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Adenosine-Dopamine interactions in the pathophysiology and treatment of CNS disorders

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Adenosine-dopamine interactions in the CNS have been studied for many years in view of their relevance for disorders of the central nervous system and their treatments. The discovery of adenosine and dopamine receptor containing receptor mosaics (higher-order receptor heteromers) in the striatum opened up a new understanding of these interactions. Initial findings indicated the existence of $A_{2A}R$-D_2R heterodimers and A_1R-D_1R heterodimers in the striatum that were followed by indications for the existence of striatal $A_{2A}R$-D_3R and $A_{2A}R$-D_4R heterodimers. Of particular interest was the demonstration that antagonistic allosteric A_{2A}-D_2 and A_1-D_1 receptor-receptor interactions take place in striatal $A_{2A}R$-D_2R and A_1R-D_1R heteromers. As a consequence, additional characterization of these heterodimers led to new aspects on the pathophysiology of Parkinson's disease, schizophrenia, drug addiction and L-DOPA-induced dyskinesias relevant for their treatments. In fact, $A_{2A}R$ antagonists were introduced in the symptomatic treatment of Parkinson's disease in view of the discovery of the antagonistic $A_{2A}R$-D_2R interaction in the dorsal striatum that leads to reduced D_2R recognition and

$G_{i/o}$ coupling in striato-pallidal GABAergic neurons. In recent years, indications have been obtained that $A_{2A}R$-D_2R and A_1R-D_1R heteromers do not exist as heterodimers, rather as receptor mosaics. In fact, A_{2A}-CB_1-D_2 receptor mosaics and A_{2A}-D_2-mGlu$_5$ receptor mosaics have been discovered using a sequential BRET-FRET technique and by using the BRET technique in combination with bimolecular fluorescence complementation. Thus, other pathogenic mechanisms beside the well-known alterations in the release and/or decoding of dopamine in the basal ganglia and limbic system are involved in Parkinson's disease, schizophrenia and drug addiction. In fact, alterations in the stoichiometry and/or topology of A_{2A}-CB_1-D_2 and A_{2A}-D_2-mGlu5 receptor mosaics may play a role. Thus, the integrative receptor-receptor interactions in these receptor mosaics give novel aspects on the pathophysiology and treatment strategies, based on combined treatments, for Parkinson's disease, schizophrenia and drug addiction.

1. Introduction

1.1 Role of dopamine and adenosine as volume transmission signals in the CNS

The first observation that led to the discovery of a mode of communication different from synaptic transmission was the appearance of extra-neuronal dopamine (DA) fluorescence around midbrain DA nerve cells following amphetamine, a catecholamine (CA) releasing drug, treatment in reserpine-nialamide-L-DOPA treated rats [1]. Locally applied CA into the striatum was also found to migrate to the neuropil [2, 3]. Such observations taken together with the existence of global monoamine terminal networks in the central nervous system (CNS, see above) and with several other observations in the literature, in particular the demonstration of large numbers of nonjunctional monoamine varicosities by Descarries [4], led Agnati and Fuxe to propose the existence of volume transmission (VT) as complementary transmission to the well-known wiring transmission (WT), or synaptic transmission, in the CNS [5]. VT in the CNS was introduced as an

extracellular fluid (ECF) and cerebrospinal fluid (CSF) form of transmission [5-11]. In this way, VT signals are chemical signals like neurotransmitters, trophic factors, ions, peptides, etc. that migrate by diffusion and convection from the source cells to the target cells in the ECF and CSF in VT channels of the extracellular space and the ventricles as a consequence of energy gradients that create migration.

Evidence exists and suggests that the main mode of communication of all the central DA neurons is short distance VT in the μm range in which DA primarily reaches extrasynaptic receptors; see [10-14]. Thus, DA via VT in the local circuits of the medium-sized striatal neurons reaches and activates extrasynaptic D_1R- and D_2R-containing Receptor Mosaics (RM) (see below) on the dendritic spines of such neurons [15]. In many regions, DA exists as a diffusing VT signal in the ECF in concentrations that vary with the pattern of DA release and has a major impact on the modulation of the polymorphic wiring networks in the CNS [16, 17]. In this way it becomes possible to understand how the DA terminal networks have such a powerful role in CNS functions involving mood, reward, fear, cognition, attention, arousal, motor function, neuroendocrine and autonomic function and indeed play a central role in neuropsychopharmacology.

Adenosine is an endogenous nucleoside and functions as a neuromodulator in many areas of the central nervous system (CNS), see [18-20]. It is a normal cellular constituent and its intracellular concentration is dependent on the breakdown and synthesis of ATP, which is metabolized to AMP. Adenosine is then formed from AMP, through the action of a 5'-nucleotidase, and the intracellular and extracellular concentrations are kept in equilibrium by means of equilibrative transporters. The two main metabolic pathways of adenosine removal depend on the enzymes adenosine deaminase (ADA) (mostly intracellular) and adenosine kinase. Extracellular adenosine concentration depends on intracellular adenosine and also on extracellular ATP (released as a neurotransmitter or as an intracellular signal, from neurons or glial cells) that is rapidly hydrolyzed to adenosine and other metabolites. However, the main source of extracellular adenosine is likely intracellular adenosine released from active cells in response to an increased metabolic demand [19, 20]. Based on its presence in and its release into the

ECF, together with the demonstration of extrasynaptic adenosine receptors, adenosine likely represents an important VT signal [18].

The two major adenosine receptors in the CNS are the adenosine A_1 and A_{2A} receptors. A_1R are widely distributed in the brain are mainly expressed in the hippocampus, cerebellum and neocortical areas. On the other hand, $A_{2A}R$ have a much more restricted brain distribution, in which the striatum contains the highest density in the brain and where they are specially concentrated in the GABAergic striato-pallidal neurons [11, 17, 18] together with the D_2R. The D_1R are instead predominantly found in the direct pathway, the striato-entopeduncular and striato-nigral GABAergic neurons, see [14, 15]).

It has been many years since the hypothesis was first introduced that adenosine-dopamine interactions in the brain primarily take place via receptor-receptor interactions in $A_{2A}R$-D_2R and A_1R-D_1R heteromers located perisynaptically at glutamate synapses on the striato-pallidal and striato-entopeduncular/nigral GABAergic neurons, respectively, see [21-25]. However, although it may be a rather infrequent event, it is possible that adenosine, via VT into DA synapses may directly modulate synaptic DA transmission via synaptic $A_{2A}R$-D_2 and A_1R-D_1R heteromers.

1.2. The concept of receptor mosaic and its implication: stoichiometry versus topology

Already in 1980 it was proposed by Fuxe and Agnati that assemblages of receptors could operate as integrative input units of membrane associated molecular circuits [26, 27]. This postulation was supported by indirect evidence on the existence of receptor-receptor interactions obtained through an analysis of the effects of neuropeptides on the binding characteristics of monoamine receptors in membrane preparations from discrete brain regions [28-30]. As a logical consequence for the indications of direct physical interactions between neuropeptide and monoamine receptors, the well-known terms heteromerization versus homomerization were introduced by the Agnati

and Fuxe teams as well as by other groups to describe this kind of interaction between different types of GPCRs, see [21, 23, 31-37].

Allosteric events were postulated to be the molecular mechanism for intra-membrane interactions in multimeric assemblages of receptors. Thus, the term receptor mosaic (RM) [8, 35, 38, 39] was introduced for assemblies of multiple receptors of the same or different kinds ($n \geq 3$) in the plasma membrane as a more meaningful term than higher-order heteromers, which nevertheless is highly relevant from a stoichiometric point of view. The term receptor mosaic indicates the "integrated output" of such an input unit, since it also stresses the concept that topology (spatial localization in the plane of the membrane) and integrative function of the receptor assemblage are deeply interconnected. In other words, the emergent properties of the receptor assemblage, or its integrated output, depend on the location and the order of activation of the participating receptors as well as on the type of allosteric interactions (entropic and/or enthalpic) within such an integrative receptor mosaic [16, 17]. Already in the 1982 [39] it was proposed that formation of a RM and/or its allosteric change could have a role in the molecular basis for the engram by leading to a transient and/or permanent change of the synaptic efficacy (i.e., the synaptic weight). The term RM maintains that allostery is any ligand-induced change in protein conformation and/or dynamics but also includes the functional characteristic of allostery, namely that one ligand alters the functional response of another ligand through a conformational change in the binding site of the second ligand, see [16, 17, 40-42]. It is then possible that a GPCR can have very different biochemical properties leading to a different pharmacology through interactions with another GPCR [15-17, 21, 35, 43-45].

The field of receptor-receptor interactions has opened up new targets for drug development [15, 17, 21, 44] and several strategies can be exploited to develop new drugs based on receptor-receptor interactions in receptor heteromers, see [46]. Higher-order receptor heteromers (receptor mosaics) also offer several additional targets for drug development. Novel drugs may be developed to modify the composition of RMs, their topography, the order of activation as well as allosteric regulators modulating the functional state of the individual receptors in the RM. Drugs may affect, e.g. (I) the synthesis and

release of receptor oligomeric building blocks from the endoplasmic reticulum, (II) the insertion of such building blocks into the plasma membrane, (III) the internalization of RMs, (IV) the adapter and scaffolding proteins organizing the RMs and (V) ligand induced receptor assembly.

The potential importance of developing allosteric modulators has also been suggested since they may, *inter alia*, substantially affect the allosteric mechanisms within the RM leading to changes in its integrative function [47-49] in addition to affects on RM assemblage, G protein, β-arrestin coupling and receptor recognition [41, 50]. An example is the discovery of an allosteric D_2R antagonist homocysteine (Hcy), which reduces D_2R agonist binding and D_2R function by its apparent binding to the third intracellular loop (IC3) of the D_2R [47, 48]. This discovery opens up the development of new antipsychotic drugs based on the development of allosteric Hcy agonist analogues and antiparkinsonian drugs based on the development of allosteric Hcy antagonist analogues.

In the present review we will focus on the $A_{2A}R$-D_2R heteromers and $A_{2A}R$-D_2R-containing RM as well as the A_1R-D_1R heteromers and the A_1R-D_1R-containing RM as targets for the development of novel treatments of CNS diseases.

2. A_{2A}-D_2-like receptor heteromers and $A_{2A}R$-D_2R containing receptor mosaics

2.1. $A_{2A}R$-D_2R heteromers

2.1.1. Biochemical and functional findings

Initially, to tackle the study of receptor heteromers traditional biochemical protocols were used and some of those methods include microscopy-based procedures, such as co-immunolocalization, and immobilized protein-protein interaction assays, such as co-immunoprecipitation. The invasive nature of these technical approaches to study protein-protein interactions still have the disadvantage of altering the natural state of the cell and therefore, may not represent its real structure. This is even more critical with membrane proteins, like GPCRs, due to their highly hydrophobic framework and the need for detergents to extract proteins

from membranes. Either working with aqueous solutions or with detergents, the composition and organization of the membrane is altered, which can be a source of false results. Nevertheless, besides the inherent technical problems associated with these methods they have been shown to give accurate results, and they are still very useful to confirm close interactions between GPCRs. During the last decade, a new set of technologies based on the use of fluorescent-fused proteins have been developed to overcome the invasive nature of the immobilized protein-protein interaction assays. These new approaches, centered on the use of various adaptations of resonance energy transfer (RET) techniques (e.g. fluorescence-RET and bioluminescence-RET), have favored the possibility of carrying out "*in vivo*" real time experiments. Thus, the use of BRET and FRET techniques has emerged as powerful tools to study GPCR oligomerization. $A_{2A}R$-D_2R heteromers [51-54] may exist in the dorsal and ventral striato-pallidal GABA pathway in which activation of $A_{2A}R$ reduce D_2R recognition, coupling, and signaling together with $A_{2A}R$ and D_2R homodimers (Fig. 1) [21, 23, 25, 51-55]. A large number of studies using the above mentioned approaches (e.g. coimmunoprecipitation, FRET and BRET), as well as biochemical binding and signaling, behavioral pharmacology, and microdialysis techniques, have corroborated the existence of $A_{2A}R$-D_2R heteromers [24, 51-53, 55-61]. Interestingly, it has been also suggested that $A_{2A}R$-D_2R heteromers may be predominantly located on the dendritic spines in the perisynaptic zones of DA terminals and glutamate synapses but also on glutamate terminals in the local circuits of the striato-pallidal GABAergic neurons [24, 55, 57, 60, 62, 63].

