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Jana Bakešová

MODULATION OF ADENOSINE A_{2A} RECEPTOR FUNCTION BY INTERACTING PROTEINS. NEW TARGETS FOR HUNTINGTON'S DISEASE.

Dissertation

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MODULACIÓN DE LAS FUNCIONES DEL RECEPTOR A_{2A} DE ADENOSINA POR INTERACCIÓN CON OTRAS PROTEÍNAS. NUEVAS DIANAS PARA LA ENFERMEDAD DE HUNTINGTON .

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Dr. Carme Lluís Biset Dr. Rafael Franco Fernández

Jana Bakešová

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To my family

In the memory of
Woody Guthrie, Americal folk singer, victim of HD

and
Prof. Václav Pelouch, my diploma thesis advisor

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ABBREVIATIONS

2-AG	2- Arachidonoylglycerol
AC	Adenylate cyclase
ADA	Adenosine deaminase
A_nR	Adenosine A_n receptor
ATP	Adenosine 5'-triphosphate
BiFC	Bimolecular fluorescence complementation
BRET	Bioluminescence resonance energy transfer
CaMK	Ca^{2+}/calmodulin dependent protein kinase
cAMP	3',5'-cyclicadenosinemonophosphate
CB_nR	Cannabinoid CB_n receptors
cDNA	Complementary deoxyribonucleic acid
CGS-21680	2-p-(2-Carboxyethyl)-phenethylamino-5'Nethylcarboxamido-adenosine
CHO	Chinese hamster ovary
CNS	Central nervous system
CP-55,940	(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol
CREB	cAMP response element-binding
DAG	Diacylglycerol
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32 kDa
DAT	Dopaminte transporter
D_c	Dimer cooperativity index
DGL	Diacylglycerol lipase
DMSO	Dimethylsulfoxide
D_nR	Dopamine D_n receptors
DOPA	3,4-Dihydroxyphenylalanine
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
ECL	Extracellular domain
EDTA	Ethylendiaminetetraacetic acid
ER	Endoplasmatic reticulum
ERK 1/2	Extracellular regulated kinase 1/2
FAHH	Fatty acid amide hydrolase
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GABA	γ-Aminobutiric acid
GFP	Green fluorescent protein
Gpe/i	Globus pallidus externum /internum
GRK	G-protein coupled receptor kinase
GST	Glutathione-S-transferase
GTP	Guanosine 5'-triphosphate
GPCR	G-protein-coupled receptor
HD	Huntington's disease
HEK	Human Embryonic Kidney
ICL	Intracellular loop
IP3	Inositol-1,4,5-triphosphate
K_D	Dissociation constant
K_{DH}	High affinity state dissociation constant

K_{DL}	Low affinity state dissociation constant
KW-6002	(E)1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-xanthine
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
mRNA	Messenger ribonucleic acid
MPTP	N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSX-2	3-(3-hydroxypropyl)-7-methyl-8-(m-methoxystyryl)-1-ropargyl-xanthine
MSN	Medium spiny neuron
NAD(P)+	Nicotinamide adenine dinucleotide (phosphate)
NMDA	N-Methyl-D-aspartate
PBP	Periplasmic binding proteins
PD	Parkinson's disease
PI3K	Phosphatidylinositol 3-kinase
PKA/B/C	Protein kinase A/B/C
PLC	Phospholipase C
PP-2A	Protein phosphatase 2
PTX	Pertussis toxin
PVDF	Polyvinylidene difluoride
QA	Quinolinic acid
RET	Resonance energy transfer
Rluc	Renilla luciferase
R-PIA	R-phenylisopropyladenosine
RT-PCR	Reverse-transcriptase polymerase chain reaction
SAM	S-adenosylmethionine
SCH-420814	2-(2-furanyl)-7-[2-[4-[4-(2-methoxyethoxy)phenyl]-1-piperazinyl]ethyl]-7H-pyrazolo[4,3-e][1,2,4]-triazolo[1,5-c]pyrimidin-5-amine
SCH-442416	2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e] [1,2,4]triazolo[1,5-c]pyrimidin-5-amine
SCH-58261	5-Amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo(4,3-e)-1,2,4-triazolo(1,5-c)pyrimidine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SN	Substancia nigra
SNc/r	Substancia nigra compacta/reticulata
SRET	Sequential resonance energy transfer
STN	Subthalamic nucleus
THC	Tetrahydrocannabinol
TKR	Tyrosine kinase receptor
TM(D)	Transmembrane (domain)
SSTR	Somatostatin receptor
VDCC	Voltage dependent Ca^{2+} channel
VER-7835	2-amino-6-(furan-2-yl)-*N*-(thiophen-2-ylmethyl)-9*H*-purine-9-carboxamide
VTA	Ventral tegmental area
YFP	Yellow fluorescent protein
ZM-241385	4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

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INTRODUCTION

1.1. GPCRs

G-protein-coupled receptors (GPCRs) or seven transmembrane domain receptors (7TM) comprise the largest superfamily of proteins in the body. In vertebrates, this family contains 1000–2000 members (more than the 1 % of the genome, 2 % in humans) including more than 1000 coding for odorant and pheromone receptors. The chemical diversity among the endogenous ligands is great; they include biogenic amines, peptides, glycoproteins, lipids, nucleotides and ions (Kolakowski et al., 1994). Furthermore, the sensation of exogenous stimuli, including light, odors, and taste, is mediated through G-protein coupled receptors (Hoon et al., 1999). It has been estimated that more than half of all modern drugs are targeted towards these receptors (Flower et al., 1999), and several ligands for GPCRs are found among the worldwide top-100-selling pharmaceutical agents. It is also evident that a small percentage of pharmacological drugs have been developed to act upon a very small number of the GPCRs, hence the potential for drug discovery within this field is still quite large.

There are two main requirements for a protein to be classified as a GPCR. The first requirement relates to seven sequence stretches, each involving between 25 to 35 consecutive amino acid residues, which show a relatively high degree of calculated hydrophobicity. These sequences are believed to represent seven α-helices that span the plasma membrane in a counter-clockwise manner, forming both a recognition and connection unit, enabling an extracellular ligand to exert a specific effect within the cell. The first crystal structure of a GPCR appeared in the year 2000, when Palczewski et al. (Palczewski et al., 2000), reported the high-resolution structure for the bovine rhodopsin receptor. With 2.3 Å resolution, it was confirmed that the α-helical transmembrane domains (TMD) rearranged in a closely packed bundle forming the transmembrane receptor core. The N-terminus of the polypeptide is located in the extracellular space, whereas the C-terminus exhibits an intracellular localization. The seven transmembrane helices are connected by six alternating intracellular (ICL) and extracellular (ECL) loops (Figure 1). The second principal requirement to be classified as a GPCR is the ability of the receptor to interact with a heterotrimeric G-protein. As shown in figure 2, some of the GPCRs

signalling pathways are dependent upon interaction with heterotrimeric G-proteins as guanine-nucleotide exchange factors; thus, upon ligand binding to a receptor, a conformational change is produced in the associated G-protein α-subunit that leads to the subsequent exchange of GDP for GTP. Subsequently, the GTP-bound form of the α-subunit dissociates from both the receptor as well as from the βγ-dimer (Marinissen et. al, 2001). Both the GTP-bound α-subunit and the released βγ-dimer modulate cellular signalling pathways. These include, among others, stimulation or inhibition of adenylate cyclase, activation of phospholipases, as well as regulation of potassium and calcium channel activity (Hamm et al., 1998). Interestingly, it has been suggested that agonist induced phosphorylation of the receptors though GRKs (G-protein-coupled receptor kinases) and the subsequent sequestration of the receptors from the cell surface (Krupnick et al., 1998) are not only important mechanisms for decreasing the signalling capacity of the receptor, but also play a key role in switching the receptor from G-protein-coupled-dependent signalling pathways to G-protein-independent signalling cascades normally associated with growth factor receptors (Luttrell et al.,1999).

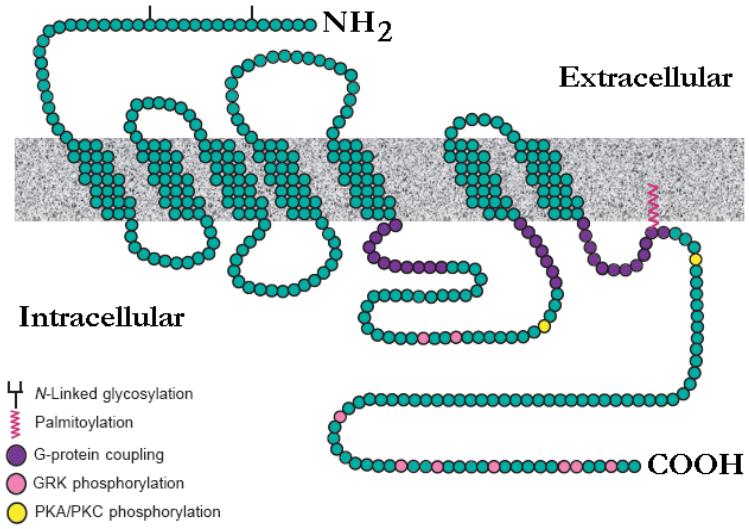

Figure 1. GPCRs. Important regions and sites are highlighted. Taken from (Lefkowitz, 2000).

The exposure of GPCRs to agonists often results in the rapid attenuation of receptor responsiveness. This receptor desensitization process is the consequence of a combination of different mechanisms. These mechanisms include the uncoupling of the receptor from

heterotrime-ric G-proteins in response to receptor phosphorylation (Hausdorff et al., 1989; Lohse et al., 1990), the internalization of cell surface receptors to various intracellular compartments (Hermans et al., 1997; Trejo et al., 1998), and the down-regulation of the total number of receptors in the cell. The latter of the three is accomplished through mechanisms to reduce receptor mRNA and protein synthesis, as well as the lysosomal degradation of pre-existing receptors (Jockers et al., 1999; Pak et al., 1999). The time frames over which these processes occur range from seconds (phosphorylation) to minutes (endocytosis) to hours (down-regulation) and the extent of receptor desensitization varies from the complete termination of receptor signalling, as observed in the visual and olfactory systems, to the attenuation of agonist potency and maximal responsiveness, such as observed for the β_2-adrenergic receptor (Sakmar et al., 1998). The extent of receptor desensitization is regulated by a number of factors that include receptor structure and cellular environment.

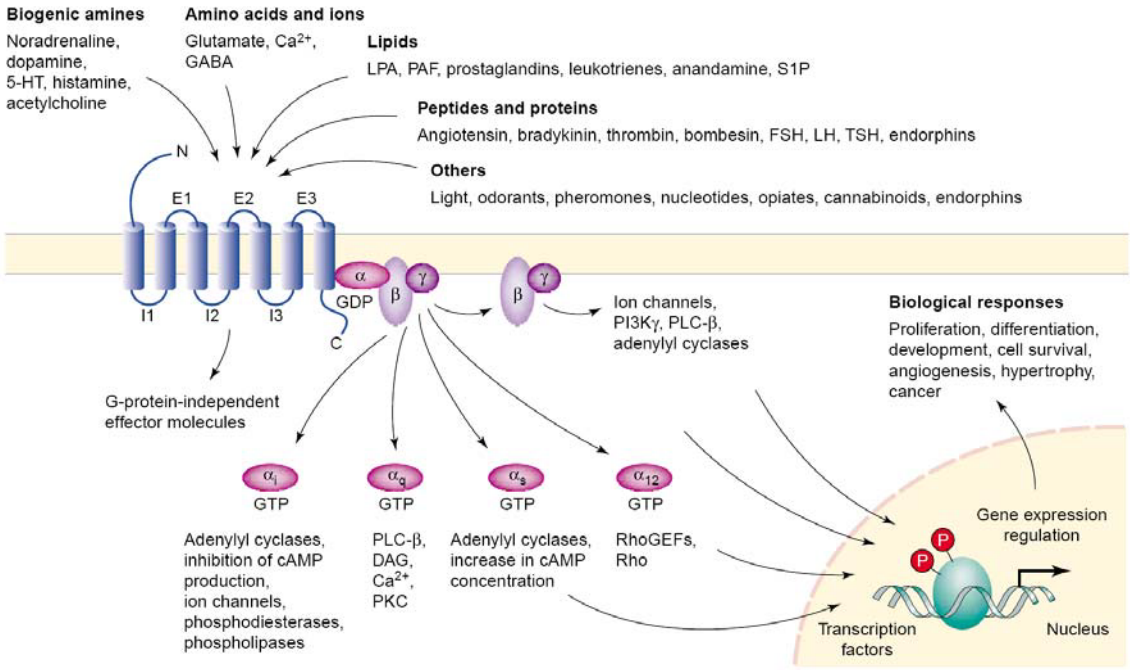

Figure 2. Diversity of G-protein-coupled receptors (GPCRs) and their signalling. A wide variety of ligands use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G-protein-dependent and -independent pathways. Such signalling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. Abbreviations: DAG, diacylglycerol; FSH, folliclesti-mulating hormone; GEF, guanine nucleotide exchange factor; LH, leuteinizing hormone; LPA, lysophosphatidic acid; PAF, plateletactivating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine-1-phosphate; TSH, thyroid-stimulating hormone. Taken from (Marinissen et al., 2001).

The most rapid means by which GPCRs are uncoupled from heterotrimeric G-proteins is through the covalent modification of the receptor through its phosphorylation by intracellular kinases (Figure 3). It is generally accepted that both second messenger-dependent protein kinases (both cAMP-dependent protein kinase (PKA) and protein kinase C (PKC)) and GRKs (G-protein coupled receptor kinases) phosphorylate serine and threonine residues within the intracellular loops and carboxylterminal tails of GPCRs (Lefkowitz et al., 1993; Krupnick et al., 1998). Second messenger-dependent protein kinases not only phosphorylate agonist-activated GPCRs, but also indiscriminately phosphorylate receptors that have not been exposed to agonist (Hausdorff et al., 1989). In contrast, GRK family members selectively phosphorylate agonist-activated receptors, thereby promoting the binding of cytosolic cofactor proteins called arrestins, which sterically uncouple the receptor from heterotrimeric G-proteins (Lohse et al. 1990).

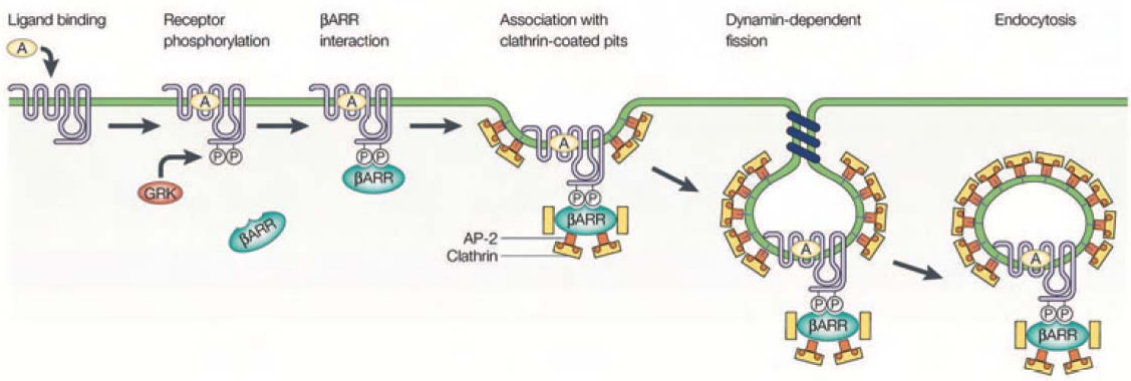

Figure 3. Desensitization and internalization model proposed for the GPCRs. After agonist (A) binding to G-protein-coupled receptors (GPCRs), GPCR kinases (GRK) phosphorylate residues in the third intracellular loop and carboxyl tail of GPCRs, leading to the recruitment of β-arrestins (βARR). The β-arrestins recruit clathrin and the AP-2 complex, which target GPCRs for clathrin-mediated endocytosis. Taken from (Pierce et al., 2001).

Internalization of GPCRs is a commonly observed response following agonist-stimulation. Over the years, numerous reports have addressed the role of receptor sequestration in desensitization and resensitization. While some evidence suggests that this phenomenon is part of the desensitization process, other evidence suggests that internalization is instead one of the means by which receptors are resensitized. Indeed, trafficking of an uncoupled receptor to

endosomal compartments allows dephosphorylation and recycling of the receptor to the cell surface (Krueger et al., 1997). This is in contrast to receptor "downregulation" observed after prolonged agonist exposure, which leads to targeting of the receptors to degradation pathways (Bohm et al., 1997). Once internalized, receptors are targeted to recycling or degradative pathways. GRKs and β-arrestins appear to be key regulatory molecules for receptor internalization since these proteins have been shown to interact with components of the clathrin-coated vesicle pathway (Figure 3). In response to GPCR activation, cytosolic β -arrestin proteins translocate to the plasma membrane and then subsequently redistribute to clathrin-coated pits bound to receptors (Figure 3). Nevertheless, not all GPCRs necessarily internalize in a β-arrestin-/clathrin-dependent manner but may also be internalized through alternative endocytic pathways. Some GPCRs have been found in cholesterol rich plasma membrane structures termed caveolae (Chun et al., 1994; Huang et al., 1997; Burgueño et al., 2003). These domains are also known as signalling domains, but appear to contain proteins involved in the formation and budding of vesicles such as the dynamin molecule. Finally, some receptors are suspected to use a third alternative endocytic pathway. No coat or adaptor proteins have been identified for the generation of these vesicles (Claing et al., 2000). However, GPCR desensitization and endocytosis can act as molecular switches coupling GPCRs to alternative signal transduction pathways. β-Arrestins not only function in the molecular switch required for GPCR desensitization and internalization, but also act as scaffolds to transduce and compartmentalize the alternative signals. In fact, β-arrestins have the ability to interact with a variety of endocytic and signalling proteins such as c-Src (Luttrell et al., 1999), MAPKs and Raf (DeFea et al., 2000).

1.1.1. GPCRs families

GPCRs do not share any overall sequence homology; the only structural feature common to all GPCRs is the presence of seven transmembrane-spanning α-helical segments connected by alternating intracellular and extracellular loops. Two cysteine residues conserved in most GPCRs (one in ECL1 and one in ECL2), form a disulfide bond, which is presumed to be important for the packaging and stabilization of a restricted number of conformations of these seven

transmembrane domains (Probst et al. 1992; Baldwin et al., 1994). Aside from wide sequence variations, GPCRs differ in the length and function of their extracellular N-terminal domain, their intracellular C-terminal domain, and the six alternating ICL and ECL loops. Each of these domains provides specific properties to the receptors. However, significant sequence homology is found within several subfamilies of the GPCR superfamily (Attwood et al., 1994). The three major subfamilies of receptors include those related to the "light receptor" rhodopsin and the β2-adrenergic receptor (family A), those related to the glucagon receptor (family B), and those related to the metabotropic neurotransmitter receptors (family C). Yeast pheromone receptors make up two minor unrelated subfamilies, family D (STE2 receptors) and family E (STE3 receptors). Finally, in *Dictyostelium discoideum*, four different cAMP receptors constitute yet another minor, but unique, subfamily of GPCRs (family F) (Kolakowski et al., 1994). A schematic representation is shown in Figure 4.

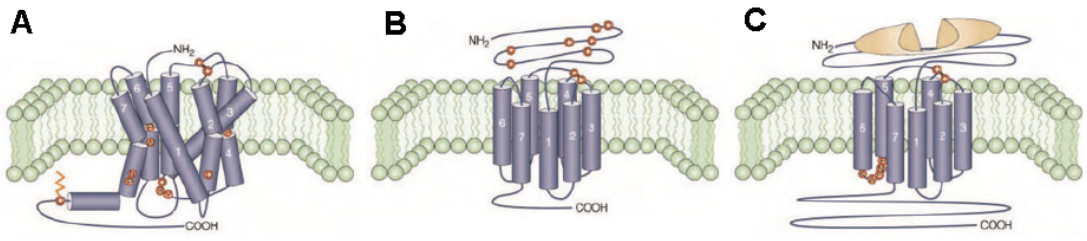

Figure 4. GPCRs classification. GPCRs can be divided into three major subfamilies A, B and C. Highly conserved key residues are indicated in red circles. Taken from (George et al., 2002).

The family A receptors, comprised of the rhodopsin/β2-adrenergic receptor-like subfamily, contain 90 % of all GPCRs and is by far the largest and most studied subfamily. The overall homology among all type A receptors is low and restricted to a number of highly conserved key amino acid residues. The high degree of conservation among these key residues suggests that they play an essential role for the structural or functional integrity of the receptors. The only residue that is conserved among all family A receptors is the arginine in the Asp-Arg-Tyr (DRY) motif, which is located on the cytoplasmic side of transmembrane (TM) segment 3 (Probst et al., 1992) and is believed to be involved in G-protein activation. To this family belong

the receptors studied in this thesis: adenosine, dopamine and cannabinoid receptor family.

Family B receptors includes approximately 20 different receptors for peptide hormones and neuropeptides, such as vasoactive intestinal peptide, calcitonin, and glucagon. Except for the disulfide bridge connecting the ECL1 and ECL2, subfamily B receptors do not contain any structural features in common with subfamily A. The DRY motif is absent. The most prominent characteristic of subfamily B receptors is a large (approximately 100 residues) extracellular amino terminus that contains several cysteine residues, presumably forming a network of disulfide bridges (Ulrich et al. 1998).

Family C receptors are characterised by an exceptionally long amino terminal domain (500–600 amino acids). This subfamily includes the metabotropic glutamate, the γ-aminobutyric acid (GABA), and the calcium receptors, among others. Similarly to families A and B, family C receptors contains two putative disulfide-forming cysteines in ECL1 and ECL2, but otherwise do not share any conserved residues. Each receptor in the subfamily C class possesses a very large extracellular domain that shares a low but significant sequence similarity to bacterial periplasmic binding proteins (PBPs). In bacteria, these proteins are involved in the transport of various types of molecules such as amino acids, ions, sugars or peptides, and are constituted of two lobes separated by a hinge region. Several studies including X-ray crystallography, indicate that the two lobes of these proteins close like a Venus flytrap upon ligand binding. The ligand-binding site of subfamily C receptors is believed to be located within the amino terminus (O'Hara et al., 1993; Conn et al., 1997).

The A-F subclassification of the GPCR superfamily is widely accepted, however, Fredriksson et al. (Fredriksson et al., 2003) after performing the first phylogenetic study of the entire superfamily of GPCRs proposed a more accurate classification. According to his classification method, also known as GRAFS, human GPCRs can be divided into five subfamilies that share a common evolutionary origin: Glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin sub-family. The A, B, C subfamilies of the older system are compatible with the new system (Attwood et al., 1994), whereas the two others are not included here.

1.1.2. Signalling pathways

G-protein coupled receptors owe their name to the interaction with heterotrimeric G-proteins, constituted of α (39-46 kDa), β (37 kDa) and γ (8 kDa). Upon ligand activation, conformational changes are induced that transmit from the receptor to G-protein and make the α subunit release GDP and bind GTP. This action permits a conformational change between the G_{α} subunit and the complex $G_{\beta\gamma}$ separating them. Both G_{α} and the $G_{\beta\gamma}$ complex acting with different effector molecules can activate or inhibit a big variety of second messengers. The signal terminates when the intrinsic GTPase activity of G_{α} hydrolyzes GTP to GDP and phosphate (Bourne et al., 1991). Four big families of G_{α} subunits exist in mammals, characteristic by their primary structure and a signalling cascade which they activate (Milligan et al., 2006). $G_{\alpha s}$ family stimulates adenylate cyclase, $G_{\alpha i/o}$ inhibits adenylate cyclase, $G_{\alpha q/11}$ activates phospholipase C_{β} (PLC_{β}) and $G_{\alpha 12/13}$ regulates Rho proteins.

Two typical examples of signalling cascades initiated by GPCRs are these that lead to formation of inositol-1,4,5-triphosphate (IP_3/DAG) and cAMP as second messangers. The effector protein target of $G_{\alpha q}$ subunit is PLC, enzyme that hydrolyses membrane phosphoinositols and generates IP_3 and DAG as second messangers. IP_3 increments intracellular concentration of calcium depleting its intracellular deposits, meanwhile DAG activates PKC. The effector molecule of $G_{\alpha s}$ and $G_{\alpha i}$ subunits is adenylate cyclase (AC), enzyme catalyzing conversion of ATP to cAMP, meanwhile $G_{\alpha s}$ stimulates it and $G_{\alpha i}$ inhibits it. cAMP activates PKA that as PKC phosphorylizes multitude of diverse proteins (receptors, ion channels, enzymes or transcription factors) regulating thus the functions of the cell.

Many responses mediated by GPCRs do not consist only of stimulation of conventional second messangers, but are a result of integration of different signalling networks among which MAPKs and JNKs can be included. The activation of MAPK via GPCRs was little studied till the last decade. It was known that this mechanism involved a *Bordetella pertussis* toxin sensible G-protein ($G_{\alpha i/o}$) and dependent strongly of the $G_{\beta\gamma}$ complex and on not identified tyrosine kinases (Faure et al., 1994; Koch et al., 1994; van Corven et al., 1993). Thus it

was deduced that in the absence of ligands with tyrosine kinase receptor (TKR) activity, the activation of GPCRs could induce the stimulation of TKR generating mitogenic signals. This phenomenon was called transactivation. Once transactivated, TKR initiated a signalling cascade identical to the one generated by its proper ligand, that means MAPK activation via the Ras, Raf, MEK, ERK 1/2 pathway (Figure 5).

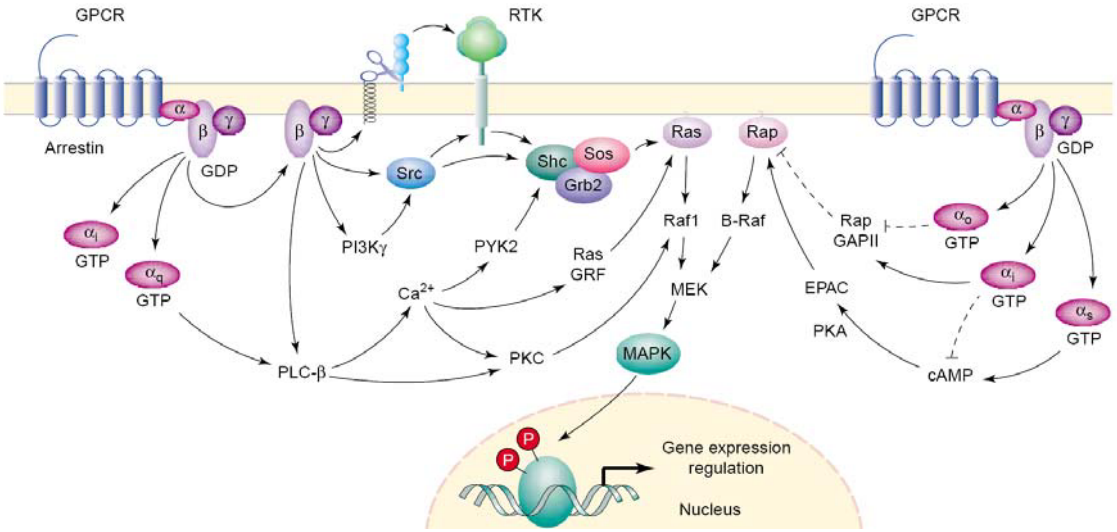

Figure 5. Scheme of multiple pathways linking GPCRs to mitogen-activated protein kinase (MAPK). Abbreviations: EPAC, exchange protein activated by cAMP; GAP, GTPase-activating protein; GRF, guanine-nucleotide releasing factor; MEK, MAPK kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PLC, phospholipase C; RTK, receptor tyrosine kinase. Taken from (Marinissen et al., 2001).

The process is initiated by the $G_{\beta\gamma}$ subunit resulting in recruitment of Sos towards the membrane. This activates the GDP/GTP exchange in the Ras protein, being this protein the intermediator connecting the signalling cascades generated by TKR transactivation and ERK 1/2 phosphorylation (Marinissen et al., 2001). Other pathway independent of transactivation exist that can lead to Ras activation. As for example, pathways dependent on intracellular calcium concentration induced by GPCRs coupled to $G_{\alpha q}$ (figure 5). Activation of ERK 1/2 requires a phosphorylation of two residues, serine and threonine, separated by only one amino acid and so can be performed only by a highly specialized enzyme, so that MEK is being considered a rate limiting step of ERK 1/2 activation. Finally, even $G_{\alpha s}$ subunit activation can lead to ERK 1/2 activation via the cAMP-PKA dependent signalling pathway. Activated ERK 1/2

is transferred to nucleus where it regulates via phosphorylation other kinases and transcription factors (Davis, 1995). On the other hand, new data revealed more of the complexity of GPCRs signalling showing that GPCRs can signal not only dependently of G-protein but also by G-protein independent mechanism which probably implicate direct union of Src and or β-arrestins to the receptors (Daaka et al., 1998; Lefkowitz, 1998; Lutrell et al., 2002).

1.1.3. GPCR interacting proteins

Many GPCRs contain sequence motifs that are known to direct protein-protein interactions and, therefore, have the theoretical capacity to interact with a wide range of other proteins. Such interactions might determine receptor properties, such as cellular compartmentalization or signalling, and can promote complexes that integrate their functions through protein scaffolding (Bockaert et al., 2004, 2006). The topology of GPCRs creates several potential regions for these interactions. Their extracellular loops are relatively short causing extracellular interactions to be dominated by the longer N-terminal sequences. However, on the intracellular face of the receptors, both the C-terminal tail and the third intracellular loop can be considerable in size. Therefore attention has been focused on these two regions. The extent of these GPCR-protein interactions varies, ranging from transitory (i.e. signalling purposes) to more stable interactions. However, GPCR-protein assemblies are considered dynamic complexes that contribute to the intricate process of downstream signalling (Franco et al., 2003). Several GPCRs interact with cytoskeletal anchoring polypeptides and can be considered a classical example of proteins that interact with GPCRs through cytoskeletal associations. This is the case of α-actinin and adenosine A_{2A} receptors (Burgueno et al., 2003), α-filamin and dopamine D_2 receptors (Lin et al. 2001), the Shank family of proteins, and several other GPCRs including type I metabotropic glutamate receptor 1 ($mGluR_1$) (Sheng et al., 2000). Apart from those interactions classically involved in signal transduction, a large number of intracellular GPCRs interactions have also been described. In the last few years, interactions between GPCRs and PDZ-domain containing proteins have been reported. PDZ-domain containing proteins play an important role as signalling modulators by defining the molecular composition of signalling complexes within microcompartments and

in the precise placement of theses complexes within the cell. The Homer-1β protein interaction with the $mGluR_1$ has been demonstrated to modulate Ca^{2+} induced mobilization of these receptors (Roche et al., 1999). However, spinophilin, another PDZ-domain containing protein, enriched in the dentritic spines (Allen et al. 1997) interacts with dopamine D_2 receptor through a novel non-PDZ domain-mediated mechanism and scaffolds it with protein phospatase 1 (PP1) (Smith et al. 1999; Richman et al. 2001). Additionally, a direct interaction of calmodulin with the third ICL3 of the dopamine D_2 receptor has been described (Bofill-Cardona et al., 2000). Calmodulin was found to bind to the human D_2 receptor at the amino acid domain 208-226 in a Ca^{2+} dependent manner and so inhibiting the G-protein activation. (Bofill-Cardona et al., 2000). In addition to the protein interactions at the intracellular domains of GPCRs, there is increasing evidence that extracellular protein-receptor interactions also might play an important role in modulating the pharmacology of GPCRs. An example of this type of protein is the enzyme adenosine deaminase that is able to interact with adenosine receptors.

1.1.3.1 ADA

Adenosine desaminase (ADA; EC.3.5.4.4, Figure 6) is a key enzyme involved in purine metabolism which catalyses the irreversible hydrolytic deamination of adenosine and 2'-deoxyadenosine to inosine or 2'-deoxyinosine and ammonia (Conway et al., 1939; Cristalli et al., 2001) (Figure 7).

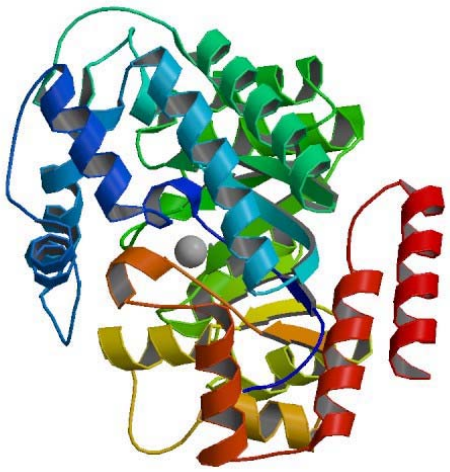

Figure 6. **ADA**. Ribbon diagram of bovine adenosine deaminase. Zinc ion visible at center. Taken from (Kinoshita et al., 2005).

ADA is distributed in all mammalian tissues (Van der Weyden et al., 1976). Although it is mainly cytosolic, it has been found on the cell surface of many cell types, including neurons (Ruiz et al., 2000); therefore it can be considered as an ecto-enzyme (Franco et al., 1998). The product codified by the human ADA gene has a molecular weight of 41 kDa and consists of 363 amino acids. There is a high degree of amino acid sequence conservation among species (Franco et al., 1998) being the bovine ADA the most similar to the human ADA (Daddona et al., 1984).

ADA plays a central role in the maintenance of immune system competence. Congenital defects of ADA lead to severe combined immuno-deficiency (SCID), which is characterised by the absence of functional T and B lymphocytes in affected individuals (Franco et al., 1998; Hershfield et al., 2003). Neurological abnormalities, which are less life threatening than immunological abnormalities, have also been described in some patients (Hirschhorn et al., 1985). Neurological alterations may be secondary to infections, or may be due to the accumulation of adenosine and its derivatives in the brain.

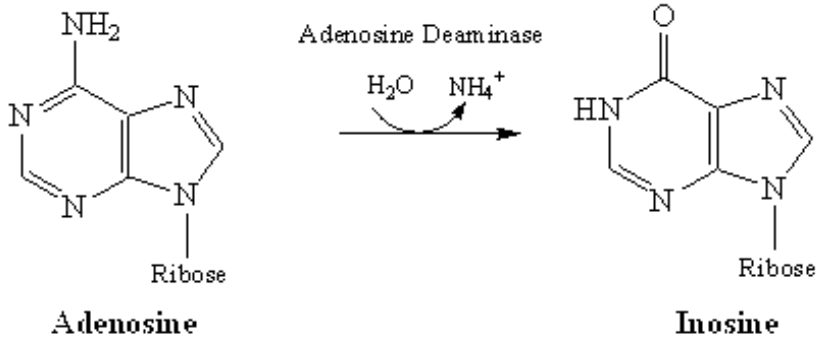

Figure 7. Deamination of adenosine catalysed by ADA.

Since ecto-ADA is a peripheral membrane protein it needs integral membrane proteins to be anchored to the membrane. CD26, a multifuncional transmembrane glycoprotein expressed on the T-cell surface was identified as an anchoring protein of ecto-ADA (Kameoca et al., 1993) and this interaction was seen critical for the regulation of adenosine signalling and for the potentiation of T-cells proliferation (Dong et al., 1996; Pacheco et al., 2005). In addition, it has been shown that ADA anchored to CD26 expressed on T-cells could bind to dendritic cells via adenosine A_{2B} receptors (A_{2B}Rs) expressed on their

surface, which led to important co-stimulation and enhancement of the immune response (Pacheco et al., 2005; Martinez-Navio et al., 2009; Climent et al., 2009).

Indeed, the second class of ecto-ADA binding proteins includes adenosine receptors, as up to now was demonstrated for A_1 adenosine receptors (A_1R) (Ciruela et al., 1996; Saura et al., 1996; Sun et al., 2005) and $A_{2B}R$ (Herrera et al., 2001). It was shown that ADA played and important role as an allosteric modulator of these receptors. The binding of both catalytically active and inactive ADA to A_1R and $A_{2B}R$ increased the agonist and antagonist affinity, potentiated signalling (Herrera et al., 2001) and modulated the desensitization of both receptors (Ciruela et al., 1996; Saura et al., 1998; Gracia et al., 1998). The radioligand binding assay with human striatum membrane preparations showed that the presence of ADA abolished the negative cooperativity in the A_1R homodimer (Gracia et al., 1998). Thus, ADA operates by two mechanisms upon adenosine receptors, indirectly by reducing the adenosine concentration preventing the receptors desensitization and directly by its binding increasing the affinity for their ligands. In the end, the physiological role of ecto-ADA interaction is to make these receptors more sensitive to adenosine (Gracia et al., 1998). In the brain in general, and specifically in the striatum, the action of ecto-ADA is very important as it modulates the adenosine receptors which are involved in the control of synaptic transmission regulating the motor actions by this brain area.

1.2. GPCRs heteromers

From the half of 90s several studies demonstrated oligomerization of numerous GPCRs (George et al., 2002) and today it is generally accepted as common feature of these receptors biology with the possibility of homodimer, heterodimer and or superior order oligomers formation (Bouvier et al., 2001; Devi et al., 2001; Agnati et al. 2003; Franco et al. 2003; Terrillon et al., 2004; Agnati et al., 2005; Prinster et al., 2005; Milligan et al., 2006; Pin et al. 2007; Carriba et al., 2008; Ferré et al., 2009). Homodimerization is defined as the physical association between identical proteins, whereas heteromerization is defined as the association between non-identical proteins. Protein-protein association can occur between two monomers forming dimers or between multiple monomers forming receptor oligomers. As

currently available techniques do not allow to distinguish between dimers or higher-order oligomers, the term dimers is often used, being the simplest form of an oligomer's functional unit that could explain the observations. Dimers/ oligomers present distinct functional characteristics compared to the single receptors they are constituted of, so that oligomerization brings new properties and functions to GPCRs. This phenomenon gives rise to a new complexity that rules over the signalling and regulation of these proteins.

1.2.1. Techniques used to identify GPCRs dimers

The first indirect evidence for the existence of GPCR dimers was provided by pharmacological studies. Complex radioligand-binding data demonstrating either positive or negative cooperativity, hinted at the possibility of physical interactions between receptors (Mattera et al., 1985). Radioligand binding assay remains to be a very important experimental tool to identify the presence of heteromers in native tissues after a so-called "molecular fingerprints" have been discovered from the studies with membrane preparations of transfected cells.

To demonstrate the formation of heteromers, experiments with chimeric (Maggio et al., 1993) or dominant-negative receptor mutants were performed. Chimeric α2-adrenergic/M3 muscarinic receptors were developed composed of the first five transmembrane domains of one receptor and the last two transmembrane domains of the other receptor. When either chimera was expressed alone, no binding or signalling could be detected, but coexpression of the two different chimeras restored binding and signalling to both muscarinic and adrenergic ligands. Such functional transcomplementations were interpreted as intermolecular interactions between inactive receptors in a way that restored both ligand binding and signalling domains within a heterodimeric complex (Maggio et al., 1993). Also, consistent with this idea, several receptor mutants behaved as dominant-negative mutants when expressed together with their cognate wildtype receptor (Benkirane et al., 1997; Bai et al., 1998; Zhu et al., 1998). In these cases, the observed blunted response was explained by invoking dimerization between wild-type and the inactive receptor. Similar experiment was very recently performed *in vivo* using a binding-deficient and signalling-deficient luteinizing hormone receptor in

$KO^{-/-}$ mice model background where these receptors could reestablish normal lueteinizing hormonal function through intermolecular functional complementation (Rivero-Muller et al., 2010).

To the first commonly used biochemical technique to investigate GPCR dimerization belongs the coimmunoprecipitation of differentially epitope-tagged receptors. The first study of this kind was performed in 1996 and demonstrated the specific interactions between α2-adrenergic receptors (Hebert et al., 1996). Since then, similar strategies have been used to document the homodimerization of the dopamine D_2 (Ng et al., 1996) and D_1 receptors (George et al. 1998), the $mGlu_5$ receptor (Romano et al., 1996), the CB_1 receptor (De Jesus et al., 2006), among others. Coimmunoprecipitation experiments also served to demonstrate the existence of heterodimers between closely related receptor subtypes, such as $GABA_BR1$ and $GABA_BR2$ (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) or δ- and κ-opioid receptors (Jordan et al., 1999), as well as more distantly related receptors including the adenosine A_1 and dopamine D_1 receptors (Gines et al., 2000), the adenosine A_1 and $GluR_1$ receptors (Ciruela et al., 2001) , the adenosine A_{2A} and $mGluR_5$ receptors (Ferre et al., 2002), or the CB_1 and D_2 receptors (Kearn et al., 2005). Although commonly used to study protein-protein interactions, coimmunoprecipitation of membrane receptors requires their solubilization using detergents, which may be problematic when studying highly hydrophobic proteins such as GPCRs that could form artifactual aggregates upon incomplete solubilization. So, the general acceptance of these complexes awaited a direct confirmation of GPCR dimerization living cells. This was made possible with the development and utilization of biophysical methods based on light resonance energy transfer.

The theory of resonance energy transfer was formulated in 1948 by Theodor Förster (Förster, 1948). This phenomenon is based on non-radiative energy transfer from a chromophore in excited state (donor) to a close molecule that absorbs it (acceptor). Techniques based on resonance energy transfer are applicable on living cells using a pair of fusion fluorescent/ luminiscence proteins cloned, most commonly, to the intracellular C-termini of GPCRs, that are transiently expressed in the cell of interest. In FRET (Fluorescence Resonance Energy Transfer, Figure 8) both donor and acceptor are

fluorescent molecules meanwhile in BRET (Bioluminiscence Resonance Energy Transfer, Figure 8) the donor is bioluminiscent and acceptor fluorescent.

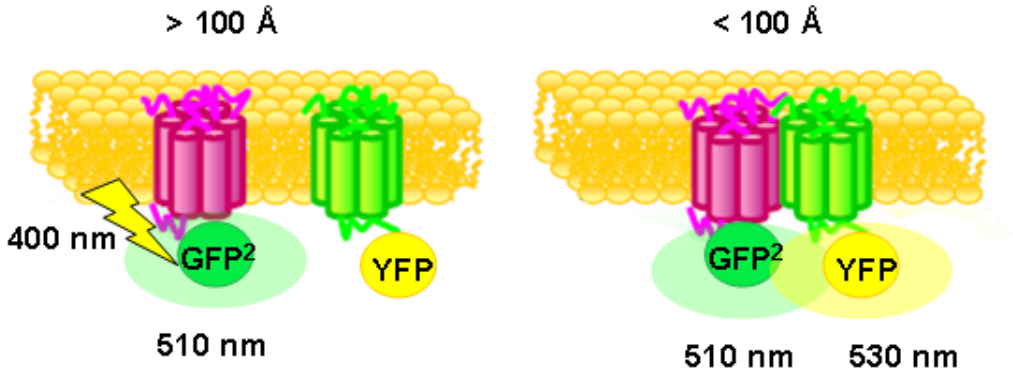

Figure 8. Schematic representation of FRET. For receptors closer than 100 Å, emission light of excited eGPF is able to excite YFP that emits light**.** Adapted from Dr. Gemma Navarro.

Luminiscence is a phenomenon occurring naturally in several marine animals such as medusas e.g. *Aequorea victoria* or sea pansy Renilla *reniformis*, of which *Rluc* (Renilla luciferase) was isolated. For to the resonance energy transfer could take place two requisites have to be fulfilled. The first is that the emission spectrum of the donor and excitation spectrum of the acceptor overlay in a manner that the emission energy of the donor can transfer directly to the acceptor fluorophore that emits light as if it was excited directly. The second requisite is the close proximity of donor and acceptor in space (equal or less than 100 Å or 10 nm). The efficiency of energy transfer decreases with the sixth potency of distance. It has to be noted that the major part or multiprotein complex in the cell is constituted between 10 and 100 Å (Stryer, 1978; Sheng et al., 2007).

Importantly, RET techniques can detect protein dimerization in living cells without disturbing the cellular environment by this phenomenon. BRET technique exists in two variants $BRET^1$ and $BRET^2$ (Figure 9). In $BRET^1$ the substrate of luciferase *Rluc* is coelenterazine H which when catalytically oxidised emits light of 480 nM what allows excitation of YFP that emits at 530 nM. In $BRET^2$ the donor of light is oxidised DeepBlueC emitting at 400 nM and acceptor GFP emitting at 510 nM (Figure 9).

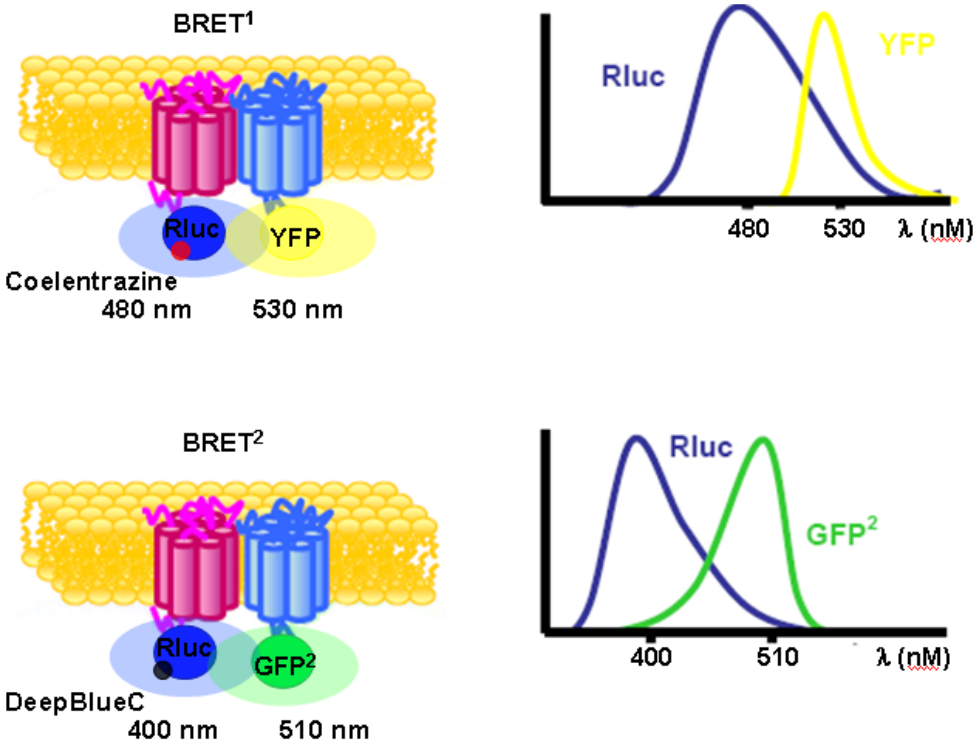

Figure 9. Schematic representation of BRET^1 and BRET^2 with the corresponding emission spectra. Adapted from Dr. Gemma Navarro.

Applying RET techniques, existence of different homodimers was discovered, for example β_2-adrenergic homodimer (Angers et al., 2008), δ-opioids (McVey et al., 2001), A_{2A} receptors (Canals et al., 2004); and heterodimers, for example somatostatin $SSTR_{2A}$ and $SSTR_{1B}$ (Rocheville et al., 2000b), A_{2A} and D_2 receptors (Canals et al., 2003), A_1 and A_{2A} receptors (Ciruela et al., 2006), A_{2A} and CB_1 receptors (Carriba et al., 2007), D_1 and H_3 histamine receptors and D_2 and H_3 (Ferrada et al., 2008, 2009) or D_1 and D_3 receptors (Marcellino et al., 2008), among others.

Our group has recently developed a combined resonance energy transfer method called SRET or sequetial BRET-FRET that permits identification of heterotrimers in living cells. In SRET, the oxidation of a Renilla luciferase (*Rluc*) substrate by a *Rluc*-fusion protein triggers acceptor excitation of the second fusion protein by BRET and subsequent FRET to the third fusion protein. Applying BRET^1 or BRET^2 gives rise to SRET^1 and SRET^2. Briefly, the in BRET^1 using coelenterazine H (485 nm) or BRET^2 using DeepBlueC (400 nm) the emission from Rluc allows energy transfer to a nearby YFP or GFP^2 acceptor, respectively. These acceptors emit 530-nm (YFP) or 510-nm

(GFP^2) light, which can result in a second energy transfer to DsRed or YFP, respectively, and concomitant emission of light at 590 nm or 530 nm. SRET will only occur between these fusion proteins if the two coupling pairs, *Rluc*/GFP^2 and GFP^2/YFP or *Rluc*/YFP and YFP/DsRed, are at a distance of less than 10 nm (Navarro et al., 2008). SRET technique allowed for the first time to identify the existence of $A_{2A}R$-D_2R-CB_1R heterotrimer in transiently transfected HEK cells (Navarro et al., 2008).

In recent years a more variants of FRET technique has been developed, like Photobleaching FRET and Time-resolved FRET (Pfleger et al., 2005) as well as a series of complementation resonance energy transfers methods like Biomolecular fluorescence complementation (BiFC) using two fragments of sYFP (nYFP, N- terminal fragment and cYFP, C-terminal fragment) that upon its spontaneous reconstitution when located in proximity (6 nM) become fluorescent (Hu et al., 2002). Similarly, fragments of *Rluc* that can reconstitute the enzymatic activity were generated (Paulmurugan et al., 2003). Finally also a combined complementation of two fluorescent fluorescent proteins can be used (Gehl et al., 2009) or combination of complementation of *Rluc* and YFP (Gandia et al., 2008).

Many of the studies characterising dimerization have been carried out in heterologous expression systems. A structural study performed by Palczewski using atomic force microscopy has demonstrated for the first time that rhodopsin exists as an array of dimers in the native retina, revealing an oligomeric organization of a GPCR *in vivo* (Palcewski et al., 2003).

1.2.2. Architecture of GPCRs dimers

Distinct intermolecular interactions have been found to be involved in various GPCRs homo- and heteromers. Disulphide bonds are important for the dimerization of the calcium-sensing and metabotropic glutamate receptor (Romano et al., 1996; Bai et al., 1998), coiled-coil interactions between the carboxyl tails are involved in the formation of the $GABA_BR1$-$GABA_BR2$ heteromer (Margeta-Mitrovic et al., 2000) and, finally, interactions between transmembrane domains have been proposed to be involved in the formation of β_2-adrenergic receptor and dopamine receptors

homodimers (Hebert et al., 1996; Ng et al., 1996). However, these proposed modes, rather than reflecting different strategies used by receptors of different classes, indicate that multiple sites of interaction are involved in the assembly and stabilization of receptor formed dimers. Computational studies proposed two alternative 3D models that could describe the assembly of GPCR dimers. Both models support the involvement of the 5th and 6th TM helices, and an important role for the third IC loop (Figure 10).

The first model is a "domain-swapping model", in which each functional unit within the dimer is composed of the first five transmembrane domains of one polypeptide chain and the last two of the second. This model would rationalize the functional complementation observed when mutant or chimeric receptors were studied. The second model is the "contact model", in which each polypeptide forms a receptor unit that touches the other through interactions involving transmembrane domains five and six (Gouldson et al., 2000).

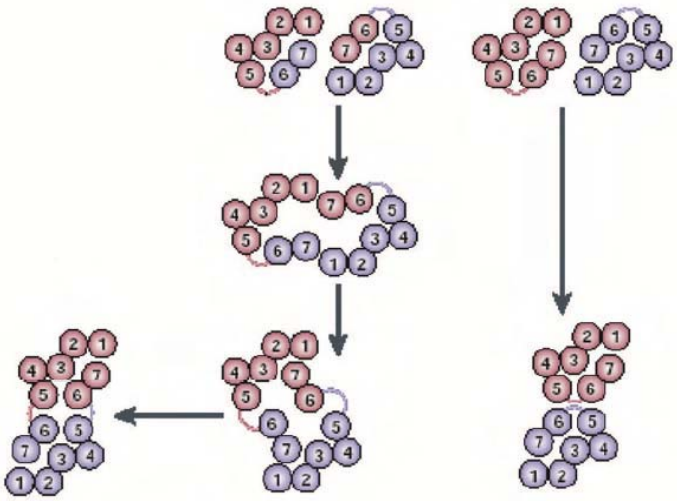

Figure 10. Alternative three-dimensional models showing dimers of GPCRs: "domain swapped" model (left) and "contact" model (right). Modified from (Gouldson et al., 2000).

Validation of these models awaits additional studies, the most direct of which would come from the resolution of the structure of a GPCR receptor dimer. Oligomeric assembly of proteins, allowing expanded diversity with a limited number of modular elements, is the rule rather than the exception in biology. When considering the nervous system, the existence of homo- and heterodimers of neurotransmitter GPCRs raises the hypothesis that could underlie the

high degree of diversity and plasticity that is characteristic of such a highly organised and complex system.

1.2.3. GPCRs heteromerization: functional consequences

The availability of a variety of techniques to study GPCR dimers has greatly facilitated the examination of a role for dimerization in regulating receptor function. This regulation has been found to exist on different levels, from modulation of receptor expression on the cell surface to adquisition of new pharmacological properties at the ligand binding level and signalling of receptor express in a dimer. It provides new perspective for the future development of drugs acting through GPCRs.

Even if in many cases the physiological relevance is not completely understood, several studies performed in heterologous expression systems suggested distinct functional roles of oligomerization of GPCR (Figure 11).

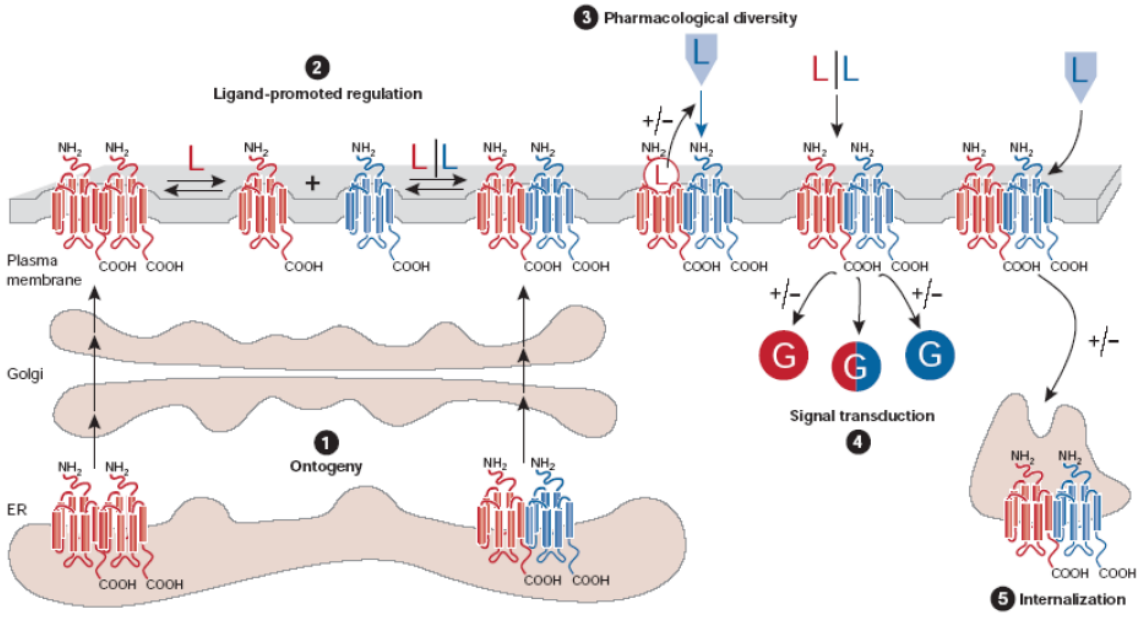

Figure 11. Proposed functional roles for GPCR oligomerization. ER, endoplasmatic reticulum, L, ligand. Taken from (Ellis et al., 2004).

For example, oligomerization can be implicated in the ontogenesis of GPCR, that means, in the control of protein folding and membrane targetting of the newly synthetised receptors as well as

their internalization (Law et al., 2005; Bulenger et al., 2005; Breit et al., 2004). Similarly, in some cases, it was observed that formation or separation of oligomers in the plasma membrane can be regulated by ligand activation. Concerning ligand binding, oligomerization confers new pharmacological properties to the receptor owing to the phenomenons of cooperativity and crosstalk, when binding of one ligand can modify the binding properties of the second receptor of the homo- or heterodimer (Ferré et al., 2007; Franco et al., 2008b), as will be discussed later. Concerning signalling via intereaction with G-protein, oligomerization can modify the signalling properties of a receptor leading to signalling potentiation, attenuation or even inverted signalling due to induction of coupling to other G-protein type. Finally, endocytotic pattern was seen to be affected by oligomerization (Terrillon et al., 2004).

One of the most significant observations to indicate that GPCR dimerization might be important in receptor folding and transport to the cell surface came from the studies of the metabotropic γ-amino-butyric acid receptor, $GABA_BR$. Three studies published simultaneously in 1998 (Jones et al., 1998; Kaupmann et al. 1998; White et al., 1998) demonstrated that co-expression of two isoforms of the $GABA_B$ receptor, $GABA_BR1$ and $GABA_BR2$, was a prerequisite for the formation of a functional $GABA_B$ receptor at the cell surface. The analysis of this phenomenon showed that the isoform $GABA_BR1$ is retained intracellularly as an immature glycoprotein and that, by contrast, the $GABA_BR2$ isoform is transported to the cell surface but cannot bind GABA or promote any intracellular signalling. Subsequently it was demonstrated that $GABA_BR2$ served as a chaperone that is essential for the proper folding and cell-surface transport of $GABA_BR1$. $GABA_BR1$/$GABA_BR2$ dimerization masks the ER-retention signal, thereby allowing ER transport and plasma membrane targeting of the dimer (Margeta-Mitrovic et al., 2000).

The paradigm that some GPCRs need to form heterodimers to reach the cell surface stems from the fact that dimers might correspond to the functional GPCR signalling unit, at least for signalling events involving G-protein activation. Increasing evidence indicates that class A GPCR dimer/oligomer biogenesis occurs at an early time point during receptor biosynthesis and processing in the ER and Golgi, where this could have an important role in the quality

control of newly synthesised receptors (Herrick-Davis et al., 2006). Once receptor oligomers are trafficked to the plasma membrane, both experimental and theoretical considerations suggest that dimeric GPCRs represent the basic functional receptor unit that engages heterotrimeric G-proteins. In addition, it is becoming increasingly clear that 'non-obligatory' heterodimers can also exist that display pharmacological and functional characteristics that differ from those of their constituent monomers can also exist (Bulenger et al., 2005).

Receptors within heterodimers may have different internalization mechanisms, heteromerization may also modulate agonist-induced trafficking properties of GPCRs. For example, the somatostatin receptor $SSTR_1$-$SSTR_5$ heterodimer is internalised despite the internalization-resistance of the $SSTR_1$ monomer (Rocheville et al., 2000a). Most family A GPCRs respond to agonist challenge by rapidly becoming internalised away from the cell surface and studies have indicated that the receptor internalizes as a dimer or oligomer (Yesilaltay et al, 2000).

Radioligand-binding studies have provided some insight into the physiological relevance of GPCR homodimers and heterodimers, since they can result in the generation of sites with novel ligand-binding properties. The first report about a heterodimer with distinct properties from their constituent receptors was the discovery of κ-δ-opioid receptors heterodimer (Jordan et al., 1999). κ-δ-receptor heterodimers do not exhibit high affinity binding for either κ- or δ-opioid receptor-selective ligands but, on the other hand, these heterodimers show high affinity for partially selective ligands. Heteromerization of GPCRs not only results in changes in the affinity of the receptors for various ligands, but binding of one ligand can be able to modify the efficacy or potency of another ligand binding to the neighbouring receptor. For example in A_1-D_1 receptor heteromer, A_1R agonists induced the disappearance of the high affinity binding sites of the D_1 receptor (Gines et al., 2000). In $A_{2A}R$-D_2R heteromer, binding of $A_{2A}R$ agonist was seen to reduce the efficiency of D_2R ligands binding by a negative cross-talk (Ferré et al., 1991), similarly as in $A_{2A}R$-A_1R heteromer, where A_1R agonist binding led to reduction of efficiency of $A_{2A}R$ agonist binding (Ciruela et al., 2006).

One of the first evidence that dimers formed a complex signalling unit demonstrated that the disruption of the dimer by a peptide fragment from the 6th TM domain inhibited the agonist-induced cAMP production. This was seen in the β2-adrenergic receptor homodimers (Hebert et al., 1996). In addition, heteromerization of two receptors may enhance the signalling of the first and inhibit the signalling of the second, as happened with angiotensin AT_1 and bradykinin B_2 receptor heterodimer (AbdAlla et al., 2000). Taking this into consideration, one of the main issues in elucidating the functional role of GPCR homodimers is to know whether agonist binding to a single subunit of the homodimer is sufficient for G-protein activation or whether both subunits in a ligand-loaded state are required. It has been described that agonist occupation of a single subunit in a dimer is sufficient for G-protein activation, results that are consistent with a number of studies demonstrating trans-complementation between a receptor defective in ligand binding and a receptor defective in G-protein activation (Carrillo et al., 2003; Milligan et al., 2005). Some current views of the physical organization of GPCRs and associated G-proteins favor a model in which GPCR dimers provide for the proper binding of a single heterotrimeric G-protein (Baneres et al., 2003; Fotiadis et al., 2004). Functional studies using the glutamate receptor demonstrated that only one receptor subunit per receptor dimer could reach a fully active state at a time (Goudet et al., 2005; Hlavackova et al., 2005)

1.2.4. Two state dimer model

Traditionally, when trying to analyse the ligand binding, GPCRs were considered as monomeric species. For this reason, up to now, a series of models considering a monomeric receptor as a basic unit were developed. When the fit of experimental binding data generates linear Scatchard plots, one binding site model can be considered that allows calculate the K_D (dissociation constant) values for the unique binding site. Nevertheless, the agonist binding to GPCRs often generates non-linear Scatchard plots, and in these cases, the results were fit traditionally to equations derived from two independent site models considering the existence of two independent states of receptor (non interconvertibles states): a high affinity state (or G-protein coupled) and a low-affinity state (or G-protein uncoupled). The data fit according to this model allows a calculation of two K_D values: one

for the high affinity state (K_{DH}) and other for the for the low affinity state (K_{DL}). Nevertheless, it was observed that the agonist can induce changes in the proportion of so called high affinity and low affinity states what indicated that these two states cannot exist separately but are interconnected (Wong et al., 1986). Working with adenosine A_1 receptors it was demonstrated that an agonist can induce an apparent change in the proportion of receptors in the high and low affinity state and this apparent interconversion between states is independent of G-protein (Casado et al., 1991). If an agonist is able to change the proportion of high and low affinity state, these two forms should be in equilibrium and consequently, the two independent states cannot adequately represent the behaviour of these receptors.

Given that nowadays it is known that GPCRs form dimers, the biphasic binding isoterms (non linear Scatchard representations) and the biphasic competition curves can be interpreted in a more direct way and can evidently be explained as a phenomenon of cooperativity. The positive or negative cooperativity can be explained naturally assuming that the binding of the first ligand molecule to one of the monomers in the homodimer modifies the binding parameters of the second ligand binding to the other monomer in the homodimer, as also occurs in the case of enzymes.

Recently, models considering a homodimer as a basic unit have been developed (Durroux, 2005; Franco et al., 2005, 2006; Albizu et al., 2006). Our group developed the so called "Two-State Dimer Receptor Model" (Franco et al., 2005, 2006). This model considers that the conformation change induced by a ligand binding to one of the dimer components is transmitted to the other component via the phenomenon of cooperativity and allows to calculate a parameter that measures the degree of cooperativity ($D_{c,}$ dimer cooperativity index). Assuming the receptor isomerization between inactive and active species, the model is able to explain the behaviour of GPCRs for which the Scatchard representation is not linear (Franco et al., 2005, 2006). Our group developed the equations from Two-State Dimer Receptor Model to fit binding data and to calcuate the macroscopic constant values from saturation and competition experiments, i.e., the macroscopic dissociation constant corresponding to the ligand binding to the non-occupied dimer (K_{D1}) and the macroscopic dissociation constant corresponding to the ligand binding to the semi-occupied

dimer (K_{D2}). These equations also allow the determination of the cooperativity index (D_c) that measures the structural changes sensed by one receptor when the other receptor in the homodimer is occupied by a ligand (Casadó et al., 2007).

