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ABBREVIATIONS LIST|

PD	Parkinson's Disease
CNS	Central Nervous System
SNc	Substantia Nigra Pars Compacta
GPCRs	G-Protein Coupled Receptors
GPCR	G-Protein Coupled Receptor
NPY	neuropeptide Y
BRET	Bioluminescence Resonance Energy Transfer
FRET	Fluorescence Resonance Energy Transfer
GFP	Green Fluorescent Protein
CFP	Cyan Fluorescent Protein
YFP	Yellow Fluorescent Protein
SH2	Src Homology 2
SH3	Src homology 3
PDZ	PSD95/DlgA/Zo-1
YTH	Yeast Two Hybrid
cDNA	Complementary DNA
MYTH	Membrane Yeast Two Hybrid
ATP	Adenosine-5'-triphosphate
TF	Transcription Factor
UBPs	Ubiquitin-specific Processing Proteases
AC	Adenylate Cyclase
DA	Dopamine
D_1R	Dopamine D_1 Receptor
PSD	Postsynaptic Density
cAMP	Cyclic adenosine monophosphate
$_L$-DOPA	L-3,4-Dihydroxyphenylalanine
GPe	Globus Pallidus External Segment
GPi	Globus Pallidus Internal Segment
STN	Subthalamic Nucleus Neurons

SNr	Substantia Nigra Pars Reticulata
MSNs	Medium-Sized Spiny Neurons
SSM	Striatal Spine Module
BBB	Blood-Brain Barrier
PLC	Phospholipase C
mGluR	Metabotropic Glutamate Receptor
MPTP	1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyrimidine
MPEP	2-Methyl-6-(Phenylethynyl)-Pyridine
TGF-β	Transforming Growth Factor-Beta
A_1R	Adenosine A_1 Receptors
PKA	Camp-Dependent Protein Kinase
IP3	Inositol Triphosphate
6-OHDA	6-Hydroxydopamine
CSC	8-(3-Chlorostyryl) Caffeine
SRET	Sequential Ressonance Energy Transfer

ABSTRACT| Parkinson's disease is a chronic and progressive condition of the central nervous system (CNS) that affects around 1% of the world population – it is the second most common neurodegenerative disease among elderly people, being only superseded by Alzheimer's disease. The symptoms of the disease are originated by a selective death of dopaminergic neurons from a particular region of basal ganglia, but the etiology of this event remains unknown. In the last few years, neurotransmission pathways of glutamate, dopamine and adenosine in the CNS have been frequently involved in the pathophysiology of many neurological and psychiatric diseases, indicating that it might be of interest to study the plasma membrane receptors that are involved in these neurotransmission systems. Accordingly, the intention of this project is to determine whether the molecular and functional interactions between glutamate, dopamine and adenosine receptors might play a key role in neurotoxic situations produced in the course of neurodegenerative diseases like Parkinson's.

RESUMO| A doença de Parkinson é uma condição crónica e progressiva do sistema nervoso central (SNC) que afecta cerca de 1% da população mundial – é a segunda doença neurodegenerativa mais comum dentre a população idosa, sendo apenas superada pela doença de Alzheimer. Os sintomas da doença são causados pela morte selectiva de neurónios dopaminérgicos de uma região particular do gânglio basal, embora a etiologia deste evento permaneça desconhecido. Nos últimos anos, as vias de neurotransmissão de glutamato, dopamina e adenosina no SNC têm sido frequentemente envolvidas na patofisiologia de muitas doenças neurológicas e psiquiátricas, indicando que pode haver interesse em estudar receptores da membrana plasmática involvidos nestes sistemas de neurotransmissão. De acordo com isto, a intenção deste projecto consiste em determinar se as interacções moleculares e funcionais entre os receptores de glutamato, dopamina e adenosina podem desempenhar um papel chave em situações neurotóxicas produzidas no decurso de doenças neurodegenerativas como a de Parkinson.

CONTENTS|

Chapter 1

Introduction: a general view

INTRODUCTION: A GENERAL VIEW| “Shaking palsy", a condition where patients gradually lose control over their limbs, was a disease well known to physicians for hundreds of years. The first scientific description of this disease was written in 1817 by Dr. James Parkinson (1755–1824), a man of many interests – besides being an English physician; he also was a geologist, palaeontologist and political activist. For his research on this disease, "shaky palsy" was ultimately renamed Parkinson's disease in his honour (García Ruiz, 2004).

Parkinson’s disease (**PD**) belongs to the family of neurodegenerative disorders with a multifactorial etiology. It is a common, chronic and progressive condition of the Central Nervous System (**CNS**), with an increasing prevalence as the population grows older and is associated with substantial morbidity and increased mortality (Weintraub et al., 2008).

The main symptoms of this disease include bradykinesia (slowness of movement – the extreme of bradykinesia is akinesia, which consists in the inability to initiate a movement), resting tremor, muscular rigidity and abnormalities in posture, which is usually the last symptom to appear, reflecting an advanced stage of the illness. Nonmotor symptoms such as depression, dementia and psychosis are also commonly manifested by PD patients (Weintraub et al., 2008).

The neuropathologic hallmark of PD is a slow progressive degeneration of dopaminergic neurons from the *substantia nigra pars compacta* (**SNc**), resulting in the depletion of striatal dopamine (Stephens et al., 2005). This neurotransmitter regulates excitatory and inhibitory outflow of the basal ganglia, so a neurochemical imbalance (involving neurotransmitters like dopamine, glutamate and adenosine) is induced in PD, which affects the functions controlled by this brain region, namely motor control, cognition, attention and some forms of associative and visual learning (Day et al., 2006; Weintraub et al., 2008).

So far, there is no cure for PD or a known way to prevent this disease, only a number of treatments that temporarily alleviate its symptoms, but that lead to the appearance of adverse secondary effects with the continuity of treatment.

The ineffective protection against neuronal degeneration and the undesirable effects of conventional drugs targeting the dopaminergic system have prompted investigators to search for alternative strategies. The new therapies are expected to act downstream on the loss of

neurons and to modulate basal ganglia circuitry, restoring the neurotransmission balance without inducing adverse side effects. It was in this "searching-for-a-new-approach" scenario, that G protein-coupled receptors emerged as potential candidates.

G protein-coupled receptors (**GPCR**s) have traditionally been thought to act as monomers, but in the last few years several research groups have changed this view, showing that primary functional GPCR signaling unit may after all consist of homodimers, heterodimers, and higher order oligomers on the plasma membrane (Prinster et al., 2005). These new functional entities display different biochemical characteristics from the individual components of the heteromer and may modulate cell signaling and trafficking, as well as change ligand binding characteristics. This new concept might force the re-interpretation of classical GPCRs pharmacodynamics (Ferré et al., 2007a).

Preliminary results recently obtained by our group in this matter, showed that dopamine, glutamate and adenosine receptors do interact in the striatal spine module (further explained bellow), a site that plays a key role in controlling basal ganglia neurotransmission (Ferré et al., 2007b).

Also, functional consequences of these interactions at the levels of ligand binding, GABA release and parkinsonian motor dysfunction have been identified, revealing new properties of these GPCRs and raising the possibility of a new rational approach of drug therapy for PD, targeting these receptors interactions (Popoli et al., 2001; Díaz-Cabiale et al., 2002).

Based on this, the aim of the major project in which the work presented here is inset, is **to establish the implications in Parkinson's disease of functional and molecular interactions of glutamate, dopamine and adenosine receptors in the basal ganglia.** Within this purpose, some specific objectives are expected to be achieved: to identify the molecular determinants of the oligomeric interactions - here, in the case of indirect interactions being found, it is intended to identify, validate and characterize proteins that interact with glutamate, dopamine and adenosine receptors and that may be potential targets for a pharmacological intervention in PD; to study the molecular plasticity of the oligomeric complexes and to establish the functionality of these complexes and their relationship with PD.

Hopefully, the results achieved with this project might be relevant to the development of combined therapies involving glutamate/dopamine/adenosine complexes that would be more efficient in the treatment of Parkinson's patients, comparatively with the results obtained with the actual drugs.

1.1 G PROTEIN-COUPLED RECEPTORS|

In the early 1980's, the primary structure of the first identified G protein-coupled receptor (**GPCR**), rhodopsin, was published. However, it was only in 2001, with the help of the so far sequenced human genome, that a deeper understanding of the presently known 800 members of the superfamily of human GPCRs was accomplished (Lagerström et al., 2008). Two years later, on the basis of phylogenetic criteria, these receptors were divided into five main families: Rhodopsin, Adhesion, Frizzled/Taste2, Secretin and Glutamate (Fredriksson et al., 2003).

All GPCRs are structurally characterized by seven hydrophobic transmembrane α-helical domains, which are connected by three extracellular and three intracellular loops, while the amino-terminal is extracellular and the carboxyl-terminal is intracellular (Prinster et al., 2005; Szidonya et al., 2008; Gurevich et al., 2008). The other characteristic common to all GPCRs is their ability to interact with and activate a heterotrimeric G protein.

1.1.1 GPCRs SIGNALING| The interaction of an agonist with a GPCR binding site induces or stabilizes a conformational change in the receptor's transmembrane domains – this alteration allows the receptor to associate with heterotrimeric G proteins and initiate a signaling cascade that may lead to a wide range of biological responses (figure 1) (Prinster et al., 2005).

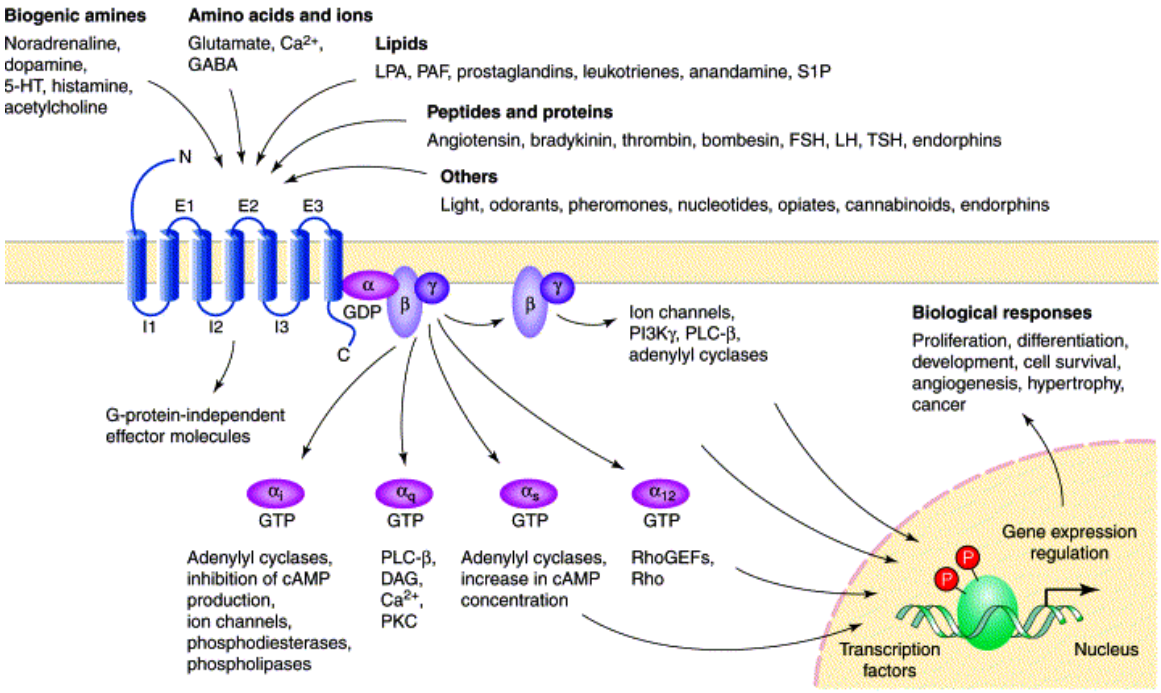


Figure 1 - **Diversity of G protein-coupled receptors (GPCR) signaling pathways.** A wide variety of ligands use GPCR to stimulate membrane, cytoplasmic and nuclear targets through heterotrimeric G protein-dependent and –independent pathways. Such signaling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. Abbreviations: DAG, diacylglycerol; FSH, follicle-stimulating hormone; GEF, guanine nucleotide exchange factor; LH, luteinizing hormone; LPA, lysophosphatidic acid; PAF, platelet activating factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; S1P, sphingosine-1-phosphate; TSH, thyroid-stimulating hormone. (Taken from Marinissen and Gutkind, 2001)

GPCRs interact with heterotrimeric G proteins composed of α, β and γ subunits that are GDP bound in the resting state. Upon ligand binding, a conformational change of the GPCR occurs, leading to the subsequent exchange of GDP for GTP in the G protein α-subunit and to the dissociation of this GTPbound subunit from the Gβγ dimer (Marinissen and Gutkind, 2001). Each of these entities can modulate several signaling pathways that include, among others, stimulation or inhibition of adenylate cyclases, activation of phospholipases, as well as regulation of potassium and calcium channel activity (Hamm, 1998).

The exposure of GPCR to agonists often results in the rapid attenuation of receptor responsiveness. This receptor desensitization process is the result of a combination of different mechanisms that include 1) the uncoupling of the receptor from heterotrimeric G proteins caused by 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 3) the down-regulation of the total number of receptors in the cell, mediated by mechanisms that 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 extent of receptor desensitization is regulated by a number of factors that include receptor structure and cellular environment.

The importance of this type of receptors and their signaling pathways is illustrated by the fact that these receptors mediate the neural, endocrine and paracrine signaling, and play a pivotal role in most senses, such as vision (due to opsin, of the Rhodopsin family), smell (through the olfactory receptors) and taste (participation of many Glutamate family members) (Lagerström et al., 2008). Furthermore, about half of the drugs used in clinical practice act through the direct or indirect modulation of GPCRs (Prinster et al., 2005; Szidonya et al., 2008; Gurevich et al., 2008).

1.1.2 MULTIMERIZATION OF GPCR| In the early 1980s Agnati and Fuxe introduced the concept of intramembrane receptor-receptor interactions and provided the first experimental observations for their existence in crude membrane preparations from various brain regions and from spinal cord (Agnati et al., 1980). After this, the demonstration of the existence of receptor-receptor interactions mainly by using radioligand-binding approaches came up quickly, for instance the existence of interactions between neurotensin and dopamine D_2 receptors or neuropeptide Y (**NPY**) and α2 adrenergic receptors (for review see Agnati et al., 2003). In the middle eighties, some studies using photo-affinity labeling, radiation inactivation, cross-linking experiments and hydrodynamic analysis held up the dimerization idea (Avissar et al., 1983; Fraser et al., 1982; Herberg et al., 1984; Peterson et al., 1986), but it was not until the 1990s when first evidences of GPCR homodimerization were accomplished by means of electrophoretic mobility, co-immunoprecipitation and transcomplementation assays. By using the SDS-PAGE and Western blot approach it was possible to visualize GPCR homodimers as they were resistant to denaturing conditions (e.g. SDS and heat treatment) and the analyzed receptors presented twice the expected molecular weight.

Still in the middle nineties, this approach was used to demonstrate the existence of adenosine A_1 and dopamine D_2 receptors homodimers in brain extracts, providing the first compelling evidence for the GPCR oligomerization phenomenon under physiological conditions (Ciruela et al., 1995; Zawarynski et al. 1998). Since then, there has been an enormously growing number of studies and observations supporting this phenomenon of dimerization for many receptors. Nowadays, mainly due to the development of new biophysical techniques like Bioluminescence and Fluorescence Resonance Energy Transfer (**BRET** and **FRET**, respectively), it is clear that, in living cells, most GPCR seem to have the tendency to dimerize with themselves – homodimerization – while others seem to be associated with different receptors – heteromerization (Agnati et al., 2003; Prinster et al., 2005; Franco et al., 2007)

Due to its relevance to this project, the FRET technique will be briefly introduced.

1.1.2.1 The Fluorescence Resonance Energy Transfer Technique| In the late 1940s, Theodor Förster formulated the principle of FRET, Fluorescence Resonance Energy Transfer (Förster, T., 1948). This phenomenon may occur when two different fluorescent chromophores (donor and acceptor) with overlapping emission/absorption spectra are separated by a suitable orientation and a distance in the range 10–80 Å (Truong et al., 2001). The principle is depicted in figure 2, using as examples the now widely used Green Fluorescent Protein (**GFP**) variants - Cyan Fluorescent Protein (**CFP**) and Yellow Fluorescent Protein (**YFP**). As shown, excitation of CFP, which is best achieved with light of a wavelength of 436 nm, normally leads to emission of the typical cyan fluorescence, with a peak at a wavelength of 480 nm. If, however, a second fluorophore such as YFP is close by, then – under appropriate circumstances – energy can be transferred to this molecule and a yellow emission with a peak wavelength of 535 nm occurs. In this transfer of energy, CFP acts as the donor and YFP as the acceptor (Lohse et al., 2007).