In the striato-pallidal GABAergic neuron, this heteromer may exist in equilibrium on the neuronal surface membrane together with $A_{2A}R$ and D_2R homomers. It seems possible that higher-order A_{2A}-D_2 RM of unknown stoichiometry and topology may also exist and contain e.g., D_2R homodimers and $A_{2A}R$ homodimers. In such a case, antagonistic $A_{2A}R$-D_2R interactions can still take place by assuming that the $A_{2A}R$ can enhance the negative cooperativity in such participating D_2R homodimers. Such events may also take place in the $A_{2A}R$-D_3R and $A_{2A}R$-D_4R heteromers (see below). A major component of the interface in the $A_{2A}R$-D_2R heteromer is the electrostatic interaction between the positively charged arginine-rich epitope in the N-

terminal domain of the IC3 of the D_2R and negatively charged epitopes in the C-terminal tail of the $A_{2A}R$, especially the epitope (aa 370-378) containing a phosphorylatable serine (Fig. 2) [52, 64]. Thus, phosphorylation events may modulate the strength of the receptor-receptor interactions within the $A_{2A}R$-D_2R heteromer and RM. These results were also supported by studies using D_1R-D_2R chimeras [65]. In addition, microdialysis experiments indicate that in awake, freely moving rats the $A_{2A}R$ agonist CGS 21680 when intrastriatally co-perfused with the D_2R-D_3R agonist quinpirole (10 μM), was able to fully counteract the quinpirole-induced reduction of extracellular GABA levels in the globus pallidus, see [61], where CGS 21680 itself did not produce any significant effects on its own.

The antagonistic $A_{2A}R$-D_2R interaction in the brain has been demonstrated in many publications including at the level of D_2R agonist recognition and animal behavior (see [23, 24, 55, 60, 63, 66-69]. These results make it likely that the $A_{2A}R$-D_2R heteromer strongly modulates the excitability in the striato-pallidal GABAergic neurons via its ability to counteract D_2R signaling to multiple effectors. The $A_{2A}R$-induced counteraction of the D_2R-induced inhibition of the Ca^{2+} influx over the L-type voltage dependent Ca^{2+} channels (Cav 3.1 channels) via the activation of phospholipase C and protein phosphatase-2B (calcineurin) [70] may be of special importance [15, 60]. The G protein involved may be $G_{i/o}$ and/or $G_{q/11}$ with the release of the βγ subunits. The counteraction of this cascade by $A_{2A}R$ leads to increased phosphorylation of this calcium channel and its increased opening favoring an upstate of the striato-pallidal GABAergic neuron, see [71]. $A_{2A}R$ activation also has been shown to counteract D_2R-induced intracellular calcium responses in cotransfected mouse fibroblast and human neuroblastoma cell lines [72, 73]. Moreover, the D_2R agonist-induced reduction of firing rates in the DA denervated striatum was enhanced by $A_{2A}R$ antagonists and attenuated by $A_{2A}R$ agonists [69]. The D_2R in the $A_{2A}R$-D_2R heteromers may also be coupled to $G_{i/o}$ since in cultured striatal neurons $A_{2A}R$ agonists can counteract the D_2R induced inhibition of forskolin stimulated cyclic adenosine monophosphate (cAMP) production without being active when given alone [15, 24, 53, 55, 60]. It is also likely that $A_{2A}R$ activation through inhibition of the $G_{i/o}$ coupling of the D_2R with the $G_{i/o}$ trimer remaining

at the D_2R will also interfere with the protein kinase B (Akt)-Glycogen synthase kinase 3 (GSK-3) signaling cascade induced by D_2R stimulation through its ß-arrestin 2 signaling [74]. Thus, ß -arrestin 2 can no longer become effectively linked to the D_2R, since the $G_{i/o}$ trimer is not sufficiently split and removed from the D_2R in the presence of $A_{2A}R$ activation (Fig.3). It should also be considered that the $A_{2A}R$-D_2R receptor-receptor interaction also leads to a conformational state of the D_2R less able to bind and activate the ß-arrestin 2 as is the case for $G_{i/o}$.

There also exists a reciprocal interaction between $A_{2A}R$-D_2R in as much as D_2R can inhibit the $A_{2A}R$-induced increase in cAMP accumulation via $G_{i/o}$ at the level of the adenylate cyclase (AC), an interaction which also can take place between A_{2A} and D_2 homomers, see [24, 55, 75]. Removal of the D_2R brake on the $A_{2A}R$ signaling would therefore, also lead to increased striatal excitability since it will result in increased protein kinase A (PKA) activity causing increased phosphorylation of □-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartic acid receptors (NMDA) and of dopamine and cAMP regulated neuronal phosphoprotein (DARPP-32) at Thr34 position with inhibition of protein phosphatase-1 further enhancing the phosphorylation and the activity of these ion channel receptors, see [60, 76-80]. Such events would also favor the up-state of the striato-pallidal GABAergic neurons. Based on the above, it seems likely that the $A_{2A}R$-D_2R heteromer, via its antagonistic receptor-receptor interaction, plays a crucial role in moving the striato-pallidal GABAergic neurons towards the up-state by the antagonism of D_2R signaling, see [15].

2.1.2. Relevance to Parkinson's disease and its treatment

Based on the antagonistic $A_{2A}R$-D_2R interaction described above, the development of $A_{2A}R$ antagonists to target these $A_{2A}R$-D_2R heteromers in the dorsal striato-pallidal GABA pathway was initiated [24, 59, 63, 81, 82]. It was also demonstrated that the antagonistic $A_{2A}R$-D_2R interaction remained and was even increased in the striatal membranes from rat models of Parkinson's disease (PD) [68, 81]. In a number of PD models, $A_{2A}R$ antagonists, like SCH 58261, have been found to dose-dependently increase locomotor activity in

combination with sub-threshold doses of L-DOPA and D_2R agonists in reserpinized mice. In fact, $A_{2A}R$ antagonists consistently reverse Parkinsonian deficits in non-human primates and rodents; see [60, 61, 68, 81]. It should be noted that in contrast to caffeine [83], subchronic $A_{2A}R$ antagonist treatment in models of Parkinson's disease does not result in tolerance development [84, 85], which represents a prerequisite for clinical development. Therefore, similar antiparkinsonian effects may be seen with acute and chronic treatment of $A_{2A}R$ antagonists.

Such results could elegantly be explained by the hypothesis that $A_{2A}R$ antagonists target the $A_{2A}R$-D_2R heteromer and increase D_2R signaling in these heteromers. These $A_{2A}R$-D_2R heteromers have been found to be constitutive [51] and thus exist in the absence of agonist activation of the receptors. Therefore, it is not likely that $A_{2A}R$ antagonist treatment will disrupt the $A_{2A}R$-D_2R heteromers but conformational changes may develop in the heteromer thereby altering its integrative activity. So far it has not been possible to see differences in the affinity and blocking activity of $A_{2A}R$ antagonists at $A_{2A}R$ belonging to $A_{2A}R$-D_2R heteromers or to $A_{2A}R$ homomers (unpublished data). The $A_{2A}R$ antagonist istradefylline (KW-6002) has been used in clinical trials and found to have interesting anti-Parkinsonian and anti-dyskinetic properties [60, 68, 78, 86]. Initial clinical studies using KW-6002 showed symptomatic but rather modest improvement in relatively advanced PD patients with dyskinetic complications [87, 88]. However, bradykinesia, muscle rigidity and resting tremor in PD patients may be improved after $A_{2A}R$ blockade.

Based on our hypothesis, treatment with an $A_{2A}R$ antagonist alone should in fact produce only modest effects in PD except during early stages of PD when DA is still being released from remaining DA terminals. Instead the $A_{2A}R$ antagonist should ideally be given in Parkinsonian patients in combination with close to threshold doses of L-DOPA and/or D_2R agonist. Clinical results observed so far would be in agreement with this view. Thus, the $A_{2A}R$ antagonist would be targeting the $A_{2A}R$-D_2R heteromer to enhance D_2R signaling.

$A_{2A}R$ antagonists, by acting on the $A_{2A}R$-D_2R heteromer will enhance D_2R signaling at the soma-dendritic level and lead to a reduction in the activity

of the striato-pallidal GABA/enkephalin pathway. In this way, motor inhibition will be reduced and the motor drive will be partly restored. A combined treatment with L-DOPA will still be optimal since it will restore D_1R activity in the direct pathway which helps motor initiation. The direct pathway becomes integrated with the indirect pathway in the globus pallidus interna and zona reticulata of the substantia nigra to optimally inhibit their GABA projections to the motor thalamus. In this way, the GABA inhibition of the excitatory glutamate thalamo-cortical pathway to the motor cortices will be removed and movements restored

To understand the role of $A_{2A}R$ antagonists in PD it becomes important to note that there also exists a reciprocal interaction by which D_2R inhibits the $A_{2A}R$ signaling at the level of AC as discussed above, either in the $A_{2A}R$-D_2R heteromer or between $A_{2A}R$ homomers and D_2R homomers (see above). In this way, we can understand why $A_{2A}R$ antagonists alone can counteract haloperidol-induced catalepsy: namely, by blocking the excessive $A_{2A}R$ signaling that results from the removal of D_2R-induced inhibition of AC by haloperidol. This mechanism can also help to explain some therapeutic effects of $A_{2A}R$ antagonists in advanced PD, see [60].

When discussing the role of $A_{2A}R$ and D_2R we should also consider the balance between $A_{2A}R$ homomers versus D_2R homomers and $A_{2A}R$-D_2R heteromers (Fig.1). L-DOPA treatment may lead to a disruption of the balance between them that ultimately leads to increases in $A_{2A}R$ signaling versus D_2R signaling. This would help to explain the reduction of the therapeutic effects of L-DOPA and the appearance of L-DOPA-induced dyskinesias after prolonged treatment. Our hypothesis is that a L-DOPA-induced co-internalization of $A_{2A}R$-D_2R heteromers and D_2R homomers leads to a compensatory up-regulation of $A_{2A}R$ and an increase in $A_{2A}R$ homomers. To support this hypothesis, increases of $A_{2A}R$ mRNA and $A_{2A}R$ immunoreactivity (IR) have also been demonstrated in both animal models of L-DOPA-induced dyskinesias and in dyskinetic PD patients, see [89]. The resulting up-regulation of $A_{2A}R$ leads to increases in PKA and phosphorylated DARPP-32 at Thr34 and increased inhibition of PP-1. This will result in an increase in protein phosphorylation including ion channels, which may help stabilize pathological RM formed under the influence of the transcriptional panorama

caused by the L-DOPA-induced excessive D_2R activation [52, 64]. This may lead to a repeated appearance of the abnormal pattern of firing in the striato-pallidal GABA pathway and contribute to dyskinesias. This hypothesis can help explain the reported anti-dyskinetic effect of $A_{2A}R$ antagonists, which cannot be explained by the enhancement of D_2R signaling in the heteromer since this would worsen dyskinesias. We have also postulated, based on this hypothesis, that $A_{2A}R$ antagonists will help to counteract the disappearance of the therapeutic effects of L-DOPA after long-term treatment, see [60, 68].