1.3. Dopamine receptors

1.3.1. Dopamine

Dopamine (3,4-dihydroxyphenethylamine, Figure 12) constitutes approximately 80 % of the catecholamines present in the brain. Similar to other neurotransmitters, dopamine is unable to cross the blood brain barrier; however, its precursors, both phenylalanine and tyrosine readily cross this barrier allowing for its subsequent biosynthesis within neurons.

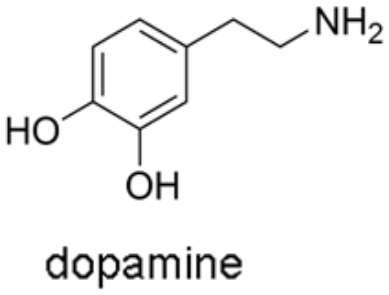

Figure 12. Dopamine. Chemical structure.

The biosynthesis of dopamine occurs within the cytosol of the nerve terminal after which the release of synthesised dopamine into the synaptic cleft leads to the subsequent sequence of events (Fuxe et al., 1965; Levitt et al., 1965). Calcium ion influx, via voltage-dependent calcium channels, triggers the fusion of the dopamine filled vesicles with the presynaptic membrane. A pore is formed and dopamine is then released into the synaptic cleft. Through diffusion it crosses the synapse and binds to dopamine receptors located pre- and postsynaptically. Upon binding, a conformation change in the receptor is induced that triggers a complex chain of intracellular events. The final outcome of dopamine release is either the activation or inhibition of the postsynaptically located neuron. Finally, the dopaminergic signalling is terminated through the re-uptake of dopamine by specific dopamine transporters (DATs) from the synaptic cleft to the

presynaptic terminal where dopamine can be stored and subsequently reused (Cooper et al., 1996; Amara et al., 1993).

Although the number of neurons that use dopamine as a neurotransmitter is rather small, this system of neurotransmission plays a very important role in many functions. Dopamine interacting with its central receptors in mammals influences a wide range of functions including movement, motivation, attention, cognition, affect, and control of pituitary hormone secretion (Missale, et al., 1998). The most prominent dopamine mediated function is the regulation of motor behavior. In the absence of dopaminergic tone, mammals are akinetic, or do not move. Increasing dopaminergic stimulation above the basal tone results in increased locomotion, and further increases the appearance of species-typical stereotyped motor patterns. In rats, stereotyped patterns take the form of focused sniffing, licking, or gnawing, and are used to determine overstimulation of dopaminergic signalling pathways. In recent years the dopaminergic system has become of great interest because of the relationship between deregulation of this system and several diseases such as Parkinson's disease, schizophrenia, Tourette syndrome, hyperprolactinemia and drug addiction (Missale et al., 1998, Vallone et al., 2000).

1.3.2. Dopamine receptors characteristics

Dopamine exerts its function via interaction with dopamine receptors which belongs to GPCRs of subfamily A: D_1R, D_2R, D_3R, D_4R, D_5R (Table 1). In 1978 dopamine receptors were first classified according to their activation or inhibition of adenylate cyclase (Spano et al., 1978). They were later classified in two subfamilies, D_1-like, which comprises D_1 and D_5, and D_2-like, including D_2, D_3, D_5 receptors. D_1-like receptors produce an increase of cAMP levels via $G_{s/olf}$ which stimulates AC and their localization is mostly postsynaptic in synaptic terminals (Civelli et al., 1993). D_2-like receptors inhibit AC via $G_{i/o}$ coupling, in addition activate K^+ channels and reduce Ca^{2+} entry through voltage-gated channels (Nicola et al., 2000). D_2-like receptors can be located on both presynaptic and postsynaptic terminals (Dal Toso, et al., 1989). D_1-like receptors contain a carboxyterminal domain about seven times longer than D_2-like receptors, while the latter have a very long third intracellular loop,

a common feature in many protein-coupled receptors G_i (Missale, et al. 1998).

Family	**D_1-like**		**D_2-like**		
Subtype	**D_1R**	**D_2R**	**D_3R**	**D_4R**	**D_5R**
G-protein	$G_{s/olf}$	$G_{s/olf}$	$G_{i/o}$	$G_{i/o}$	$G_{i/o}$
Mechanism of signal transduc-tion	+ AC + PLC	+ AC	- AC + PLC - channels Ca^{2+} + channels K^+	- AC + PLC - channels Ca^{2+} + channels K^+	- AC + PLC
Effector Molecules	↑AMPc ↑PKA ↑IP_3	↑AMPc	↓AMPc ↑IP_3 ↓Ca^{2+} ↑K^+	↓AMPc ↑IP_3 ↓Ca^{2+} ↑K^+ ↑Na^+/K^+ exchange	↓AMPc ↑arachid. acid ↑Na^+/K^+ exchange
Affinity for dopamine Kd (nM)	2340	261	2.8 - 274	4 - 27	28 - 450
Agonist **Kd (nM)**	SKF-38393 1 -150	NPA 187	Quinpirole 4.8 - 474	Bromocrip-tine 5 - 7.4	(-) Apomor-fine 4
Antagonist Kd (nM)	SCH-23390 0.11 - 0.35	SCH-23390 0.11 - 0.54	Raclopride 1 - 5	UH 232 2.9 - 9.2	Clozapine 9 - 42

Table 1. Dopamine receptors characteristics

The carboxyterminal, in both families, contains phosphorylation and palmitoylation sites that are believed to play an important role in receptor desensitization and formation of a fourth intracellular loop, respectively. All of the dopamine receptors subtypes share several conserved residues within their TMDs, which are believed to be the

minimal requirements for catecholamine binding. The two serine residues in the fifth TMD are thought to be involved in recognition of the two hydroxyl groups of catecholamines, and the aspartic acid residue located within the third TMD is thought to act as a counter ion for the amine group in biogenic amines (Hibert et al. 1991). D_1-like receptors contain two glycosylation sites at the amino terminal and the ECL2, while D_2 and D_3 receptors may have multiple (at least 4) glycosylation sites in their extracellular domains. To study the pharmacological properties of dopamine receptor, ligands that easily discriminate between D_1-like and D_2-like receptors are available, however, they are not selective for members of each subfamily. A remarkable difference inside the D_1-like receptors subfamily is the affinity for dopamine since the D_5 receptor is ten times more affine than the D_1 receptor (Missale, et al., 1998). Within the D_2-like subfamily, the D_3 receptor is the one with the highest affinity for dopamine (about 20 times higher compared to the D_2 receptor.

1.3.3. Dopamine D_2 receptor

Dopamine D_2 receptor (D_2R) is primarily found in brain tissue, including the caudate-putamen, olfactory tubercle and nucleus accumbens, where it is expressed by GABAergic neurons coexpressing enkephalins. In addition, the mRNA of this receptor is also found in the substantia nigra (SNr) and in the ventral tegmental area (VTA), the nuclei that give rise to the major dopaminergic pathways of the brain, indicating the role of D_2R as one of the main dopamine receptors to directly control the activity of dopamine containing neurons. However, the D_2R is also found outside the central nervous system, in the anterior and intermediate lobes of the pituitary gland, which indicates that it is also a primary dopamine receptor for regulating hormone release (Vallone et al., 2000).

The alternative splicing of the sixth exon generates the D_{2L} and D_{2S} isoforms (Figure 13). Studies performed using dopamine $D_{2L}R$ knock-out mice indicate a preferential involvement of D_{2L} receptors in postsynaptic responses while the $D_{2S}R$ appears to be preferentially expressed by midbrain dopaminergic neurons acting as an inhibitory autoreceptor (Mercuri et al., 1997; Rouge-Pont et al. 2002; Lindgren et al., 2003). Previous studies have shown that D_{2L} and D_{2S} receptors bind to distinct G-proteins, most likely due to their structural

differences. However, both isoforms function by binding to the pertussis toxin-sensitive G-proteins G_i or G_z, both of which have an inhibitory effect on adenylate cyclase that seems to be the predominant signalling pathway utilised by D_2Rs in the central nervous system (Kebabian et al., 1971; Enjalbert et al., 1983; Leck et al., 2006).

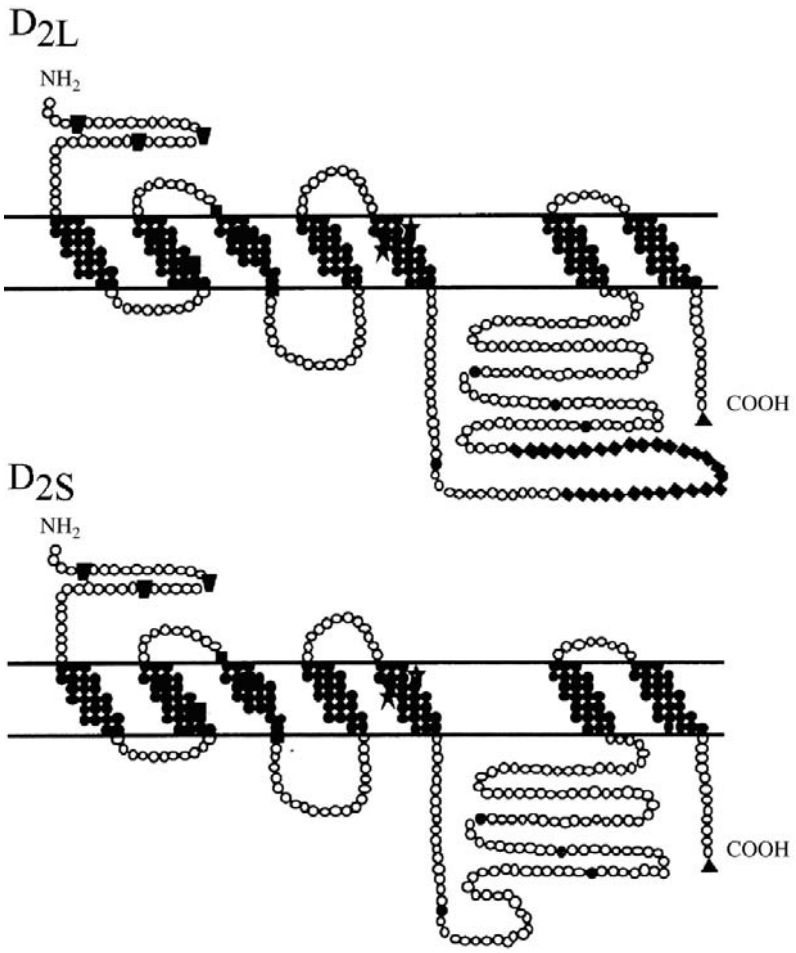

Figure 13. Schematic secondary structure of dopamine D_2 long (D_{2L}) and D_2 short (D_{2S}) receptors isoforms. Adapted from (Pivonello et al., 2007).

The D_2R G-protein coupling has also been described to modulate the activation of K^+ currents leading to cell hyperpolarization (Missale et al., 1998). Suppression of Ca^{2+} currents through L-type voltage dependent Ca^{2+} channels (L-type VDCC) is one of the best established adenylate cyclase-independent signalling pathways of D_2 receptors demonstrated in cell lines and striatal neurons (Ghahremani et al., 1999; Figure 14).

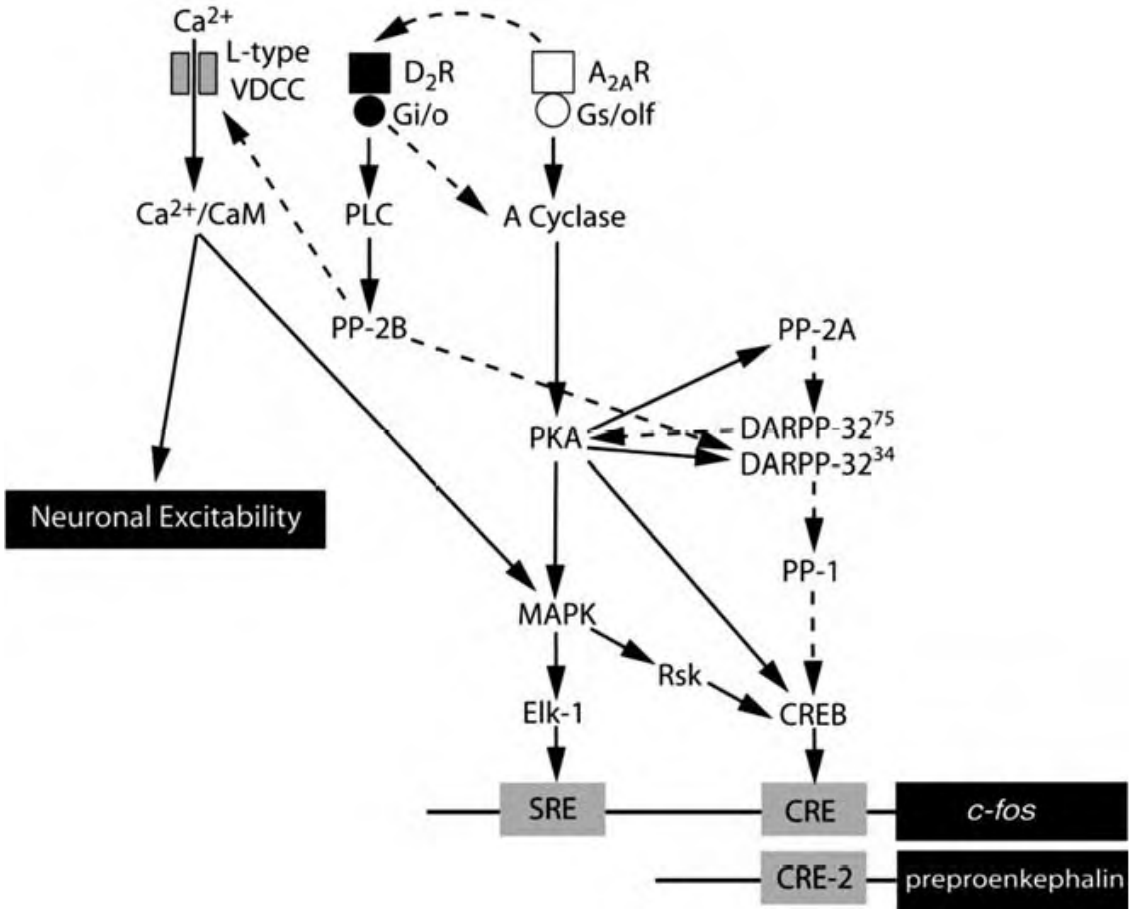

Figure 14. Signal transduccion of D_2R and its connection with $A_{2A}R$ signalling. Full lines represent stimulatory effects and dashed line inhibitory effects. Taken from (Ferré et al., 2004).

This pathway seems to involve the βγ subunits of the G-protein, PLC activation, IP3 dependent-intracellular Ca^{2+} mobilization and activation of the Ca^{2+} dependent serine-threonine phosphatase calcineurin (PP-2A) (Hernandez-Lopez et al., 2000). In addition, calcineurin is the main phosphatase involved in dephosphorylation of DARPP-32. Therefore, D_2 receptor activation produces DARPP-32 dephosphorylation both by inhibiting adenylate cyclase activity and by a Ca^{2+}/calcineurin-dependent and adenylate cyclase-independent mechanism (Nishi et al., 1997; Hakansson et al., 2006). Finally, dopamine D_2Rs stimulation has also been described to induce mitogen-activated protein kinase (MAPK) and cAMP response element-binding protein (CREB) phosphorylation in neurons (Yan et al., 1999). CaMK, PKC, DARPP-32 and elevated Ca^{2+} appears to be important in this pathway. Activation of MAPK has been proposed to play an important role in dopamine-induced regulation of gene

expression and long term neuronal adaptation in the striatum (Ferguson et al., 2003; Lee et al., 2006).

Baik et al. generated the D_2R knockout mice in 1995 (Baik et al., 1995). These mice showed a striking impairment of motor behavior (parkinsonian-like phenotype) supporting an essential role for these receptors in the dopaminergic control of movement. Increase in the density of postsynaptic D_2 receptors was observed in schizofrenia (Joyce et al., 1988) and in Parkinson's disease patients not treated with L-DOPA (Seeman et al., 1990). In mouse models of PD, dopamine depletion caused a loss of endocannabinoid-dependent LTD at excitatory synapses onto indirect pathway MSNs (Kreitzer et al., 2007; Shen et al., 2008b).

1.3.4. Dopamine D_1 receptor

Dopamine D_1 receptor (D_1R) is the most widespread dopamine receptor and is expressed at higher levels than any other dopamine receptor (Dearry et al., 1990). D_1R mRNA and protein have been found in the striatum, dorsally and ventrally in both the nucleus accumbens and the olfactory tubercle. In addition, D_1Rs have been detected in the limbic system, hypothalamus, and thalamus. D_1Rs are preferentially localised in striatal GABAergic neurons of indirect pathway, in MSN co-expressing substance P, mainly postsynaptically (Gerfen et al., 1990). D_1Rs couple with the heterotrimeric G-proteins $G_{\alpha s}$ and $G_{\alpha olf}$, which cause sequential activation of adenylate cyclase, cyclic AMP-dependent protein kinase, and the protein phosphatase-1 inhibitor DARPP-32. D_1R also signals via phospholipase C-dependent and cyclic AMP-independent mobilization of intracellular calcium (Neve et al., 2004). Increase of D_1R levels was observed in schizofrenia and altered signalling was detected in Parkinson's disease (Seeman et al., 1990).

1.3.5. Basal ganglia

The basal ganglia are located in the telencephalon and consist of several interconnected nuclei: the striatum, globus pallidus external segment (Gpe), globus pallidus internal segment (Gpi), substantia nigra (SN), and the subthalamic nucleus (STN; Figure 15).

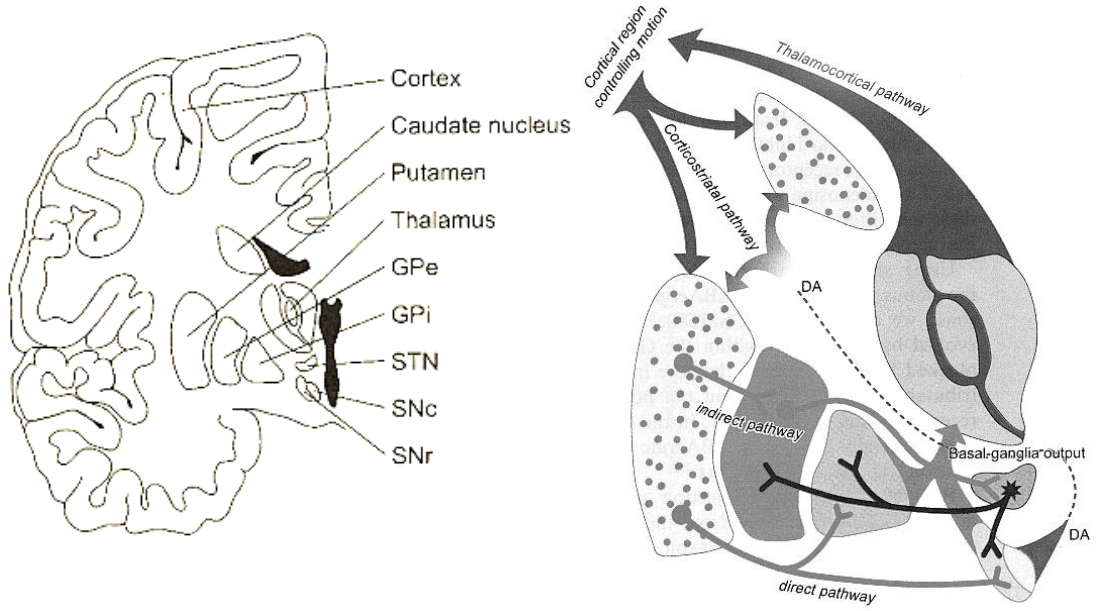

Figure 15. Basal ganglia. Left: Anatomy of the human basal ganglia structures and their localization in the brain. Right: Motor circuit of the basal ganglia, direct and indirect pathway. GPe – globus pallidus externum, GPi – globus pallidus internum, STN – subthalamic nucleus, SNc – substantia nigra pars compacta, SNr – substantia nigra pars reticulata; DA – dopamine. Taken from (Kase, 2001)

The striatum is a major component of basal ganglia (Kase, 2001). Striatum can be divided in dorsal and lateral part. In primates, the dorsal striatum is divided by the internal capsule into the medially located caudate nucleus and the laterally positioned putamen. Putamen (the dorsomedial striatum) receives inputs primarily from association cortex (Goldman et al.,1977, Ragsdale et al., 1981) and caudate nucleus (dorsolateral striatum) receives inputs from sensorimotor cortex (Kunzle et al., 1975, Liles et al., 1985). The ventral striatum, or nucleus accumbens, represents a third subdivision of the striatum (Nicola, 2007). The ventral striatum, like the patches of the dorsal striatum, receives glutamatergic inputs from frontal cortex and limbic regions (Brog et al., 1993). However, the dopaminergic innervation of the ventral striatum derives from the ventral tegmental area, a separate midbrain nucleus adjacent to the substantia nigra parts compacta, SNc (Fields et al., 2007). Dorsal striatum (caudate nucleus and putamen) is implicated in execution of learning or complex motor behaviour, ventral striatum (nucleus accumbens) participates in conversion of motivation to action. The receptors for neurotransmitters can be expressed differently in distinct anatomical parts of striatum: For example, cannabinoid CB_1Rs receptors are highly expressed in

nucleus accumbens and caudate nucleus, but not in putamen (Herkenham et al. 1991).

Medium spiny projection neurons (MSNs) are the most numerous in the dorsal striatum, with at least 75 % of neurons belonging to this type in primates (Graveland et al., 1985a; Tepper et al., 2010), and up to 95 % in rodents and in the cat (Kemp et al.,1971; Graveland et al., 1985b). The second class of neurons present in the dorsal striatum are interneurons (GABAergic or cholinergic), that are typically spiny, and unlike the medium spiny neurons, do not send projections outside the striatum (Phelps et al., 1985; Cowan et al., 1990; Kawaguchi, 1993; Kawaguchi et al., 1995; Wu et al., 2000; Tepper et al., 2010). Finally, the striatum also contains a small amount of dopaminergic intrinsic neurons. Although the number of these neurons is almost vestigial in normal rodent striatum, it is more prevalent in the primate striatum (Dubach et al., 1987; Ikemoto et al., 1996).

The medium spiny neurons:
- Form dendritic spines via that make synaptic connections (Kreitzer et al., 2009)
- are inhibitory using the neurotransmitter gama-amino-butyric acid
- express DARPP-32
- obtain glutamatergic innervation from cortex and dopaminergic innervation form substancia nigra
- have a unique firing pattern (Kreitzer et al., 2009).

The medium spiny neurons can be divided into two types (Figure 16) according to expression of different peptides and neuro-transmitter receptors. MSNs of direct pathway express dynorphin and substance and D_1 receptor coupling to stimulatory G_s. They are also called striatonigral MSNs. They project directly to GPi and SNr (Figure 16). Stimulation of the direct pathway results in motor activation (Figure 17). Cortical glutamatergic synapses connecting to striatonigral neurons express adenosine $A_{2A}R$ forming, almost in part, heteromers with adenosine A_1R receptor. MSNs of indirect pathway express enkephalin and D_2 receptors coupling to inhibitory Gi. They are also called striatopallidal MSNs. They project to GPe (Figure 16). Stimulation of MSN of indirect pathway results in motor inhibition (Figure 17).

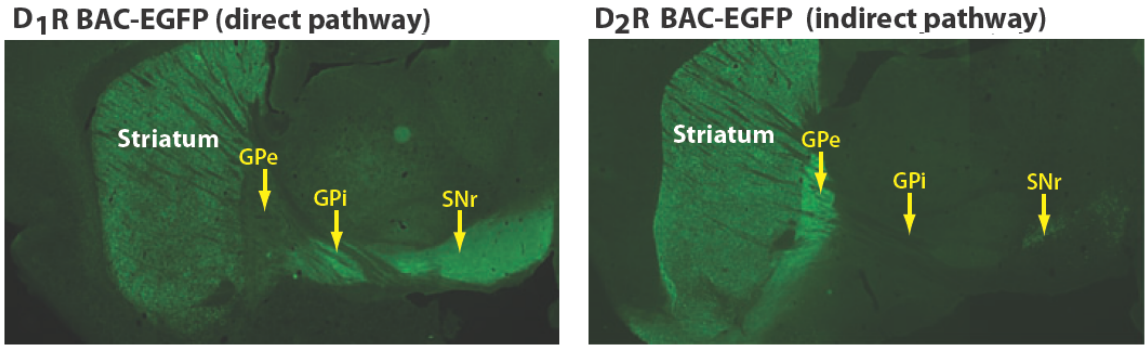

Figure 16. Visualisation of the direct and indirect pathways in fluorescent BAC mice models. Fluorescent imaging of a brain section from a mouse expressing enhanced green fluorescent protein (eGFP) under regulation of the D_1R promoter shows D_1R-expressing MSNs in the striatum that project axons through the GPe, which terminate in the GPi and GPe. Fluorescent imaging of a D_2R-eGFP mouse shows that labeled MSNs provide axonal projections that terminate in the GPe but do not extend to the GPi or SNr. Taken from review (Gerfen et al., 2011), original article by (Gong et al., 2003).

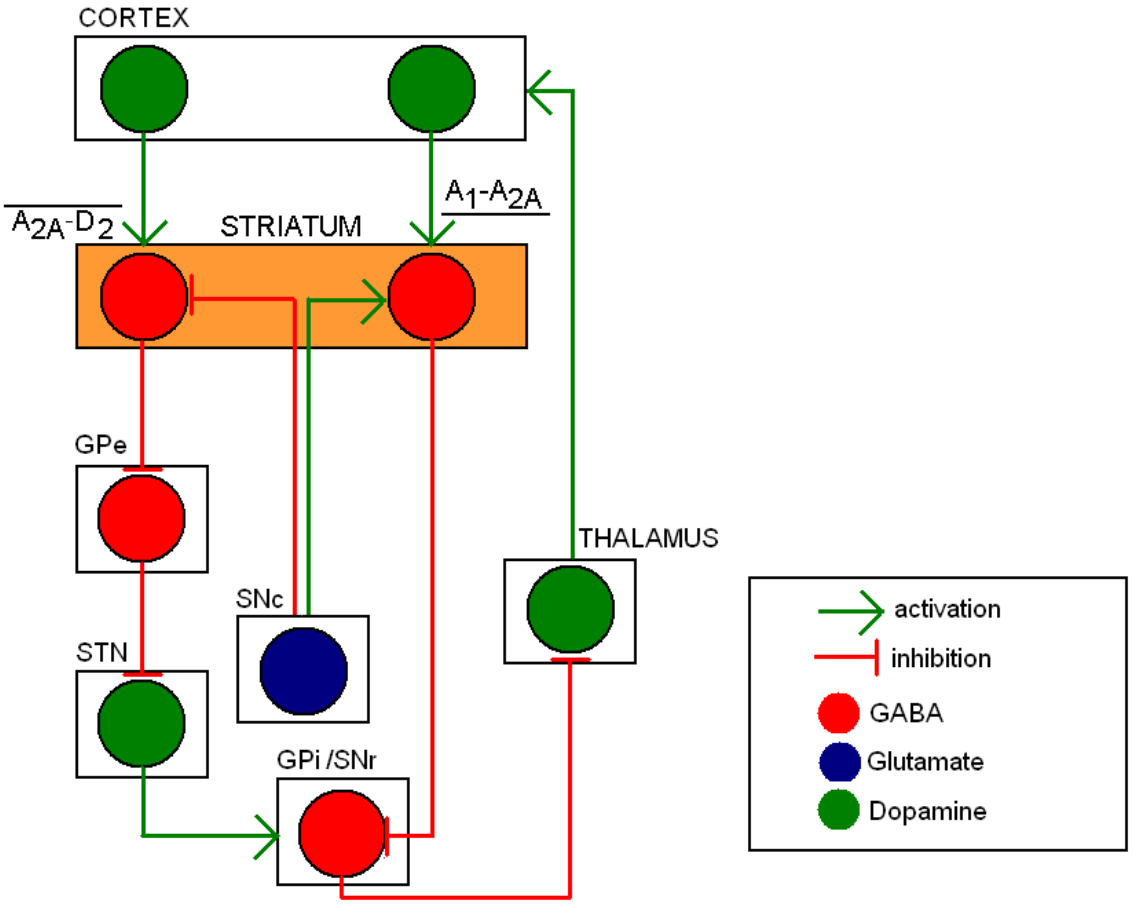

Figure 17. Motor circuit of basal ganglia, direct and indirect pathway. Direct pathway induces movement and indirect pathway inhibits movement. $A_{2A}R$-D_2R heteromers are located in the postsynaptic terminal of the corticostriatal synapsis of the indirect pathway and A_1R-$A_{2A}R$ in the presynaptic terminal of both direct and indirect pathway. Adapted from Dr. Sergi Ferré.

In HD striatopallidal neurons degenerate first are their dysfunction or degeneration leads to hyperkinetic movements. In striatopallidal MSNs D_2R is coexpressed with adenosine A_{2A} receptor (Schiffmann et al., 1991, 1993, 2007; Svenningsson et al., 1999) in the postsynaptic part of corticostriatal synapses. The excitability of both neuronal types is slightly different: D_2-containing MSN are more excitable (Day et al., 2008) and contain more tree branching (Gertler et al. 2008).

Direct and indirect pathways converge in Gpi/SNr, the main output of basal ganglia motor circuit (Figure 16). Gpi/SNr neurons are inhibitory, GABAergic and project to thalamus. Thalamus projects back to cortex with glutamatergic efferents. So according to both inputs from direct and indirect pathway the final outcome is transmitted back to cortex. Direct pathway tends to activate voluntary movements and indirect pathway inhibits involuntary components of movement. An adequate equilibrium between both pathways produces normal movements. Dopamine, produced by neurons from substancia nigra compacta, is the key regulator of the correct functioning of basal ganglia, it induces motor activation via activation of D_1R in striatopalidal neurons of direct pathway and inhibition of D_2R in striatonigral neurons of indirect pathway, that means potentiating the stimulatory direct pathway and depressing the inhibitory indirect pathway. Dopamine thus stimulates movement acting upon both pathways. In Parkinson's disease, due to depletion of dopamine because of the degeneration of nigrostriatal neurons, the movement depression or hypokinesia is experienced. Excess of dopaminergic stimulation would lead to hyperkinesia. In Huntington's disease, hyperkinetic choreic movements are due to a gradual disappearence of the contribution of the indirect inhibitory pathway, but at the end, the subsequent degeneration of the direct pathway and the nigrostriatal neurons finally leads to disappearance of movement (Glass et al., 2000).

The classification of MSNs projection in direct and indirect pathway belongs to the classical vision of basal ganglia motor circuitry, as it was defined in mid 1980s (Penney et al., 1986; Albin et al., 1989; DeLong, 1990). Recently the vision of basal ganglia circuit becomes to change and is not seen as simple "go through" structure anymore, where the connectivity and functional interactions occur

unidirectionally, but other microcircuits appear and more extensive reciprocal innervation is observed and the issue becomes more complex (Obeso et al., 2011).

1.4 Adenosine receptors

There are four known subtypes of adenosine receptors (ARs) – referred to as A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R (Figure 18)– each of which has a unique pharmacological profile, tissue distribution and effector coupling (Table 2).

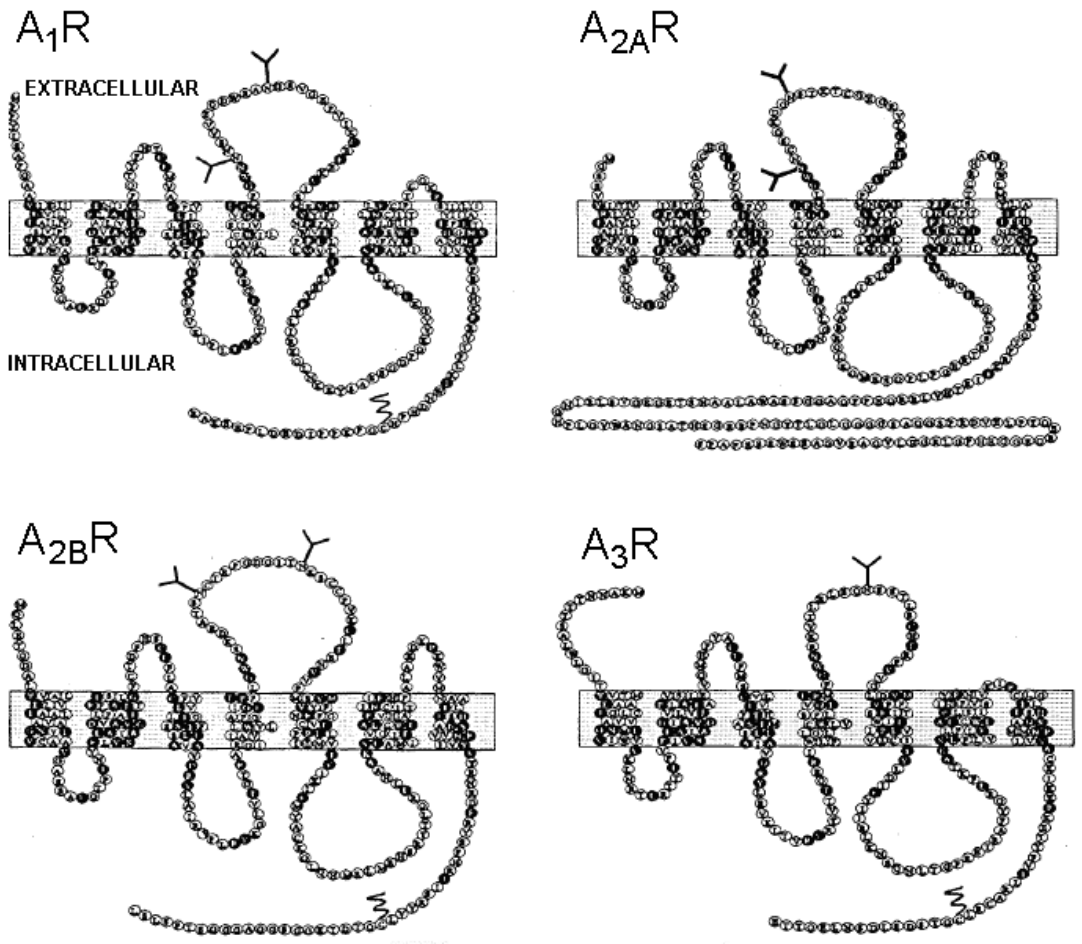

Figure 18. Scheme of adenosine receptors. Very long C-terminal tail of the $A_{2A}R$ lacking palmitoylation site. Glycosylation sites in EC2 of all adenosine receptors. Taken from PhD thesis of Dr. Francisco Ciruela.

All possess glycosylation sites and all but $A_{2A}R$ a palmitoylation site near the carboxyl terminus, that would allow another insertion in the membrane generating a fourth intracellular loop that has been

suggested to participate in the coupling of the receptor to the G-protein (Bouvier et al. 1995). A_1R, $A_{2A}R$, and A_3R have a molecular weight of 36.7, 36.4 and 36.6 kDa respectively, whereas due to its long C-terminal tail $A_{2A}R$ has a molecular weight of 45 kDa (Palmer et al., 1995). Originally only two adenosine receptors were known and they were classified on their effect on cAMP levels in different tissues, these were A_1R (inhibitory) and $A_{2A}R$ (stimulatory). A_1R and $A_{2A}R$ show high affinity for their natural agonist adenosine and with major expression profiles in the CNS and periphery. Later, $A_{2B}R$ and A_3R were discovered. Their activation becomes more relevant in states of notoriously incremented adenosine levels, at micromolar adenosine concentration (Fredholm et al., 2001) according to their lower affinity for agonists. A_1Rs and A_3Rs couple to $G_{i/o}$ and $A_{2A}R$ and $A_{2B}R$ couple to G_s.

Experiments with chimeric A_1/A_{2A} receptors indicate that structural elements in both the third intracellular loop and the carboxyl terminus influence coupling of A_1Rs to G_i, whereas elements in the third intracellular loop but not the carboxyl terminus contribute to $A_{2A}R$ coupling to G_s (Tucker et al., 2000). The homology between receptor subtypes is quite low, about 45 % (Stehle et al., 1992) with areas of high homology located within the transmembrane regions. These regions, together with the second extracellular loop, were proposed to be mainly involved in ligand binding (Rivkees et al. 1999), while interaction with G-proteins occurs within the third intracellular loop. Moreover, adenosine receptors contain several features common to all G-protein coupled receptors: Cys residues on the extracellular loop that may be involved in disulfide bond formation and confer a conformational stability to receptors after insertion to the plasma membrane (Dohlman et al., 1990). All the cloned adenosine receptors present a “DRY” sequence which has been suggested to mediate G-protein activation. Each of the adenosine receptors possesses consensus sites for N-linked glycosylation on their second extracellular loops that is involved in membrane targeting (Klotz et al., 1986). Intracellular domains phosphorylation sequences consensus are present and phosphorylation is implicated in receptors desensitisation (Ramukar et al., 1991; Palmer et al., 1994; Saura et al., 1998).

Subtype	**A_1R**	**$A_{2A}R$**	**$A_{2B}R$**	**A_3R**
G-protein	$G_{i/o}$	$G_{s/olf}$	$G_{s/q}$	$G_{i/q}$
Mechanism of signal transduction	- AC + PLC - channels Ca^{2+} + channels K^+	+ AC - channels Ca^{2+}	+ AC + PLC	- AC + PLC
Effector molecules	↓AMPc ↑IP3 ↓Ca^{2+} ↑K^+	↑AMPc ↑IP3 ↑$Ca2^+$	↑AMPc ↑IP3 ↑Ca^{2+}	↑AMPc ↑IP3 ↑Ca^{2+}
Affinity for adenosine Kd (nM)	70	150	5100	6500
Selective Agonist	R-PIA	CGS-21680	-	IB-MECA
Selective Antagonist	DPCPX	ZM-241385	MRS 1706	L-268605
Physiologic action	Inhibition of synaptic transmission and motor activity. Hyperpolarization. Ischemic preconditioning.	Facilitates the liberation of neurotransmitters. Integration motorsensorial.	Modulation of calcium channels.	Ischemic preconditioning.

Table 2. Adenosine receptors characteristics

1.4.1. Adenosine

Adenosine is an endogenous nucleoside formed by a purinic base adenine bound to a ribose by a β-N-glycosilic bond (Figure 19).

Adenosine and its derivatives are essential constituent of all living cells. It plays a structural role as a building block of nucleic acids, in cellular metabolism (energy storage: ATP), as intracellular regulators (cofactors: NAD+, NADP+, FAD; second messenger in cellular signalling: cAMP) (Arch et al., 1978; Pull et al., 1972) and as neuromodulator in the control of synaptic transmission acting on adenosine receptors (Cobbin et al., 1974).

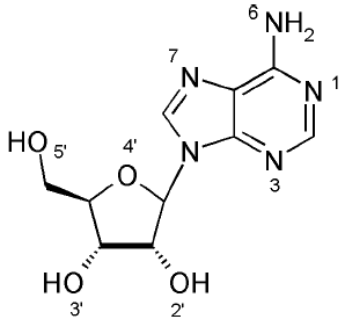

Figure 19. Adenosine. Chemical structure

Under normal conditions, adenosine is continuously formed intracellularly as well as extracellularly. Basal concentrations of this nucleoside reflect an equilibrium between synthesis and degradation (Frendholm et al., 2001). The intracellular production is mediated by intracellular 5'-nucleotidase, which dephosphorylates AMP (Schubert et al., 1979; Zimmermann et al., 1998) and by hydrolysis of S-adenosyl-homocysteine (Broch et al., 1980). Adenosine generated intracellularly is transported into the extracellular space mainly via equilibrative transporters. The dephosphorylation of extracellular AMP to adenosine, mediated by ecto-5'-nucleotidase, is the last step in the breakdown of extracellular adenine nucleotides, such as ATP, to adenosine (Dunwiddie et al., 1997; Zimmermann et al., 1998). Adenosine can also be released into the extracellular space after neuronal activation with specific neurotrans-mitter ligands. Glutamatergic agonists, such as NMDA or kainate, dose-dependently increase adenosine levels (Carswell et al., 1997; Delaney et al., 1998). Dopamine D_1 receptors enhance adenosine release via an NMDA receptor-dependent increase in extracellular adenosine levels (Harvey et al., 1997). Extracellular levels of adenosine are decreased by specific adenosine transporters and by extracellular adenosine deaminase (Fredholm et al.,1994b, Lloyd et al., 1995). Under physiological conditions, extracellular adenosine concentrations remain very low (20-300 nmol/L, Delaney et al., 1996), whereas

traumatic or hypoxic events, stressful situations and increased neurotransmitter release lead to a several 100-fold increase of extracellular adenosine levels (Latini et al., 2001).

In the brain, which expresses high levels of adenosine receptors, adenosine is secreted by the majority of cells, including neurons and glia, and neuromodulates the activity of CNS in both normal and pathophysiological processes, acting on pre- post- and/or extra-synaptic sites. Accordingly, adenosine was seen to play a role in the inhibition of excitatory neurotransmitters release (Phillis et al., 1979; Ciruela et al., 2006), inhibition of spontaneous motor activity, neuronal differentiation and migration (Rivkees et al., 1995; Canals et al., 2005), memory and learning (Wei et al., 2011), regulation of sleep (Antle et al., 2001), anxiety (Johansson et al., 2001) and excitation, and neuroprotection during hypoxia/ischemia (Pedata et al., 2005). It was related with Alzheimer's disease (Maia et al., 2002), Parkinson's disease (Schwarzschild et al., 2002), schizofrenia (Ferré et al., 1997), epilepsy (Rebola et al., 2005b), drug addiction (Brown et al. 2008) and finally Huntington's disease (Reggio et al., 1999), as will be further discussed in this thesis.

Caffeine is a weak, virtually non-selective adenosine receptor antagonist. It served as a structural model in organic synthesis of more potent and selective antagonists. So the first adenosine receptor antagonists were xanthine (caffeine and theophylline) derivatives (to this groups belongs MSX-2 and KW-6002), followed by second class of complex nitric heterocycles (pyrazolo-thiazolo-pyrimidin-like: ZM-241385, SCH-58261, SCH-442416; Figure 20), and more recently by structurally unrelated compounds found by library compound screening (Jacobson et al., 2006, Cristalli et al., 2007).

The pharmacology of caffeine is still investigated. Studies of the affinity of caffeine for adenosine receptors in the brain tissue brought variable results: showing either no difference, a preferential affinity for $A_{2A}R$, or a preferential affinity for A_1R. In the studies using cloned transfected receptors, caffeine displays higher affinity for $A_{2A}R$ than for A_1R (Ciruela et al., 2006). Interestingly, it was found that the affinity of caffeine for the $A_{2A}R$ depends on the presence of co-transfected receptors. In HEK cells expressing $A_{2A}R$-D_2R heteromer the affinity of caffeine for $A_{2A}R$ was the same as in the cells

expressing only $A_{2A}R$ but considerably decreased in cells expressing A_1R-$A_{2A}R$ heteromers (about 12 times). In A_1R-$A_{2A}R$ heteromer the affinity of caffeine was the same for both adenosine receptors (Ciruela et al., 2006). This discovery supports the fact that the heteromerization changes the receptors pharmacology.

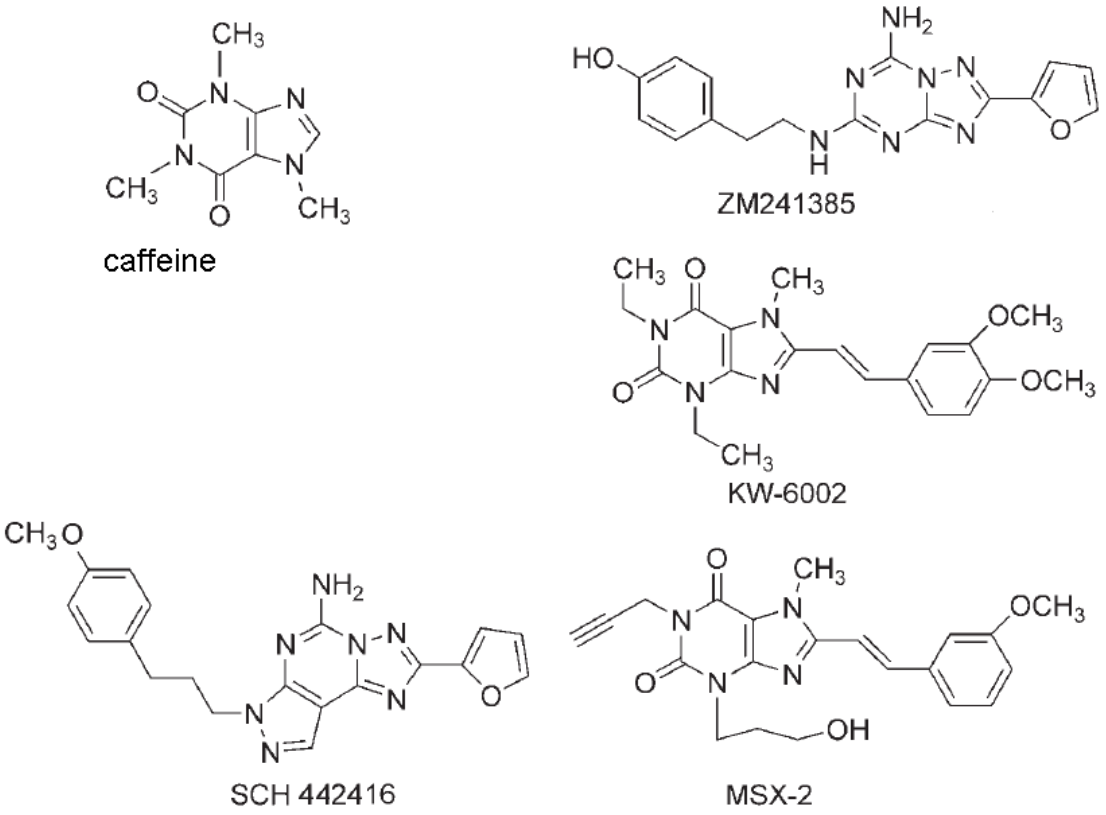

Figure 20. $A_{2A}R$ antagonists. Formula of natural $A_{2A}R$ antagonist caffeine and some selected synthetic compounds.

1.4.2 Adenosine A_{2A} receptors

From the first studies of distribution of $A_{2A}R$ performed with autoradiography using ^{3}H-CGS-21680 high levels of staining were seen in striatum, both in dorsal (caudate-putamen) and ventral striatum (nucleus accumbens), in addition in olfactory tubercule and globus pallidus externum in rat (Jarvis et al., 1989; Wan et al., 1990) and human brain (Martinez-Mir et al., 1991, Glass, 2000). From northen blot (Fink et al., 2002; Peterfreund et al., 1996) and in situ hybridization studies (Schiffman et al., 1991; Fink et al., 1992; Svenningsson et al., 1998, 1999) it was further evident that striatal $A_{2A}R$ was almost exclusively expressed in the medium spiny neurons

of the indirect pathway expressing enkephalin and co-expressing D_2 receptor and not (or at best only to a limited extend) in MSN of direct pathway. By means of more sensible techniques, like immuno-histochemistry or radioligand binding, lower levels of $A_{2A}R$ were also detected in other parts of the brain, as amygdala, hippocampus, hypothalamus, thalamus and cerebellum (Rosin et al., 1998ab; Rebola et al., 2005). It is not only expressed on neurons, but also on the vessel walls where they mediate vasodilation (Coney et al., 1998), and on glial cells. In the peripheral tissues, the $A_{2A}R$ can be found in spleen, thymus, heart, lung, kidney, leucocytes and blood platelets (Moreau et al., 1999).

In 2008 crystal structure of the human A_{2A} adenosine receptor was determined in complex with its high-selective antagonist ZM-241385. Not containing the canonical palmitoylation site found in the majority of GPCRs, a small helix that does not cross the cell membrane is located at the membrane cytoplasm interface (helix VIII) stabilizes the structure by interacting with helix I. The extracellular surface properties of the $A_{2A}R$ are largely dictated by its ECL2, which in $A_{2A}R$ lacks the prominent secondary structural elements, such as β-sheet and α-helix, as in the rhodopsin and β-adrenegric receptors, respectively. Instead, the ECL2 of the $A_{2A}R$ is mainly a spatially constrained random coil having three disulfide linkages with ECL1. Two of the three disulfide bonds (Cys71–Cys159 and Cys74–Cys146) are unique to the $A_{2A}R$; the third (Cys77–Cys166) is conserved among many class A GPCRs. In addition, a fourth intraloop disulfide bond is formed in ECL3 between Cys259 and Cys262 with the sequence Cys-Pro-Asp-Cys (CPDC), which creates a kink in the loop that constrains the position of ECL3 and orients His264 at the top of the ligand-binding site. The extensive disulfide bond network forms a rigid, open structure exposing the ligand-binding cavity to solvent, possibly allowing free access for small molecule ligands. Four amino acid residues are crucial for the ligand binding and their mutation was reported to disrupt antagonist and/or agonist binding, i.e. Glu169 in EC2, His250 and Asn253 in helix VI and Ile274 in helix VII (Jaakola et al., 2008).

Last year a crystal structure of $A_{2A}R$ bound to agonist (UK-432097, “conformationally selective ligand”) was obtained. Relative to inactive, antagonist-bound $A_{2A}R$, the agonist-bound

structure displays an outward tilt and rotation of the cytoplasmic half of helix VI, a movement of helix V, and an axial shift of helix III, resembling the changes associated with the active-state opsin structure. Additionally, a seesaw movement of helix VII and a shift of extracellular loop 3 are likely specific to $A_{2A}R$ and its ligand. The availability of both agonist- and antagonist-bound $A_{2A}R$ structures now provides the opportunity to solve the basic question of how ligand binding at the extracellular side of the receptor triggers conformational changes at the intracellular side, where G-protein and other effectors bind and initiate the cascade of downstream signalling pathways (Xu et al., 2011).

A_{2A} receptors can be found both pre- and postsynaptically: presynaptically on the corticostriatal glutamatergic projections (Hettinger et al., 2001) postsynaptically on the GABAergic striatopallidal neurons projecting to the globus pallidus, containing the peptide enkephalin, and enriched with dopamine D_2 receptors (Schiffmann et al., 1991). According to a study of Rebola et al., in the striatum, A_{2A} receptors are more abundantly located outside the active zone and A_{2A} receptors present in nerve terminals were most densely located in the postsynaptic density fraction, although they could also be identified in the presynaptic active zone fraction. (Rebola et al., 2005). In addition there is a recent evidence that presynaptic $A_{2A}Rs$ are preferentially localised in cortical glutamatergic terminals that contact striatal neurons of the direct pathway rather than of indirect pathway (Quiroz et al., 2009), so that there is a segregation of $A_{2A}R$ in the corticostriatal synapses, being presynaptic $A_{2A}R$ expressed in projections of cortical neurons to the MSNs of the direct pathway and postsynaptic $A_{2A}R$ expressed in striatopallidal MSNs of the indirect pathway. As we will see later, $A_{2A}R$ plays a different role in each location.

The major signal transduction pathway used by $A_{2A}Rs$ (Figure 21) includes the activation of adenylate cyclase by means of G_s in general or G_{olf} in the striatum (Kull et al., 1999, 2000). G_{olf} activates adenylate cyclase generating cAMP, which activates the AMP-dependent protein kinase (PKA). PKA regulates the state of phosphorylation of various proteins, importantly DARPP-32 (dopamine and cAMP regulated phosphoprotein, 32 kDa) which is expressed in very high concentration in the GABAergic efferent

neurons (Kull et al., 1999, 2000). Under basal conditions, DARPP-32 is phosphorylated at Thr75 and inhibits PKA (Nishi et al., 2000). This inhibition can be rescued by dephosphorylation promoted by protein phosphatase-2A, also activated by PKA (Nishi et al., 2000). By PKA activation DARPP-32 is activated by phosphorylation at Thr34 and it becomes a potent selective inhibitor of protein phosphatase-1 (PP-1) (Kull et al., 2000). PP-1 inhibits the activation of CREB in the nucleus. CREB is an important point of convergence of the $A_{2A}R$ signalling and it can be activated through pathways activated by G_{α} or $G_{\beta\gamma}$ subunits, that means by cAMP dependent or independent pathway and involving ERKs signalling or not. In cells expressing B-Raf (CHO cells or striatum), the cAMP dependent activation of CREB occurs through PKA–Src–Rap1–B-Raf–MEK–MAPK pathway. Here CREB is only one of many targets of MAPK. Or also by direct phosphorylation by PKA or via actions of DARPP-32 not including MAPK activation. The cAMP-independent pathway can occur via activation of Ras–Raf1–MEK–MAPK (Vossler et al., 1997). In cells not expressing B-Raf, e.g. HEK, activation of MAPK happen via Ras (Seidel et al., 1999). This signalling pathway is also induced by PLCβ activation (Wirkner et al., 2000) or phospha-tidylinositol 3-kinase (PI3K)-Akt signalling (Lee et al., 2001).

ERK 1/2 activation in the dorsal striatum is necessary for action-outcome learning and performance of goal-directed actions. In the ventral striatum, ERK 1/2 is necessary for the motivating effects of reward-associated stimuli on instrumental performance. Dysregulation of ERK 1/2 signalling in the striatum by repeated drug exposure contributes to the development of addictive behavior (Shifflet et al., 2011). ERK 1/2 influences gene expression through its interaction with transcriptional regulators, such as ribosomal s6 kinase (RSK), mitogen- and stress-activated protein kinase-1 (MSK1) as well as the transcription factor elk-1 (Kelleher et al., 2004). Furthermore, treatments that interfere with ERK signalling such as the MEK/ERK inhibitors e.g. U0126, impaired long-term memory retention (Shifflet et al., 2011). ERK 1/2 likely enables corticostriatal plasticity, in part, through regulation of transcription factors such as cAMP response element binding (CREB) protein, as disruption of CREB signalling in the striatum prevents striatal LTP and LTD induction (Pittenger at al., 2006).

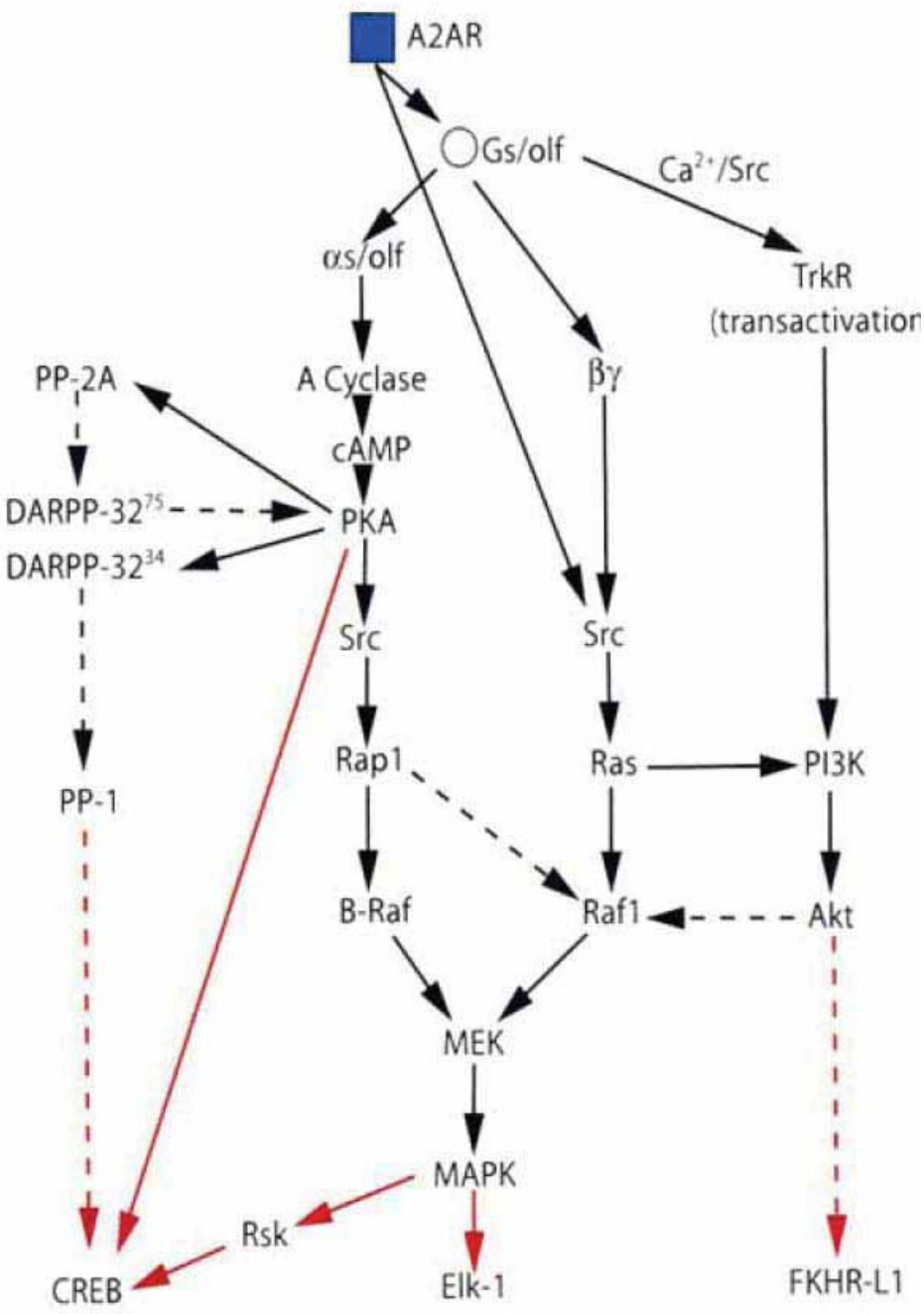

Figure 21. $A_{2A}R$ signal transduction. Full lines represent stimulatory effects and dashed line inhibitory effects. Red lines indicate signalling taking place in nucleus. Taken from (Ferré et al., 2003).

Upon agonist stimulation, the $A_{2A}R$ response "quickly" desensitises within a time frame of less than an hour. Desensitisation (attenuation of adenylate cyclase stimulation) has been described in various cellular systems expressing both endogenous and recombinant $A_{2A}R$ (Ramukar et al., 1991; Palmer et al., 1994) This rapid desensitization involves $A_{2A}R$ phosphorylation mostly by GRK in the proximal portion of the C-terminus (Thr-298) of $A_{2A}R$ (Palmer et al.

1994). Selective activation of G-proteins by stimulation of the $A_{2A}R$ is predominantly dictated by its 3IL (in its N-terminal portion) (Olah et al., 1997). The C-terminal segment seems to be required for the transition of the $A_{2A}R$ to the activated state, since its truncation blunts constitutive activity (Klinger et al., 2002). Finally, the C-terminal segment of the $A_{2A}R$ seems to be involved in the formation of $A_{2A}R$-D_2R heteromeric complexes and to the interaction of $A_{2A}R$ with the actin cytoskeleton. A longer agonist exposure induces receptor internalization, which has been shown to be a necessary step for either resensitization or down-regulation of A_{2A} receptor through clathrin-coated vesicles (Palmer et al., 1994).

A_{2A} receptor-deficient mice were viable with normal development, suggesting that $A_{2A}R$ function may not be critical during neurogenesis. However, they displayed behaviors reflecting increased anxiety and agression in males (Ledent et al., 1997). Interestingly, $A_{2A}R^{-/-}$ mice were more susceptible than wild type mice to striatal degeneration and weight loss caused by a low dosage of 3NP which did not induce glutamate-related excitotoxicity (Blum et al., 2003).

1.4.3. A_{2A} receptor heteromers

1.4.3.1. Postsynaptic A_{2A} receptor heteromers

The postsynaptic $A_{2A}R$ is found in enkephalin MSNs of indirect pathway. It is mainly found perisynaptically to the postsynaptic density in the neck of dendritic spines, adjacent to dopaminergic synapses (Ferré et al., 2007) where it can be found in different populations of heteromers (Figure 22). It can be expressed as $A_{2A}R$-$A_{2A}R$ homodimer, as $A_{2A}R$-D_2R heteromer (Ciruela et al., 2006; Hillion et al., 2002; Orrú et al., 2011b), as $A_{2A}R$-D_2R-$mGlu_5R$ heterotrimer (Cabello et al., 2009), as $A_{2A}R$-CB_1R heteromer (Carriba et al., 2007) and as $A_{2A}R$-CB_1R-D_2R heterotrimer (Navarro et al., 2008).

The antagonistic interactions between the $A_{2A}R$ and D_2R receptors were demonstrated at the biochemical, functional and behavioural level. The first indication about an antagonist relationship was obtained from analysis of behaviour of animal models of Parkinson's disease (Fuxe et al., 1974). The use of naturally present

$A_{2A}R$ antagonists caffeine and theophylline in combination with L-DOPA and dopamine agonists led to an increase of motor activity produced by dopaminergic compounds. The first direct clue about the interaction of $A_{2A}R$ with D_2R was brought by experiments in membrane preparations from rat striatum, where stimulation of $A_{2A}Rs$ produced a decrease in the affinity of D_2Rs for agonists due to conformational modification in the D_2R binding site (Ferré et al., 1991). This interaction pointed towards the possible existence of $A_{2A}R$-D_2R heteromer. Similar results were seen in different co-transfected cell lines (Dasgupta et al., 1996; Kull et al., 1999; Salim et al., 2000). This was eventually confirmed in 2001 by coimmunoprecipitation and colocalization experiments in transfected cells and primary neuronal cultures and it was observed that a prolonged exposition of $A_{2A}R$ and D_2R agonists led to co-aggregation and co-internalization and co-desensitization of both receptors (Hillion et al., 2002). Finally, in living cotransfected cells by BRET and FRET experiments it was proved that $A_{2A}R$ and D_2R indeed heteromerized (Canals et al., 2003). From the $A_{2A}R$ and D_2R negative cross-talk, the use of A_{2A} antagonist in Parkinson disease was proposed.

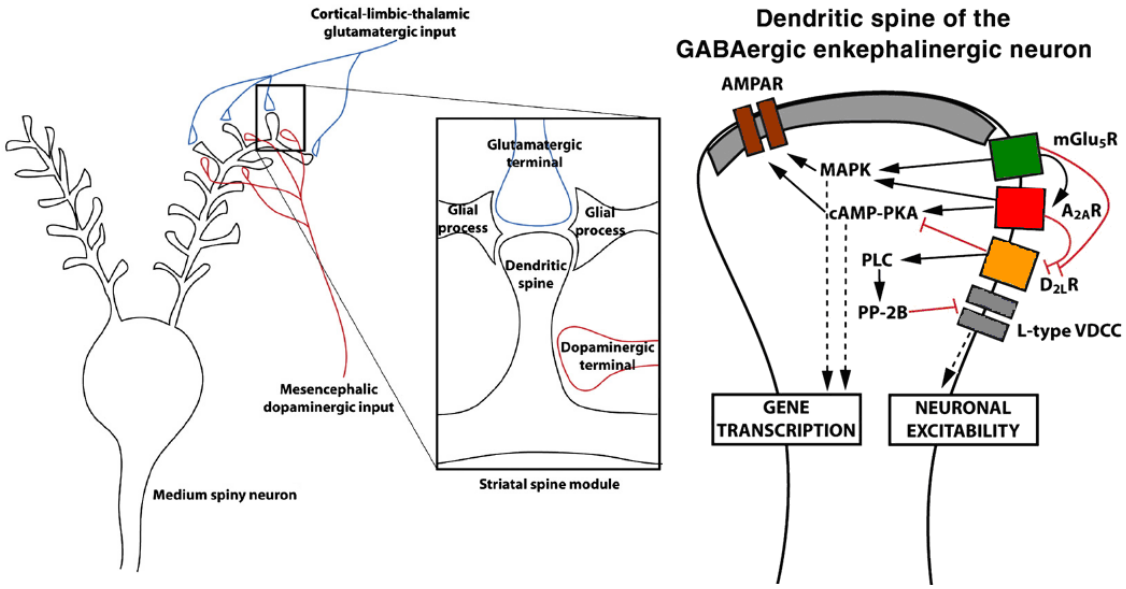

Figure 22. Striatal spine module. Left: Connectivity of dendritic spines with glutamatergic projections on the head and dopaminergic projections on the neck of the spines. Right: $A_{2A}R$ in the postsynaptic terminal**.** $A_{2A}R$ forming heteromers with D_2R and $mGlu_5R$ and consequences of these interactions. Taken from (Ferré et al., 2007).