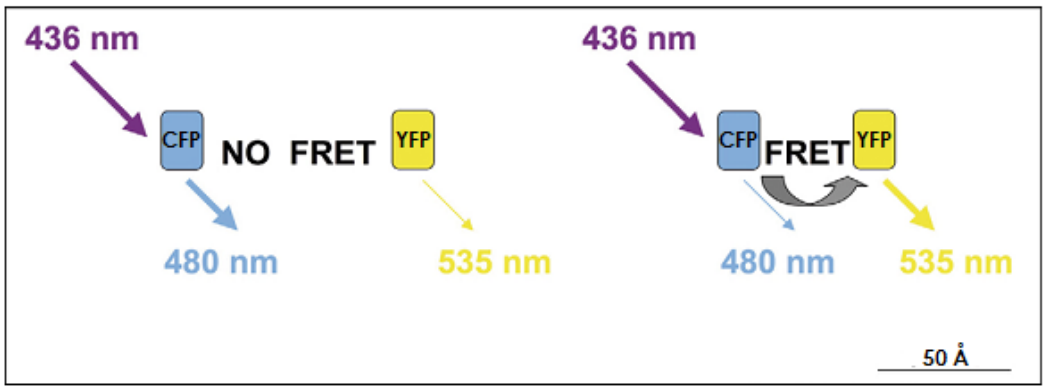


Figure 2 - Principle of FRET using CFP and YFP as a donor–acceptor pair. FRET occurs when the donor (CFP) is close enough (<80 Å) to the acceptor (YFP), so that upon excitation of CFP (with light at a wavelength of 436 nm), energy is transferred to YFP and causes yellow emission (at 535 nm), while the cyan emission (at 480 nm) decreases. (Adapted from Lohse et al., 2007)

From the early 1970s until over the past decade, FRET was essentially a spectroscopic ruler, used as a tool for structure studies (Stryer, 1978). This "structure elucidator" has, however, lost most of its value in recent years with the appearance of techniques such as X-ray crystallography or NMR spectroscopy. Recently, though, the introduction of GFP to FRET-

based imaging microscopy brought a whole new importance to this technique, which is now a unique sensitive probe of protein–protein interactions and protein conformational changes in living cells. This reinvented technique marked the beginning of real-time imaging of dynamic molecular events *in vivo*, providing researchers with crucial insight into the biological mechanisms and the physiological functions of a cell (Bastiaens et al., 2001).

The donor and acceptor fluorophores that allow FRET to occur can be placed either in two different locations of the same protein (*intramolecular* FRET) or on two separate proteins (*intermolecular* FRET) (figure 3). The first strategy records conformational changes that result in changes in either the distance or the orientation of the fluorophores. The latter strategy, on the other hand, allows the monitoring of protein–protein interactions, since FRET will occur when the two proteins carrying the different fluorescent molecules associate, and will be reduced or disappear when the two proteins dissociate (Lohse et al., 2007)

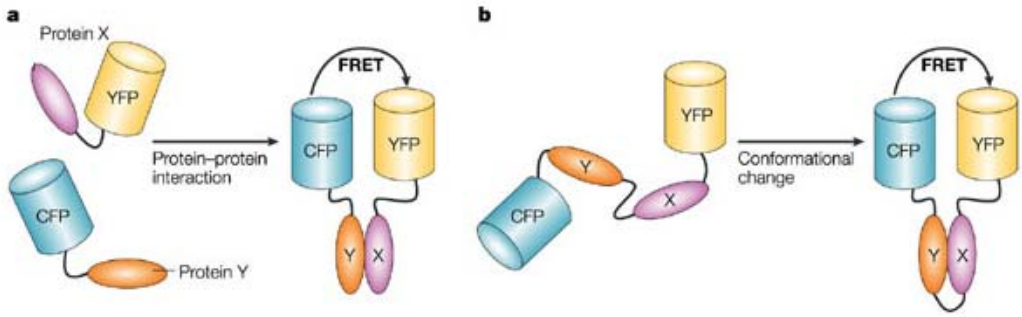


Figure 3 – Intermolecular and intramolecular FRET. **a)** An intermolecular FRET-based probe can occur between one molecule (protein Y) fused to the donor (CFP) and another molecule (protein X) fused to the acceptor (YFP). Upon proteins interaction, the fluorophores are brought into close proximity, causing an increase of FRET efficiency. When they dissociate, FRET diminishes. **b)** An intramolecular FRET-based probe may occur when both fluorophores are fused to the same host molecule, which undergoes a conformational change - for instance, a transition between 'open' and 'closed' conformations. (Taken from Zhang et al., 2002)

In conclusion, fluorescent fusion protein-based FRET is an invaluable tool in the detection of protein-protein interactions and protein conformational changes at a single-cell level, unveiling both temporal and spatial information on molecular structures in living cells. The

new research opportunities are immense: it brings the possibility of establishing the relationship between a conformational change and its kinetics with specific downstream signaling. In fact, in a not far future, it is likely that this technique will allow the simultaneous observation of a series of reversible molecular processes in living cells, by monitoring, for instance, a signaling cascade using different markers. Also, it allows the localization of receptors signaling – particularly, specific sites of synaptic activity might be identified, or compartmentalization of receptor activation and second messenger production might become visible. At last, the ultimate and more ambitious objective will be to transfer the applications of this FRET imaging microscopy into more physiological settings, including ultimately true *in vivo* imaging – something that giving the techniques available today is not so far away from reality and that will certainly unveil many of the aspects that lay behind receptor signaling complexity (Lohse et al., 2007).

1.1.3 MECHANISMS OF GPCR OLIGOMERS FORMATION| When the time comes of explaining the GPCR oligomerization phenomena, two possibilities can be considered: either the interaction occurs by the direct contact between the receptors, or it happens in an indirect way, implying the participation of a third protein that establishes the "bridge" between receptors.

1.1.3.1 Direct Interactions| Due to the great structural complexity that characterizes the GPCRs superfamily, a general and unifying model explaining the dimerization mechanism of these receptors does not exist. Among many other theories, there are currently two prevailing mechanisms that try to explain the formation of GPCR dimers and higher order oligomers: on the one hand there is the "**contact dimer**" or "lateral packing" model, in which GPCRs directly contact each other via interaction sites on the exterior of the transmembrane domains, leading to the formation of receptor oligomers – in this model the integrity of the receptor monomers is maintained. On the other hand, there is the "**domain-swapping**" model, in which two independent folding units (transmembrane domains) of the

receptor separate and recombine between GPCR monomers, which integrity is altered in this mechanism (Szidonya et al., 2008).

In theory, both models of dimerization may co-exist and explain the formation of higher-order oligomeric structures. However, from an energetic output point of view, the first model is more likely to happen, since the formation of contact dimers does not require the rearrangements of the transmembrane domains between the receptors (Szidonya et al., 2008).

1.1.3.2 Indirect Interactions| Protein-protein interactions are in the base of the formation of macromolecular complexes responsible for all biological structures, playing a major role in cellular growth and development. This fact makes only natural that the domains responsible for these interactions, such as the SH2 (*Scr-homology* 2), SH3 or PDZ, have been highly conserved throughout evolution. Most of GPCRs contain sequence motifs capable of interacting with these domains giving them the potential to interact with a wide range of other proteins. The topology of GPCR creates several potential regions for these interactions to occur. On the extracellular side, only the N-terminal portion is long enough to sustain possible interactions. On the intracellular face of the receptors, however, both the C-terminal tail and the third intracellular loop can be of considerable length, drowning the attention of interaction studies to these two regions (figure 4).

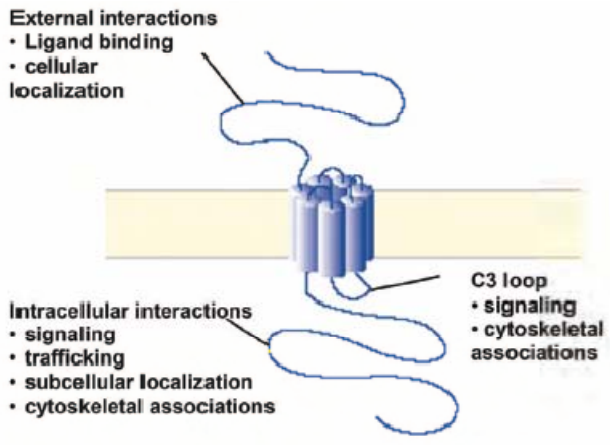


Figure 4 - GPCR regions with identified interacting proteins. The generic functions of proteins that interact with these regions of GPCRs are indicated.

These indirect interactions are the basis of a protein network associated with GPCR, which includes scaffolding (or scaffold) proteins containing one or more interacting domains, signaling proteins and proteins of the cytoskeleton. These proteins normally develop important functions: they can act as an anchor, localizing GPCR to plasma membrane and allowing the formation of macromolecular complexes, or they can work as signaling modulators, facilitating or inhibiting the receptors signal transduction and/or their internalization.
Most of the proteins that were proved to interact with the receptors were identified by the Yeast Two Hybrid system, briefly enlightened bellow.

1.1.3.3 Yeast Two Hybrid System| The rationale of the Yeast Two-Hybrid (**YTH**) system, a powerful tool in the study of protein-protein interactions, relies on the fact that in most eukaryotic transcription factors, the activating and binding domains are modular and can function in close proximity to each other without direct binding. This means that even if the transcription factor is split into two fragments, it can still activate transcription when the two fragments are indirectly connected. As a result, if a screening is to be performed in order to identify which proteins expressed from a complimentary DNA (**cDNA**) library interact with a protein of interest (bait), the bait is fused to a DNA-binding domain, while the cDNA library-encoded proteins are fused to a transcriptional activation domain. When a protein encoded by the cDNA library binds to the bait, meaning that they interact, both activities of the transcription factor are reunited and transcription from a reporter gene is started (Miller et al., 2004).

Despite of the large amount of information that can be extracted from the YTH approach, this technique has a key technical limitation: due to the complex chemical properties of membrane-protein interactions, namely their hydrophobic nature, in a screening using a GPCR, for instance, only the soluble domains of the receptor (e.g. intracellular loops) could be used in this two-hybrid assay, so merely the interactions between soluble proteins would be detected, excluding all the membrane proteins that could potentially be interacting with the receptor too - this is a major handicap when studying the partners of a membrane protein such as the GPCR (Fetchko et al., 2004).

Due to the pivotal role of membrane proteins in many cellular functions and biological processes that include cell signalling, transport of membrane-impermeable molecules, cell-to-cell communication, and cell adhesion, there is a strong demand from the scientific community to gain further insight into pathways and interactions involving these proteins. Also, because of their extracellular accessibility, membrane protein-protein interactions might represent potential drug targets. In order to understand the ground rules that govern these processes, the identification and the study of the intervenient partners that interact with certain proteins of interest is necessary.

Taking the limitations of the classic YTH approach into account, the group of Dr. Igor Stagljar (Stagljar et al., 1998) has developed a new genetic method for the *in vivo* detection of membrane protein interactions in *Saccharomyces cerevisiae.* This new strategy uses the split-ubiquitin approach first described by Johnsson and Varshavsky (1994) which is based on the detection of the *in vivo* processing of a reconstituted split ubiquitin (figure 5). Ubiquitin is a small, conserved protein of 76 aminoacids involved in protein degradation (Glickman et al., 2002). The attachment of this molecule to a cellular protein represents a signal for the degradation of the target protein by the 26S proteasome, an ATP-dependent multisubunit protease. The new mentioned system is based on the ability of the N- and C-terminal halves of ubiquitin, Nub and Cub, to reassemble into a quasi-native ubiquitin (split-ubiquitin). In this assay, the halves of ubiquitin are fused to two interacting proteins, X and Y, at least one of which is membrane bound (figure 5). Upon interaction of these two proteins, the halves of ubiquitin are brought together, and the transcription factor that is fused to the membrane protein is cleaved and released, after which it enters the nucleus and activates the transcription of reporter genes, enabling easy detection (Thaminy et al., 2003).

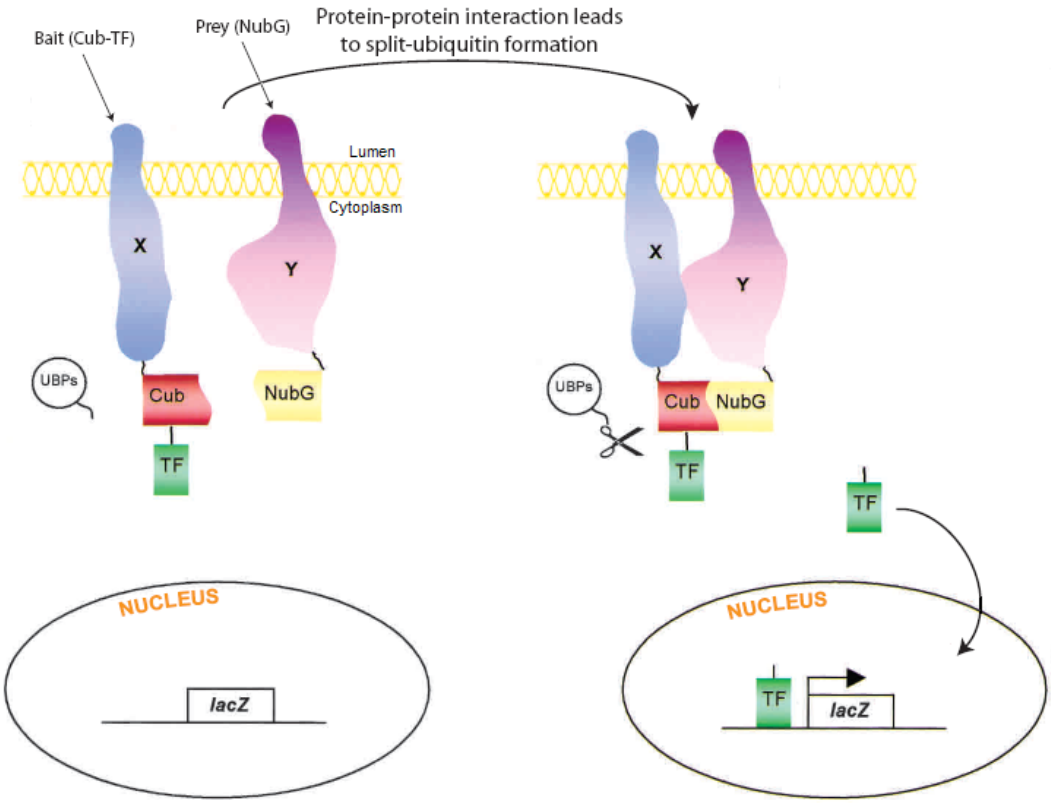


Figure 5 - Outline of the membrane-based YTH system. A membrane bait protein of interest X is fused to Cub followed by an artificial transcription factor (TF), while another membrane (or cytoplasmic) protein Y is fused to the NubG domain (Y-NubG). In case interaction between X and Y occurs, it results in the assembly of split-ubiquitin and the proteolytic cleavage of the TF by ubiquitin-specific proteases (UBPs), present in the cytosol and nucleus of all eukaryotic cells, which allows TF to enter the nucleus, leading to the activation of the yeast reporter genes. (Adapted from Thaminy et al., 2003)

In this manner, and in contrast to the conventional YTH system in which interactions occur in the nucleus, the membrane-based YTH (**MYTH)** system represents an *in vivo* assay that detects interactions between membrane proteins in their natural environment. It is also possible to use this technology as a screening system for membrane proteins, allowing the identification of novel binding partners of a membrane protein of interest. Giving the extracellular accessibility of these proteins, the split-ubiquitin membrane YTH technology beholds a major potential for its application in the pharmacological field.

1.1.4 FUNCTIONS OF GPCRs MULTIMERIZATION| When a ligand binds to a receptor unit in a heteromer, that conformational change may be sensed by another unit in the complex, resulting in an intermolecular crosstalk that leads to a different cell signaling from that obtained with the activation of the single monomer. An example of this transmodulation phenomenon can be found in heteromers of A_{2A} and D_2 receptors, where the dopamine receptor ligands induce the heterologous desensitization of the adenosine receptor (Hillion et al., 2002). This property, led Ferre and his group to classify the neurotransmitter receptor heteromers as "processors of computations", able to modulate cell signaling (Ferré et al., 2007a).