Taken together, the demonstrated anti-Parkinsonian effect of $A_{2A}R$ antagonists in clinical studies has given 'proof of concept' that intramembrane receptor-receptor interactions in receptor heteromers can lead to the development of novel therapies. The $A_{2A}R$ in the $A_{2A}R$-D_2R heteromer in the dorsal striato-pallidal GABAergic neurons is the major target for $A_{2A}R$ antagonists when used in treatment of PD.

$A_{2A}R$ antagonists may have not only anti-Parkinsonian properties but also neuroprotective and anti-dyskinetic properties. Recently it has been found that inactivation of forebrain $A_{2A}R$ fully counteracts nigral DA nerve cell degeneration in mouse model of Parkinson's disease associated with a block also of nigral gliosis [90]. In line with these results, an attenuation of the DA nerve cell degeneration and of the gliosis was observed after treatment with the $A_{2A}R$ antagonist SCH58261 [90]. However, the molecular mechanism for its neuroprotective effect on DA cells, as found by Schwartzschild *et al*., see [60, 68], is still unclear, although an increase in retrograde trophic signaling from the striatum has been proposed by Fuxe *et al*. [60].

When discussing the potential neuroprotective effects of $A_{2A}R$ antagonists in Parkinson's disease the epidemiological evidence should also be considered. There exists an inverse association between intake of coffee and caffeine in Japanese-American men and the risk of development of Parkinson's disease [91]. These results were strengthened by a study in a larger and more ethnically diverse cohort of prospectively followed men showing again an inverse relationship between consumption of caffeinated but not decaffeinated coffee and the risk of development of Parkinson's disease [92]. This link between consumption of caffeine in coffee and lower incidence of Parkinson's disease strongly favor the view that $A_{2A}R$ antagonists

may have neuroprotective actions in Parkinson's disease. Thus, it is generally believed that caffeine exerts its psychoactive actions through blockade of A_1R and $A_{2A}R$ in the CNS [20, 83]. Overall, $A_{2A}R$ antagonists therefore clearly offer a realistic opportunity to improve PD treatment.

2.1.3. Relevance to schizophrenia and its treatment

The indicated existence of $A_{2A}R$-D_2R heteromers with antagonistic $A_{2A}R$-D_2R interactions in the ventral striato-pallidal GABA pathway has also introduced the strategy of using $A_{2A}R$ agonists for the treatment of schizophrenia. In this way, based on these antagonistic interactions, $A_{2A}R$ agonist treatment would lead to a reduction in the high affinity state of the D_2R and a reduction of its $G_{i/o}$ coupling [24, 25, 55, 93]. It is also known that the common feature of antischizophrenic drugs of the D_2R antagonist type is the antagonism of the D_2R-ß-arrestin 2 interaction and thus of Akt-GSK-3 signaling [94, 95]. As discussed, the observations suggest that $A_{2A}R$ agonists may also interfere with the D_2R-ß-arrestin 2 interaction (Fig. 3). Also it has been shown that the $A_{2A}R$ agonist CGS-21680 reduces protein phosphatase 2A activity in the murine heart [96] which increases Akt activation and thereby blocks the GSK-3 signaling (Fig. 3).

The classical treatment in schizophrenia is the use of DA receptor antagonists such as haloperidol (a typical antipsychotic) to block the D_2R on both nigro-striatal and mesolimbic DA neurons [5, 97-101]. This leads to motor and mental effects through the blockade of excessive D_2R-mediated DA transmission in the nigro-striatal and meso-limbic DA systems, respectively; see [25, 55, 93]. Atypical antipsychotic drugs, like benzamides, that display reduced motor side effects may at least in the case of remoxipride block only subpopulations of D_2R [102]. The reason for this may be that remoxipride-sensitive D_2R receptors are part of a particular receptor heteromer [15, 17, 24, 45] that provide a unique pharmacology to these D_2R and make them remoxipride-sensitive. According to the Seeman hypothesis of schizophrenia [101], the major error in psychosis is an increased proportion of D_2R in the high affinity state that results in the development of D_2R supersensitivity. This makes our proposal on the antipsychotic potential of $A_{2A}R$ agonists of special interest since they can, via the $A_{2A}R$-D_2R heteromer,

preferentially reduce the high affinity agonist state of the D_2R in both the dorsal and ventral striatum.

However, the current combined glutamate/DA hypothesis of schizophrenia states that the meso-limbic DA neurons are hyperactive due to reduced NMDA receptor function of cortical glutamate systems. This resultsyh7 in the reduced activity of the descending cortical glutamate projections to the ventral tegmental area (VTA) giving rise to the meso-cortical and meso-limbic DA systems. In this way, the VTA GABA interneurons that inhibit the firing of the meso-limbic DA neurons have reduced activity and as a consequence the meso-limbic DA neurons become hyperactive and inhibition of the ventral striato-pallidal GABA pathway becomes increased [15, 45, 103]. The ventral striato-pallidal GABAergic neurons integrate and transfer the emotional information from the limbic system via the medial dorsal (MD) nucleus to the prefrontal cortex (PFC) [15]. The increased activity of these D_2R reduces the glutamate drive to the prefrontal cortex and further worsens the hypoglutamatergic state in schizophrenia. As it was postulated early on [104], DA receptors in meso-limbic DA transmission are a major target for antipsychotic drugs that improve the emotional state of the schizophrenic patients.

We have proposed that the $A_{2A}R$ agonists may be used as anti-schizophrenic drugs through their antagonism of D_2R signaling in the $A_{2A}R$-D_2R heteromer in the soma-dendritic region of the ventral striato-pallidal GABA pathway. This would help to reestablish the glutamate drive from the mediodorsal thalamic nucleus to the prefrontal cortex [15]. In fact, $A_{2A}R$ agonists strongly reduce the D_2R agonist binding affinity in the nucleus accumbens shell and core [105] diminishing both D_2R recognition and G protein coupling. The $A_{2A}R$-D_2R heteromer also exists on the glutamate terminals of the local circuits in the striato-pallidal GABAergic neurons and counteracts D_2R-induced inhibition of glutamate release upon $A_{2A}R$ activation. This increase in glutamate release after $A_{2A}R$ agonist treatment will also contribute to enhance the excitability of the ventral striato-pallidal GABA pathway and thereby add to the antipsychotic activity of $A_{2A}R$ agonists. It is important to note that D_2 autoreceptors are not directly modulated by $A_{2A}R$ agonists since $A_{2A}R$ does not exist in the DA terminal networks. In this way,

$A_{2A}R$ agonist treatment will not affect the function of the D_2 autoreceptor to further contribute in lowering DA release.

The anti-schizophrenic potential of $A_{2A}R$ agonists is further underlined through behavioral analysis in the amphetamine and phencyclidine rat models of schizophrenia [106] and in the *Cebus apella* monkey model of schizophrenia [107] in which it demonstrated an atypical antipsychotic profile. Therefore, $A_{2A}R$ agonist treatment represents a new strategy for the treatment of schizophrenia, especially in combination with very low doses of atypical and/or typical D_2R antagonists, to further reduce the development of extrapyramidal side effects, see [15, 45].

Disturbances in the $A_{2A}R$ molecular mechanisms especially in the $A_{2A}R$-D_2R receptor heteromer in schizophrenia should be considered since deficits in the operation of the antagonistic $A_{2A}R$-D_2R interaction may increase the vulnerability to develop the disease [15]. However, it should be noted that treatment with $A_{2A}R$ antagonists in PD has so far not led to an increase in psychotic episodes in PD patients, see [68], possibly due to a low adenosine and in particular DA tone in the nucleus accumbens and other parts of the ventral striatum in the PD patients on $A_{2A}R$ antagonist monotherapy.

Dual-probe microdialysis evidence from the Tanganelli group (Ferraro *et al*., unpublished data (see [15]), in awake, freely moving rats supports the $A_{2A}R$ agonist treatment strategy in schizophrenia. It was demonstrated that the D_2R-D_3R agonist quinpirole (10 μM) when superfused in nucleus accumbens was able to reduce accumbal extracellular GABA levels while increased extracellular GABA levels in the MD nucleus, medial division. The actions of the D_2R agonist were counteracted by the $A_{2A}R$ agonist CGS 21680 (1 μM) when co-superfused with quinpirole into the nucleus accumbens. These results provide for the first time the functional evidence for a connection between the ventral striato-pallidal GABA pathway with the MD medial division which is known to innervate the prefrontal cortex via widespread glutamate projections via the ventral pallidal-MD GABA pathway, see [108].

Furthermore, in agreement, Ferraro *et al*. (unpublished data) found using dual probe microdialysis that extracellular levels of glutamate in the prefrontal cortex were reduced after accumbal superfusion with quinpirole (10 μM), see

[15]. The $A_{2A}R$ agonist CGS 21680 (1 μM) when co-superfused with the D_2R agonist in the nucleus accumbens not only counteracted the D_2R agonist induced reduction of prefrontal glutamate levels, but even resulted in a small rise of extracellular levels of glutamate in the prefrontal region. These results give functional neurochemical evidence that A_{2A}-D_2 receptor-receptor interactions in the nucleus accumbens have substantial relevance for the activity of the MD glutamate projections to the prefrontal cortex and schizophrenia in view of hypoglutamatergia and hypofunction in the dorsolateral prefrontal cortex of schizophrenic patients, see [109].

Within the nucleus accumbens, quinpirole, upon local superfusion, reduced the extracellular levels of accumbal glutamate probably via inhibition of glutamate release from cortico-striatal glutamate terminals known to possess D_2R. These glutamate terminals may also possess $A_{2A}R$ that interact with D_2R and are known to release glutamate [110]. In agreement, the $A_{2A}R$ agonist CGS 21680 superfused into the nucleus accumbens in the present experiments produced a substantial and prolonged release of glutamate (Ferraro *et al*., unpublished data); see [15]. Interestingly when CGS 21680, at a concentration by itself ineffective on cortical extracellular glutamate levels, was co-perfused in the nucleus accumbens with quinpirole, it significantly antagonized the reduction of extracellular glutamate levels induced by quinpirole in the prefrontal cortex (Ferraro *et al*., unpublished data), see [15].

Taken together, these results give evidence of the importance of antagonistic $A_{2A}R$-D_2R interactions in both the ventral striato-pallidal GABA pathway and in the cortico-striatal glutamate terminals in the control of the prefrontal glutamate projections via the ventral pallidum and MD, a loop with major disturbances in schizophrenia. Jones, Popken and colleagues [109] have demonstrated that there exists a subnucleus specific loss of nerve cells in the medial thalamus of schizophrenics. Through stereological counts, a 30% loss of nerve cells was demonstrated in the MD nucleus primarily confined to the parvocellular and densocellular subnuclei. It is of substantial interest that the parvocellular part projects to the dorsolateral parts of the prefrontal cortex and other regions known to be compromised in schizophrenia, see [109]. These results underline the relevance of the present strategy of targeting the $A_{2A}R$-D_2R heteromer in the nucleus accumbens by

$A_{2A}R$ agonist treatment alone or in combination with low doses of D_2R antagonists to help restore the glutamate drive from MD to the dorsolateral prefrontal cortex.