From the selective $A_{2A}R$ antagonists, KW-6002 was the most interesting compound. It already underwent full clinical evaluation with hopeful results, but it still needs further investigation before it will be able to be approved. After first positive results obtained from animal models, rodents and monkeys (Fenu et al., 1997; Kanda et al., 1998, 2000), it was started to be taken by patients on clinical trials. The first, proof of concept trial, with KW-6002 was performed in 2003 (Bara-Jimenez et al., 2003). Its development under the name of Istradefylline was financed by Japanese pharmaceutical company Kyowa. Results from first smaller clinical tests (Bara-Jimenez et al, 2003; Hauser et al., 2003; Guttman et al., 2006) showed certain positive results. KW-6002 alone provided no antiparkinsonian response in moderately advanced PD patients, in contrast to the normalization of locomotor function that had been observed in primates. However, consistent with the primate studies was the observation that the antiparkinsonian response could be maintained with less dyskinesia by using istradefylline with lower levodopa doses. In addition, the prolongation of the efficacy half-time following discontinuation of a levodopa infusion suggested that istradefylline might reduce "off-time" in patients with motor fluctuations on levodopa. ("Off-time" refers to periods of the day when the medication is not working well, causing worsening of Parkinsonian symptoms, these periods become more often as the PD progresses). Larger clinical trials brought similar results (Lewitt et al., 2008; Fernandez et al, 2008). To resume it, KW-6002 reduced the "off time" in moderate to advanced PD patients already receiving dopaminergic therapy, with an increase in non-troublesome dyskinesia. However, the effect on motor function has not been statistically significant in all studies and other side effects were present in some patients (Jenner et al., 2009). Despite the positive findings, in 2008 Kyowa received a “Not Approvable” letter from the U.S. Food and Drug Administration (FDA), suspended its development in US, but later on decided to perform a further studies. Importantly, in PD patients KW-6002 has yet to be tested under similar circumstances to those which revealed positive effects in rodent and primate studies. For example, KW-6002 may be co-administered with a sub-optimal dose of dopamine agonists or L-DOPA, instead of with the optimal doses used so far in clinical trials and to be proven in patients with less advances PD. Besides KW-6002, other $A_{2A}R$ antagonists have also

entered clinical trials, for example SCH-420814 (Merck-Schering), SYN-115 (Roche), vipadenant, ST-1535 (Armentero et al., 2011).

Similar approaches were used to demonstrate the $A_{2A}R$ intereaction with metabotropic glutamate receptor type 5 ($mGlu_5R$). $A_{2A}R$ co-immunoprecipitates with $mGlu_5R$ in cotransfected cells and colocalizes in striatal tissue (Ferré et al., 2002, 2003). Radioligand binding assays in rat striatum membranes showed a similar effect of $mGlu_5R$ upon D_2R that means, stimulation of $mGlu_5Rs$ also produced a decrease in the affinity of D_2Rs for its agonists. Moreover when $A_{2A}R$ and $mGlu_5R$ were simultaneously stimulated the inhibitory effect on D_2R was stronger than the reduction induced by stimulation of either receptor alone (Popoli et al., 2001), indicating a possible existence of $A_{2A}R–D_2R–mGlu_5R$ heteromer.

In addition to the cross-talk at the level of ligand binding, there is a strong antagonistic interaction between $A_{2A}Rs$ and D_2Rs at the second messenger level, that may not depend on the heteromerization. Stimulation of D_2Rs, which are coupled to inhibitory $G_{i/o}$, counteracts adenylate cyclase activation induced by stimulatory G_{olf}-coupled $A_{2A}Rs$ (Kull et al., 1999; Hillion et al., 2002). Stimulation of $A_{2A}R$ activates adenylate cyclase with consequent activation of the protein kinase A (PKA) signalling pathway and induction of the expression of different genes, such as c-fos and preproenkephalin, by the constitutive transcription factor CREB (Ferré et al., 1997, 2005). In addition, $A_{2A}R$-mediated activation of PKA can induce the phosphorylation of AMPARs (Hakansson et al., 2006), which is important for the development of plastic changes at glutamatergic synapses, including recruitment of AMPARs to the postsynaptic density (PSD) (Song et al., 2002). However, under basal conditions, stimulation of $A_{2A}Rs$ poorly activates cAMP-PKA signalling or increases gene expression owing to strong tonic inhibition of adenylate cyclase by D_2R stimulation with endogenous dopamine (Ferré et al., 1997, 2005; Lee et al., 2002). In accordance, systemic administration of selective $A_{2A}R$ agonists did not lead to an increase of striatal c-fos expression in rats (Karcz-Kubicha et al., 2003). Nevertheless, stimulation of $mGlu_5Rs$ allows $A_{2A}Rs$ to override the D_2R-mediated inhibitory effects. Thus, central co-administration of $A_{2A}R$ and $mGlu_5R$ agonists did induce an increase in striatal expression of c-fos (Ferré et al., 2002). $mGlu_5R$ also activated MAPK

in transfected cells and striatal slices (Ferré et al., 2002) and potentiated $A_{2A}R$ signalling in an MAPK-dependent manner (Nishi et al., 2003), so $A_{2A}R$–$mGlu_5R$ and possibly $A_{2A}R$–D_2R–$mGlu_5R$ heteromers are able to modulate plastic changes in the striatum. Indeed, pharmacological or genetic inactivation of $A_{2A}Rs$ or $mGlu_5Rs$ impaired corticostriatal long-term potentiation (d'Alcantara et al., 2001; Gubellini et al., 2003).

Even if the signalling of $A_{2A}R$ under normal conditions is depressed by endogenous dopamine levels, it can have an effect upon D_2R ligand binding as described above and consequently upon its signalling via PLC implicated in the activation of L-type voltage dependent L-VDCC channels (Nicola et al., 2000). This in fact is the main role of $A_{2A}R$ inhibitory regulation of D_2R stimulation in striatum and is independent of cAMP-PKA signalling (Dasgupta et al., 1996; Ferré et al., 2008). According to Azdad et al. the D_2R-mediated suppression of NMDA-induced depolarized plateau is mediated by the suppression of L-VDCC calcium channels (type Cav1.3a) current through the D_2R activation of PLC signalling cascade involving the activation of calcineurin and dephosphorylation of these channels. The $A_{2A}R$ is able to counteract this D_2R mediated suppression of NMDA-induced depolarized plateau via a direct $A_{2A}R$–D_2R interaction at the membrane level through heteromerization (Azdad et al., 2009). This consequently leads to firing of the indirect pathway MSNs, that is, to motor inhibition. Thus, central or local administration of $A_{2A}R$ agonists produced a pronounced decrease in motor activity (Ferré et al., 1997). Furthermore, $A_{2A}R$ agonists and antagonists selectively counteract and potentiate, respectively, the motor activation and decrease in neuronal firing and neurotransmitter release that are induced by dopamine D_2R agonists (Ferré et al., 1993, 1997; Stromberg et al., 2000). In different behavioral models, $mGlu_5R$ agonists and antagonists produced similar effects as $A_{2A}R$ agonists and antagonists, respectively, upon the D_2R motor control. So a selective $mGlu_5R$ agonist preferentially inhibited motor activation induced by D_2R agonists (Popoli et al., 2001), whereas $mGlu_5R$ antagonists counteracted the effects of D_2R antagonists (Ossowska et al., 2001). Furthermore, $A_{2A}R$ and $mGlu_5R$ agonists and $A_{2A}R$ and $mGlu_5R$ receptor antagonists also showed synergistic effects at the behavioral level (Popoli et al., 2001; Ferré et al., 2002; Kachroo et al., 2005). $A_{2A}R$–D_2R–$mGlu_5R$ receptor interactions provide the rationale for the

possible application of $mGlu_5R$ antagonists or combined $A_{2A}R$ and $mGlu_5R$ antagonists in Parkinson's disease (Ferré et al., 1992, 1997; Ossowska et al., 2001; Jenner et al., 2005; Kachroo et al., 2005).

1.4.3.2 Presynaptic A_{2A} receptor heteromers

At presynaptic level $A_{2A}R$ is found in glutamatergic terminals innervating dynorphinergic MSNs of direct pathway (Rosin et al., 2003; Quiroz et al., 2009). Here it forms heteromers with other presynaptically located receptors, importantly with A_1R (Ciruela et al., 2006; Figure 23). It was surprising at first to consider a heterodimer controled by the same neurotransmitter, adenosine, formed by a stimulatory and an inhibitory receptor. As A_1R and $A_{2A}R$ are coupled to $G_{i/o}$ and $G_{s/olf}$-proteins, respectively (Kull et al., 1999; Fredholm et al., 2001), stimulation of presynaptic A_1Rs receptors decreases the probability of neurotransmitter release, whereas activation of presynaptic $A_{2A}Rs$ enhances neurotransmitter release (Yawo et al., 1993; Wu et al., 1997; O'Kane et al., 1998; Lopes et al., 2002; Quarta et al., 2004).

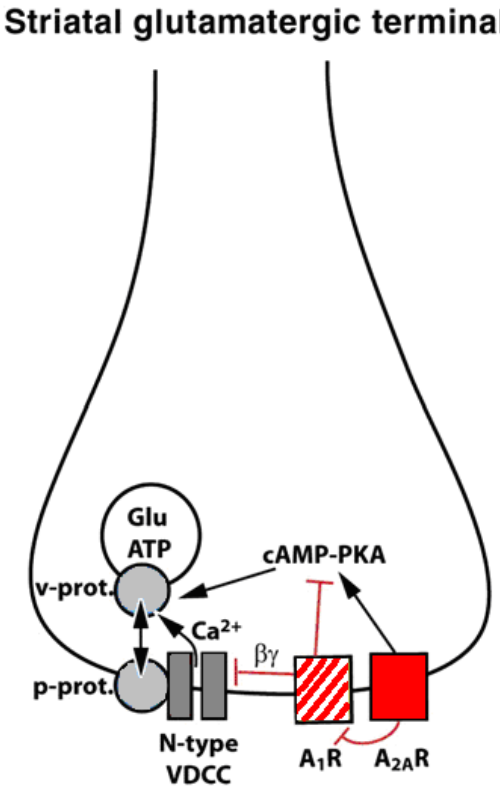

Figure 23. $A_{2A}R$ in the presynaptic terminal. $A_{2A}R$ forming heteromers with A_1R and regulating glutamate relaese in the corticostriatal synapse**.** Taken from (Ferré et al., 2007).

The evidence for functional antagonistic interactions between A_1R and $A_{2A}R$ modulating glutamate release in the striatum and hippocampus was provided earlier by many studies (O'Kane et al., 1998; Lopes et al., 2002; Quarta et al., 2004). The physical interaction was demonstrated by resonance energy transfer methods BRET and TR-FRET and coimmunoprecipitation in 2006 (Ciruela et al., 2006) and it was elucidated that the A_1R-$A_{2A}R$ heteromer indeed played a very important role in the glutamate release control in the striatum and that it was the concentration of extracellular adenosine which decided about the outcome. Because of that, the role of A_1R-$A_{2A}R$ heteromer is also called a concentration dependent switch. As mentioned before the affinity of A_1R to adenosine is better (K_D = 70 nM) than its affinity to $A_{2A}R$ (K_D = 150 nM) (Fredholm et al., 2001). The physiological concentration of adenosine lies inside of this range (Delaney et al., 1996) and is sufficient to activate both receptors, if they are abundantly expressed (Fredholm et al., 2007). The extracellular levels of adenosine increase locally as a function of neuronal firing and synaptic activity (Schiffman et al., 2007). So, under basal conditions, the relatively low extracellular levels of adenosine preferentially bind to and stimulate A_1Rs and this preferential A_1R stimulation in the A_1R-$A_{2A}R$ heteromer inhibits glutamatergic neurotransmission. Under conditions of stronger adenosine release, A_{2A} receptor activation in the A_1R-$A_{2A}R$ heteromer would block A_1R-mediated function, with the overall result a facilitation of the evoked release of glutamate (Ciruela et al., 2006). The inhibitory effect of A_1R on striatal glutamate release probably involves inhibition of N- and P/Q-type VDCCs by $G_{\beta\gamma}$ protein subunits; this is the most commonly reported mechanism for inhibition of neurotransmitter release by $G_{i/o}$-coupled receptors, including A_1Rs (Yawo et al.,1993; Jarvis et al., 2001). The stimulatory effect of $A_{2A}Rs$ on striatal glutamate release is probably related to their ability to activate cAMP-PKA signalling as this mechanism has been shown for $A_{2A}R$ induced acetylcholine release in the striatum, GABA release in the globus pallidus and serotonin release in the hippocampus (Gubitz et al., 1996; Shindou et al., 2002; Okada et al., 2001). This effect is related to the ability of PKA to phosphorylate different elements of the machinery that is involved in vesicular fusion (Leenders et al, 2005).

The postsynaptic $A_{2A}R$ can also control the glutamate release indirectly, and that happens in the indirect pathway. In enkephaliner-

gic MSNs the postsynaptic $A_{2A}R$ can control the production of endocannabinoids acting on presynaptic CB_1R, which coupling to G_i controls glutamate release in different brain areas (Freund et al. 2003; Piomelli et al. 2003) (see chapter 1.5). Using targeted whole-cell recordings from direct- and indirect-pathway MSNs Lerner et al. demonstrated that $A_{2A}R$ antagonists potentiated 2-AG release and induced a long-term depression in indirect-pathway MSNs, but not direct-pathway MSNs. This suggested that $A_{2A}R$ antagonists can produce locomotor activation by disinhibiting a tonic $A_{2A}R$-mediated inhibition of D_2R-mediated endocannabinoid release in the enkephalinergic MSNs (Lerner et al., 2010).

1.4.4. Adenosine receptors in Huntington's disease

1.4.4.1. Huntington's disease and huntingtin

Huntington's disease (HD) is an autosomally dominant inherited progressive neurodegenerative disorder characterised by motor, cognitive and psychiatric impairments. It was named after Dr. George Huntington who first described it in 1872. Patients typically present motor disturbances as chorea – jerky, random, and uncontrollable dance-like movements – which explains the middle age name for HD, the St.' Vitus dance (Walker, 2007).

HD affects about 5 individuals per 100.000 and the primary cause is a mutation of the huntingtin gene that leads to an aberrant aplification of CAG repeat resulting in a longer polyQ tract in the N-terminus of the huntingin protein, a fact that leads to serious pathological consequences. Normal alleles at this site contain up to 35 CAG repeats, but when they reach 41 or more, the disease manifests. With 36 – 40 repeats the disease sometimes manifests and sometimes not. The huntingtin gene (denominated IT15) located in short arm of chromosome 4 (4p16.3) was found in 1993 (HDCRG, 1993). HD generally starts to manifest in the fourth life decade, but juvenile form (onset before 20 but as early as 1 year of age) also exists and occurs with a very high number of the CAG repeats.

In the prediagnostic phase, individuals might become irritable, multitasking becomes difficult and forgetfulness and anxiety mount. In diagnostic phase the affected individuals show distinct chorea,

incoordination and motor impersistence. Patients with early-onset Huntington's disease might not develop chorea, or it might arise only transiently during their illness. Most individuals have chorea that initially progresses but then, with later onset of dystonia and rigidity, it becomes less prominent (Walker, 2007). Cognitive dysfunction in HD often spares long-term memory but impairs executive functions, such as organising, planning, checking, or adapting alternatives, and delays the acquisition of new motor skills. These features worsen over time; speech deteriorates faster than comprehension. As motor and cognitive deficits become severe, patients eventually die, usually from complications of falls, inanition, dysphagia, or aspiration. Typical latency from diagnosis to death is 20 years (Walker, 2007; Muñoz, 2006). In spite of a unique and known origin of the HD, a monogenic disease, no effective treatment to influence the onset or the progression is presently available.

In 1976 the first induced excitotoxic (Coyle et al., 1976) and in 1996 the first transgenic mouse model (Mangiarini et al., 1996) were developed and several drugs passed to clinical trials with a large investigation going on. At the laboratory level HD is usually studied in cell cultures (Lunkes et al., 1998) and in animal models as *Caenorhabditis elegans* (Faber et al., 1999), *Drosophila* (Marsh et al., 2003) and rat (von Hörsten et al., 2003). The fly and mouse models consistently show neuronal polyglutamine inclusions and indicate that pathology is dependent on polyglutamine length, is late onset, progressive and degenerative, with neuronal dysfunction followed by neuronal death. The most studied and best characterised transgenic mice models up to date are the R6/1 and R6/2 model. R6/1 mice express one copy of N-terminal fragment of human huntingtin with 115 CAG repetitions and R6/2 express three copies with about 150 CAG repetitions. R6/1 are sometimes compared with the adult and R6/2 with the juvenile form of HD (Margiarini et al., 1996). In 2009 a simian model (Yang et al, 2008) and sheep model were developed (Jacobsen et al., 2010).

Huntingtin is a large completely soluble protein of about 3,144 amino acids and 348 kDa. It is ubiquitously expressed, with the highest levels in CNS neurons and the testes (Ferrante et al., 1997; Fusco et al., 1999). It is found mostly in the cytoplasm, although in lesser amounts also in the nucleus (Kegal et al., 2002). In cytoplasm it

is associated with various organels, endoplasmic reticulum, Golgi complex, both clathrin-coated and non-coated endocytic and autophagic vesicles, endosomal compartments, plasma membrane, microtubules and mitochondria (DiFiglia et al., 1995; Kegel et al., 2002). It is found in soma, dendrites, axons and in nerve terminals (Li et al., 2003).

The crystal structure of huntingtin is not known yet and there are only some identified motifs in the primary amino acid sequence with a defined function. At the very N-terminus beginning at the 18th amino acid lies the critical polyQ region. In unaffected individuals, contains from 7 to 35 glutamine residues (HDCRG, 1993). Perutz et al. showed that this portion forms a polar zipper structure, and suggested that its physiological function is to bind transcription factors that contain a polyQ region (Perutz et al., 1994). It has now been shown that wild-type huntingtin interacts with several partners and that the polyQ tract is a key regulator of such binding (Harjes et al., 2003; Li et al., 2004; Goehler et al., 2005).

PolyQ region is followed by a number of prolines, polyP stretch, which might help with solubility (Steffan et al., 2004). Downstream of these regions lie several so-called HEAT repeats, of about 40 amino acids, which are involved interactions with other proteins (Neuwald et al., 2000). There are two targetting sequences: nuclear export signal and nuclear localization signal (Xia et al., 2003). Both wild type and mutated huntingtin can be proteolytically cleaved by caspases (-2, -3 and -6) and calpains (Wellington et al., 1998; Gafni et al. 2002) in different sites, not all well defined yet, and it is known that some cleavages occur preferentially in striatum and other in cortex (Mende-Mueller et al., 2001). The contribution of huntingtin proteolysis to cell function is not clear. However, modifications in the activity of caspase and calpain reduce the proteolysis and toxicity of the mutant protein, and delay disease progression (Wellington et al., 2000).

It is not fully understood if HD is caused by the absence of the normal functions of huntingtin or by acquired pathological functions of the mutated protein (Cattaneo et al., 2001), probably a combination of both. The functions of normal huntingtin are many. Huntingtin protein is essential for normal embryonic development (Nasir et al., 1995; Wexler et al., 1987), it is also important for neuronal survival

(Dragatsis et al., 2000) and neuroprotection (Rigamonti et al., 2000; Leavitt et al., 2006, Zeron et al., 2002; Cattaneo et al., 2001; Gervais et al., 2002; Humbert et al., 2002). At synaptic terminals, huntingtin is involved in the control of synaptic transmission (Sun et al., 2001; Zeron et al., 2002; Smith et al., 2005). Many of these functions are lost or affected when the mutation is present. In addition, the mutated huntingtin brings toxicity that cannot be explained by the lost of the physiological functions. The late-onset of the disease reminds Alzheimer's disease since it is produced an accumulation of toxic protein fragments (Temussi et al., 2003). Aggregates of cleaved or entire mutated protein accumulate in different conformations, together with bound proteases in the cytoplasm and also in the nucleus (Wellington et al., 1998) of all the cells in the brain and body. It is not known if these aggregates are toxic (Cooper et al. 1998; Hackam et al. 1998; Yang et al., 2002), are not harmful (Kuemmerle et al., 1999) or are even neuroprotective (Arrasate et al. 2004; Truant et al., 2008), if the fragments are more or less toxic than the entire mutated protein. Possibly the smaller fragments but not the larger aggregates are toxic and have negative effects on synaptic transmission (Li et al., 2003).

The mutation of huntingtin leads to neurodegeneration of specific brain areas as it is clearly visible in postmortem HD individuals' brain. These regions comprise the striatum and, in a lesser extent, cortex, hippocampus and cerebellum (Vonsattel et al., 1998). Interestingly, the first and most affected neurons are the medium spiny neurons (MSNs) that are selectively or preferentially vulnerable in HD (Graveland et al., 1985c). It is interestingly to note the enkephalinergic MSNs are more vulnerable that the dynorphinergic ones (Reiner et al., 1988; Albin et al., 1992; Richfield et al., 1995) (Figure 24). The primary dysfunction and latter degeneration of enkephalinergic neurons leads to the manifestation of typical hyperkinetic symptoms of HD, chorea and dyskinetic movements as is logically attributed to the gradual diminution of the inhibitory output of the indirect pathway. Later, when dynorphinergic MSNs and nigrostriatal neurons also degenerate, chorea is replaced by a lack of movements due to the total dysfunction of basal ganglia circuitry (Glass et al., 2002). It is important to note that it is not fully elucidated why MSNs are the most affected neurons in HD.

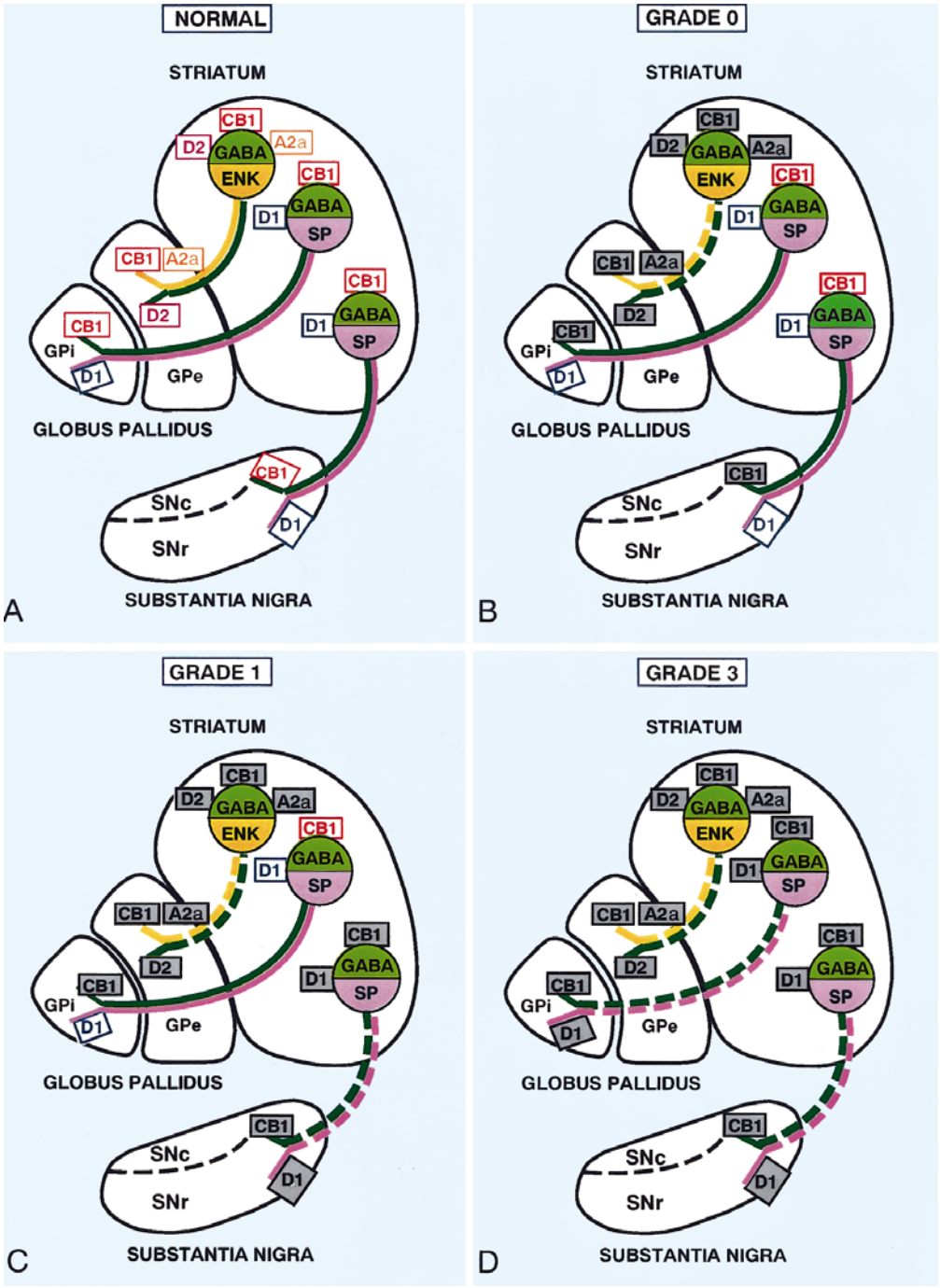

Figure 24. Degeneration of striatal neurons in HD. Gradual degeneration of striatal neurons in the first (grade 0) and late grades (3) of the HD compared with a healthy state (normal). Disappearance of the nervous projection in a dashed line. Disappearance of the $A_{2A}R$, D_1R, D_2R, CB_1R receptors staining: (grey boxes). ENK – enkephalin, SP – substance P, SNc, SNr – substantia nigra pars compacta and pars reticulata. Taken from (Glass et al., 2000).

The degenerative process of MSNs was described by Ferrante et al. (Ferrante et al. 1991). Degenerative changes were characterised by truncated dendritic arbors, spine loss, and irregular focal swellings along dendrites. Dendritic arbors are highly dynamic structures,

exhibiting frequent branch additions and retractions and maintenance of synaptic input is critical for dendritic stability (Coleman et al., 1968, Jones et al., 1962, Sfakianos et al. 2007) Inversely, lack of synaptic input leads to dendritic loss. Nevertheless, the dendritic loss can also be caused by an excessive synaptic stimulation. This phenomenon is called excitotoxicity. Striatal MSN receive a strong glutamatergic input from cortex, and glutamate receptos agonists reproduced the symptoms of HD in excitotoxic HD animal models (Beal et al., 1986; Popoli et al., 1994). The sensitivity to glutamate depends in part on the NMDA receptors. NMDA receptors of MSNs contain higher amounts of NR2B in their subunits composition than do neurons in other parts of the brain. This isoform is more susceptible to glutamate activation. Enkephalin neurons interestingly express even higher amounts NR2B than the dynorphin neurons (Cepeda et al., 2001; Jarabek et al., 2004), which could also contribute to their higher exicitotoxic vulnerability.

1.4.4.2. Adenosine A_{2A} receptors in HD

A comparative study of cannabinoid CB_1, dopamine D_1 and D_2, adenosine A_{2A} and $GABA_A$ receptor expression in the basal ganglia of graded HD revealed a complex pattern of degeneration. While loss in dopamine receptors appears to correlate with cell death progression, A_{2A} and CB_1 receptors exhibit a much more pronounced reduction in all regions suggesting that their dysfunction is occurring prior to cell death (Glass et al., 2000; Figure 23).

From autoradiography experiment using the $A_{2A}R$ agonist [^{3}H]CGS-21680 performed in post mortem brain slices of patients in early (grade 0), intermediate (grade 1, 2) and late (grade 3) neuropathological grades of Huntington's disease, it was demonstrated that the expression of $A_{2A}R$ gradually decreases in the basal ganglia of HD patients (Figure 25). In control brains $A_{2A}R$ binding was fairly homogeneous within the caudate nucleus and putamen. A dramatic loss of $A_{2A}R$ binding was observed in grade 0 (35% of controls), it was a further dramatic decrease in A_{2A} receptor binding in grade 1 (12 % of controls) and more advanced cases showed no detectable $A_{2A}R$ binding. As for the dopamine receptors, the binding appeared to decline in a heterogeneous fashion, with irregular shaped patches of receptors declining slightly more rapidly than the receptors in the

surrounding regions. In the globus pallidus, A_{2A}Rs were present only within the Gpe where a dramatic and total loss occurred in the very earliest stages of HD (Glass et al., 2000).

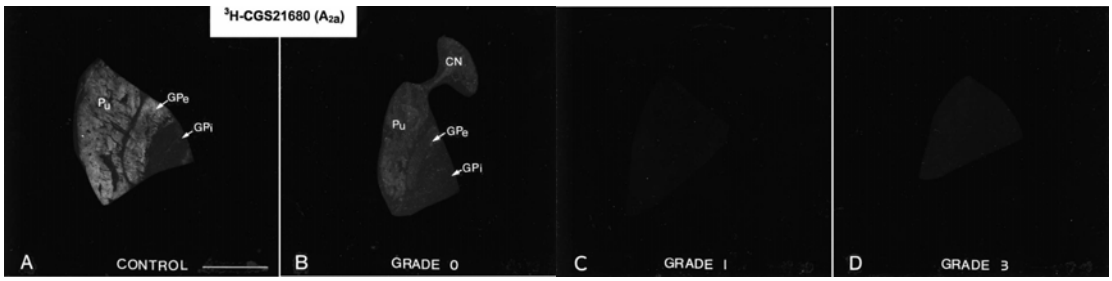

Figure 25. A_{2A}R in HD. Gradual disappearance of the A_{2A}R, stained by ^{3}H-CGS21680 binding, in the slices of human striatum in gradual states of the progression of the disease; Pu – putamen, GPe, GPi – globus pallidus externum and internum, CN – caudate nucleus (Glass et al., 2000).

Similar experiments were performed in transgenic HD mouse model by Cha et al. (Cha et al., 1999) in brain slices of R2/6 mice in different stages of disease (at 2, 4, 8 and 12 postnatal weeks). Compared to the wild type mice, A_{2A}R levels were normal at two weeks of age, but significantly decreased by 4 week and they were only about 10 % of the control at 12 week, when R6/2 develop neurological symptoms but without evidence of neuronal loss (Mangiarini et al. 1996). Similar results were also seen with R6/1 transgenic mice. D_2R and A_{2A}R binding was decreased as early as three months of age, that means before the R6/1 animals become symptomatic (15 and 21 weeks of age) (Cha et al., 1999). From the above mentioned autoradiography experiments is not possible to discriminate between loss of agonist binding and loss in agonist affinity. When the A_{2A}R expression was determined using the A_{2A}R antagonist [^{3}H]ZM-241385 in saturation binding experiments and R6/2 mouse striatal membrane, an initial increase in B_{max} at the postnatal day 9-14 followed by a decrease before the postnatal day 21 was detected without differences in the antagonist ZM-241385 binding affinity (Tarditi et al., 2006). Giving more complexity, B_{max} and ZM-241385 binding affinity was increased in human peripheral blood cells from both symptomatic and presymptomatic HD patients, (Varani et al., 2003). Similar results were observed using the A_{2A}R antagonist SCH-58261 and striatal cell line expressing a mutant huntingtin (Varani et al., 2001).

The role of A_{2A}Rs in HD is being recently investigated. It was demonstrated that total A_{2A}R knock-out mice were more susceptible than wild type mice to striatal degeneration and weight loss caused by a low dosage of 3-nitropropionic (3NP) intoxication that did not induce glutamate-related excitotoxicity (Blum et al. 2003). Very recently, the pathophysiological consequences of genetic deletion of A_{2A}Rs in HD have been studied by crossing an A_{2A}R knockout mice with the N171-82Q HD transgenic model of HD. Knockout of A_{2A}Rs moderately but significantly worsens motor performances and survival of N171-82Q mice and leads to a decrease in striatal enkephalin expression. These results support that early and chronic blockade of A_{2A}Rs might not be beneficial in HD (Mievis et al., 2011) but, with this model, it cannot be discarded additional alterations in other protein/receptors expression due to genetic manipulation that can influence the results.

1.4.4.3. A_{2A} receptor antagonists in HD treatment

In recent years several works have been published studying the effects of A_{2A}R antagonists in animal models of HD but they did not bring clear results. It was observed that A_{2A}R agonists (CGS-21680) as well as antagonists (SCH-58261, ZM-241385) were able to improve the HD pathology. It was observed that the benefit of the effect depended on the dose used, including absolutely opposing outcome. The heteromers specificity was not considered in any of the cases and it was concluded that the mixture of pre- and postsynaptic effects is always present and cannot be eliminated (Popoli et al., 2002; Gianfriddo et al., 2004; Chou et al, 2005; Domenici et al., 2007; Martire et al., 2007; Minghetti et al., 2007; Scattoni et al., 2007, Cipriani et al., 2008).

First experiments were made in excitotoxic animal models. A_{2A}R antagonist SCH-58261 showed neuroprotective effects in an excitotoxic HD rat model. The main mechanism of its effect was the inhibition of quinolinic acid (QA)-evoked increase in extracellular glutamate. SCH-58261 administered at low doses, but not at high doses, before the striatal injection of QA reduced the effects of QA on motor activity, electroencephalographic changes and striatal gliosis (Popoli et al., 2002). This result was confirmed in transgenic R6/2 mice, where SCH-58261 administered through microdialysis into the

striatum significantly decreased the glutamate outflow (Gianfriddo et al., 2004). The beneficial effect of this antagonist was also evaluted in the same mice model but in the presymptomatic phase. One week treatment with SCH-58261 before the appearance of symptomatic phenotype prevented emotional/anxious behavior and electrophysiological alterations but tended to exacerbate the impairment in motor coordination in R6/2 mice (Domenici et al., 2007). The authors speculate that SCH-58621 administration between the 5th and the 6th week of life might interfer with the modulatory role of A_{2A}Rs on the activity of other systems and/or receptors. When the effect of SCH-58261 in R6/2 animal model was studied in symptomatic phase, the administration of SCH-58261 for 2 weeks R6/2 mice did not alleviate motor coordination alterations and only led to modest motor improvement in the inclined plane test (Cipriani et al., 2008).

The A_{2A}R agonist CGS-21680 5-week treatment (starting from the 7th week) in R6/2 mice improved motor coordination, reduced the loss of brain weight and the size of neuronal intranuclear inclusions in R6/2 mice (Chou et al., 2005), suggesting that in the frankly symptomatic phase of the disease the treatment with the A_{2A}R agonist, rather than the antagonist, could be beneficial. In agreement with these results, Martire et al. (2007) have shown a beneficial effects of CGS-21680 but only in symptomatic phases of HD in R6/2 mice. Interestingly, upon the activation of A_{2A}R opposite modulation of NMDA-induced toxicity occurred in wild type versus HD mice. In 12–13 weeks old animals the CGS-21680 treatment helped the recovery from the NMDA induced toxicity (measuring the recovery of extracellular field potentials) in the striatum in HD but worsened the recovery in wild type mice. In early symptomatic (7–8 weeks) mice, no differences were observed between wild type and HD animals in terms of basal synaptic transmission and response to NMDA (Martire et al., 2007).

1.5. Cannabinoid receptors

1.5.1. CB_1R

Cannabis, or marijuana, has been used for centuries, but its major psychoactive constituent, Δ^9-tetrahydrocannabinol (Δ^9-THC), was not identified until the 1960s (Gaoni et al., 1964). In 1990 the first

cannabinoid receptor was identified and named CB_1R (Matsuda et al., 1990) three years later followed by CB_2R (Munro et al., 1993). CB_2R was initially thought to be expressed mainly in the immune system (Pertwee et al., 1997) nevertheless its presence was larter described in glial cells (Sanchez et al., 2001; Walter et al., 2003; Nuñez et al., 2004). More recently several works reporting its expression in neuronal cells appeared (Ashton et al., 2006; Gong et al., 2006; Brusco et al., 2008ab). The endogenous agonists of cannabinoid receptors are endocannabinoids. The first identified endocannabinoid was anadamide, making reference to the Sanskrit name for bliss or happiness, ananda (Devane et al., 1992). Later described, 2-arachidonoylglycerol (2-AG) was found to be 200 times more abundant than anandamide (Sugiura et al., 1995; Stella et al., 1997). Apart of anandamide and 2-AG, other endocannabinoids were identified, as noladin ether (Hanus et al., 2001), virodhamin (Porter et al., 2002), N-arachinodoyldopamine (Huang et al., 2002). All the endogenous ligands are lipidic and are biosynthesized from distinct phospholipidic precursors present in cell membranes by Ca^{2+}-dependent synthetizing enzymes also located at the membrane (Bisogno et al., 2003; Okamoto et al., 2004). The enzymes necessary for the biosynthesis of anandamide are the Ca^{2+}-dependent N-acyltransferase and N-acyl-phosphatidylethanolaminephospholipase D. For the biosynthesis of 2-AG, the main enzymes involved are the Ca^{2+}-dependent and G_q-11-coupled receptor-activated phospholipase C and diacylglycerol lipase (DGL). The fact that the biosynthesis comes from membrane phospholipids indicates that these compounds are not stored in vesicles like other neurotransmitters but upon demand are synthetized, liberated to the extracellular space and cross the membrane due to their lipidic nature althorough there are several pieces of evidence that both liberatory and recapturing transporters exist (Beltramo et al., 1997; Hillard et al., 1997), but up to now it was not possible identify them (Ligresi et al., 2004). Interestingly, activation of D_2 receptor in the striatum increases the anadamide but not 2-AG production (Giuffrida et al., 1999; Ferrer et al., 2003). Similarly, activation of NMDA receptors in cortical neurons was shown to increase the 2-AG liberation whereas only simultaneous activation of NMDA and alpha-7 nicotinic receptors led to anadamine liberation (Stella et al., 2001). Endocannabinoids are degraded by presynaptically located monoacylglycerol lipase (Dinh et al., 2002) and postsynaptically located fatty acid amide hydrolase (FAHH) (Egertova et al., 2006).

The effects of cannabinoids include euphoria, relaxation, hypolocomotion or even catalepsy, tachycardia, vasodilatation, hypotermia, immunosupression and increase of appetite (Ameri et al., 1999; Piomelli et al., 2003; Di Marzo et al., 2004). The generalized effect of cannabinoid application in the brain leads to motor suppression.

CB_1 receptor (CB_1R) belongs to A family of GPCRs (Figure 26). Different from other receptors of this family, CB_1R does not have a disulphuric bridge in EC2, but it does contain one in EC3, and it does not contain Pro residue in TM5. It can be glycosylated in three loci and so its molecular weight can differ up to 10 kDa (Nie at al., 2001). Cannabinoid CB_1R is the most abundant G-protein-coupled receptor in the brain (Katona et al., 2008). Within the CNS, CB_1Rs are densely distributed in the basal ganglia, hippocampus, cerebral cortex and cerebellum, with low to moderate expression in the diencephalon, brainstem and spinal cord (Herkenham et al., 1990; Glass et al., 1997)

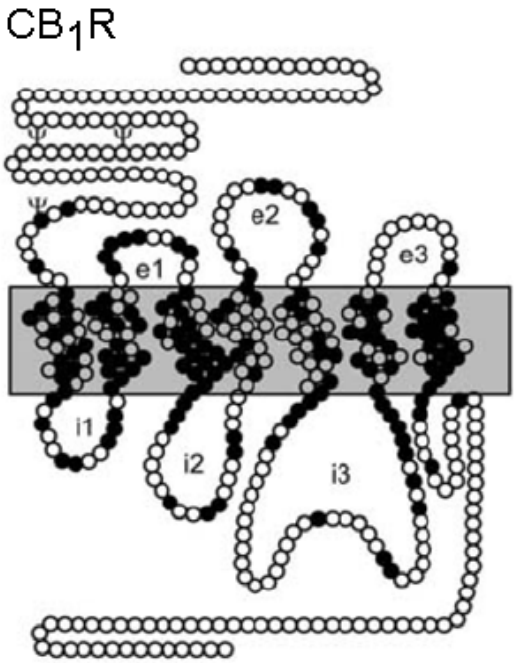

Figure 26. Schematic representation of CB_1R. Ψ marks a glycosylation sites, black balls are conserved in both CB_1R and CB_2R. Taken from (Sagredo et al., 2005).

The main CB_1R signalling pathway is mediated by coupling to inhibitory $G_{i/o}$ proteins, thus inhibiting adenylate cyclase (Howlett et al., 1986). Interestingly, under some condition, CB_1R can also couple to stimulatory G_s proteins, being a promiscuous G-protein coupling

receptor. This occurs, for example upon pretreatment with PTX (Bonhaus et al., 1998) or in CB_1R-D_2R heteromer (Glass et al., 1997; Bonhaus et al., 1998; Jarrahian et al., 2004; Kearn et al, 2005). D_2R normally couples do G_i as well as CB_1 but when they form heteromer, the receptor pair couples to only one G-protein which is G_i (Jarrahian et al., 2004). This was seen to happen constitutively (Jarrahian et al., 2004) but some works reported that $G_{s/olf}$ protein-dependent adenylate cyclase activation needs co-stimulation of both receptors (Glass et al., 1997; Kearn et al., 2005). CB_1R stimulation leads to activation of ERK 1/2 via both PKA dependent ($G_{\alpha s}$) and independent pathways (via $G_{\beta\gamma}$ or independently of G-protein via β-arrestin). In PC12 cells, upon PTX pretreatment, CB_1R signalling was via ERK 1/2 even when signalling via G_i was blocked. It is interesting to note, that this signalling pattern only occurs with the agonist HU210 (Scotter et al., 2010). Recent work showed that a single amino acid in IL2 is responsible for the preferential G_i or G_s coupling (Chen et al., 2010).

The CB_1R-mediated $G_{i/o}$-dependent signalling via ERK 1/2 was seen neuroprotective in a HD cellular model. Conversely, G_s-mediated signalling induced by "promiscuous" agonist, i.e. HU210 upon PTX pretreatment or by increasing cAMP levels led to an increase of huntingtin aggregates associated with cellular death (Scotter et al., 2010). The CB_1R-mediated activation of the ERK 1/2 pathway, c-Fos, and Krox-24 was seen strongly implicated in the protection against glutamate toxicity (Marsicano et al., 2003). Several in vivo studies have shown a robust up-regulation of c-Fos and Krox-24 in specific neuronal populations within the striatum (Glass et al., 1995, Valjent et al., 2001) and hippocampus (Marsicano et al., 2003; Valjent et al., 2001; Derkinderen et al., 2003) following cannabinoid treatment. Marsicano et al. (2003) showed that CB_1Rs knocked out specifically within the hippocampal glutamatergic neurons results in elevated glutamate toxicity leading to severe seizures and death. Krox-24 and c-Fos are physiologically regulated by CB_1Rs in specific neuronal cells and are likely involved in the long term neuronal changes induced by cannabinoids. Krox-24 has been associated with important biological functions such as the stabilization of long lasting long-term potentiation (Dragunov et al., 1996; Hughes et al., 1998), cell differentiation (Sukhatme et al., 1988; Krishnaraju et al., 1995; Pignatelli et al., 1999), as well as cell survival or death signal in neuronal cells (Pignatelli et al., 1999, 2003). CB_1Rs also activate

PKB/Akt and phosphoinositol-3-kinase signalling pathways (Bouaboula et al., 1995; Gomez del Pulgar et al., 2000; Pertwee et al., 1997). CB_1Rs inhibit voltage dependent calcium channels of both type N and P/Q and stimulate rectifying potassium channels (McAllister et al., 2002).

In the striatum, CB_1 receptors are mainly localized at synapses established between glutamatergic terminals and GABAergic (both enkephalinergic and dynorphinergic) neurons (Martin et al., 2008) and play a pivotal role in the inhibitory control of motor behaviour (Katona et al., 2008; Pazos et al., 2008). In the corticostriatal synapse CB_1Rs are mainly localized presynaptically but in lesser amount also postsynaptically (Rodriguez et al., 2001; Pickel et al., 2004; 2006; Kofalvi et al., 2005; Matyas et al., 2006; Uchigashima et al., 2007). The main physiological function of cannabinoids at these synapses is to regulate the neurotransmitters release (Freund et al., 2003; Marsicano et al., 2003; Katona et al., 2006). One of the best studied functions of endocannabinoids is their retrograde signalling with stimulation of presynaptic CB_1Rs and the consequent inhibition of neurotransmitter release. In both hippocampus and cerebellum it was shown that activation of postsynaptic neurons resulted in the release of endocannabinoids from these neurons (Ohno-Shossaku et al., 2001; Wilson et al., 2001; Kreitzer et al., 2001). Then, the endogenous ligands act as retrograde signalling molecules to inhibit presynaptic calcium influx in axonal terminals and, subsequently, reduce the release of neurotransmitter. 2-AG, rather than anandamide, seems to be mainly responsible for endocannabinoid-mediated retrograde signalling in the striatum and, probably in other brain areas (Hashimotodani et al., 2007).

Synthetic cannabinoid receptor agonists were classified into 4 families according to their chemical structure. The classical agonists, including HU-210, show similar structure to THC. The non-classical agonists, e.g. CP-55,940, are similar to the classical ligands but they do not contain the anillopyran ring. The aminoalquilindol family agonists, e.g. WIN-55,212-2, present a different chemical structure and, as it was described, bind to the receptor in a distinct binding site compared to the other agonists. The last family of eicosanoids, e.g. ACEA, bears a structure very similar to endocannabinoids (Lambert et al., 2005).

1.5.2. CB_1 receptor heteromers

In the striatal MSNs, CB_1R can be found in different heteromers at the pre- and postsynaptic level in the corticostriatal synapse. These heteromers are different in the direct and the indirect pathway according to the differential distribution of the partner receptors, namely $A_{2A}R$ and D_2R. To the described CB_1R heteromers belong the CB_1R-CB_1R homodimers (Wager-Miller et al., 2002), $A_{2A}R$-CB_1R (Carriba et al., 2007) and CB_1R-D_2R heterodimers (Marcellino et al., 2008), and $A_{2A}R$-CB_1R-D_2R heterotrimers (Navarro et al., 2008). The exact distribution of these receptor heteromers and their contribution to the control of motor function is being investigated and is not yet fully elucidated, although many pieces of partial knowledge are available. As previously mentioned, at the presynaptic level CB_1Rs activation leads to inhibition of glutamate release via inhibition of calcium channels thus suppressing the neurotrasmission in these synapses. This can theoretically lead to motor inhibition when suppressing the glutamate release in the direct pathway but could also lead to motor activation when suppressing the glutamate release in the indirect pathways. The final motor outcome is a combination of these effects and depends on the neurotransmitter concentrations i.e. the concentration of endocannabinoids, adenosine and dopamine, receptor levels activated by these neurotransmitters and importantly on the heteromerization between receptors which can induce changes in the neurotransmitters affinity. Importantly, not only presynaptic but also postsynaptic CB_1Rs participate on the motor control. At the pre-synaptic level at the synapses of direct pathway, CB_1Rs can heteromerize with $A_{2A}Rs$ and form CB_1R-$A_{2A}R$ heterodimer (Ferré et al., 2010). Recent work showed that presynaptic $A_{2A}R$ inhibits the CB_1R-mediated synaptic effects and that this occurs probably via cAMP-PKA pathway (Martire et al., 2011) and may or not be dependent on the formation of CB_1R-$A_{2A}R$ heterodimer, as it could also occur at the level of signalling.

On the other hand, in neuroblatoma cells endogenously expressing CB_1Rs and $A_{2A}Rs$, CB_1R signalling via $G_{\alpha i}$ is dependent on the $A_{2A}R$ activation (Carriba et al., 2007). The $A_{2A}R$ antagonist ZM-241385 inhibited the CB_1R agonist-induced decrease of forskolin-stimulated cAMP levels and antagonized the motor depressant effect of CB_1R activation, which seems contradictory to the $A_{2A}R$-CB_1R

relationship at the presynaptic level. Other recent work described that A_{2A}Rs activation, most probably the postsynaptic A_{2A}R activation, potentiated the synaptic effects of CB_1R (Tebano et al., 2010); and that the CB_1R induced depression of synaptic transmission was prevented by pharmacological or genetic inactivation of A_{2A}R (Soria et al., 2004; Yao et al., 2006). According to Tebano et al. (Tebano et al., 2010) in this case the postsynaptic mechanism would depend on the interaction between A_{2A}R and CB_1R receptors in the enkephalinergic MSN and probably also on the interaction with D_2R as some of the effects of CB_1R-A_{2A}R interactions seem to depend on D_2R function (Andersson et al., 2005). CB_1R-D_2R-A_{2A}R receptor heterotrimers have been detected in HEK cells by our group (Navarro et al., 2008), and they are likely to occur in the striatum (Ferré et al., 2009). Thus it seems that distinct CB_1Rs–containing heteromers differentially located at presynaptic or postsynaptic membranes can account for the described diverse relationship between adenosine and cannabinoids.

1.5.3. CB_1R in HD

The caudate nucleus and putamen showed a moderately low level of cannabinoid CB_1 receptor binding in the normal brain. The grade 0 of HD exhibited a moderate decrease in CB_1R binding (50 %) as compared to controls (Figure 27).

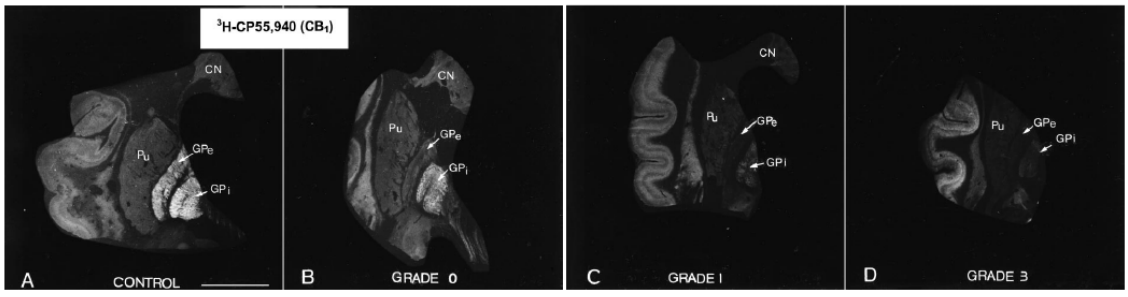

Figure 27. **CB_1R in HD.** Gradual disappearance of the CB_1R, stained by ^{3}H-CGS21680 binding, in the slices of human striatum in gradual states of the progression of the disease; Pu – putamen, GPe, GPi – globus pallidus externum and internum, SNc, SNr – substantia nigra pars compacta and pars reticulata, CN – caudate nucleus (Glass et al., 2000).

The CB_1R binding decreased dramatically in all HD patients with more advanced pathology, reaching binding values similar to background levels in grade 2 and 3. Very high densities of CB_1R

binding sites were seen in the globus pallidus of the control brains. The highest densities were present in the GPi and moderate densities were present throughout the rostrocaudal extent of the GPe. Cannabinoid receptor binding was decreased dramatically in both pallidal segments in all cases of HD (Figure 26). Within the very early stages of HD (grade 0), the loss of CP-55,940 binding was pronounced, being 9 % of normal. In contrast, the density of CB_1R binding in GPi had reduced to 19 % of normal. However, in the more advanced cases of HD, CB_1R binding in both segments had dramatically decreased to an average of between 3–7 % of normal levels. CB_1R labelling within the substantia nigra was very dense and discreetly localized to the pars reticulata. The levels of CB_1R binding showed a marked decrease in grade 0 (19 % of normal), and even greater decreases by grade 1 (10 % of normal). By grade 2, binding was undetectable above background levels (Glass et al., 2000).

The downregulation of CB_1 receptor expression observed in HD patients and animal models seems to occur at early stages of the disease and prior to the appearance of clinical symptoms, neurodegeneration and changes in other neurochemical parameters (Maccarrone et al., 2007; Pazos et al., 2008). The loss of CB_1Rs in mutant huntingtin transgenic mice is brain region-specific, as it occurs in the lateral striatum and, to a lesser extent, in the medial striatum, but not in the cortex (Denovan-Wright et al., 2000; McCaw et al., 2004). This early progressive loss subsequently contributes to the hyperkinesia observed in the initial phases of the disease (Denovan-Wright et al., 2000). A significant downregulation of CB_1 receptor binding and messenger RNA levels has been documented in the basal ganglia of HD patients (Glass et al., 2000) and animal models (Denovan-Wright et al., 2000; Lastres-Becker et al., 2002; McCaw et al., 2004).

The CB_1R activation was seen protective against mutant huntingtin-induced death via $G_{i/o}$- mediated inhibition of cAMP and phosphorylation of ERK 1/2 in an undifferentiated PC12 cell model of HD transfected with CB_1R. Nevertheless, CB_1Rs activation with some ligands (HU-210, but not WIN-55212-2 or BAY-59-3074) was in some conditions (upon PTX pretreatment) also capable of coupling to G_s and, stimulation of cAMP, and resulted in enhanced aggregate formation associated with cell death in this system (Scotter et al., 2010).

In 2011 a double-mutant mouse model expressing human mutant huntingtin exon 1 in CB_1R-null background was developed, so it was possible to study the role of CB_1R in HD (Blazquez et al., 2011). CB_1R deletion aggravated the symptoms in R6/2 mouse model. Administration of THC to R6/2 mice exerted a therapeutic effect and ameliorated neuropathology and molecular pathology. Experiments conducted in striatal cells showed that the mutant huntingtin-dependent receptor down-regulation involved the control of the CB_1R gene promoter by repressor element 1 silencing transcription factor and sensitizing cells to excitotoxic damage. *In vitro* and *in vivo* evidence supported the CB_1R control of BDNF expression and the decrease in BDNF levels concomitant with CB_1R loss, which may contribute significantly to striatal damage in Huntington's disease. The impact of CB_1R down-regulation on HD pathology is associated, at least in part, to a loss of wild-type huntingtin function process, and that the huntingtin-mediated control of CB_1R gene expression relies on REST, a transcriptional repressor that regulates the expression of a large network of neuronal proteins (Johnson et al., 2009). In addition, several reports support that CB_1Rs confer neuroprotection by enhancing BDNF expression, although the molecular basis of this connection remains unknown (Galve-Roperh et al., 2008). It is thus conceivable that the decrease of BDNF levels concomitant with CB_1R loss contributes significantly to striatal damage in HD (Zuccato et al., 2007) and CB_1R–evoked neuroprotection (Galve-Roperh et al., 2008). These results support the notion that CB_1R down-regulation is a key pathogenic event in HD, and suggest that activation of these receptors in patients with HD may attenuate disease progression (Blazquez et al., 2011). Thus, CB_1R genetic ablation in mice aggravates HD's symptoms and pathology, while CB_1R pharmacological activation attenuates them. Likewise, CB_1R down-regulation sensitizes striatal cells to excitotoxic damage, while enhanced CB_1R expression renders striatal cells more resistant to excitotoxic damage. Besides this pivotal role of CB_1Rs, the participation of other endocannabinoid system elements in Huntington's disease pathology might also be considered. Specifically, the striatal expression of the anandamide degrading enzyme FAAH is upregulated in symptomatic disease in HD-like mice as well as in patients with HD, most likely reflecting - like in other neuropathologies - a process of astroglial activation (Benito et al., 2003, 2007). Accordingly, the levels of anandamide and palmitoylethanolamide (another FAAH substrate) have been shown to

decline in the striata of symptomatic, but not pre-symptomatic, R6/2 mice (Bisogno et al., 2008). This decrease in endocannabinoid and endocannabinoid-like messengers might contribute to the aggravation of HD symptomatology at late stages of the disease.

Pharmacological activation of CB_1Rs in patients with early-stage HD might thus be beneficial in attenuating disease progression in these subjects. The first controlled trial conducted with a cannabis component (cannabidiol) reported no effect on chorea severity in 15 patients with Huntington's disease (Consroe et al., 1991). However, cannabidiol, although structurally similar to THC, is not a cannabinoid receptor agonist. The only double-blind, placebo-controlled, cross-over study of CB_1R agonist (specifically nabilone) in HD has been recently reported (Curtis et al., 2009). This 44-patient trial has shown improvements in total motor score, chorea, cognition, behaviour and neuropsychiatric inventory upon cannabinoid treatment, which was safe and well tolerated.

AIMS

G-protein-coupled receptors interact with distinct proteins in the extracellular and intracellular domains of the cell and also they interact each other forming homo- and heterodimers or higher oligomers at the membrane level. Since these protein-protein interactions implicate important changes in the functionality of these receptors, the knowledge of the new pharmacological and signalling properties of these protein complexes is found very useful to understand neuronal transmission and in the search of pharmaceutical compounds of high efficiency in neurological diseases. In this frame, the general aim of this Thesis is *to investigate the pharmacological and functional consequences of adenosine A_{2A} receptor interaction with other proteins***.** To reach this geneneral aim, three particular aims were formulated.

At the cell surface, the enzyme ADA, in addition to regulate the neurotransmission by metabolizing the neuromodulator adenosine, has an important relevance as an allosteric modulator of adenosine receptors. As it was previously described in our research group, ADA is an allosteric modulator of A_1 and A_{2B} adenosine receptors, but it was not known if ADA modulates the A_{2A} receptor function. Thus, the first aim of this Thesis was to study the molecular and functional interaction between ADA and A_{2A} receptors.

AIM 1. To study the molecular and functional interaction between ADA and adenosine A_{2A} receptors

The A_{2A} receptors heteromerize with adenosine A_1 receptors at the presynaptic level in the corticostriatal synapse of the direct pathway GABAergic neurons and with dopamine D_2 receptors at the postsynaptic level in the indirect pathway GABAergic neurons, having A_{2A} receptors a different and specific physiological role in each of these two heteromers. Taking into account that a receptor heteromer is a macromolecular complex composed of at least two functional receptor units with biochemical properties that are demonstrably different from those of its individual receptors, in the second aim of this Thesis we investigated the pharmacological characteristics of ligand binding to A_{2A} receptors in these two heteromers. In this aim,

we wanted to find an A_{2A} receptor antagonist more selective for A_{2A}-A_1 receptor heteromer. This type of compounds could be used to lower the neutrotrasmission of the direct pathway whose dominant functioning is the cause of the appearance of hiperkinetic movements in patients with Huntington´s disease.

AIM 2. To search for more selective antagonists of A_{2A} receptor for presynaptic A_1-A_{2A} receptor heteromers *versus* postsynaptic A_{2A}-D_2 receptor heteromers that can be useful for the treatment of neurological diseases, particularly in Huntington´s disease

In addition to heteromerize with adenosine A_1 and dopamine D_2 receptors, A_{2A} receptors also interact with cannabinoid receptors CB_1. CB_1 receptors are found pre- and postsynaptically in both the direct and indirect pathways and might modulate the A_{2A} receptor function in the striatum. In fact, the A_{2A}-CB_1 receptor heteromers were previously described by our research group and, although it was known that activation of A_{2A} receptors was necessary for CB_1 receptor signalling in neuroblastoma cell line where the heteromers were expressed, the pharmacological and functional characteristics of these heteromers are not known. In this Thesis, we wanted to study the pharmacological and functional characteristics of A_{2A}-CB_1 receptor heteromers and also to determine whether selective A_{2A} receptor antagonists show different selectivity for A_{2A} receptors or A_{2A}-CB_1 receptor heteromers in order to characterize pharmacological tools able to block A_{2A} receptors forming or not forming heteromers with CB_1 receptors. To do this, we formulated the third objective of this Thesis:

AIM 3. To investigate the pharmacological and functional properties of A_{2A} receptors in the A_{2A}-CB_1 receptor heteromers and to determine whether selective A_{2A} receptor antagonists show different selectivity for A_{2A} receptors or A_{2A}-CB_1 receptor heteromers

RESULTS

La unión y la señalización de ligandos del receptor A_{2A} de adenosina están moduladas alostéricamente por la adenosina desaminasa.

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Los receptores A_{2A} de adenosina están presentes de forma abundante en el estriado, zona principal del control motor en el sistema nervioso central. Técnicas de transferencia de energía por resonancia bioluminis-cente (BRET) mostraron que los homómeros de los receptores A_{2A} pueden actuar como proteínas de anclaje del enzima adenosina desaminasa (ADA; EC 3.5.4.4) en la superficie celular. La unión del ADA modificó la estructura cuaternaria de los receptores A_{2A} de adenosina, presentes en la superficie celular, e incrementó tanto la afinidad de agonistas como de antagonistas en experimentos de unión de radioligandos a membranas estriatales, en las que se coexpresan ambas proteínas. La ADA también incrementó la fosforilación mediada por ERK 1/2. En conjunto, todos estos experimentos muestran que la ADA, además de regular la concentración extracelular de adenosina, puede actuar como modulador alostérico incrementando de forma considerable la afinidad de sus ligandos y la funcionalidad de los receptores. Esta poderosa regulación puede tener implicaciones en la fisiología y farmacología de los receptores A_{2A} de adenosina neuronales.

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A_{2A} adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase

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A_{2A}Rs (adenosine A_{2A} receptors) are highly enriched in the striatum, which is the main motor control CNS (central nervous system) area. BRET (bioluminescence resonance energy transfer) assays showed that A_{2A}R homomers may act as cell-surface ADA (adenosine deaminase; EC 3.5.4.4)-binding proteins. ADA binding affected the quaternary structure of A_{2A}Rs present on the cell surface. ADA binding to adenosine A_{2A}Rs increased both agonist and antagonist affinity on ligand binding to striatal membranes where these proteins are co-expressed. ADA also increased receptor-mediated ERK1/2 (extracellular-signal-regulated kinase 1/2) phosphorylation. Collectively, the results of the present study show that ADA, apart from regulating the concentration of extracellular adenosine, may behave as an allosteric modulator that markedly enhances ligand affinity and receptor function. This powerful regulation may have implications for the physiology and pharmacology of neuronal A_{2A}Rs.

Key words: adenosine deaminase, adenosine receptor, allosteric interaction, G-protein-coupled receptor, protein–protein interaction, receptor binding parameter.

INTRODUCTION

Self-association of proteins to form dimers and higher-order oligomers and/or interaction with other proteins are key factors in cell signalling [1–3]. A paradigmatic example are adenosine receptors. The nucleoside adenosine exerts a modulatory action in many areas of the CNS (central nervous system) via its four GPCR (G-protein-coupled receptor) subtypes: A_1Rs (adenosine A_1 receptors) and A_3Rs (adenosine A_3 receptors) that are negatively coupled to the adenylate cyclase, and A_{2A}Rs (adenosine A_{2A} receptors) and A_{2B}Rs (adenosine A_{2B} receptors) that mediate the stimulation of adenylate cyclase activity [4]. Along the plasma membrane (horizontal plane), A_1Rs and A_{2A}Rs may form homo-oligomers [5–7] and heteromers with other receptors [8–11], and the oligomerization generates new and unique biochemical and functional characteristics by modulating the binding properties, G-protein coupling and receptor trafficking [3,12,13]. Across the membrane (vertical to the plane of the membrane), A_1Rs interact with intracellular proteins that are not directly involved in the signalling cascade, such as the Hsc73 (heat-shock cognate 73 stress protein), and this direct interaction is relevant for receptor function [14]. Also across the membrane, both A_1Rs and A_{2B}Rs interact with a protein that has an extracellular topology, ADA (adenosine deaminase) [15–18].

ADA is an enzyme involved in purine metabolism which catalyses the hydrolytic deamination of adenosine and 2′-deoxyadenosine to inosine or 2′-deoxyinosine and ammonia. Congenital defects of ADA lead to SCID (severe combined immunodeficiency), which is characterized by the absence of functional T- and B-lymphocytes in affected individuals [19,20]. Neurological abnormalities, which are less life threatening than immunological abnormalities, have also been described in a portion of patients [21]. Neurological alterations may be secondary to infections, or may be due to the accumulation of adenosine and derivatives in brain. Although the location of ADA is mainly cytosolic, it has been found on the cell surface of many cell types, including neurons [22]; therefore it can be considered as an ecto-enzyme [19]. Apart from A_1Rs and A_{2B}Rs, another class of ecto-ADA-binding protein is CD26, a multifunctional transmembrane glycoprotein, acting as a receptor and a proteolytic enzyme [23]. It has been shown that ADA anchored to the dendritic cell surface, probably by the A_{2B}R, binds to CD26 expressed on the surface of T-cells, triggering co-stimulation and enabling an enhanced immune response [24–26].

