornykiewicz 1962, 1964; Olanow et al. 2004; Hornykiewicz 2006). Based on the early work of Fuxe and Ungerstedt (1974), on translational research and on data from clinical trials (Mizuno et al. 2013b; Saki et al. 2013; Kondo et al. 2015), a selective $A_{2A}R$ antagonist, istradefylline (Nouriast™), was approved in Japan for adjunctive antiparkinsonian therapy. The underlying idea was to reduce the dose of levodopa (or the dopamine-receptor-related medication) to diminish the side effects. In fact, long-term treatment with levodopa may lead to uncontrolled movements.

Cumulative evidence along decades, in different laboratories and under a variety of experimental setups, led to find a dopamine-adenosine antagonism in striatum. Even assuming that receptors are expressed individually (and not as heteromers), activation of adenosine A_1 and dopamine D_1 receptors in the direct pathway (or A_{2A} and D_2 in the indirect pathway) would lead to opposite effects as one of the receptors is coupled to G_i and another to G_s. Solid reviews describing the molecular basis of the antagonism may be found in the literature. As the complete list of reviews is quite notable, we here suggest the following ones that arise from different laboratories and/or present different but complementary perspectives (Bibbiani et al. 2003; Tanganelli et al. 2004; Schwarzschild et al. 2006; Ferré et al. 2007a, 2009b, 2010a, Fuxe et al. 2007, 2010, 2015; Simola et al. 2008; Armentero et al. 2011; Beggiato et al. 2014; Navarro et al. 2016a; Borroto-Escuela et al. 2017b).

In vivo experimental data on the potential of $A_{2A}R$ ligands to i) affect striatal dopaminergic neurotransmission and striatal plasticity and ii) to be efficacious in the unilateral 6-hydroxydopamine rat model of Parkinson's disease were provided by *inter alia* Pinna et al. (1997, 2007), Strömberg et al. (2000), Agnati et al. (2004). $A_{2A}R$ knockout (KO) mice have been used to ensure that a lack of $A_{2A}R$-mediated signaling (and of any A_{2A}Het-mediated signaling) provides data that reinforces the antiparkinsonian potential of receptor blockade (Kachroo 2005).

Reviews on the role of Hets in the pathogenesis of Parkinson's disease and their potential as therapeutic targets of the disease appeared soon after the discovery of GPCR heteromers. Reviews with titles reflecting the relevance of purinergic signaling and/or receptor heteromerization were provided by Maggio et al. (2010), Navarro et al. (2016a, 2017), Borroto-Escuela et al. (2017a). However, the list of relevant reviews on the subject is quite broad. From such list we would recommend the following reviews (and references therein): (Schwarzschild et al. 2002; Morelli et al. 2007; Ferre et al. 2008; Fuxe et al. 2008, 2015; Ferré et al. 2009b).

We also believe that the paper by Short et al. (2006) provides a solid account on an interdependence between dopamine and adenosine receptors disclosed from characterizing receptor expression in adenosine and dopamine receptor KO mice (single KOs, i.e., only one receptor gene knocked out in each of the transgenic lines). This supports the early work of Fuxe and Ungerstedt (1974). Authors concluded that "*the existence of functional interactions between dopaminergic and*

purinergic systems in these reward and motor-related brain regions" (Short et al. 2006).

Therefore, antagonists of adenosine receptors were soon proposed to increase dopamine action in Parkinson's disease, which consists of the depletion of dopamine in striatum due to nigral neurodegeneration. In summary, the conceptual approach was to use adenosine receptor antagonists to increase the dopaminergic action in striatal GABAergic neurons.