2.1.4. Relevance to cocaine addiction and its treatment

An increase in $A_{2A}R$ in the nucleus accumbens has been observed after extended cocaine self-administration, see [111]. Following a 10-day cocaine self-administration procedure, one that increased accumbal D_1R, D_2R and D_3R signaling through a cocaine-induced increase in extracellular DA levels via its well-known blockade of the DA transporter, gave rise to a compensatory up-regulation of $A_{2A}R$ that diminished during a cocaine withdrawal period. Behavioral pharmacological results have demonstrated that $A_{2A}R$ antagonists reinstate cocaine self-administration [112] and that $A_{2A}R$ agonists diminish the reinforcing effects of cocaine [113]. Furthermore, $A_{2A}R$ agonists counteract the development and expression of sensitization to the locomotor activation effects of cocaine [114]. In fact, antagonistic $A_{2A}R$-D_2R interactions have been demonstrated in the nucleus accumbens at both the binding and behavioral level and at the level of neuronal function [24, 55, 60, 93, 105].

A direct action of cocaine on the $A_{2A}R$ is not involved in producing this probable rise of accumbal $A_{2A}R$ signaling in the animals with cocaine still present in the brain [111]. The disappearance of the $A_{2A}R$ rise during the withdrawal period may help to explain the increased reinforcing efficacy of cocaine in animals after 7-days of cocaine withdrawal [115]. This could involve a compensatory mechanism to increase signaling via accumbal D_2R and D_3R by reducing the $A_{2A}R$ brake on D_2R and D_3R signaling [111]. The results indicate a putative role of antagonistic $A_{2A}R$-D_2R interactions at the membrane (in $A_{2A}R$-D_2R heteromers) and cytoplasmatic level in the nucleus accumbens in the prevention of development of cocaine addiction. These results open up the possibility that $A_{2A}R$ agonists can represent cocaine antagonists to be used in the prevention of cocaine addiction. This is not supported by the fact that the lack of $A_{2A}R$ signaling reduces the reinforcing

efficacy of cocaine [116] in $A_{2A}R$ knockout mice but may be explained by a reorganization of the D_2R containing RM in these transgenic mice.

The observed rise of $A_{2A}R$ in the nucleus accumbens after extended cocaine self-administration may depend on the existence of an atypical cAMP response element (CRE) in the core promoter of the $A_{2A}R$ gene [117]. CREB (CRE binding protein) diminishes cocaine reward in this region [118] and is enabled by increased activation of the extracellular signal-related kinase [119]. $A_{2A}R$ and D_2R are collocated in the ventral striato-pallidal GABAergic pathway [120-122] and cocaine-induced activation of the D_2R can produce an increase in CREB phosphorylation via several intracellular mechanisms, see [15, 60, 111].

It should also be considered that the $A_{2A}R$ up-regulation reflects not only increases in e.g. $A_{2A}R$-D_2R heteromers but also increased formation of $A_{2A}R$ homomers that further increase the excitability of the striato-pallidal GABAergic neurons [24, 55, 60, 89] by counteracting D_2R-mediated inhibition of these neurons. Another mechanism could be that persistent D_2-like receptor activation sensitizes $A_{2A}R$ signaling at the level of the AC via the release of βγ dimers from the activated G_i proteins [24, 55, 60, 89, 123-125]. It is of interest that the increased density of $A_{2A}R$ in the nucleus accumbens after cocaine self-administration demonstrated a reduced affinity of the $A_{2A}R$ antagonist binding sites. This may, *inter alia,* reflect the formation of novel A_{2A}/D_2-like RM with a different stoichiometry and/or topology that produces conformational changes in the receptors of this RM, which lead to altered allosteric interactions and changes in the affinity of the $A_{2A}R$.

2.2. $A_{2A}R$-D_3R and $A_{2A}R$-D_4R heteromers

2.2.1. Biochemical and functional findings

A study by Torvinen *et al*. [126] demonstrated a specific and high FRET efficiency in cells transiently co-transfected with $A_{2A}R$-YFP and D_3R-GFP[2] receptors providing evidence that $A_{2A}R$ and D_3R receptors form an $A_{2A}R$-D_3R heteromer (Fig. 2). Also similar to the D_2R, the D_3R contains the arginine-rich epitope in its IC3 that makes possible an electrostatic interaction with the carboxyl terminus of the $A_{2A}R$ [55]. Evidence was also obtained in

membranes prepared from stably transfected CHO cell lines, in which $A_{2A}R$ activation reduces D_3R agonist binding and D_3R signaling. This provided the evidence for an antagonistic $A_{2A}R$-D_3R interaction in $A_{2A}R$-D_3R heteromers similar to the antagonistic interaction observed in the $A_{2A}R$-D_2R heteromer. $A_{2A}R$-D_3R heteromers may therefore exist in the nucleus accumbens where the $A_{2A}R$ and D_3R are co-distributed provided that they are co-expressed in the same neuron. In view of the existence of D_3R dimers and tetramers in brain [127] the existence of higher order $A_{2A}R$-D_3R heteromers (A_{2A}-D_3 RM) should be considered.

The existence of $A_{2A}R$-D_4R heteromers has also been postulated based on the existence of the arginine-rich epitope in the IC3 loop of the D_4R, which can interact with the negatively charged epitopes in the $A_{2A}R$ carboxyl terminus (Fig. 2) [55]. Recently, it has also been possible to demonstrate the existence of $A_{2A}R$-D_4R heteromers through BRET experiments in transiently co-transfected $A_{2A}R$-D_4R in cell lines (Borroto-Escuela *et al*. unpublished data). The $A_{2A}R$ and D_4R may be codistributed especially in the island (striosome, patch) striato-nigral GABAergic system at the soma-dendritic level [128], where it is postulated that $A_{2A}R$-D_4R heteromers may exist. The striatal island system is involved in cognitive, reward and motivational functions, see [129, 130], which may be modulated by the postulated $A_{2A}R$-D_4R heteromers.

2.2.2. Relevance to CNS diseases and their treatments

The D_3R is being considered a target for novel anti-schizophrenic drugs that display a D_3R antagonist profile [131, 132]. Therefore A_{2A}-D_3 RM offer possibilities for novel treatment strategies of this disease that might include the combined use of novel D_3R antagonists and $A_{2A}R$ agonists in order to reduce D_3R signaling.

Indirect indications have been obtained for a rise in D_3R density in the nucleus accumbens in cocaine self-administering animals after experiencing cocaine withdrawal [111] which are in line with previous results that demonstrated increases in D_3R binding after cocaine withdrawal that was also associated with an increase in cocaine-seeking behavior [133, 134]. D_3R antagonists counteract cocaine seeking and cocaine enhanced reward and

may be used in treatment of cocaine addiction [131, 135, 136]. Furthermore, an up-regulation of D_3R mRNA levels was found in reward networks of human cocaine fatalities [137]. Therefore, the reduction not only of the antagonistic $A_{2A}R$-D_2R interaction but also of the antagonistic $A_{2A}R$-D_3R interaction in animals after 7-days of cocaine withdrawal [111] may contribute to the increased motivation to self-administer cocaine [115]. The $A_{2A}R$ may also have a role in cocaine addiction through its potential modulation of D_4R in the island striato-nigral DA system in view of the demonstration of $A_{2A}R$-D_4R heteromers in cotransfected cell lines (Borroto-Escuela *et al*., unpublished data).

2.3. $A_{2A}R$-D_2R containing receptor mosaics

2.3.1. A_{2A}-D_2RmGlu_5 receptor mosaic

2.3.1.1. Biochemical and functional findings

The existence of functional $A_{2A}R$-D_2R-$mGlu_5R$ oligomers in the GABAergic striato-pallidal neuron has often been discussed based on the high and selective co-expression of $mGlu_5R$, D_2R and $A_{2A}R$ in these particular cells, on the demonstration of $A_{2A}R$-D_2R heteromers (see above) and $A_{2A}R$-$mGlu_5R$ [138] heteromers and on the existence of strong multiple interactions between the three receptors [15]. The existence of neurotransmitter receptor heteromers in general and $A_{2A}R$-D_2R heteromers in particular is now broadly accepted and reinforced by the fact that the functional meaning of heteromerization is being revealed. Thus, the heteromerization of neurotransmitter receptors and their existence as RM have been demonstrated in neuronal cells as functional entities that possess different biochemical characteristics with respect to the individual components of the RM. Therefore, the heteromer might be considered as a molecular switch that fine-tunes the information flow between neurons, thus the signaling mediated by a single stimulated receptor within the heteromer might be, from a qualitative and/or quantitative point of view, different to that expected when all the receptors are simultaneously stimulated. Interestingly, the existence of RM or higher-order receptor heteromers has been recently demonstrated [139, 140].

Taking advantage of the recent fluorescence-based approaches to study protein-protein interactions, we have recently demonstrated the existence of higher-order $A_{2A}R$-D_2R-$mGlu_5R$ oligomers or RM (Fig. 4). Initially, by using bimolecular fluorescence complementation (BiFC), we visualized for the first time the occurrence of $mGlu_5R$-D_2R heterodimers in living cells [139]. Furthermore, the combination of BiFC and BRET techniques allowed us to detect the existence of receptor oligomers containing more than two protomers, namely $A_{2A}R$-D_2R-$mGlu_5R$ higher-order oligomers or RM (Fig. 4) [139]. Thus, this new experimental approach has allowed the study of the quaternary structure of A_{2A}-D_2-$mGlu_5$ RMs.

Interestingly, by using triple-labeling post-embedding immunogold and detection at the electron microscopic level, the precise simultaneous distribution of $A_{2A}R$, D_2R and $mGlu_5R$ in striatal neurons has been performed. It is noticeable that these three receptors co-distributed in post-synaptic structures along the extra-synaptic and peri-synaptic plasma membrane of spines that establish asymmetrical, putative glutamatergic, synapses with axon terminals [139]. Overall, this is the first direct anatomic evidence for $mGlu_5R$, D_2R and $A_{2A}R$ co-distribution in the same neuronal compartment and supports the notion of that these receptors form a RM in GABAergic striatopallidal neurons.

2.3.1.2. Relevance to CNS diseases and treatments

The A_{2A}-D_2-mGlu5 RM may mainly be in operation to produce activation of the cortico-striatal glutamate synapse and the striato-pallidal GABAergic neurons when motor inhibition of certain movements is required [15]. The increased firing in the glutamate terminals will release glutamate and co-stored ATP that will result in an increased formation of adenosine and lead to the increased activation of both pre- and postjunctional $A_{2A}R$ and $mGlu_5R$ receptors that synergize to counteract the D_2R signaling in the glutamate terminals and in the striato-pallidal GABAergic neurons. In this way, the firing of the striato-pallidal GABAergic neurons can develop without being restrained by the D_2R signaling that is aimed to silence the striato-pallidal GABAergic neurons. Once the firing in the cortico-striatal glutamate pathways

slows down the balance between glutamate and DA signaling, the RM will reach another set-point dependent on the movements to be initiated.

Parkinson's disease. The development of $mGlu_5R$ antagonists are yet another strategy for treatment of PD based on their ability to enhance D_2R recognition and signaling in these RM in the dorsal striato-pallidal GABAergic pathway by the removal of the antagonistic $mGlu_5R$-D_2R interaction. In addition, $mGlu_5R$ antagonists block the ability of $mGlu_5R$ to enhance NMDA receptor signaling, which will also favor anti-Parkinsonian actions by reducing the excitability of the striato-pallidal GABAergic neurons and thus their ability to cause motor inhibition, see [141].