We have also demonstrated that binding of enzymatically active or inactive ADA to A_{2B}R increases its affinity and signalling by a protein–protein interaction [17]. In the case of A_1Rs, the ADA–A_1R interaction is very relevant since the enzyme potentiates signal transduction and modulates the desensitization of A_1Rs [15,18,27]. Despite the well-established positive modulation exerted by ADA on A_1Rs and A_{2B}Rs, it is not known whether the enzyme is able to modulate the A_{2A}R subtype. There is currently a major interest in the ability of central A_{2A}Rs to control synaptic plasticity at glutamatergic synapses due to a combined ability of these receptors to facilitate the release of glutamate and the activation of NMDA; furthermore, A_{2A}Rs also control glial function and brain metabolic adaptation, and are important in controlling the demise of neurodegeneration [28]. In

Abbreviations used: A_{2A}R, adenosine A_{2A} receptor; A_{2B}R, adenosine A_{2B} receptor; ADA, adenosine deaminase; BCA, bicinchoninic acid; BRET, bioluminescence resonance energy transfer; CHO, Chinese-hamster ovary; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HEK-293T, HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40); PEI, polyethylenimine; Rluc, *Renilla* luciferase; SCID, severe combined immunodeficiency; TM, transmembrane domain; YFP, yellow fluorescent protein.

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the present paper we report the molecular interaction between ADA and $A_{2A}R$ that results in ADA-induced conformational changes in the quaternary structure of $A_{2A}Rs$ homodimers and in the pharmacological and functional characteristics of brain striatal $A_{2A}Rs$. A fine-tune regulation exerted by ADA probably has important implications for the physiology and pharmacology of neuronal $A_{2A}Rs$.

EXPERIMENTAL

Fusion proteins and expression vectors

The human cDNA for the $A_{2A}Rs$ or $GABA_{B2}$ (γ-aminobutyric acid B2) receptors cloned into pcDNA3.1 were amplified (removing stop codons) using sense and antisense primers harbouring either unique EcoRI or KpnI sites. The fragments were then subcloned to be in-frame with Rluc (*Renilla* luciferase) into the EcoRI and KpnI restriction site of an Rluc-expressing vector (pRluc-N1; PerkinElmer), or into the EcoRI and KpnI or BamHI restriction site of the variant of GFP (green fluorescent protein) (EYFP-N3; enhanced yellow variant of GFP; Clontech), to give the plasmids that express $A_{2A}Rs$ or $GABA_{B2}$ receptors fused to Rluc or YFP (yellow fluorescent protein) on the C-terminal end of the receptor ($A_{2A}R$–Rluc, $A_{2A}R$–YFP or $GABA_{B2}R$–Rluc). As previously reported [9,11], when analysed by confocal microscopy, it was observed that all fusion proteins showed a similar membrane distribution as naïve receptors, and fusion of Rluc and YFP to $A_{2A}Rs$ did not modify receptor function, as determined by cAMP assays.

Transient transfection

HEK-293T [HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40)] cells were grown in DMEM (Dulbecco's modified Eagle's medium; Gibco) supplemented with 2 mM L-glutamine, 100 units/ml penicillin/streptomycin and 5 % (v/v) heat-inactivated FBS (fetal bovine serum) (all supplements were from Invitrogen). HEK-293T cells growing in six-well dishes were transiently transfected with the corresponding fusion protein cDNA using the PEI (polyethylenimine; Sigma) method. Cells were incubated (for 4 h) with the corresponding cDNA together with PEI (5.47 mM nitrogen residues) and 150 mM NaCl in a serum-starved medium. After 4 h, the medium was changed to a fresh complete culture medium. At 48 h after transfection, cells were washed twice in quick succession in HBSS (Hanks balanced salt solution) with 10 mM glucose, detached and resuspended in the same buffer containing 1 mM EDTA. To control the cell number, the protein concentration of the sample was determined using the BCA (bicinchoninic acid) method (Pierce) using BSA dilutions as standards.

Generation of a CHO (Chinese-hamster ovary) cell clone expressing $A_{2A}Rs$

CHO cells were maintained at 37 °C in an atmosphere of 5 % CO_2 in αMEM (α-minimal essential medium) without nucleosides (Invitrogen), containing 10 % FBS, 50 μg/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine (300 μg/ml). CHO cells were transfected with the cDNA corresponding to human $A_{2A}R$ and cloned into a pcDNA3.1/Hygro vector with a hygromycin-resistance gene using the Lipofectamine™ (Invitrogen) method following the manufacturer's instructions. At 1 day after transfection, the selection antibiotic was added at a concentration that was previously determined using a selection antibiotic test. The antibiotic-resistant clones were isolated and cultured in six-well plates in the presence of the selection antibiotic. After an appropriate number of days/passages, a stable line expressing 6 ± 1 pmol/mg of protein, with an affinity constant for the $A_{2A}R$ antagonist ZM 241385 of 1 ± 0.3 nM, was selected and cultured in the presence of hygromycin (300 μg/ml).

BRET (bioluminescence resonance energy tranfer)

HEK-293T cells were co-transfected with 0.15 μg of cDNA corresponding to $A_{2A}R$–Rluc acting as a BRET donor, and increasing amounts of cDNA corresponding to $A_{2A}R$–YFP (0.8–3 μg of cDNA) acting as a BRET acceptor. As a negative control, HEK-293T cells were co-transfected with 0.15 μg of $A_{2A}R$–Rluc and increasing amounts of cDNAs corresponding to the $GABA_{B2}$–YFP receptor (0.3–3 μg of cDNA). After 48 h of transfection, the cell suspension (20 μg of protein) was dispensed in duplicate into 96-well black microplates with a transparent bottom (Porvair), and the fluorescence was measured using a Mithras LB940 fluorescence-luminescence detector (Berthold) with an excitation filter of 485 nm and an emission filter of 535 nm. For BRET measurement, 20 μg of cell suspension was distributed in duplicate into 96-well white opaque microplates (Porvair), and coelenterazine H (Molecular Probes) was added at a final concentration of 5 μmol/l. After 1 min the readings were collected in a Mithras LB 940 instrument which allows the integration of the signals detected in the short-wavelength filter at 485 nm (440–500 nm) and the long-wavelength filter at 530 nm (510–590 nm). The same samples were incubated for 10 min, and the luminescence was measured to quantify the donor. The BRET ratio is defined as:

$$[(\text{emission at } 510-590)/(\text{emission at } 440-500)] - \text{Cf}$$

where Cf corresponds to (emission at 510–590)/(emission 440–500) for the A_{2A}–Rluc construct expressed alone in the same experiment. Curves were fitted to a non-linear regression equation, assuming a single phase with GraphPad Prism software (San Diego, CA, U.S.A.).

Immunostaining

Wild-type CHO cells and $A_{2A}R$-expressing CHO cells, grown on glass coverslips, were washed with PBS and fixed with 2 % paraformaldehyde and 60 mM sucrose (pH 7.4) for 15 min at room temperature (25 °C). Cells were washed twice with PBS containing 15 mM glycine, and treated with 1 % BSA, 20 mM glycine and 0.05 % sodium azide for 20 min before the addition of the antibodies. Then, cells were labelled for 45 min either with 100 μg/ml of the anti-$A_{2A}R$ antibody [14,29] or 50 μg/ml of the anti-ADA antibody [30], both conjugated with FITC as described previously [14]. Cells were washed with PBS containing 1 % BSA, 20 mM glycine and 0.05 % sodium azide, and placed on coverslips for the subsequent fluorescence microscopy analysis in a Leica TCS 4D confocal laser-scanning microscope (Leica Lasertechnik).

Brain striatal membrane preparation and protein determination

Sheep brains were obtained from the local slaughterhouse. Membrane suspensions from sheep brain striatum were prepared as described previously [31]. Tissue was disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica) for three 5 s periods in 10 vol. of 50 mM Tris/HCl buffer (pH 7.4), containing a protease inhibitor cocktail (Sigma, 1:1000). After eliminating cell debris by centrifugation at 1000 ***g*** for 10 min, membranes were obtained by centrifugation at 35 000 rev./min (40 min at

4 °C; rotor type 90 Ti, Beckman) and the pellet was resuspended and recentrifuged under the same conditions. The pellet was stored at − 80 °C and was washed once more as described above and resuspended in 50 mM Tris/HCl buffer for immediate use. Protein was quantified using the BCA method (Pierce) using BSA dilutions as the standard.

Enzyme activity of ADA and ADA inhibition by Hg^{2+}

Bovine ADA (Roche) enzyme activity was determined at 25 °C with 0.1 mM adenosine as the substrate in 50 mM Tris/HCl buffer (pH 7.4). The decrease in the absorbance at 265 nm ($\Delta\varepsilon = 7800\ M^{-1} \cdot cm^{-1}$) was monitored in an Ultrospec 3300 pro spectrophotometer (Biochrom); 1 ml cuvettes with a 1 cm light pathlength were used. Hg^{2+}-inactivation of bovine ADA was performed by a pre-incubation (2 h), of 15 units/ml desalted ADA with 100 μM $HgCl_2$, and removal of free Hg^{2+} by gel filtration as described previously [16]. No residual activity was found after a 4 h incubation with 0.1 mM adenosine and a high excess (10 μg/ml) of inhibited enzyme in the conditions described above.

Radioligand-binding experiments

ADA dose-dependent curves were obtained by incubating (2 h) sheep brain striatal membrane suspensions (0.3 mg of protein/ml) with the indicated concentration of $A_{2A}R$ agonist [^{3}H]CGS 21680 (42.7 Ci/mmol; PerkinElmer) or $A_{2A}R$ antagonist [^{3}H]ZM 241385 (27 Ci/mmol; American Radiolabelled Chemicals) in the presence or the absence of the indicated amounts of desalted bovine ADA at 25 °C in 50 mM Tris/HCl buffer (pH 7.4), containing 10 mM $MgCl_2$.

Saturation experiments were performed by incubating striatal membrane suspensions (0.3 mg of protein/ml) with increasing concentrations of the $A_{2A}R$ antagonist [^{3}H]ZM 241385 (triplicates of ten different concentrations, from 0.1 to 27 nM), at 25 °C in 50 mM Tris/HCl buffer (pH 7.4), containing 10 mM $MgCl_2$, in the absence or the presence of 0.2 i.u./ml (1 μg/ml) ADA.

Competition experiments were performed by incubating striatal membrane suspensions (0.3 mg of protein/ml) with a constant amount of [^{3}H]CGS 21680 or [^{3}H]ZM 241385 and it was increasing concentrations of CGS 21680 (triplicates of ten different concentrations from 1 nM to 10 μM; Tocris) or ZM 241385 (triplicates of 11 different concentrations, from 0.01 nM to 10 μM; Tocris) in the absence or presence of 0.2 i.u./ml (1 μg/ml) desalted ADA at 25 °C in 50 mM Tris/HCl buffer (pH 7.4), containing 10 mM $MgCl_2$, providing sufficient time to achieve equilibrium for the lowest radioligand concentration (5 h). In all experiments, non-specific binding was determined in the presence of 10 μM CGS 21680 or 10 μM ZM 241385 and it was confirmed that the value was the same as calculated by extrapolation of the competition curves. Free and membrane-bound ligand were separated by rapid filtration of 500 μl aliquots in a cell harvester (Brandel) through Whatman GF/C filters embedded in 0.3 % PEI, which were subsequently washed for 5 s with 5 ml of ice-cold Tris/HCl buffer (pH 7.4). The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics) overnight at room temperature, and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer) with an efficiency of 62 % [14].

Binding-data analysis

Since $A_{2A}Rs$ are expressed as dimers or higher-order oligomers [6,13], radioligand competition curves were analysed by non-linear regression using the commercial Grafit curve-fitting software (Erithacus Software), by fitting the specific binding data to the mechanistic two-state dimer receptor model [32,33]. This model considers a homodimer as the minimal structural unit of the receptor. To calculate the macroscopic equilibrium dissociation constants from saturation binding experiments the following equation previously deduced [34] was considered (eqn 1):

$$A_{bound} = (K_{DA2} \times A + 2A^2) \times R_T / (K_{DA1} \times K_{DA2} + K_{DA2} \times A + A^2) \quad (1)$$

where A represents the free radioligand (the $A_{2A}R$ antagonist [^{3}H]ZM 241385) concentration, R_T is the total amount of receptor dimers, and K_{DA1} and K_{DA2} are the macroscopic dissociation constants describing the binding of the first and the second radioligand molecule to the dimeric receptor.

When binding of A to the dimer is non-co-operative, $K_{DA2}/K_{DA1} = 4$ (see [32,33] for details) and, therefore, K_{DA1} is enough to characterize the binding. In this case, the above equation can be reduced to (eqn 2):

$$A_{bound} = 2A \times R_T / (2K_{DA1} + A) \quad (2)$$

To calculate the macroscopic equilibrium dissociation constants from competition binding experiments the following equation previously deduced [34,35] was considered (eqn 3):

$$A_{total\ bound} = (K_{DA2} \times A + 2A^2 + K_{DA2} \times A \times B/K_{DAB}) \times R_T / [K_{DA1} \times K_{DA2} + K_{DA2} \times A + A^2 + K_{DA2} \times A \times B/K_{DAB} + K_{DA1} \times K_{DA2} \times B/K_{DB1} + K_{DA1} \times K_{DA2} \times B^2/(K_{DB1} \times K_{DB2})] + A_{non-specific\ bound} \quad (3)$$

Here A represents free radioligand (the $A_{2A}R$ agonist [^{3}H]CGS 21680 or the $A_{2A}R$ antagonist [^{3}H]ZM 241385) concentration, B represents the assayed competing compound (CGS 21680 or ZM 241385) concentration, and K_{DB1} and K_{DB2} are, respectively, the macroscopic equilibrium dissociation constants of the first and second binding of B; K_{DAB} is the hybrid equilibrium radioligand/competitor dissociation constant, which is the dissociation constant of B binding to a receptor dimer semi-occupied by A.

Binding to GPCRs can display negative co-operativity and in these circumstances $K_{D2}/K_{D1} > 4$. On the other hand, for positive co-operativity, $K_{D2}/K_{D1} < 4$ [34]. To measure the degree of co-operativity, the two-state dimer receptor model also introduces a co-operativity index (D_C). The dimer co-operativity index for the radioligand A ([^{3}H]ZM 241385) or the competing ligand B (CGS 21680 or ZM 241385) was calculated as [13,34,35] (eqn 4):

$$D_{CA} = \log(4K_{DA1}/K_{DA2});\ D_{CB} = \log(4K_{DB1}/K_{DB2}) \quad (4)$$

D_C measures the affinity modifications occurring when a protomer senses the binding of the same ligand molecule to the partner protomer in a dimer. The way the index is defined is such that its value is '0' for non-co-operative binding, positive values of D_C indicate positive co-operativity, whereas negative values imply negative co-operativity [13,34,35].

In the experimental conditions when both the radioligand A ([^{3}H]CGS 21680 or [^{3}H]ZM 241385) and the competitor B (CGS 21680 or ZM 241385) show non-co-operativity ($D_C = 0$), it results that $K_{DA2} = 4K_{DA1}$ and $K_{DB2} = 4K_{DB1}$, and eqn (3) was simplified

to (eqn 5):

$$A_{\text{total bound}} = (4K_{DA1} \times A + 2A^2 + 4K_{DA1} \times A \times B/K_{DAB}) \times R_T/\left(4K_{DA1}^2 + 4K_{DA1} \times A + A^2 + 4K_{DA1} \times A \times B/K_{DAB} + 4K_{DA1}^2 \times B/K_{DB1} + K_{DA1}^2 \times B^2/K_{DB1}^2\right) + A_{\text{non-specific bound}} \quad (5)$$

When both the radioligand A ([^{3}H]CGS 21680 or [^{3}H]ZM 241385) and the competitor B are the same compound and the binding is non-co-operative, eqn (5) simplifies to (eqn 6):

$$A_{\text{total bound}} = (4K_{DA1} \times A + 2A^2 + A \times B) \times R_T/\left(4K_{DA1}^2 + 4K_{DA1} \times A + A^2 + A \times B + 4K_{DA1} \times B + B^2\right) + A_{\text{non-specific bound}} \quad (6)$$

Goodness of fit was tested according to a reduced χ^2 value given by the non-linear regression program. The test of significance for two different population variances was based upon the *F*-distribution (see [32] for details). Using this *F* test, a probability greater than 95 % ($P < 0.05$) was considered the criterion to select a more complex equation to fit binding data over the simplest one. In all cases, a probability of less than 70 % ($P > 0.30$) resulted when one equation to fit binding data was not significantly better than the other. Results are given as parameter values ± S.E.M. of three to four independent experiments.

ERK (extracellular-signal-regulated kinase) phosphorylation assay

A_{2A}R-expressing CHO cells were cultured in serum-free medium for 16 h before the addition of any agent. Cells were treated (for 1 h at 37 °C) with medium or the indicated concentration of ADA before the addition of the A_{2A}R agonist CGS 21680 for a further incubation of 5 min. Cell were washed with ice-cold PBS and lysed by the addition of 500 μl of ice-cold lysis buffer [50 mM Tris/HCl (pH 7.4), 50 mM NaF, 150 mM NaCl, 45 mM 2-glycerophosphate, 1 % Triton X-100, 20 μM phenyl-arsine oxide, 0.4 mM sodium orthovanadate and protease inhibitor cocktail]. Cell debris was removed by centrifugation at 13 000 ***g*** for 5 min at 4 °C and the protein was quantified using the BCA method using BSA dilutions as standards. To determine the level of ERK1/2 phosphorylation, equivalent amounts of protein (15 μg) were separated by electrophoresis on denaturing SDS/PAGE (10 % gels) and transferred on to PVDF-FL membranes. Odyssey blocking buffer (LI-COR Biosciences) was then added, and membranes were rocked for 90 min. Membranes were then probed with a mixture of a mouse anti-(phospho-ERK 1/2) antibody (1:2500 dilution; Sigma) and rabbit anti-ERK 1/2 antibody (1:40 000 dilution; Sigma) for 2–3 h. Bands were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10 000 dilution; Sigma) and IRDye 680 (anti-rabbit) antibody (1:10 000 dilution; Sigma) for 1 h and scanned by the Odyssey IR scanner (LI-COR Biosciences). Bands densities were quantified using the scanner software and exported to Excel (Microsoft). The level of phosphorylated ERK1/2 isoforms was normalized for differences in loading using the total ERK protein band intensities.

RESULTS

ADA was anchored to the cell surface of A_{2A}R-expressing cells

To investigate a potential direct interaction of ADA and A_{2A}Rs, wild-type CHO cells and a CHO–A_{2A}R clone were selected, since

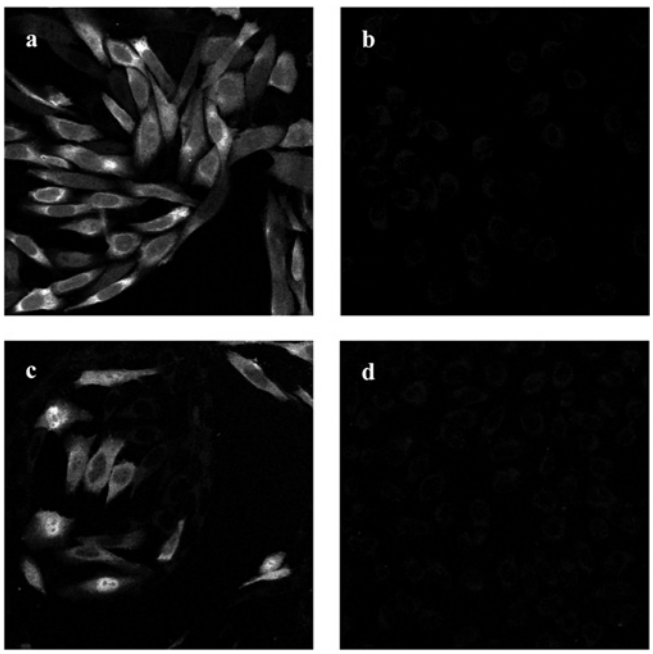

Figure 1 Expression of ADA on the cell surface of wild-type and A_{2A}R-expressing CHO cells

Non-permeabilized wild-type CHO cells (**b** and **d**) or CHO–A_{2A}R cell clone (**a** and **c**) were labelled with FITC-conjugated anti-A_{2A}R antibody (**a** and **b**) or with FITC-conjugated anti-ADA antibody (**c** and **d**). Cells were processed for confocal microscopy analysis as described in the Experimental section.

CHO cells do not constitutively express adenosine receptors and since rodent CD26 endogenously expressed in CHO cells does not interact with ADA [36]. Parental CHO cells did not express A_{2A}Rs since they could not be labelled using a specific anti-A_{2A}R antibody (Figure 1b). The CHO–A_{2A}R clone showed a marked staining for A_{2A}R (Figure 1a). ADA, which was detected in the cytoplasm using permeabilized CHO cells (results not shown), did not appear at the cell surface of parental CHO cells (Figure 1d). However, cell-surface ADA was detected in CHO–A_{2A}R cells (Figure 1c), indicating that the ADA released to the cell culture may bind to the cell surface only in cells expressing A_{2A}Rs. These results indicate that the cell-surface A_{2A}R behaved as an ADA-anchoring protein.

ADA binding affected the quaternary structure of A_{2A}Rs

To investigate the consequences of the ADA–A_{2A}R interaction, and taking into consideration that A_{2A}Rs are expressed as dimers or higher-order oligomers [6], the effect of ADA on the quaternary structure of A_{2A}R–A_{2A}R homomers was analysed by BRET experiments. Cells were co-transfected with 0.15 μg of the cDNA encoding A_{2A}R–Rluc and increasing amounts of the cDNA corresponding to A_{2A}R–YFP. At 48 h post-transfection, cells were treated (20 min at 37 °C) with medium or with 1 μg/ml ADA in medium, and BRET was measured. In the absence of ADA, the hyperbola obtained upon increasing the acceptor expression indicated a specific interaction between the two fusion proteins (Figure 2). The $BRET_{max}$ was 43 ± 3 mBU and the $BRET_{50}$ was 9 ± 2. The specificity of the A_{2A}R homomerization was confirmed by the unspecific (linear) BRET signal obtained in cells co-transfected with the cDNA corresponding to A_{2A}R–Rluc and increasing amounts of the cDNA corresponding to $GABA_{B2}$–YFP receptor (Figure 2). Interestingly, in the presence of ADA, a significant ($P < 0.01$) increase in the $BRET_{max}$ was observed

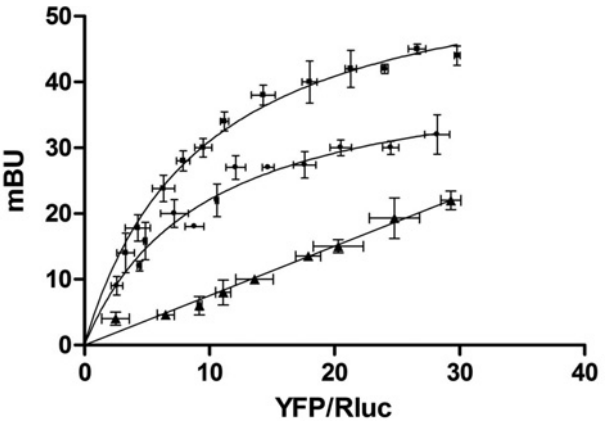

Figure 2 Effect of ADA on A_{2A}R homomerization detected by BRET experiments

BRET saturation experiments were performed as described in the Experimental section using cells transfected with 0.15 μg of cDNA corresponding to A_{2A}R–Rluc and increasing amounts of cDNA corresponding to A_{2A}R–YFP (0.8–3 μg of cDNA) (■ and ●) or to $GABA_{B2}$–YFP receptor (0.3–3 μg of cDNA) as a negative control (▲). After 48 h of transfection, cells were treated for 20 min with medium (● and ▲) or with 1 μg/ml ADA (■) before BRET determination. Both fluorescence and luminescence for each sample were measured before every experiment to confirm similar donor expressions (approximately 120 000 bioluminescence units) while monitoring the increase in acceptor expression (10 000–50 000 fluorescence units). The relative amount of BRET is given as the ratio between the fluorescence of the acceptor (YFP) and the luciferase activity of the donor (Rluc). BRET data are expressed as means ± S.E.M. of three to four different experiments grouped as a function of the amount of BRET acceptor.

(60 ± 2 mBU) without significant alterations in $BRET_{50}$ (9 ± 1). These results can be interpreted in two ways. In one, ADA led to conformational changes in A_{2A}R homomers that reduces the distance between Rluc and YFP fused to the C-terminal domain of the two A_{2A}R-containing fusion proteins. In the other, ADA increases the receptor homomerization by increasing the affinity between protomers. In this last case, a decrease in the $BRET_{50}$ values could be expected as there is binding between monomers to give homomers; $BRET_{50}$ might represent the affinity between protomers. Since the $BRET_{50}$ values were not changed in the presence of ADA we favour the first interpretation, that of ADA causing conformational changes.

ADA modulated the agonist and antagonist binding to A_{2A}Rs

The effect of ADA on ligand binding to A_{2A}Rs was first determined using A_{2A}Rs expressed in a more physiological context. For this purpose striatal membranes, which express a high amount of A_{2A}R, were selected. Isolated membranes were incubated with increasing concentrations of ADA and 17 nM of the radiolabelled A_{2A}R agonist ([^{3}H]CGS 21680, see the Experimental section). ADA enhanced in a dose-dependent manner the agonist binding to A_{2A}Rs (Figure 3a) with an EC_{50} value of 0.26 ± 0.03 ng/ml, which approximately corresponds to 6 pM. To test whether the effect of ADA was independent of its enzymatic activity, a preparation containing an irreversible-inhibited enzyme was used. ADA was inactivated using a preparation containing 100 μM Hg^{2+}; non-bound Hg^{2+} was removed by gel filtration prior to the assays (see the Experimental section). Membrane suspensions were incubated with 17 nM [^{3}H]CGS 21680 in the absence or in the presence of 1 μg/ml of active or Hg^{2+}-inactivated ADA. Both, active or Hg^{2+}-inactivated ADA enhanced to a similar extent agonist binding to striatal A_{2A}Rs (Figure 3a, inset), thus demonstrating that the effect was independent of the enzyme activity and suggesting that, in our exhaustively washed membrane preparation, there is not enough endogenous adenosine to interfere with the ligand binding

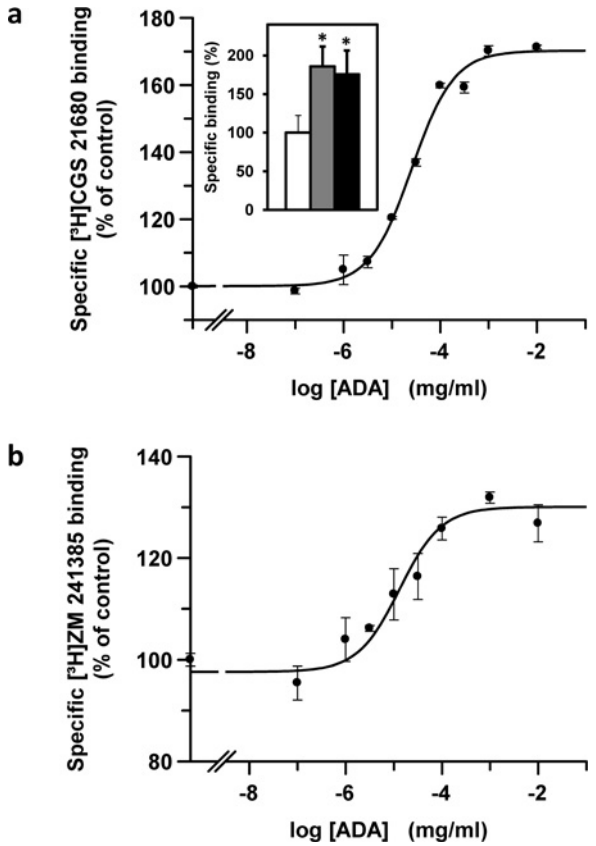

Figure 3. Effect of ADA on A_{2A}R agonist and antagonist binding to brain striatal membranes

Binding of 17 nM [^{3}H]CGS 21680 (**a**) or 1.6 nM [^{3}H]ZM 241385 (**b**) to striatal membranes (0.3 mg of protein/ml) was performed as described in the Experimental section, in the presence of increasing concentrations of ADA. Data points on the *y* axis correspond to the binding in the absence of ADA. Inset in (**a**): 17 nM [^{3}H]CGS 21680 binding in the absence (white bar) or in the presence of 1 μg/ml of active (grey bar) or Hg^{2+}-inactivated (black bar) ADA was performed as described above. Data are means ± S.E.M. ($n = 3$). Significant differences with respect to the samples in the absence of ADA were calculated by an unpaired Student's *t* test (*$P < 0.05$).

to receptors. ADA also enhanced the A_{2A}R antagonist [^{3}H]ZM 241385 binding to striatal membranes in a dose-dependent manner (Figure 3b) with an EC_{50} value of 0.13 ± 0.06 ng/ml, which is approximately equivalent to 3 pM ADA. Purified BSA (1–10 nM) did not modify agonist or antagonist binding to striatal A_{2A}Rs, showing that the ADA effect was specific (results not shown). All of these results suggest that ADA is an allosteric modulator of A_{2A}Rs.

To further investigate the modulating effect of ADA on agonist and antagonist binding, the pharmacological parameters for ligand binding to A_{2A}Rs were calculated by means of saturation and competition experiments. To investigate the modulating effect of ADA on the A_{2A}R antagonist equilibrium dissociation constants, brain striatal membranes were incubated with increasing concentrations of [^{3}H]ZM 241385 in the absence or in the presence of 1 μg/ml ADA, and saturation experiments were performed as indicated in the Experimental section. Since A_{2A}Rs are expressed as dimers or higher-order oligomers [6], radioligand saturation curves were analysed by fitting the specific binding data to the mechanistic two-state dimer receptor model [32,33], which considers a homodimer as the minimal structural unit of the receptor. In the absence or in the presence of ADA, the saturation curves (Figure 4a) were monophasic ($D_C = 0$)

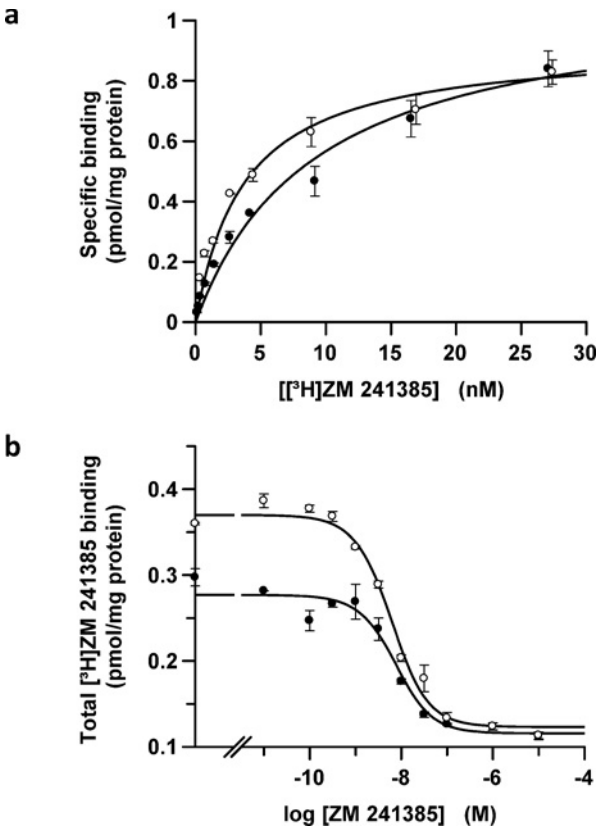

Figure 4 Effect of ADA on A_{2A}R antagonist affinity constants

(**a**) Saturation binding experiments of increasing concentrations of the radiolabelled antagonist [^{3}H]ZM 241385 (0.1–27 nM) or (**b**) competition experiments of the antagonist [^{3}H]ZM 241385 (1.6 nM) binding against increasing concentrations of ZM 241385, in the absence (●) or in the presence (○) of 1 μg/ml ADA. Data are means ± S.E.M. from a representative experiment ($n = 3$) performed in triplicate.

according to the non-co-operative behaviour of ZM 241385 binding to A_{2A}Rs [35]. The resulting equilibrium constants from fitting data to eqn (2) were 4.6 ± 0.8 nM and 1.9 ± 0.4 nM in the absence or in the presence of ADA respectively (mean ± S.E.M. of three different assays). This effect of ADA on antagonist affinity was also analysed by competition-binding experiments with 1.6 nM [^{3}H]ZM 241385 and increasing concentrations of ZM 241385 in the absence or in the presence of 1 μg/ml ADA. In the absence or in the presence of ADA, the competition curves (Figure 4b) were also monophasic ($D_C = 0$). The resulting equilibrium constants from fitting data to eqn (6) were 5.1 ± 0.7 nM and 3.3 ± 0.8 nM in the absence or in the presence of ADA respectively (mean ± S.E.M. of three different assays), not significantly different from saturation parameters. Thus ADA significantly ($P < 0.05$) increased the affinity of A_{2A}Rs for the antagonist.

To determine the modulating effect of ADA on the A_{2A}R agonist CGS 21680 equilibrium dissociation constants, we only carried out competition-binding experiments since saturation experiments with a low-affinity ligand are not reliable. Radioligand binding was therefore determined in brain striatal membranes incubated with a constant amount of [^{3}H]CGS 21680 (17 nM) and increasing concentrations of CGS 21680, in the absence or presence of 1 μg/ml ADA. As shown in Figure 5, competition curves of [^{3}H]CGS 21680 against CGS 21680 were monophasic ($D_C = 0$) according to the non-co-operative behaviour expected for CGS 21680 binding [37]. The resulting equilibrium

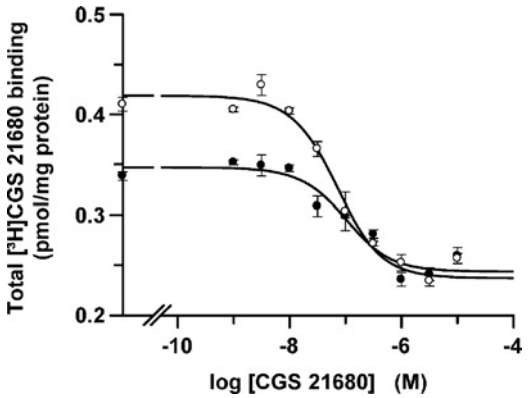

Figure 5 Effect of ADA on A_{2A}R agonist affinity constants

Competition experiments of the agonist [^{3}H]CGS 21680 (17 nM) binding against increasing concentrations of CGS 21680, in the absence (●) or in the presence (○) of 1 μg/ml ADA. Data are means ± S.E.M. from a representative experiment ($n = 3$) performed in triplicate.

constant from fitting data to eqn (6) were 90 ± 20 nM and 41 ± 4 nM in the absence or in the presence of ADA respectively (mean ± S.E.M. of three different assays). Thus ADA also significantly ($P < 0.05$) increased the affinity of A_{2A}Rs for the agonist.

Signalling consequences of the ADA–A_{2A}R interaction

To investigate the functional consequences of the interaction of ADA with A_{2A}Rs, the A_{2A}R-mediated signal transduction was determined in cells expressing the receptors. Accordingly, CHO–A_{2A}R cells were treated for 5 min at 37 °C with increasing amounts of the A_{2A}R agonist CGS 21680 in the absence or presence of 1 μg/ml ADA, and ERK1/2 phosphorylation was determined as indicated in the Experimental section. In the absence of ADA, CGS 21680 up to 200 nM dose-dependently increased ERK1/2 phosphorylation followed by a decrease of signalling at high CGS 21680 concentrations (Figure 6). The phenomenon in which previous or continued exposure of receptor to agonist results in a diminished functional response of the receptor upon subsequent or sustained agonist treatment has been defined as desensitization [38]. It has been described that A_{2A}R-mediated adenylate cyclase stimulation desensitizes rapidly in cultured cells (see [38] for a review). The results of the present study suggest that in A_{2A}R-expressing CHO cells there is also a CGS 21680-promoted desensitization of ERK1/2 phosphorylation. In the presence of ADA, a significant increase in the CGS 21680-induced ERK1/2 phosphorylation was observed, resulting in a bell-shaped concentration–response curve (Figure 6). According to an ADA-induced increase in ligand affinity for A_{2A}Rs, ADA also increased the A_{2A}R signalling, determined as ERK1/2 phosphorylation. These results show that ADA not only increased ligand affinity for A_{2A}Rs, but also was able to modulate, in a positive manner, signal transduction. ADA may then be considered an enhancer of ligand binding and of A_{2A}R-mediated signalling events.

DISCUSSION

Cell-surface ADA needs to be anchored to the plasma membrane by means of specific receptors. In the present paper we describe that ADA may bind to A_{2A}Rs on the surface of living cells. By FRET or BRET it has previously been demonstrated that A_{2A}Rs form homomers and that homomers, but not monomers, appear to be the functional species at the cell surface of transfected cells [6]. Thus the quaternary structure of A_{2A}Rs is constituted by,

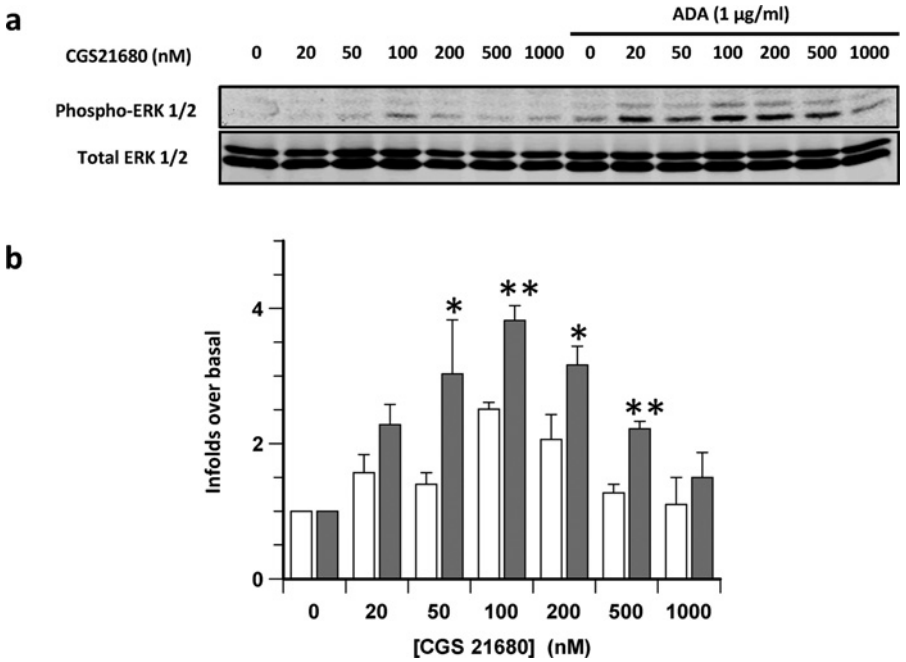

Figure 6 Effect of ADA on A_{2A}R-mediated ERK1/2 phosphorylation

A_{2A}R-expressing CHO cells were stimulated with increasing concentrations of the A_{2A}R agonist CGS 21680 in the presence or in the absence of 1 μg/ml ADA. In (**a**) a representative Western blot is shown. In (**b**) values are means ± S.E.M. of three independent experiments. Grey columns are in the presence of 1 μg/ml ADA, white columns are in the absence of 1 μg/ml ADA. Significant differences with respect to the samples in the absence of ADA were calculated by an unpaired Student's *t* test (*$P < 0.05$ and **$P < 0.01$).

at least, two protomers that form a dimer. Probably resulting from a decrease in the distance between the C-termini of the A_{2A}R protomers fused to Rluc and YFP, ADA binding led to modifications in the quaternary structure of A_{2A}R homomers that could be detected by BRET experiments. Using a similar set up Canals et al. [6] showed that A_{2A}R agonists are not able to modify the BRET signal. Therefore the ability of BRET to detect ADA-triggered conformational changes within the A_{2A}R homomers suggests that ADA exerts a control of the function of A_{2A}R homomers by a strong modification of their quaternary structure. In fact, the ADA-induced structural changes in the A_{2A}R molecule correlated with marked affinity modifications in the binding of both agonist and antagonist. Irrespective of its enzymatic activity, ADA was able to significantly decrease agonist and antagonist equilibrium dissociation constants. The ADA-induced increase in the ligand affinities indicates that ADA behaved as a positive modulator of A_{2A}Rs.

In addition to orthosteric sites, many GPCRs have been found to possess structurally distinct allosteric domains. One characteristic feature of the allosteric interaction is that the receptor is able to simultaneously bind an orthosteric and an allosteric ligand, introducing complexity into pharmacological responses by modifying the affinity or the signal imparted by the orthosteric ligand [39]. An allosteric effect results in a positive modulation if the modulator facilitates the interaction, or in a negative modulation if it inhibits the interaction of the ligand with the orthosteric-binding site [39,40]. According to these concepts, ADA is an allosteric ligand of A_{2A}Rs that positively modulates the agonist and antagonist binding to the orthosteric site of the receptor. Kreth et al. [41] have shown that an endogenous allosteric modulator leads to a reduced ligand affinity and to an impaired function of the A_{2A}R of human granulocytes in sepsis. Furthermore, some compounds have been synthesized and evaluated as positive enhancers of agonist and antagonist radioligands for the neuronal A_{2A}R [42,43]. A_{2A}Rs are allosterically modulated by sodium ions binding to an allosteric site linked to Glu^{13} in TM1 (TM is transmembrane domain) and His^{278} in TM7, and by the potassium-sparing diuretic amiloride [43–45]. The ability of allosteric modulators to fine-tune pharmacological responses has sparked interest in their potential applications in both clinical and basic science settings [40]. This interest is more relevant in the case of neurotransmitter receptor targets due to the fact that synaptic neurotransmission occurs in extremely complex circuits implicated in many neurological functions. Owing to the implication of A_{2A}Rs in many neurodegenerative diseases, such as Parkinson's and Huntington's disease, obsessive-compulsive disorders and drug addiction [46], different approaches have been tested to find allosteric modulators, i.e. a structure-based ligand-discovery methodology provided new routes for modulation of this neuronal key target [47–49]. Conceptually the allosteric interaction described in the present study is different from the one exerted by small molecules since it comes from the interaction across the membrane with a protein that has an extracellular topology. By means of the interaction with an extracellular domain of A_{2A}Rs, ADA exerts a fine-tune modulation of adenosine neuroregulation that may have important implications for the function of neuronal A_{2A}Rs, which are enriched in and play a key role in the brain striatum. The presence of ADA bound to the cell surface of neurons has been demonstrated [22], reinforcing the concept that this allosteric effect of ADA is likely to occur *in vivo*. With this in mind one may hypothesize that ADA SCID patients with ADA mutations affecting the binding of ADA to A_{2A}R may manifest neurological alterations that are predicted to be different from those resulting from mutations not affecting the ADA–A_{2A}R interface. Probably, mutations affecting the interaction would be less deleterious for striatal function since it would attenuate overactivation of A_{2A}R exerted by the elevated

adenosine levels. Irrespective of this, the results described in the present study show that ADA, apart from reducing the adenosine concentration, binds to $A_{2A}R$ behaving as an allosteric effector that markedly enhances agonist-induced signalling thought to be the MAPK (mitogen-activated protein kinase) pathway, increasing ERK1/2 phosphorylation. Thus the physiological role of the ADA–adenosine receptor interaction is to make those receptors more functional.

AUTHOR CONTRIBUTION

Eduard Gracia, Carme Lluís, Antoni Cortés, Vicent Casadó, Rafael Franco and Enric Canela conceived and designed the experiments; Eduard Gracia, Kamil Perez-Capote, Estefanía Moreno, Jana Barkesová, Josefa Mallol, Antoni Cortés and Vicent Casadó performed the experiments; Eduard Gracia, Kamil Pérez-Capote, Estefanía Moreno, Jana Barkešová, Josefa Mallol, Carme Lluís, Enric Canela, Antoni Cortés and Vicent Casadó discussed and analysed data; Carme Lluís, Rafael Franco, Antoni Cortés, Vicent Casadó and Enric Canela wrote the paper.

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Perfil pre- y postsináptico de los antagonistas del receptor de adenosina A_{2A} en el estriado

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Los receptores de adenosina A_{2A} ($A_{2A}R$) del estriado se localizan preferentemente en las neuronas espinosas de tamaño medio (MSN) de la vía eferente indirecta, dónde heteromerizan con receptores de dopamina D_2 (D_2R). Los $A_{2A}R$ se encuentran también localizados presinápticamente en las terminales corticoestriatales glutamatérgicas que contactan con las MSNs de la vía eferente directa, dónde heteromerizan con los receptores de adenosina A_1 (A_1R). Se ha formulado la hipótesis de que los antagonistas de los $A_{2A}R$ postsinápticos podrían ser útiles para la enfermedad de Parkinson, mientras los antagonistas de los $A_{2A}R$ presinápticos podrían ser beneficiosos en trastornos discinéticos como los de la enfermedad de Huntington, los trastornos obsesivos compulsivos y la adicción a drogas. El objetivo de este trabajo ha sido determinar si los antagonistas selectivos de los $A_{2A}R$ se pueden subdividir según actúen preferentemente pre- versus postsinápticamente. La potencia para bloquear la actividad motora y la liberación de glutamato inducidas por estimulación cortical eléctrica y la potencia para inducir activación motora se han utilizado como medidas de la actividad pre- y postsináptica in vivo, respectivamente. De manera significativa, los antagonistas SCH-442416 y KW-6002 mostraron perfiles preferentes pre- y postsinápticos, respectivamente, mientras que los otros compuestos evaluados (MSX-2, SCH-420814, ZM-241385 y SCH-58261)

no mostraron una preferencia clara. Se llevaron a cabo experimentos de unión de radioligandos a membranas de células que expresaban heterodímeros $A_{2A}R$-D_2R y A_1R-$A_{2A}R$ para determinar posibles diferencias en las afinidades de estos compuestos para diferentes heterodímeros de los $A_{2A}R$. La heteromerización desempeña un papel clave en el perfil presináptico del antagonista SCH-442416, ya que este compuesto se unía a los $A_{2A}R$ con una afinidad menor cuando estaban co-expresados con los D_2R que cuando estaban co-expresados con los A_1R. El KW-6002 mostró la mejor afinidad relativa por los $A_{2A}R$ co-expresados con los D_2R que co-expresados con los A_1R, lo que al menos en parte puede explicar el perfil postsináptico de este compuesto. Además, los perfiles farmacológicos in vitro de los compuestos MSX-2, SCH-420814, ZM-241385 y SCH-58261 estaban de acuerdo con sus perfiles mixtos pre- y postsinápticos detectados in vivo. Basándonos en sus acciones preferenciales pre- versus postsinápticas, los compuestos SCH-442416 y KW6002 podrían considerarse como compuestos de partida para el desarrollo de compuestos más efectivos antidiscinéticos y antiparkinsonianos, respectivamente.

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Striatal Pre- and Postsynaptic Profile of Adenosine A_{2A} Receptor Antagonists

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Abstract

Striatal adenosine A_{2A} receptors (A_{2A}Rs) are highly expressed in medium spiny neurons (MSNs) of the indirect efferent pathway, where they heteromerize with dopamine D_2 receptors (D_2Rs). A_{2A}Rs are also localized presynaptically in cortico-striatal glutamatergic terminals contacting MSNs of the direct efferent pathway, where they heteromerize with adenosine A_1 receptors (A_1Rs). It has been hypothesized that postsynaptic A_{2A}R antagonists should be useful in Parkinson's disease, while presynaptic A_{2A}R antagonists could be beneficial in dyskinetic disorders, such as Huntington's disease, obsessive-compulsive disorders and drug addiction. The aim or this work was to determine whether selective A_{2A}R antagonists may be subdivided according to a preferential pre- *versus* postsynaptic mechanism of action. The potency at blocking the motor output and striatal glutamate release induced by cortical electrical stimulation and the potency at inducing locomotor activation were used as *in vivo* measures of pre- and postsynaptic activities, respectively. SCH-442416 and KW-6002 showed a significant preferential pre- and postsynaptic profile, respectively, while the other tested compounds (MSX-2, SCH-420814, ZM-241385 and SCH-58261) showed no clear preference. Radioligand-binding experiments were performed in cells expressing A_{2A}R-D_2R and A_1R-A_{2A}R heteromers to determine possible differences in the affinity of these compounds for different A_{2A}R heteromers. Heteromerization played a key role in the presynaptic profile of SCH-442416, since it bound with much less affinity to A_{2A}R when co-expressed with D_2R than with A_1R. KW-6002 showed the best relative affinity for A_{2A}R co-expressed with D_2R than co-expressed with A_1R, which can at least partially explain the postsynaptic profile of this compound. Also, the *in vitro* pharmacological profile of MSX-2, SCH-420814, ZM-241385 and SCH-58261 was is in accordance with their mixed pre- and postsynaptic profile. 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.

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Introduction

The striatum is the major input structure of the basal ganglia [1]. More than ninety five percent of striatal neurons are γ-aminobutyric-acidergic (GABAergic) medium spiny neurons (MSNs). These neurons receive two main inputs: glutamatergic afferents from cortical, thalamic and limbic areas and dopaminergic afferents from the substantia nigra pars compacta and the ventral tegmental area [1]. MSNs are efferent neurons that give rise to the two efferent pathways of the basal ganglia, the 'direct' and 'indirect' striatal efferent pathways [1]. It is generally accepted that stimulation of the direct and indirect pathways results in motor activation and motor inhibition, respectively, and that smooth motor drive results from the counterbalanced influence of the direct and indirect pathways on the neural activity of the output structures [2,3]. Direct MSNs express dopamine receptors predominantly of the D_1 receptor (D_1R) subtype, whereas indirect MSNs are known for their high expression of dopamine D_2 receptors (D_2Rs) and adenosine A_{2A} receptors (A_{2A}Rs) [1,4,5].

There is clear evidence for the existence of postsynaptic mechanisms in the control of glutamatergic neurotransmission to the indirect MSN by at least two reciprocal antagonistic interactions between A_{2A}R and D_2R [4]. In one type of interaction, A_{2A}R and D_2R are forming heteromers and, by means of an allosteric interaction, A_{2A}R counteracts the D_2R-mediated inhibitory modulation of the effects of NMDA receptor stimulation in the indirect MSN, which includes Ca^{2+} influx, transition to the up-state and neuronal firing in the up-state [6,7]. This interaction has been suggested to be mostly responsible for the locomotor depressant and activating effects of A_{2A}R agonist and antagonists, respectively [4]. The second type of interaction

involves $A_{2A}R$ and D_2R that do not form heteromers, but most probably homomers [4]. In this interaction, which takes place at the level of adenylyl-cyclase (AC), stimulation of G_i-coupled D_2R counteracts the effects of G_{olf}-coupled $A_{2A}R$ [4]. Due to a strong tonic effect of endogenous dopamine on striatal D_2R, this interaction keeps $A_{2A}R$ from signaling through AC. However, under conditions of dopamine depletion or with blockade of D_2R, $A_{2A}R$-mediated AC activation is unleashed. This is biochemically associated with a significant increase in the phosphorylation of PKA-dependent substrates, which increases gene expression and the activity of the indirect MSN, producing locomotor depression (reviewed in ref. [4]). This interaction seems to be the main mechanism responsible for the locomotor depression induced by D_2R antagonists. Thus the motor depressant and most biochemical effects induced by genetic or pharmacologic blockade of D_2R are counteracted by the genetic or pharmacological blockade of $A_{2A}R$ [8–10].

Striatal $A_{2A}Rs$ are not only localized postsynaptically but also presynaptically, in glutamatergic terminals, where they heteromerize with A_1 receptors (A_1Rs) and where their stimulation facilitates glutamatergic neurotransmission [5,11]. Interestingly, presynaptic $A_{2A}Rs$ are preferentially localized in glutamatergic terminals of cortico-striatal afferents to the direct MSN [5]. According to the widely accepted functional basal circuitry model [2,3], blockade of postsynaptic $A_{2A}R$ localized in the indirect MSN should produce motor activation (by potentiating D_2R-mediated effects by means of $A_{2A}R$-D_2R receptor interactions). On the other hand, according to the same model, blockade of presynaptic $A_{2A}R$ localized in the cortico-striatal glutamatergic terminals that make synaptic contact with the direct MSN should decrease motor activity (by inhibiting glutamate release). The preferential locomotor-activating effects of systemically administered $A_{2A}R$ receptor antagonists can be explained by a stronger influence of a tonic adenosine and $A_{2A}R$ receptor-mediated modulation of the indirect pathway *versus* the direct pathway under basal conditions. In any case, the potency at inducing locomotor activation can be used as an *in vivo* measure of the ability of an $A_{2A}R$ antagonist to block postsynaptic striatal $A_{2A}R$. Recently we have established an *in vivo* model that evaluates the efficacy of cortico-striatal glutamatergic neurotransmission to the direct MSN, by quantifying the correlation between the current delivered into the orofacial premotor cortex and the concomitant electromyographic response elicited in the jaw muscles [5]. In this model, $A_{2A}R$ or D_1R antagonists were able to counteract the motor output induced by cortical electrical stimulation, which can only be explained by blockade of striatal presynaptic $A_{2A}R$ or postsynaptic D_1R, respectively [5,12].

Receptor heteromer is defined as a macromolecular complex composed by at least two (functional) receptor units with biochemical properties that are demonstrably different from those of its individual components [13]. Specific ligand binding characteristics are one of those properties [13,14]. The aim of the present study was, first, to investigate the possible existence of different pre- and postsynaptic profiles of several $A_{2A}R$ antagonists. The potency at blocking the motor output and striatal glutamate release induced by cortical electrical stimulation and the potency at inducing locomotor activation were used as *in vivo* measures of pre- and postsynaptic activities, respectively. Second, we wanted to evaluate if the different pre- and postsynaptic profiles could be related to different affinities that $A_{2A}R$ could have for those compounds when forming heteromers with either A_1R or D_2R. In fact, the results strongly suggest that heteromerization plays a key role in the pre- and postsynaptic profile of $A_{2A}R$ antagonists.

Materials and Methods

Ethics Statement

All animals used in the study were handled in accordance with the National Institutes of Health Animal care guidelines. The animal research conducted to perform this study was approved by the NIDA IRP Animal Care and Use Committee (under the auspices of protocol 09-BNRB-73) on 12/7/2009.

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighting between 300–350 g were used in these experiments. Rats were housed 2 per cage and they maintained at a temperature of $22\pm2^{\circ}C$ on a regular 12-h light–dark cycle. Food and water were available *ad libitum*.

Adenosine $A_{2A}R$ antagonists

The following $A_{2A}R$ antagonists were used: 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (**SCH-442416**), 2-(2-Furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (**SCH-58261**), 2-(2-furanyl)-7-[2-[4-[4-(2-methoxyethoxy)phenyl]-1-piperazinyl]ethyl]-7H-pyrazolo[4,3-e][1,2,4]-triazolo[1,5-c]pyrimidin-5-amine (**SCH-420814**), 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (**ZM-241385**), (E)-1, 3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione (**KW-6002**), (E)-3-(3-hydroxypropyl)-8-[2-(3-methoxyphenyl)vinyl]-7-methyl-1-prop-2-ynyl-3,7-dihydropurine-2,6-dione (**MSX-2**) and its water-soluble phosphate prodrug (E)-phosphoric acid mono-(3-{8-[2-(3-methoxyphenyl)vinyl]-7-methyl-2,6-dioxo-1-prop-2-ynyl-1,2,6,7-tetrahydropurin-3-yl}propyl) ester disodium salt (MSX-3). **MSX-3** is a water-soluble phosphate pro-drug of MSX-2; in vivo MSX-3 is readily converted to the $A_{2A}R$ antagonist MSX-2 (Sauer et al., 2002). For their systemic administration, the compounds were prepared as follows:

SCH-442416 and SCH 58261 were suspended in a solution of 5% dimethyl- sulfoxide (DMSO) (Sigma-Aldrich, St. Louis,MI), 5% TWEEN80 (Sigma-Aldrich, St. Louis, MI) and 90% ddH_2O; SCH-420814 was suspended in a solution of 20% PEG400, 40% β-cyclodextrin and 40% Lutrol 1% (in ddH_2O); ZM-241385 was suspended in a solution of 15% DMSO, 10% TWEEN80 and 75% ddH_2O; KW-6002 was suspended in a solution of 8% TWEEN80 and 92% ddH_2O; MSX-3 was dissolved in sterile saline (with 3 µl/ml saline of 1 M NaOH solution, final pH 7.4). All drugs but MSX-3 (Sigma-Aldrich, St. Louis, MI) were provided by CHDI Foundation Inc. (Los Angeles, CA, US). SCH-420814 was administered subcutaneously (s.c.) at 1 ml/kg and the other drugs were administered via intraperitonal (i.p.) injection at volume of 2 ml/kg.

Locomotor Activity

Locomotor activity was measured by placing the animals individually in motility soundproof chambers (50×50 centimeters; Med Associates Inc., VT). Locomotion was measured by counting the number of breaks in the infrared beams of the chambers. The animals were placed in individual acrylic chambers at noon on the day of testing. A lamp inside each chamber remained lit during this period. Following 90 min of habituation, the rats were injected i.p. with different doses of each compound or vehicle and locomotor activity was recorded for 90 min after the drug or vehicle administration. All the animals were tested only once. The effect of different doses of the $A_{2A}R$ antagonists on locomotor activity were analyzed using a one-way analysis of variance (ANOVA), followed by Newman-Keuls' *post-hoc* test.

Surgical procedures

Rats were anesthetized with 3 ml/kg of Equithesin (4.44 g of chloral hydrate, 0.972 g of Na pentobarbital, 2.124 g of $MgSO_4$, 44.4 ml of propylene glycol, 12 ml of ethanol and distilled H_2O up to 100 ml of final solution; NIDA Pharmacy, Baltimore, MD) and implanted unilaterally with bipolar stainless steel electrodes, 0.15 mm in diameter (Plastics One, Roanoke, VA), into the orofacial area of the lateral agranular motor cortex (3 mm anterior, 3 and 4 mm lateral, and 4.2 mm below bregma). The electrodes and a head holder (connected to a swivel during stimulation) were fixed on the skull with stainless steel screws and dental acrylic resin. For the experiments with electromyographic (EMG) recording, electrodes were also implanted in mastication muscles (during the same surgical procedure). Two 5 mm-long incisions were made in the skin on the upper and lower jaw areas to expose the masseter and the lateral pterygoid muscles. Two silicon rubber-coated coiled stainless steel recording electrodes (Plastics One, Roanoke, VA) were slipped below the skin from the incision in the skull until the tips showed up from the incisions in the jaw. The bare tips of the electrodes were then held in contact with the masseter and the lateral pterygoid muscles and the skin was closed with surgical staples. The other end of the recording electrodes was encased in a molded plastic pedestal with a round threaded post which was attached to an electrical swivel and then to a differential amplifier (Grass LP511, Grass Instruments, Warwick, RI). The pedestal was secured to the skull with dental cement together with the stimulation electrodes. For the *in vivo* microdialysis experiments, concentric microdialysis probes with 2-mm long dialysis membranes (Eicom Corp., Tokio, Japan) were implanted respectively into the striatum ipsilateral to the stimulation electrodes (0.0 mm AP, 4.5 ML and 7.0 mm DV).

EMG recording and power correlation analysis

Rats were placed in individual bowl chambers. Both stimulation electrodes and recording electrodes were attached using flexible shielded cabling to a four channel electrical swivel. Stimulation electrodes were connected to two-coupled constant current isolation units (PSIU6X, Grass Instruments West Warwick, RI) driven by an electrical stimulator (Grass S88X; Grass Instruments). The recording electrodes were connected to a differential amplifier (Grass LP511, West Warwick, RI). This configuration allows the rat to move freely while the stimulation and EMG recordings are taking place. After 60 min of habituation, biphasic current pulse trains (pulse of 0.1 ms at 120–200 μA; 100 Hz, 160 ms trains repeating once per 2 seconds) were delivered. The current intensity was adjusted to the threshold level, defined as the minimal level of current intensity allowing at least 95% of the stimulation pulses to elicit a positive EMG response. Positive EMG response was defined as at least 100% increase of the peak to peak amplitude respect to the background tonic EMG activity lasting more than 100 ms or at least 70% increase in the power of the EMG signal respect to the baseline. Positive EMG responses always matched observable small jaw movements. The threshold level was different for each animal but it was very stable and reproducible once established. The threshold level was in the 100 to 150 μA range for most cases and it reached 200 μA in a few (6) animals. Animals that failed to show a positive EMG response with electrical cortical stimulation intensities of 200 μA were discarded from the experimental procedure (less than 10%). Both stimulator monitoring and the amplified and filtered EMG signal (20,000 times gain, bandwidth from 10 to 1,000 Hz with a notch filter set at 60 Hz) were directed to analog-to-digital converter for recording (Lab-Trax-4, World Precision Instruments, Sarasota, FL) and backup (NI 9215, National Instruments, Austin, TX) and digitized at a sampling rate of 10,000 samples/second. Recordings of the digitized data were made using the software Data Trax2 software (World Precision Instruments) and LabVIEW SignalExpress (National Instruments). A power correlation analysis was used to quantify the correlation between the stimulation pulses of current delivered into the orofacial motor cortex (input signal; μA) and the elicited EMG response in the jaw muscles (output signal; μV). Decrease in the power correlation coefficient (PCC) between these two signals is meant to describe a decrease in the efficacy of the transmission in the neural circuit. Off-line, both signals were rectified and the root mean square (RMS) over each period of the stimulation pulses was calculated in the recorded signals using Data Trax2 software. The transformed data (RMS) from the stimulator monitor and the EMG were then exported with a time resolution of 100 samples/second to a spreadsheet file. The stimulation signal values were used as a reference to select data in a time window of 320 ms starting at the beginning of each train of pulses. This time window was chosen to ensure the analysis of any EMG response whose occurrence or length was delayed from the onset of the stimulation trains and to maximize the exclusion from the analysis of spontaneous jaw movements not associated with the stimulation. Pearson's correlation between the RMS values from the stimulation and EMG signals was then calculated for each experimental subject. PCC was calculated using the data recorded 40 min after the administration of the dose of any compound or vehicle. The effects of the different doses of $A_{2A}R$ antagonists on PCC were analyzed by a one-way ANOVA, followed by Dunnett's *post-hoc* test.

In vivo microdialysis

The experiments were performed on freely moving rats 24 h after probe implantation. An artificial cerebrospinal solution of (in mM) 144 NaCl, 4.8 KCl, 1.7 $CaCl_2$, and 1.2 $MgCl_2$ was pumped through the microdialysis probe at a constant rate of 1 μl/min. After a washout period of 90 min, dialysate samples were collected at 20-min intervals. After 60 min of collecting samples for baseline, the rats were injected either with the $A_{2A}R$ antagonists KW-6002 or SCH-442416. Both compounds were compared to vehicle controls (5% DMSO, 5% of TWEEN80 and 90% of ddH_2O). After 20 min from drug or vehicle injection, electrical stimulation pulses were applied through the electrodes implanted in the orofacial motor cortex for 20 min (pulse of 0.1 ms at 50–150 μA; 100 Hz, 160 ms trains repeating once × second) and samples were collected for 2 additional hours. Glutamate content was measured by reverse-phase HPLC coupled to a flourimetric detector (Shimadzu Inc., Tokio, Japan) [15]. Glutamate values were transformed as percentage of the mean of the three values before the drug or vehicle injection and transformed values were statistically analyzed. The effect of KW-6002, SCH-442416 and vehicle were analyzed using a one-way ANOVA for repeated measures followed by a Tukey's *post-hoc* test.