Another common feature of these "processors", are the changes in G protein coupling, dependent on coactivation of the receptor units in the receptor heteromers. For instance, D_2R usually couples to $G_{i\text{-}o}$ proteins, but in the D_1R–D_2R heteromers, when D_1R is coactivated, it switches to $G_{q/11}$ (Ferré et al., 2007a).

It is also important to notice that heteromerization has been related to change the rate of receptor internalization after the agonist binding, and to be able to modify the lateral diffusion of receptors in the plasma membrane (Ferré et al., 2007a). We may then say that the third main function of neurotransmitter receptor heteromers as processors of computations that modulate cell signaling, is the ability to alter receptor trafficking.

All in all, although many aspects of these interactions are not completely understood, it is now widely accepted that oligomerization of some GPCRs has an important influence in its expression and function, namely in the agonist binding and efficacy, G-protein selectivity and receptor trafficking (Szidonya et al., 2008).

These oligomeric interactions between GPCRs have some important physiologic implications: for instance, some GPCRs absolutely require assembly with a specific partner to be able to achieve surface expression and functional activity; otherwise, when expressed alone, these receptors are completely non-functional. This is the case of the metabotropic receptor GABAB: when the isoform GABAB1R is expressed alone, it stays intracellularly retained in the endoplasmic reticulum. On the other hand, when only the isoform GABAB2R is expressed, it reaches the plasma membrane but it cannot be activated by

GABA nor initiate signal transduction. Only when both receptors are co-expressed is the oligomer delivered to cell surface and fully functional (White et al., 1998).

1.2 DOPAMINE, GLUTAMATE AND ADENOSINE G PROTEIN-COUPLED RECEPTORS AND PARKINSON's DISEASE|

More than 90% of GPCRs are expressed in the brain, usually in four or five different anatomic regions, being the expression of some of them often restricted to this tissue (Vassilatis et al., 2003). The combination of RT-PCR and *in situ* hybridization techniques has demonstrated that these receptors expression shows differential patterns, which suggests that the combination of some of these receptors might regulate different physiological processes. An example of this differential location of a determined combination of GPCRs is the case of the adenosine A_{2A}, dopamine D_2 and glutamate $mGlu_5$ receptors, all of them localized in the dendritic spines of striatopallidal GABAergic neurons where they play a fundamental role in the functioning of these neurons (Cabello et al., 2009).

1.2.1 DOPAMINE| Dopamine (**DA**), a hormone and the precursor of noradrenaline and adrenaline, is a neurotransmitter in the mammalian brain, where it interferes in a variety of functions such as motor control, behavior and cognition, motivation and reward, food intake and endocrine regulation (Missale et al., 1998). As a hormone, DA inhibits the release of prolactin from the anterior lobe of the pituitary (Ben-Jonathan et al., 2001).

In the late 1970s, DA receptors were divided into two distinct populations, on the basis of pharmacological and biochemical evidence: the D_1 receptors (**D_1R**), associated to the stimulation of adenylate cyclase (**AC**), and the D_2 receptors (**D_2R**), negatively coupled to this effector (von Lubitz et al., 1999).

Later, after the introduction of gene cloning techniques, three novel DA receptors subtypes have been identified - these have been called D_3 (**D_3R**), D_4 (**D_4R**) and D_5 (**D_5R**) - and then were included in the previously established groups, according to if they were positively (D_5) or negatively (D_3 and D_4) coupled to AC (Missale et al., 1998).

Nowadays, it is generally accepted that D_1 and D_5 receptors are members of the so called **D1-like family** of dopamine receptors, whereas the D_2, D_3 and D_4 receptors are members of the **D2-like family** (figure 6) (Missale et al., 1998).

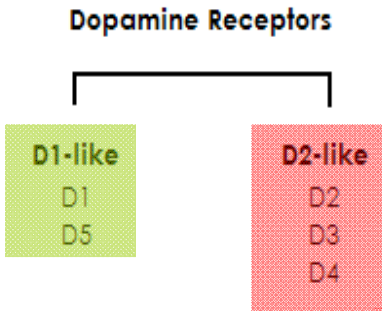


Figure 6 - Scheme of dopamine receptors

All of these receptors are coupled to a G protein and reveal seven transmembrane domains, sharing most of their structural characteristics. These receptors are located not only within the postsynaptic density (PSD) but also extrasynaptically (Missale et al., 1998).

The D_1-like receptors are characterized by a short third loop, as in many receptors coupled to G_s protein, which subsequently activates AC**,** increasing the intracellular concentration of cAMP. This increase in neurons is typically excitatory and can induce an action potential by modulating the activity of ion channels (Missale et al., 1998). Although this type of receptors has little or no effect on locomotor activity when expressed alone, they interact synergistically with D_2R, determining forward locomotion. This activity was shown to decrease when the DA release diminished, which might happen when, for instance, the D_2 autoreceptors are activated (Jackson et al., 1994).

Concerning the D_2–like receptors, they have a long third intracellular loop, a typical feature of G_i proteins-coupled receptors (Missale et al., 1998). The interaction between these and $G_{\alpha i}$ proteins, besides activating K^+ channels, causing cell hyperpolarization, it leads to an increase of phosphodiesterase activity, which breaks down cAMP, producing an inhibitory effect in neurons. This group of DA receptors has been the subject of extensive studies, which have demonstrated its participation in numerous important physiological functions, including the synthesis and release of pituitary hormones and control of motor activity. For that reason, they represent the major target of antipsychotic drugs and are involved in various neuropathological conditions such as Tourette's syndrome, drug addiction and PD. (Vallone et al., 2000). In fact, this group of DA receptors has demonstrated a notable antiparkinsonian effect – their administration in the early stages of the disease originates

significantly less dyskinesias than $_{L}$-Dopa, the drug commonly used in PD therapy. For this reason, D_2 family of receptors have been used in clinics for over three decades (Linazasoro et al., 2008).

Despite the advances made in the study of signal transduction, shading a light on how the activation of DA receptors converts into changes in neuronal function, major issues remain to be determined. Namely, the knowledge of the physiological function of some DA receptors, particularly of those discovered later, remains very incomplete. That lack of information might be due to the fact that there are still no pharmacological agents selective for each receptor subtype (Missale et al., 1998). Once that part is accomplished, the next step would be to develop new specific therapeutic agents that could be used in the treatment of diseases directly related to DA disturbances, like is PD.

In order to better understand how the selective death of dopaminergic neurons in PD leads to the movement disorders, it is important to gain a clear view of the function and circuitry (in normal and parkinsonian states) of basal ganglia.

1.2.1.1 Basal Ganglia| The basal ganglia are a group of interconnected nuclei in the brain that are associated with a variety of functions, namely the control of voluntary movements. It includes: ***caudate, putamen, nucleus accumbens, globus pallidus, substantia nigra*** **and** ***subthalamic nucleus*** (figure 7) (Rouse et al., 2000).

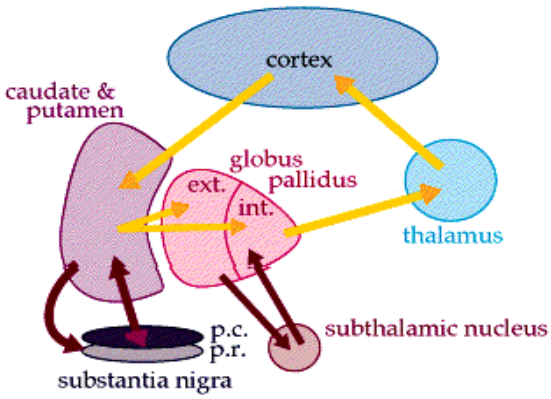


Figure 7 - Connections of the basal ganglia.

The two nuclei *caudate* and *putamen* together comprise what is called the ***striatum***. This structure receives most of the input from cerebral cortex, being the major point of entry of information into the basal ganglia, and it is very important in functions such as motor control and habit learning. The *caudate* and *putamen* are reciprocally interconnected with the *substantia nigra*, but send most of their output to the *globus pallidus* (Rouse et al., 2000; Schiffmann et al., 2007).

The *globus pallidus* can be divided into two parts: the *globus pallidus externa* (**GPe**) and the *globus pallidus interna* (**GPi**) – the last sends the major inhibitory output from the basal ganglia back to *thalamus*. Both receive input from the *caudate* and *putamen*, and both are in communication with the *subthalamic nucleus* (**STN**) (Schiffmann et al., 2007). Overactivity and burst firing of STN has been repeatedly observed in PD animal models as well as in human patients with the pathology (Blandini et al., 2000; Marino et al., 2003).

The substantia nigra can also be divided into two parts: the *substantia nigra pars compacta* (SNc) and the *substantia nigra pars reticulata* (SNr). The first receives input from the *caudate* and *putamen*, and sends back the information. SNc is a local of DA production and is crucial for normal movement - in PD this structure is damaged. The second also receives input from the two nuclei, but sends it outside the basal ganglia to control head and eye movements (Blandini et al., 2000).

The circuitry starts when information from intrinsic and extrinsic sources is provided to striatum. Here, ninety percent of neurons are medium-sized spiny neurons (**MSNs**) and receive two main striatal inputs with distinct roles: the glutamatergic afferents (from cortical, limbic and thalamic areas), responsible for triggering the striatal circuits, and the dopaminergic afferents (from the SNc), with a more modulatory role – together, the two neurotransmitters' afferents in contact with the dendritic spine of the MSN, form the most common striatal spine module (**SSM**), which is considered to be the minimum portion that operates as an integrative independent unit in the striatum (figure 8) (Ferré et al., 2007b; Schiffmann et al., 2007).

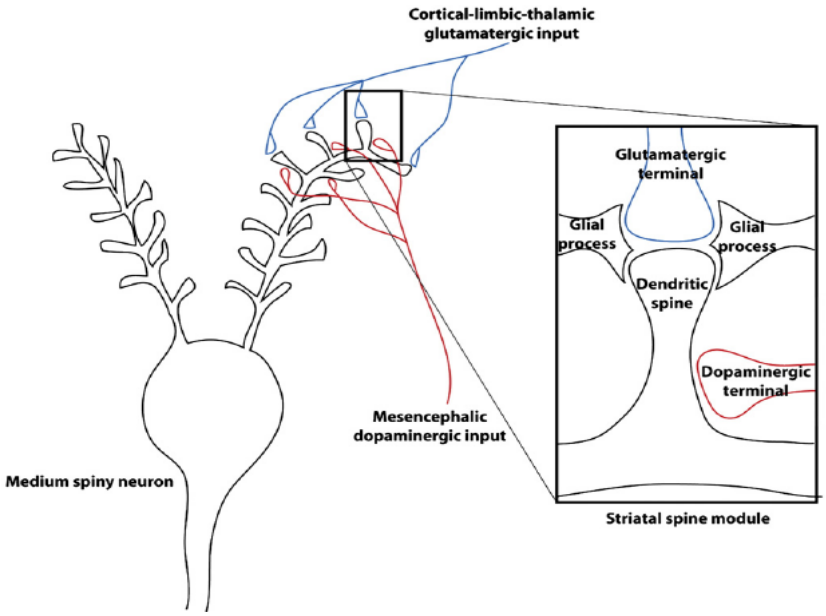


Figure 8 - Striatal GABAergic neuron, also known as striatal medium spiny neuron (MSN), is the most common neuron in the striatum and it receives two main inputs coming from different regions of the brain: glutamatergic afferents from cortical, limbic and thalamic areas, and dopaminergic afferents from the *substantia nigra pars compacta* (SNc). The magnified scheme shows the most common striatal spine module (SSM), which includes the dendritic spine of the MSN and glutamatergic and dopaminergic terminals, which make synaptic contact with the head and neck of the dendritic spine, respectively (Ferré et al., 2007b).

The morphological organization of these inputs is consistent with their functional roles: relatively to the dendritic spine, the glutamatergic terminal makes synaptic contact with the head while the dopaminergic terminal makes synaptic contact with the neck (Schiffmann et al., 2007). This close anatomical localization suggests that DA released from the SNc terminals is in a privileged position to exert a modulatory effect on glutamatergic neurotransmission coming from the cortex and thalamus (Tang et al., 2001; Schiffmann et al., 2007).

From the striatum, about half of the MSNs project directly to the basal ganglia output nuclei (GPi plus SNr), representing the inhibitory direct pathway, and another half of the striatal neurons participate in a polysynaptic projection, giving rise to the excitatory indirect pathway. In this last case, in a physiological situation, neurones from the striatum inhibit GPe which projections would, if they were not inhibited, exert an inhibitory effect on STN, a glutamatergic nucleus that sends excitatory projections to the output nuclei. As a result, when the indirect pathway is activated, the increased inhibition of GPe leads to the disinhibition of STN, resulting in the excitation of output nuclei (figure 9) (Rouse et al., 2000; Blandini et al., 2000; Marino et al., 2003).

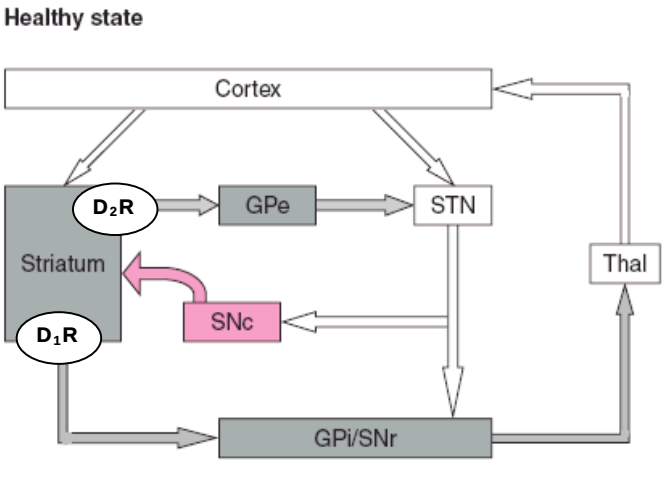


Figure 9 - Simplified schematic diagram of the basal ganglia circuit in a physiologic situation. Inhibitory GABAergic projections are indicated by filled arrows while excitatory glutamatergic projections are indicated by open arrows. The pink box and arrow indicates the modulatory dopaminergic nigrostriatal pathway. In normal conditions, dopamine stimulates the direct striatonigral pathway via D_1 receptors and inhibits the indirect striatopallidal route via D_2 receptors. **GPe** = *globus pallidus* external segment; **GPi** = *globus pallidus* internal segment; **SNc** = *substantia nigra pars compacta*; **SNr** = *substantia nigra pars reticulata*; **STN** = *subthalamic nucleus*; **Thal** = *thalamus*. (Adapted from Marino et al., 2003)

A delicate balance between inhibition of output nuclei through the direct pathway and excitation through the indirect pathway, is believed to be essential for normal motor function. When this equilibrium is disturbed, a variety of motor disorders such as PD may emerge (Blandini et al., 2000).

Given the importance of such mechanism, it is highly regulated by dopaminergic neurons coming from the SNc. This DA input to the striatum modulates the direct inhibition and indirect excitation separately, due to the presence of two types of DA receptors on MSNs. Thus, D_1 receptors are primarily expressed on MSNs that project directly to the output nuclei, while D_2 receptors essentially modulate the indirect pathway (Jackson et al., 1994; Day et al., 2006). Regarding the importance of this last group of receptors, in an *in vivo* study using two animal models of PD, it was shown that synaptic efficacy is dependent of DA and the respective D_2R, implying that these receptors are able to produce changes in striatal functions such as motor control (Tang et al., 2001).

1.2.1.2 Dopamine and Parkinson's Disease| Although the quantity of neurons that use dopamine as a neurotransmitter are few, this system of neurotransmission plays a very important role in many functions. Accordingly, dysregulation of DA neurotransmission in the CNS has been implicated in a variety of neuropsychiatric disorders, such as Tourette's syndrome, schizophrenia, drug and alcohol dependence (Kienast et al., 2006), attention-deficit hyperactivity disorder (Díaz-Heijtz et al., 2006) and, as expected, Parkinson's disease (Fuxe et al., 2006).

PD is a common neurodegenerative disorder primary characterized by the loss of DA-containing neurons in the SNc that innervate the striatum (von Lubitz et al., 1999; Rouse et al., 2000; Marino et al., 2003; Day et al., 2006). This deficient level of striatal dopamine directly affects the circuits of basal ganglia: while the inhibitory direct pathway suffers a decrease, the indirect pathway is enhanced, which leads to the disinhibition of STN and, subsequently, to the excessive drive from this structure (Marino et al., 2003; Stephens et al., 2005; Bové et al., 2005). In addition to its main targets, the STN also provides excitatory innervation to the dopaminergic neurons in the SNc. Therefore, there is a good chance that the disinhibition of STN neurons that occurs as a consequence of dopamine depletion may

also contribute to the progression of PD by glutamatergic overstimulation of SNc neurons, leading to a vicious cycle in which STN overactivity and nigral damage support each other (Rodriguez et al., 1998). The net result of these alterations is an excessive excitatory glutamatergic basal ganglia outflow, which is believed to underlie the severe motor disturbances observed in the parkinsonian state (figure 10) (Marino et al., 2003).