10.7.2 Heteromers as Targets of Antiparkinsonian Drugs

Soon after identification of A_1D_1Het and of $A_{2A}D_2$Het, these heteromers were proposed as targets or Parkinson's disease (Fuxe et al. 2003). Despite forgotten due to the usual way to develop novel drugs, i.e., by screening cells expressing individual receptors, it is evident that any antiparkinsonian medication is acting on receptors in heteromeric contexts. Thus, levodopa does not act on isolated dopaminergic receptors but on receptors forming Hets. In the case of Nouriast™, the drug is acting on those Hets identified as of today, namely, $A_{2A}D_2$Hets with or without CB_1 or $mGlu_5$ receptors. The potential of cannabinoids or mGlu5 receptor ligands has been suggested (Ferré et al. 2009b, 2010a), but the underlying reasons are out of the scope of the present article. In terms of adenosine receptors, it was suggested that A_1 receptor agonists acting on A_1R, which are expressed in the direct pathway, reduce D_1 receptor and levodopa-induced dyskinesia (see (Ferré et al. 1994; Florán et al. 2002; Franco et al. 2005; Mango et al. 2014) and references therein). It should be noted that in dyskinesia the level of the D_3 receptor and of D_1D_3Hets increase (Marcellino et al. 2008; Farré et al. 2015). These results suggest that also D_3 receptor ligands may be useful in the therapy of dyskinesia and that Hets may be considered targets for drugs able to counteract this side effect of chronic medication of levodopa and dopamine receptor agonists.

In what concerns the A_1A_{2A}Het, which is presynaptic (unlike A_1D_1 or $A_{2A}D_2$ Hets that are postsynaptic), it is not known how istradefylline (Nouriast™) is affecting its function in striatal glutamatergic terminals of patients. In healthy conditions, blockade of A_{2A}R does not seem to produce any evident effect via those heteromers. In fact, A_{2A}R antagonists are very safe, and this fits with a general rule (surely with exceptions) that receptors antagonists may be taken in chronic regimes by patients of diverse illnesses. A deeper look into the differential pharmacology of Hets has led to find that different drugs may have different "potencies" for the same receptor but in different heteromeric contexts. In brief, there is data showing that the affinity of an antagonist for A_{2A}R, or of caffeine for A_1R or A_{2A}R, is different when tested in different Hets. With data using different (pre- and postsynaptic) Hets and different antagonists, Orru et al. (2011) have suggested that "*on the basis of their preferential pre- versus postsynaptic actions, SCH-442416 and KW-6002 may be used as lead compounds to obtain more effective antidyskinetic and antiparkinsonian compounds,*

respectively." SCH-442416 is a broadly studied $A_{2A}R$ selective antagonist, whereas KW-6002 is another one (also known as istradefylline).

Interestingly, a recent report has linked early-onset Parkinson's disease cases to a point mutation in the gene of the A_1R (ADORA1). The mutation leads to the substitution of a conserved amino acid in transmembrane 7 (Jaberi et al. 2016). Based on current data and in the proposed models for receptor Hets, this mutation would not affect interacting interfaces of homo- or heteromers; then alternative explanations include altered binding of adenosine or altered signaling.

Taking into account the successful case of Nouriast™, one wonders why it is relevant to consider A_{2A}Hets as targets. On the one hand, the adenosine-dopamine antagonism is evidenced at the Het level, i.e., it is a significant print of the adenosine-dopamine Hets. Therefore, the "intracellular" antagonism due to counterbalancing second messenger cAMP levels is complemented with antagonism at the receptor level within the A_{2A}-D_2Het context. The added value of having those Hets in a very precise location, the striatal spine module, also plays a role, as pointed out by Fuxe et al. (1998, 2007), Tanganelli et al. (2004), Ferré et al. (2007a, 2009b, 2010a), Beggiato et al. (2014) and as deduced by its role in controlling striatal glutamatergic neurotransmission (Ferré et al. 2007b).

Unlike for the A_1-A_{2A}Het, no detailed structural model exists for adenosine-dopamine Hets. Allosteric interactions within the quaternary structure are essential for Het function, i.e., for integrating the dopamine and adenosine inputs (Fuxe et al. 2010). Remarkably, the $A_{2A}D_2$Het has been a paradigm to detect electrostatic interactions that are key for the functional activity of the signaling unit. Apart from the consensus on the involvement of transmembrane domains in Het formation, it was demonstrated that strings of amino acid residues with opposite charges do interact, do it tightly, and are important for quaternary structure and function (Borroto-Escuela et al. 2010). One example is provided by the epitope-epitope interactions involving arginine residues in the N-terminal part of the third intracellular loop of the D_2R and acidic residues in the C-terminal end of the $A_{2A}R$ (Ciruela et al. 2004). Complexes formed by synthetic peptides mimicking the interaction are even resistant to mass spectrometry processing thus demonstrating the strength of the epitope-epitope intraction. Finally, it should be noted that structure may be affected by phosphorylation, i.e., whereas serine would not participate on epitope-epitope interactions, a negatively charged phosphorylated serine would. One of the properties of Hets is a differential traffic respect to individually expressed receptors; apart from co-internalization and particular processing of internalized receptors that may be target to degradation or recycled back to the cell surface, there is involvement of both ß-arrestin/clathrin- and caveolin-dependent pathways (Escriche et al. 2003; Genedani et al. 2005; Franco et al. 2007; Borroto-Escuela et al. 2011)