Cell clones

To obtain CHO cells expressing single receptors or co-expressing $A_{2A}R$ and A_1R or $A_{2A}R$ and D_2R, the human cDNAs for A_1R or D_2R cloned in pcDNA3.1 vector (containing a geneticin resistance gene) were used. The human $A_{2A}R$ was cloned into a pcDNA3.1/Hygro vector with a hygromycin resistance gene. For single transfections, CHO cells were transfected with the cDNA corresponding to $A_{2A}R$, A_1R or D_2R using lipofectamine (Invitrogen, Carlsbad, USA) method following the instructions of the supplier. 24 h after transfection the selection antibiotic was added at a concentration that was previously determined by a selection antibiotic test. Antibiotic resistant clones were isolated in the presence of the selection antibiotic (1200 μg/ml geneticin or

1000 μg/ml hygromycin). After an appropriate number of days/passes, several stable lines were selected and cultured in the presence of the selection antibiotic (600 μg/ml geneticin or 300 μg/ml hygromycin). To obtain clones co-expressing $A_{2A}R$ and A_1R or $A_{2A}R$ and D_2R, CHO cells expressing high affinity $A_{2A}R$ (obtained as above described) were transfected with the human cDNAs for A_1R or D_2R cloned in pcDNA3.1 vector using lipofectamine. After an appropriate number of days/passes stable lines were selected and cultured in the presence of the selection antibiotic. The receptor(s) expression in the cell clones was first detected by dot-blot of cell lysates using commercial available antibodies and wild-type CHO cells lysates as negative basal staining. Positively moderated stained clones were grown to obtain membranes in which the receptor expression was quantified by radioligand-binding experiments (see Results).

Bioluminescence Resonance Energy Transfer (BRET) assays

The fusion proteins $A_{2A}R$-*Renilla Luciferase* ($A_{2A}R$-RLuc), A_1R-Yellow Fluorescence Protein (A_1R-YFP) and D_2R-YFP were prepared and characterized as described elsewhere [16]. The cDNA encoding serotonin $5HT_{2B}$-YFP receptor was kindly provided by Dr. Irma Nardi (University of Pisa, Italy). CHO cells were transiently transfected with the corresponding fusion protein cDNA (see Figure legends) using lipofectamine. Cells were incubated (4 h) with the corresponding cDNA together with lipofectamine and Opti-MEM medium (Invitrogen). After 4 hours, the medium was changed to a fresh complete culture medium. Twenty-four hours after transfection, cells were washed twice in quick succession in HBSS with 10 mM glucose and scraped in 0.5 ml of the same buffer. To control the cell number, sample protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions as standards. To quantify fluorescence proteins, cells (20 μg protein) were distributed in 96-well microplates (black plates with a transparent bottom) and fluorescence was read at 400 nm in a Fluo Star Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter. Receptor-fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing protein-Rluc alone. For BRET measurements, the equivalent of 20 μg of cell protein were distributed in 96-well microplates (Corning 3600, white plates; Sigma) and 5 μM coelenterazine H (Molecular Probes, Eugene, OR) was added. After 1 minute of adding coelenterazine H, the readings were collected using a Mithras LB 940, which allows the integration of the signals detected in the 485 nm-short- (440–500 nm) and the 530 nm-long-(510–590 nm) wavelength filters. To quantify receptor-Rluc expression luminescence readings were performed after 10 minutes of adding 5 μM coelenterazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the Rluc construct expressed alone in the same experiment.

Radioligand binding experiments

Cells were disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland) for two 5 s-periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4 containing a proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA). Cell debris was removed by centrifugation at 1,500 *g* for 5 min at 4°C and membranes were obtained by centrifugation at 105,000 *g* (40 min, 4°C). Membranes were resuspended and centrifuged under the same conditions. The pellet was stored at −20°C, washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Membrane protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standard. For competition experiments, membrane suspensions (0.2 mg of protein/ml) were incubated for 2 h at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and 0.2 U/ml of adenosine deaminase (ADA, EC 3.5.4.4; Roche, Basel, Switzerland) with the indicated free concentration of the A_1R, $A_{2A}R$, or D_2R antagonist [^{3}H]DPCPX (GE Healthcare, UK), [^{3}H]ZM-241385, or [^{3}H]YM-09151-2, respectively (NEN Perkin Elmer, Wellesley, MA, USA) or the A_1Ragonist [^{3}H](R)-PIA (Moravek Biochemicals Inc., Brea, CA, USA) and increasing concentrations of DPCPX, ZM-241385, YM-09151-2, the $A_{2A}R$ agonist CGS-21680 or the tested $A_{2A}R$ antagonist (all provided by CHDI Foundation Inc.). Non-specific binding was determined in the presence of 11 μM of the corresponding non-radiolabelled ligand. Free and membrane-bound ligand were separated by rapid filtration of 500 μl aliquots in a cell harvester (Brandel, Gaithersburg, MD, USA) through Whatman GF/C filters embedded in 0.3% polyethylenimine that were subsequently washed for 5 s with 5 ml of ice-cold 50 mM Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA, USA) overnight at room temperature and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer, Boston, MA, USA) with an efficiency of 62% [17]. All displacers were dissolved in DMSO and diluted in the binding medium. The DMSO concentration in the binding incubates was less than 0.5% and, at this concentration, it did not affect agonist or antagonist affinity for their respective receptors.

Binding data analysis

Radioligand competition curves were analyzed by nonlinear regression using the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK), by fitting the binding data to the mechanistic two-state dimer receptor model [18,19]. Since there is now abundant evidence for GPCR oligomerization, including A_1R, $A_{2A}R$ and D_2R [20–23] and the minimal functional unit of GPCRs in biological tissues seems to imply dimerization [23], this model considers a homodimer as the minimal structural unit of the receptor. Here, we also consider the possibility of a homodimer as the minimal structural unit of a receptor forming homomers or forming heteromers with another receptor. To calculate the macroscopic equilibrium dissociation constants the following equation for a competition binding experiment deduced previously [19,24] was considered:

$$\begin{aligned} A_{\text{total bound}} = {} & \left(K_{DA2}A + 2A^2 + K_{DA2}AB / K_{DAB}\right) R_T / \\ & \left(K_{DA1}K_{DA2} + K_{DA2}A + A^2 + \right. \\ & K_{DA2}\,AB / K_{DAB} + K_{DA1}K_{DA2}B / K_{DB1} \\ & \left. + K_{DA1}K_{DA2}B^2 / (K_{DB1}K_{DB2})\right) \\ & + A_{\text{non-specific bound}} \end{aligned} \quad (1)$$

where A represents free radioligand (the adenosine A_1R or $A_{2A}R$ or dopamine D_2R antagonist [^{3}H]DPCPX, [^{3}H]ZM-241385 or [^{3}H]YM-09151-2, respectively or the A_1R agonist [^{3}H](R)-PIA) concentration, R_T is the total amount of receptor dimers and K_{DA1} and K_{DA2} are the macroscopic equilibrium dissociation constants describing the binding of the first and the second radioligand molecule (A) to the dimeric receptor; B represents the assayed

competing compound concentration, and K_{DB1} and K_{DB2} are, respectively, the macroscopic equilibrium dissociation constants for the binding of the first ligand molecule (B) to a dimer and for the binding of the second ligand molecule (B) to the semi-occupied dimer; K_{DAB} is the hybrid equilibrium radioligand/competitor dissociation constant, which is the dissociation constant of B binding to a receptor dimer semi-occupied by A.

When the radioligand A shows non-cooperative behaviour, eq. (1) can be simplified to eq. (2) due to the fact that $K_{DA2} = 4K_{DA1}$ [19,25] and, therefore, K_{DA1} is enough to characterize the binding of the radioligand A:

$$\begin{aligned} A_{\text{total bound}} = {} & \left(4K_{DA1}A + 2A^2 + 4K_{DA1}AB / K_{DAB}\right)R_T / \\ & \left(4K_{DA1}{}^2 + 4K_{DA1}A + A^2\right. \\ & + 4K_{DA1}AB / K_{DAB} + 4K_{DA1}{}^2B / K_{DB1} \\ & \left. + 4K_{DA1}{}^2B^2 / (K_{DB1}K_{DB2})\right) \\ & + A_{\text{non-specific bound}} \end{aligned} \quad (2)$$

Binding to GPCRs quite often displays negative cooperativity. Under these circumstances $K_{D2}/K_{D1}>4$ and then K_{D1} and K_{D2} represent the "high-affinity" and the "low-affinity" binding sites, respectively. On the other hand, for positive cooperativity, $K_{D2}/K_{D1}<4$ and then K_{D2} represents the "high-affinity" and K_{D1} represents the "low-affinity"binding sites [25]. The two-state dimer model also introduces a cooperativity index (D_{CB}). The dimer cooperativity index for the competing ligand B is calculated as [19,25]:

$$D_{CB} = \log\left(4K_{DB1} / K_{DB2}\right)$$

The way the index is defined is such that its value is "0" for non-cooperative binding, positive values of D_C indicate positive cooperativity, whereas negative values imply negative cooperativity [14,19].

In experimental conditions when both the radioligand A and the competitor B (i.e., most adenosine A_{2A} receptor antagonist tested in the present study) show non-cooperativity, it results that $K_{DA2} = 4K_{DA1}$ and $K_{DB2} = 4K_{DB1}$, and eq. (1) can be simplified to:

$$\begin{aligned} A_{\text{total bound}} = {} & \left(4K_{DA1}A + 2A^2 + 4K_{DA1}AB / K_{DAB}\right) R_T / \\ & \left(4K_{DA1}{}^2 + 4K_{DA1}A + A^2\right. \\ & + 4K_{DA1}\,AB / K_{DAB} + 4K_{DA1}{}^2B / K_{DB1} \\ & \left. + K_{DA1}{}^2B^2 / K_{DB1}{}^2\right) + A_{\text{non-specific bound}} \end{aligned} \quad (3)$$

When both the radioligand A and the competitor B (DPCPX, ZM241385, SCH 23390 or YM-09151-2) are the same compound and the binding is non-cooperative, eq. (3) simplifies to:

$$\begin{aligned} A_{\text{total bound}} = {} & \left(4K_{DA1}A + 2A^2 + AB\right)R_T / \\ & \left(4K_{DA1}{}^2 + 4K_{DA1}A + A^2\right. \\ & \left. + AB + 4K_{DA1}B + B^2\right) \\ & + A_{\text{non-specific bound}} \end{aligned} \quad (4)$$

Goodness of fit was tested according to reduced χ^2 value given by the nonlinear regression program. The test of significance for two different population variances was based upon the F-distribution (see ref. [25] for details). Using this *F* test, a probability greater than 95% ($p<0.05$) was considered the criterion to select a more complex equation to fit binding data over the simplest one. In all cases, a probability of less than 70% ($p>0.30$) resulted when one equation to fit binding data was not significantly better than the other. Results are given as parameter values ± S.E.M. of three-four independent experiments.

Results

Striatal pre- *versus* postsynaptic profile of A_{2A} receptor antagonists

Dose-response experiments with the six $A_{2A}R$ antagonists indicated that four compounds (SCH-420814, SCH-58261, MSX-3 and ZM-241385) had a similar potency (similar minimal significant effective doses) at inducing locomotor activation (Fig. 1) and at reducing PCC (Fig. 2). The other two compounds had a very different profile: KW-6002 produced a strong locomotor activation already at the dose of 0.3 mg/kg i.p., while it did not reduce PCC at the highest tested dose (10 mg/kg i.p.). On the other hand, SCH-442416 produced a very weak locomotor activation, only significant at doses higher than 3 mg/kg i.p., while it significantly decreased PCC already at the dose of 0.1 mg/kg i.p.

In vivo microdialysis with cortical electrical stimulation was used as an additional *in vivo* evaluation of the preferential pre- and postsynaptic activity of SCH-442416 and KW-6002, respectively. SCH-442416 significantly counteracted striatal glutamate release induced by cortical stimulation at a dose that strongly reduced PCC but did not induce locomotor activation (1 mg/kg i.p.; Fig. 3). On the other hand, KW-6002 did not modify striatal glutamate release induced by cortical stimulation at a dose that produced a pronounced locomotor activation but did not reduce PCC (1 mg/kg i.p.; Fig. 3).

Development of CHO cell-lines expressing A_1-A_{2A} or A_{2A}-D_2 receptor heteromers

Cell clones expressing $A_{2A}R$, A_1R-$A_{2A}R$ heteromers or $A_{2A}R$-D_2R heteromers and control clones expressing A_1R or D_2R were generated (see Materials and Methods). First of all, the ability of $A_{2A}R$ to form heteromers with A_1R or D_2R in CHO cells was demonstrated by BRET experiments in cells transiently co-expressing $A_{2A}R$-Rluc and A_1R-YFP or $A_{2A}R$-Rluc and D_2R-YFP. A positive BRET signal for energy transfer was obtained (Fig. 4). The BRET signal increased as a hyperbolic function of the concentration of the YFP-fusion construct added reaching an asymptote. As a negative control the BRET pair formed by $A_{2A}R$-Rluc and 5-$HT_{2B}R$-YFP was used. As shown in Figure 4, the negative control gave a linear non-specific BRET signal. The significant and hyperbolic BRET signal found for these fusion proteins indicates that the intermolecular interaction between $A_{2A}R$ and A_1R or $A_{2A}R$ and D_2R in CHO cells is specific.

$A_{2A}R$-D_2R and A_1R-$A_{2A}R$ heteromerization in stably transfected CHO cells was shown by ligand binding experiments. This is an indirect approach for the identification of a receptor heteromer in native tissues or cells [13]. In the $A_{2A}R$-D_2R heteromer, an allosteric interaction between both receptors in the heteromer has been described, in which the dopamine D_2R agonist affinity decreases in the presence of an $A_{2A}R$ agonist [14]. In CHO cells stably expressing $A_{2A}R$ and D_2R, the affinity of the D_2R for dopamine was determined by competition experiments of the D_2R antagonist [^{3}H]YM-09151-2 *versus* dopamine in the presence (Fig. 5a) or in the absence (Fig. 5b) of the $A_{2A}R$ agonist

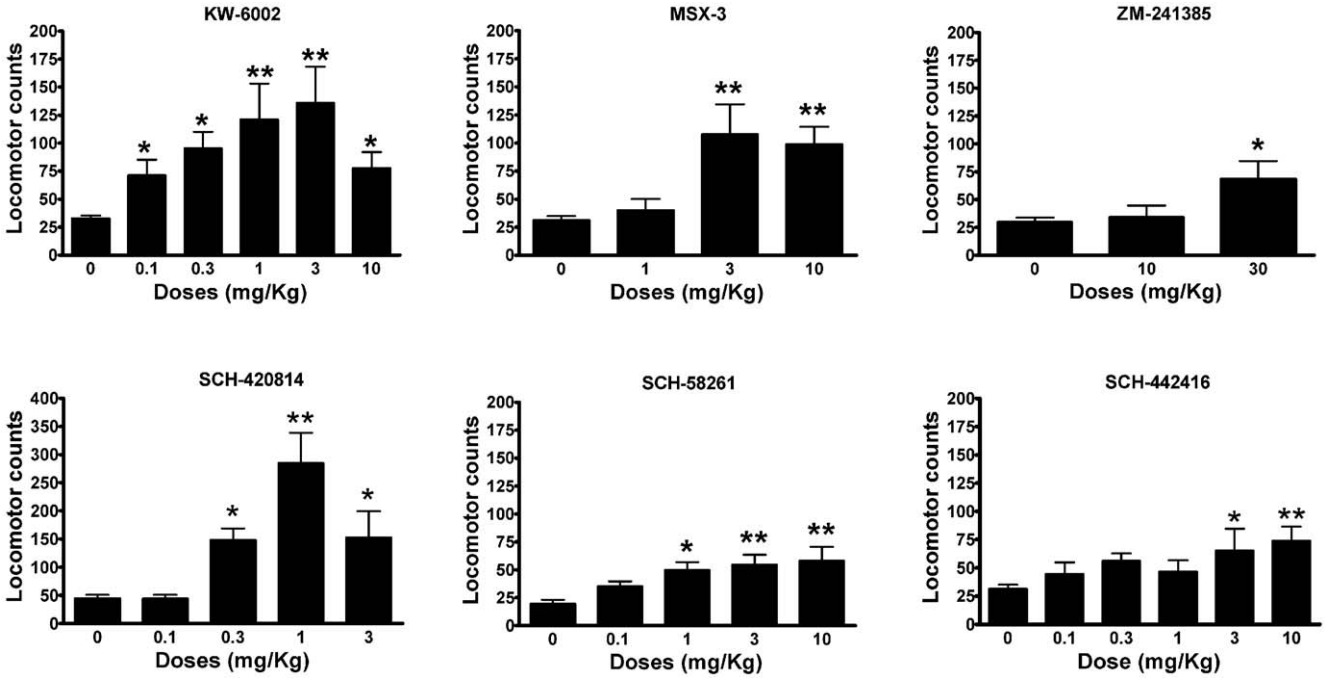

Figure 1. Locomotor activation in rats induced by A_{2A}R antagonists. Data represent means ± S.E.M. of the locomotor activity (distance traveled, in cm, of total accumulated counts) in habituated rats (90 min) during 90 min following the drug administration (n = 6–8 per group). * and **: $p<0.05$ and $p<0.01$, respectively in comparison to vehicle-treated animals (0 mg/kg); ANOVA with *post-hoc* Newman–Keuls' comparisons, $p<0.5$ and $p<0.01$, respectively).
doi:10.1371/journal.pone.0016088.g001

CGS-21680 (200 nM). By fitting data obtained in the absence of CGS-21680 to eq. 3 (Methods; considering $K_{DA1} = 2.9$ nM see below) the calculated K_{DB1} was 9±2 μM. In the presence of CGS-21680, 5 μM of dopamine was unable to decrease the radioligand bound and more than 50% of radioligand bound was found in the presence of 100 μM of dopamine (Fig. 5b). A K_{DB1} >30 μM was

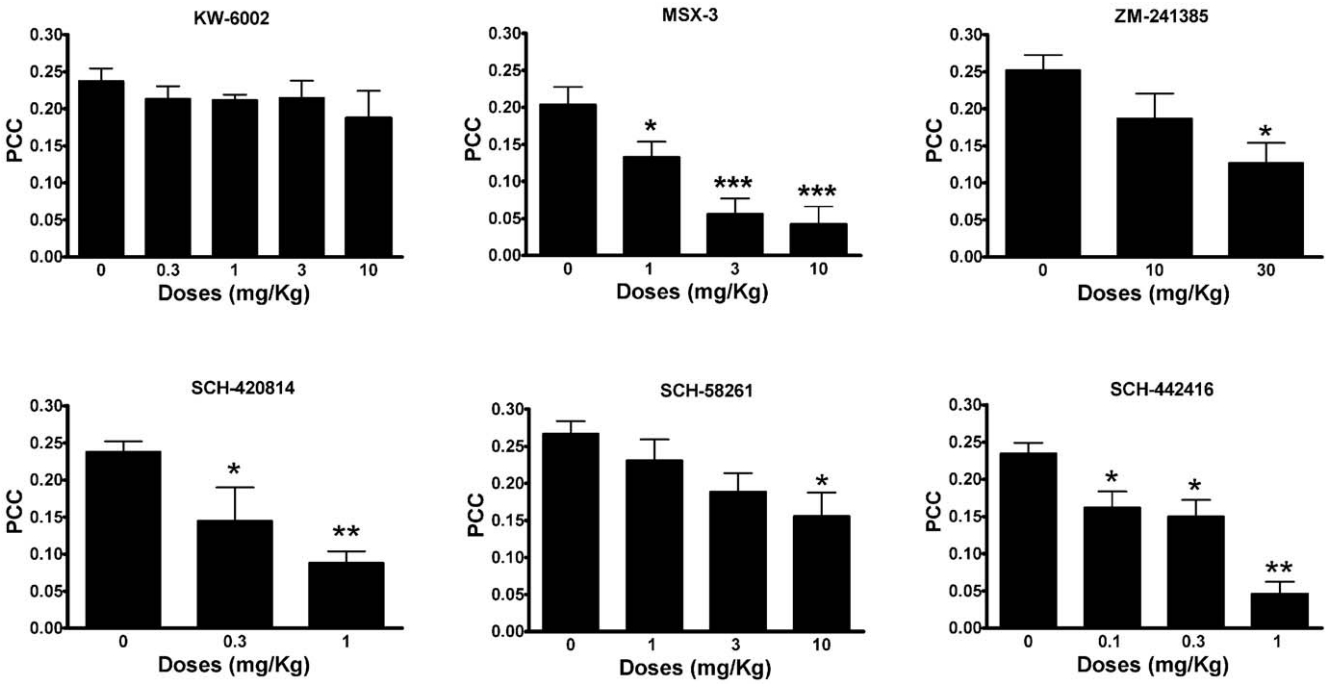

Figure 2. Blockade by A_{2A}R antagonists of the motor output induced by cortical electrical stimulation. Dose-dependent decrease in the Power Correlation Coefficient (PCC) induced by the administration of different A_{2A}R antagonists. Results represent means ± S.E.M. (n = 5–6 per group). * and **: $p<0.05$ and $p<0.01$, respectively in comparison to vehicle-treated animals (0 mg/kg); ANOVA with *post-hoc* Dunnett' comparisons, $p<0.5$ and $p<0.01$, respectively).
doi:10.1371/journal.pone.0016088.g002

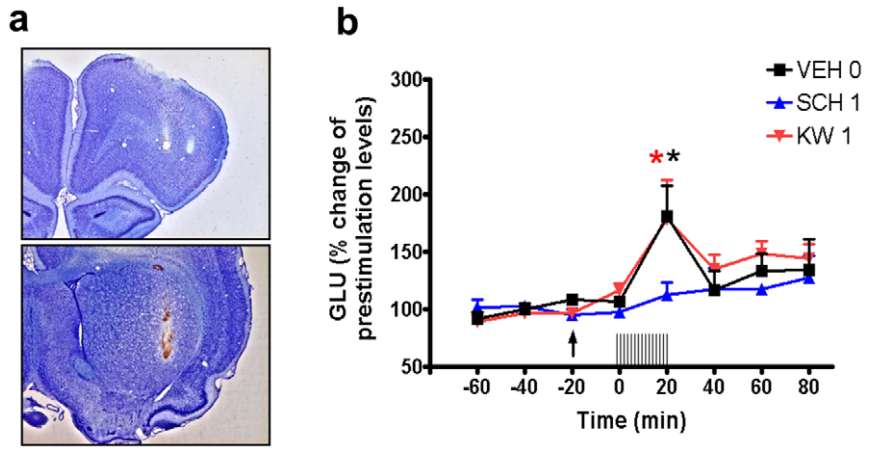

Figure 3. Blockade by A_{2A}R antagonists of striatal glutamate release induced by cortical electrical stimulation. (a) Representative coronal sections of a rat brain, stained with cresyl violet, showing the tracks left by the bipolar stimulation electrode in the orofacial area of the lateral agranular motor cortex (top) and by the microdialysis probe in the lateral striatum (bottom). (b) Effect of systemic administration of the A_{2A}R antagonists SCH-442416 and KW-6002 (1 mg/kg, i.p., in both cases) on the increase in glutamate extracellular levels in the lateral striatum induced by cortical electrical stimulation. Results are expressed as means ± S.E.M. of percentage of the average of the three values before the stimulation (n = 5–7 per group). Time '0' represents the values of the samples previous to the stimulation. The arrow indicates the time of systemic administration. The train of vertical lines represents the period of cortical stimulation. *: $p<0.05$ compared to value of the last sample before the stimulation (repeated-measures ANOVA followed by Tukey's test).
doi:10.1371/journal.pone.0016088.g003

estimated and it was shown that CGS-21680 induced a decrease in the dopamine affinity for D_2R. An allosteric interaction in the A_1R-A_{2A}R heteromer has also been described, in which the A_1R agonist affinity decreases in the presence of an A_{2A}R agonist [11]. As shown in Figure 6a, the displacement of the A_1R agonist [^{3}H]R-PIA by CGS21680 was significantly ($p<0.001$) better fitted by a biphasic than by a monophasic curve. At low CGS-21680 concentrations, when it binds preferentially to A_{2A}R (at concentrations of CGS-21680 <500 nM, the direct binding of CGS-21680 to A_1R is <1%, according to the calculated affinity of A_1R for CGS-21680), CGS-21680 decreased the binding of [^{3}H]R-PIA to the A_1R with an IC_{50} value of 386±35 nM (n = 3). At high CGS-21680 concentrations (>10 μM), the [^{3}H]R-PIA binding displacement reflects the binding of CGS-21680 directly to the A_1R and the competition between CGS-21680 and R-PIA for the binding to the A_1R. In fact, in the control clone expressing only A_1R, the displacement by CGS-21680 of [^{3}H]R-PIA only occurred at CGS-21680 concentrations higher than 10 μM (Fig. 6b).

A pharmacological characterization of selected cell clones was performed with competition experiments of radio-labeled antagonists of A_1, A_{2A} and D_2 receptors *versus* selective agonists or antagonists. In all cases, the competition curves of the A_{2A}R antagonist [^{3}H]ZM-241385 (2 nM) *versus* ZM-241385 (0.1 nM to 11 μM), the D_2R antagonist [^{3}H]YM-09151-2 (0.2 nM) *versus* YM-09151-2 (0.01 nM to 11 μM) or the A_1R antagonist [^{3}H]DPCPX (2 nM) *versus* DPCPX (0.1 nM to 11 μM), were monophasic, indicating the absence of cooperativity (see Materials and Methods). By fitting the binding data to eq. 4 (Materials and Methods), the K_D (K_{D1}) values obtained for the antagonists ZM-241385 or YM-09151-2 were 8±3 nM and 2.9±0.3 nM, respectively, for the chosen A_{2A}R-D_2R clone, the K_D values obtained for the A_1R and A_{2A}R antagonists were 8±2 nM (DPCPX) and 1.8±0.4 nM (ZM-241385), respectively, for the chosen A_1R-A_{2A}R cell clone and the K_D value obtained for A_{2A}R antagonist (ZM-241385) was 0.9±0.3 nM for the chosen A_{2A}R cell clone. Also by fitting the binding data to eq. 4 (Materials and Methods), the K_D value obtained for the A_1R antagonist (DPCPX) was 8.6±0.9 nM for the A_1R cell clone and the K_D value obtained for the D_2R antagonist (YM-09151-2) was 0.23±0.08 nM for the D_2R cell clone. These values were then used to determine the affinity constants showed in Tables 1 and 2. The agonists affinity in each selected clone was determined by competition experiments using the A_{2A}R antagonist [^{3}H]ZM-241385 (2 nM) *versus* the agonist CGS-21680 (1 nM to 50 μM), the D_2R antagonist [^{3}H]YM-09151-2 (0.2 nM), *versus* the agonist quinpirole (0.1 nM to 30 μM), or the A_1R antagonist [^{3}H]DPCPX (2 nM), *versus* the agonist R-PIA (1 nM to 50 μM). As it is shown in Tables 1 and 2, the agonist affinity for A_{2A}R in A_{2A}R, A_{2A}R-D_2R or in A_{2A}R-A_1R cells is in the same range as that reported for brain striatum or for cells expressing human A_{2A}R (between 30 and 250 nM) [7]. Nevertheless, the affinity of the A_{2A}R for the selective agonist CGS-21680 was slightly but significantly lower when co-expressed with D_2R (see Table 2). A_1R (but not A_{2A}R or D_2R) agonist binding showed negative cooperativity (negative D_{CB} values, see Materials and Methods), both in cells expressing A_1R and in cells co-expressing A_1R and A_{2A}R (Tables 1 and 2).

Screening of A_{2A}R antagonists on cells expressing A_1-A_{2A} or A_{2A}-D_2 receptor heteromers

To test if selected A_{2A}R antagonists display different selectivity for A_1R-A_{2A}R or A_{2A}R-D_2R heteromers, competition experiments with these ligands were performed using CHO cells expressing A_{2A}R, A_1R-A_{2A}R or A_{2A}R-D_2R. We found that none of the six A_{2A}R antagonists first tested in the *in vivo* models were able to bind with moderate affinity to A_1R or to D_2R in CHO cells expressing A_1R or D_2R (data not shown), indicating that these compounds are specific ligands for A_{2A}R. Competition experiments of [^{3}H]ZM-241385 (2 nM) binding *versus* increasing concentrations of each A_{2A}R antagonist (1 nM to 100 μM) were performed as indicated in Methods and binding data from competition experiments were fitted assuming that receptors are dimers and statistically (F test, see Materials and Methods) testing whether the competitor (A_{2A}R antagonists) binding was cooperative (biphasic competition curves; fitting to eq. 2) or non-cooperative (monophasic competition curves;

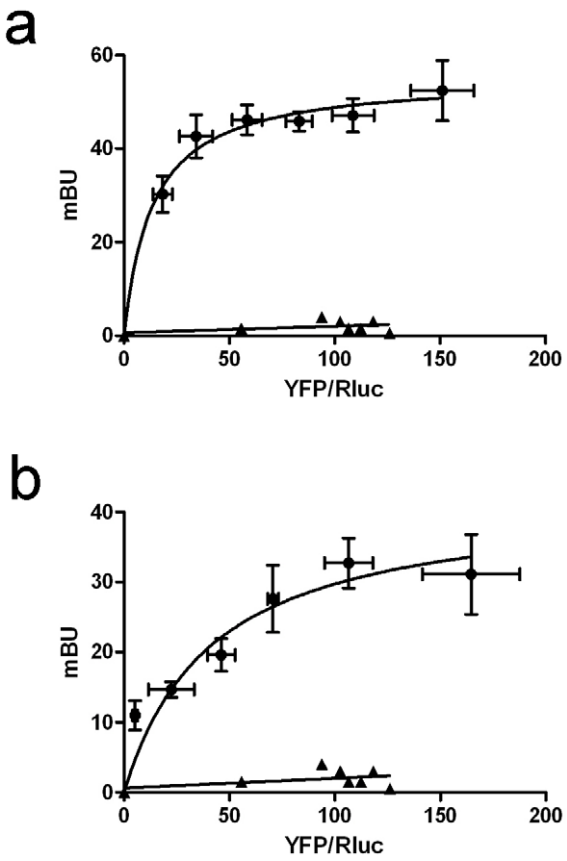

Figure 4. Identification of receptor heteromers in CHO cells by BRET saturation curve. BRET experiments were performed with CHO cells co-expressing A_{2A}R-*RLuc* and A_1R-YFP (A) or A_{2A}R-*RLuc* and D_2R-YFP (B). Co-transfections were performed with increasing amounts of plasmid–YFP (0.25 to 4 μg cDNA corresponding to A_1R-YFP and 0.5 to 8 μg corresponding to D_2R-YFP) whereas the A_{2A}R-*RLuc* construct was maintained constant (0.5 μg cDNA). Both fluorescence and luminiscence of each sample were measured before every experiment to confirm similar donor expressions (about 100,000 luminescent units) while monitoring the increase acceptor expression (10,000–25,000 fluorescent units). As a negative control, linear BRET was obtained in cells expressing equivalent luminescence and fluorescence amounts corresponding to A_{2A}R-*RLuc*, (0.5 μg transfected cDNA) and serotonin $5HT_{2B}$-YFP (0.5 to 8 μg transfected cDNA) receptors. The relative amount of acceptor is given as the ratio between the fluorescence of the acceptor minus the fluorescence value of cells expressing the donor alone (YFP) and the luciferase activity of the donor (Rluc). BRET data are expressed as means ± S.D. of 4–6 different experiments grouped as a function of the amount of BRET acceptor.
doi:10.1371/journal.pone.0016088.g004

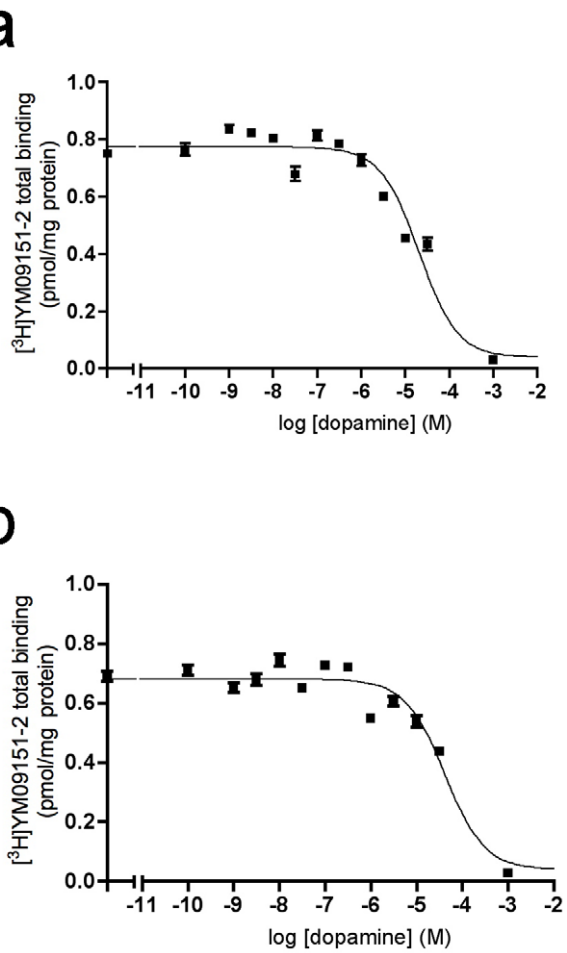

Figure 5. Allosteric interaction between A_{2A}R and D_2R in A_{2A}R-D_2R CHO cells. Competition experiments were performed in membrane preparations from CHO cells expressing A_{2A}R and D_2R with 0.5 nM [^{3}H]YM-09151-2 and increasing concentrations of dopamine (from 0.1 nM to 30 μM) in the absence (a) or in the presence (b) of 200 nM CGS-21680 as indicated in Methods. Data represent means ± S.E.M. of a representative experiment performed with triplicates.
doi:10.1371/journal.pone.0016088.g005

fitting to eq. 3). Since the screened compounds are A_{2A}R antagonists, competition curves were expected to be monophasic, assuming that antagonist binding is not cooperative. In fact, in all cell clones, MSX-2, KW-6002, SCH-420814, ZM-241385 and SCH-58261 gave monophasic competition curves (fitting binding data to eq. 2 was not better than fitting to eq. 3; see Methods and Fig. 7 a–c as an example). Accordingly, the pharmacological characterization for these compounds gave $D_{CB} = 0$ and $K_{DB2} = 4K_{DB1}$ (see Table 3). For all compounds, co-transfection with A_1R did not significantly modify their affinity for A_{2A}R. On the other hand, co-transfection with D_2R significantly reduced the affinity of A_{2A}R for MSX-2, SCH-420814, SCH-58261 and ZM-241385, from two to about nine times, and did not significantly modify the affinity of A_{2A}R for KW-6002 (Table 3).

For SCH-442416, a careful statistically-based analysis of the monophasic or biphasic nature of the competition curves led to an unexpected finding: in A_{2A}R-D_2R cells, competition curves of [^{3}H]ZM-241385 (2 nM) binding *versus* increasing concentrations of SCH-442416 were biphasic (fitting to eq. 2 improves the fitting to eq. 3; see Methods) (Fig. 7d). Table 4 shows the deduced pharmacological parameters from competition experiments of [^{3}H]ZM-241385 *versus* SCH-442416 in cells expressing A_{2A}R, A_1R-A_{2A}R and A_{2A}R-D_2R. In A_{2A}R and A_1R-A_{2A}R cells the curves were monophasic. Accordingly, the pharmacological characterization gave a D_{CB} values of 0 and a $K_{DB2} = 4K_{DB1}$. In contrast, as mentioned above, in cells expressing A_{2A}R-D_2R, competition curves were biphasic, and binding data were then fitted to eq. 2 (Methods) and robust parameters were obtained (Table 4). Thus, in A_{2A}R-D_2R cells, SCH-442416 binding showed a strong negative cooperativity and, consequently, with a marked loss of affinity (an increase of 600 times in K_{DB2}) respect to cells expressing A_{2A}R. This is reflected by the B_{50} value (concentration competing 50% of radioligand binding), which was more than 40 times higher in A_{2A}R-D_2R cells than in A_1R-A_{2A}R cells or A_{2A}R cells.

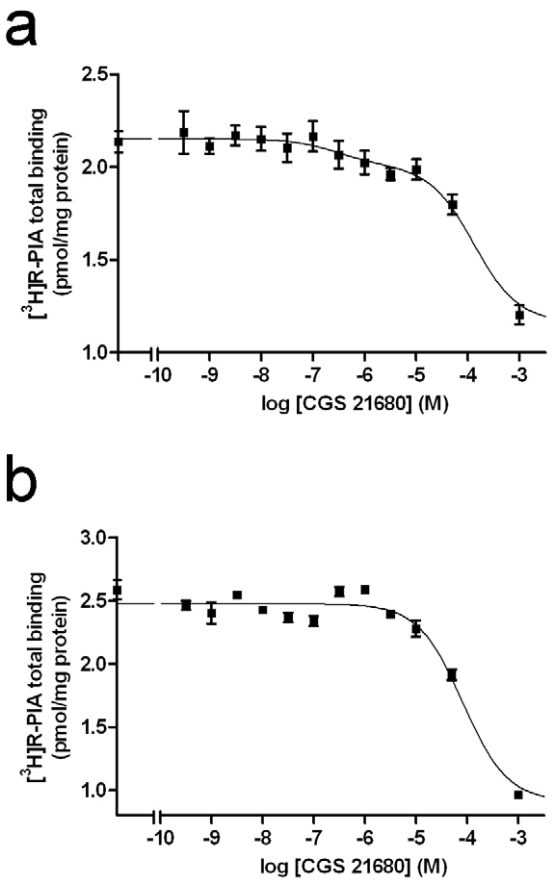

Figure 6. Allosteric interaction between A_1R and $A_{2A}R$ in A_1R-$A_{2A}R$ CHO cells. Competition experiments were performed in membrane preparations from CHO cells expressing A_1R or A_1R and $A_{2A}R$ with 12 nM [^{3}H]R-PIA *versus* increasing concentrations of the $A_{2A}R$ agonist CGS-21680 as indicated in Methods. Data represent means ± S.E.M. of a representative experiment performed with triplicates.
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Discussion

An important finding of the present study is that several $A_{2A}R$ antagonists previously thought as being pharmacologically similar present different striatal pre- and postsynaptic profiles. Six compounds already known as selective $A_{2A}R$ antagonists were first screened for their ability to block striatal pre- and postsynaptic $A_{2A}Rs$ with *in vivo* models. Locomotor activation was used to evaluate postsynaptic activity while PCC reduction was used to determine presynaptic activity (see Introduction). Two compounds, SCH-442416 and KW-6002, showed preferential pre- and postsynaptic profiles, respectively, and four compounds, MSX-3, SCH-420814, SCH-58261 and ZM-241385, showed mixed pre-postsynaptic profiles. Combining *in vivo* microdialysis with cortical electrical stimulation was used as an additional *in vivo* evaluation of presynaptic activity of SCH-442416 and KW-6002. In agreement with its preferential presynaptic profile, SCH-442416 significantly counteracted striatal glutamate release induced by cortical stimulation at a dose (1 mg/kg i.p.) that strongly reduced PCC but did not induce locomotor activation. On the other hand, according to its preferential postsynaptic profile, KW-6002 did not modify striatal glutamate release induced by cortical stimulation at a dose (1 mg/kg i.p.) that produced a pronounced locomotor activation but did not counteract PCC. In a previous study, we reported that intrastriatal perfusion of MSX-3 almost completely counteracted striatal glutamate release induced by cortical electrical stimulation [5], which agrees with its very effective reduction of PCC shown in the present study.

Table 1. Pharmacological parameters for agonist binding to A_1R, $A_{2A}R$ and D_2R in A_1R, $A_{2A}R$ and D_2R CHO cells.

Parameters	$A_{2A}R$ cells	A_1R cells	D_2R cells
K_{DB1}	90±30 nM	13±3 nM	120±60 nM
K_{DB2}	360±120 nM	1±0.3 mM	480±240 nM
D_{CB}	0	−1.3	0
B_{50}	180±60 nM	110±30 nM	240±120 nM

Binding data from competition experiments were fitted assuming that receptors form homodimers, and cooperativity ($D_{CB} \neq 0$, fitting to eq. 2; Materials and Methods) or non-cooperativity ($D_{CB} = 0$, fitting to eq. 3; Materials and Methods) in competitor ligand binding was statistically tested (F test). K_{DB1} and K_{DB2} are, respectively, the equilibrium dissociation constants of the first and second binding of B (the A_1R, $A_{2A}R$, or D_2R agonists: R-PIA, CGS-21680 or quinpirole, respectively) to the dimer. D_{CB} is the "dimer cooperativity" index for the binding of the ligand B, and B_{50} is the concentration providing half saturation for B. Data are mean ± S.E.M. values of three experiments.
doi:10.1371/journal.pone.0016088.t001

Another important finding of the present study is that at least part of these pharmacological differences between $A_{2A}R$ antagonists can be explained by the ability of pre- and postsynaptic $A_{2A}R$ to form different receptor heteromers, with A_1R and D_2R, respectively [4–6,11,14]. Radioligand-binding experiments were performed in CHO cells stably expressing $A_{2A}R$, $A_{2A}R$-D_2R heteromers or A_1R-$A_{2A}R$ heteromers to determine possible differences in the affinity of these compounds for different $A_{2A}R$ heteromers. Co-expression with A_1R did not significantly modify the affinity of $A_{2A}R$ for the different ligands, but co-expression with D_2R decreased the affinity of all compounds, with the exception of KW-6002. The structural changes in the $A_{2A}R$ induced by heteromerization with the D_2R could be detected not only by antagonists but also by agonists. Indeed, the affinity of the selective $A_{2A}R$ agonist CGS-21680 was reduced in cells co-

Table 2. Pharmacological parameters for agonist binding to A_1R-$A_{2A}R$ and $A_{2A}R$-D_2R CHO cells.

Parameters	$A_{2A}R$-D_2R cells		$A_{2A}R$-A_1R cells	
	$A_{2A}R$	D_2R	$A_{2A}R$	A_1R
K_{DB1}	200±40 nM*	1.2±0.6 μM	70±10 nM	0.7±0.3 nM
K_{DB2}	0.8±0.4 μM	4.8±2.4 μM	280±40 nM	1.1±0.5 μM
D_{CB}	0	0	0	−2.6
B_{50}	0.4±0.08 μM	2.4±1.2 μM	140±20 nM	30±10 nM

Binding data from competition experiments were fitted assuming that receptors (also when heteromerizing) form homodimers, and cooperativity ($D_{CB} \neq 0$, fitting to eq. 2; Materials and Methods) or non-cooperativity ($D_{CB} = 0$, fitting to eq. 3; Materials and Methods) in competitor ligand binding was statistically tested (F test). K_{DB1} and K_{DB2} are, respectively, the equilibrium dissociation constants of the first and second binding of B (the A_1R, $A_{2A}R$, or D_2R agonists: R-PIA, CGS-21680 or quinpirole, respectively) to the dimer. D_{CB} is the "dimer cooperativity" index for the binding of the ligand B, and B_{50} is the concentration providing half saturation for B. Data are mean ± S.E.M. values of three experiments.
*: $p<0.05$ compared to K_{DB1} values in A_1R-$A_{2A}R$ and $A_{2A}R$ cells (Table 1); one-way ANOVA, followed by Newman-Keuls test.
doi:10.1371/journal.pone.0016088.t002

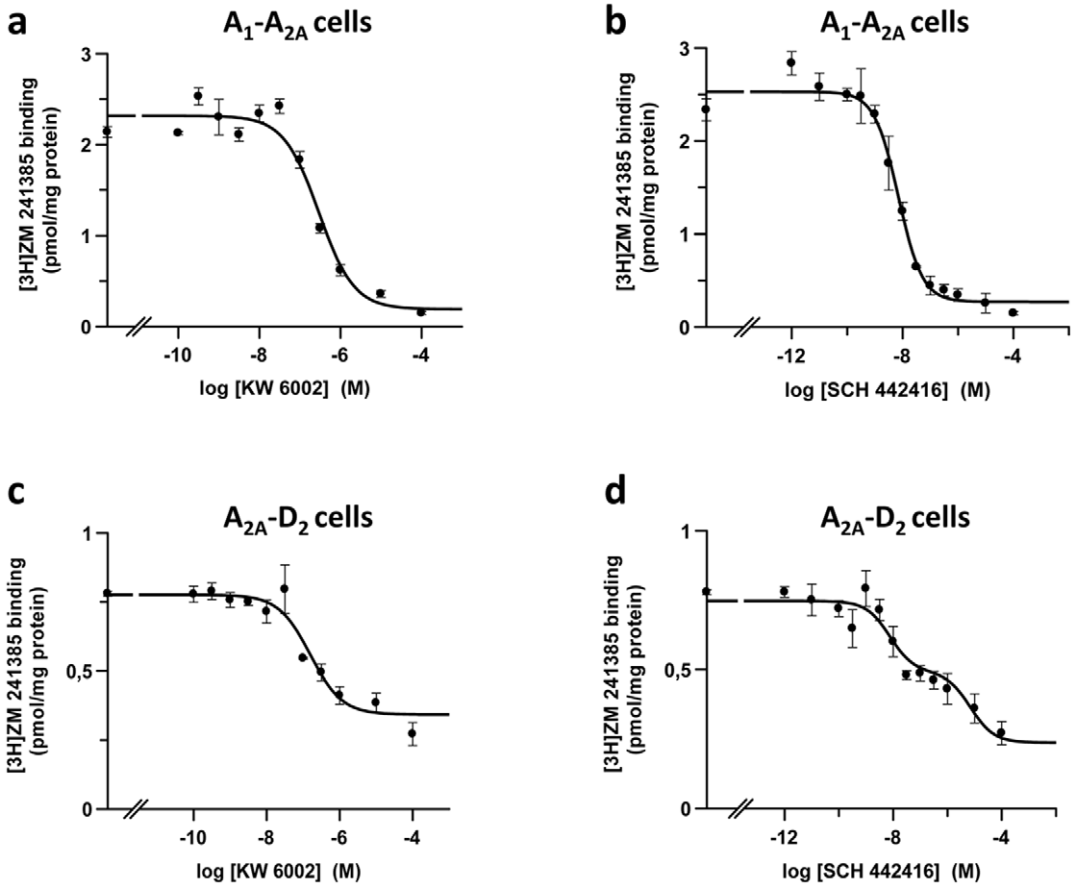

Figure 7. Binding of the A_{2A}R antagonists KW-6002 and SCH-442416 to A_1R-A_{2A}R and A_{2A}R-D_2R CHO cells. Competition experiments of [^{3}H]ZM-241385 (2 nM) *versus* increasing concentrations of KW-6002 (a and c) or SCH-442416 (b and d) were performed as indicated in Methods in membrane preparations from CHO cells expressing A_1R and A_{2A}R (a and b) or A_{2A}R and D_2R (c and d). Data are means ± S.E.M. of a representative experiment performed with triplicates.
doi:10.1371/journal.pone.0016088.g007

Table 3. Pharmacological parameters for A_{2A}R antagonist binding to A_{2A}R, A_1R-A_{2A}R and A_{2A}R-D_2R CHO cells.

K_{D1} (nM)	A_{2A}R cells	A_1R-A_{2A}R cells	A_{2A}R-D_2R cells
ZM241385	0.9±0.3	1.8±0.4	8±3*
SCH58261	3.3±0.3	4.7±0.6	23±8*
MSX2	3.2±0.2	4.2±0.3	7±2*
KW6002	100±10	100±20	160±70
SCH420814	0.5±0.1	1.1±0.1	2.7±0.8*

Competition experiments of [^{3}H]ZM-241385 (2 nM) binding *versus* increasing concentrations of A_{2A} receptor antagonists were performed as indicated in Methods in membrane preparations from CHO cells expressing A_{2A}R or A_1R and A_{2A}R or A_{2A}R and D_2R. Binding data were fitted assuming that receptors (also when heteromerizing) form homodimers, and cooperativity ($D_{CB} \neq 0$, fitting to eq. 2; Materials and Methods) or non-cooperativity ($D_{CB} = 0$, fitting to eq. 3; Materials and Methods) for competitor ligand binding was statistically tested (F test). Only K_{DB1} values (equilibrium dissociation constant of the first binding of B: ZM-241385, MSX-2, SCH-58261, SCH-420814 or KW-6002) are shown, since the analysis demonstrated non-cooperativity for the five A_{2A}R antagonists. Data are mean ± S.E.M. values of three experiments.
*: $p<0.05$ compared to K_{DB1} values in A_{2A}R cells; one-way ANOVA, followed by Newman-Keuls test.
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Table 4. Pharmacological parameters for SCH-442416 binding to A_{2A}R, A_1R-A_{2A}R and A_{2A}R-D_2R CHO cells.

Parameters	A_{2A}R cells	A_1R-A_{2A}R cells	A_{2A}R-D_2R cells
K_{DB1}	2.0±0.3 nM	2.4±0.4 nM	7±4 nM
K_{DB2}	8±2 nM	10±2 nM	5±2 µM**
D_{CB}	0	0	−2.3
B_{50}	4.0±0.6 nM	4.8±0.8 nM	190±80 nM**

Competition experiments of [^{3}H]ZM-241385 (2 nM) binding *versus* increasing concentrations of SCH-442416 were performed as indicated in Methods in membrane preparations from CHO cells expressing A_{2A}R or A_1R and A_{2A}R or A_{2A}R and D_2R. Results were fitted assuming that receptors (also when heteromerizing) form homodimers, and cooperativity ($D_{CB} \neq 0$, fitting to eq. 2; Materials and Methods) or non-cooperativity ($D_{CB} = 0$, fitting to eq. 3; Materials and Methods) of SCH-442416 binding was statistically tested (F test). K_{DB1} and K_{DB2} are, respectively, the equilibrium dissociation constants of the first and second binding of B (SCH-442416) to the dimer. D_{CB} is the "dimer cooperativity" index for the binding of the ligand B, and B_{50} is the concentration providing half saturation for B. Data are mean ± S.E.M. values of three experiments.
**: $p<0.01$, respectively compared to the K_{DB2} and B_{50} values in A_2R and A_1R-A_{2A}R cells; Kruskal-Wallis, followed by Dunn's test.
doi:10.1371/journal.pone.0016088.t004

transfected with the D_2R. When trying to explain the differential action of SCH-442416 observed *in vivo*, it is interesting to note that SCH-442416 showed a much higher affinity for the $A_{2A}R$ in a presynaptic-like than in a postsynaptic-like context. The binding of SCH-442416 to the $A_{2A}R$-D_2R heteromer displayed a strong negative cooperativity, phenomenon that was not observed for the binding of SCH-442416 to the A_1R-$A_{2A}R$ heteromer. This negative cooperativity explains the pronounced decrease in affinity of $A_{2A}R$ in cells expressing $A_{2A}R$-D_2R heteromers (B_{50} values 40 times higher in cells expressing $A_{2A}R$-D_2R than A_1R-$A_{2A}R$ heteromers).

The loss of affinity of $A_{2A}R$ upon co-expression of D_2R was much less pronounced for ZM-241385, SCH-58261, MSX2 or SCH-420814, for which the affinity was reduced from two to about nine fold. Taking into account that these $A_{2A}R$ antagonists behave similarly than the $A_{2A}R$ agonist CGS-21680 in terms of binding to A_1R-$A_{2A}R$ and $A_{2A}R$-D_2R heteromers, it is expected that these four compounds compete equally for the binding of the endogenous agonist at pre- and at postsynaptic sites. This would fit with the *in vivo* data, which shows that these compounds have a non-preferred pre-postsynaptic profile. Yet, KW-6002 was the only antagonist whose affinity was not significantly different in cells expressing $A_{2A}R$, A_1R-$A_{2A}R$ heteromers or $A_{2A}R$-D_2R heteromers. Thus, KW-6002 showed the best relative affinity for $A_{2A}R$-D_2R heteromers of all coumpounds, which can at least partially explain its preferential postsynaptic profile.

The present results support the notion that receptor heteromers may be used as selective targets for drug development. Main reasons are the very specific neuronal localization of receptor heteromers (even more specific than for receptor subtypes), and a differential ligand affinity of a receptor depending on its partner (or partners) in the receptor heteromer. In the striatum, $A_{2A}R$ provides a particularly interesting target, eventually useful for a variety of neuropsychiatric disorders. $A_{2A}R$-D_2R and A_1R-$A_{2A}R$ heteromers are segregated in different striatal neuronal elements. While $A_{2A}R$-D_2R heteromers are located postsynaptically in the dendritic spines of the indirect MSNs [4–6,14], A_1R-$A_{2A}R$ receptor heteromers are located presynaptically in glutamatergic terminals contacting the MSNs of the direct pathway [5,11,14]. Blocking postsynaptic $A_{2A}R$ in the indirect MSN should potentiate D_2R-mediated motor activation, which is a strategy already used in the development of anti-parkinsonian drugs [26–28]. However, blocking $A_{2A}R$ in glutamatergic terminals to the direct MSN could potentially be useful in dyskinetic disorders such as Huntington's disease and maybe in obsessive-compulsive disorders and drug addiction [5]. The present results give a mechanistic explanation to the already reported antiparkinsonian activity of KW-6002 [27,28] and suggest that SCH-442416 could be useful in dyskinetic disorders, obsessive-compulsive disorders and in drug addiction. Medicinal chemistry and computerized modeling should help understanding the molecular properties that determine the particular pharmacological profile of SCH-442416 and KW-6002, which may be used as lead compounds to obtain more effective antidyskinetic and antiparkinsonian compounds, respectively. It will also be of importance to take into account potential changes in the expression of pre- and postsynaptic $A_{2A}R$s and in their respective heteromers which can occur in those mentioned neuropsychiatric disorders. For instance, dopamine denervation seems to differentially modify the expression of striatal $A_{2A}R$, A_1R and D_2R [28–31]. This could be addressed by applying the *in vivo* methodology here described to animal models.

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Author Contributions

Conceived and designed the experiments: MO JB MB CQ VB SRG CL AC RF VC EIC SF. Performed the experiments: MO JB MB CQ AC VC. Analyzed the data: MO CQ CL AC VC SF. Wrote the paper: MO CL AC VC SF.

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Figure 1

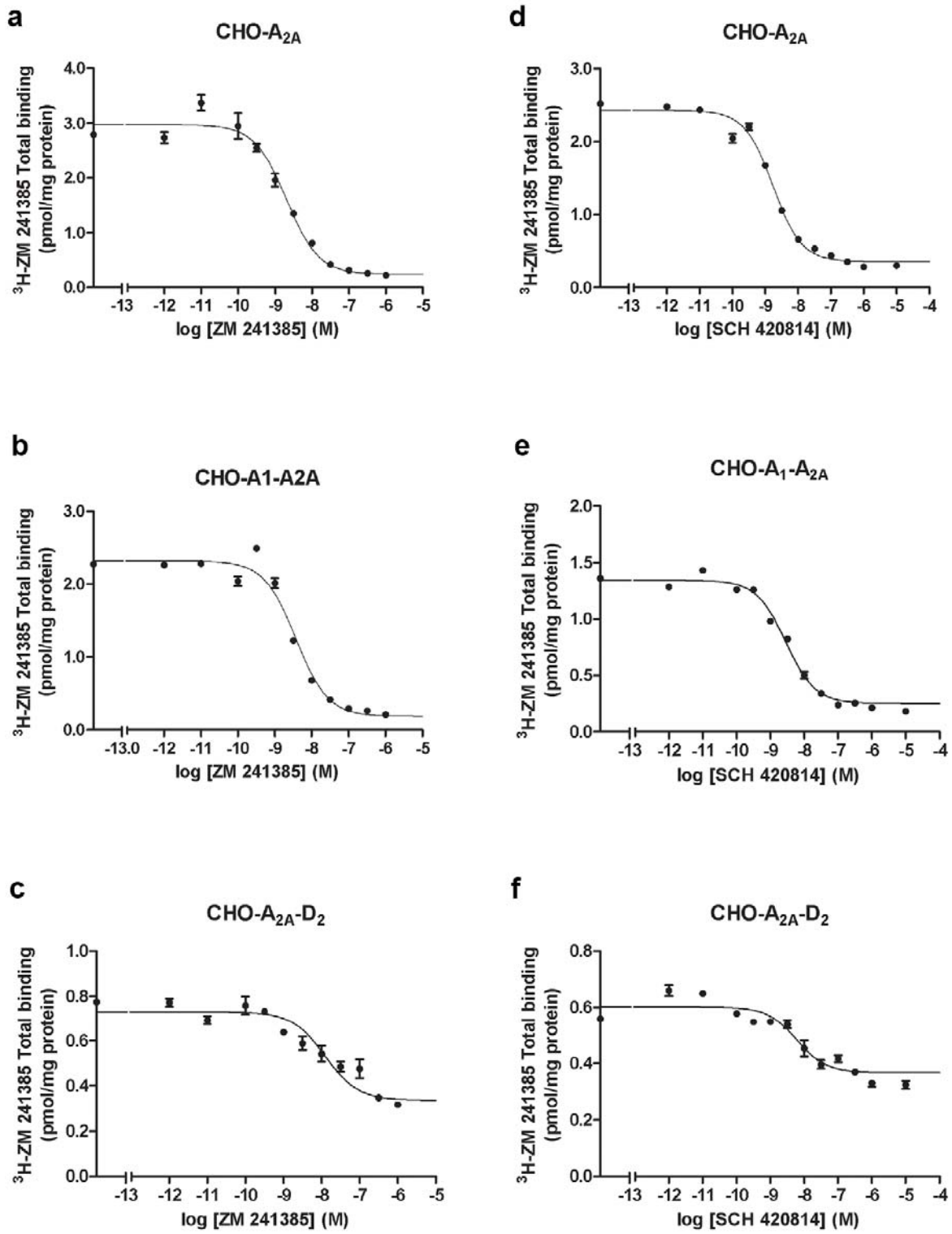
a
CHO-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
log [ZM 241385] (M)
d
CHO-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
log [SCH 420814] (M)
b
CHO-A1-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
log [ZM 241385] (M)
e
CHO-A1-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
log [SCH 420814] (M)
c
CHO-A2A-D2
3H-ZM 241385 Total binding (pmol/mg protein)
log [ZM 241385] (M)
f
CHO-A2A-D2
3H-ZM 241385 Total binding (pmol/mg protein)
log [SCH 420814] (M)

Figure 2

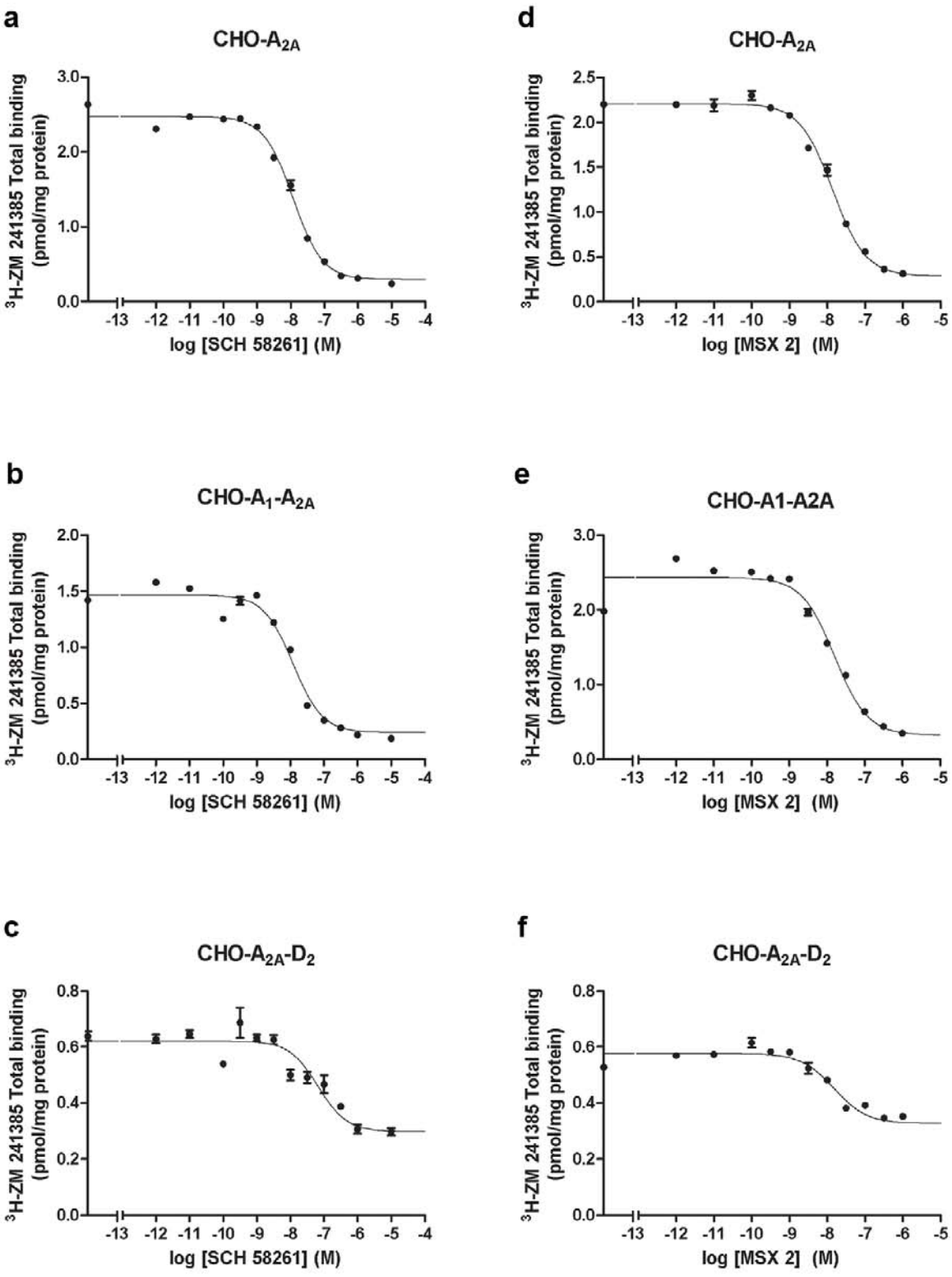
a
CHO-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
log [SCH 58261] (M)
d
CHO-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
log [MSX 2] (M)
b
CHO-A1-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
log [SCH 58261] (M)
e
CHO-A1-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
log [MSX 2] (M)
c
CHO-A2A-D2
3H-ZM 241385 Total binding (pmol/mg protein)
log [SCH 58261] (M)
f
CHO-A2A-D2
3H-ZM 241385 Total binding (pmol/mg protein)
log [MSX 2] (M)

Figure 3

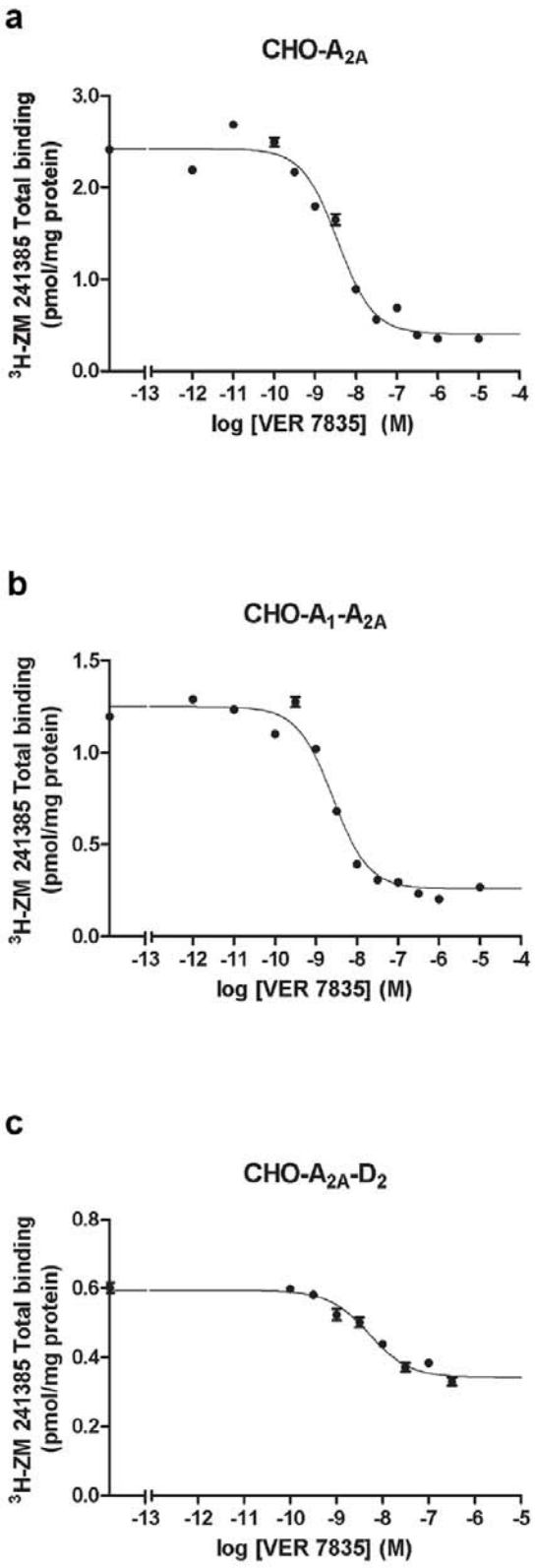
a
CHO-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
3.0
2.0
1.0
0.0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
log [VER 7835] (M)
b
CHO-A1-A2A
3H-ZM 241385 Total binding (pmol/mg protein)
1.5
1.0
0.5
0.0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
log [VER 7835] (M)
c
CHO-A2A-D2
3H-ZM 241385 Total binding (pmol/mg protein)
0.8
0.6
0.4
0.2
0.0
-13 -12 -11 -10 -9 -8 -7 -6 -5
log [VER 7835] (M)

Figure legends

Fig. 1. Competition curves corresponding to the values of displacement with ZM-241385 and SCH-420814 stated in table 3

Fig. 2. Competition curves corresponding to the values of displacement with SCH-58261 and MSX-2 stated in table 3

Fig. 3. Competition curves correcponiding to the values of displacement with VER-7835 stated in Supplementary table 1.