It should be noted that the ability of $mGlu_5R$ antagonists to produce motor activation requires both $A_{2A}R$ and D_2R, which underlines their interdependence and supports the concept of $A_{2A}R$-D_2R-$mGlu_5R$ RM [142]. The synergism of $A_{2A}R$ and $mGlu_5R$ antagonists to increase locomotion in reserpinized mice [142, 143] can be elegantly explained by the existence of A_{2A}-D_2-$mGlu_5$ RM where $A_{2A}R$-$mGlu_5R$ synergize to counteract D_2R signaling, see [68].

The postulated A_{2A}-D_2-$mGlu_5$ RM in the striato-pallidal GABAergic neurons with multiple receptor-receptor interactions is therefore, a novel target for anti-Parkinsonian drugs. The above results have led to the proposal that $mGlu_5R$ antagonists especially in combination with $A_{2A}R$ antagonists or drugs with both $A_{2A}R$ and $mGlu_5R$ antagonist properties are symptomatic anti-Parkinsonian drugs of special value particularly in view of their neuroprotective properties, see also [15, 60].

Schizophrenia. The postulated A_{2A}-D_2-$mGlu_5$ RM may also exist in the ventral striato-pallidal GABAergic neurons and in the glutamate terminal networks of the ventral striatum. In fact, $A_{2A}R$ and $mGlu_5R$ agonists synergize when co-superfused into the nucleus accumbens to increase GABA release in the ventral pallidum [144]. Evidence for a role of $mGlu_5R$ in schizophrenia-related behavior in rodents such as prepulse inhibition has also been obtained [145]. Therefore, combined treatment with $A_{2A}R$ and $mGlu_5R$ agonist drugs or drugs with combined $A_{2A}R$ and $mGlu_5R$ agonist properties may be an effective novel strategy for treatment of schizophrenia based on the synergistic $A_{2A}R$-$mGlu_5R$ interaction which should be able to override the pathologically increased D_2R

signaling in this RM that may potentially be present in schizophrenia. The addition of a low dose of a D_2R antagonist to the $A_{2A}R$-$mGlu_5R$ agonist treatment should also be considered.

2.3.2. A_{2A}-CB_1-D_2 receptor mosaic

2.3.2.1. Biochemical and functional findings

In previous work [146, 147] indications were obtained for the existence of cannabinoid-dopamine CB_1R-D_2R heteromers in co-transfected HEK-293 cells based on FRET analysis and for an antagonistic CB_1R-D_2R interaction based on D_2R binding analysis. In 2005, studies using co-immunoprecipitation in HEK-293 cells gave further indications for CB_1R-D_2R heteromers with an enhanced formation after concurrent activation of the two receptors. It was noticed that in this heteromer CB_1R signaling, in part, switches from the inhibition of the AC to a pertussis toxin-insensitive activation of AC [148], see also [149].

Colocalization of D_2R and CB_1R in striatum was first observed in 2002-2003 [150, 151] and have been later found particularly in cortico-striatal glutamate terminals, in the soma and dendrites of ventral striato-pallidal GABAergic neurons and in local collaterals of the striato-pallidal GABAergic neurons, see [152, 153]. Therefore, a chemical anatomical basis for CB_1R-D_2R interactions exists and results indicate that endocannabinoids, via inhibitory feedback, can counteract D_2R-mediated responses [154-156]. Recently, novel evidence has been obtained for the existence of CB_1R-D_2R heteromers based on FRET analysis in HEK-293 cells [157]. Thus, FRET data show a strong and specific FRET signaling in cells co-transfected with cDNA of vectors encoding for D_2R-GFP^2 and CB_1R-YFP but not in various controls including mixtures of cells expressing D_2R-GFP^2 or CB_1R-YFP or co-transfections with GFP^2 or YFP without being tagged to the corresponding receptor. Of substantial interest is the finding of antagonistic D_2R binding modulation by CB_1R agonists that reduce D_2R agonist affinity in striatal membranes. Similar results were also observed in the nucleus accumbens shell demonstrated by quantitative receptor autoradiography [157]. In agreement, behavioral analysis revealed that CB_1R agonists can counteract

D_2R agonist-induced hyperlocomotion, an effect that was blocked by rimonabant, a CB_1R antagonist, which also can enhance the action of the D_2-like receptor agonist quinpirole [157]. These results clearly suggest that antagonistic intramembrane CB_1R-D_2R interactions exist in CB_1R-D_2R heteromers in the ventral and dorsal striatum and lead to reduced D_2R signaling, increased excitation and firing of the striato-pallidal GABAergic neurons and counteraction of D_2R-induced hyperlocomotion.

Of special interest in this behavioral analysis was the observation that the $A_{2A}R$ antagonist, MSX-3, could prevent the ability of the CB_1R agonist CP 55,940 to counteract the D_2R agonist-induced hyperlocomotion [157]. These results indicated the involvement of $A_{2A}R$ that is also known to exist in the cortico-striatal glutamate terminals and the soma-dendritic regions of the striato-pallidal GABAergic neurons in the antagonistic intramembrane CB_1R-D_2R interaction (see above). In line with these results, $A_{2A}R$-CB_1R heteromers were also demonstrated in transiently transfected HEK-293 cells and in neuroblastoma cells in which CB_1R signaling was entirely dependent upon $A_{2A}R$ activation [158]. Finally, it was also shown that motor depression caused by CB_1R agonists was blocked by $A_{2A}R$ antagonists. From the accumulating evidence, the existence of A_{2A}-CB_1-D_2 RM was therefore postulated [157].

The sequential BRET-FRET (SRET) technique was developed specifically for the identification of trimeric receptor mosaics [140]. Through a combination of BRET and FRET, trimeric receptor mosaics could finally be identified. This method was the essential technique to finally identify receptor mosaics, [15-17, 35, 43, 157]. Using the SRET technique, the A_{2A}-CB_1-D_2 RM was the first RM to be identified in living cells, and this discovery is in line with the indications for its existence obtained in previous work on the brain, see [157]. This RM is an integrator of DA, adenosine and endocannabinoid signals.

The present hypothesis for the operation of this putative A_{2A}-CB_1-D_2 RM in the brain states that the antagonistic CB_1R-D_2R interaction activated by the D_2R-induced release of endocannabinoids into the extracellular fluid removes the D_2R brake on $A_{2A}R$ signaling to AC, see [15], by an inhibitory feedback mechanism by the activation of CB_1 receptors in putative A_{2A}-CB_1-D_2 RM in the ventral striato-pallidal GABAergic neurons and in cortico-striatal

glutamate terminals. The increase in $A_{2A}R$ signaling in the ventral striato-pallidal GABAergic neurons will, via DARPP-32 phosphorylation at Thr34, strongly contribute to the markedly increased activity in ventral striato-pallidal GABAergic neurons by the inhibition of protein phosphatase-1, which will enhance the phosphorylation of ion channels and ion channel-linked receptors. The simultaneous release of $A_{2A}R$ signaling in striatal glutamate terminals will increase glutamate release and therefore lead to an increase in the glutamate drive of the ventral and dorsal striato-pallidal GABAergic neurons. Such an operation of this RM may be the molecular basis for the observed blockade of the D_2R agonist-induced locomotor hyperactivity and contribute to CB_1R agonist-induced motor inhibition [157]. It seems likely that the former is mainly in operation as an inhibitory feedback mechanism to reduce an exaggerated and prolonged activation of D_2R that will produce a prolonged silencing of the striato-pallidal GABAergic neurons. The release of the D_2R brake on $A_{2A}R$-induced activation of AC probably plays a major role in making this possible.

2.3.2.2. Relevance to CNS diseases and their treatments

Parkinson's disease. The results from Marcellino *et al*. [157] clearly suggest that CB_1R antagonists may represent novel symptomatic anti-Parkinsonian drugs by enhancing D_2R signaling to uphold its brake on $A_{2A}R$ signaling at the level of AC in the putative A_{2A}-CB_1-D_2 RMs. These molecular events may explain the enhancement of D_2R agonist-induced hyperlocomotion by the CB_1R antagonist and its ability to counteract the CB_1R agonist-induced inhibition of D_2R-induced hyperlocomotion.

It also seems likely that low doses of CB_1R and $A_{2A}R$ antagonists may synergize to enhance D_2R-mediated anti-Parkinsonian actions in early Parkinson's disease where DA release remains to a substantial degree from the remaining DA nerve terminal networks. The $A_{2A}R$ antagonist will also act by interfering with the ability of $A_{2A}R$ to inhibit D_2R signaling in the A_{2A}-D_2-$mGlu_5$ RM as discussed above. In contrast, in late PD with a very low DA tone, it becomes necessary to add low threshold doses of L-DOPA or D_2R agonists to achieve substantial therapeutic activity from CB_1R and $A_{2A}R$

antagonists. Through the possibility to use low doses of the two and/or three drugs, side effects such as dyskinesias may be effectively reduced.

In line with these results, CB_1R antagonists can strongly enhance the stereotypies caused by combined treatment with D_1R and D_2R agonists [159]. Furthermore, increased CB_1R binding and G protein-coupling has been observed in the basal ganglia of patients with Parkinson's disease and in the MPTP marmoset model of Parkinson's disease [160]. These neurochemical effects are counteracted by L-DOPA therapy suggesting that the CB_1R changes observed, represent a CB_1R receptor up-regulation in response to reduced D_2R signaling in PD that fails to elicit the release of endocannabinoids [161] and thus fails to activate the inhibitory feedback via the CB_1R. Similar results have been observed by Strömberg, Andersson and colleagues [162] after chronic haloperidol blockade of the D_2R. This mechanism can also help explain the reduced expression of cannabinoid CB_1R mRNA in the basal ganglia of postmortem brain of Parkinsonian patients [163] as a result of the dopaminergic treatment. The CB_1R antagonists should therefore act, as postulated above, to counteract the D_2R-activated inhibitory feedback activation of the CB_1R in the A_{2A}-CB_1-D_2 RM with the aim to bring down D_2R signaling. The enhancement of D_2R signaling in this RM should be optimized by a combined treatment with CB_1R and $A_{2A}R$ antagonists in order to block the two allosteric mechanisms of antagonizing the D_2R in A_{2A}-CB_1-D_2 RM.

Schizophrenia. Antagonistic CB_1R-D_2R interactions may also exist in postulated A_{2A}-CB_1-D_2 RM in the ventral striato-pallidal GABAergic neurons and in the cortico-accumbal glutamate terminals. The possibility is open that CB_1R agonists may possess antipsychotic properties by their ability to reduce D_2R signaling in this pathway and in the afferent glutamate terminals that will lead to a reduction of positive symptoms of schizophrenia. This is however, in apparent disagreement with the fact that □9-tetrahydrocannabinol (THC) is reported to exacerbate psychotic symptoms in schizophrenia, see [164]. However, these actions may be exerted at CB_1R in other brain regions *inter alia* the cerebral cortex.

These novel observations may help explain the findings that the increased CSF levels of anandamide found in schizophrenic patients are

inversely correlated with psychotic symptoms [165]. Thus, an over activity of D_2R-mediated DA transmission in the ventral striatum may lead to an increased formation of the endocannabinoid anandamide with an increased inhibitory feedback on the D_2R signaling via the CB_1R-D_2R antagonistic interaction in the A_{2A}-D_2-CB_1 RM on the glutamate terminals and in the ventral striato-pallidal GABAergic neurons. In this way, the excessive D_2R-mediated inhibition of the ventral striato-pallidal GABAergic neurons may be reduced by anandamide, which can act as an agonist in the postulated A_{2A}-CB_1-D_2 RMs.The precipitation of psychotic periods by cannabis use may also be related to the reduction of anandamide signaling in the brain [165] that leads to a reduction in the antagonistic CB_1R-D_2R interaction.