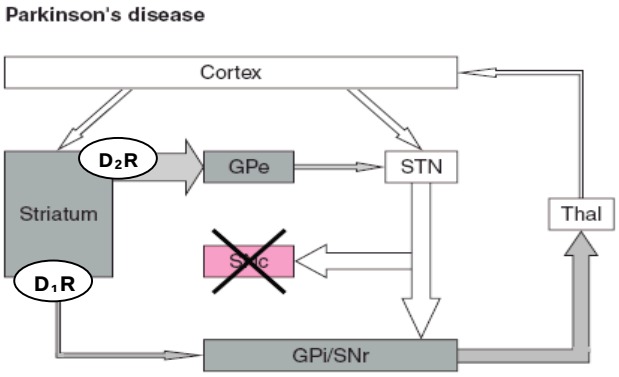


Figure 10 - Simplified schematic diagram of the basal ganglia circuit in the parkinsonian state. Inhibitory GABAergic projections are indicated by filled arrows, excitatory glutamatergic projections are indicated by open arrows. Thickness of arrows indicates the degree of activation of the pathway. The dopamine depletion of the striatum results in an imbalance between the direct and indirect output pathways. More concretely, there is an inhibition of direct GABAergic striatonigral pathway, excessive activation of the indirect striatopallidal pathway, disinhibition of the STN and increased excitation of GPi/SNr neurons. The subsequent excessive inhibition of thalamocortical neurons produces the characteristic reduction of movements of PD. **GPe =** *globus pallidus* external segment; **GPi =** *globus pallidus* internal segment; **SNc** = *substantia nigra pars compacta*; **SNr =** *substantia nigra pars reticulata*; **STN** = *subthalamic nucleus*; **Thal** = *thalamus*. (Adapted from Marino et al., 2003)

Many studies have been carried out in an attempt to understand the mechanisms that are linking DA depletion, loss of dendritic spines from MSNs (Stephens et al., 2005), changes in indirect pathway and the alterations produced in motor function. One worth mentioning was undertaken by Day, M. et al. (2006). Using multiphoton imaging approaches, these authors showed that DA depletion leads to a rapid and profound loss of spines and glutamatergic synapses on striatopallidal MSNs (indirect pathway), by a mechanism involving the

enhanced Ca^{2+} entry through L-type channels containing a Cav1.3α1 subunit. As expected, the blockade of these intraspine channels or the deletion of the subunit prevented the selective death of MSNs. Striatonigral neurons (direct pathway) were not affected, highlighting the asymmetric effect of DA depletion in the two pathways – this could be due to the different distribution of DA receptors types. In this way, the authors suggest that the loss of D_2R modulation, following DA depletion, disinhibits intraspine Cav1.3 channels, which destabilizes spines and synaptic contacts (Day et al., 2006).

On the basis of the classical model explained earlier, a dopamine reduction would be expected to stimulate the activity of D_2R expressed in striatopallidal neurons. The results obtained by this group bring a new interesting detail: DA depletion, besides inducing the selective elimination of part of striatopallidal MSNs, also seems to increase their excitability, resulting in an enhanced responsiveness to the remaining synapses (Day et al., 2006).

Regarding the therapeutical side of DA, after realizing that the main pathology responsible for the manifestation of PD symptoms was the localized degeneration of dopaminergic neurons in the striatum, scientists begun to develop DA replacement therapies for the treatment of the disease (Ahlskog, 2001).

Since DA cannot cross the blood-brain barrier (**BBB**), if it was administrated as a drug to PD patients, it would not produce any visible effect. The solution to increase the amount of dopamine in these brains was to use L-3,4-dihydroxyphenylalanine (**$_L$-DOPA**), which is the precursor of DA and is able to cross the BBB (Ahlskog, 2001).

Nowadays, $_L$-DOPA is still the most effective treatment for PD, providing dramatic improvement of the motor symptoms in the first years of treatment. However, none of the currently approved anti-parkinsonian drugs has been found to prevent the underlying death of dopaminergic neurons. Moreover, prolonged treatment with dopamimetic agents produces a variety of cognitive (e.g. dementia and psychosis) and motor severe side effects – for instance, chronic administration of $_L$-DOPA can itself trigger the involuntary jerky movements (dyskinesias) usually seen in PD patients (Ahlskog, 2001).

The ineffective protection against neuronal degeneration and the adverse effects of drugs targeting the dopaminergic system have prompted researchers to look for alternative approaches that would target dopaminergic neurotransmission downstream of the lost neurons and modulate basal ganglia motor circuitry. Their aim is to find a novel therapy capable of restoring the balance between direct and indirect pathways, with a reduced risk of side effects (Jackson et al., 1994; Marino et al., 2003; Coccurello et al., 2004; Schwarzschild et al., 2006).

1.2.2 GLUTAMATE| Glutamate is the major excitatory neurotransmitter in CNS, acting through both ligand gated ion channels (ionotropic receptors), mostly located in the postsynaptic density, and G-protein coupled (metabotropic) receptors, mainly localized post and extrasynaptically (Ferré et al., 2007b).

Activation of these receptors is responsible for basal excitatory synaptic transmission and interferes in many different aspects of CNS physiology, such as motor control and coordination, sensory perception and pain transmission, learning and memory processes and developmental plasticity (Rouse et al., 2000; Iacovelli et al., 2004). Glutamate is also a potent endogenous neurotoxic agent that is proposed to play a critical role in the development or progression of diverse neurological disorders.

The **ionotropic glutamate** (**iGlu**) **receptors** are multimeric assemblies of four or five subunits, and are subdivided into three groups (AMPA, NMDA and Kainate receptors) based on their pharmacology and structural similarities. These receptors are associated to the mediation of fast excitatory synaptic transmission, mainly its facilitation, although there is also evidence that show a clear inhibitory action of these receptors (Pinheiro et al., 2008).

Metabotropic glutamate (**mGlu**) **receptors**, widely distributed throughout the CNS, form a family of eight GPCRs subtypes, that have been divided into three groups (I, II and III), based on sequence similarity, pharmacology and intracellular signaling mechanisms - G-protein coupling and association with the respective second-messenger (figure 11) (Conn et al., 1997; Rouse et al., 2000). These receptors, which have been reported to be expressed in the striatum, modulate neuronal excitability at the postsynaptic level and control

neurotransmitter release through their coupling to G proteins and the subsequent recruitment of second messenger systems (Conn et al., 1997; Anwyl, 1999; Pinheiro et al., 2008).

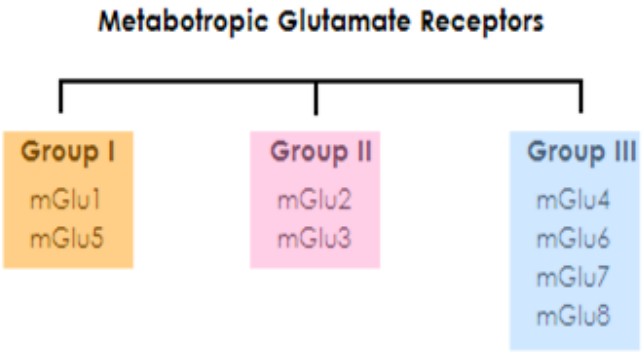


Figure 11 – Scheme of glutamate metabotropic receptors

Group I mGlu receptors, usually localized next to postsynaptic density (Lagerström et al., 2008) and at dopaminergic synapses, act through G_q-like proteins coupled to effector molecules such as phospholipase C (**PLC**), while group II and group III receptors, which have been described at presynaptic level on excitatory corticostriatal terminals and GABAergic output fibers, are negatively attached to AC, though the coupling to $G_{i/0}$-like proteins – these receptors function as autoreceptors and their stimulation inhibits glutamate release throughout the brain, by a mechanism involving the depression of P/Q-type Ca^{2+} channels, although their activation in physiological conditions have only been seen in a few synapses (Ferré et al., 2007b; Pinheiro et al., 2008). In general, the group I mGlu receptors most often serve as postsynaptic receptors involved in increasing neuronal excitability, whereas the group II and group III mGlu receptors normally have a presynaptic distribution and are involved in reducing neurotransmitter release, although there are some exceptions.

In conclusion, iGluRs and mGluRs, following activation by endogenous glutamate, interfere in the modulation of both excitatory and inhibitory neurotransmission, directly affecting higher brain functions, such as long-term memory and spatial and motor learning. In order to better understand the laying mechanisms and function of these receptors in normal health

and disease conditions, future research, combined with the development of new cellular imaging techniques, is needed.

1.2.2.1 Glutamate and Parkinson's Disease| According to the previously mentioned model of basal ganglia circuitry, the DA neuronal loss leads to hyperactivity of the STN, which culminates in an increased glutamatergic excitatory drive onto the internal part of the *globus pallidus* (Breysse et al., 2003).

With the realization of the pathological changes that occur in the basal ganglia circuitry in a PD condition, and considering the consequent hyperactivity of glutamatergic neurotransmission (Rouse et al., 2000), scientists began to think that glutamatergic pathways had to play a major role in the expression of the motor disturbances and neurodegeneration observed in this disease (Bonsi et al., 2007).

Based on this, and due to the severe side effects associated with currently available dopaminergic therapies, efforts have been made in an attempt to counteract glutamatergic hyperactivity, either by surgical inactivation of the STN or through the pharmacological blockade of this nervous transmission system, providing an effective therapy for part of PD pathology (Marino et al., 2003; Breysse et al., 2003).

In this way, several recent studies sustain that both iGluRs and the mGluRs are therapeutical targets for this illness.

Concerning the ionotropic group, the most encouraging results have been obtained with the NMDA receptors. Namely, the focal administration of this receptor's antagonists was shown to prevent nigral degeneration in an animal model of PD (Blandini et al., 2001), and when applied into the striatum, it reduced the occurrence of parkinsonian symptoms (Hallett et al., 2004).

A commonly used primate model in the study of PD's pathology is the MPTP-lesioned primate, where the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyrimidine (**MPTP**) selectively destroys dopamine neurons. The finding that in this animals there is a blocking of mitochondrial respiration, due to the binding of the active metabolite of MPTP to complex I

of the respiratory chain, led to the discovery of a deficit in this complex in PD patients, namely in platelets and *substantia nigra* (Bonsi et al., 2007; Parker et al., 2008).

Combining this finding with the knowledge of a potential role of glutamate in the pathology of PD, a hypothesis involving an indirect excitotoxic effect of this neurotransmitter has been proposed to explain, in part, the degeneration of dopamine neurons in PD (Marino et al., 2003). According to this hypothesis, mitochondrial or oxidative defects lead to an energy deficit and a deteriorating membrane potential. The resulting depolarization induces a relief of voltage-sensitive Mg^{2+} block of the NMDA receptors, leading to a continuous activation of these receptors and justifying the toxicity of high levels of endogenous glutamate (Beal et al., 1993).

Despite the apparent neuroprotection and antiparkinsonian benefits conferred by some NMDA antagonists, it's utilization in clinical trials has been limited due to adverse side effects such as severe cognitive deficits, ataxia, and hallucinations, probably a result of the widespread distribution of this glutamate receptors (Bonsi et al., 2007; Parker et al., 2008).

The failure of pharmacological approaches targeting NMDA receptors in PD therapy turned the interest of scientists to metabotropic glutamate receptors, which have the ability to regulate the striatal function and therefore are now viewed as new potential candidates (Rouse et al., 2000). Supporting the fact that these receptors may bear direct responsibility for the expression of some of PD symptoms, results of recent studies indicate that mGlu receptors are heavily expressed throughout the basal ganglia, where they have several important functions in regulating neuronal excitability and synaptic transmission (Marino et al., 2002; Marino et al., 2003). In fact, there is a growing number of studies demonstrating that the pharmacological modulation of mGluRs can ameliorate parkinsonian symptoms, like motor abnormalities, in several experimental models of PD (Rouse et al., 2000; Marino et al., 2003; Breysse et al., 2003; Ossowska et al., 2005; Bonsi et al., 2007). Specifically, while group I mainly induces depolarisation of neurons in CNS (actually, evidence suggest that only mGluR5 mediates the direct depolarization of STN neurons), group II and III of these receptors decrease excitatory transmission at the cortical-striatal synapse (Marino et al., 2003).

Starting with group I mGluR, behavioral studies involving the injection of this group agonists, combined with measurements of c-*fos* expression in the STN, suggest that activation of this receptors enhances the transmission through the indirect pathway. This means that it is likely that antagonists of this group may have a great therapeutic potential in the treatment of PD, by selectively reducing activity through this pathway (Rouse et al., 2000; Breysse et al., 2003). Regarding mGlu1 receptors, they are expressed by dopaminergic neurons of the SNc where, upon activation, they can either hyperpolarize or depolarize (Mercuri et al., 1992; Fiorillo et al., 1998,) these neurons, possibly depending on the intensity and duration of mGlu receptor activation.

Considering mGluR5 privileged expression pattern within the basal ganglia and their agonists' excitatory role in the STN (enhancing the indirect pathway), it is not surprising that these receptors' antagonists have the power to decrease the imbalance between direct and indirect pathways in PD, ameliorating the visible symptoms (Marino et al., 2003; Breysse et al., 2003). These effects have already been reported: among other features, antagonists like 2-methyl-6-(phenylethynyl)-pyridine (**MPEP**) proved to be able to increase reaction times in a rodent model (Breysse et al., 2002) and reduce dyskinesias in a primate model of PD (Hill et al., 2001). Similar results were obtained with the hemiparkinsonian rat (Breysse et al., 2003; Battaglia et al., 2003), another widely used animal model with certain neuropathological features very similar to those that can be found in post-mortem tissue of parkinsonian patients (Battaglia et al., 2003; Stephens et al., 2005).

Regarding group II mGluR, agonists of this group (concretely, the LY379268) were shown to be able to decrease the extent of nigro-striatal degeneration in MPTP-treated mice, showing that activation of this group can be an alternative to the inhibition of group I in the perspective of a potential PD therapy (Hill et al., 2001). In the striatum, specifically at the STN–SNr synapse, activation of mGlu2 and mGlu3 receptors reduces excitatory transmission (Cartmell et al., 2000) by inhibiting glutamate release, which reduces the excitatory drive from the indirect pathway to the basal ganglia output nuclei.

Group II mGlu receptors activation could, therefore, have a similar effect to that of dopamine on overall transmission through the indirect pathway, although it exerts this effect at the final synapse of this circuit, which is well downstream of the striatum, where dopamine acts.

The neuroprotective actions of these receptors can also be exerted by an additional mechanism, involving their activation at the level of astrocytes, inducing the production and release of glial transforming growth factor-beta (**TGF-β**), which has been reported to protect neurons in mixed cortical cultures exposed to NMDA excitotoxicity (Bruno et al., 1998).

About the group III mGlu receptors, subtypes mGlu4 and mGlu7 are presynaptically localized in the basal ganglia, and negatively modulate both glutamate and GABA release (Corti et al., 2002; Matsui et at., 2003, Valenti et al. 2003). In fact, the suppression of GABA transmission at the striatopallidal synapse due to activation of presynaptic mGlu4 receptors is one of the most important functions of this group (Valenti et al., 2003). The striatopallidal synapse is the first synapse in the indirect pathway and is thought to be highly regulated by striatal dopamine and, for that reason, mGlu4 receptors activation might act downstream of dopamine neurons, exerting a dopamine-like effect by reducing transmission at this synapse. Accordingly, in a recent study, agonists of group III mGluRs were injected into specific brain regions of reserpine-treated rats, and anti-akinetic effects have been reported, although the mechanism in question remains unexplained (MacInnes et al., 2004).

Because of the ubiquitous distribution of glutamatergic synapses, mGlu receptors have the potential to intervene in a wide variety of functions in the CNS. The fact that these receptors subtypes are so diverse and so heterogeneously distributed, makes them perfect targets to pharmacological agents that selectively interact with mGlu receptors involved in only one or a limited number of CNS functions. Therefore, gaining a detailed understanding of the specific roles of mGlu receptors and by identifying the mechanisms by which the modulation of these receptors activity exerts their beneficial effects in animal models of PD, could have a dramatic impact on development of novel treatment strategies for a variety of psychiatric and neurological disorders, more efficient and without the undesirable complications of conventional PD treatment. Because of this, a great deal of effort has been

focused on developing small molecules that selectively activate or inhibit specific mGlu receptor subtypes.