KD_1 (nM)	$A_{2A}R$ cells	A_1R-$A_{2A}R$ cells	$A_{2A}R$-D_2R cells
VER-7835	**1.1 ± 0.15 nM**	**1.5 ± 0.22 nM**	**2.7 ± 0.10 nM**

Supplementary Table. 1. Binding of $A_{2A}R$ antagonist VER-7835 to A_1R-$A_{2A}R$ and $A_{2A}R$-D_2R CHO cells. Competition experiments of [3H]ZM-241385 (2 nM) binding versus increasing concentrations of A_{2A} receptor antagonists were performed as indicated in Methods in membrane preparations from CHO cells expressing $A_{2A}R$ or A_1R and $A_{2A}R$ or $A_{2A}R$ and D_2R. Binding data were fitted assuming that receptors (also when heteromerizing) form homodimers, and cooperativity (D_{CB} ≠? 0, fitting to eq. 2; Materials and Methods) or non-cooperativity (DCB = 0, fitting to eq. 3; Materials and Methods) for competitor ligand binding was statistically tested (F test). Only $K_{DB}1$ values (equilibrium dissociation constant of the first binding of B: VER-7835) are shown, since the analysis demonstrated non-cooperativity for the five $A_{2A}R$ antagonists. Data are mean ± S.E.M. values of three experiments. One-way ANOVA, followed by Newman-Keuls test.

Caracterización farmacológica y funcional de los heterómeros de receptores de adenosina A_{2A} y de cannabinoides CB_1

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Running title: Heterómeros de receptores de adenosina A_{2A} y cannabinoides CB_1.

La expresión del heterómero formado por receptor de adenosina A_{2A} y cannabinoides CB_1 en células co-transfectadas y en el estriado de rata ha sido previamente descrita. A pesar de que se conoce que la activación de los receptores A_{2A} es necesaria para la señalización de los receptores CB_1 en neuroblastomas dónde estos heterómeros se expresan, las características farmacológicas y funcionales de estos heterómeros no se conocen. En este trabajo hemos caracterizado el heterómero de receptores A_{2A}-CB_1. Examinando la señalización dependiente de la proteína G, determinamos que aunque los receptores A_{2A} se acoplan a proteínas G_s cuando se expresan individualmente, en el heterómero ambos receptores, A_{2A} y CB_1, se acoplan a proteína G_i observándose un cross-talk sinérgico a nivel de la activación de la proteína G cuando los dos receptores se coactivan. Estudiando las vías de señalización de MAPK, observamos que la fosforilación de ERK

1/2 está controlada principalmente por el receptor CB_1 en el heterómero. También estudiamos la unión de agonistas y antagonistas a ambos receptores y demostramos que no se produce un efecto alostérico a nivel de unión de ligandos al hetorómero de receptores A_{2A}-CB_1. Sin embargo, dos antagonistas específicos del receptor A_{2A}, KW-6002 y VER-7835, mostraron una afinidad menor por el receptor A_{2A} cuando éste forma heterómeros A_{2A}-CB_1. Nuestros resultados ponen de manifiesto que los heterómeros de receptores A_{2A}-CB_1 constituyen una unidad singular para la señalización de adenosina y cannabinoides, introduciendo una diversidad a la señalización del receptor A_{2A} que puede ser terapéuticamente relevante en enfermedades neurológicas que involucran las neuronas estriatales.

Pharmacological and functional characterization of adenosine A_{2A}-cannabinoid CB_1 receptor heteromers

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Runnig title: Adenosine A_{2A}-cannabinoid CB_1 receptor heteromers

Summary

We previously reported the adenosine A_{2A}-cannabinoid CB_1 receptor heteromer expression in co-transfected cells and in rat striatum. Although it was known that activation of A_{2A} receptors was necessary for CB_1 receptor signaling in neuroblastoma cell line where the heteromers were expressed, the pharmacological and functional characteristics of these heteromers are not known. Here we characterized the A_{2A}-CB_1 receptor heteromer. Looking at the G protein dependent signaling, we observed that, although the A_{2A} receptors are coupled to G_s protein when expressed alone, both A_{2A} and CB_1 receptors are coupled to G_i protein in the heteromer and a synergistic cross-talk in G protein activation was observed when both receptors are co-activated. Looking at the MAPK pathway, we observed that CB_1 receptor was mainly controlling ERK 1/2 phosphorylation in the heteromer. We also studied the agonist and antagonist binding to both receptors and demonstrated that there is not an allosteric effect on ligand binding for A_{2A}-CB_1 receptor heteromers but two specific A_{2A} receptor antagonists, KW-6002 and VER-7835, lost affinity for A_{2A} receptors when expressed in A_{2A}-CB_1 heteromers. Our results have shown that A_{2A}-CB_1 receptor heteromers constitute a singular unit for adenosine and cannabinoids signaling, introducing diversity in A_{2A} receptor signaling that can be therapeutically relevant in neurological diseases involving striatal neurons

Introduction

Adenosine A_{2A} receptors are the most abundant subtype of adenosine receptors present in the striatum (Albin et al. 1989; Svenningsson et al. 1999) where they influence dopaminergic and glutamatergic neurotransmission, regulate motor activity and modulate excitotoxic mechanisms (for review see Svenningsson et al. 1999; Popoli et al. 2004, 2007; Cunha 2005; Fredholm et al. 2005; Schiffmann et al. 2007). A_{2A} receptors can be found both post- and presynaptically. Postsynaptically, A_{2A} receptors are found in the striatal efferent GABAergic neuron, also called the medium spiny neuron (MSN). These neurons constitutes more than 95% of the striatal neuron population (Gerfen, 2004) and are classified in two main subtypes, the enkephaline expressing MSNs projecting to the globus pallidus (the indirect pathway), expressing adenosine A_{2A} receptors and the dynorphin expressing MSNs (the direct pathway) predominantly expressing adenosine receptors of the A_1 subtype

(Ferré et al., 1997; Agnati et al., 2003; Gerfen et al., 2004). Presynaptically, A_{2A} receptors are found on the corticostriatal glutamatergic projections (Hettinger et al., 2001). There is recent evidence that presynaptic A_{2A} receptors are preferentially localized in cortical glutamatergic terminals that contact striatal neurons of the direct pathway rather than of indirect pathway (Quiroz et al., 2009). At the postsynaptic level in the indirect pathway, the activation of A_{2A} receptors leads to counteraction of dopamine D_2 receptor-mediated suppression of NMDA-induced depolarization and, thus, increases the action of the indirect pathway leading to motor depression (Azdad et al., 2009). This phenomenon was demonstrated to occur through adenosine A_{2A}-dopamine D_2 receptor heteromer formation in which A_{2A} receptor agonists decreased the affinity of D_2 receptor for dopamine (Azdad et al., 2009). At the presynaptic level in the direct patway, the activation of A_{2A} receptors increases glutamate release enhancing the glutamatergic neurotransmission and inducing motor activation (Ciruela et al., 2006). This phenomenon was demonstrated to occur through adenosine A_1-adenosine A_{2A} receptor heteromers (Ciruela et al., 2006).

Cannabinoid CB_1 receptors are the most abundant G-protein in the brain (Katona et al., 2006) and are also densely distributed in the striatum (Herkenham et al., 1990; Glass et al., 1997). In the striatum CB_1 receptors are localized in both types of MSNs, in enkephalinergic and dynorphinergic neurons of indirect and direct pathways respectively (Hohmann and Herkenham, 2000; Fusco et al., 2004). At this postsynaptic localization, CB_1 receptors negatively modulate locomotion (Ferré et al., 2010; Monory et al., 2007). Furthermore, striatal CB_1 receptors are localized in parvalbumin-expressing GABAergic interneurons (Hohmann and Herkenham, 2000; Fusco et al., 2004) and presynaptically are found in glutamatergic and GABAergic terminals (Rodriguez et al., 2001; Köfalvi et al., 2005; Pickel et al., 2004, 2006; Mátýas et al., 2006). The major physiological function of presynaptic CB_1 receptors is to regulate the release of various neurotransmitters (Katona et al., 2006; Freund et al., 2003; Marsicano et al., 2003). Very high expression of A_{2A} and CB_1 receptors in the striatum suggests that direct or indirect interactions between A_{2A} and CB_1 receptors are involved in the modulation of motor activity and goal-directed behaviours. It is known that A_{2A} receptors regulate CB_1 receptor action on both pre- and postsynaptic levels (Martire et al., 2011 Andersson et al., 2005; Tebano et al., 2009). A recent work showed that presynaptic A_{2A} receptors inhibits the CB_1 receptor-mediated synaptic effects and that this occurs probably via cAMP-PKA pathway (Martire et al., 2011),

this may be or not dependent on a physical interaction between both receptors, as it could also occur at the level of signalling. It seems that interactions between A_{2A} and CB_1 receptors localized in glutamatergic terminals that contact dynorphinergic MSNs are primarily involved in the hypolocomotor and rewarding effects of THC. However, it has been also suggested that postsynaptic mechanisms are involved in striatal A_{2A} receptor-dependent CB_1 receptor function (Andersson et al., 2005; Tebano et al., 2009). In fact, we have previously demonstrated that A_{2A} and CB_1 receptors form heteromers in HEK cells and in human neuroblastoma (Carriba et al., 2008) and CB_1 receptors co-localize and co-immunoprecipitate with A_{2A} receptors in the rat striatum (Carriba et al. 2007). In a human neuroblastoma cell line, CB_1 receptor signaling was found to be completely dependent on A_{2A} receptor activation. Accordingly, blockade of A_{2A} receptors counteracted the motor depressant effects produced by the intrastriatal administration of a cannabinoid CB_1 receptor agonist (Carriba et al., 2007). Although the effect of A_{2A} receptor activation on CB_1 receptor function was studied, the effect of CB_1 receptor on A_{2A} receptor function is not known. As heteromerization with CB_1 receptors can exert a fine tune modulation of the A_{2A} receptor pre- and postsynaptic behaviour, in this paper we wanted to characterize the A_{2A}-CB_1 receptor heteromers in order to know how the CB_1 receptors modulate the pharmacological and functional characteristics of the A_{2A} receptors. Moreover, the aim of this work was also to determine whether selective A_{2A} receptor antagonists show different selectivity for A_{2A} receptors or A_{2A}-CB_1 receptor heteromers in order to characterize pharmacological tools able to block A_{2A} receptors forming or not forming heteromers with CB_1 receptors.

Material and Methods

Receptor ligands

The following A_{2A} receptor antagonists were used: 4-(2-[7-Amino-2-(2-furyl) [1,2,4] triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol (**ZM-241385**, Tocris, Bristol, UK), 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4] triazolo[1,5-c]pyrimidin-5-amine (**SCH-442416**, Tocris, Bristol, UK), (E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione (**KW-6002**, Axon Medchem, Groningen, Netherlands), 2-amino-6-(furan-2-yl)-N-(thiophen-2-ylmethyl)-9H-purine-9-carboxamide (**VER-7835,** kindly provided by

Dr. Sergi Ferré**)**. As A_{2A} receptor agonist, 4-[2-[[6-Amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9*H*-purin-2-yl]amino]ethyl]benzene propanoic acid hydrochloride (CGS-21680, Sigma, Saint Louis, MO, USA) was used. As CB_1 receptor agonist (-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethyl-heptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP-55,940, Tocris, Bristol, UK) was used.

Cell clones and cell culture

CHO cell lines were maintained in α-MEM medium without nucleosides, containing 10% fetal bovine serum, and the indicated antibiotics. Cells were maintained at 37ºC in an atmosphere of 5% CO_2, and were passaged when they were 80-90% confluent, twice a week.

To obtain CHO cells expressing A_{2A} receptors, the human cDNAs for A_{2A} receptor was cloned into a *pcDNA3.1/Hygro* vector with a hygromycin resistance gene. CHO cells were transfected with this cDNA using lipofectamine (Invitrogen, Carlsbad, USA) method following the instructions of the supplier. 24 h after transfection the selection antibiotic was added at a concentration that was previously determined by a selection antibiotic test. Antibiotic resistant clones were isolated in the presence of the selection antibiotic (1000 μg/ml hygromycin). After an appropriate number of days/ passes, several stable lines were selected and cultured in the presence of the selection antibiotic (300 μg/ml hygromycin) and a CHO cell clone (CHO-A_{2A}) expressing high affinity A_{2A} receptors (see Results) was selected.

To obtain CHO cells co-expressing A_{2A} and CB_1 receptors, the human cDNA of CB_1 receptor cloned in *pcDNA3.1* was amplified without its stop codon using sense and antisense primers harboring unique BamHI and EcoRI. The amplified fragment was subcloned to be in-frame into restriction sites of the multiple cloning sites of *pEYFP-N1* vector, with a geneticin resistance gene (Clontech, Heidelberg, Germany), to give the plasmids corresponding to CB_1-YFP receptor fusion protein. CHO-A_{2A} cells (obtained as above described) were transfected with the cDNA for CB_1-YFP receptor construct using the lipofectamine method. After an appropriate number of days/passes, stable lines were selected and cultured in the presence of the selection antibiotic (300 μg/ml hygromycin, 1200 μg/ml geneticin). Expression of CB_1 receptors was first detected by monitoring its own fluorescence emission at 530 in a fluorescent microscope Zeiss Axiovert 25 (Göttingen, Germany) and fluorescent

clones were cultured in presence of the selection antibiotic (300 μg/ml hygromycin, 600 μg/ml geneticin). Moderately fluorescent clones (500-5000 fluorescent units in 0.2 mg/ml protein solution), were selected by fluorescence reading in a Fluostar Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using an excitation filter at 485 nm and emission filter at 530 nm.The selected clone (CHO-A_{2A}-CB_1 cells) was grown to obtain membranes in which the receptor expression was quantified by radioligand-binding experiments (see Results).

ERK 1/2 (extracellular-signal-regulated kinase) phosphorylation assay

CHO-A_{2A} or CHO-A_{2A}-CB_1 cells were cultured in serum-free medium containing 0.2 U/ml of adenosine deaminase (ADA, EC 3.5.4.4; Roche, Basel, Switzerland) for 16 h before the addition of any agent. Cells were stimulated at 37ºC in the same fresh medium for 5 min with the indicated concentrations of A_{2A} receptor agonist CGS-21680 and/or the CB_1 receptor agonist CP-55,940. Cells were washed with ice-cold PBS and lysed by the addition of 500 μl of ice-cold lysis buffer [50 mMTris/HCl (pH 7.4), 50 mM NaF, 150 mM NaCl, 45 mM 2-glycerolphosphate, 1% Triton X-100, 20 μM phenyl-arsine oxide, 0.4 mM sodium orthovanadate and protease inhibitor cocktail]. Cell debris was removed by centrifugation at 13.000 g for 5 min at 4 ºC and the protein was quantified using the BCA method using BSA dilutions as standards. To determine the level of ERK 1/2 phosphorylation, equivalent amounts of protein (15 μg) were separated by electrophoresis on denaturing SDS/PAGE (10 % gels) and transferred on to PVDF-FL membranes. Odyssey blocking buffer (LI-COR Biosciences) was then added, and membranes were rocked for 60 min. Membranes were then probed with a mixture of a mouse anti-(phospho-ERK 1/2) antibody (1:2500 dilution; Sigma) and rabbit anti-ERK 1/2 antibody (1:40000 dilution; Sigma) overnight. Bands were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody (1:10000 dilution; Sigma) and IRDye 680 (anti-rabbit) antibody (1:10000 dilution; Sigma) for 1 h, washed with PBS and scanned by the Odyssey IR scanner (LI-COR Biosciences). Bands densities were quantified using the scanner software and exported to Excel (Microsoft). The level of phosphorylated ERK 1/2 isoforms was normalized for differences in loading using the total ERK 1/2 protein band intensities.

CellKey label-free assays.

The CellKey system provides a universal, label-free, cell-based assay platform that uses cellular dielectric spectroscopy (CDS) to measure endogenous and transfected receptor activation in real time in live cells (Schroder et al., 2011). Changes in the complex impedance (DZ or dZ) of a cell monolayer in response to receptor stimulation were measured. Impedance (Z) is defined by the ratio of voltage to current as described by Ohm's law (Z=V/I). CHO-A_{2A} or CHO-A_{2A}-CB_1 cells were grown to confluence in a CellKey Standard 96 well microplate that contains electrodes at the bottom of each well. For untreated cells or for cells preincubated (overnight at 37ºC) with pertussis toxin (PTx 10 ng/ml) or cholera toxin (ChTx 100 ng/ml), medium was replaced by HBSS buffer (Gibco) suplemented with 20mM HEPES 30 minutes prior to running the cell equilibration protocol. A baseline was recorded for 5 minutes and then cells were treated with increasing concentrations of A_{2A} or CB_1 receptor agonists alone or in combination and data was acquired for the following 10 minutes. To calculate the impedance, small voltages at 24 different measurement frequencies were applied to treated or non-treated cells. At low frequencies, extracellular currents (iec) that pass around individual cells in the layer were induced. At high frequencies, transcellular currents (itc) that penetrate the cellular membrane were induced and the ratio of the applied voltage to the measured current for each well is the impedance. The data shown refer to the maximum complex impedance induced extracellular currents (Ziec) response to the ligand addition.

Radioligand binding experiments

Cells were disrupted with a Polytron homogenizer (PTA 20 TS rotor, setting 3; Kinematica, Basel, Switzerland) for two 5 s-periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4 containing a proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA). Cell debris was removed by centrifugation at 1,500 g for 5 min at 4ºC and membranes were obtained by centrifugation at 105,000 g (40 min, 4ºC). Membranes were resuspended and centrifuged under the same conditions. The pellet was stored at -20ºC, washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use. Membrane protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin dilutions as standard. For competition experiments, membrane suspensions (0.2 mg of

protein/ml) were incubated for 2 h at 25ºC in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$, 0.2 U/ml of ADA and 1mg/ml fatty acid free BSA (Sigma-Aldrich, St. Louis, MI, USA) for CB_1 receptor binding, with the indicated free concentration of the A_{2A} receptor antagonist [^{3}H]ZM-241385 (NEN Perkin Elmer, Wellesley, MA, USA), the A_{2A} receptor agonist [^{3}H]CGS-21680 (NEN Perkin Elmer, Wellesley, MA, USA) or the CB_1 receptor agonist [^{3}H]CP-55,940 (NEN Perkin Elmer, Wellesley, MA, USA) and increasing concentrations of ZM-241385, CGS-21680, SCH-442416, KW-6002 or VER-7835 in the absence or, when indicated, in the presence of a constant concentration of the compound acting as a modulator. Nonspecific binding was determined in the presence of 11 μM of the corresponding non-radiolabelled ligand. Free and membrane bound ligand were separated by rapid filtration of 500 ml aliquots in a cell harvester (Brandel, Gaithersburg, MD, USA) through Whatman GF/C filters embedded in 0.3% polyethylenimine containing 1mg/ml fatty acid free BSA for CB_1 receptor binding, that were subsequently washed (or washed twice for CB_1 receptor binding) for 5 s with 5 ml of ice-cold 50 mM Tris-HCl buffer containing 1mg/ml fatty acid free BSA for CB_1 receptor binding. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA, USA) overnight at room temperature and radioactivity counts were determined using a Tri-Carb 1600 scintillation counter (PerkinElmer, Boston, MA, USA) with an efficiency of 62% (Sarrió et al., 2000). When necessary, displacers were dissolved in DMSO and diluted in the binding medium. The DMSO concentration in the binding incubates was less than 0.5% and, at this concentration, it did not affect agonist or antagonist affinity for their respective receptors.

Binding data analysis

Radioligand competition curves were analyzed by nonlinear regression using the commercial Grafit curve-fitting software (Erithacus Software, Surrey, UK), by fitting the binding data to the mechanistic two-state dimer receptor model (Casadó et al., 2007; 2009a). Since there is now abundant evidence for GPCR oligomerization, including A_{2A} and CB_1 receptors (Bouvier et al., 2001; Devi et al., 2001; Franco et al. 2003;,Carriba et al., 2006; 2007), this model considers a homodimer as the minimal structural unit of the receptor. Here, we also consider the possibility of a homodimer as the minimal structural unit of a receptor forming homomers or forming heteromers

with another receptor. To calculate the macroscopic equilibrium dissociation constants the following equation for a competition binding experiment deduced previously (Franco et al., 2006) was considered:

$$A_{total\ bound} = (K_{DA2}A + 2A^2 + K_{DA2}AB / K_{DAB})\ R_T / (K_{DA1}K_{DA2} + K_{DA2}A + A^2 + K_{DA2}\ AB/\ K_{DAB} + K_{DA1}K_{DA2}B\ /K_{DB1} + K_{DA1}K_{DA2}B^2\ /\ (K_{DB1}K_{DB2})) + A_{non\text{-}specific\ bound}$$

eq. 1

where A represents free radioligand (the A_{2A} or CB_1 receptor ligands [^{3}H]ZM-241385, [^{3}H]CGS-21680 or [^{3}H]CP-55,940) concentration, R_T is the total amount of receptor dimers and K_{DA1} and K_{DA2} are the macroscopic equilibrium dissociation constants describing the binding of the first and the second radioligand molecule (A) to the dimeric receptor; B represents the assayed competing compound concentration, and K_{DB1} and K_{DB2} are, respectively, the macroscopic equilibrium dissociation constants for the binding of the first competitor molecule (B) to a dimer and for the binding of the second competitor molecule (B) to the semi-occupied dimmer; K_{DAB} is the hybrid equilibrium radioligand/competitor dissociation constant, which is the dissociation constant of B binding to a receptor dimer semi-occupied by A.

When both the radioligand A and the competitor B are the same compound, eq. (1) simplifies to eq. 2 due to the fact that $K_{DA1} = K_{DB1}$, $K_{DA2} = K_{DB2}$ and $K_{DAB} = K_{DA2}$ (Gracia et al., 2012 in preparation)

$$A_{total\ bound} = (K_{DA2}\ A + 2A^2 + A\ B)\ R_T / (K_{DA1}\ K_{DA2} + K_{DA2}\ A + A^2 + A\ B + K_{DA2}\ B + B^2) + A_{non\text{-}specific\ bound}$$

eq. 2

When the radioligand A shows non-cooperative behaviour, eq. (1) can be simplified to eq. (3) due to the fact that $K_{DA2} = 4K_{DA1}$ (Casadó et al., 1990a, 2009a); and, therefore, K_{DA1} is enough to characterize the binding of the radioligand A:

$$A_{total\ bound} = (4K_{DA1}A + 2A^2 + 4K_{DA1}AB / K_{DAB})\ R_T / (4K_{DA1}{}^2 + 4K_{DA1}A + A^2 + +4K_{DA1}AB / K_{DAB} + 4K_{DA1}{}^2B / K_{DB1} + 4K_{DA1}{}^2B^2 / (K_{DB1}K_{DB2})) + A_{non\text{-}specific\ bound}$$

eq. 3

In experimental conditions when both the radioligand A and the competitor B (i.e., most adenosine A_{2A} receptor antagonist tested in the present study) show non-cooperativity, it results that $K_{DA2} = 4K_{DA1}$ and $K_{DB2} = 4K_{DB1}$, and eq. (1) can be simplified to:

$$A_{total\ bound} = (4K_{DA1}A + 2A^2 + 4K_{DA1}AB / K_{DAB})\ R_T / (4K_{DA1}^2 + 4K_{DA1}A + A^2 + 4K_{DA1}AB / K_{DAB} + 4K_{DA1}^2B / K_{DB1} + K_{DA1}^2B^2 / K_{DB1}^2) + A_{non\text{-}specific\ bound}$$

eq. 4

When both the radioligand A and the competitor B are the same compound and the binding is non-cooperative, eq. (4) simplifies to: (Casadó et al., 2009b)

$$A_{total\ bound} = (4K_{DA1}A + 2A^2 + AB)\ R_T / (4K_{DA1}^2 + 4K_{DA1}A + A^2 + AB + 4K_{DA1}B + B^2) + A_{non\text{-}specific\ bound}$$

eq. 5

Goodness of fit was tested according to reduced χ^2 value given by the nonlinear regression program. The test of significance for two different population variances was based upon the F-distribution. Using this *F* test, a probability greater than 95% ($p < 0.05$) was considered the criterion to select a more complex equation to fit binding data over the simplest one. In all cases, a probability of less than 70% ($p > 0.30$) resulted when one equation to fit binding data was not significantly better than the other. Results are given as parameter values ± S.E.M. of three-four independent experiments.

Results

Pharmacological characterization of cells expressing A_{2A} or A_{2A} and CB_1 receptors

Using energy transfer experiments and co-immunoprecipitation, we previously described that A_{2A} and CB_1 receptors form heteromers when expressed in cells or in the brain striatum (Carriba et al., 2007, 2008). To compare the functional characteristics of A_{2A} receptors when expressed alone or forming heteromers with CB_1 receptors, we first generated CHO cell clones expressing A_{2A} receptors (CHO-

A_{2A}) or A_{2A}-CB_1 receptors (CHO-A_{2A}-CB_1) as indicated in Materials and Methods. The pharmacological characterization of A_{2A} receptors in these cells was performed by radioligand binding experiments. Competition experiments of the A_{2A} receptor antagonist [^{3}H]ZM-241385 (1.5 nM) *versus* ZM-241385 (0.01 nM to 11 mM) or the A_{2A}R agonist [^{3}H]CGS-21680 (19 nM) *versus* CGS-21680 (0.1 nM to 100 μM) using CHO-A_{2A} cell membranes gave the competition curves shown in Figure 1. Binding data were fitted assuming that receptors are dimers and statistically (F test, see Materials and Methods) testing whether the competitor (A_{2A} receptor antagonist or agonist) binding was cooperative (biphasic competition curves; fitting to eq. 2) or non-cooperative (monophasic competition curves; fitting to eq. 5). Fitting data from Figure 1 a or b to eq. 2 was not better than fitting data to eq. 5 according to the monophasic nature of both competition curves and indicating that agonist and antagonist binding to A_{2A} receptors is non-cooperative. The equilibrium dissociation constant (K_{DA1}) values for the antagonist ZM-241385 and the agonist CGS-21680 appears in Table 1. The A_{2A} receptor ligand binding parameters were also determined as described above using CHO-A_{2A}-CB_1 cell membranes. Competition curves of [^{3}H]ZM-241385 (1.6 nM) *versus* ZM-241385 (0.01 nM to 11 mM) or [^{3}H]CGS-21680 (19 nM) *versus* CGS-21680 (0.1 nM to 100 μM) are shown in Figure 2. Using CHO-A_{2A}-CB_1 cell membranes competition curves were also monophasic and K_{DA1} values (Table 1) were obtained by fitting data to non-cooperative binding equation (eq. 5). The agonist and antagonist dissociation constants were very similar in both cell lines and are comparable with the data obtained from analogous experiments performed using native tissues (For review see Cristalli et al., 2007).

The pharmacological characterization of CB_1 receptors were performed by competition experiments using CHO-A_{2A}-CB_1 cell membranes. The competition curve of 0.7 nM [^{3}H]CP-55,940 versus CP-55,940 (0.001 nM to 10 μM) appears in Figure 2c. Data fitting to eq. 2 (cooperative binding) was not better than fitting data to eq. 5 (non-cooperative binding) according to the monophasic nature of the curve and indicating that agonist binding to CB_1 receptors is non-cooperative. The equilibrium dissociation constant (K_{DA1}) value for the CP-55,940 binding obtained by fitting data to eq. 5 appears in Table 1. The dissociation constant is comparable to the data obtained from native tissues (For review see Pertwee et al., 1997).

Functional characteristics of A_{2A}-CB_1 receptor heteromers

One of the reported specific characteristics of A_{2A}-CB_1 receptor heteromers is that CB_1 receptor signaling via cAMP pathway is dependent on A_{2A} receptor activation (Carriba et al., 2007). We used this characteristic as a fingerprint to check the A_{2A}-CB_1 receptor heteromerization in CHO-A_{2A}-CB_1 cells and to further characterize the heteromer signaling. Since cAMP is a signal pathway under G protein activation, we looked for the heteromer fingerprint measuring the cross-talk between A_{2A} and CB_1 receptors on G protein activation by the CellKey label-free assay (see Materials and Methods). In CHO-A_{2A} cells the A_{2A} receptor agonist CGS-21680 (10nM) induced a G_s profile (decreases in impedance) that was completely blocked when cells were treated with cholera toxin (ChTx) but not significantly modified upon pertussis toxin (PTx) treatment (Figure 3a) according to A_{2A} receptors coupling to a G_s protein (Kull et al., 1999, 2000; for review see Fredholm et al., 2001). Surprisingly, in CHO-A_{2A}-CB_1 cells the A_{2A} receptor agonist CGS-21680 (10 nM to 1 μM) did not produce decreases in impedance and a moderate increase in impedance, corresponding to a G_i profile, was observed at the high CGS-21680 concentrations. According to a G_i profile, the impedance increase was reverted by PTx treatment (Figure 3b). These results indicate that A_{2A} receptors are coupled to G_i protein in these cells. As expected for a receptor coupled to a G_i protein, like CB_1 receptors, activation of CHO-A_{2A}-CB_1 cells with the CB_1 receptor agonist CP-55,940 (10 nM to 1 μM) showed increases in impedance corresponding to a G_i profile that were completely blocked when cells were treated with PTx (Figure 3b). Interestingly, in CHO-A_{2A}-CB_1 cells co-activated with a suboptimal concentration of CGS-21680 (100 nM) and a suboptimal concentration of CP-55,940 (100 nM) a synergistic increase in impedance was observed that was blocked by PTx. On one hand, the synergistic cross-talk between both receptors indicates that A_{2A} and CB_1 receptors form heteromers in CHO-A_{2A}-CB_1 cells and on the other hand, the G protein profile indicates that both receptors are coupled to G_i protein in the heteromer.

Apart from G protein-mediated signaling, many GPCRs are able to signal in a G protein-independent way (Valjent et al., 2000; Shenoy et al., 2003, 2006; Beaulieu et al., 2005; deWire et al., 2007). ERK 1/2 phosphorylation is one of the MAPK pathways that has been described to be activated in a G protein-independent and arrestin-dependent mechanism (DeWire et al., 2007). In this context, we sought to

study if heteromer formation might also influence A_{2A} or CB_1 receptor-mediated ERK 1/2 signaling. CHO-A_{2A} or CHO-A_{2A}-CB_1 cells were activated for 5 min with 200 nM of A_{2A}R agonist CSG-21680, 100 nM of CB_1R agonist CP-55,940 or both and ERK 1/2 phosphorylation was determined as indicated in Materials and Methods. As it can be seen in Figure 4 and as expected, CP-55,940 only induced ERK 1/2 phosphorylation in CHO-A_{2A}-CB_1 cells. Activation with CGS-21680 induced ERK 1/2 phosphorylation in CHO-A_{2A} cells and only a moderate effect in CHO-A_{2A}-CB_1 cells. Interestingly, when CHO-A_{2A}-CB_1 cells were co-activated with both agonist, the ERK 1/2 phosphorylation was not significantly different from the signaling induced by CP-55,940 alone, indicating that is CB_1 receptor controlling the ERK 1/2 signaling under the heteromer.

Pharmacological characteristics of A_{2A}-CB_1 receptor heteromers

Receptor heteromer is defined as a macromolecular complex composed by at least two receptor units with biochemical properties that are demonstrably different from those of its individual components (Ferré et al., 2009). Not only functional characteristics, as described above, but also specific ligand binding characteristics are one of those properties (Ferré et al., 2007, 2009). For a receptor heteromer, the ligand binding to one protomer can induce changes in the ligand binding to the other protomer through an allosteric phenomenon driven by a molecular interaction between protomers in the heteromer (Ferré et al., 2009). To this end, we investigated the effect of agonist binding to CB_1 receptors on the agonist affinity for A_{2A} receptors and *vice versa* (the effect of agonist binding to A_{2A} receptors on agonist affinity for CB_1 receptors) in CHO-A_{2A}-CB_1 cell membranes. Competition experiments of the A_{2A}R agonist [^{3}H]CGS-21680 (19 nM) *versus* CGS-21680 (0.1 nM to 100 μM) were performed in the presence of the CB_1 receptor agonist CP-55,940 (300 nM) and competition experiments of the CB_1 agonist [^{3}H]CP-55,940 (0.7 nM) versus CP-55,940 (0.001 nM to 10 μM) were performed in the presence of CGS-21680 (100 nM). In both cases, competition curves (Figure 5) were monophasic and K_{DA1} values (Table 1) were obtained by fitting data to non-cooperative binding equation (eq. 5). Since no significative changes were observed in K_{DA1} values in the presence or in the absence of the agonist for the partner receptor we can conclude that there is not an allosteric effect on ligand binding for A_{2A}-CB_1 receptor heteromers.

Screening of A_{2A} receptor antagonists on cells expressing A_{2A} receptors or A_{2A}-CB_1 receptor heteromers

It is now accepted that A_{2A} receptor containing heteromers are the true targets for some brain diseases (Bara-Jimenez et al., 2003; Orru et al., 2011; for review see Armentero et al., 2011). Under a pharmacological perspective, it is interesting to find compounds showing different pharmacological properties between receptors and receptor heteromers. A_{2A} receptor antagonists are interesting candidates. To test if selected A_{2A} receptor antagonists display different selectivity for A_{2A} receptors or A_{2A}-CB_1 receptor heteromers, competition experiments with some A_{2A} receptor antagonists as displacers were performed using CHO-A_{2A} or CHO-A_{2A}-CB_1 cell membranes. Competition experiments were performed displacing both A_{2A}R antagonist [^{3}H]ZM-241385 (1.5 nM) or A_{2A}R agonist [^{3}H]CGS-21680 (19 nM) with increasing of A_{2A} receptor antagonists SCH-442416 (0.01 nM to 100 μM) , KW-6002 (0.1 nM to 100 μM) or VER-7835 (0.01 nM to 100 μM), as indicated in Materials and Methods. Competition curves are shown in Figure 6. In all cases, fitting binding data to a cooperative binding equation (biphasic competition curves; eq. 3) was not better than to fit binding data to a non-cooperative binding equation (monophasic competition curves, eq. 4) according to the monophasic nature of curves in Figure 6. The equilibrium dissociation constant values (K_{DB1}) for the displacer A_{2A} receptor antagonists were deduced from fitting data to eq. 4 (considering the K_{DA1} values from Table 1) and appear in Table 2. Taking into account both [^{3}H]ZM-241385 and [^{3}H]CGS-21680 displacement curves, the A_{2A} receptor antagonist SCH-442416 shows similar affinity for A_{2A} receptors or A_{2A}-CB_1 receptor heteromers (Table 2). Interestingly, the K_{DB1} values of both KW-6002 and VER-7835 significantly ($p < 0.05$) increased when A_{2A} receptors form heteromers with CB_1 receptors. This decrease in affinity was higher when KW-6002 or VER-7835 displaced the A_{2A} receptor agonist [^{3}H]CGS-21680, being the K_{DB1} values almost 10 or 4 times higher respectively for A_{2A}-CB_1 heteromers than for A_{2A} receptors (Table 2). These results indicate a decrease in affinity for KW-6002 or VER-7835 to bind A_{2A} receptors in A_{2A}-CB_1 heteromers.

Discussion

G protein-coupled receptors cannot only be considered as single functional units, but as forming part of multimolecular aggregates localized in the plane of the plasma membrane (Bouvier, 2001; Marshall, 2001; Pin et al. 2007; Ferré et al., 2009). In fact, it seems that most members of this family can exist as homomers or heteromers (Albizu et al., 2010; Birdsall et al., 2010, Fuxe et al., 2010). A receptor heteromer is a macromolecular complex composed of at least two functional receptor units with biochemical properties that are demonstrably different from those of its individual receptors (Ferré et al., 2009). We reported that cannabinoid CB_1 and adenosine A_{2A} receptors form heteromers in co-transfected cells and rat striatum where they co-localize in fibrilar structures (Carriba et al, 2007). Although it was known that activation of A_{2A} receptors was necessary for CB_1 receptor signaling in a human neuroblastoma cell line where the heteromers were expressed (Carriba et al., 2007), the pharmacological and functional characteristics of these heteromers are far from being completely studied. Here we report several major conclusions on the biochemical characteristics of A_{2A}-CB_1 receptor heteromers. First, by measuring G protein activation by the CellKey label-free assay, we demonstrated that A_{2A} and CB_1 receptors are coupled to G_i protein in the heteromer. Second, we observed a synergistic cross-talk in G protein activation when both receptors are co-activated but it is mainly the CB_1 receptor controlling the ERK 1/2 signaling under the heteromer. Third, we demonstrated that there is not an allosteric effect on ligand binding for A_{2A}-CB_1 receptor heteromers and finally we found that two specific A_{2A} receptor antagonist, KW-6002 and VER-7835 lost affinity for A_{2A} receptors when expressed in A_{2A}-CB_1 heteromers.

Receptor heteromers provide functional entities that possess different biochemical properties with respect to the individual components of the heteromer. A receptor unit in the heteromer can display several biochemical properties that can be simply dependent on the presence of the other unit or on co-stimulation of the two receptor units in the heteromer. Changes in G protein coupling only by the presence of the partner receptor are a common characteristic of neurotransmitter receptor heteromers. In opioid receptors (OR) δOR–μOR heteromer, the receptor units in the heteromer couple to G proteins other than those usually associated with the individually expressed receptors. Thus, signaling by stimulating the receptor units in

the δOR–μOR heteromer (which causes inhibition of adenylate cyclase) is not sensitive to pertussis toxin, suggesting a G protein switch from G_i to G_z (George et al., 2000, Levac et al., 2000]. There are examples of changes in G-protein coupling that are dependent on co-activation of the receptor units in the receptor heteromer. Dopamine D_2 receptor normally couples to G_{i-o} proteins, but in the dopamine D_1–D_2 receptor heteromer it switches to $G_{q/11}$ when D_1 receptor is co-activated. In this way, the D_1–D_2 receptor heteromer provides a selective mechanism by which dopamine activates phospholipase C-mediated calcium signaling (Rashid et al., 2007). Here we demonstrated that adenosine A_{2A} receptors, coupled to G_s protein when expressed alone, switch to G_i protein in A_{2A}-CB_1 receptor heteromers in the absence or in the presence of CB_1 receptor agonists. Thus A_{2A}-CB_1 receptor heteromer provides a selective mechanism by which cannabinoid receptor blocks the A_{2A} receptor-mediated cAMP production.

Frequently, activation of one receptor unit in the heteromer implies intermolecular cross-talk involving conformational changes sensed by the other receptor unit in the heteromer. These conformational changes lead to modulation of ligand binding and/or signaling of the partner receptor. In some cases, stimulation of one receptor unit decreases the affinity and signaling of the other receptor unit as it has been described for adenosine A_1–A_{2A} and adenosine A_{2A}–dopamine D_2 receptor heteromers, that show antagonistic allosteric interactions and a negative cross-talk (Ferré, 1991, Hillion et al., 2002, Canals et al., 2003, Ciruela et al., 2006). In other cases stimulation of one receptor unit increases the signaling and the affinity of the other receptor unit for endogenous or exogenous ligands as occurs for dopamine D_2–somatotatine SST5 receptor heteromer in which stimulation of D_2 receptors significantly increases the affinity of SST5 receptors for agonists (Rocheville et al., 2000). For the A_{2A}-CB_1 receptor heteromers, ligand binding to CB_1 receptors did not modify the ligand binding to A_{2A} receptors and vice versa, indicating a lack of allosteric interactions for this heteromer. However, a synergistic increase in G_i protein activation was observed when both receptors were co-activated. This is in accordance with the fact that activation of A_{2A} receptors was necessary for CB_1 receptor signaling in neuroblastoma cell line previously described (Carriba et al., 2007). Our results imply a processing of information, at the membrane level, of the signals impinging on the A_{2A}-CB_1 heteromers. In this case, the neurotransmitter receptor heteromer

functions as a processor of computations that modulates G protein-mediated signaling because quantitative or qualitative aspects of the signaling generated by stimulation of either receptor unit in the heteromer are different from those obtained during co-activation. Apart from G protein-mediated signaling, many GPCRs are able to signal in a G protein-independent way (Shenoy et al., 2003, 2006; Beaulier et al. 2005; DeWire et al., 2007; Valjent et al., 2000). ERK 1/2 phosphorylation is one of the MAPK pathways that has been described to be activated in a G protein-independent and arrestin-dependent mechanism (DeWire et al., 2007). Looking at the ERK 1/2 phosphorylation when both receptors in the A_{2A}-CB_1 heteromers are co-activated it seems that is mainly the CB_1 receptor controlling the ERK 1/2 signaling under the heteromer. Since ERK 1/2 phosphorylation is related to plasticity (Shiflett et al., 2011) it seems that cannabinoids are controling changes in ERK 1/2-mediated plasticity in cells where the heteromers are expressed.

Striatal adenosine A_{2A} receptors are highly expressed in MSNs of the indirect efferent pathway and are also localized presynaptically in cortico-striatal glutamatergic terminals contacting MSNs of the direct efferent pathway and in both localizations A_{2A} receptors codistributed with CB_1 receptors (see Introduction). It has been hypothesized that postsynaptic $A_{2A}R$ antagonists should be useful in Parkinson's disease, while presynaptic $A_{2A}R$ antagonists could be beneficial in dyskinetic disorders, such as Huntington's disease, obsessive-compulsive disorders and drug addiction (Orru et al 2011a, 2011b; Blum et al., 2003; Armentero et al., 2011). Thus, to look for A_{2A} antagonist selectively targeting A_{2A} receptors or A_{2A}-CB_1 receptor heteromers can have relevance for therapeutical purpose. Here we performed competition experiments using the A_{2A} receptor antagonists SCH-442416, KW-6002 or VER-7835. We have previously described SCH-442416 targets presynaptic A_{2A} receptors whilst KW-6002 targets postsynaptic A_{2A} receptors (Orru et al., 2011a) and VER-7835 probably does not discriminate between both receptor localizations (unpublished results). We found that SCH-442416 affinity was similar for A_{2A} receptors and A_{2A}-CB_1 receptor heteromers suggesting that both can be blocked presynaptically by SCH-442416 and indicating that A_{2A}-CB_1 receptor heteromers must be considered to analyze the antidyskinetic effects of SCH-442416. Interestingly, we found a decrease in affinity for KW-6002 or VER-7835 to bind A_{2A}-CB_1 heteromers compared to A_{2A} receptors. It seems that at postsynaptic level KW-6002 or VER-7835 would block A_{2A} receptors more selectively than A_{2A}-CB_1

receptor heteromers and this has to be taken into account to analyze the antiparkinsonian effect of KW-6002. In summary, our results show that A_{2A}-CB_1 receptor heteromers facilitate the understanding the role of interactions between adenosine and cannabinoids in the brain, since A_{2A}-CB_1 receptor heteromers act as processors of computations that modulate cell signaling and can be therapeutical targets in neurological diseases involving striatal neurons.

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Figure Legends

Fig. 1. A_{2A} receptor agonist and antagonist binding to CHO-A_{2A} cell membranes. Competition experiments were performed in membranes preparations (0.2 mg protein/mg) from CHO-A_{2A} cells using 1.5 nM of the A_{2A} receptor antagonist [^{3}H]ZM-24135 and increasing concentrations (0.01 nM to 10 μM) of non-radiolabeled ZM-24135 (a) or 19 nM of the A_{2A} receptor agonist [^{3}H]CGS-21680 and increasing concentrations (0.1 nM to 100 μM) of non-radiolabeled CGS-21680 (b) as described in Methods. Data are means ± S.E.M. of a representative experiment performed with triplicates.

Fig. 2. A_{2A} and CB_1 receptor ligand binding to CHO-A_{2A}-CB_1 cell membranes. Competition experiments were performed in membranes preparations (0.2 mg protein/mg) from CHO-$A_{2A}CB_1$ cells using 1.6 nM of A_{2A} receptor antagonist [^{3}H]ZM-24135 and increasing concentrations (0.01 nM to 10 μM) of non-radiolabeled ZM-24135 (a), 19 nM of A_{2A} receptor agonist [^{3}H]CGS-21680 and increasing concentrations (0.1 nM to 100 μM) of non-radiolabeled CGS-21680 (b) or 0.7 nM of CB_1 receptor agonist [^{3}H] CP-55,940 and increasing concentrations (0.001 nM to 10 μM) of non-radiolabeled CP-55,940 (c), as described in Materials and Methods. Data are means ± S.E.M. of a representative experiment performed with triplicates.

Fig. 3. G_i-dependent signaling of A_{2A}-CB_1 receptor heteromers. CellKey label-free assays were performed in CHO-A_{2A} cells (a) or in CHO-A_{2A}-CB_1 cells (b) as indicated in Materials and Methods. Cells were treated with medium (Buffer), PTx (10 ng/ml) or ChTx (100 ng/ml) and were stimulated or not with 10 nM (a) or increasing concentrations (b) of A_{2A} receptor agonist CGS-21680, increasing concentrations of CB_1 receptor agonist CP-55,940 or both (b). Results are mean ± S.E.M from 3 to 4 independent experiments. Statistical significance was calculated by one way ANOVA followed by Dunnett multiple comparison test; *$p<0.05$, **$p<0.01$, ***$p<0.005$ compared with the respective control (buffer or buffer plus toxin), $^{\&\&\&}p<0.005$ compared with cells treated only with one agonist.

Fig. 4. A_{2A}-CB_1 receptor heteromer-mediated ERK 1/2 phosphorylation. CHO-A_{2A} or CHO-A_{2A}-CB_1 cells were stimulated with the A_{2A} receptor agonist CGS-21680 (200 nM, black columns) or the CB_1 receptor agonist CP-55,940 (100 nM, white columns) alone or in combination (dashed column) and ERK 1/2 phosphorylation was determined as indicated in Materials and Methods. Values are represented in-fold respect to basal levels in absence of agonist and are means ± S.E.M. of three independent experiments. On the bottom a representative Western blot is shown for samples in duplicates. Statistical significance was calculated by one way ANOVA followed by Dunnett multiple comparison test; *p<0.05,**p<0.01, ***p<0.005 compared with the respective basal.

Fig. 5 Lack of allosteric interaction between A_{2A} and CB_1 receptors in A_{2A}-CB_1 receptor heteromers. Competition experiments were performed in membranes preparations (0.2 mg protein/mg) from CHO-A_{2A}-CB_1 cells using 19 nM of A_{2A} receptor agonist [^{3}H]CGS-21680 and increasing concentrations (0.1 nM to 100 μM) of non-radiolabeled CGS-21680 in the presence (triangles, solid line) or in the absence (dotted line) of CB_1 agonist CP-55,940 (300 nM) (a) or using 0.7 nM [^{3}H] CP-55,940 and increasing concentrations (0.001 nM to 10 μM) of non-radiolabeled CP-55,940 in the presence (triangles, solid line) or in the absence (dotted line) of 100 nM CGS-21680 (b), as described in Materials and Methods. Data are means ± S.E.M. of a representative experiment performed with triplicates.

Fig. 6. Binding of the A_{2A} receptor antagonists SCH-442416, KW-6002 and VER-7835 to A_{2A} receptors and to A_{2A}-CB_1 receptor heteromers. Competition experiments of 19 nM [^{3}H]CGS-21680 (a to c) or 1.5 nM [^{3}H]ZM-24135 (d to f) versus increasing concentrations of SCH-442416 (a and d), KW-6002 (b and e) or VER-7835 (c and f) were performed as indicated in Materials and Methods using membrane preparations (0.2 mg protein/ml) from CHO-A_{2A} cells (circles, solid line) or CHO-A_{2A}-CB_1 cells (triangles, dashed line). Data are means ± SD. of a representative experiment performed with triplicates.

Tables

Table 1. Equilibrium dissociation constant (K_{DA1}) of ligand binding to CHO-A_{2A} or CHO-A_{2A}-CB_1 cell membranes

Ligand	CHO-A_{2A}	CHO-A_{2A}-CB_1
CGS-21680	110 ± 30 nM	90 ± 30 nM
		120 ± 50 nM (treated with CP-55,940)
ZM-241358	3.9 ± 0.9 nM	5.0 ± 2.0 nM
CP-55,940		5.0 ± 2.0 nM
		2.9 ± 1.7 nM (treated with CGS-21680)

Table 2. Equilibrium dissociation constant (K_{DB1}) of A_{2A} receptor antagonists binding to CHO-A_{2A} or CHO-A_{2A}-CB_1 cell membranes

Displacer	[^{3}H]CGS-21680		[^{3}H]ZM-241385	
	CHO-A_{2A}	CHO-A_{2A}-CB_1	CHO-A_{2A}	CHO-A_{2A}-CB_1
SCH-442416	382 ± 110 nM	570 ± 170 nM	51 ± 11 nM	59 ± 20 nM
KW-6002	424 ± 74 nM	3700 ± 1100	121 ± 21 nM	229 ± 28 nM
VER-7835	59 ± 20 nM	181 ± 65 nM	4.2 ± 0.7 nM	6.8 ± 0.7 nM

Figures

Figure 1

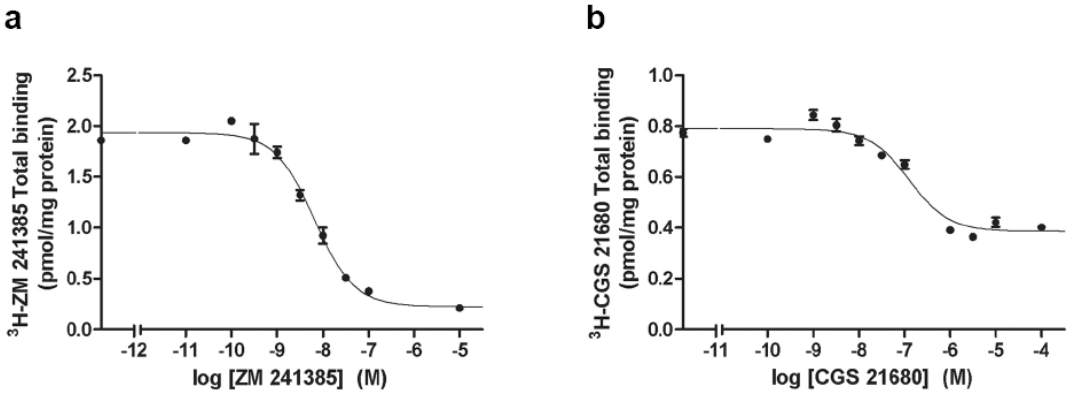


Figure 2

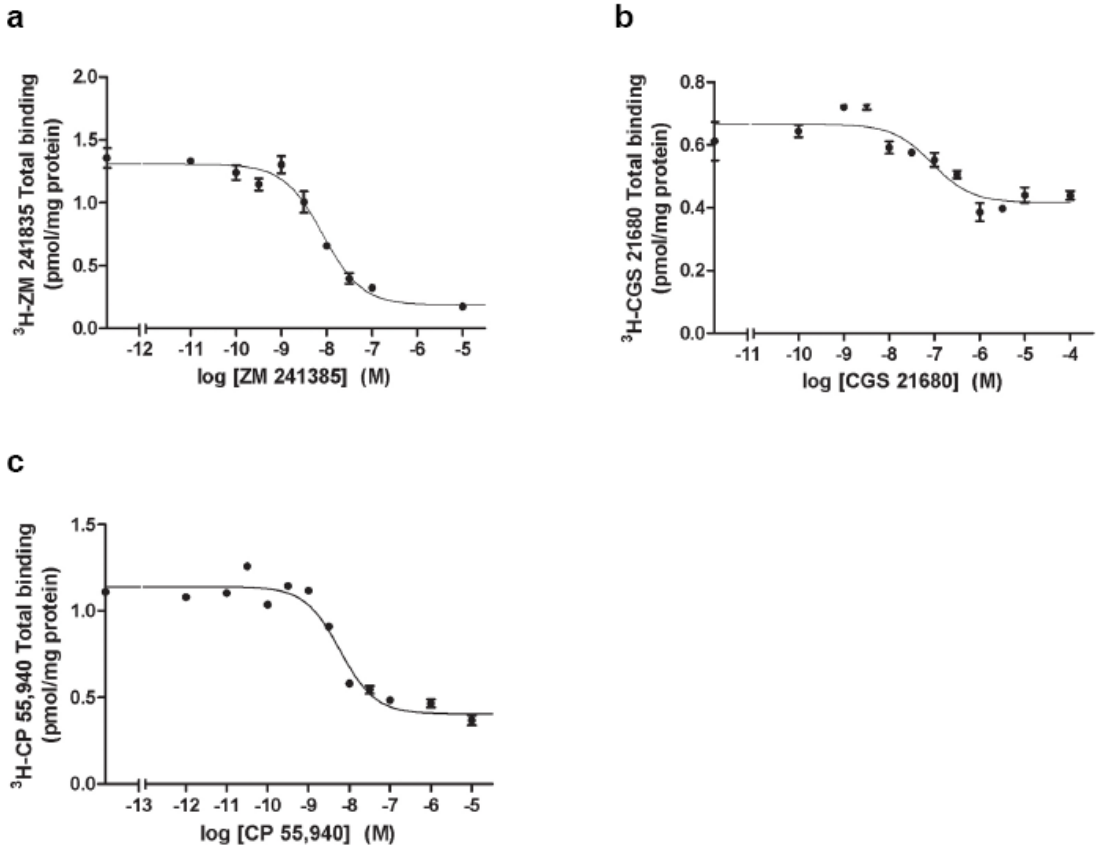
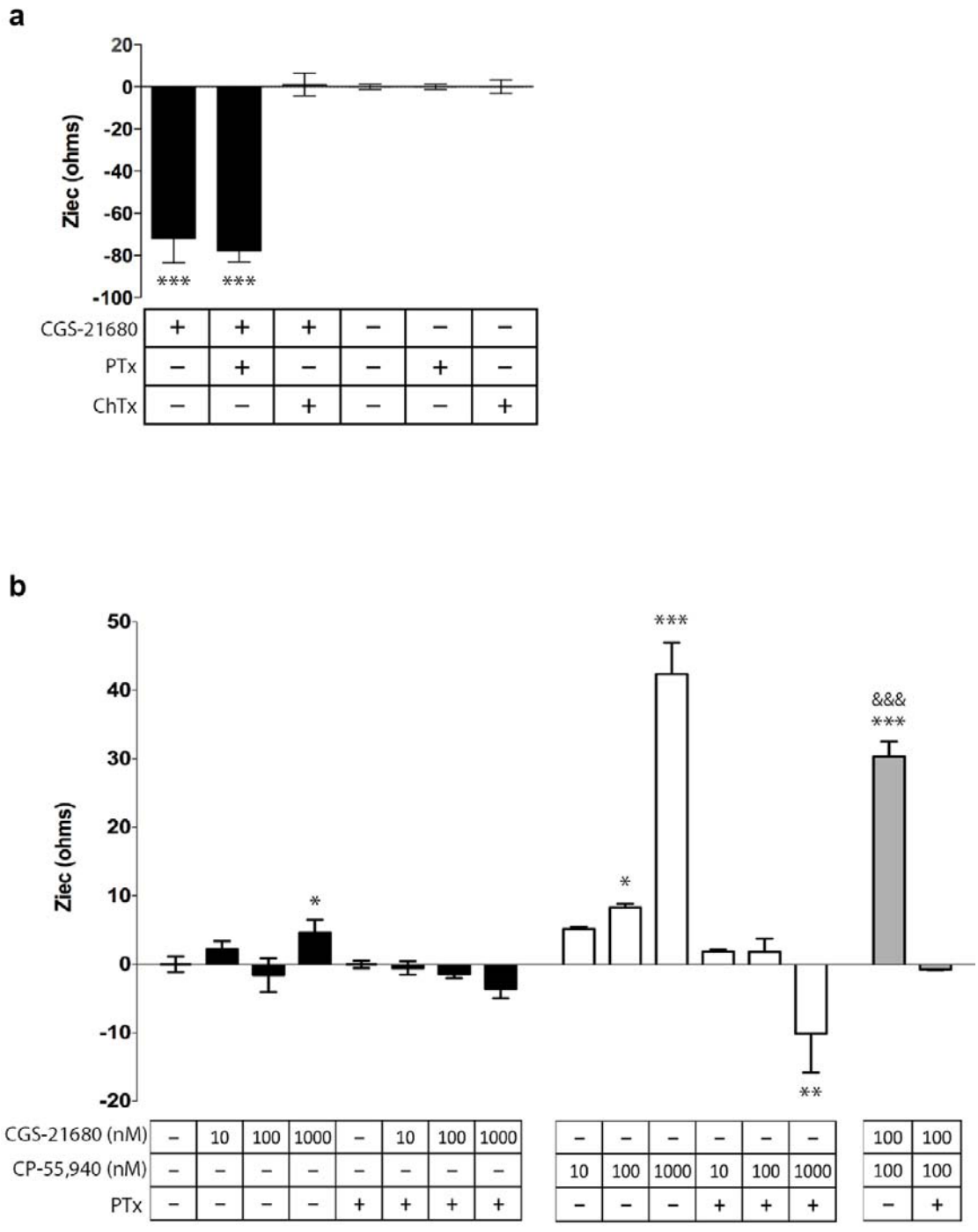


Figure 3


a
Ziec (ohms)
20
0
-20
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CGS-21680 + + + – – –
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ChTx – – + – – +
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Ziec (ohms)
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Figure 4

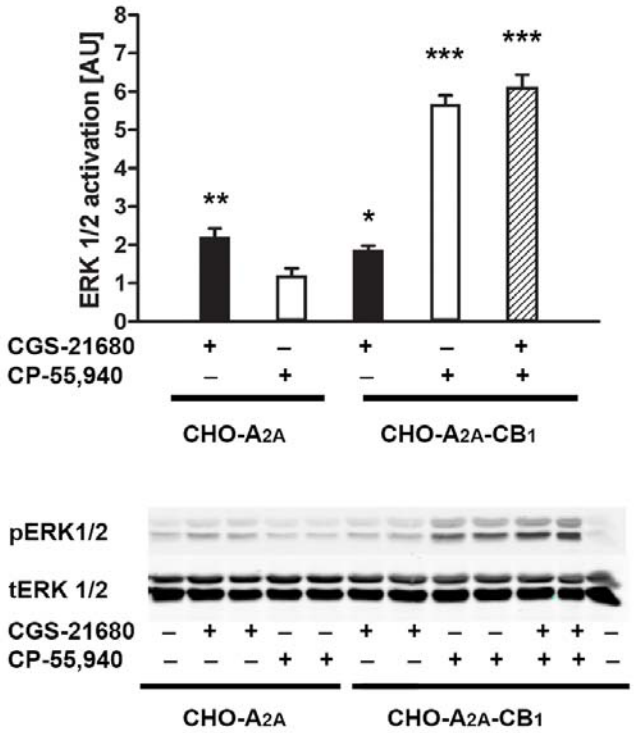


Figure 5

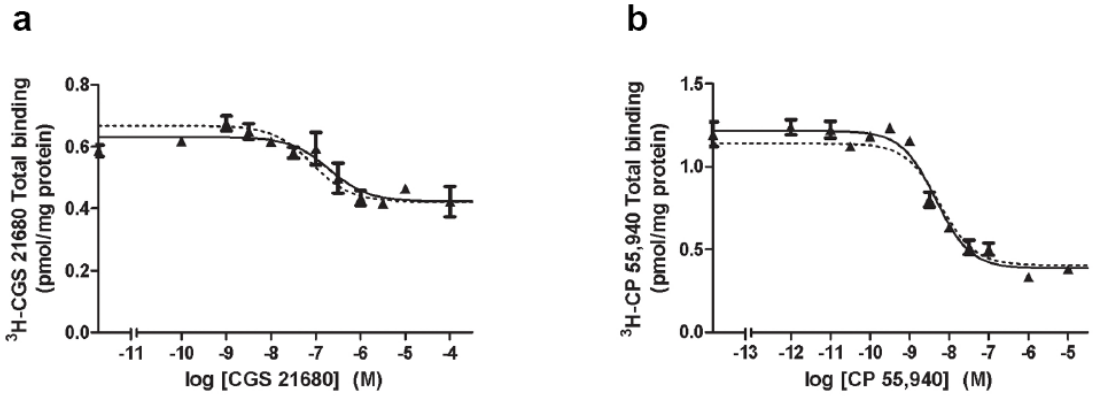
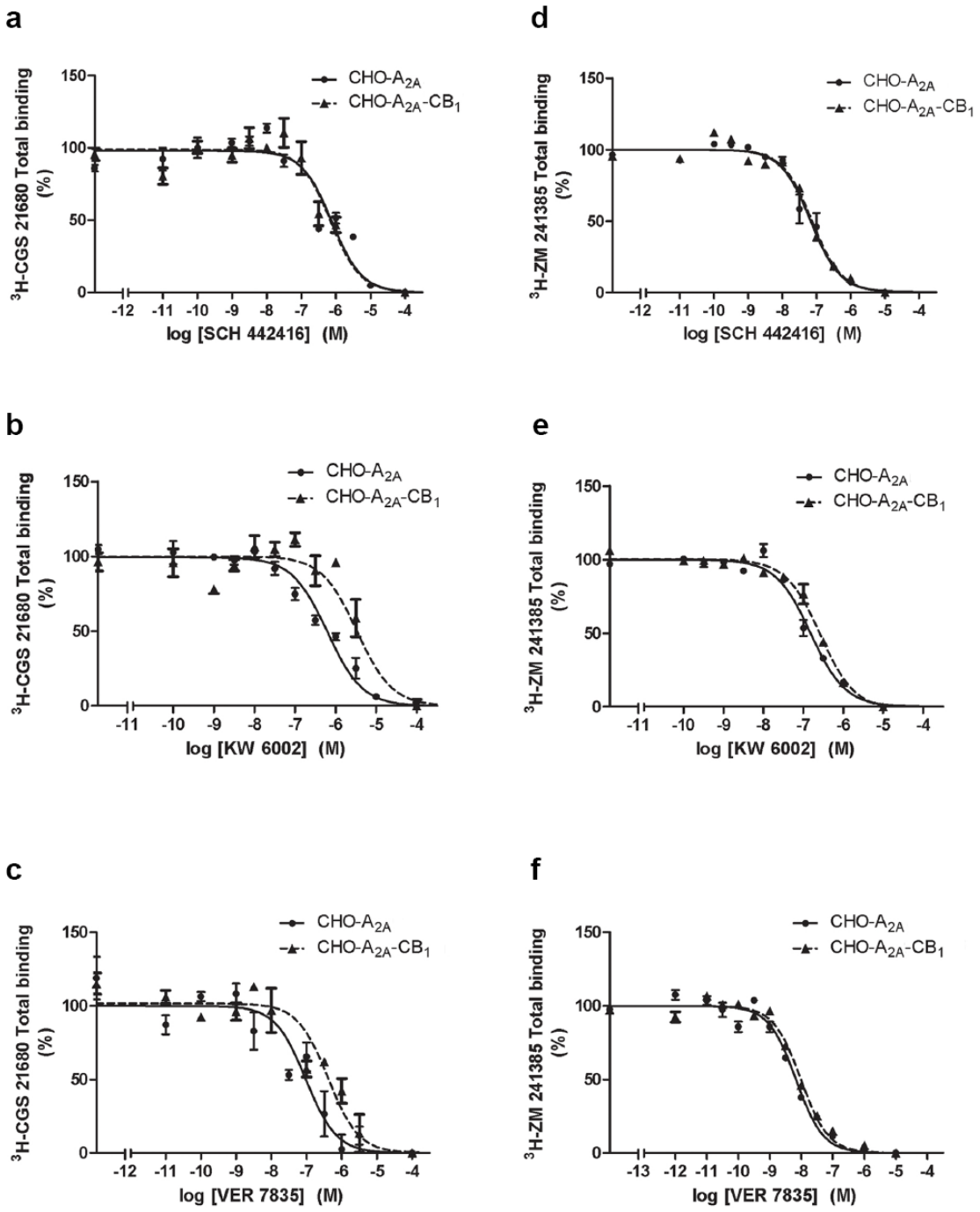


Figure 6


a
d
b
e
c
f
CHO-A2A
CHO-A2A-CB1
3H-CGS 21680 Total binding (%)
3H-ZM 241385 Total binding (%)
log [SCH 442416] (M)
log [KW 6002] (M)
log [VER 7835] (M)

Cannabinoid CB_1 receptor forms heteromers with dopamine D_1 and adenosine A_1 receptor in transfected HEK cells

Cannabinoid CB_1 receptors (CB_1R) are the most abundant neutrotransmitter receptors in the brain and are found pre- and postsynaptically (Katona et al., 2006). Interaction of CB_1R with others neurotransmitter receptors in the enkephalinergic motor spiny neurons (MSNs) of the indirect pathway was previously demonstrated, first for dopamine D_2 receptors forming CB_1-D_2 receptor heteromers (Kearn et al., 2005), later for adenosine A_{2A} receptors forming A_{2A}-CB_1 receptor heteromers (Carriba et al., 2007), and, more recently, it has been described A_{2A}-D_2-CB_1 receptor heteromers in the striatum (Navarro et al., 2008). Apart from MSNs of the indirect pathway, CB_1R could colocalize with adenosine A_1 receptors (A_1R) at the presynaptic level in the corticostriatal synapse of direct pathway where A_1R are found, or colocalize with both A_1R and dopamine D_1 receptors (D_1R) at the postsynaptic level in dynorphinergic MSNs of the direct pathway (Ferré et al., 1997; Gerfen, 2004). Based on these colocalizations we wanted to find out if CB_1R can physically interact with A_1R or D_1R forming heteromers in living cells.