Cocaine addiction. The present indications of antagonistic CB_1R-D_2R interactions in RM in the ventral striato-pallidal GABAergic pathway may help explain the ability of the CB_1R agonist WIN 55,512-2 to counteract the rewarding actions of cocaine in intracranial self-stimulation experiments [166]. Thus, D_2R participate in mediating cocaine reward by inhibiting the activity in this reward-regulating pathway, see [111]. In line with this hypothesis, the CB_1R agonist WIN 55,212-2 can also reduce cocaine self-administration [167]. Our interpretation is that the CB_1R agonist, via the antagonistic CB_1R-D_2R interaction, leads to an increased activity in the ventral striato-pallidal GABAergic neurons thereby reducing the reward value of cocaine. As stated above, A_{2A}R agonists can also reduce cocaine self-administration. Therefore, we postulate that combined treatment with A_{2A}R agonists and CB_1R agonists that preferentially activates the A_{2A}R and CB_1R in A_{2A}-CB_1-D_2 RM should represent an interesting and novel strategy for preventing the development of cocaine abuse.

3. A_1R-D_1R heteromers and A_1R-D_1R containing receptor mosaics

3.1. A_1R-D_1R heteromers

3.1.1. Biochemical and functional findings

A_1R and D_1R were shown to co-immunoprecipitate in co-transfected Ltk- fibroblast cells [168], a phenomenon that appeared specific, since co-immunoprecipitation was not detected in A_1R-D_2R co-transfected Ltk- fibroblast cells. The A_1R-D_1R co-immunoprecipitation was observed in the

absence of A_1R or D_1R receptor agonist exposure, thereby indicating their constitutive formation. However, the A_1R-D_1R co-immunoprecipitation was substantially reduced after a 1 h treatment with the D_1R agonist SKF 38393, giving evidence that D_1R activation leads to disruption of the A_1R-D_1R heteromeric receptor complex. This disruption did not occur if combined treatment with SKF 38393 and the A_1R agonist *R*-PIA was applied. Thus, these initial results indicated that A_1R and D_1R form receptor heteromers at least in cell lines. Later, co-immunoprecipitation experiments indicated that they may also exist in striatum [169]. Further evidence that an A_1R-D_1R heteromer exists was recently obtained, since specific BRET and FRET signals can be detected between fluorophore-tagged A_1R and D_1R upon transient co-transfection in cell lines [170]. The A_1R-D_1R heteromer may exist on the cell surface membrane together with A_1R homodimers [171] and D_1R homodimers [172], and it is not known if the A_1R-D_1R heteromer is preferred.

Competition experiments with the D_1R antagonist [^{3}H]-SCH 23390 versus dopamine were performed in striatal membrane preparations as well as in membrane preparations from an A_1R-D_1R co-transfected Ltk- cell line [173, 174]. The A_1R agonist CPA, in the nanomolar range, caused a marked reduction in the proportion of D_1R in its high affinity state. These effects were mimicked by the GTP analogue Gpp (NH)p (100 μM). These results make it likely that A_1R activation in the A_1R-D_1R heteromer leads to an uncoupling of the D_1R to its $G_{s/olf}$ protein.

The possible role of the G_i protein in the A_1R-D_1R interaction at the binding pocket level was studied using pertussis toxin, since it inactivates the G_i protein coupled to the A_1R. It was found that pertussis toxin treatment blocked the effects of low but not high concentrations of the A_1R agonist on D_1R binding characteristics [174]. However, it could not be determined if the D_1R modulation by the high 10 μM concentration of CPA was due to activation of the low affinity A_1R receptors or to the activation of the remaining high affinity A_1R, since pertussis toxin-induced ribosylation of the G_i protein was not complete.

This problem could however be solved by involving adenosine deaminase (ADA) in the analysis, which metabolizes adenosine to inosine. This enzyme binds to A_1R as an ectoenzyme [175] and is necessary to obtain

the high affinity binding state of A_1R [176]. An irreversible inhibitor of ADA, deoxycoformycin (DCF), was found to fully counteract the effects of high and low concentrations of CPA on the binding characteristics of the D_1R. The blockade of the enzymatic activity of ADA was shown not to be involved in this action of DCF. These results give evidence that it is the high affinity state of A_1R that is responsible for the interaction with D_1R, at least at the level of the binding pocket [177]. Thus, ADA may directly bind to the A_1R, which is necessary for the A_1R high affinity state to develop. This state has a protein conformation such that the A_1R antagonistically interacts with the D_1R. It follows that functional A_1R-D_1R heteromer requires ADA to be bound to A_1R, underlining the important role of receptor interacting proteins in heteromers [24, 170].

In the A_1R-D_1R co-transfected fibroblast cell line, the expected antagonistic interaction at the level of AC level was observed after A_1R and D_1R co-activation [174]. The antagonistic A_1R-D_1R interaction found at both the recognition and AC levels was found to be correlated, indicating that the inhibitory interaction in the A_1R-D_1R heteromer was involved in causing the A_1R-mediated inhibition of D_1R signaling.

The available evidence suggests that there exist antagonistic intramembrane A_1R-D_1R interactions in the dorsal and ventral striatum and in the prefrontal cortex [24, 25, 170, 178]. This involves also an ability of A_1R agonists to antagonistically modulate D_1R antagonist binding sites in the nucleus accumbens and the prefrontal cortex that cause a reduction of their affinity.

Agonist-induced co-aggregation and co-desensitization of A_1R-D_1R heteromers

Permeabilized cells were used and the A_1R agonist *R*-PIA (100 nM, 1 h) caused aggregates of A_1R-D_1R, while the D_1R agonist SKF 38393 (10 μM, 1 h) caused clusters of D_1R alone, in line with the D_1R agonist induced disruption of the A_1R-D_1R heteromers [168]. It is of substantial interest that combined treatment with the two agonists, which maintains the heteromerization of A_1R and D_1R, reduced the co-aggregation of A_1R-D_1R. In this case, a clear-cut decrease in D_1R signaling to the AC was observed. Thus, upon co-activation of A_1R and D_1R in the heteromer, with no formation

of co-aggregates and maintenance of A_1R-D_1R heteromers, a desensitization of D_1R signaling occurs. This desensitization may involve an uncoupling of the D_1R in the A_1R-D_1R heteromer to the G_s protein. In contrast, A_1R-D_1R co-aggregates (activation of A_1R alone) or D_1R aggregates (activation of D_1R alone) did not result in desensitization of D_1R signaling [168]. In contrast to the A_1R-D_1R co-transfected fibroblast cell line, combined treatment with A_1R and D_1R agonists produced co-aggregates in cortical nerve cells in culture. Such differential actions may be caused by differences in the stoichiometry and in adapter and scaffolding proteins interacting with the A_1R-D_1R heteromers but neverless indicate an important role of A_1R and D_1R agonists in modulating the cotrafficking of A_1R-D_1R heteromers in the forebrain and thus in D_1R function.

Function. An A_1R antagonistic modulation of D_1R signaling was observed in the regulation of transcription factors in the striatum after DA terminal denervation based on analysis of the immediate early gene NGFI-A and *c-fos* mRNA levels and of GABA release in the striato-entopeduncular GABAergic pathway as studied with microdialysis [178]. The same year behavioral indications for antagonistic A_1R-D_1R receptor interactions were also obtained with adenosine A_1R antagonists potentiating the motor effects of D_1R agonists [179]. Collectively, this work in the 1990s indicated the existence of antagonistic intramembrane A_1R-D_1R interactions reducing D_1R signaling in the direct pathways (see [24]).

3.1.2. Relevance to CNS diseases and their treatments

The antagonistic interaction in A_1R-D_1R heteromers, where D_1R is the crucial receptor in view of its important behavioral role, offers a new way to modulate D_1R signaling, namely to reduce striatal D_1R signaling with A_1R agonists and enhance it with A_1R antagonists (see [24, 25]). This offers a therapeutic potential for A_1R antagonists in Parkinson's disease as seen also by A_1R antagonist enhancement of D_1R-induced locomotion. Often, A_1R drugs in low doses by themselves have only weak effects in the behavioral models used but can strongly modulate the D_1R signaling [179, 180]. This is also beautifully illustrated through microdialysis studies and through the induction

of immediate early genes (IEG) (see above and [181]). In addition, A_1R agonists can strongly counteract the D_1R agonist-induced oral dyskinesias in rabbits [173] indicating a therapeutic potential of A_1R agonists in L-DOPA-induced dyskinesias in parkinsonian patients by targeting the striatal A_1R-D_1R heteromer.

A_1R agonists also counteract D_1R agonist-induced electroencephalography (EEG) arousal in rats [182] probably by targeting the postulated A_1R-D_1R heteromers in the frontal cortex. A_1R antagonists targeting these heteromers may therefore, have a potential therapeutic role in attention deficit hyperactivity disorders (ADHD) by increasing EEG arousal provided it will lead to increased attention. This proposal is supported by the demonstration of sedative-hypnogenic properties of adenosine analogues that could in part be mediated via A_1R-D_1R heteromers in the frontal cortex. In fact, the A_1R agonist CPA but not the $A_{2A}R$ agonist CGS 21680 prevents EEG arousal due to D_1R activation [182].

3.2. Putative A_1-D_1-D_3 receptor mosaics

As mentioned earlier, a large array of experimental approaches utilizing heterologous expression systems have been used to demonstrate receptor-receptor interactions. Interestingly, by using some of these approaches, namely BRET, FRET and acceptor photobleaching FRET, a D_1R-D_3R interaction has been demonstrated [183]. Interestingly, in membrane preparations from bovine striatum the D_3R agonist R(+) 7-OH-DPAT was found to shift the D_1R agonist competition curve to the left ([^{3}H]-SCH23390 versus SKF-38393) demonstrating that D_3R activation increases the affinity of the D_1R agonist binding sites [183]. These results suggest the existence of synergistic intramembrane D_3R-D_1R interactions at the level of D_1R recognition in striatal D_3R-D_1R heteromers. In line with these findings, the D_3R agonist PD 128907 is also shown to enhance the actions of the D_1R agonist SKF 38393 on locomotion in reserpinized mice [183].

Based on previous work by the Schwartz and Sokoloff group [184-186], see also [23, 131, 173] and in line with the recent findings of Marcellino *et al.* [183] it seems likely that D_3R-D_1R synergism in their receptor heteromer in the

direct striatal pathway may contribute to L-DOPA-induced dyskinesias. D_3R antagonists acting on the D_3R-D_1R heteromer may therefore have anti-dyskinetic properties. In fact, induction of D_3R expression may be one mechanism for behavioral sensitization to L-DOPA [187]. D_3R antagonists blocking D_3R-D_1R synergism may also have anti-cocaine reward properties in view of the D_3R up-regulation found e.g. in reward circuitries of human cocaine fatalities [188] and where both D_1R and D_3R are involved in cocaine actions [187, 189].

Interestingly, Surmeier *et al*. [190] found that around 50% of the striato-nigral and striato-entopeduncular GABAergic neurons show D_3R expression. There is the possibility that at least some of these neurons also express A_1R and that not only A_1R-D_1R heteromers but also A_1-D_1-D_3 RM may be formed in these neurons of the direct pathway. Such a RM composed of three different GPCR types may be an especially important integrative center for transmitter and modulator signals with D_1R as the hub receptors and the other two as receptors important for proper D_1R function. Therefore, it may be the case that combined treatment with D_3R antagonists and A_1R agonists could be rational to reduce D_1R signaling especially in combination with low doses of D_1R antagonists. Overall, this approach may bring about an improved treatment of L-DOPA-induced dyskinesias and of cocaine addiction with reduced side effects in view of the lower doses of D_1R antagonists that can be used, see [15].