1.2.3 ADENOSINE| Since it is not stored in vesicles nor released as a bolus in response to depolarization of a presynaptic membrane, more than a neurotransmitter, adenosine is a neuromodulator. It accumulates in extracellular fluids as a result of cell physiology, influenced by the breakdown and synthesis of ATP, being released from both neurons and glial cells (Ferré et al., 2007b).

Adenosine regulates many physiological processes, particularly in excitable tissues such as the heart and brain, where it either reduces the activity of excitable tissues (e.g. by slowing the heart rate) or increases the delivery of metabolic substrates (e.g. by inducing vasodilation), helping to couple the rate of energy expenditure to the energy supply. It also plays a variety of different roles as an intercellular messenger, particularly in the brain, where high concentrations of adenosine receptors were shown to interfere in both normal and pathophysiological processes that include regulation of sleep, arousal, neuroprotection and epilepsy (Popoli et al., 2004).

GPCRs for adenosine were first identified around 1970 (Zezula et al., 2008). These receptors, which could either inhibit (A_1) or stimulate (A_2) AC, were later classified in different sub-types of adenosine receptors: A_1, A_{2A}, A_{2B} and A_3 (Missale et al., 1998), with A_1 and A_3 receptors coupled with subfamilies $G_{i\,(1\text{-}3)}$ and G_0 of G proteins, and A_{2A} and A_{2B} interacting with the G_s (figure 12) (Zezula et al., 2008; Cieslak et al., 2008).

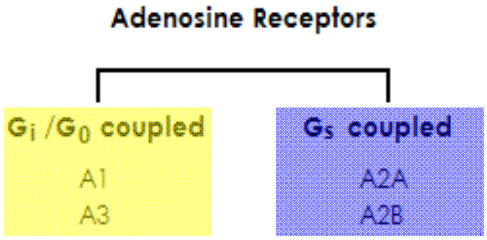


Figure 12 – Scheme of adenosine receptors.

A_1 and A_{2A} are the two of more significance as neurotransmitters since they have a higher affinity for adenosine and because A_{2B} and A_3 subtypes are mainly located in peripheral tissues outside the brain and show significantly lower affinity for adenosine (von Lubitz et al., 1999; Cieslak et al., 2008). Between the A_1 and A_{2A} subtypes, the effects of A_1 receptors (**A_1Rs**) greatly predominate, due to their superior number and higher affinity for adenosine (von Lubitz et al., 1999).

Adenosine is highly associated with glutamate neurotransmission: postsynaptically, activation of NMDARs induces adenosine release; presynaptically, increase of the frequency of impulses arriving at the glutamatergic nerve terminal induces a rapid increase in the concentration of adenosine at the glutamatergic synapse (Cunha, 2001). On the other hand, parallel to the regulation of adenosine release by glutamate neurotransmission, both A_1Rs and A_{2A}Rs can be found at glutamatergic terminals, where they modulate glutamate (and other neurotransmitters) release (Ferré et al., 2007b).

A_1Rs have the highest abundance of adenosine receptors in the brain, occurring in structures like the hippocampus, cerebellum, spinal cord, thalamus and striatum (Cieslak et al., 2008). As a result of their coupling to Gi or Go containing G-proteins, A_1Rs are the main mediators of the inhibitory neuromodulation by adenosine (Fredholm et al., 2005). Their activation leads to a decrease of cAMP levels and exerts a stimulatory effect on phospholipase C (von Lubitz et al., 1999). It also affects the currents of ionic channels: on the one side, there is activation of inwardly rectifying K^+ channels and, on the other side; there is the closing of N and Q-type calcium channels, causing hyperpolarization (von Lubitz et al., 1999; Cieslak et al., 2008; Boison 2008).

The net result of the actions of A_1Rs when these receptors accumulate on presynaptic membranes, is the inhibition of the release of different brain neurotransmitters, including glutamate, GABA, dopamine, serotonin and acetylcholine (figure 13) (von Lubitz et al., 1999; Ferré et al., 2007b; Boison 2008).

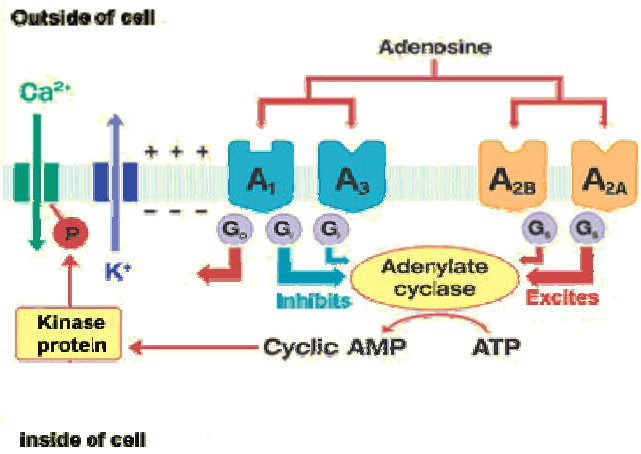


Figure 13 – Schematic diagram of intracellular signal mechanisms of adenosine receptors. Adenosine mediates its varied effects by activating specific subtypes of membrane receptors. Currently, four adenosine receptor subtypes have been identified: A_1, A_{2A}, A_{2B} and A_3. Each receptor is coupled to a G protein, which can either stimulate (G_s) or inhibit (G_i) the enzyme adenylate cyclase, respectively raising or diminishing the levels of cyclic AMP (cAMP). Changes in the levels of cAMP regulate the activity of intracellular protein kinases that phosphorylate intracellular proteins or transmembrane ion channels (i.e. calcium). A_1 receptor, which is coupled to a Go protein, can also directly modulate the transport of potassium. (Taken from http://www.aderis.com/img/art_adenosine.gif)

Regarding adenosine A_2Rs, the G_s/Golf proteins to which they are coupled activate AC (Kull et al., 2000; Vu, 2005), which converts ATP into cAMP that activates the cAMP-dependent protein kinase (PKA), which in turn regulates the state of phosphorylation of various substrate proteins. In contrast to A_1Rs, the A_2 subtype has an excitatory action (Sebastião et al., 1996), and their stimulation results in a calcium-dependent release of glutamate and acetylcholine, by a mechanism that may involve P-type channels (von Lubitz et al., 1999). In addition to the preferential G_s/adenylate cyclase pathway, A_{2A} receptors have also been shown to use other second messenger signaling pathways including PLC (Wirkner et al., 2000), phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK (Seidel et al., 1999; Schulte et al., 2003; Canals et al., 2005). However, due to the fact that A_1Rs predominate in number and have greater affinity for adenosine than A_{2A}, A_1Rs effects prevail – reduced excitotoxicity by reducing glutamate release (figure 8) (von Lubitz et al., 1999).

Interestingly, many studies have provided evidence for the existence of functional antagonistic interactions between A_1Rs and A_{2A}Rs that modulate glutamate release in the striatum and hippocampus (Lopes et al., 2002; Quarta et al., 2004). The coexistence of both stimulatory A_{2A}Rs and inhibitory A_1Rs in the same terminal is intriguing, particularly in view of their opposite functional effects. Recently, using radioligand-binding, co-immunoprecipitation, BRET and time-resolved FRET techniques, Ciruela et al. (2006) demonstrated the existence of A_1R–A_{2A}R heteromers in mammalian cells and in striatal glutamatergic nerve terminals, where the A_{2A}R activation is able to reduce the affinity of the A_1R for agonists, providing a switch mechanism by which low and high concentrations of adenosine inhibit and stimulate, respectively, glutamatergic neurotransmission.

A_{2A} and A_{2B} receptors differ in their location: in the brain, A_{2B}Rs are widely spread, while A_{2A} subtype is primarily located in the dopamine-rich areas of basal ganglia, as well as in *nucleus accumbens* and olfactory bulb (Missale et al., 1998). Inhibitors of this last receptor subtype were shown to exert strong neuroprotective functions and to prevent apoptosis (Silva et al., 2007).

Adenosine A_3 receptors are not as well known as the others. It is known that its concentration in the brain is 10-30 times lower than the respective concentration of A_1 and A_2 subtypes in the cortex and striatum, and that the stimulation of this receptor leads to the formation of inositol triphosphate (**IP3**), resulting in an increase of calcium in cells (von Lubitz et al., 1999; Cieslak et al., 2008).

1.2.3.1 Adenosine and Parkinson's Disease| In part due to the unique CNS distribution of adenosine A_{2A}Rs, highly concentrated in striatopallidal neurons, in contrast to the widespread expression of the other adenosine receptors, antagonists of this subtype have recently emerged as potential candidates of non-dopaminergic anti-parkinsonian agents (Schwarzschild et al., 2006; Boison 2008). A number of recent evidences supports this therapeutic role of selective A_{2A}R antagonists, which were shown to attenuate the

overactivity of the striatopallidal pathway observed in PD condition (Kanda et al., 1998; Morelli et al., 2001).

On the one hand, there are studies showing the adverse effects of adenosine $A_{2A}R$ agonists, which were shown to induce a marked decrease of spontaneous and DA agonist-induced motor activity (Morelli et al., 2007). In behavioural studies using another $A_{2A}R$ agonist (CGS 21680), a reduction of the contralateral turning induced by DA agonists in hemi-parkinsonian rats was observed (Morelli et al., 1994).

On the other hand, when blockers of $A_{2A}Rs$ were administrated to the same unilaterally 6-Hydroxydopamine (**6-OHDA**) lesioned animal model, there was a clear augment of the number of contralateral rotations induced by $_{L}$-DOPA or by stimulation of DA receptors (Fenu et al., 1997).

Also in rats to which striatal bilateral 6-OHDA was injected, selective $A_{2A}R$ antagonists CSC potently reversed the akinetic deficits produced (Coccurello et al., 2004).

When tested in primates treated with MPTP, $A_{2A}R$ antagonists produced motor stimulant effects and potentiated $_{L}$-DOPA-mediated effects (Kanda et al., 1998; Grondin et al., 1999; Coccurello et al., 2004; Morelli et al., 2007).

Besides the akinesias, other distinctive motor anomalies of PD have also been shown to improve with A_{2A} blockade; namely, A_{2A} antagonists improved parkinsonian tremulous movements in a rodent model (Correa et al., 2004). Moreover, muscle rigidity (increased resistance to passive movement) induced by the DA-depleting agent **reserpine** was ameliorated by an A_{2A} antagonist, or totally eliminated by the synergistic combination of this antagonist plus $_{L}$-DOPA (Wardas et al., 2001).

All together, these results strongly suggest that blockade of $A_{2A}R$, which apparently potentiates DA transmission, might greatly improve the motor disabilities observed in different models of PD.

The mechanism by which $A_{2A}R$ antagonists ameliorate PD symptoms is still not completely understood, although investigators suspect that it might be the result of the blockade of an

altered responsiveness of striatopallidal neurons responsible for the imbalance between direct and indirect pathways (Schwarzschild et al., 2006; Morelli et al., 2007).

Moreover, some of the consequences that result from the activation of A_{2A}Rs are the enhancement of glutamate release (in glutamatergic nerve terminals), leading to neurotoxicity, and the facilitation of neuroinflammation, producing brain injuries, when the activation occurs on glial cells. These examples make it easier to understand how the blockade of A_{2A}R by the respective antagonists exerts a neuroprotective effect on dopaminergic neurons (Hauser et al., 2003; Schwarzschild et al., 2006; Morelli et al., 2007). In effect, the blockade of adenosine A_{2A}R not only ameliorates parkinsonian symptoms, as illustrated above, but also exerts a neuroprotective activity that might be able to hold back the progression of the disease *(*Ikeda et al., 2003)*.* In addition, given the fact that A_{2A}Rs are also located in the limbic system, hippocampus, and amygdala, it is likely that drugs acting on these compounds might also have encouraging effects in psychic symptoms, such as those expressed in PD (e.g. depression and psychosis) (Cieslak et al., 2008).

The ability of A_{2A} antagonists to reverse parkinsonian motor imbalances in the mentioned PD animal models, particularly in the non-human primates, led to clinical trials in PD patients, where the effect of these antagonists, in a combined action with $_{L}$-DOPA, was assessed.

These studies demonstrated symptomatic improvement in patients with relatively advanced PD (already receiving the DA substitute), even in those who had already developed dyskinetic motor complications as a result of the chronic administration of $_{L}$-DOPA (Schwarzschild et al., 2006; Linazasoro et al., 2008).

More concretely, the most promising results have been obtained with the antagonist KW6002, also known as **istradefylline**, which was shown to potentiate the antiparkinsonian response when paired with a low-dose of $_{L}$-DOPA, but with less dyskinesias (Bara-Jimenez et al., 2003; Jenner 2005; LeWitt 2008) – still, in other studies where a placebo group was included, dyskinesias were about twice as frequent with the antagonist as with the control (Pourcher et al., 2006).

Overall, this line of research revealed that despite a less pronounced than expected antiparkinsonian effect of this agent, istradefylline is safe, well tolerated by the majority of patients and capable of offering a clinically meaningful reduction of "off-time" (period of time when symptoms of PD return), suggesting that this drug might hold a major potential for the treatment of PD, though further research is still needed (Heijtz et al., 2997; Linazasoro et al., 2008; Cieslak et al., 2008). In fact, the positive results obtained in phase II of some clinical trials (Kanda et al., 1998) were considered promising enough to justify a future phase III of trials (Schapira et al., 2006).

Despite the encouraging initial results, the scenario of a possible development of tolerance to adenosine antagonists when used as therapeutical drugs cannot be excluded – many of the effects of the non-selective adenosine receptor antagonist **caffeine** exhibit tolerance after merely a few days of administration in both animals and humans (Fredholm et al., 1999).

After the repeated administration of an antagonist, it might also happen that "agonist-like" effects are induced, resulting in what Coccurello, R. *et al*. (2004) called the "inversion effect".

Nevertheless, these authors also provided the first evidence that the chronic A_{2A}Rs blockade, simulated by a 3-week treatment with the highly selective antagonist **CSC** (8-(3-chlorostyryl) caffeine), is able to enhance motor control without eliciting any side effects nor tolerance (Coccurello et al., 2004; Cieslak et al., 2008).

The mentioned studies all point to a further investigation of the role of A_{2A}Rs antagonists in the treatment of PD. Regardless of the major breakthroughs achieved in the last few years in this matter, it is still too early to say if adenosine-based therapies will ever be applied in PD patients as a substitute to or as a complementary of conventional treatments. It is crucial to unravel the molecular mechanisms that lie beyond the A_{2A} antagonists-mediated protection against motor disorders, in order to develop new, alternative and improved drugs to treat this disease that affects about 1% of the world population.

1.3 DOPAMINE, GLUTAMATE AND ADENOSINE RECEPTORS OLIGOMERIZATION: IMPLICATIONS IN PARKINSON's DISEASE|

Since the seventies that dopamine (DA) replacement therapies reveal the most effective action in the treatment of PD, providing a rapid and profound amelioration of motor symptoms; nevertheless, chronic administration of $_{L}$-Dopa and other dopamimetic drugs tends to lose efficiency and leads to a variety of motor and cognitive adverse effects (Jankovic, 2002).

Therefore, the major goal of PD pharmacological research at the present time is the development of drugs capable of modulating dopaminergic transmission by means of an indirect action exerted at the level of DA receptors. Hopefully, these drugs will be able to improve motor deficits without inducing the severe side-effects of standard dopaminergic treatments.

The demonstration of functional and molecular interactions between GPCRs, namely dopamine, glutamate and adenosine receptors, is a recent finding that brought a new perspective over the origin of neurodegenerative diseases such as PD. Consequently, also the possibility of a new pharmacological target to the treatment of these infirmities has emerged – based on the GPCR oligomerization phenomenon, it is likely that a therapeutical intervention based in a combined drug treatment, directly targeted at the receptors present at the oligomer, reveals itself as highly efficient in the amelioration of symptoms.

1.3.1 INTERACTIONS BETWEEN $A_{2A}R/D_2R$| As mentioned before, one of the most important functions mediated by D_2R is the movement control, a complex function also regulated by other neurotransmitters and respective receptors, usually located in the basal ganglia. That is the case of adenosine, a neuromodulator able to regulate the release of neurotransmitters in striatum. Accordingly, the functional cross-talk between dopamine and adenosine receptors has been implied in a number of neurodegenerative diseases, mainly PD, and other phenomena related to motor control, drug addiction and neuropsychiatric disorders (Agnati et al., 2003).