In order to investigate the A_1R-CB_1R and CB_1R-D_1R heteromer expression, BRET assays in co-trasfected HEK cells were performed. The human version of fusion proteins CB_1-*Renilla Luciferase* (CB_1R-*RLuc*), A_1R-Yellow Fluorescence Protein (A_1R-YFP) and D_1-Yellow Flurescent Protein (D_1-YFP) were prepared and characterized as described elsewhere (Navarro et al., 2009). The cDNA encoding for dopamine $D_{4.4}$-YFP receptor was obtained and cloned as described previously (Gonzalez et al., 2012). HEK 293T cells were transiently transfected with the corresponding fusion protein cDNAs (see Figure legends) using the polyethyleneimine (PEI, Sigma, Steinheim, Germany) transfection protocol. Cells were incubated (4 h) with the corresponding cDNA, 5.47 mM (in nitrogen residues) PEI and 150 mM NaCl in a serum free DMEM medium (Invitrogen, Carlsbad, USA). After 4 hours, the medium was changed to a fresh complete culture DMEM. Forty-eight hours after transfection, cells were washed with HBSS containing 10 mM glucose, raised and centrifugated 5 min at 1500 g. To control the cell number, sample protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions as standards. To

quantify fluorescence proteins, cells (20 mg protein) were distributed in 96-well microplates (black plates with a transparent bottom) and fluorescence was read at 400 nm in a Fluo Star Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10 nm bandwidth excitation filter. Receptor fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing protein-Rluc alone. For BRET measurements, the equivalent of 20 mg of cell protein were distributed in 96-well microplates (Corning 3600, white plates; Sigma) and 5 mM coelenterazine H (Molecular Probes, Eugene, OR) was added. After 1 minute of adding coelenterazine H, the readings were collected using a Mithras LB 940, which allows the integration of the signals detected in the 485 nm-short- (440–500 nm) and the 530 nm-long-(510–590 nm) wavelength filters. To quantify receptor-Rluc expression, luminescence readings were performed after 10 minutes of adding 5 mM coelenterazine H. The BRET ratio was defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(long-wavelength emission) / (short-wavelength emission)] for the Rluc construct expressed alone in the same experiment.

A positive and saturable BRET signal was found for the pairs A_1YFP and CB_1*Rluc* (Figure 1A). From the saturation curve, a $BRET_{max}$ of 44.9 ± 3.7 mBU and $BRET_{50}$ of 13.1 ± 4.0 mBU were calculated. A positive and saturable BRET signal was also found for the pair CB_1*Rluc* and D_1YFP (Figure 1B) giving a $BRET_{max}$ of 38.2 ± 2.6 mBU and a $BRET_{50}$ of 22.5 ± 7.7 mBU. The negative control (CB_1*Rluc* and $D_{4.4}$YFP pair) gave a low nonspecific BRET signal.

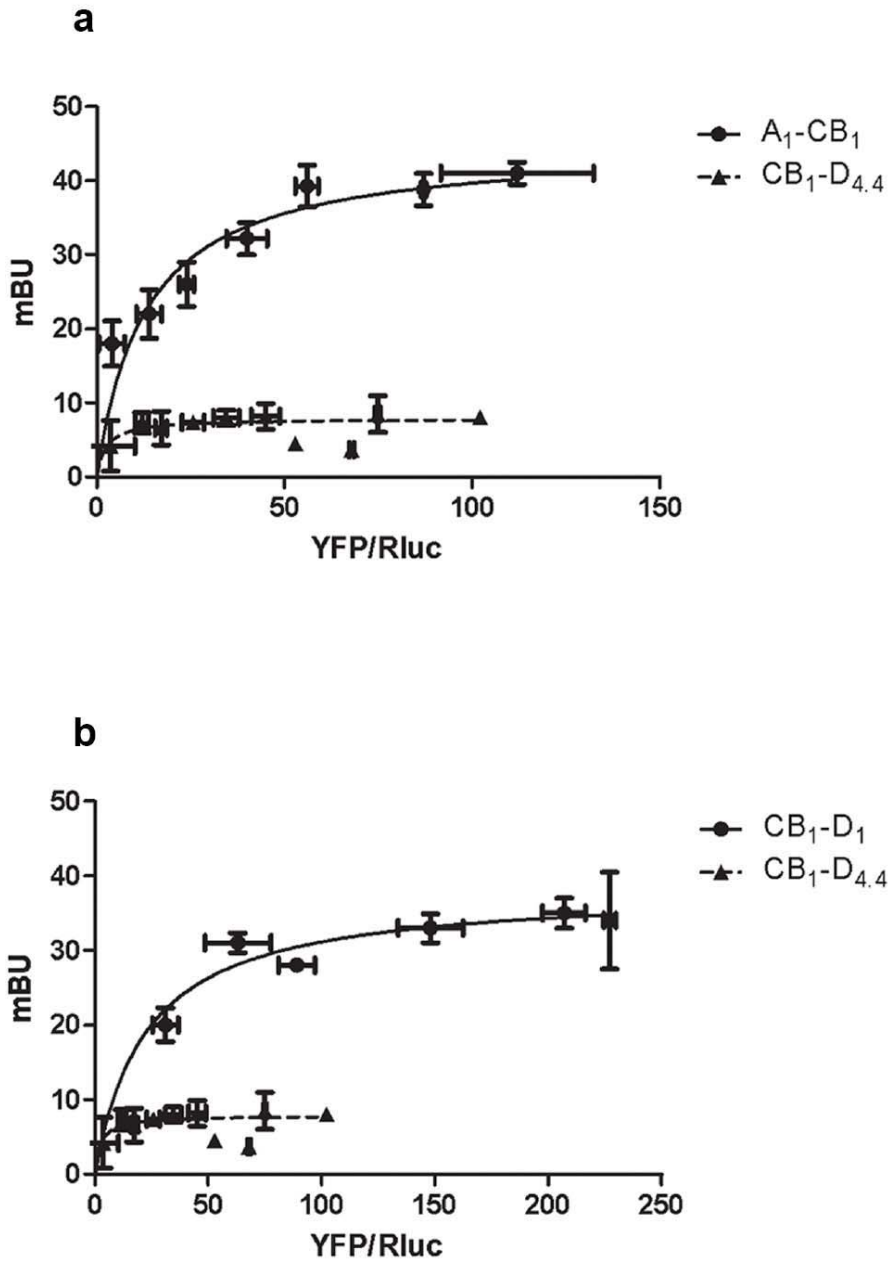
Here, applying the BRET technique in transfected HEK cells, we demonstrate, for the first time, that CB_1R can indeed physically interact with A_1R and D_1R. As all these three receptors play an important role in the striatum it can be possible that heteromers contribute to the complex control of the motor functions in this region. Further functional studies are needed to bring more insight into the nature of these complex intereactions and their pharmacological consequences.

Figure Legends

Fig.1. **A_1R-CB_1R and CB_1R-D_1R heteromers detected by BRET**. BRET saturation curves (full lines) were obtained using HEK-293 cells co-transfected with: (A) 0.3 μg of cDNA corresponding to CB_1*Rluc* (90,000 bioluminiscence units) and increasing amounts of cDNA corresponding to A_1YFP (0.1-1.1 μg; 500-10.000 fluorescence units) or (B) 0.2 μg of cDNA corresponding to CB_1*Rluc* (80.000 bioluminiscence units) and increasing amounts of cDNA corresponding to D_1YFP (0.1 -1.2 μg; 1000-20.000 fluorescence units). As negative control, cells were co-transfected with 1.0 μg of cDNA corresponding to CB_1*Rluc* and increasing amount (0.1-6 μg) of cDNA corresponding to $D_{4.4}$YFP (dashed line, 90.000 bioluminiscence units). BRET data are expressed as mili BRET Units (mBU) and is BRET ratio x 1000. Values are means ± S.D. of three different experiments grouped as a function of the amount of BRET acceptor. Curves were fitted by using a non-linear regression equation assuming a single phase, with the GraphPad Prism software.

Figures

Figure 1



SUMMARY OF RESULTS AND DISCUSSION

The G-protein-coupled receptors (GPCRs) are no longer considered as separate functional units, but as multimolecular complexes where they physically interact with each other as well as with other proteins in the horizontal and vertical plain of the membrane (Bouvier, 2001; Franco et al., 2003; Ferré et al., 2009; Albizu et al., 2006, 2010; Birdsall et al., 2010, Fuxe et al., 2010). Considering protein-protein interactions at the vertical plain respect to the membrane, adenosine A_1 (A_1R) and A_{2B} ($A_{2B}R$) receptors interact with the extracellular enzyme ADA (ecto-ADA). In the pharmacology of A_1R and $A_{2B}R$, ecto-ADA was found to be a potent positive allosteric modulator of agonist and antagonist binding to adenosine receptors that was not dependent of its enzymatic activity but only of its direct protein-protein interaction (Saura et al., 1996; Herrera et al., 2001). Recently a big interest lies in understanding of the capacity of adenosine A_{2A} receptors ($A_{2A}R$) to control the neurotransmission and synaptic plasticity in glutamatergic synapses due to the combined ability of these receptor to control the glutamate release and activate NMDA receptors in normal and pathological states (Cunha et al., 2008). Indeed, targeting $A_{2A}R$ was seen neuroprotective in some neurodegenerative diseases (Gomes et al., 2011). Thus, considering the importance of $A_{2A}R$ and the importance ADA in the cognate adenosine receptors' pharmacology, in this Thesis we first studied the molecular and functional interaction between ADA and $A_{2A}R$.

In the paper ***"A_{2A} adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase"*** here presented**,** we report that ADA could bind to plasma membrane of CHO cells transfected with $A_{2A}R$ but not to wild type CHO cells that do not express this receptor. This indicates that ADA is able to interact with $A_{2A}R$. It has previously been demonstrated by FRET and BRET techniques that $A_{2A}Rs$ form homomers and that homomers, but not monomers, appear to be the functional species at the cell surface of transfected cells (Canals et al., 2004). Thus, the quaternary structure of $A_{2A}Rs$ is constituted by, at least, two protomers that form a dimer. ADA binding to $A_{2A}Rs$ led to the modification of the quaternary structure of $A_{2A}R$ homodimers, since ADA induced an increase of energy transfer between $A_{2A}R$ protomers detected by BRET experiments (reflected by the increase in the $BRET_{max}$). It is interesting to note that this modification is of considerable magnitude,

since the binding of agonists to $A_{2A}R$ was not able to modify the BRET signal, as it was previously demonstrated by Canals et al. using a similar set up (Canals et al., 2004). Therefore the ability of BRET to detect ADA triggered conformational changes within the $A_{2A}R$ homomers suggests that ADA exerts a control of the function of $A_{2A}R$ homomers by a strong modification of their quaternary structure.

In order to investigate if the ADA-induced structural changes in the $A_{2A}R$ homomers correlates with modifications in the $A_{2A}R$ pharmacological properties, we performed radioligand binding assays with $A_{2A}R$ agonists and antagonists in the absence or presence of ADA. We demonstrated that ADA significantly decreases the dissociation equilibrium constants of $A_{2A}R$ agonists and antagonists. This general increase of affinity induced by ADA was independent of its enzymatic activity and indicates that ADA behaves as an allosteric ligand. In fact, in addition to orthosteric sites, many GPCRs have been found to possess structurally distinct allosteric domains. One characteristic feature of the allosteric interaction is that the receptor is able to simultaneously bind an orthosteric and an allosteric ligand, introducing complexity into pharmacological responses by modifying the affinity or the signal imparted by the orthosteric ligand (May et al., 2007). An allosteric effect results in a positive modulation if the modulator facilitates the interaction, or in a negative modulation if it inhibits the interaction of the ligand with the orthosteric-binding site (May et al., 2007; Conn et al., 2009). According to these concepts, ADA is an allosteric ligand of $A_{2A}Rs$ that positively modulates the agonist and antagonist binding to the orthosteric site of the receptor. The existence of $A_{2A}R$ allosteric ligands has been already reported. Kreth et al. (Kreth et al., 2009) have shown that a certain endogenous allosteric modulator led to a reduced ligand affinity and to an impaired function of human granulocytes $A_{2A}R$ in sepsis. Furthermore, some compounds have been synthesized and evaluated as positive enhancers of agonist and antagonist radioligands for the neuronal $A_{2A}R$ (van den Nieuwendijk et al., 2004; Gao et al., 2005). $A_{2A}Rs$ are also allosterically modulated by sodium ions binding to an allosteric site linked to Glu13 in transmembrane domain 1 (TM1) and His278 in TM7, and by the potassium-sparing diuretic amiloride (Gao et at., 2000, 2005; Göblyös et al., 2011).

Having demonstrated the positive effects of ADA upon the $A_{2A}R$ agonists binding, we wanted to study if this improved $A_{2A}R$ pharmacology had also an improved functional outcome. To test this, we developed a stable CHO cell line expressing human $A_{2A}R$ receptor and analyzed signal transduction by observing the effect of ligands and ADA on MAPK (mitogen-activated protein kinase) phosphorylation. Indeed, the presence of ADA significantly increased the ERK 1/2 phosphorylation in accordance with the previously indicated increase in the agonist CGS-21680 binding affinity.

The new discovered ability of allosteric modulators to fine-tune pharmacological responses has sparked interest in their potential applications in both clinical and basic science settings (Conn et al., 2009). This interest is the most relevant in the case of neurotransmitter receptor targets due to the fact that synaptic neurotransmission occurs in extremely complex circuits implicated in many neurological functions. Owing to the implication of $A_{2A}Rs$ in many neurodegenerative diseases, such as Parkinson's and Huntington's disease, obsessive-compulsive disorders and drug addiction (Stone et al., 2009), different approaches have been tested to find allosteric modulators, i.e. a structure-based ligand discovery methodology provided new routes for modulation of this neuronal key target (Stone et al., 2009; Cristalli et al, 2008; Carlsson et al., 2010; Katritch et al., 2010). Conceptually, the allosteric interaction described in the present study is different from the one exerted by small molecules since it comes from the interaction across the membrane with a protein that has an extracellular topology. By means of the interaction with an extracellular domain of $A_{2A}Rs$, ADA exerts a fine-tune modulation of adenosine neuroregulation that may have important implications for the function of neuronal $A_{2A}Rs$, which are enriched in and play a key role in the striatum. The presence of ADA bound to the cell surface of neurons has been demonstrated (Ruiz et al., 2000), reinforcing the concept that this allosteric effect of ADA is likely to occur *in vivo*. With this in mind one may hypothesize that ADA SCID patients with ADA mutations affecting the binding of ADA to $A_{2A}R$ may manifest neurological alterations that are predicted to be different from those resulting from mutations not affecting the ADA–$A_{2A}R$ interface. Probably, mutations affecting the interaction would be less deleterious for striatal function since it would attenuate the over-activation of $A_{2A}R$ exerted by the elevated adenosine levels.

In summary, the results described in this study show that ADA, apart from reducing the adenosine concentration, binds to $A_{2A}R$ behaving as an allosteric effector that markedly enhances agonist-induced signalling throught to be the MAPK pathway, increasing ERK 1/2 phosphorylation. Thus, the physiological role of the ADA–adenosine receptor interaction is of a great importance because it makes those receptors more functional.

The synaptic transmission controled by GPCRs operating via their binding to neurotrasmitters is extremely complex and it is not only regulated by neurotrasmitters receptors intereacting proteins, i.e. previously mentioned ADA, but, interestingly, also by interacting with other receptors forming receptor heteromers. The existence of heteromers between receptors for different neurotrasmitters or neuromodulators has already become a generally accepted fact in the scientific community. This acceptance brought changes in the way of understanding the neurotransmission and its complex control in different parts of the brain. Importantly, receptor heteromerization confers diverse biochemical properties to the heteromer that are different to those of its individual components, as changes in their functionality and pharmacology (Terrillon et al., 2004, Ferré et al 2009). If receptors are expressed as heteromers, heteromers but not monomers must be considered as the actual targets in drug discovery. This novel approach to GPCRs' biochemistry opened a big field of new possibilities in the search of more effective compounds applicable in the treatment of neurological diseases. At the beginning of this Thesis it was known that $A_{2A}R$ formed heteromers with dopamine D_2 receptors (D_2R, Hillion et al., 2002), A_1R (Ciruela et al., 2006) or cannabinoid CB_1 receptors (CB_1R, Carriba et al., 2008) and that these heteromers were differentially expressed in different synaptic domains in the striatum and that played a different role in motor control. In the striatum, in the corticostriatal synapse formed by glutamatergic cortical efferents and GABAergic medium spiny neurons (MSN), $A_{2A}R$ heteromerize with A_1R at the presynaptic level in the direct pathway and with D_2R at the postsynaptic level in the indirect pathway (Ciruela et al., 2006; Hillion et al., 2002; Quiroz et al. 2009). In conditions of higher extracellular concentrations of adenosine, as can happen either in higher neuronal activation in normal conditions or in several pathological conditions (e.g. ischemia, Huntington's disease (HD), the presynaptic $A_{2A}R$ activation increases glutamate

release. This leads to increase of excitatory output from basal ganglia and increases the motor functions. In addition, high concentrations of glutamate can lead to excitotoxity, which is considered as one of the possible causing mechanisms HD. In HD the over-activation of the direct pathway leads to choreic movements. Thus, to block the presynaptic $A_{2A}R$ function seems twice important: to reduce the movement and to reduce excitotoxicity. On the other hand, activation of the postsynaptic $A_{2A}R$ found in indirect pathway also potentiates the neurotransmission but here with a consequence of motor depression. Inhibition of postsynaptic $A_{2A}R$ would therefore lead to motor activation and worsen the choreic movements. Thus, in HD investigation, an important branch of research focused on the assessment of possible beneficial effects of $A_{2A}R$ agonists and antagonists considering these receptors as individual entities meanwhile the possibility that a selected $A_{2A}R$ heteromer could play a role in the disease has never been considered up to now. Since it was previously discovered in our group that caffeine, a natural adenosine receptors antagonist, showed significantly different affinity in different $A_{2A}R$ heteromers, we decided to explore a set of synthetic $A_{2A}R$ antagonist with hope to find preferences in their binding affinities for $A_{2A}R$ forming heteromers with A_1R and D_2R. In the paper ***"Striatal Pre- and Postsynaptic Profile of Adenosine A_{2A} Receptor Antagonists"*** we chose to evaluate six compounds already known as selective $A_{2A}R$ antagonists: MSX-2, SCH-420814, SCH-442416, SCH-58261, KW-6002, and ZM-241385 for their presynaptic *versus* postsynaptic affinity.

First, changes in rat locomotion, distance travelled, were measured after the subcutaneous (s.c.) or intraperitonal (i.p) systemic application of $A_{2A}R$ antagonist. Increase of locomotions reflected the potency of postsynaptic action of $A_{2A}R$ antagonists. Second, to measure the presynaptic action of $A_{2A}R$ antagonists, we performed "power correlation coefficient (PCC) reduction assays" consisted in measuring electromyographic (EMG) recordings upon motor cortical stimulation. The rats were again administered s.c. or i.p. with $A_{2A}R$ antagonists and were implanted with stimulation electrodes into one region of motor cortex and recording electrodes were implanted in mastication muscles. The stimulation current intensity was adjusted to the threshold level to an intensity allowing at least 95 % of the stimulation pulses to elicit a positive EMG response. Positive EMG

responses always matched observable small jaw movements. A power correlation analysis was used to quantify the correlation between the stimulation pulses of current delivered into the orofacial motor cortex (input signal; mA) and the elicited EMG response in the jaw muscles (output signal; mV). Decrease in PCC between these two signals is meant to describe a decrease in the efficacy of the transmission in the neural circuit, in other words the potency of presynaptic action of the $A_{2A}R$ antagonist. Applying these two protocols, two compounds from the tested $A_{2A}R$ antagonists: SCH-442416 and KW-6002, showed preferential pre- and postsynaptic profiles, respectively, and four compounds: MSX-3, SCH-420814, SCH-58261 and ZM-241385, showed mixed pre-postsynaptic profiles. In the third protocol, additional *in vivo* evaluation of presynaptic activity of the two preselected compounds, SCH-442416 and KW-6002, was considered, combining *in vivo* microdialysis with cortical electrical stimulation. The ability to counteract the glutamate release was measured as follows. The rats were implanted microdialysis probe for injection of $A_{2A}R$ antagonists. After 20 min from drug or vehicle injection, electrical stimulation pulses were applied through the stimulation electrodes and dialysate samples were collected at 20-min intervals and glutamate content was measured by reverse-phase HPLC. The ability to counteract the glutamate release is another way to reflect the potency of presynaptic action of the $A_{2A}R$ antagonist.

The results obtained from all of these evaluations were in agreement in that KW-6002 showed preferential postsynaptic profile while SCH-442416 showed preferential presynaptic profile. This last antagonist significantly counteracted striatal glutamate release induced by cortical stimulation at a dose (1 mg/kg i.p.) that strongly reduced PCC but did not induce locomotor activation. On the other hand, KW-6002 did not modify striatal glutamate release induced by cortical stimulation at a dose (1 mg/kg i.p.) that produced a pronounced locomotor activation but did not counteract PCC. Our results on MSX-3 compoud corroborate with a previously published study which reported that its intrastriatal perfusion almost completely counteracted striatal glutamate release induced by cortical electrical stimulation (Quiroz et al., 2009), a fact that agrees with its very effective reduction of PCC shown in our study.

One important hypothesis of our study was that at least part of these pharmacological differences between $A_{2A}R$ antagonists might be explained by the ability of pre- and postsynaptic $A_{2A}R$ to form different receptor heteromers, with A_1R and D_2R, respectively (Ferre et al., 2009; Quiroz et al., 2009; Azdad et al., 2009, Ciruela et al., 2006: Ferre et al, 2007). To investigate this hypothesis, we created a series of stable CHO (Chinese hamster ovary) cell lines expressing $A_{2A}R$, A_1R, or D_2R or expressing A_1R-$A_{2A}R$ or $A_{2A}R$-D_2R heteromers. With the best selected cellular lines, radioligand binding experiments were performed in order to determine possible differences in the affinity of these compounds for $A_{2A}R$ present in A_1R-$A_{2A}R$ and $A_{2A}R$-D_2R heteromer in comparison with $A_{2A}R$ not forming heteromers.

Concerning the behaviour of $A_{2A}R$ in the presynaptic A_1R-$A_{2A}R$ heteromer, the co-expression of adenosine $A_{2A}R$ with adenosine A_1R did not significantly modify the affinity of $A_{2A}R$ for the different ligands, meanwhile looking at the postsynaptic $A_{2A}R$-D_2R heteromer, the co-expression of $A_{2A}R$ with D_2R decreased the affinity of all compounds, with the exception of KW-6002. The structural changes in the $A_{2A}R$ induced by heteromerization with the D_2R could be detected not only by antagonists but also by agonists. Indeed, the affinity of the selective $A_{2A}R$ agonist CGS-21680 was also reduced in cells co-expressing D_2R. When trying to explain the differential action of SCH-442416 observed *in vivo*, it is interesting to note that SCH-442416 showed a much higher affinity for the $A_{2A}R$ in a presynaptic-like than in a postsynaptic-like context. The binding of SCH-442416 to the $A_{2A}R$-D_2R heteromer displayed a strong negative cooperativity, phenomenon that was not observed for the binding of SCH-442416 to the A_1R-$A_{2A}R$ heteromer, and was visually apparent as a biphasic displacement curve in the radioligand binding assays. The negative cooperativity detected in SCH-442416 binding to the $A_{2A}R$-D_2R heteromer explains the pronounced decrease in affinity of $A_{2A}R$ in cells expressing $A_{2A}R$-D_2R heteromers (B_{50} values 40 times higher in cells expressing $A_{2A}R$-D_2R than A_1R-$A_{2A}R$ heteromers).

Although the other evaluated compounds did not shown negative cooperativity, a loss of affinity was observed. The loss of affinity of $A_{2A}R$ upon co-expression of D_2R was much less pronounced for ZM-241385, SCH-58261, MSX2 or SCH-420814, for

which the affinity was reduced from two to about nine fold. Taking into account that these $A_{2A}R$ antagonists behave similarly as the $A_{2A}R$ agonist CGS-21680 in terms of binding to A_1R-$A_{2A}R$ and $A_{2A}R$-D_2R heteromers, it is expected that these four compounds compete equally for the binding of the endogenous agonist at pre- and at postsynaptic sites. This would fit with the *in vivo* data, which show that these compounds have a non-preferred pre-postsynaptic profile. Yet, KW-6002 was the only antagonist whose affinity was not significantly different in cells expressing $A_{2A}R$ or A_1R-$A_{2A}R$ or $A_{2A}R$-D_2R heteromers. Thus, KW-6002 showed the best relative affinity for $A_{2A}R$-D_2R heteromers of all compounds, which can at least partially explain its preferential postsynaptic profile.

Taking all into account, the present results support the notion that receptor heteromers may be used as selective targets for drug development. Main reasons are the very specific neuronal localization of receptor heteromers (even more specific than for receptor subtypes), and a differential ligand affinity of a receptor depending on its partner (or partners) in the receptor heteromer. In the striatum, $A_{2A}R$ provides a particularly interesting target, eventually useful for a variety of neuropsychiatric disorders. $A_{2A}R$-D_2R and A_1R-$A_{2A}R$ heteromers are segregated in different striatal neuronal elements. While $A_{2A}R$-D_2R heteromers are located postsynaptically in the dendritic spines of the indirect MSNs (Ferré et al., 2009; Quiroz et al., 2009; Azdad et al., 2009; Ferré et al., 2007), A_1R-$A_{2A}R$ receptor heteromers are located pre-synaptically in glutamatergic terminals contacting the MSNs of the direct pathway (Quiroz et al., 2009; Ferre et al., 2007; Ciruela et al., 2006). Blocking postsynaptic $A_{2A}R$ in the indirect MSN should potentiate D_2R-mediated motor activation, which is a strategy already used in the development of anti-parkinsonian drugs (Jenner, 2003; Stacy et al., 2008). However, blocking $A_{2A}R$ in glutamatergic terminals to the direct MSN could potentially be useful in dyskinetic disorders such as Huntington's disease and maybe in obsessive-compulsive disorders and drug addiction (Quiroz et al., 2009). The present results give a mechanistic explanation to the already reported antiparkinsonian activity of KW-6002 (Kaasinen et al., 2000; Stacy et al., 2008) and suggest that SCH-442416 could be useful in dyskinetic disorders, obsessive-compulsive disorders and in drug addiction. Medicinal chemistry and computerized modeling should help understanding the molecular properties that determine the

particular pharmacological profile of SCH-442416 and KW-6002, which may be used as lead compounds to obtain more effective antidyskinetic and antiparkinsonian compounds, respectively.

When trying to target $A_{2A}R$ in the striatum, also cannabinoid CB_1 receptors (CB_1R) must be considered for two reasons. One, CB_1Rs are the most abundant pre- and postsynaptic neutrotransmitter receptors in the brain (Katona et al., 2006) and the other, CB_1R and adenosine $A_{2A}R$ form heteromers in co-transfected cells and rat striatum (Carriba et al., 2007). The newly emerging $A_{2A}R$ antagonists potentially useful in PD or HD should be analysed in detail looking at the possible pharmacological modulation of $A_{2A}R$ by CB_1R to bring more insight to their future application. Thus, in the last part of this Thesis, in the work: **"*Pharmacological and functional characterization of adenosine A_{2A}-cannabinoid CB_1 receptor heteromers*"** we wanted to investigate if the presence of CB_1R can modulate the pharmacology and functionality of $A_{2A}R$.

CB_1R can form heteromers with dopamine and adenosine receptors. We studied the interactions of CB_1R with dopamine D_1 and adenosine A_1 receptors by the technique of BRET and we saw that A_1-CB_1 and CB_1-D_1 receptor heteromers were formed in transfected HEK cells (see Supplement II). As already mentioned above our group previously reported that CB_1R and $A_{2A}R$ form heteromers in co-transfected cells and rat striatum where they co-localize in fibrilar structures (Carriba et al., 2007). We focused on this last pair because, although it was known that activation of $A_{2A}R$ was necessary for CB_1 receptor signalling in a human neuroblastoma cell line where the heteromers were expressed (Carriba et al., 2007), the pharmacological and functional characteristics of these heteromers were not completely studied. In this paper by measuring G-protein activation by the CellKey label-free assay, we demonstrated that $A_{2A}R$ and CB_1R are coupled to G_i protein in the heteromer. We also observed a synergistic cross-talk in G-protein activation when both receptors are co-activated but it is mainly the CB_1R controlling the ERK 1/2 signalling under the heteromer. Although we demonstrated that there is not an allosteric effect on ligand binding for $A_{2A}R$-CB_1R heteromers, we found that two specific $A_{2A}R$ antagonist, KW-6002 and VER-7835 lost affinity for $A_{2A}R$ when expressed in $A_{2A}R$-CB_1R heteromers.

Changes of biochemical properties of one receptor in the heteromer can be simply dependent on the presence of the other receptor unit or on co-stimulation of the two receptor units in the heteromer. Changes in G-protein coupling induced only by the presence of the partner receptor are a common characteristic of neurotransmitter receptor heteromers. In opioid receptor δOR–μOR heteromer, the receptor units in the heteromer couple to G-proteins other than those usually associated with the individually expressed receptors. Thus, signalling by stimulating the receptor units in the δOR–μOR heteromer (which causes inhibition of adenylate cyclase) is not sensitive to pertussis toxin, suggesting a G-protein switch from G_i to G_z (George et al., 2000, Levac et al., 2000). There are also examples of changes in G-protein coupling that are dependent on co-activation of both receptors in the receptor heteromer. For example, dopamine D_2 receptor normally couples to G_{i-o} proteins, but in the dopamine D_1R–D_2R heteromer it switches to $G_{q/11}$ when D_1R is co-activated. In this way, the D_1R–D_2R heteromer provides a selective mechanism by which dopamine activates phospholipase C-mediated calcium signalling (Rashid et al., 2007). Here we demonstrated that $A_{2A}R$, coupled to G_s protein when expressed alone, switches to G_i protein in $A_{2A}R$-CB_1R heteromers in the absence or in the presence of CB_1R agonists. Thus $A_{2A}R$-CB_1R heteromer provides a selective mechanism by which cannabinoid receptor blocks the $A_{2A}R$-mediated cAMP production.

Frequently, activation of one receptor unit in the heteromer implies intermolecular cross-talk involving conformational changes sensed by the other receptor unit in the heteromer. These conformational changes lead to modulation of ligand binding and/or signalling of the partner receptor. In some cases, stimulation of one receptor unit decreases the affinity and signalling of the other receptor unit as it has been described for adenosine A_1–A_{2A} and adenosine A_{2A}–dopamine D_2 receptor heteromers, that show antagonistic allosteric interactions and a negative cross-talk (Ferré 1991, Hillion et al., 2002, Canals et al., 2003, Ciruela et al., 2006). In other cases stimulation of one receptor unit increases the signalling and the affinity of the other receptor unit for endogenous or exogenous ligands as occurs for dopamine D_2–somatotatine SST5 receptor heteromer in which stimulation of D_2Rs significantly increases the affinity of SST5Rs for agonists (Rocheville et al., 2000). For the $A_{2A}R$-CB_1R

heteromers, ligand binding to CB_1R did not modify the ligand binding to $A_{2A}R$ and *vice versa*, indicating a lack of allosteric interactions for this heteromer. However, a synergistic increase in G_i protein activation was observed when both receptors were co-activated. This is in accordance with the fact that activation of $A_{2A}Rs$ was necessary for CB_1R signalling in neuroblastoma cell line previously described (Carriba et al., 2007). Our results imply a processing of information, at the membrane level, of the signals impinging on the $A_{2A}R$-CB_1R heteromers. In this case, the neurotransmitter receptor heteromer functions as a processor of computations that modulates G-protein-mediated signalling because quantitative or qualitative aspects of the signaling generated by stimulation of either receptor unit in the heteromer are different from those obtained during co-activation.

Apart from G-protein-mediated signalling, many GPCRs are able to signal in a G-protein-independent way (Shenoy et al., 2003, 2006; Beaulier et al. 2005; DeWire et al., 2007; Valjent et al., 2000). ERK 1/2 phosphorylation is one of the MAPK pathways that has been described to be activated in a G-protein-independent and arrestin-dependent mechanism (DeWire et al., 2007). Looking at the ERK 1/2 phosphorylation, when both receptors in the $A_{2A}R$-CB_1R heteromers are co-activated it seems that the CB_1R controls the ERK 1/2 signalling under the heteromer. Since ERK 1/2 phosphorylation is related to plasticity (Shiflett et al., 2011) it seems that cannabinoids control changes in ERK 1/2-mediated plasticity in cells where the heteromers are expressed.

As discussed above, it has been hypothesized that postsynaptic $A_{2A}R$ antagonists should be useful in Parkinson's disease, while presynaptic $A_{2A}R$ antagonists could be beneficial in dyskinetic disorders, such as Huntington's disease, obsessive-compulsive disorders and drug addiction (Orru et al., 2011a, 2011b; Blum et al., 2003; Armentero et al., 2011). Thus, it seems that to look for the role of CB_1R expression on the affinity of the $A_{2A}R$ antagonists that we previously found to be pre- and postsynaptically selective, SCH-442416 and KW-6002, can have relevance for therapeutical purpose. We chose the A_{2A} antagonists SCH-442416 and KW-6002 and also VER-7835, which probably does not discriminate between both receptor localizations (unpublished results shown in the Supplement I), and performed radioligand competition experiments.

We found that SCH-442416 affinity was similar for $A_{2A}R$ and $A_{2A}R$-CB_1R heteromers suggesting that both can be blocked presynaptically by SCH-442416 and indicating that $A_{2A}R$-CB_1R heteromers must be considered to analyse the antidyskinetic effects of SCH-442416. Interestingly, we found a decrease in affinity for KW-6002 or VER-7835 to bind $A_{2A}R$-CB_1R heteromers compared to $A_{2A}Rs$. It seems that at postsynaptic level KW-6002 or VER-7835 would block A_{2A} receptors more selectively than $A_{2A}R$-CB_1R heteromers and this has to be taken into account to analyse the antiparkinsonian effect of KW-6002.

In summary, $A_{2A}R$-CB_1R heteromers facilitate the understanding of the role of interactions between adenosine and cannabinoids in the brain, since $A_{2A}R$-CB_1R heteromers act as processors of computations that modulate cell signalling and can be a therapeutical target relevant in neurological diseases involving striatal neurons.

CONCLUSION

Conclusions derived from aim 1

- Adenosine A_{2A} receptor acts as a membrane anchoring protein of ADA. ADA binds to A_{2A} receptor homomers and induces a strong modification of their quaternary structure.

- ADA was found to be an allosteric ligand of human adenosine A_{2A} receptors. Irrespective of its enzymatic activity, ADA positively modulated the agonist and antagonist binding to the orthosteric site of the receptor. In addition, ADA markedly enhances A_{2A} receptor signalling, increasing the A_{2A} receptor agonist-induced ERK 1/2 phosphorylation.

- In summary, this powerful regulation exerted by ADA might have important implications in the physiology and pharmacology of neuronal A_{2A} receptors that are implicated in the striatal motor regulation.

Conclusions derived from aim 2

- Based on *in vitro* and *in vivo* approaches, the A_{2A} receptor antagonists ZM-241385, MSX-2, SCH-420814, and SCH-58261 showed no clear presynaptic or postsynaptic preference.

- Based on *in vitro* and *in vivo* approaches, the compound SCH-442416 was classified as a preferential presynaptic A_{2A} receptor antagonist, and the compound KW-6002 was classified as a preferential postsynaptic A_{2A}R antagonist.

- The physical presence of dopamine D_2 receptor in the A_{2A}-D_2 receptor heteromer induced a strong negative cooperativity in the A_{2A} receptors that was detected by SCH-442416. This cooperativity indicates that A_{2A}-A_{2A} receptor homodimers are present in the A_{2A}-D_2 receptor heteromers.

- In summary, on the basis of their preferential pre- versus postsynaptic actions, SCH-442416 can be used as a lead compound in the development of antidyskinetic drugs in

Huntington's disease, meanwhile KW-6002 can be beneficial in Parkinson's disease.

Conclusions derived from aim 3

- Adenosine A_{2A} receptor changes its G-protein coupling from stimulatory G_s to inhibitory G_i when it forms heteromer with CB_1 receptor and a synergistic cross-talk in G-protein activation is observed when both receptors are co-activated.

- CB_1 receptor mainly controls the ERK 1/2 signalling under the A_{2A}-CB_1 receptor heteromer.

- The A_{2A}-CB_1 receptor heteromers do not show allosteric effects at the ligand binding level.

- The two specific A_{2A} receptor antagonist, KW-6002 and VER-7835 lost affinity for A_{2A} receptors when expressed in A_{2A}-CB_1 receptor heteromers.

- In summary, it is shown that A_{2A}-CB_1 receptor heteromers constitute a singular unit for adenosine and cannabinoids signalling, introducing diversity in A_{2A} receptor signalling that can be therapeutically relevant in neurological diseases involving striatal neurons.

ANNEX

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Production of functional recombinant G-protein coupled receptors for heteromerization studies

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ABSTRACT

G-protein-coupled receptors (GPCRs) represent a diverse protein family of receptors that transduce signals from the extracellular surrounding to intracellular signaling molecules evoking various cellular responses. It is now widely accepted that GPCRs are expressed and function as dimers or most probably as oligomers of more than two receptor protomers. The heteromer has different biochemical and pharmacological characteristics from the monomers, which increases the functional responses of GPCRs. GPCRs are involved in many diseases, and are also the target of around half of all modern medicinal drugs. In the case of Parkinson's disease, a degenerative process caused by gradual disappearance of dopaminergic nigrostriatal neurons, it is suspected that the targets for treatment should be dopamine-receptor-containing heteromers. Technologies based on the use of fluorescent- or luminescent-fused receptors and adaptations of resonance energy transfer (RET) techniques have been useful in investigating the functional inter-relationships between receptors in a heteromer. In this study functional recombinant adenosine A_{2A}-Rluc, dopamine D_2-GFP[2] and histamine H_3-YFP receptor fusion proteins were successfully cloned and characterized, producing the essential basis for heteromerization studies between these receptors. This might provide a better insight into their pharmacological and functional inter-relationships in the brain and enable the design and evaluation of new therapeutic strategies for Parkinson's disease.

1. Introduction

G-protein-coupled receptors (GPCRs) represent a diverse protein family of receptors that transduce signals from the extracellular surrounding to intracellular signaling molecules evoking various cellular responses. The chemical diversity of the ligands that bind and activate GPCRs is exceptional and they stimulate cytoplasmic and nuclear targets through heterotrimeric G-protein-dependent and independent pathways. Since the early 1980s, experimental data has accumulated suggesting that GPCRs may be expressed and function as dimers or most probably as oligomers of more than two receptor protomers (Fuxe et al., 2008). Both homo- and heteromers were found in a variety of studies (Bulenger et al., 2005). The heteromers have different biochemical and pharmacological characteristics from the monomers (Ferre et al., 2007), which considerably increases the possible functional responses of GPCRs affecting all aspects of receptor physiology and pharmacology.

G protein-coupled receptors are involved in many diseases, but are also the target of around half of all modern medicinal drugs (Gilchrist, 2010). Nevertheless, currently the developed drugs target only a small number of GPCRs, and the potential for drug discovery within this field is enormous. The discovery of physiologically relevant GPCR heteromers suggested that new, more selective, drugs can be developed by targeting the heteromers instead of the monomers thus increasing the breadth and depth of receptors available for therapeutic interventions. Such "designer" drugs currently include allosteric regulators, inverse agonists, and drugs targeting hetero-oligomeric complexes (Panetta and Greenwood, 2008).

When considering the nervous system, the existence of heteromers of neurotransmitter GPCRs contributes to the high degree of plasticity characteristic for such a highly organized and complex system. Neurotransmitter receptors are no more considered as single functional units, but as forming part of multimolecular aggregates localized in the plane of the plasma membrane which can contain other interacting proteins (Franco et al., 2007). In the case of Parkinson's disease, a degenerative process caused by a gradual disappearance of the dopaminergic nigrostriatal neurons, it is suspected that the real targets for treatment should be dopamine-receptor-containing heteromers, among which

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adenosine receptors have been extensively studied (Franco, 2009). At present there are different clinical trials in which synthetic A_{2A} receptor ($A_{2A}R$) antagonists are under evaluation, since there is a functional antagonism between $A_{2A}R$ and dopamine D_2 receptor (D_2R) in the striatum (Canals et al., 2003). Also, histamine H_3 receptor (H_3R) has been proposed as a promising candidate (Leurs et al., 2005; Ferrada et al., 2008; Moreno et al., 2011).

To fully appreciate the contribution of these heteromers to normal physiology of the brain and use them for selective drug targeting, it is necessary to investigate the functional inter-relationships between the receptors in a heteromer. Technologies based on the use of fluorescent-fused proteins and different adaptations of resonance energy transfer (RET) techniques have been very useful. RET consists of a nonradiative (dipole–dipole) transfer of energy from a chromophore in an excited state fused to receptor A (known as the "donor"), to a chromophore fused to receptor B (the "acceptor"). This results in reduction of the donor emission and a consequent increase of fluorescence emission by the acceptor. In fluorescence RET (FRET) the molecules are fluorescent, whereas in bioluminescence RET (BRET) the donor is a bioluminescent enzyme which excites the acceptor fluorophore in the presence of a substrate. If two receptors are positioned at a distance beneath 10 nm (i.e., form a heteromer) effective energy transfer can occur. To evaluate the existence of higher-order oligomers, a sequential BRET–FRET technique, called SRET, to identify heteromers formed by the physical interaction of three different proteins in living cells has recently been developed (Carriba et al., 2008).

GPCRs oligomerization is difficult to analyze in native cells, therefore, many cell lines in which receptor proteins can be efficiently expressed have been widely used as an accepted model. Cloning different GPCRs into fluorescent or luminescent vectors enables their easy detection and tracking, as well as the employment of various techniques for investigation of receptor heteromerization at the intramembrane (RET, radioligand binding. . .) and intracellular signaling level (cAMP, ERK. . .).

The aim of this study was the production and characterization of recombinant $A_{2A}R$-Rluc, D_2R-GFP^2 and H_3R-YFP receptors, which would in the future be used to provide better insight into their pharmacological and functional inter-relationships in the brain, and lead to the design of new drugs for the treatment of Parkinson's disease.

2. Materials and methods

2.1. Preparation of vectors and insert DNA

Renilla luciferase expressing vector pRluc-N1 and Green Fluorescent Protein 2 expressing vector pGFP^2-N3(h) were obtained from PerkinElmer, Boston, MA. The Enhanced Yellow variant of green Fluorescent Protein vector pEYFP-N1 was obtained from Clontech, Heidelberg, Germany. The lyophilized plasmid DNAs were reconstituted according to manufacturer's instructions.

The cDNA of human $A_{2A}R$ from a host plasmid pcDNA3.1 (10 ng/μL) was amplified without its stop codon using sense and antisense primers (10 μM) harboring EcoRI and BamHI sites, to be in-frame with Rluc in the pRluc-N1 vector. The cDNA of human D_2R from pcDNA3.1 (10 ng/μL) was amplified without its stop codon using sense and antisense primers (10 μM) harboring HindIII and KpnI, to be in-frame with GFP^2 in the pGFP^2-N3(h) vector. The cDNA of human H_3R from a host plasmid (Johnson & Johnson Pharmaceutical Research & Development, L.L.C., San Diego, CA, USA) was amplified without its stop codon using sense and antisense primers harboring EcoRI and BamHI, to be in frame with eYFP in the pEYFP-N1 vector. The obtained PCR products were analyzed by electrophoresis on a 1% agarose gel, and the detected bands were purified using PCR clean-up/gel extraction NS® Extract II kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany).

2.2. Cutting and ligation

The purified PCR products and target vectors were cut in separate tubes by the appropriate restriction enzymes, i.e., $A_{2A}R$ and pRluc-N1 with EcoRI and BamHI, D_2R and pGFP^2-N3(h) with HindIII and KpnI, and H_3R pEYFP-N1 with BamHI and EcoRI. The products were run on a 1% agarose gel with 2 mM guanosine. The obtained linear vectors and cDNAs were extracted from the gel using PCR clean-up/gel extraction NS® Extract II kit, and their concentrations were determined using NanoDrop spectrophotometer (ThermoFisher Scientific). Typically 100 ng of target vector DNA and a 3 to 6-fold molar excess of inserts were used with 100 IU of T4 DNA ligase (Promega, WI, USA) in a total volume of 10 μL in an overnight temperature gradient according to the manufacturer's recommendation.

2.3. Bacterial transformation

The ligation mixture was used to transform 100 μL of Dh5α chemically competent cells (Invitrogen, Paisley, UK). The tubes were incubated on ice for 30 min, heat shocked at 42 °C for 45 s and then returned to ice. After 5 min, 900 μL of Luria-Bertani (LB) medium was added to each transformation mixture and the tubes were placed in an incubator at 37 °C for 75 min, with shaking. After centrifugation (2500 rpm 2 min) the supernatant was discarded and 100 μL of fresh LB added to dissolve the pellet. The cultures were spread onto LB plates containing appropriate antibiotics (for pRluc-N1 kanamycin 100 μg/mL, for pGFP^2-N3(h) zeocin 25 μg/mL and for pEYFP-N1 kanamycin 100 μg/mL) and incubated at 37 °C overnight.

2.4. Plasmid DNA preparation

Several colonies were picked from each LB plate, and 5 mL of LB solution containing appropriate antibiotics was inoculated with a single colony in separate 15 mL falcons. The suspensions were grown at 37 °C for 6 h with shaking, then transferred to 250 mL of LB containing appropriate antibiotics in erlenmayers and further grown at 37 °C overnight with shaking. The next day, the suspensions were centrifuged for 20 min at 7500 rpm at room temperature, and plasmid DNA was isolated from the pellet using PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen, Paisley, UK). After obtaining DNA in water solution, the samples were quantified, aliquoted and stored at −20 °C for further use.

All constructs were verified by restriction digestion and positive samples were sequenced to confirm the correct insertion of cDNA into the vectors.

2.5. Cell culture

Human embryonic kidney cells (HEK-293T, American Type Tissue Culture, Manassas, VA) and Chinese hamster ovary cells (CHO, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Paisley, UK) and in minimum essential medium (αMEM, Gibco, Paisley, UK), respectively, supplemented with 2 mM L-glutamine, 100 UI/mL penicillin/streptomycin and 5% (v/v) heat-inactivated fetal bovine serum (FBS) (all supplements were from Invitrogen, Paisley, UK). Cells were maintained at 37 °C in an atmosphere of 5% CO_2, and were passaged twice a week.

2.6. Transient transfection

Cells were grown in six-well dishes or 25 cm^2 flasks to 80% confluence and were transiently transfected with cDNA of $A_{2A}R$, $A_{2A}R$-Rluc, D_2R, D_2R-GFP^2, H_3R or H_3R-YFP depending on the experiment, using the PolyEthylenImine (PEI, Polysciences, Eppelheim, Germany) method. Cells were incubated with a mix containing cDNA, 5.47 mM PEI and 150 mM NaCl in a serum starved medium. After 4 h, medium was changed to a fresh complete medium, and further experiments were performed at appropriate times after transfection.

2.7. Expression of constructs

48 h after transfection, HEK-293 T cells were rapidly washed twice in Hanks' balanced salt solution (HBSS – 137 mM NaCl, 5 mM KCl, 0.34 mM $Na_2HPO_4{\cdot}12H_2O$, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2{\cdot}2H_2O$, 0.4 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4) with 10 mM glucose, detached by gently pipetting and resuspended in the same buffer. To control the cell number, sample protein concentration was determined using a Bradford assay kit (Bio-Rad, Munich, Germany) using bovine serum albumin dilutions as standards. To quantify the luminescence of $A_{2A}R$-Rluc, cell suspension (20 μg of protein) was distributed in triplicates in 96-well microplates (Corning 3600, white plates with white bottom). 5 mM coelenterazine H (Invitrogen Molecular Probes, Eugene, OR, USA) was added and the luminescence of Rluc quantified after 10 min using a Mithras LB 940 fluorescence-luminescence detector (Berthold Technologies, DLReady, Germany) detecting the light emitted by the Rluc (440–500 nm). To quantify the fluorescence of H_3R-YFP, cell suspension (20 μg of protein) was distributed in duplicates into 96-well black microplates with a transparent bottom (Porvair, King's Lynn, UK) and read in a Mithras LB940 equipped with an excitation filter of 485 nm and an emission filter of 530 nm. To quantify the fluorescence of D_2R-GFP^2, plates were read in a Fluostar Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high energy xenon flash lamp, using a 10-nm bandwidth excitation filter at 400 nm (393–403 nm), and 10-nm bandwidth emission filters corresponding to 506–515 nm filter (Ch 1) and 527–536 nm filter (Ch 2). Rluc, YFP or GFP^2 data was calculated as the signal of the sample minus the signal of non-transfected cells, and is given in relative luminescence (RLU) and relative fluorescence (RFU) units.

2.8. Confocal microscopy

HEK-293T cells transiently transfected with various amounts of cDNA of $A_{2A}R$-Rluc, D_2R-GFP^2 and H_3R-YFP were grown in six-well dishes on 15-mm glass coverslips. At 60% confluence, cells were rinsed with phosphate-buffered saline PBS, fixed in 4% paraformaldehyde for 15 min, and washed with PBS containing 20 mM glycine to quench the aldehyde groups. Cells were permeabilized with PBS containing 20 mM glycine, 1% bovine serum albumin (BSA) (buffer A) and 0.05% Triton X-100 during 5 min. After that the cells were blocked with buffer A for 1 h at room temperature. Cells were labeled for 1 h with the primary mouse monoclonal anti-Rluc antibody (Millipore, Bedford, USA). The cover slips were then washed and stained for 1 h with the secondary antibody cyanine 3-conjugated affinity purified donkey antimouse IgG (Jackson ImmunoResearch, West Grove, USA). Negative control of the secondary antibody was performed for each sample. The coverslips were rinsed for 30 min in buffer A and fixed with Mowiol mounting medium. Microscopic observations of the pattern of expression of the fusion proteins were made using Olympus FV 300 confocal scanning laser microscope (Leica Lasertechnik, Leica Microsystems, Mannheim, Germany). Detection of the D_2R-GFP^2 and H_3R-YFP constructs was performed using their fluorescent properties.

2.9. ERK phosphorylation assay

CHO cells were transiently transfected with 0.5 μg of $A_{2A}R$-Rluc cDNA, 1 μg of D_2R-GFP^2 cDNA and 2.5 μg of H_3R-YFP cDNA. Cells were treated (or not) with 1 μM ZM241385 ($A_{2A}R$ antagonist), 1 μM YM091502 (D_2R antagonist) or 1 μM thioperamide (H_3R antagonist) for 30 min before the addition of the agonists 200 nM CGS21680, 1 μM quinpirole or 50 nM (R)-α-methylhistamine (RAMH), respectively, for 5 min. All drugs were provided by CHDI Foundation Inc. (Los Angeles, CA, US). At the end of the incubation periods, cells were rinsed with ice-cold PBS and lysed by the addition of 500 μL of ice-cold lysis buffer (50 mM Tris–HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 45 mM, β-glycerophosphate, 1% Triton X-100, 20 mM phenylarsine oxide, 0.4 mM $NaVO_4$ and protease inhibitor cocktail). The cellular debris was removed by centrifugation at 13,000 × *g* for 5 min at 4 °C, and the proteins were quantified by the bicinchoninic acid (BCA) method using bovine serum albumin dilutions as standard (Pierce Chemical Co., Rockford, IL, USA). To determine the level of ERK1/2 phosphorylation, equal amounts of protein (10 μg) were separated by electrophoresis on a denaturing 10% SDS-polyacrylamide gel and transferred onto Immobilon-FL PVDF membrane (Millipore, Bedford, USA). After blocking the membranes in Odyssey Blocking Buffer (LI-COR, Lincoln, NE, USA) they were probed with a combination of mouse anti-phosphoERK1/2 antibody (Sigma, 1:2500) and rat anti-ERK1/2 antibody (Sigma, 1:40,000) that recognizes both phosphorylated and non-phosphorylated ERK1/2 in order to rule out that the differences observed were due to the application of unequal amounts of lysates.

The 42 and 44 kDa bands corresponding to ERK 1 and ERK 2 were visualized by the addition of a mixture of IRDye 800 (anti-mouse) antibody and IRDye 680 (anti-rabbit) antibody (1:10,000, Sigma) for 1 h and scanned by the Odyssey Infrared Scanner (LI-COR Biosciences, Lincoln, NE, USA). Bands densities were quantified using the Odyssey V3.0 software and exported to Excel (Microsoft, Redmond, WA, USA). The level of phosphorylated ERK1/2 isoforms was normalized for differences in loading using the total ERK protein band intensities. As a basal value (standardized to 1) activation of ERKs in transfected nontreated cells was used, while non-transfected cells were used as blank.

3. Results and discussion

The progress of the cloning process was monitored electrophoretically (Fig. 1). PCR products of insert cDNA were run on a 1% agarose gel, and distinctive bands around 1.3 kb corresponding to $A_{2A}R$, D_2R and H_3R were visible on the gel as expected (Fig. 1A). After purification, the PCR products and vectors were subjected to double digestion with the corresponding restrictive enzymes and subsequently ligated. After transformation of Dh5α cells with the ligation mixtures intact circular plasmids were obtained, with $A_{2A}R$ inserted into pRluc-N1, D_2R into $pGFP^2$-N3 and H_3R into pEYFP-N1 (Fig. 1B). All constructs were verified by double digestion, and the products were analyzed on 1% agarose gel. Clones were regarded as positive if they produced two bands, of 1.3 kb for $A_{2A}R$ and 4.9 kb for linearized pRluc-N1, 1.3 kb for D_2R and 4.3 kb for linearized $pGFP^2$-N3 and 1.3 kb for H_3R and 4.7 kb for linearized pEYFP-N1 (Fig. 1C). The correct insertion of cDNA into the vectors of the positive samples was confirmed by sequencing. Nucleotide sequences showed that the obtained constructs $A_{2A}R$-Rluc, D_2R-GFP^2 and H_3-YFP express Rluc, GFP^2 and EYFP on the C-terminal ends of the receptors, respectively. One confirmed clone for each receptor was

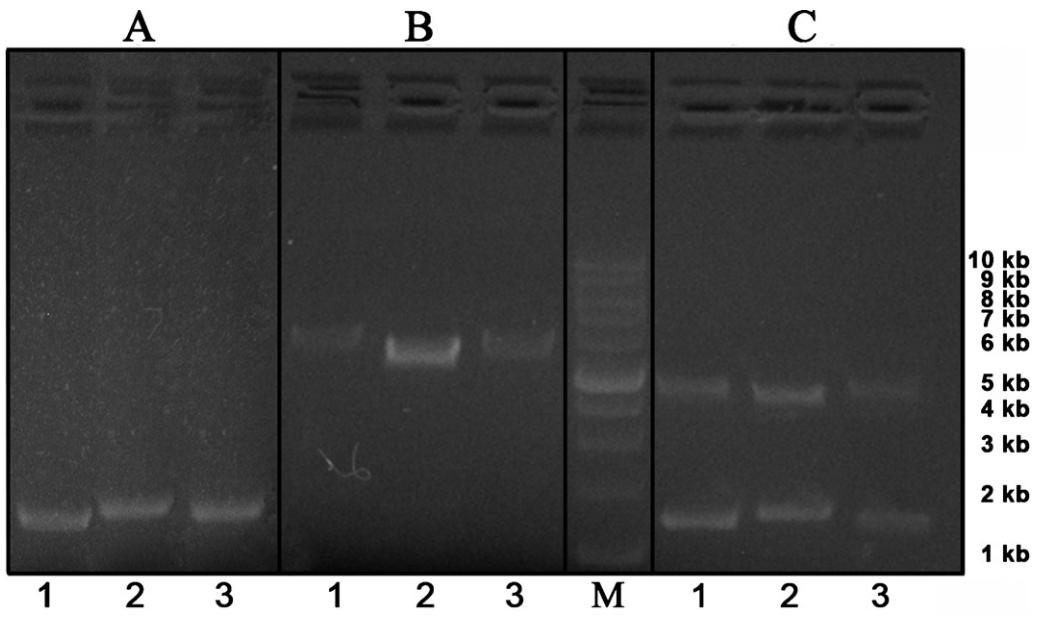

Fig. 1. Agarose gel electrophoresis of starting PCR products (A), intact plasmids (B) and digested plasmids (C). (A1) $A_{2A}R$, (A2) D_2R, (A3) H_3R; (B1) $A_{2A}R$-Rluc-N1, (B2) D_2R-GFP^2-N3, (B3) H_3R-EYFP-N1; (C1) $A_{2A}R$ and linearized pRluc-N1, (C2) D_2R and linearized pGFP^2-N3, (C3) H_3R and linearized pEYFP-N1; M, molecular weight marker.

used for transfection into HEK-293T and CHO cells. All expression and functional experiments were initially performed in both HEK-293T and CHO cell lines, to verify the validity of the obtained data. The same pattern of results for the localization, level of expression and functionality of the constructs was obtained in both cell lines. Expression and localization data from HEK cells is presented, as they consistently gave lower fluorescence and luminescence background signals. In the case of ERK1/2 phosphorylation studies, data obtained from CHO cells is presented, as they showed less variability of the level of ERK phosphorylation in repeated assays.

After transient transfection into HEK-293T cells increasing expression levels of the cloned receptors were detected (Fig. 2). Results are given in relative luminescence or relative fluorescence units by subtracting the value of untransfected cells and represent mean ± s.e.m. of three to five independent experiments. The receptor constructs gave increasing signals of Rluc (Fig. 2A), GFP^2 (Fig. 2B) and YFP (Fig. 2C) with increasing amounts of transfected cDNA, usually reaching a plateau at some higher concentration. From the curves, it was determined that the optimal amounts for transfection were 0.5 μg of cDNA for $A_{2A}R$-Rluc, 1 μg of cDNA for D_2R-GFP^2 and 2.5 μg of cDNA for H_3R-YFP, so the signal they produce in cells would be sufficient for detecting, while minimizing overexpression. These amounts of the constructs were determined as optimal to decrease the likelihood of false positive/negative results in further heteromerization studies.

Spatial expression of the receptors was observed by confocal microscopy at different times after transient transfection into HEK-293T cells. Representative images from three to four inde-

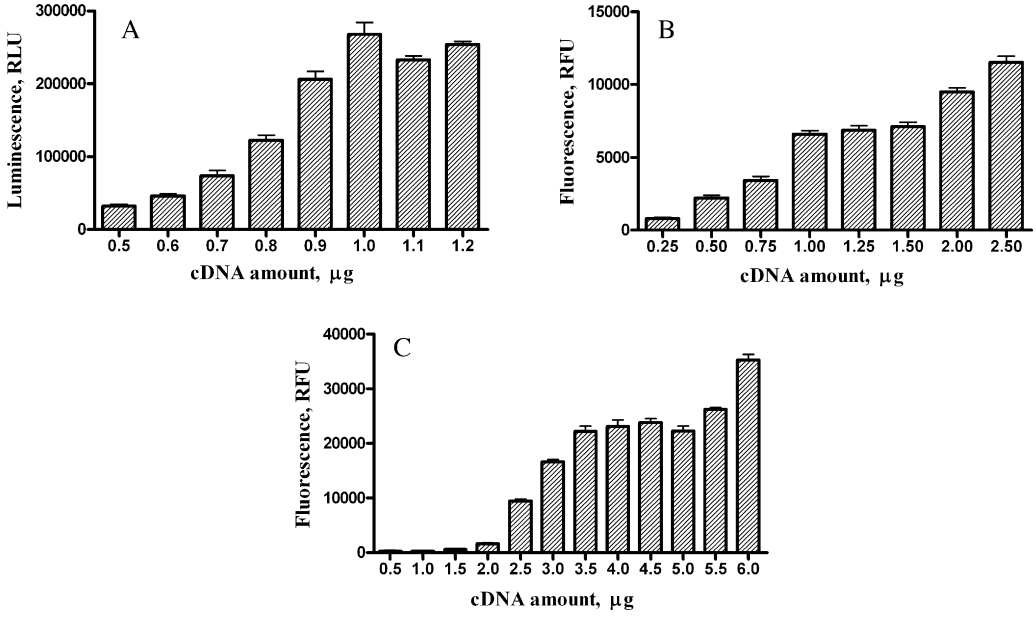

Fig. 2. Expression of $A_{2A}R$-Rluc (A), D_2R-GFP^2 (B) and H_3R-YFP (C) constructs. Results are obtained by subtracting the value of untransfected cells and represent mean ± s.e.m. of three to five independent experiments. RLU, relative luminescence units, RFU, relative fluorescence units.