4. Final comments

Dopamine receptors in general, and D_2R and D_1R in particular, may be considered as the functionally most important receptors in several types of striatal GPCR heterodimers. Interestingly, in view of their strong actions on multiple effectors in the striato-pallidal and striato-entopeduncular/nigral GABAergic neurons, it is postulated that these dopamine receptors-containing oligomers are mainly located outside the glutamate and DA synapses (see above). The standard treatment, e.g. with L-DOPA and/or DA agonists [191] in PD builds on the activation of these D_2R and D_1R by moderate to high doses, while the accessory $A_{2A}R$ and A_1R and others in the different heterodimers and RM are not targeted.

A novel principle may now be used to develop D_2R agonists for treatment of PD based on the existence of various D_2R containing heterodimers (two receptors) and RM (three or more receptors; higher-order oligomers) since the conformational state of the D_2R and likely its pharmacology probably differs from one receptor assembly to another. It may also be due to different receptor-protein interactions, and also influenced by the local molecular histology of the surface membrane of discrete striato-pallidal nerve cell populations and of striatal glutamate and DA nerve terminal networks. Thus, the full or partial agonist pharmacology of D_2R in terms of potency and efficacy may show substantial differences among various types of RM such as A_{2A}-D_2-$mGlu_5$ RM versus A_{2A}-CB_1-D_2 RM versus D_2 monomers and homodimers, and $A_{2A}R$-D_2R and CB_1R-D_2R heterodimers etc. The development of specific D_2R agonists, specifically for the D_{2S} autoreceptor may be especially hopeful since the D_{2S} autoreceptor participates in unique RM versus the postjunctional D_{2L} receptors. In this way, RMs of D_{2S} autoreceptors and non-α7 nicotinic receptors have been found in the striatal DA nerve terminals [192], see also [193] as well as direct protein-protein interactions of the D_{2S} autoreceptor with the DA transporter which are disrupted in schizophrenia [194]. This should give exciting new possibilities to develop novel and more selective D_2R agonist drugs for treatment of Parkinson's disease by preferentially acting on certain postjunctional RM in the striato-pallidal GABAergic neurons and their glutamate inputs.

The same principle may also be used to develop novel D_2R antagonists for the treatment of schizophrenia. Thus, the potency and efficacy of full and partial D_2R antagonists and their inverse D_2R agonist activity may vary among the different D_2R assemblies, due to differences in the conformational state of the participating D_{2L} postjunctional and D_{2S} autoreceptors which give them differences in D_2R antagonist pharmacology. The major target may be the postjunctional D_2R in the ventral striato-pallidal GABAergic neurons inhibiting the glutamate drive to the prefrontal cortex in view also of likely increases in meso-limbic DA activity in schizophrenia, see [15, 17, 24, 25, 103]. It should be mentioned that the atypical antipsychotic drug remoxipride, a selective D_2R antagonist, unlike haloperidol *in vivo* blocks only a subpopulation of D_2R in nigro-striatal and meso-limbic/cortical regions

as evaluated by the protection against the N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) induced decreases in D_2R binding due to its irreversible inactivation of D_2R [102]. It was suggested that the blockade of this D_2R subpopulation *in vivo* was the basis for its antipsychotic activity and its atypical antipsychotic profile with reduced extrapyramidal side effects. The underlying mechanism may be that this selective D_2R antagonist can only bind and block D_2 receptors in distinct receptor heterodimers and receptor mosaics present in these regions due to their unique D_2R antagonist pharmacology. This may now be tested by studying how the potency and efficacy of remoxipride to block D_2 receptors varies in cell lines upon co-transfection with other receptors known to form heterodimers and receptor mosaics with the D_2R. This approach may also be valid for the treatment of cocaine addiction (see above) where novel and more selective D_2R antagonists for certain D_2R-containing heterodimers and RM in the ventral striatum may offer improvement of treatment.

The other novel principal strategy based on the different D_2R- and D_1R-containing receptor mosaics is the targeting of their accessory receptors that antagonistically interact via receptor-receptor interactions with the D_2R (key receptor) involving e.g. the $A_{2A}R$, and the D_1R and the A_1R, which can involve combined treatment with very low doses of D_2R agonist/antagonists and D_1R agonist/antagonists, respectively.

In PD, a combined treatment will make possible the use of very low to low doses of L-DOPA and D_2R agonists since in combination with $A_{2A}R$ antagonists the inhibitory impact of these receptors on D_2R is removed [15, 17, 24, 60]. Thus, a reduction of the collateral effects of L-DOPA and D_2R agonists may be observed along with improvement of hypokinesia, resting tremor and rigidity [68]. In early PD, it may be possible to substantially delay the onset of L-DOPA and D_2R agonist treatment by introducing monotherapy or combined therapy with $A_{2A}R$ or CB_1R antagonists which also may delay the neurodegeneration of the nigral DA cells, see [15, 17].

In schizophrenia, low doses of D_2R antagonists with reduced collateral effects may be used since a combined treatment with low doses of $A_{2A}R$ agonists will synergistically reduce D_2R signaling at the membrane and the cytoplasmatic level in the ventral striato-pallidal GABAergic neurons [15, 17,

45, 62]. Thus, with this combined treatment anti-schizophrenic actions may be obtained with reduced extrapyramidal side effects and monotherapy with $A_{2A}R$ agonists should also be considered in view of their atypical antipsychotic profile [15, 106]. A similar combined treatment strategy may also be used against development of cocaine addiction.

5. Future research

It becomes important to discover the $A_{2A}R$-D_2R and A_1R-D_1R heterodimers and RM *in vivo* within the brain and their functional role. This may be accomplished through the generation of BAC transgenic mice [195] with fluorescently tagged wild-type adenosine and dopamine receptors together with mice that express mutant adenosine and dopamine receptor that cannot heteromerize but remain functional. Therefore the heteromers demonstrated can then be analyzed, e.g. in mice models of Parkinson's disease, schizophrenia, etc., in order to understand their role in the pathogenesis and treatments of neurological and mental diseases.

The biochemical *in vitro* work should unravel the pharmacology of the various adenosine and dopamine heterodimers and RM assisted by computerized modeling of the heteromers and RM and bioinformatic analysis of the participating receptors. In this way, adenosine and dopamine receptor interfering drugs may be discovered that preferentially interact with distinct heterodimers and RM of adenosine and dopamine receptors [15, 17].

It is crucial to characterize the receptor interfaces of the various heteromers and RM through a combined biochemical and bioinformatic analysis that includes mathematical approaches. In this way, novel drugs can be developed that directly target the receptor interface in this way mimicking the adenosine-dopamine receptor-receptor interactions that take place via the receptor interfaces through allosteric mechanisms.

Finally, it becomes important to also establish and characterize the role of allosteric modulators in the adenosine and dopamine receptor heterodimers and RM [47] and how they become integrated with the receptor-receptor interactions to modulate the orthosteric sites and the coupling of the receptors to G proteins and β-arrestins. The understanding of such integrative processes is also vital for drug development in neuropsychopharmacology.

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Figure Legends

Figure 1. Schematic illustration of $A_{2A}R$ and D_2R homodimers and $A_{2A}R$-D_2R heterodimer. The striato-pallidal GABAergic neurons might co-express $A_{2A}R$ and D_2R homo- and heterodimers (dashed box) at the plasma membrane. Adenosine and dopamine can potentially interact with both homo- and heterodimers converging in the control of adenylate cyclase function an integrated cellular response is generated. The functional balance between these three oligomers determines the final adenylate cyclase output and thus the eventual cellular response. The antagonistic allosteric $A_{2A}R$-D_2R interaction in the heterodimer (dashed box) is shown (filled black arrow) as well as the negative and positive coupling of D_2R and $A_{2A}R$ to the adenylate cyclase, respectively.

Figure 2. Illustration of positively charged arginine-rich epitopes ^{215}VLRRRRKRVN^{224} (D_2R), ^{216}KQRRRKRI^{223} (D_3R), and ^{377}TRRRRRAK^{385} (D_4R) in the N-terminal part of the third intracellular loop of D_2R, D_3R, D_4R, electrostatically interacting with negatively charged C-terminal epitopes of the $A_{2A}R$ (^{370}SAQEpSQGNT^{378}, ^{388}HELKGVCPEPPGLDDPLAQDGAGVS^{412}). The most important residues in the $A_{2A}R$ appear to be the phosphorylated serine in the ^{370}SAQEpSQGNT^{378} in the C-terminal epitope of the $A_{2A}R$ (see [52, 55, 64]). These electrostatic interactions represent important hot spots in the receptor interface of the $A_{2A}R$-D_2R, $A_{2A}R$-D_3R and $A_{2A}R$-D_4R heteromers. The prototype was the $A_{2A}R$-D_2R heteromer (dashed box).

Figure 3. Schematic representation of protein kinase B/glycogen synthase kinase 3 (Akt/GSK3) signalling networks regulated by dopamine D_2R. (left) The stimulation of D_2R lead to an initial change in receptor conformation that mediate the activation of $G_{i/o}$ protein, leading to inhibition of adenylyl cyclase, subsequently to receptor phosphorylation by G-protein receptor kinase and the recruitment of b-arrestin. The recruitment of b-arrestin results in the formation of a signalling complex that comprises at least b-arrestin, PP2A and Akt. The formation of this complex result in the deactivation of Akt by protein phosphatise 2A (PP2A) and the subsequent stimulation of GSK-3 that mediates dopamine-dependent behaviors. (right) The antagonistic allosteric $A_{2A}R$-D_2R interaction in the heterodimer could reduce b-arrestin recruitment, resulting in an enhancement of Akt phosphorylation, thus inhibiting GSK3 (see text).

Figure 4. Schematic representation of putative A_{2A}-D_2-$mGlu_5$ RM and $A_{2A}R$-D_2R heterodimer in the striato-pallidal GABAergic neurons (A) and A_1-D_1-D_3 RM and A_1R-D_1R heterodimer in the striato-entopeduncular/nigral GABAergic neurons (B). In panel A, the antagonistic $A_{2A}R$-D_2R and $mGlu_5R$-D_2R interactions in the A_{2A}-D_2-$mGlu_5$ RM are shown as well as the antagonistic $A_{2A}R$-D_2R interactions in the heterodimer. The interactions at the level of adenylate cyclase are also indicated in which D_2R inhibits and $A_{2A}R$ activates this enzyme. D_2R signalling from the putative RM and/or the heterodimer control the excitability of the striato-pallidal GABAergic neurons by gating ion channels. In panel B, the antagonistic A_1R-D_1R and facilitatory D_1R-D_3R interactions in the putative A_1-D_1-D_3 RM are shown as well as antagonistic A_1R-D_1R interactions in the heterodimer. The interactions at the level of adenylate cyclase are also indicated in which D_1R activates and A_1R and possibly D_3R inhibits this enzyme. The activation of PKA contributes not only to activate intracellular pathways that lead to the phosphorylation of DARPP-32, MEK, and CREB, but also to phosphorylation events that lead to an increase in the activity of cation channels.