In striatum, adenosine A_{2A}Rs are co-localized with D_2 dopaminergic receptors, which creates the opportunity for the two neurotransmitters to interact antagonistically (Cieslak et al., 2008).

In the early 1990s, the group of Ferré et al. (1991) provided the first indirect evidence for a membrane-delimited interaction between A_{2A} and D_2 receptors. From binding experiments, the authors observed that activation of A_{2A}R reduced the binding affinity of DA to the D_2R, and also interfered with the coupling of these receptors to its associated G proteins (Ferré et al., 1991; Ferré et al., 1997).

However, it was not until 2001 that studies using co-immunoprecipitation and co-localization techniques have confirmed that adenosine A_{2A} and dopamine D_2 receptors do form heteromeric complexes, which probably constitute the molecular basis for the functional antagonism observed between the two receptor types *in vitro* and *in vivo*, modulating the function of striatopallidal neurons (Ferré et al., 1991; Hillion et al., 2002; Canals et al., 2003; Fuxe et al., 2003; Ciruela et al., 2004). The final confirmation of the existence of A_{2A}R/D_2R heteromers came with the resonance energy transfer techniques in *in vivo* cells (Canals et al., 2003).

This intramembrane interaction between the two receptors seems to involve a D_2R-$G_{q/11}$-PLC signalling pathway, as well as a strong antagonism at the second messenger level, where stimulation of G_i-coupled D_2R counteracts the activation of AC induced by stimulation of A_{2A}R (Ferre et al., 2007).

The heteromerization of A_{2A}R and D_2R is of great relevance for PD. Despite the fact that analysis of PD patients' brains has not revealed any changes in their amount of adenosine, it seems that simultaneous activation of D_2Rs and inhibition of A_{2A}Rs could produce a superior enhancement of patients' mobility than the sum of the effects of each drug on its own (Cieslak et al., 2008).

Again, it was the group of Ferre et al. (1992) the first to suggest that these interactions could provide a new therapeutic strategy for PD, mostly based on the co-administration of A_{2A}R antagonists with $_L$-Dopa or other DA receptor agonists (Ferré et al., 1992; Ferre et al., 2007). In this scenario, A_{2A}R antagonists would target the A_{2A}/D_2 heteromer, blocking the

antagonistic effects of endogenous adenosine on the heteromer, which would lead to the enhance of D_2R-mediated signalling. This combined therapy, using both A_{2A}R antagonists and D_2R agonists, has already been showed to potentiate the antiparkinsonian actions of $_L$-Dopa, not only in animal models (Kanda et al., 1998), but also in PD patients (Bara-Jimenez et al., 2003; Hauser et al., 2003).

It is worth pointing out that the interaction between A_{2A} and D_2 receptors does not always result in a mutual antagonism (Zezula et al., 2008). Under certain conditions, a synergistic signal may be induced, and according to Yao et al., (2002) this has important implications in the rewarding action of adenosine in the *nucleus accumbens* (Yao et al., 2002; Cieslak et al., 2008).

1.3.2 INTERACTIONS BETWEEN A_{2A}R/mGluR5| Adenosine A_{2A}R are highly expressed in the striatum, *nucleus accumbens*, olfactory tubercle, and the *globus pallidus* where group I mGlu receptors were also found to be strongly expressed (Coccurello et al., 2004).

A few years ago, Ferré et al. (1999) followed by Popoli et al. (2001), provided evidences that A_{2A} and group I mGlu receptor agonists could synergistically reduce the affinity of the D_2 agonist-binding sites in striatal membranes (Ferré et al., 1999; Popoli et al., 2001).

More recently, a high degree of A_{2A} and mGlu5 co-localization was detected in striatal neurons in primary cultures (Fuxe et al., 2003) and in glutamatergic nerve terminals in the striatum (Rodrigues et al., 2005).

Together with some co-immunoprecipitation studies (Ferré et al., 2002), these results strongly suggest that these receptors may interact and form heteromers with different functionalities from those they had as individual components.

Further studies have indicated that the molecular mechanism underlying synergistic interaction between A_{2A} and mGlu5 receptors does not occur at the level of second messengers (Ferré et al., 2002), but at the level of c-fos expression, MAP kinases and phosphorylation of DA- and cAMP- regulated phosphoprotein DARPP-32 (Schwarzschild et

al., 2006). Given the enriched location of this last protein in MSN neurons in the striatum, it is strongly suggested that A_{2A}R-mGlu5R complexes play a major role in striatal plasticity and, more concretely, in DA signalling (Ferre et al., 2002; Nishi et al., 2003; Schwarzschild et al., 2006; Morelli et al., 2007)

Evidence showing the effects of these interactions on specific motor deficits such as akinesia has been recently publicized by Coccurello et al. (2004), who found that "chronic" co-administration of subthreshold doses of A_{2A} and mGlu5 receptors antagonists alleviate motor deficits in the 6-OHDA-lesioned rat model of PD. This finding also emphasizes that the combined blockade of the two receptor types is more effective in reversing akinesia than each treatment administered separately (Coccurello et al., 2004).

Moreover, a similar effect was demonstrated after "acute" co-administration of A_{2A} and mGlu5 receptors antagonists, SCH58261 and MPEP, respectively, which was found to synergistically diminish the parkinsonian muscle rigidity in rats (Wardas et al., 2001; Ossowska et al., 2005).

All together, these results sustain that combined blockade of A_{2A} and mGlu5 receptors, whether it is a chronic or acute administration, potentiates their beneficial effect in PD models. More recently, Kachroo et al. (2005), demonstrated that the antiparkinsonian effect of treatment with the mGlu5R antagonists requires the functional integrity of both glutamate and adenosine receptors, suggesting the existence of a functional interaction between them.

Hence, a combined pharmacological blockade of both receptors might provide a novel and promising non-dopaminergic strategy for the treatment of motor symptoms in PD.

1.3.3 INTERACTIONS BETWEEN D_2R/mGluR5/A_{2A}R| Recent findings suggest that besides adenosine A_{2A} and dopamine D_2, also the glutamate mGlu5 receptors are co-localized postsynaptically in the striatopallidal GABAergic neurons – as previously referred, these play a key role in the pathophysiology of basal ganglia disorders such as PD (Ferré et al., 2002; Conn et al., 2005). Similarly to previous cases, this co-localization in the dendritic spines of the indirect striatopallidal GABA neurons suggests that physical and multiple

functional interactions occur between A_{2A}, D_2 and mGlu5 receptors, and that these interactions modulate the function of the striatal GABA output neurons (figure 14) (Morelli et al., 2007).

As mentioned before, mGluR5 has been shown to physically associate with $A_{2A}R$ in the striatum. On the other hand, stimulation of mGluR5 antagonizes the binding of DA to the D_2R, which suggests that it also heteromerizes with D_2R, although a physical interaction between these two receptors has not yet been proved. Furthermore, stimulation of mGlu5 receptors potentiates the antagonistic effect of $A_{2A}Rs$ on D_2R binding, suggesting the existence D_2R-mGluR5-$A_{2A}R$ complex heteromers (Ferre et al., 2007).

The additional finding that mGlu5 antagonist-induced motor stimulation requires A_{2A} and D_2 receptors highlights the interdependence of these three receptors in modulating motor function (Kachroo et al., 2005).

This functional antagonistic relationship is further supported by a study already mentioned in this work, where Popoli et al. (2001), besides demonstrating that the stimulation of mGlu5Rs inhibited the D_2R-induced turning behaviour in 6-OHDA-lesioned rats, this effect was greatly potentiated by a combined administration with an A_{2A} receptor agonist (Popoli et al., 2001). In fact, other studies also corroborate that $A_{2A}R$ and $mGlu_5R$ agonists and $A_{2A}R$ and $mGlu_5R$ antagonists show synergistic effects at the behavioral level (Ferre et al., 2002; Kachroo et al., 2005).

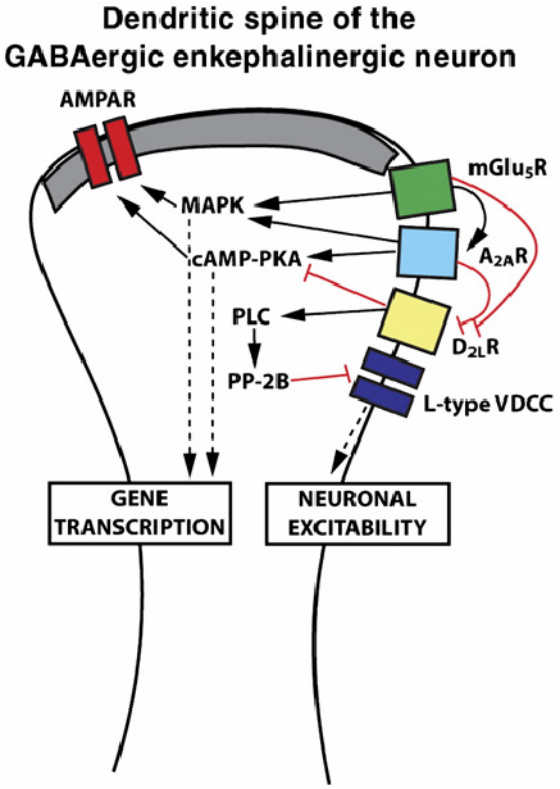


Figure 14 - Heteromers of A_{2A} ($A_{2A}R$), D_2 (D_2R) and $mGlu_5$ ($mGlu_5R$) receptors in the dendritic spine of the striatopallidal neuron. The differential stimulation of the units of the receptor heteromers determines the predominant signaling pathway and the consequent changes in neuronal excitability and gene transcription, with implications for plastic changes in the glutamatergic synapse, such as phosphorylation and recruitment of AMPA receptors (AMPARs) to the postsynaptic density. Under conditions of weak glutamatergic neurotransmission, there is a predominant effect of D_2 receptor signaling, with decreased neuronal excitability and gene transcription. On the other hand, when glutamatergic neurotransmission predominates, adenosine is released and both glutamate and adenosine receptors are strongly stimulated, inhibiting D_2R signaling and leading to an increased neuronal excitability and gene transcription. In black and red, stimulatory and inhibitory effects, respectively. Abbreviations: VDCC, voltage-dependent calcium channel; MAPK, mitogen-activated protein kinases; PKA, protein kinase A; PLC, phospholipase C; PP-2B, protein phosphatase 2B or calcineurin. (Taken from Ferre et al., 2007)

Such oligomeric complexes containing the three mentioned receptors only very recently were proved to exist in living cells, by immunodetection, co-immunoprecipitation, BRET and SRET (Sequential Ressonance Energy Transfer) techniques, among others (Cabello et al., 2009). The obtained results in this work also strongly suggest that the heteromer $A_{2A}R$–D_2R–$mGlu_5R$ can be found in GABAergic striatopallidal neurons, site known to play a key role in controlling basal ganglia neurotransmission and, consequently, motor learning and movement control.

The data presented above, sustaining the existence of physical and functional interactions between $A_{2A}R$–D_2R–$mGlu_5$ receptors, provides the rationale for the application of $A_{2A}R$ antagonists and possible application of $mGlu_5$ receptor antagonists in PD (Ferre et al. 1992; Ferre et al. 1997; Ossowska et al. 2001; Kachroo et al. 2005; Jenner, 2005). This opens new perspectives from a therapeutic point of view, giving rise to the development of novel strategies for treatment of Parkinson's disease and other neurodegenerative conditions with similar etiology, like schizophrenia or Tourette's syndrome.

1.4 OBJECTIVES|

Overall, the background and state of the art of scientific knowledge in this matter, briefly presented here, provides the basis to the initial hypothesis of this project, which is focused on the role of the molecular and functional interactions of glutamate, dopamine and adenosine receptors in Parkinson's disease. Giving the pioneering nature of this major project, some initial fundamental steps are required to be completed before the intricate oligomerization studies can be initiated. Therefore, in the work here presented, the main focus was on adenosine A_{2A} receptor, around which two major objectives were delineated: to find membrane proteins interacting with this receptor, that can modulate its function and participate in its oligomeric associations, and to build and characterize the necessary tools to perform the future FRET-based studies involving the $A_{2A}R$. Also, in a more therapeutic-oriented research, it is intended to perform tissue-level studies in a PD animal model, in order to characterize the expression of $A_{2A}R$, D_2R and mGlu5R, and determine the oligomerization degree of these receptors in a normal *versus* pathological condition. The specific objectives of this work are listed as follows:

I. To determine the existence of membrane proteins interacting with the C-terminal tail of the human adenosine A_{2A} receptor, using the MYTH system.

II. To clone a construct of the A_{2A} receptor tagged with the fluorophore CFP in the N-terminal of the receptor.

III. To assess the correct expression and full function of the clone, by means of western blotting, confocal microscopy and cAMP accumulation assays.

IV. To verify the suitability of the construct for FRET experiments.

V. To analyze the kinetics of the interaction between $A_{2A}R^{CFP}$ construct and the ligand that is intended to be used in future FRET assays.

VI. To determine the relative quantification of glutamate mGlu5, dopamine D_2 and adenosine A_{2A} receptors in striatum of hemiparkinsonian rat model.

VII. To determine the oligomerization degree between these three receptors.

Chapter 2

Materials and Methods

2.1 MATERIALS|

2.1.1 ANTIBODIES| In this project, for Western blot and co-immunoprecipitation experiments, the following antibodies were used: rabbit anti-human dopamine D_2 receptor polyclonal antibody (Millipore Corporation, Billerica, USA, 1:1000), mouse anti-adenosine A_{2A} receptor monoclonal antibody (Upstate; clone 7F6-G5-A2; 1:1000), rabbit anti-glutamate mGlu5 receptor polyclonal antibody (Upstate; 1:1000) and rabbit anti-actinin polyclonal antibody (Santa Cruz Biotechnology, Santa. Cruz, CA). The secondary antibodies used were goat anti-rabbit IgG peroxidase conjugated (Pierce ThermoScientific, Rockford, USA, 1:30000) and rabbit anti-mouse IgG, peroxidase conjugated (Pierce ThermoScientific, Rockford, USA, 1:10000). For immunofluorescence assays it was used the mouse anti-A_{2A}R monoclonal antibody, detected with the Cy5-conjugated donkey anti-mouse IgG antibody (Sigma-Genosys).

2.2 MEMBRANE YEAST TWO HYBRID|

The *Saccharomyces cerevisiae* strain AP4 was sequentially transformed with pCCW-A_{2A}R bait vector (kindly provided by Dr. Igor Stagljar from University of Toronto) and a human brain cDNA library constructed in the pPR3-N NubG-X vector (Dualsystems Biotech Ag, Schlieren, Switzerland) according to the manufacturer's instructions. Transformants were plated onto minimal yeast medium lacking histidine, tryptophan, lysine and adenine and supplemented with 25 mM 3-aminotriazole. Plates were incubated at 30°C for 5 days, and yeast colonies that grew on this selective medium were re-streaked onto fresh plates and assayed for β-galactosidase activity following the manufacturer's instructions. Prey plasmids were isolated from yeast and transformed into *Escherichia coli* "HB101 Competent Cells" (Invitrogen). The 5′-end of each clone was sequenced using a vector primer and analyzed by BLAST search.

2.3 DNA CLONING|

To perform the FRET-based studies of adenosine A_{2A} receptor ($A_{2A}R$), a human $A_{2A}R$ construct containing a stable version of the CFP protein (CFP cerulean) was cloned. The new construct can act as a donor in a FRET process if the proper acceptor (e.g. Alexa 488) is in close proximity. In this topic it will be explained how the DNA insert of synthetic $A_{2A}R$ gene was amplified by polymerase chain reaction (PCR), digested with restriction enzymes and inserted into CFP-PTHR vector in order to obtain the new construct $A_{2A}R^{CFP\text{-}N}$. All used primers in this work were from biomers.net, Ulm, Germany.

2.3.1 DNA Amplification by PRC| In order to amplify the $A_{2A}R$ DNA sequence (PCR1) we used the primer FA_{2A}BamHI and the RA_{2A}Xho). This amplification originated a DNA fragment with 100-200 bp. To amplify the CFP DNA sequence the following primers were used: F_{CFP}Xho and R_{CFP}Eco. The PCR1 reaction consisted of a prepared mixture of 25 µL of iProof High Fidelity Master Mix (Bio-Rad, containing 2 x HF or GC Buffer; 0,04 U/µL iProof; 400 µM dNTP), 2,5 µL of each used primer, 10 ng/mL of DNA and autoclaved Milli-Q water till 50 µL of final volume, developed in a thermal cycler iCycler (Bio-Rad), with the conditions described in table I.