Finally, it is worth mentioning the role of Ca^{2+} in heteromer-mediated signaling. It is likely that changes in the concentration of the ion may alter the quaternary structure of Hets in which electrostatic interactions are relevant. In fact, Ca^{2+} and/or calcium-binding proteins (e.g., calmodulin) modulate structure and function of $A_{2A}D_2$Hets (Ferré et al. 2010b; Woods et al. 2008; Navarro et al. 2009). These results

explain, at least in part, the elusive relationship between dopaminergic transmission and calcium ions.

10.7.3 How Levodopa-Induced Dyskinesia Affects Heteromerization

Parkinson's disease and adenosine-receptor-containing Hets constitute another paradigm due to the fact that their relationships have been investigated in healthy conditions and in the disease before and after chronic medication. On the one hand, the presence of $A_{2A}D_2$Hets and of Hets also including CB_1 receptors was demonstrated in rodent models of the disease (Pinna et al. 2014a, b). In our opinion, these results are important, as they show that these Hets are indeed targets of the dopamine replacement therapy. On the other hand, A_{2A}-CB_1-D_2 receptor heteromerization is disrupted after chronic levodopa administration (Pinna et al. 2014a, b). Remarkably, these results obtained in a rodent model were confirmed in a non-human primate model (Bonaventura et al. 2014), thus pointing to their validity for patients. Also consistent with those findings are the results showing in A_{2A}R knockout animals a reduction in levodopa-induced dyskinesia as reported by Xiao et al. (2011). Interestingly, similar results were obtained upon deletion of the A_1R (Xiao et al. 2011). While it is not known whether heteromer disruption is cause or consequence of chronic medication, these results show that the target of the antiparkinsonian medication changes with time. To our understanding, these results may provide the basis for the design of optimal therapeutic approaches, i.e., varying the medication and/or the dose at different stages of the disease may reduce the side effects that for Parkinson's are not only dyskinesias but cognition deficits.

10.7.4 Adenosine Receptor and Adenosine-Receptor-Containing Heteromers and Neuroprotection

The success of approval of istradefylline for the therapy of Parkinson's disease provides further hopes for other neurodegenerative diseases. In vivo assays in animal models demonstrate the usefulness of A_{2A}R antagonists in acute neural damage, for instance, in hypoxia (Chen and Pedata 2008; Melani et al. 2015; Boia et al. 2017). In the case of (chronic) neurodegenerative diseases (Parkinson's, Alzheimer's, etc.), the real issue is to know whether A_{2A}R antagonists are addressing symptoms or are also affecting disease progression. Animals lacking expression of A_{2A}R are more resistant to neuronal death in an α-synuclein model of Parkinson's disease (see (Kachroo and Schwarzschild 2012) and references therein). Surely one of today's challenges is to demonstrate whether A_{2A}R antagonists are neuroprotective, i.e., they prevent neuronal death (see Franco and Navarra 2018 and references therein).

Apart from the issue of demonstrating whether a given compound is neuroprotective in humans (Kieburtz and Olanow 2015; Olanow et al. 2017), there is evidence of microglia involvement in both promoting neuroinflammation, neuronal death and the release of factors that prevent neuronal death. In fact, after an insult and microglia cell recruitment and activation, there are two possible phenotypes: M1 or proinflammatory and M2 or neuroprotective (see (Franco and Fernández-Suárez 2015) and references therein). Due to the expression of adenosine receptors in resting and reactive microglia, it is suggested that adenosine receptor ligands may be protective (Corriden and Insel 2012; Koizumi et al. 2013; Beamer et al. 2016; Pedata et al. 2016; Woods et al. 2016). However, data is missing on how ligands acting on adenosine receptors may produce M2-skewed cells and on how adenosine-receptor-containing heteromers may contribute to the inflammatory/neuroprotective balance.

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