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Figure 1- Fuxe *et al.*

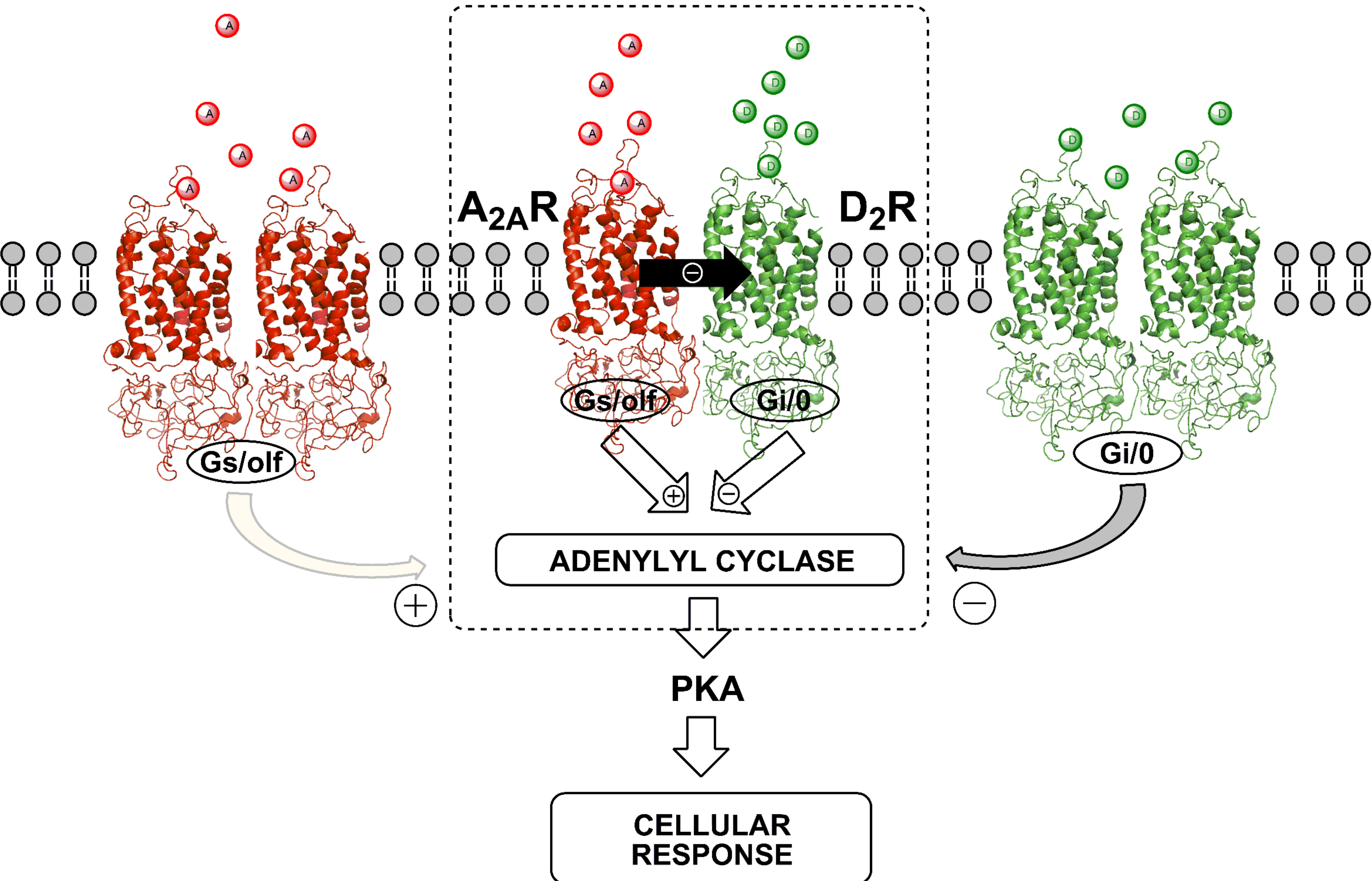
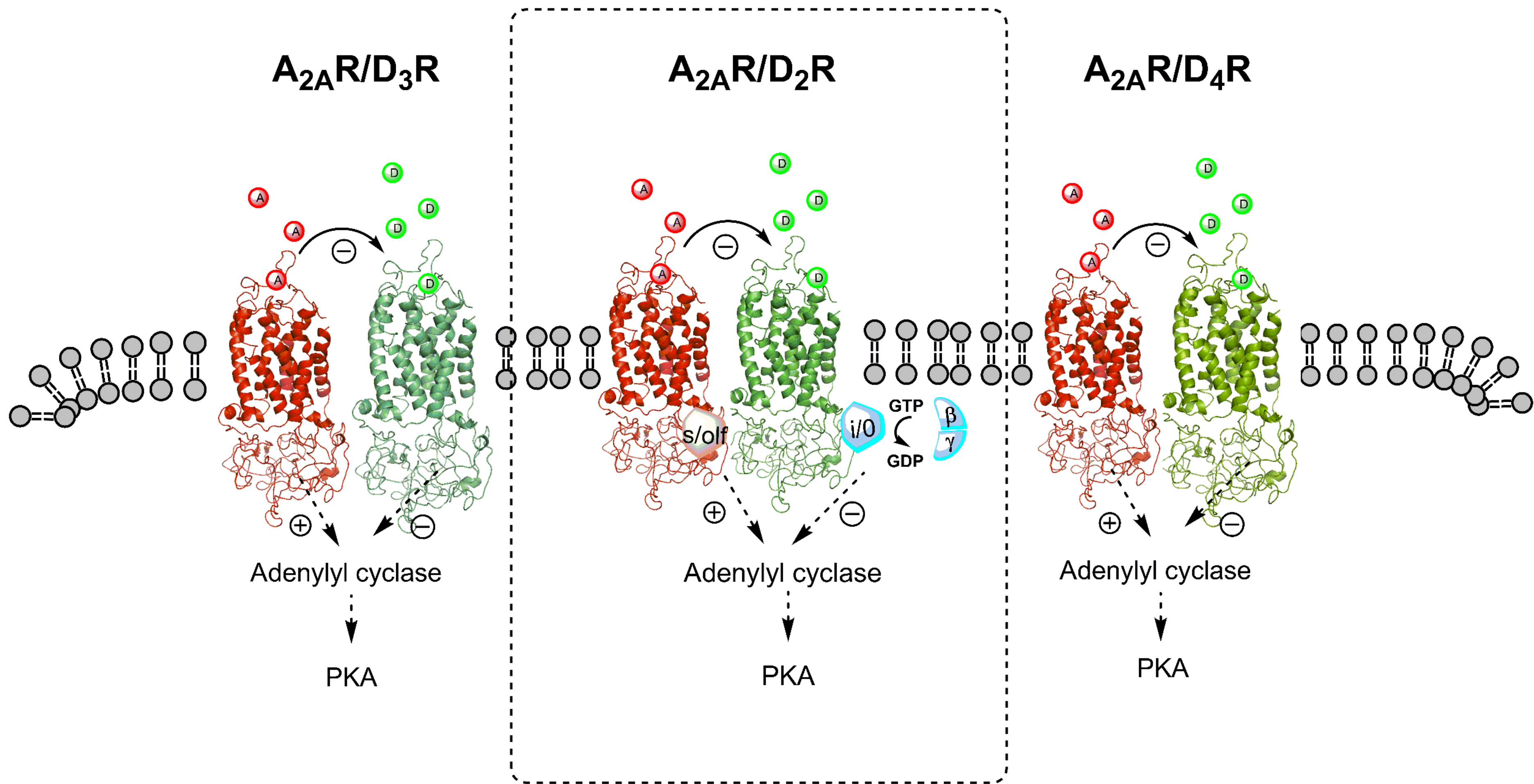


Figure 2- Fuxe *et al.*



D_3R-IC3: [216]-KQRRRKRI-[223] **D_2R-IC3:** [215]-VLRRRRKRVN-[224] **D_4R-IC3:** [377]-TRRRRRAKI-[385]

$A_{2A}R$-CT: [370]-SAQEpSQGNT-[378]

$A_{2A}R$-CT: [388]-HELKGVCPEPPGLDDPLAQDGAGVS-[412]

Figure 3- Fuxe *et al.*

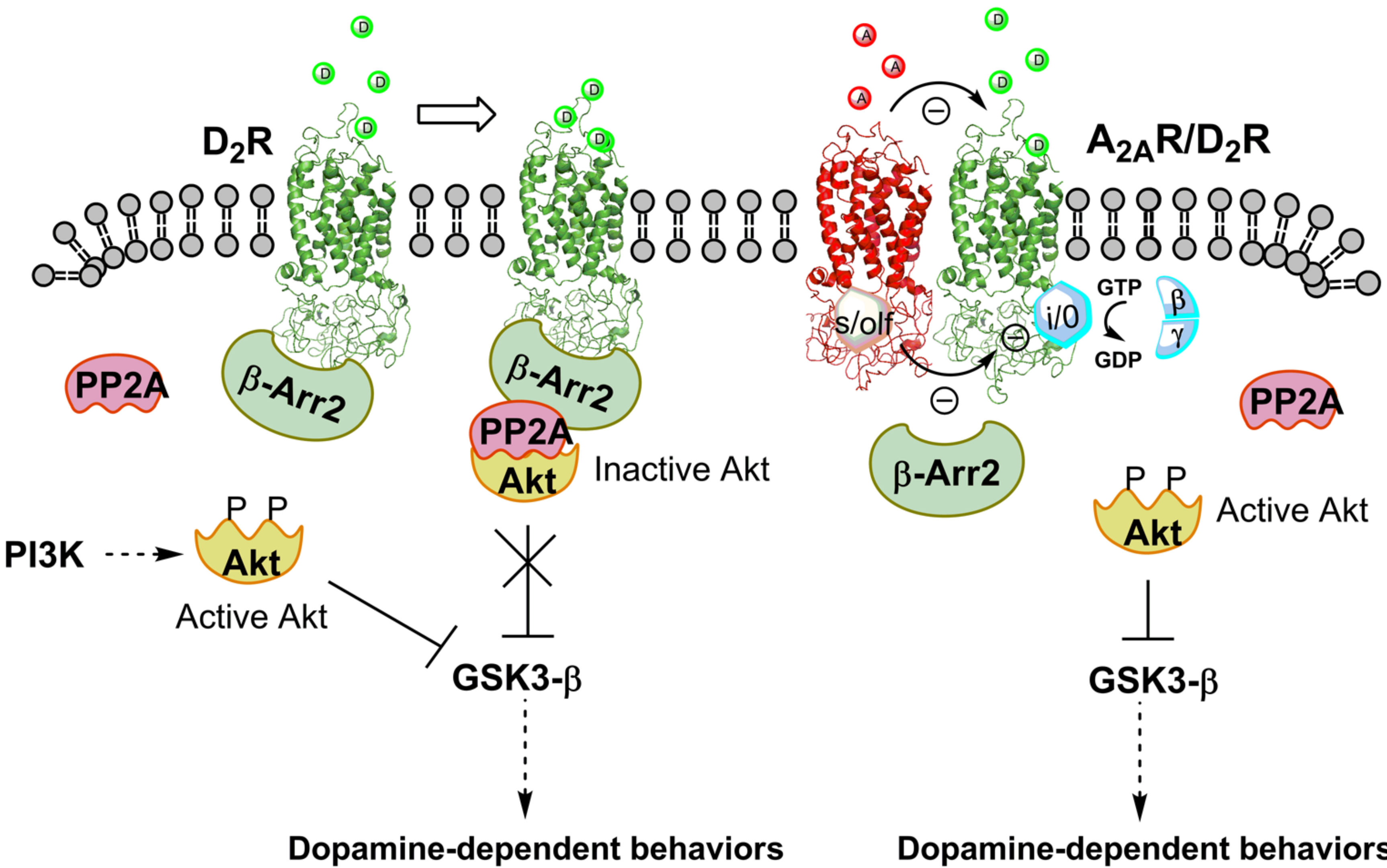


Figure 4- Fuxe *et al*