Table I - Defined conditions for the PCR1 using iProof. The loop depicted in grey was performed 35 times (35 cycles).

Cycle Step	Temperature (ºC)	Time
Initial denaturation	98	4 min
Denaturation	98	10 sec
Annealing	50	30 sec
Extension	72	30 sec / kb
Final extension	72	7 min

The PCR products were purified with Jet quick PCR purification Spin kit/250 (Genomed), with a final step of DNA elution with 50 µL autoclaved Milli-Q water.

2.3.2 Electrophoresis in Agarose Gel| This technique was used in this work whether to check the size of a DNA fragment, or to proceed to the cutting of an agarose gel slice, to purify a DNA sample. Concerning the cloning process of $A_{2A}R^{CFP\text{-}N}$, in order to verify the amplified DNA fragments from PCR1, a 1% agarose gel was prepared, using a 1kb molecular weight leader (Biootools). The agarose was melted in TBE buffer (Tris 0,04M, Boric acid 0,114% (v/v), Guanosine 1mM, EDTA 1mM, pH 8). For simple DNA segment checking, the electrophoresis was developed at 120V, while for DNA sample extraction it ran at 80V, to avoid DNA damage. The DNA samples were ressuspended in loading buffer 6x (0,25% (w/v) bromophenol blue, 0,25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll in H2O), before applied into the gel. DNA obtained by cutting the slice of agarose gel was purified with Jet Sorb Gel Extraction kit/300 (Genomed).

2.3.3 Digestion with Restriction Enzymes| Restriction endonucleases are enzymes that recognize small DNA sequences and catalyze double strand DNA excision in specific sites inside or near the recognized sequence. Inserts and vector DNA were digested with specific restriction enzymes using the recommended buffer during 2 hours (depending on the enzymatic efficiency) at a 37ºC incubator (incubator NÜVE Inc., Ankara, Turkey). The corresponding DNA fragments were verified by agarose gel electrophoresis (2.3.2), purified and quantified. $A_{2A}R$ and CFP-PTHR vector were digested with *Bam*HI and *Xho*I. The enzymes and recommended buffers were purchased from Promega Corporation (Madison, USA).

2.3.4 Ligation| This technique consists basically in the ligation between two cDNA fragments (insert and vector sequences) by the action of a DNA ligase. A mixture containing 100 ng of digested vector, 1 μL of DNA ligase, 10x ligase buffer, a desired concentration of digested insert (determined by the following expression) and autoclaved Milli-Q water to a final volume of 10 μL, was prepared.

$$\text{ng insert} = \text{ratio insert / vector} \times [(\text{X ng vector} \times \text{kb insert})$$

The ratio between insert and vector used was of 3/1. In order to achieve the optimal temperature for the ligation reaction (~14ºC), an overnight temperature gradient (4-22ºC) was performed.

2.3.5 Bacterial Transformation| Nucleic acids require assistance to traverse the outer and inner membranes of the bacteria and reach an intracellular site where they can be expressed and replicated. To achieve this goal, *Escherichia coli* "HB101 Competent Cells" (Invitrogen), were used as a host to incorporate the desired DNA, by means of a chemical heat shocking process. This method generally yield transformants at frequencies > 108 colonies/µg of supercoiled plasmid DNA. Briefly, the necessary aliquots of HB101 strain were defrosted at 4ºC for 30 min. Next, a 10% (v/v) of DNA was incubated with the competent cells for another 30 min at 4ºC, after which followed the thermal shock, performed by incubating this mixture for 45 sec at 42ºC in a water bath (Precisterm). To allow cell recovering, 1 mL of LB medium (10 g/L triptone, 5 g/L levedure extract, 10 g/L NaCl) was added and the mixture was incubated at 37ºC in a 180 rpm shaker (Shaker Infors-HT, Bottmingen, Switzerland) for 2 hours. Finally, the transformed bacteria were inoculated in selective antibiotic plaques and incubated overnight at 37ºC. These plaques had previously been prepared with LB medium and agar (15 g/L), then sterilized in autoclave and added the selective antibiotic ampicilin (0,1 g antibiotic/L medium).

2.3.6 PCR Screening| To confirm the correct incorporation of the insert into the vector, grown bacterial colonies were checked using the PCR screening method. 10 chosen colonies were resuspended in 10 µL of water and 5 µL of this mixture were used to perform a 25 µL PCR (PCR2) containing the following reagents: 12,5 µL of GoTaq Green Master Mix 2x (Promega Corporation, Madison, USA), 1,25 µL of two specific primers (T7 vector primer and RA_{2A}XhoI) and 5 µL of autoclaved Milli-Q water. The PCR was developed using the conditions described in table II and the PCR products were checked by agarose gel electrophoresis. Meanwhile, the remaining 5 µL of resuspended colonies were incubated in a 37ºC shaker with LB medium and selective antibiotic. After checking, for correctly cloned inserts into the vector, MAXI preps were performed and DNA sequence checked.

Table II - Defined conditions for PCR2. The loop depicted in grey was performed 20 times (20 cycles).

Cycle Step	Temperature (ºC)	Time
Initial denaturation	95	4 min
Denaturation	95	30 sec
Annealing	50	30 sec
Extension	72	1 min / kb
Final extension	72	7 min

2.3.7 DNA Sequencing| The final step in the cloning process was to confirm the exact DNA sequence. For this, the sequencing technical service of Barcelona University was requested, using the *Big Dye Terminator v1.1 Cycle* sequencing kit. Samples of the new fusion proteins were delivered with the specific primers to the sequencing procedure. For the confirmation of $A_{2A}R^{CFP\text{-}N}$ construct sequence, three PCRs using different primers were developed: one with the forward T7 vector primer, in order to observe the CFP sequence; and two others, one using the primer FA_{2A}BamHI and the other the RA_{2A}XhoI, with the intention to check the sequence of the $A_{2A}R$ gene. The obtained sequence was then checked using the bioinformatics tool BLAST (Basic Local Alignment Search Tool; Altschul et al., 1997) and also manually.

2.4 CELL CULTURE|

The cell-based studies performed in this project were mostly developed in human embryonic kidney cells (HEK-293 cells), obtained from American Type Tissue Culture (ATTC, Manassas, USA). First described in 1977 (Graham et al., 1977), this is a very stable, low cost, widely-used cell line in cell bioresearch. Only in the [3H]-cAMP accumulation assays a different cell line (kindly provided by Dr. Kjell Fuxe) was used, the AtT20, a line of mouse anterior pituitary-derived cells, widely used to study the

biosynthesis and secretion of peptide hormones and peptide-processing enzymes. One day before transfection, both cell lines were cultured in DMEM medium supplemented with 5% heat-inactivated FBS, 1% antibiotic cocktail and L-glutamine 2 mM, and kept at 37ºC in a humidified 5% CO_2 atmosphere incubator (Water-Jacketed 3250 incubator; Forma Scientific, Ohio, USA). When the cells were confluent, tripsine was added to detach them. Then the cells were seeded at a density of 150000 cells/ml into 25 cm^2 flasks or into 6-well plates (for immunoblotting and radioligand binding assays, respectively). For immunofluorescence and FRET experiments, cells were seeded in cover slips introduced into 6-well plates, at a density of 75000 or 150000 cells/ml, respectively.

2.5 TRANSIENT TRANSFECTION|

Transfection deals with the introduction of foreign DNA into eukaryotic cells by means of a nonviral-based method. In this work, two transfection methods were used: Calcium Phosphate method and TransFectin method, briefly described below.

2.5.1 CALCIUM PHOSPHATE METHOD| Briefly, this well-known transfection method consists in the addition of high concentrations of calcium chloride ($CaCl_2$) to a solution containing phosphate ions in order to generate a calcium phosphate precipitate. Thus, the DNA to be transfected forms a calcium precipitate, readily observed under the microscope (a few hours after transfection), which cells incorporate through an endocytosis process. For western blotting and immunochemistry experiments, HEK cells were transiently transfected using this approach. Optimal conditions for this Calcium Phosphate method were defined through years. Briefly, 186 µL of a 1 M $CaCl_2$ solution were added to 750 µL of Milli-Q water containing 10 µg of the desired DNA and incubated for 2 minutes at room temperature. Next, 750 µL of HEPES (50mM; pH 7.05) were added and the solution vigorously mixed for 1 minute, after which a 1:10 final volume of the transfection solution was added to the cell culture (45-75% confluent). Cells were harvested after 48 hours of incubation in order to ensure the proper expression of the desired protein. In case of co-transfection of more than one DNA, the final amount of DNA (10 µg) was completed with the empty vector pcDNA3.1.

2.5.2 TRANSFECTIN METHOD| For radioligand binding assays and FRET experiments, transfections were carried out with TransFectin ™ Lipid Reagent (Bio-Rad) as described in the manufacturer's instructions. Briefly, the procedure begins with the preparation of two solutions: one containing 0,8 µg of plasmid DNA for each 50 µL of serum-free medium; and other containing 0,8 µL of TransFectin Reagent also for each 50 µL of serum-free medium. The two solutions are then gently mixed together and incubate for 20 minutes at room temperature. Next, since we were using 6-well plates for these experiments, instructions were to directly add 250 µL of the DNA-TransFectin solution to each well, already containing 750 µL of serum-containing medium. Similarly to the previous method, cells were harvested after 48 hours of incubation at 37ºC in a 5% CO_2 atmosphere. This procedure is illustrated in figure 15.

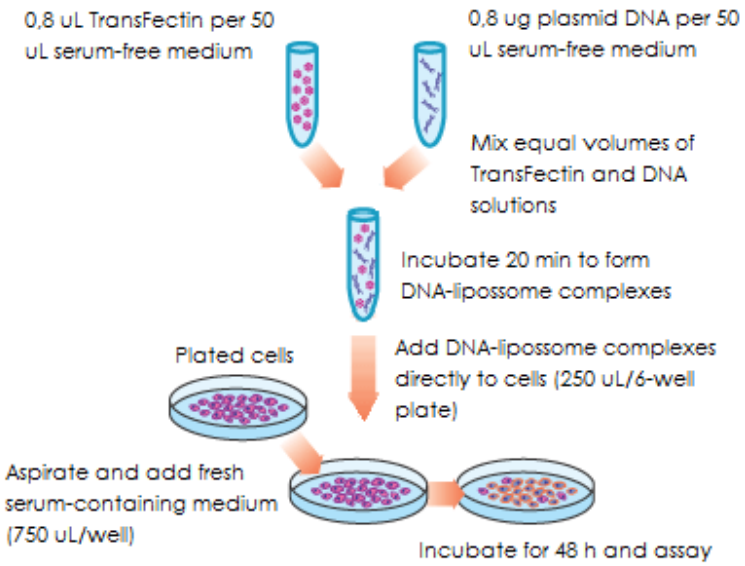


Figure 15 – Scheme of transfection with TransFectin ™ Lipid Reagent method (adapted from www.bio-rad-com).

2.6 PREPARATION OF MEMBRANE EXTRACTS|

For Western blot analysis or co-immunoprecipitation assays, membrane suspensions from transfected HEK-293 cells or from rat striatum were obtained as described below: Transiently transfected HEK-293 cells were detached with 150 µL of EDTA 500mM, centrifuged for 5 minutes at 1000 rpm and washed twice with ice-cold PBS (Phosphate

Buffered Saline; 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.9 mM $NaH_2PO_4 \cdot 2H_2O$, 154 mM NaCl, pH 7.4). From here on, the protocol for membranes preparation is the same for both cells and tissue. The samples were resuspended in 1,5mL of Tris-HCl 50mM pH 7,4 containing a protease inhibitor cocktail (1/100), homogenized with a polytron (2 x 20 seconds so the samples would not heat) and centrifuged for 30 minutes at 13200 rpm at 4ºC. The resulting pellet corresponds to total cell membranes. The pellet was then resuspended in ice-cold TRIS buffer and centrifuged twice at 4ºC for washing. Protein concentration of the samples was determined by a BCA protein assay kit (Pierce Biotechnology, Rockford, USA), using BSA dilutions as standards. After a final centrifugation, the pellets from samples were resuspended in a 4x concentrated Laemmli sample buffer (3g Tris-HCl pH 6.8 (250mM), 40mL 40% (v/v,) Glycerol, 5g 5% (p/v) SDS, 5mg 0.005% (p/v) Bromophenol Blue, 200mM DTT 2%) and heated at 100ºC for 5 minutes.

2.7 WESTERN BLOT ANALYSIS|

This common molecular biology technique, defined by Towbin et al., (1979) and Burnette (1981), allows the identification of proteins both on the basis of its detection with a specific antibody and on its relative molecular weight.

Denatured samples were analyzed by SDS-PAGE-based Laemili method (Laemmli, 1970). Thus, a 7-10% polyacrilamide gel was prepared and 20-40µg of proteins were resolved with a constant voltage of 80V (stacking phase), followed by 120V until samples ran completely, in an electrophoresis buffer (25 mM Trisbase, 0,19 M glicine, 0,1% SDS).

After the electrophoresis, proteins were transferred into a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Biosciences, Uppsala, Sweden) using a semidry transfer system (Trans-blot SD Semi-Dry Transfer Cell, BioRad), at a constant voltage of 20 V during 30 minutes. Membranes were blocked for 1 hour in a 5% milkblot (PBS with 0.05% Tween20 (PBS-Tween20) and 5% non-fat dry milk) and then incubated with the primary antibody overnight at 4°C. Membranes were then washed three times (10 minutes each wash) with PBS-Tween20 and incubated with the corresponding secondary antibody, also diluted in 5% milkblot. After additional washes, the blots were developed using a chemiluminescent detection kit (SuperSignal West Pico, Pierce Biotechnologies) in a luminescent analyzer LAS-3000 (Fujifilm, Tokio, Japan). The density of the bands on the

membrane was analyzed with Multi Gauge software. Whenever the membranes were to be reprobed, a striping solution (Re-Blot Plus Strong Solution 10x, Chemicon International) was used to break all previously bonds between primary and secondary antibodies. The membrane was incubated for 15 minutes at room temperature with the referred solution, followed by blocking and western blot protocol.

2.8 IMMUNOFLUORESCENCE|

Transiently transfected HEK-293 cells were harvested, rinsed with PBS and then fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were washed with PBS containing 20 mM glycine (buffer A) to quench aldehyde groups and permeabilized for 5 minutes with PBS/20mM glycine containing 0,05% Triton X-100. After two washes, blocking was performed for 1 hour using buffer A containing 1% BSA (buffer B), and cells were labeled with the indicated primary antibody, for 1 hour at room temperature. After another 30 minutes of washing in buffer B, cells were stained with the corresponding secondary antibodies for another hour. The antibodies used were diluted in PBS/20mM glycine/1% BSA. Finally, cover slips were mounted with mounting medium Vectashield H-1000 (Vector Laboratories, Inc. Burlingame, USA) and confocal microscope observations were performed in a Compact Laser Scanning Confocal Microscope.

2.9 MEASUREMENT OF cAMP ACCUMULATION|

The accumulation of cAMP was measured with a [3H]-cAMP assay system (Amersham Biosciences) as described in the manufacturer's manual. 24 hours after transfection with $A_{2A}R^{CFP\text{-}N}$, Adenosine deaminase (ADA) was added (0,5 u/mL) to fresh minimum essential medium without serum, and cells were left overnight at 37ºC in a humidified 5% CO_2 atmosphere incubator. Before the determination of cAMP levels, a sample (50 μM) of the phosphodiesterase inhibitor Zardaverine (Calbiochem, San Diego, CA) was added to the media 10 min before the agonist treatment. Then, A_{2A} receptor agonist adenosine (Sigma-Aldrich) 10 μM or forskolin (Sigma-Aldrich) 10 μM (in control experiments) were added to certain wells and incubated for 10 min at 37ºC, followed by the cAMP levels' determination assay.

2.10 CO-IMMUNOPRECIPITATION ASSAY|

For co-immunoprecipitation assays, membranes were first solubilized in ice-cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (1/100) to a final concentration of 2mg protein in 1mL. 200µL of lysate were reserved and precipitated with 1mL of cold acetone, left at -20ºC overnight. The remaining supernatant was incubated overnight with 5µg of the indicated primary antibody, under constant rotation at 4 °C. Then, 50 µl of a suspension of TrueBlot anti-rabbit Ig immunoprecipitation beads (eBioscience, Boston, MA) were added and the mixture was incubated for 2 hours on a rocking platform at 4 °C. After a 1 minute spin at 13600 rpm, pelleted beads were washed 3 times with 500 µl of RIPA buffer and the supernatant was discarded. The acetone precipitates were centrifuged at 13600 rpm for 20 minutes at 4ºC and the acetone was aspirated and the remains left to evaporate. Then, samples were prepared for SDS-PAGE, by adding 100 µl Laemmli buffer (with DTT 2%) to the bead pellet, followed by vortex and heat to 90-100°C for 5 minutes. After a spin of 3 minutes, supernatant was collected and loaded onto the gel. Immunoblottings were performed using the appropriate antibodies.