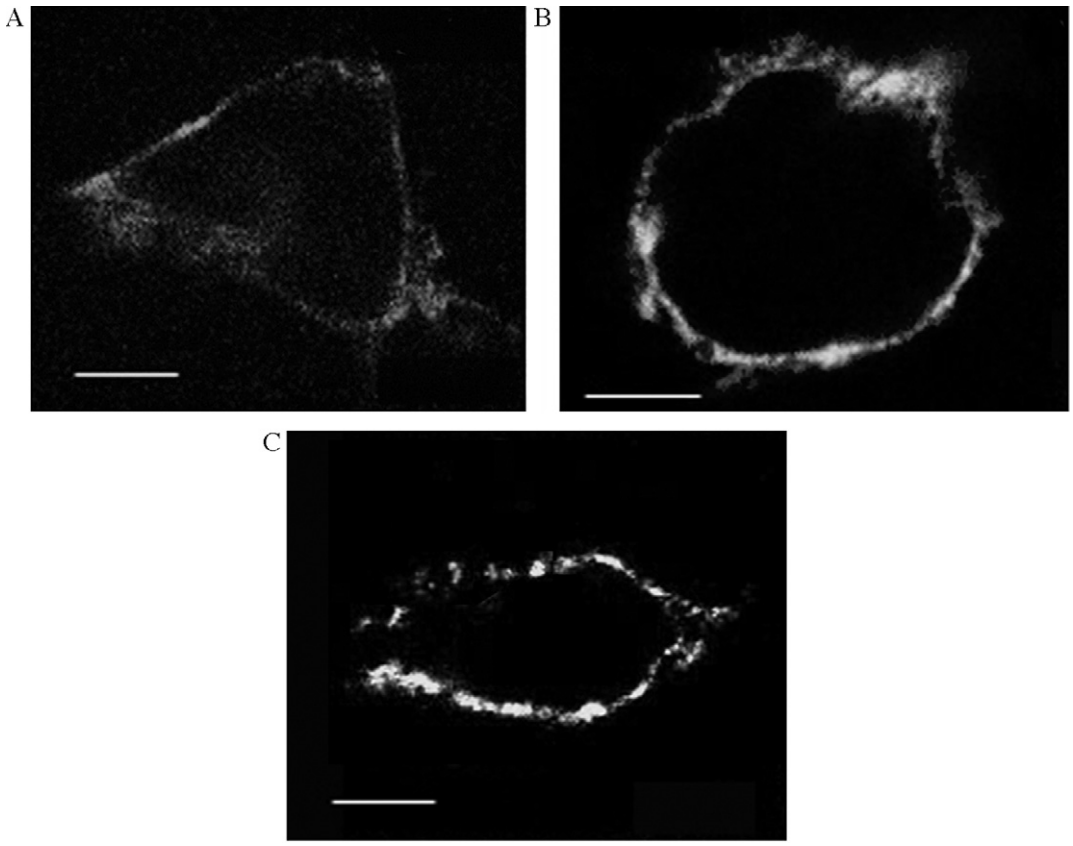

Fig. 3. Confocal microscopy images of HEK-293 T cells expressing A_{2A}R-Rluc (A, 0.5 μg of cDNA), D_2R-GFP^2 (B, 1 μg of cDNA) and H_3R-YFP (C, 2.5 μg of cDNA) constructs. Representative images from three to four independent experiments are shown. Scale bars, 10 μm.

pendent experiments for each construct are shown, considering the fact that the localization of the constructs was the same in over 90% of cells in each experiment (Fig. 3). The localization of A_{2A}R (Fig. 3A) was determined by detecting its Rluc tag with a monoclonal anti-Rluc antibody labeled with Cy3 dye, while the localization of D_2R (Fig. 3B) and H_3R (Fig. 3C) was determined by detecting their fluorescent GFP^2 and YFP tags, respectively. Controls using non-transfected cells and cells transfected only with empty vectors pRluc-N1, pGFP^2-N3 and pEYFP-N1 were employed in each experiment. While nontransfected cells gave no specific fluorescent signal, empty-vector transfected cells gave a fluorescent signal which was not localized in the plasma membrane, even after 72 h. When the cloned A_{2A}R-Rluc, D_2R-GFP^2 and H_3R-YFP were transfected, it was noted that 24 h after transfection the receptors were abundant in the endoplasmatic reticulum, but also beginning to migrate to the plasma membrane. The optimal time needed for their expression in the plasma membrane was determined to be 48 h after transfection. This time frame was used in all subsequent experiments, to be certain that the GPCRs have been correctly placed in the plasma membrane. Using confocal microscopy it was shown that the cloned receptors express correctly in the cells and travel to the plasma membrane as their corresponding wild-type couples.

The functionality of the constructs was compared to their respective non-fluorescent couples measuring the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) upon ligand stimulation in transiently transfected CHO cells. Controls using non-transfected cells and cells transfected only with empty vectors stimulated with ligands for A_{2A}, D_2 and H_3 receptors were employed in each experiment. No specific increase in ERK1/2 phosphorylation upon agonist stimulation was observed in neither non-transfected (Fig. 4) nor empty-vector transfected cells, which suggested that CHO cells did not have a significant level of endogenously expressed A_{2A}, D_2 and H_3 receptors. Cells transfected with cDNA of A_{2A}R (Fig. 5A) or A_{2A}R-Rluc (Fig. 5B) were treated or not with the A_{2A}R agonist CGS21680 in the presence or absence of the A_{2A}R antagonist ZM241385. Cells transfected with cDNA of

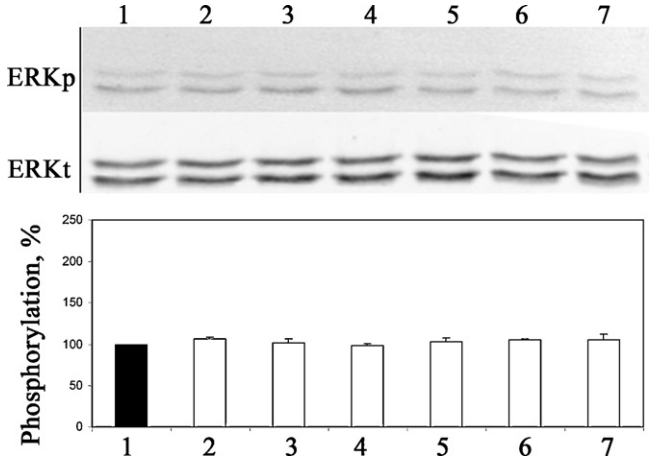

Fig. 4. Ligand-induced ERK phosphorylation in non-transfected CHO cells. Non-transfected CHO cells were treated or not (1) with the A_{2A}R agonist CGS21680 (200 nM) in the absence (2) or in the presence (3) of the A_{2A}R antagonist ZM241385 (1 μM), and with the D_2R agonist quinpirole (1 μM) in the absence (4) or in the presence (5) of the D_2R antagonist YM091502 (1 μM), and with the H_3R agonist RAMH (50 nM) in the absence (6) or in the presence (7) of the H_3R antagonist thioperamide (1 μM). In the top panel a representative Western blot is shown. In the bottom panel band density quatification results are expressed as a percentage of phosphorylation of the non-treated cells and represent mean ± s.e.m. of three independent experiments.

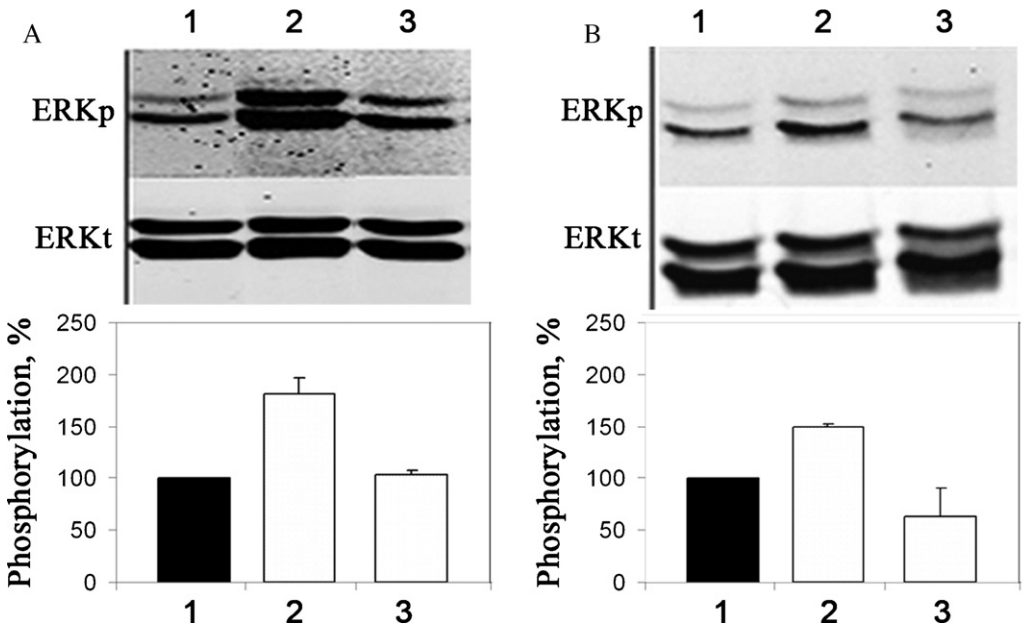

Fig. 5. $A_{2A}R$ (A) and $A_{2A}R$-Rluc (B) agonist-induced ERK phosphorylation. CHO cells transfected with 0.5 μg of cDNA of $A_{2A}R$ or $A_{2A}R$-Rluc were treated or not (1) with the $A_{2A}R$ agonist CGS21680 (200 nM) in the absence (2) or in the presence (3) of the $A_{2A}R$ antagonist ZM241385 (1 μM). In the top panel representative Western blots are shown. In the bottom panel band density quatification results are expressed as a percentage of phosphorylation of the non-treated cells and represent mean ± s.e.m. of three independent experiments.

D_2R (Fig. 6A) or D_2R-GFP^2 (Fig. 6B) were treated or not with the D_2R agonist quinpirole in the presence or absence of the D_2R antagonist YM091502. Cells transfected with cDNA of H_3R (Fig. 7A) or H_3R-YFP (Fig. 7B) were treated or not with the H_3R agonist RAMH in the presence or absence of the H_3R antagonist thioperamide. After quantification and normalization of data for differences in loading, results were expressed as a percentage of the value of transfected untreated cells and represent mean ± s.e.m. of three to five independent experiments. Upon stimulation with the corresponding agonists, cells expressing the wild type and the cloned receptors were able to induce ERK1/2 phosphorylation, and this effect was blocked by the corresponding antagonists. As this effect

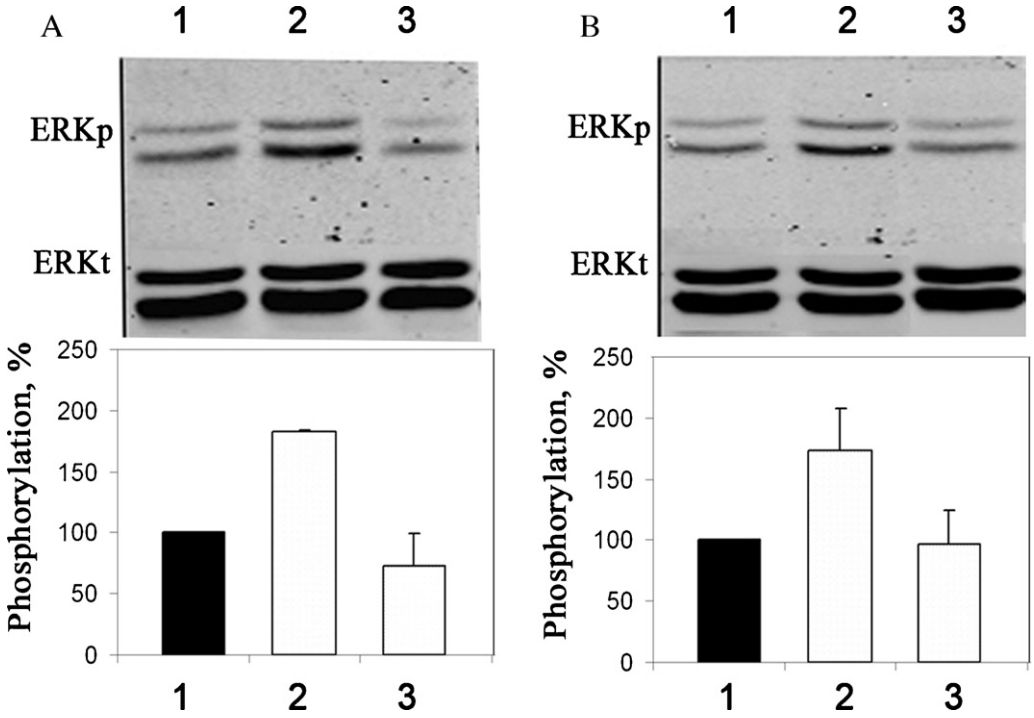

Fig. 6. D_2R (A) and D_2R-GFP^2 (B) agonist-induced ERK phosphorylation. CHO cells transfected with 1 μg of cDNA of D_2R or D_2R-GFP^2 were treated or not (1) with the D_2R agonist quinpirole (1 μM) in the absence (2) or in the presence (3) of the D_2R antagonist YM091502 (1 μM). In the top panel representative Western blots are shown. In the bottom panel band density quatification results are expressed as a percentage of phosphorylation of the non-treated cells and represent mean ± s.e.m. of three independent experiments.

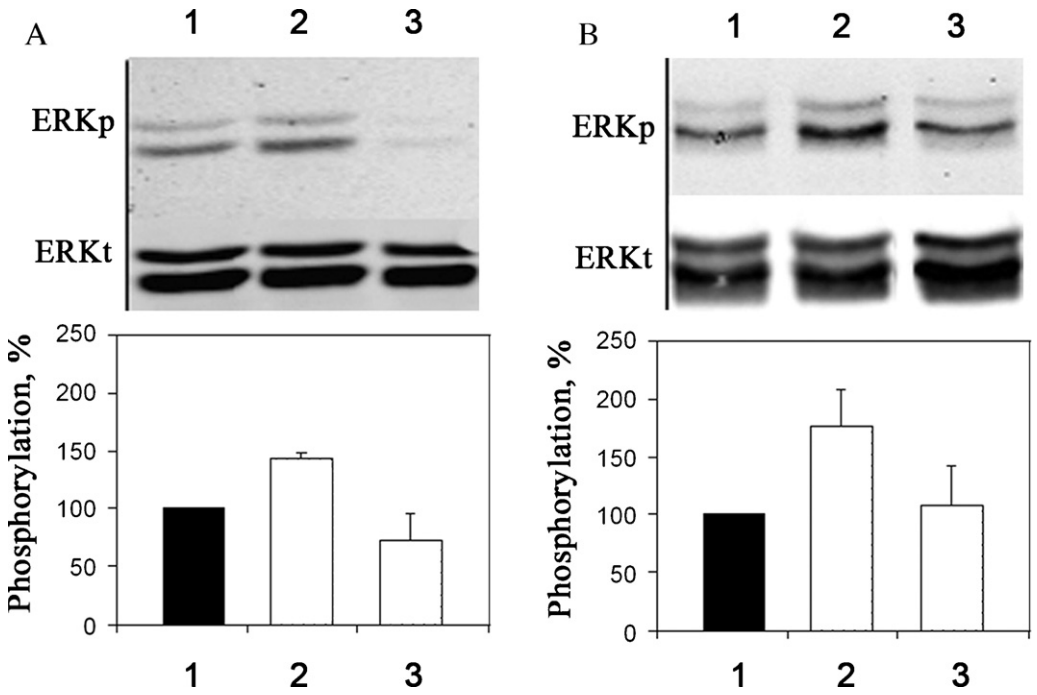

Fig. 7. H_3R (A) and H_3R-YFP (B) agonist-induced ERK phosphorylation. CHO cells transfected with 2.5 μg of cDNA of H_3R or H_3R-YFP were treated or not (1) with the H_3R agonist RAMH (50 nM) in the absence (2) or in the presence (3) of the H_3R antagonist thioperamide (1 μM). In the top panel representative Western blots are shown. In the bottom panel band density quatification results are expressed as a percentage of phosphorylation of the non-treated cells and represent mean ± s.e.m. of three independent experiments.

was not observed in non-transfected nor empty-vector transfected cells, it was concluded that specific changes in ERK1/2 phosphorylation were induced by the expression of the transfected receptors. Also, the cloned constructs exhibited a similar pattern (potency) of phosphorylation of ERKs upon ligand stimulation of $A_{2A}R$-Rluc, D_2R-GFP2 and H_3R-YFP and $A_{2A}R$, D_2R and H_3R, respectively. These results indicated that the cloned receptors are functional and can be used in experiments for determining the response of cells to different stimuli that affect the signaling pathway of ERK kinases. This method is extensively used to determine the impact of receptor heteromerization on cellular signaling response upon multiple stimulation.

4. Conclusion

The previous experiments confirmed that the cloned $A_{2A}R$-Rluc, D_2R-GFP[2] and H_3R-YFP constructs are functional, express correctly in the plasma membrane and can be used in further experiments to elucidate the pharmacological and functional inter-relationships between adenosine A_{2A}, dopamine D_2 and histamine H_3 receptors in the brain. Hopefully, this will enable the design and evaluation of new therapeutic strategies for Parkinson's disease.

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RESUMEN EN CASTELLANO

1. INTRODUCCIÓN

Los receptores de siete dominios transmembrana o acoplados a proteína G (G-protein coupled receptors o GPCR) forman una de las familias de proteínas más importantes en vertebrados. Se estima que constituyen más de un 2 % del genoma humano, y sus ligandos presentan una gran diversidad química, desde aminas biogénicas, péptidos, glicoproteínas, lípidos, nucleótidos, iones, hasta proteasas (Kolakowski et al., 1994). Independientemente de la diversidad existente, todos los GPCR son proteínas integrales de membrana con una estructura tridimensional similar. Estos receptores contienen siete dominios transmembrana hidrofóbicos, en una conformación de hélice α, unidos por tres bucles intracelulares y tres extracelulares, con el extremo N-terminal situado en la cara extracelular de la membrana y el extremo C-terminal en el lado citoplasmático (Lefkowitz et al., 2000). Una segunda característica importante que comparten todos los GPCR es su capacidad de interaccionar con las proteínas G, proteínas heterotriméricas (subunidades αβγ) que actúan como intercambiadores de nucleótidos de guanina. Una vez que el ligando interacciona con el receptor, las subunidades α y βγ modulan diversas vías de transducción de señal dentro de la célula (Marinissen et. al, 2001). Dentro de la gran familia de los receptores acoplados a proteínas G, esta Tesis se ha centrado en el estudio de los receptores de adenosina, concretamente del subtipo A_{2A} ($A_{2A}R$) y sus intereactiones con los receptores de adenosina A_1 (A_1R), dopamina D_2 (D_2R) y cannabinoides CB_1 (CB_1R), así como también con un importante enzima extracelular, la adenosina desaminasa (ADA).

Adenosina es un nucleósido endógeno formado por la base purínica adenina y una ribosa y, entre otras funciones, actúa como un neuromodulador en el control de la transmisión sináptica al unirse a los receptores de adenosina (Cobbin et al., 1974). Se conocen cuatro subtipos de receptores de adenosina: A_1, A_{2A}, A_{2B} y A_3, clasificados así en función de sus características moleculares, bioquímicas y farmacológicas (Bouvier et al. 1995). De hecho, mientras los subtipos A_1 y A_3 se acoplan principalmente a proteínas G de tipo G_i, los receptores de los subtipos A_{2A} y A_{2B} lo hacen a proteínas G de tipo G_s, teniendo así como principal vía de señalización la inhibición o activación de la adenilato ciclasa, respectivamente (Fredholm et al., 2001). La acción de la adenosina a través de sus receptores específicos

desempeña papeles importantes en la modulación de muchas funciones celulares. En el sistema nervioso central es un importante neuromodulador. La ADA, que es un enzima del metabolismo purínico que degrada adenosina o desoxiadenosina a inosina o desoxiinosina y amoníaco, se encuentra en el citoplasma pero también actúa como ecto-enzima. La ecto-ADA se une a la superficie celular mediante su interacción con proteínas de membrana, entre ellas los receptores A_1 y A_{2B} de adenosina (Saura et al., 1996; Herrera et al., 2001) la unión de la enzima a los receptores induce a éstos una mayor afinidad por los ligandos, considerándose la enzima como un modulador alostérico de los receptores

De los cuatro subtipos de receptores de adenosina, los $A_{2A}R$ han sido objeto de intensa investigación en el campo de las neurociencias debido, principalmente, a su relación con patologías derivadas de desórdenes en el movimiento como es la enfermedad de Parkinson y la enfermedad de Huntington (Schwarzschild et al., 2002; Reggio et al., 1999). Los $A_{2A}R$ presentan una elevada expresión en estriado, núcleo accumbens y tubérculo olfatorio (Martinez-Mir et al., 1991, Glass 2000). Mediante técnicas de alta sensibilidad se ha detectado la expresión en otras zonas del sistema nervioso y en tejidos periféricos (Rosin et al., 1998ab; Moreau et al., 1999; Rebola et al., 2005). La principal vía de transducción de señal de los $A_{2A}R$ es la activación de la enzima adenilato ciclasa por parte de la proteína $G_{s/olf}$ (Kull et al., 1999, 2000), ello produce el aumento de los niveles de AMPc y la activación de la proteína quinasa PKA, la cual regula el estado de fosforilación de una gran variedad de proteínas. Los $A_{2A}R$ también activan otras vías independientes de AMPc, como la activación de PLC y MAPK. En el estriado los $A_{2A}R$ se encuentran a nivel pre- y postsináptico. Presinápticamente en las proyecciones corticostriatales glutamatérgicas de la vía directa y postinápticamente en las neuronas GABAérgicas estriatopalidales de la vía indirecta (Schiffmann et al., 1991; Hettinger et al., 2001; Quiroz et al., 2009). Cómo veremos más adelante, los $A_{2A}R$ desempeñan un papel distinto en cada una de estas localizaciones.

La dopamina es un neurotransmisor que ejerce su efecto actuando sobre sus receptores específicos situados en la membrana celular. Los receptores de dopamina se clasifican en función de sus propiedades moleculares, bioquímicas y farmacológicas, en dos

familias: la familia D_1-*like*, que cuenta con los receptores D_1 y D_5 acoplados a proteínas del tipo G_s, activadoras, entre otras, de la enzima adenilato ciclasa y la familia D_2-*like*, que cuenta con los receptores D_2, D_3 y D_4, acoplados a proteínas del tipo $G_{i/o}$, las cuales inhiben a la adenilato ciclasa y activan otras vías como la de PLC o inhiben las corrientes de Ca^{2+} (Spano et al., 1978). Dentro de la familia de los receptores de dopamina, el D_2R ha sido objeto de un gran número de estudios debido a su participación en muchas funciones fisiológicas de gran importancia como el control de la actividad motora. Además, los D_2R también están implicados en diversos estados neuropatológicos como las enfermedades de Parkinson y Huntington o en la addición a drogas. Representan la principal diana de fármacos antisicóticos (Missale, et al., 1998, Vallone et al., 2000). Los D_2R se encuentran principalmente en el sistema nervioso central, concretamente en el tubérculo olfatorio, caudado-putamen y en el núcleo accumbens donde se expresa en neuronas GABAérgicas que coexpresan encefalina. Además, aunque en menor cantidad, también se encuentra mRNA de este receptor en otras zonas, como la sustancia negra, el área tegmental ventral y en el sistema nervioso periférico (Vallone et al., 2000). La principal vía de transducción de señal descrita para los D_2R es la mediada por la inhibición de la enzima adenilato ciclasa a través de la subunidad α_i de las proteínas del tipo $G_{i/o}$, pero también se han descrito vías de señalización independientes de los niveles de AMPc, como la inhibición de las corrientes de Ca^{2+} o la activación de la PLC (Lee et al., 2006).

Los endocanabinoides, como anandamina y 2-araquidonilglicerol, son lípidos endógenos biosintetizados a partir de distintos precursores fosfolipídicos presentes en la membrana celular (Devane et al., 1992; Sugiura et al., 1995). Sus efectos sobre el sistema nervioso central incluyen euforia, relajación y depresión motora. Estos efectos están mediados por la acción agonista sobre los receptores de canabionoides CB_1 y CB_2 (Ameri et al., 1999; Piomelli et al., 2003; Di Marzo et al., 2004). La vía de señalización más común para los CB_1R está mediada por la proteina $G_{i/o}$ inhibiendo, así, la adenilato ciclasa (Howlett et al., 1986). Curiosamente, bajo ciertas condiciones, los CB_1R también pueden acoplarse a proteínas activadoras G_s siendo así un receptor promiscuo cuando se trata de la unión a la proteína G (Bonhaus et al., 1998). En el estriado, los CB_1R se encuentran mayoritariamente en las sinapsis establecidas entre los terminales

glutamatérgicos y GABAérgicos de las neuronas espinosas de tamaño medio (MSN) tanto encefalinergicas como dinorfinérgicas y tienen un papel clave en el control del comportamiento motor (Martin et al., 2008; Katona et al., 2008; Pazos et al., 2008). En las sinapsis corticostriatales, los CB_1R están localizados a nivel presináptico pero en menor medida también a nivel postsináptico. De similar manera como la adenosina, la función fisiológica principal de los cannabionoides en estas sinapsis es regular la liberación de otros neurotransmisores (Marsicano et al., 2003; Katona et al., 2006).

A partir de la mitad de los años 90 empezaron a aparecer varios estudios que demostraban la oligomerización de diversos GPCR y en la actualidad este fenómeno ya se acepta como una característica común de su biología. Así, los GPCR se pueden encontrar expresados como homodímeros, heterodímeros u oligómeros de orden superior (Bouvier et al., 2001; Devi et al., 2001; George et al., 2002; Agnati et al. 2003; Franco et al. 2003; Terrillon et al., 2004; Agnati et al., 2005; Prinster et al., 2005; Milligan et al., 2006; Pin et al. 2007; Carriba et al., 2008; Ferré et al., 2009). La homodimerización se define como la asociación física entre proteínas idénticas, mientras que la heteromerización es la asociación entre proteínas distintas. La asociación proteína-proteína puede ocurrir entre dos monómeros que forman dímeros o entre varios monómeros que forman oligómeros de receptores. Como en la actualidad las técnicas disponibles no permiten distinguir entre los dímeros u oligómeros de orden superior, el término dímero se utiliza a menudo para expresar de la forma más simple la unidad funcional de un oligómero. Los dímeros u oligómeros presentan distintas características funcionales en comparación con las de los receptores individuales, así la oligomerización confiere nuevas propiedades y funciones a los GPCR. Este fenómeno da lugar a una nueva diversidad en la señalización y regulación de estos receptores. Un ejemplo de lo anteriormente mencionado lo constituyen los heterómeros entre $A_{2A}R$, A_1R, D_2R y CB_1R. Los $A_{2A}R$ forman heterómeros con los A_1R y regulan la liberación de glutamato en las terminales glutamatérgicas corticoestriatales que inervan a las neuronas espinosas de tamaño medio (MSN) que constituyen la vía directa del circuito motor de los ganglios basales (Rosin et al., 2003; Ciruela et al., 2006; Quiroz et al., 2009). Los $A_{2A}R$ forman también heterómeros con los D_2R (Hillion et al., 2002) lo que regula la activación de las MSN que expresan encefalina y constituyen la vía

indirecta del circuito motor de los ganglios basales (Ferré et al, 2007). Los $A_{2A}R$ también pueden formar heterómeros con los CB_1R (Carriba et al., 2007) expresados a nivel pre- y postsináptico en ambas vías directa e indirecta (Rodriguez et al., 2001; Pickel et al., 2004; 2006; Kofalvi et al., 2005; Matyas et al., 2006; Uchigashima et al., 2007). A nivel postsináptico se ha descrito la formación de heterotrímeros $A_{2A}R$-CB_1R-D_2R (Carriba et al., 2008). Los heterómeros A_1R-$A_{2A}R$, al controlar la liberación de glutamato, pueden estar implicados en aquellas disfunciones causadas por excitotoxicidad de glutamato. Un ejemplo lo constituye la enfermedad de Huntington (HD), una enfermedad neurodegenerativa progresiva, hereditaria de manera autosómica dominante, que se caracteriza por trastornos motores, congitivos y siquiátricos. La causa de HD es una simple mutación en la proteína huntingtina. Se sabe que la mutación de huntingtina conlleva una progresiva neurodegeneración que empieza en el estriado, en las MSN que constituyen la vía indirecta del circuito motor de los ganglios basales (Graveland et al., 1985c; Reiner et al., 1988; Albin et al., 1992; Richfield et al., 1995; Vonsattel et al., 1998). La disfunción de los receptores expresados en la sinapsis corticostriatal de esta vía, y su siguiente desapareción, lleva a un descontrol motor y la aparación de moviementos hipercinéticos denominados coreas. Una de las teorías formuladas sobre una de las causas de la HD es la excitotoxicidad de glutamato (Beal et al., 1986; Popoli et al., 1994); por tanto, los heterómeros A_1R-$A_{2A}R$ pueden ser diana para HD.

2. OBJETIVOS

Los receptores acoplados a proteína G interaccionan con diversas proteínas en la parte extracelular e intracelular de la membrana y también interaccionan entre ellos formando homo- y heterodímeros u oligomeros de grado superior a nivel de la membrana. Dado que estas interacciones proteína-proteína implican cambios importantes en la funcionalidad de estos receptores, el conocimiento de las nuevas propiedades farmacológicas y funcionales de estos complejos proteicos puede ser muy útil para entender la trasmisión neuronal y para la búsqueda de fármacos de alta eficiencia en las enfermedades neurológicas. En este marco, el objetivo general de esta Tesis *es investigar las consecuencias farmacológicas y funcionales de*

la interacción del receptor A_{2A} de adenosina con otras proteínas. Para alcanzar este objetivo geneneral formulamos tres objetivos concretos.

En la superficie celular, la enzima ADA, además de regular la neurotransmisión metabolizando el neuromodulador adenosina, tiene una relevancia importante como modulador alostérico de los receptores de adenosina. En estudios previos, nuestro grupo de investigación describió que la ADA es un modulador alostérico de los receptores de adenosina A_1 y A_{2B}, pero no se conoce si la ADA modula la función de los receptores A_{2A}. Por ello, el primer objetivo de esta Tesis ha sido estudiar la interacción molecular y funcional entre la ADA y de los A_{2A}R.

OBJETIVO 1. Estudiar la interacción molecular y funcional entre la ADA y los receptores de adenosina A_{2A}.

Los A_{2A}R heteromerizan con los A_1R a nivel presináptico en las terminaciones glutamatérgicas corticoestriatal que contactan con las neuronas GABAérgicas de la vía directa y con los D_2R a nivel postsináptico en las neuronas GABAérgicas de la vía indirecta, teniendo un papel fisiológico específico y distinto en cada de esos dos heterómeros. Teniendo en cuenta que un heterómero de receptores es un complejo macromolecular compuesto al menos por dos unidades de receptores funcionales con propiedades bioquímicas diferentes a las de los receptores individuales, en el segundo objetivo de esta Tesis hemos investigado las características farmacológicas de los A_{2A}R en estos dos heterómeros, con el objetivo de encontrar antagonistas de los A_{2A}R que fuesen más selectivos para el heterómero A_{2A}R-A_1R. Este tipo de compuestos son interesantes para reducir la neutrotrasmisón de la vía directa, cuyo funcionamiento dominante podría incrementar la aparición de movimientos hipercinéticos en pacientes con la enfermedad de Huntington.

OBJETIVO 2. Buscar antagonistas más selectivos para el receptor A_{2A} en el heterómero de receptores A_1-A_{2A} presináptico *versus* al heterómero de receptores A_{2A}-D_2 postsináptico, que puedan ser útiles para el tratamiento de enfermedades neurológicas, en particular para la enfermedad de Huntington.

Además de heteromerizar con los A_1R y los D_2R, los receptores A_{2A} también interaccionan con receptores de cannabinoides CB_1. Los CB_1R se encuentran pre- y postsinápticamente en las vías directa e indirecta y podrían modular la función de los $A_{2A}R$ en el estriado. De hecho, el heterómero $A_{2A}R$-CB_1R ha sido previamente descrito en nuestro grupo de investigación y aunque ya se sabía que la activación de los $A_{2A}R$ es necesaria para la señalización de los CB_1R en una línea celular derivada de neuroblastoma dónde estos heterómeros se expresaban, las características farmacológicas y funcionales de esos heterómeros no son conocidas. Un objetivo de esta Tesis ha sido estudiar las características farmacológicas y funcionales de los heterómeros $A_{2A}R$-CB_1R y determinar si los antagonistas de los $A_{2A}R$ muestran una selectividad distinta por los $A_{2A}R$ o por los heterómeros $A_{2A}R$-CB_1R. Para ello, hemos formulado el tercer objetivo de esta Tesis:

OBJETIVO 3. Investigar las características farmacológicas y funcionales de los receptores A_{2A} en los heterómeros $A_{2A}R$-CB_1R y determinar si algunos antagonistas selectivos del receptor A_{2A} muestran una selectividad diferencial por los receptores A_{2A} o los heterómeros $A_{2A}R$-CB_1R.

3. RESUMEN DE RESULTADOS Y DISCUSIÓN

Los receptores acoplados con la proteína G (GPCR) ya no son considerados como unidades funcionales separadas, sino como complejos multimoleculares donde interactúan físicamente entre sí, así como con otras proteínas en el plano horizontal y vertical de la membrana (Bouvier, 2001; Franco et al., 2003; Ferré et al., 2009; Albizu et al., 2006, 2010; Birdsall et al., 2010, Fuxe et al., 2010). Teniendo en cuenta las interacciones proteína-proteína en el plano vertical respecto a la membrana, los receptores de adenosina A_1 (A_1R) y A_2B ($A_{2B}R$) interactúan con la enzima extracelular ADA (ecto-ADA). La ecto-ADA, de manera independiente de su actividad enzimática, es un potente modulador alostérico positivo de la unión de agonistas y antagonistas (Saura et al., 1996; Herrera et al., 2001). Existe un gran interés en investigar la capacidad de los receptores de adenosina A_{2A} ($A_{2A}R$) para controlar la neurotransmisión y la plasticidad sináptica en las sinapsis glutamatérgicas debido a que estos

receptores controlan la liberación de glutamato en estados normales y patológicos (Cunha et al., 2008). De hecho, los $A_{2A}R$ actúan como neuroprotectores en algunas enfermedades neurodegenerativas (Gomes et al., 2011). Por lo tanto, teniendo en cuenta la importancia de los $A_{2A}R$ y el papel de la ADA en la farmacología de los receptores de adenosina estudiados, en esta Tesis se investigó, por primera vez, la interacción molecular y funcional entre la ADA y los $A_{2A}R$.

En el artículo: "***A_{2A} adenosine receptor ligand binding and signaling is allosterically modulated by adenosine deaminase***" se describe que la ADA puede unirse a la membrana plasmática de células CHO transfectadas con $A_{2A}R$, pero no de las células *wild type* que no expresan este receptor. Esto indica que la ADA es capaz inteaccionar con $A_{2A}R$. Se ha demostrado previamente a través de técnicas de FRET y BRET que los $A_{2A}R$ forman homómeros y que los homómeros, pero no los monómeros, parecen ser la especie funcional en la superficie de células transfectadas (Canals et al., 2004). Así, la estructura cuaternaria de los receptores A_{2A} está constituida por, al menos, dos protómeros que dan lugar a un dímero. La unión de la ADA dio lugar a la modificación de la estructura cuaternaria de los homodímeros de $A_{2A}R$ ya que la ADA indujo un aumento en la transferencia de energía detectado por los experimentos de BRET (reflejado por el incremento en el $BRET_{max}$). Es interesante señalar que esta modificación es de magnitud considerable, ya que la unión de agonistas a los $A_{2A}R$ no es capaz de modificar la señal de BRET (Canals et al., 2004). Por lo tanto la capacidad de la técnica de BRET para detectar los cambios conformacionales en los homómeros $A_{2A}R$ provocados por ADA sugiere que la ADA puede modular la función de los homómeros de $A_{2A}R$.

Para investigar si los cambios estructurales inducidos por la ADA en los homómeros de $A_{2A}R$ correlacionaban con modificaciones en las características farmacológicas de los $A_{2A}R$ hemos realizado ensayos de unión de radioligandos con agonistas y antagonistas en ausencia o presencia de ADA. Demostramos que la ADA disminuye significativamente las constantes del equilibrio de disociación de los agonistas y antagonistas de $A_{2A}R$. Este incremento general de afinidad inducido por la ADA era independiente de su actividad enzimática e indica que la ADA se comporta cómo un ligando alostérico. De hecho, se ha demostrado que, además de los centros ortostéricos, muchos

GPCR poseen dominios alostéricos estructuralmente distintos. Uno de los rasgos característicos de las interacciones alostéricas es que el receptor es capaz de unir simultáneamente un ligando ortostérico y otro alostérico, proporcionando una mayor complejidad de las respuestas farmacológicas al modificar la afinidad o la señal impartida por el ligando ortostérico (May et al., 2007). Un efecto alostérico produce una modulación positiva si el modulador facilita la interacción o, por el contrario, da lugar a una modulación negativa si éste inhibe la interacción del ligando a través del centro de unión ortostérico (May et al., 2007; Conn et al., 2009). De acuerdo con estos conceptos, la ADA es un ligando alostérico de los receptores A_{2A} que modula positivamente la unión de agonistas y antagonistas al centro ortostérico del receptor. Además de los efectos positivos de la ADA sobre la unión de agonistas a $A_{2A}R$, se observó que también mejoraba su funcionalidad. Para este estudio desarrollamos una línea estable a partir de las células CHO que expresaba receptores A_{2A} humanos y analizamos la transducción de señal estudiando el efecto de los ligandos y la ADA sobre la fosforilación de MAPK (proteínas quinasas activadas por mitógenos). Efectivamente, la presencia de ADA incrementó la fosforilación de ERK 1/2 de acuerdo con el anteriormente indicado incremento en afinidad del agonista CGS-21680.

La capacidad de los moduladores alostéricos para controlar finamente las respuestas farmacológicas ha despertado interés por su potencial aplicación en la investigación básica y clínica (Conn et al., 2009). Este interés es más relevante en el caso de los receptores para neurotransmisores debido al hecho de que la neurotransmisión sináptica acontece en circuitos de extrema complejidad implicados en numerosas funciones neurológicas. Debido a la implicación de A_{2AR} en diversas enfermedades neurodegenerativas como Parkinson, Huntington, desórdenes obsesivo-compulsivos y adicción a drogas (Stone et al., 2009), diversas aproximaciones han sido probadas para encontrar moduladores alostéricos (Stone et al., 2009; Cristalli et al., 2008; Carlsson et al., 2010, Katritch et al., 2010). Conceptualmente, las interacciones alostéricas descritas en nuestro trabajo son diferentes a aquellas que se ejercen a través de moléculas de bajo peso molecular, ya que se producen como consecuencia de la interacción, a través de la membrana, con una proteína que tiene una topología extracelular. Así, a través de la interacción con un dominio

extracelular del receptor A_{2A}, la ADA ejerce una modulación fina en la neuroregulación inducida por adenosina que puede tener implicaciones importantes en la función de los A_{2AR} en el estriado cerebral. Se ha demostrado la presencia de ADA unida a la superficie de las neuronas (Ruiz et al., 2000), lo que refuerza el concepto de que este efecto alostérico del enzima sea muy probable que ocurra *in vivo*. Teniendo en cuenta todo lo descrito hasta ahora, se podría hipotetizar que los pacientes que padecen el síndrome de inmunodeficiencia severa combinada (SCID), en los que las mutaciones de la ADA afectan a la interacción ADA-A_{2A}R, podrían presentar alteraciones neurológicas que posiblemente serían diferentes a aquellas en las que las mutaciones no afecten a la interacción entre las dos proteínas. Probablemente, las mutaciones que afectan esta interacción serían menos perjudiciales para la función del estriado, ya que atenuarían la sobreactivación de A_{2A}R ejercida por la elevación de los niveles de adenosina.

En resumen, los resultados descritos en este trabajo indican que la ADA, además de reducir la concentración de adenosina endógena, interacciona con el receptor A_{2A} de adenosina comportándose como un efector alostérico que incrementa de forma notable la señalización a través de la vía de las MAPK incrementando la fosforilación de las ERK 1/2. Así, el papel fisiológico de la interacción ADA-receptor de adenosina sería de gran importancia porqué hace estos receptores más funcionales.

La función de los GPCR es extremadamente compleja y no sólo es regulada por las proteínas que interaccionan con los receptores de neurotrasmisores, como con la anteriormente mencionada ADA, si no también mediante la interacción con otros receptores para la formación de heterómeros. La existencia de heterómeros entre los receptores de diferentes neurotrasmisores o neuromoduladores se ha convertido en un hecho generalmente aceptado por la comunidad científica. Esta aceptación ha introducido cambios en la forma de entender la neurotransmisión y su complejo control en diferentes partes del cerebro. Es importante destacar que el heterómero tiene propiedades bioquímicas diversas que son distintas a las de sus componentes individuales (Terrillon et al., 2004; Ferré et al., 2009). Si los receptores se expresan como heterómeros, éstos deben ser considerados como dianas reales en el descubrimiento de fármacos. Este nuevo enfoque en la bioquímica de los GPCR ha abierto un gran

campo de nuevas posibilidades en la búsqueda de compuestos más eficaces aplicables en el tratamiento de enfermedades neurológicas. Al iniciarse esta Tesis se sabía que $A_{2A}R$ formaba heterómeros con los receptores de dopamina D_2 (D_2R, Hillion et al., 2002), adenosine A_1R (Ciruela et al., 2006) o los receptores cannabinoides CB_1 (CB_1R, Carriba et al., 2008) y que estos heterómeros se expresan en diferentes dominios sinápticos en el estriado y que desempeñaban un papel diferente en el control motor. En el estriado, en la sinapsis glutamatérgica corticoestriatal formada por eferentes corticales y neuronas GABAérgicas espinosas de tamaño medio (MSN), los $A_{2A}R$ heteromerizan con A_1R a nivel presináptico en la vía directa y con D_2R a nivel postsináptico en la vía indirecta (Ciruela et al., 2006; Hillion et al., 2002; Quiroz et al. 2009). En condiciones de altas concentraciones extracelulares de adenosina, como puede suceder durante una mayor activación neuronal en condiciones normales o en varias condiciones patológicas (por ejemplo en isquemia o enfermedad de Huntington), la activación presináptica de $A_{2A}R$ aumenta la liberación de glutamato. Las altas concentraciones de glutamato pueden llevar a excitotoxicidad, que es considerada como uno de los posibles mecanismos que pueden ocurrir en la HD. En HD la sobreactivación de la vía directa lleva a movimientos coreicos. Por lo tanto, bloquear la función de $A_{2A}R$ presináptico puede ser beneficioso por dos motivos: reducir el movimiento y reducir la excitotoxicidad. Por otro lado, la activación del $A_{2A}R$ postsináptico que se encuentra en vía indirecta también potencia la neurotransmisión, pero aquí con una consecuencia de depresión motora. La inhibición del $A_{2A}R$ postsináptico, por tanto, conduce a la activación motora y agrava los movimientos coreicos. Así en la investigación de HD se evaluaron los posibles efectos beneficiosos de los agonistas y antagonistas de $A_{2A}R$, considerando estos receptores como entidades individuales. La posibilidad de que un heterómero de $A_{2A}R$ desempeñe un papel en la enfermedad no se había considerado hasta ahora. Por ello, decidimos explorar una serie de antagonistas de $A_{2A}R$ sintéticos con la esperanza de encontrar preferencias en su afinidad por $A_{2A}R$ formando heterómeros con A_1R o D_2R. En el artículo ***"Striatal Pre- and Postsynaptic Profile of Adenosine A_{2A} Receptor Antagonists"*** hemos estudiado seis compuestos ya conocidos como antagonistas selectivos de $A_{2A}R$: MSX-2, SCH-420814, SCH-442416, SCH-58261, KW-6002 y ZM-241385 para evaluar su afinidad presináptica *versus* postsináptica.

La acción postsináptica de los antagonistas de $A_{2A}R$ se determinó midiendo cambios en la locomoción de ratas (distancia recorrida) después de una aplicación sistémica por vía subcutánea (s.c.) o intraperitoneal (i.p.) de los antagonistas de $A_{2A}R$. El aumento en la locomoción en función de la concentración de antagonistas refleja la potencia de la acción postsináptica de los antagonistas de $A_{2A}R$. Para evaluar la acción presináptica de los antagonistas de $A_{2A}R$ se realizaron experimentos de cuantificación del movimiento de los músculos de masticación inducidos por una estimulación cortical motora en ratas a las que se les administraron los antagonistas de $A_{2A}R$ por vía s.c. o i.p. Aplicando estos dos protocolos, dos antagonistas de $A_{2A}R$: SCH-442416 y KW-6002, mostraron perfiles preferentes pre- o postsinápticos, respectivamente, y cuatro compuestos: MSX-3, SCH-420814, SCH-58261 y ZM-241385, mostraron perfiles mixtos pre-postsinápticos. En un tercer protocolo se consideró una evaluación adicional *in vivo* de la actividad presináptica de los dos compuestos preseleccionados: SCH-442416 y KW-6002, combinando microdiálisis *in vivo* con la estimulación eléctrica cortical. La capacidad de contrarrestar la liberación de glutamato inducida por estimulación cortical es otra manera de reflejar la potencia de la acción presináptica de un antagonista de $A_{2A}R$. El conjunto de los resultados mostró que el KW-6002 tiene un perfil preferente postsináptico, mientras que SCH-442416 tiene un perfil preferente presináptico.

Al menos una parte de las diferencias farmacológicas encontradas entre los antagonistas $A_{2A}R$ evaluados podía explicarse por la capacidad del $A_{2A}R$ formar heterómeros pre- y postsinápticos con A_1R y D_2R, respectivamente. Para llegar a esta conclusión, se desarrollaron una serie de líneas celulares que expresaban de manera estable $A_{2A}R$, A_1R, D_2R solos o para que expresaran heterodímeros A_1R-$A_{2A}R$ o $A_{2A}R$-D_2R. Con los mejores clones seleccionados se realizaron ensayos de unión de radioligandos para determinar las posibles diferencias en la afinidad de estos compuestos para el $A_{2A}R$ presente en heterómero A_1R-$A_{2A}R$ o $A_{2A}R$-D_2R en comparación con $A_{2A}R$ que no formaba heterómeros. La coexpresión de $A_{2A}R$ con A_1R no modificó significativamente la afinidad por los ligandos de $A_{2A}R$ evaluados, mientras que la co-expresión de $A_{2A}R$ con D_2R disminuyó la afinidad por todos los compuestos analizados con la única excepción del compuesto KW-6002. Los cambios estructurales en el

$A_{2A}R$ inducidos por la heteromerización con el D_2R se pudieron detectar no sólo por los antagonistas, sino también por los agonistas. De hecho, la afinidad del agonista de $A_{2A}R$ CGS-21680 también se redujo en las células que co-expresaban D_2R. La unión de SCH-442416 al heterómero $A_{2A}R$-D_2R mostró una fuerte cooperatividad negativa en la unión de radioligandos, fenómeno que no se observó para la unión de SCH-442416 al heterómero A_1R-$A_{2A}R$. La cooperatividad negativa detectada en la unión de SCH-442416 al heterómero $A_{2A}R$-D_2R explica la pronunciada disminución en la afinidad por $A_{2A}R$ en células que expresan $A_{2A}R$-D_2R (valores de B_{50} 40 veces mayores en células que expresan $A_{2A}R$-D_2R comparado con las que expresan el heterómero A_1R-$A_{2A}R$).

A pesar de que los otros compuestos evaluados no mostraron una cooperatividad negativa, se observó una pérdida de afinidad por el $A_{2A}R$ coexpresado con el D_2R. Teniendo en cuenta que estos antagonistas de $A_{2A}R$ se comportaron de manera similar al agonista de $A_{2A}R$ CGS-21680 en cuanto a la unión a los heterómeros A_1R-$A_{2A}R$ y $A_{2A}R$-D_2R, se espera que estos cuatro compuestos compitan de manera igual en la unión con un agonista endógeno a nivel pre- y postsináptico. Esto encajaría con los datos obtenidos *in vivo* que demuestran que estos compuestos tienen un perfil dual pre-postsináptico. Sin embargo, KW-6002 fue el único antagonista cuya afinidad no fue significativamente diferente en las células que expresaban $A_{2A}R$ o sus heterómeros A_1R-$A_{2A}R$ o $A_{2A}R$-D_2R. Por lo tanto, KW-6002 mostró la mejor afinidad relativa por $A_{2A}R$-D_2R de todos los compuestos evaluados, lo que, al menos en parte, puede explicar su perfil preferente postsináptico.

El conjunto de resultados apoya la idea de que los heterómeros de receptores deben considerarse al investigar dianas selectivas para el desarrollo de fármacos. Las razones principales son la muy específica localización neuronal de los heterómeros de receptores (aún más específica que la que tienen los subtipos de receptores), y una afinidad distinta para el ligando en función de su pareja (o parejas de receptores) en el heterómero. En el estriado, $A_{2A}R$ representa una diana de especial interés, potencialmente útil para una variedad de trastornos neurosiquiátricos. Los heterómeros $A_{2A}R$-D_2R y A_1R-$A_{2A}R$ están segregados en diferentes elementos neuronales del estriado. Mientras que los heterómeros $A_{2A}R$-D_2R se encuentran

postsinápticamente en las espinas dendríticas de las MSN de la vía indirecta (Ferré et al, 2007, 2009; Quiroz et al., 2009;. Azdad et al, 2009) los heterómeros A_1R-$A_{2A}R$ se encuentran presinápticamente en los terminales glutamatérgicos que contactan con las MSN de la vía directa (Ciruela et al., 2006; Ferré et al., 2007; Quiroz et al., 2009). Bloquear el $A_{2A}R$ postsináptico en las MSN de vía indirecta debería potenciar activación motora mediada por D_2R, lo que es una estrategia ya utilizada en el desarrollo de fármacos antiparkinsonianos (Jenner et al, 2003; Stacy et al, 2008). Sin embargo, bloquear el $A_{2A}R$ de los terminales glutamatérgicos en las MSN de vía directa podría ser útil en trastornos discinéticos como en la enfermedad de Huntington y tal vez en trastornos obsesivos compulsivos y la adicción a drogas (Quiroz et al., 2009). Así, los resultados descritos dan una explicación mecanística de la actividad antiparkinsoniana ya reportada para el compuesto KW-6002 (Kaasinen et al., 2000; Stacy et al., 2008) y sugieren que el compuesto SCH-442416 podría ser útil en los trastornos de discinesia, trastornos obsesivos compulsivos y en la adicción a drogas. La química médica y modelos computarizados tendrían que ayudar en la comprensión de las propiedades moleculares que determinan los perfiles farmacológicos particulares del SCH-442416 y KW-6002, que podrían considerarse como compuestos de partida para el desarrollo de compuestos más efectivos antidiscinéticos y antiparkinsonianos, respectivamente.

Cuando se considera los $A_{2A}R$ cómo diana en el estriado, los receptores de cannabinoides (CB_1R) también deben ser considerados por dos razones. Primero, los CB_1R son los receptores de neutrotransmisores más abundantes en el en cerebro (Katona et al., 2006) y se encuentran localizados pre- y postsinápticamente. Los nuevos antagonistas de A_{2A} emergentes potencialmente útiles en la enfermedad de Parkinson o Huntington deberían ser analizados con detalle considerando la posible modulación por CB_1R a todos los niveles de interacción para aportar más entendimiento a su futura aplicabilidad. Así en el trabajo: **"*Pharmacological and functional characterization of adenosine A_{2A}-cannabinoid CB_1 receptor heteromers*"** investigamos si la presencia del CB_1R puede modular la farmacología y funcionalidad del $A_{2A}R$.

CB_1R puede heteromerizar con receptores de dopamina y adenosina. Estudiamos las interacciones del CB_1R con receptores de

adenosina A_1 y de dopamina D_1 a través de la técnica de BRET y vimos que se formaron heterómeros A_1R-CB_1R y CB_1R-D_1R en células HEK transfectadas (Anexo al trabajo mencionado). Nuestro grupo había descrito previamente que CB_1R y $A_{2A}R$ forman heterómeros en células cotransfectadas y en el estriado de rata dónde colocalizan en estructuras fibrilares (Carriba et al., 2007). Nos centramos en esta última pareja porqué, aunque se conocía que la activación de los $A_{2A}R$ es necesaria para la señalización de los receptores CB_1 en el neuroblastoma humano dónde estos heterómeros se expresan (Carriba et al., 2007), las características farmacológicas y funcionales de estos heterómeros no habían sido estudiadas. En este artículo, midiendo la activación de proteínas G demostramos que el $A_{2A}R$ y CB_1R en el heterómero están acoplados a proteína G_i. A la vez también observamos un cross-talk sinérgico a nivel de la activación de proteína G cuando ambos receptores se coactivan y observamos que mayoritariamente es el receptor CB_1 el que controla la señalización de ERK 1/2 en el heterómero. A pesar de que demostramos que no hay un efecto alostérico sobre la unión de ligandos en el heterómero $A_{2A}R$-CB_1R encontramos que dos antagonistas específicos de $A_{2A}R$, KW-6002 y VER-7835 perdieron afinidad por los receptores A_{2A} en los heterómeros $A_{2A}R$-CB_1R.

Los cambios en las propiedades bioquímicas de un receptor en el heterómero pueden simplemente depender de la presencia de otra unidad de receptor o de la coestimulación de ambos receptores en el heterómero. Cambios en el acoplamiento a proteína G inducidos solamente por la presencia del otro receptor son comunes en heterómeros formados por receptores de neurotrasmisores. En el heterómero de los receptores de opioides δOR–μOR, los receptores se acoplan a proteínas G diferentes a las asociadas a los receptores expresados individualmente (George et al., 2000; Levac et al., 2000). Hay también ejemplos de cambios en el acoplamiento a proteínas G que dependen de la coactivación de las unidades de receptor en el heterómero. Por ejemplo, el receptor de dopamina D_2 se acopla normalmente a proteína $G_{i\text{-}o}$ pero en el heterómero D_1R-D_2R se acopla a $G_{q/11}$ mientras el D_1R está coactivado (Rashid et al., 2007). Aquí demostramos que los $A_{2A}R$, que es acoplan a la proteína G_s cuando están expresados solos, se acoplan debilmente a proteína G_i en el heterómero $A_{2A}R$-CB_1R, en ausencia o en presencia de los agonistas del CB_1R. Así el heterómero $A_{2A}R$-CB_1R constituye un mecanismo

selectivo mediante cual el receptor de cannabinoides bloquea la producción de AMPc mediada por el $A_{2A}R$.

Frecuentemente, la activación de una unidad de receptor en el heterómero implica un cross-talk intermolecular que involucra cambios conformacionales detectados por la otra unidad del receptor en el heterómero. Estos cambios conformacionales conllevan una modulación en la unión de ligando y/o señalización del otro receptor. En algunos casos, la estimulación de un receptor disminuye la afinidad y señalización del otro receptor como se ha descrito para los heterómeros de los receptores de adenosina A_1-A_{2A} y adenosina y dopamina A_{2A}-D_2, que muestran interacciones alostéricas negativas (Ferré et al., 1991; Hillion et al., 2002; Canals et al., 2003; Ciruela et al., 2006). En otros casos la estimulación de un receptor incrementa la señalización y la afinidad de otro receptor por ligandos endógenos o exógenos como ocurre en el heterómero de receptores de dopamina D_2-somatostatina SST5 dónde la estimulación de los D_2R significativamente incrementa la afinidad de los SST5R por agonistas (Rocheville et al., 2000). Para los heterómeros $A_{2A}R$-CB_1R, la unión de ligandos a los CB_1R no modificó la unión de ligandos a los $A_{2A}R$ y *vice versa*, indicando una ausencia de interacciones alostéricas en este heterómero. No obstante se observó un incremento sinérgico en la activación de proteína G cuando ambos receptores fueron coactivados. Eso está de acuerdo con el hecho de que la activación de los $A_{2A}R$ es necesaria para la señalización del CB_1R en la línea celular derivada del neuroblastoma previamente descrita (Carriba et al., 2007).

Aparte de la señalización mediada por la proteína G, muchos GPCR son capaces de señalizar de manera independiente de la proteína G (Shenoy et al., 2003, 2006; Beaulier et al. 2005; DeWire et al., 2007; Valjent et al., 2000). La fosforilación de ERK 1/2 es una de las vías que puede ser activada por un mecanismo independiente de proteína G y dependiente de arrestina (DeWire et al., 2007). Cuantificando la fosforilación de ERK 1/2 en los heterómeros $A_{2A}R$-CB_1R cuando ambos receptores están coactivados, parece que son mayoritariamente los receptores CB_1 los que controlan la señalización de ERK 1/2. Cómo la fosforilación de ERK 1/2 está relacionada con plasticidad (Schiflett et al., 2011) parece que los cannabinoides controlan los cambios en la plasticidad mediada por ERK 1/2 en células dónde estan estos heterómeros expresados.

Como hemos mencionado más arriba, los antagonistas selectivos de $A_{2A}R$ postsinápticos tendrían que ser útiles en la enfermedad de Parkinson, mientras los antagonistas de $A_{2A}R$ presinápticos podrían ser beneficiosos en los trastornos discinéticos y la addición a drogas (Orru et al 2011a, 2011b; Blum et al., 2003; Armentero et al., 2011). Así pues parece que buscar el papel que tiene la expresión del CB_1R en la afinidad de los agonistas de $A_{2A}R$, SCH-442416 y KW-6002, puede tener una relevancia para fines terapeúticos. Elegimos los antagonistas de $A_{2A}R$, un presináptico, SCH-442416, un postsináptico, KW-6002, y un mixto, VER-7835 (véase el Anexo a este trabajo), y llevamos a cabo experimentos de competición de unión de radioligandos. Encontramos que la afinidad de SCH-442416 es similar por $A_{2A}R$ y para los heterómeros $A_{2A}R$-CB_1R sugiriendo que ambos pueden ser bloqueados presinápticamente con SCH-442416 e indicando que los heterómeros $A_{2A}R$-CB_1R debe ser considerados a la hora de analizar de los efectos discinéticos de SCH-442416. Curiosamente, encontramos un decremento en la afinidad por KW-6002 o VER-7835 uniéndose al heterómero $A_{2A}R$-CB_1R comparado con los $A_{2A}R$. Parece que a nivel postsináptico KW-6002 o VER-7835 bloquearían los receptores A_{2A} más selectivamente que los heterómeros $A_{2A}R$-CB_1R y esto debe tenerse en cuenta a la hora de analizar los efectos antiparkinsonianos de KW-6002.

En resumen, los heterómeros $A_{2A}R$-CB_1R facilitan el entendimiento del papel de las interacciones entre adenosina y cannabinoides en el cerebro, ya que los heterómeros $A_{2A}R$-CB_1R actúan como procesadores computacionales que modulan la señalización celular y pueden ser una diana terapeúticamente relevante en las enfermedades neurológicas que involucran las neuronas estriatales.

4. CONCLUSIONES

Conclusiones derivadas del objetivo 1

- El receptor de adenosina A_{2A} actúa como una proteína de anclaje a la membrana de la ADA. La ADA se une a los homómeros de los receptores A_{2A} e induce una fuerte modificación en su estructura cuarternaria.

- La ADA es un ligando alostérico de los receptores de adenosina A_{2A}. Independientemente de su actividad enzimática, la ADA modula positivamente la unión de agonistas y antagonistas al sitio ortostérico del receptor. Además la ADA aumenta notablemente la señalización del receptor A_{2A} incrementando la fosforilación de ERK 1/2 inducida por el agonista de este receptor.

- En resumen, esta potente regulación ejercida por la ADA podría tener implicaciones importantes para la fisiología y la farmacología de los receptores A_{2A} neuronales que están implicados en la regulación motora ejercida en el estriado.

Conclusiones derivadas del objetivo 2

- Basándonos en los resultados obtenidos *in vitro* e *in vivo*, los antagonistas del receptor A_{2A} ZM-241385, MSX-2, SCH-420814 y SCH 58261 no mostraron ninguna preferencia clara presináptica ni postsináptica.

- Basándonos en los resultados obtenidos *in vitro* e *in vivo* el compuesto SCH-442416 se comporta como un antagonista del receptor A_{2A} preferentemente presináptico, y el compuesto KW-6002 es un antagonista de $A_{2A}R$ preferentemente postsináptico.

- La presencia física del receptor de dopamina D_2 en el heterómero $A_{2A}R$-D_2R induce una fuerte cooperatividad negativa en los receptores A_{2A} que se detecta mediante el compuesto SCH-442416. Esta cooperatividad indica que los homodímeros $A_{2A}R$-$A_{2A}R$ están presentes en los heterómeros $A_{2A}R$-D_2R.

- En resumen, basándose en sus acciones preferenciales pre-*versus* postsinápticas, SCH-442416 puede ser utilizado como un compuesto de partira para el desarrollo de fármacos antidiscinéticos para la enfermedad de Huntington, por su parte KW-6002 puede ser beneficioso en la enfermedad de Parkinson.

Conclusiones derivadas del objetivo 3

- El receptor de adenosina A_{2A} cambia su acoplamiento a proteína G de una G_s estimuladora a una G_i inhibidora cuando heteromeriza con el receptor CB_1 y se observa un cross-talk sinérgico a nivel de activación de proteína G cuando ambos receptores están coactivados.

- El receptor CB_1 controla la señalización de ERK 1/2 en el heterómero $A_{2A}R$-CB_1R.

- El heterómero $A_{2A}R$-CB_1R no muestra efectos alostéricos a nivel de unión de ligandos.

- Dos antagonistas específicos del receptor A_{2A}, KW-6002 y VER-7835 pierden afinidad por los receptores A_{2A} en los heterómeros $A_{2A}R$-CB_1R.

- En resumen, se muestra que los heterómeros $A_{2A}R$-CB_1R constituyen una unidad singular en la señalización mediada por adenosina y cannabinoides, introduciendo diversidad en la señalización a través del receptor $A_{2A,}$ lo que puede ser terapéuticamente relevante en enfermedades neurológicas que involucran neuronas estriatales.

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La Tesis Doctoral de Jana Bakešová, titulada "MODULATION OF ADENOSINE A_{2A} RECEPTOR FUNCTION BY INTERACTING PROTEINS. NEW TARGETS FOR HUNTINGTON'S DISEASE" se presenta como un compendio de publicaciones.

El manuscrito "***A_{2A} adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase***" ha sido publicado en la revista Biochemical Journal con un factor de impacto 5.0, situada dentro del primer cuartil en el área de Bioquímica y Biología Molecular. El manuscrito "***Striatal Pre- and Postsynaptic Profile of Adenosine A_{2A} Receptor Antagonists***" ha sido publicado en la revista Plos One con un factor de impacto 4.4, situada dentro del primer cuartil en el área de Biología. El manuscrito "***Pharmacological and functional characterization of adenosine A_{2A}-cannabinoid CB_1 receptor heteromers***" está en vías de preparación para ser enviado a la revista Journal of Neurochemistry con un factor de impacto de 4.3, situada dentro del primer cuartil en el área de Neurociéncias.

En el trabajo "***A_{2A} adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase***" la doctoranda ha desarrollado y caracterizado el clon estable de células CHO que expresan el receptor de adenosina A_{2A} y ha participado en experimentos de señalización por fosforilación de ERK 1/2. En el trabajo "***Striatal Pre- and Postsynaptic Profile of Adenosine A_{2A} Receptor Antagonists***" la doctoranda ha clonado el vector de expresión pcDNA3.1/Hygro-A_{2A}R, ha desarrollado y caracterizado las líneas estables CHO-A_{2A}, CHO-A_1 y CHO-A_1-A_{2A}, ha llevado a cabo los experimentos de BRET para determinar heterómeros A_1R-A_{2A}R y ha efectuado experimentos de desplazamiento de radioligandos con los antagonistas de receptor A_{2A} de adenosina. En

el trabajo **"*Pharmacological and functional characterization of adenosine A_{2A}-cannabinoid CB_1 receptor heteromers*"** exeptuando los experimentos de determinación de la señalización dependiente de proteína G, ha llevado a cabo la totalidad del trabajo experimental.

El Dr. Eduardo Gracia ha utilizado el manuscrito **"*A_{2A} adenosine receptor ligand binding and signalling is allosterically modulated by adenosine deaminase*"** para la elaboración de su tesis doctoral. Y el Dr. Marcu Orru ha utilizado el manuscrito **"*Striatal Pre- and Postsynaptic Profile of Adenosine A_{2A} Receptor Antagonists*"** para la elaboración de su tesis doctoral.

Barcelona, 15 de marzo 2012.

Dra. Carme Lluís Biset

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