2.11 LIVE-CELL IMAGING: FRET ASSAYS|

Cells were seeded into 18 mm diameter glass coverslips and transfected the next day. For the measuring of cAMP response, cells were transiently transfected with $A_{2A}R^{N\text{-}CFP}$ and cAMP sensor $Epac1^{CFP/YFP}$ (Nikolaev et al., 2004) for the recording of ligand-induced changes, cells were transfected with the $A_{2A}R^{N\text{-}CFP}$ alone. FRET assays were performed 20–48 hours post-transfection. Cells maintained in Hank's Buffered Salt Solution (**HBSS**) were placed at room temperature on a Zeiss inverted microscope (Axiovert135) equipped with an oil immersion x63 Plan-Neofluar objective and a dual-emission imaging acquisition system coupled to a CoolSNAP-HQ charge-coupled device camera (Visitron Systems, Puchheim, Germany). Single cells were excited with light from a polychrome IV (TILL Photonics) and the illumination time was typically set to 25 ms applied with a frequency of 40 Hz. All binding events were recorded in real-time, while cells were continuously

superfused with HBSS pH 7,4. Detected FRET signals were digitalized with an AD converter (Digidata1322A, Axon Instruments, Union City, CA) and stored on a personal computer by using CLAMPEX 9.0 software (Axon Instruments).

Chapter 3

Results and Discussion

3.1 MEMBRANE YEAST TWO HYBRID| A whole series of proteins were found to interact with the A_{2A} receptors.

Using the "split16 ubiquitin Membrane Yeast Two Hybrid (MYTH)" system, developed by Dr. Igor Stagljar, a screening using the C-terminal tail region of the A_{2A} receptor as bait was performed, giving rise to a whole series of membrane proteins (table III).

Table III - List of some of the proteins that in the performed screening were positive for the interaction with the A_{2A} receptor.

Homo sapiens melanoma antigen family H, 1 (MAGEH1), mRNA
Homo sapiens spinster homolog 1 (Drosophila) (SPNS1), mRNA
Rhomboid domain-containing protein 2
Homo sapiens immunoglobulin superfamily containing leucine-rich repeat (ISLR), transcript variant 1, mRNA
Homo sapiens heat shock 70kDa protein 8 (HSPA8), transcript variant 1, mRNA
Homo sapiens Yip1 interacting factor homolog B (S. cerevisiae) (YIF1B), transcript variant

The found proteins may, on the one hand, play a role in the function of these receptors and, on the other hand, may also interact with other GPCRs (such as mGlu5Rs and D_2Rs), contributing to their heteroligomerization and playing a major role in the molecular and functional plasticity of these GPCRs complexes.
Similar to the classic version, the split-ubiquitin MYTH system is prone to false positives and thus, interactions that have been identified in this screen should be further confirmed in an independent system. Co-immunoprecipitation or pull-down assays, using recombinant expressed proteins or protein fragments, are commonly used to this purpose, although both methods are problematic when used with integral membrane proteins.
Alternatively, methods that are more suitable for use with membrane proteins, such as co-localization experiments or FRET, may also be used to confirm an interaction.

3.2 CLONING OF $A_{2A}R^{CFP-N}$| A construct of adenosine A_{2A} receptor containing the cyan fluorescent protein CFP tagged to the N-terminal was successfully cloned.

In order to study the kinetics of agonist binding to $A_{2A}R$ and determine the effect of this receptor oligomerization on receptor binding using the real-time FRET approach, the first step was to generate a receptor construct that contained in its N-terminal tail the fluorescent protein CFP (figure 16), that can act as a donor in a FRET process if the proper acceptor is in close proximity. To this end, classical molecular biology techniques were used (as described in 2.3), in order to produce the $A_{2A}R^{CFP-N}$ construct.

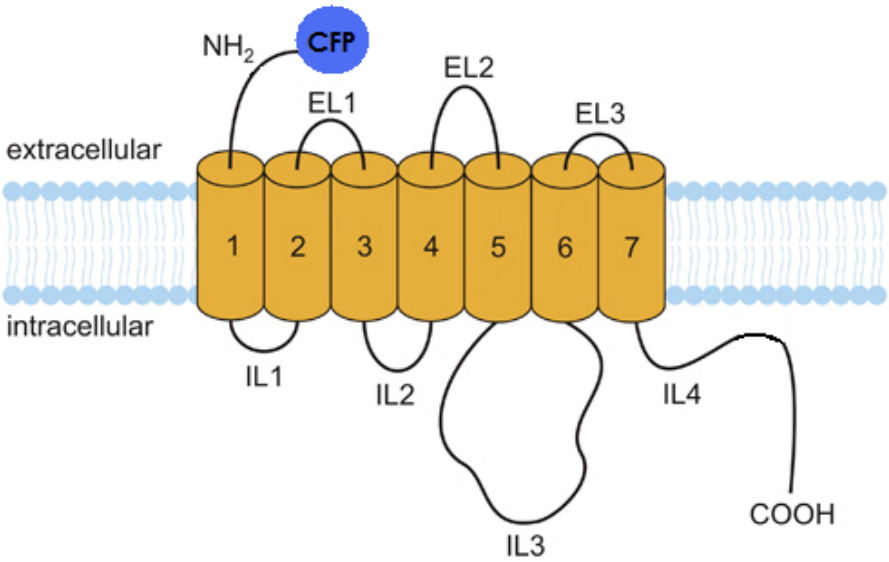


Figure 16 – Scheme of the $A_{2A}R^{CFP-N}$ construct (adapted from http://www.fz-juelich.de/isb/isb-1/GPCR)

Briefly, the synthetic $A_{2A}R$ genes were amplified by PCR with the primers FA_{2A}BamHI and RA_{2A}XhoI, purified and digested with the restriction enzymes BamHI and XhoI, as described in 2.3.3, and inserted into the vector CFP-PTHR, already digested with the same enzymes (2.3.4). After bacterial transformation with thermal choking process using competent *Escherichia coli* cells (2.3.5) the colonies that have grown in LB-agar-ampicilin plaques were selected (the control plaque contained the vector alone) and checked by PCR screening (2.3.6). Once confirmed the presence of the insert, two positive chosen colonies were purified by MAXI preps. Finally, $A_{2A}R^{CFP-N}$ construct successful cloning was

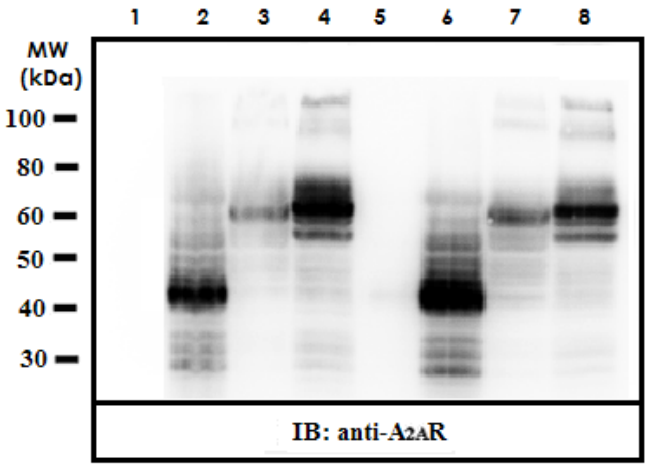
confirmed by a PCR sequencing protocol (2.3.7) using the T7 vector primer, FA_{2A}BamHI and RA_{2A}XhoI.

The sequences were checked by BLAST, a bioinformatics algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences. This final step confirmed that the insert was correctly inserted into the vector and that the construct $A_{2A}R^{CFP\text{-}N}$ was ready to use.

3.2.1 EXPRESSION OF THE RECEPTOR CONSTRUCT| Clone $A_{2A}R^{CFP\text{-}N}$ is properly expressed in HEK-293 cells and mainly distributed in the plasma membrane.

The correct expression of the receptor construct was analysed by western blot and confocal microscopy techniques. For both approaches, the fusion protein $A_{2A}R^{CFP\text{-}N}$ was transiently transfected into HEK-293 cells, as previously described in section 2.5.1.

Shown in figure 16 is the expression pattern of the $A_{2A}R^{CFP\text{-}N}$ immunodetected with the mouse anti-$A_{2A}R$ antibody and with the rabbit anti-GFP antibody, at the expected molecular weight ~67kDa, which results from the ~42 kDa of the $A_{2A}R$ plus the ~25 kDa of the CFP protein. Cells were also transfected with the empty pcDNA3.1 vector (as a negative control), $A_{2A}R^{WT}$ and $A_{2A}R^{YFP}$ (as a positive control).



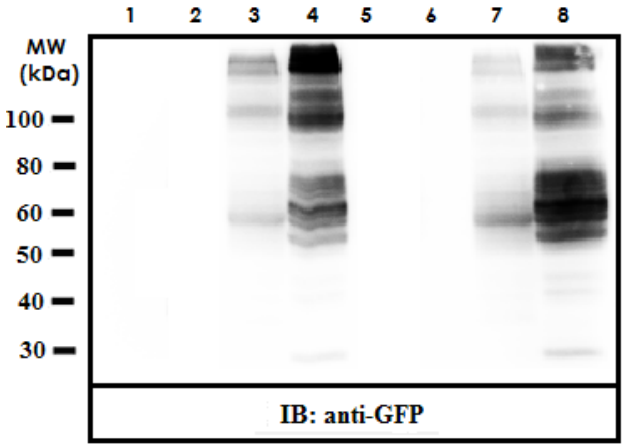


Figure 16 – Detection of $A_{2A}R^{CFP-N}$ construct by Western-blot technique. 40 µg of protein were applied in a 7% polyacrylamide gel by means of SDS-PAGE, followed by Western-blot and developed with specific antibodies. HEK-293 cells, transiently expressing the empty vector pcDNA3.1 (lane 1 and 5), $A_{2A}R^{WT}$ (lane 2 and 6), $A_{2A}R^{CFP-N}$ (lane 3 and 7) or $A_{2A}R^{YFP}$ (lane 4 and 8), were washed and membranes processed for immunoblotting, which was developed with mouse anti-$A_{2A}R$ monoclonal antibody (upper panel) or with rabbit anti-GFP polyclonal antibody (lower panel). The figure is representative of 4 assays independently performed.

The upper panel was obtained with the mouse anti-$A_{2A}R$ monoclonal antibody, while the lower one was probed with the rabbit anti-GFP polyclonal antibody. Both of the antibodies revealed specificity for $A_{2A}R$ and GFP, since the bands were not visible on the lanes that contained pcDNA3.1 (in both cases) and $A_{2A}R^{WT}$ (in the second probe).

A construction of $A_{2A}R^{YFP}$ was included so as to provide a molecular weight to expect and compare to the $A_{2A}R^{CFP}$. Although both constructions were detected, as predicted, at ~67kDa, the presence of two bands in the blot of the $A_{2A}R^{YFP}$ was a little unexpected. The appearance of two distinct bands at the expected molecular weight may be due to a number of events such as glycosylations, as is it known that GPCRs can be extensively N-glycosylated. In order to remove the N-linked glycans and consequently deglycosylate the protein, another step would have to be performed: the samples had to be previously treated with the peptide N-Glycosidase F, an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins.

Another difference between the $A_{2A}R^{YFP}$ and $A_{2A}R^{CFP}$ worth to be discussed is the unmistakable higher intensity of bands of the former. The hypothesis that this could be due to differences in the quantity of sample loaded onto the gel was rapidly eliminated, since the results showed here were replicated in the 4 assays performed. A difference in the success rate of DNA transfection was also considered, but that proposition was discarded when the immunofluorescence experiments were performed (images displayed above), since there was a similar number of fluorescent cells in both cases. It is then likely that a higher intensity of the $A_{2A}R^{YFP}$ band means that this receptor is more synthesized and expressed in the cell since, as shown bellow, although a higher distribution of the $A_{2A}R^{CFP}$ clone in the cell membrane was observed by confocal microscopy, higher protein levels of $A_{2A}R^{YFP}$ were detected in the blot.

The expression and cellular distribution of $A_{2A}R^{CFP\text{-}N}$ is represented in the confocal microscopy images shown below (figure 17), as well as of the $A_{2A}R^{WT}$ and $A_{2A}R^{YFP}$ used in the previous immunoblotting. It is demonstrated by the confocal microscopy technique that the construct $A_{2A}R^{CFP\text{-}N}$ is essentially distributed in the plasma membrane of cells.

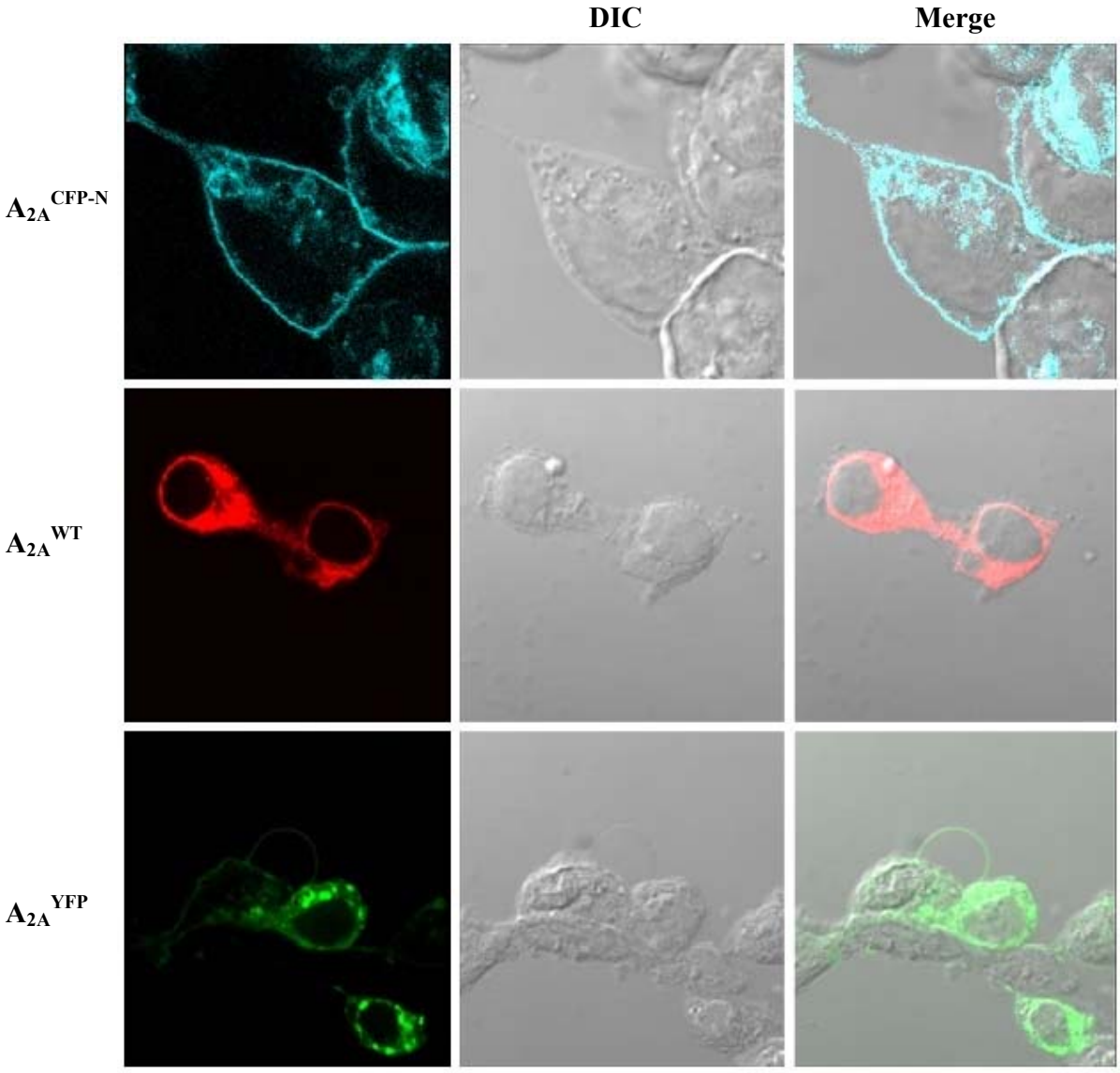


Figure 17 - Expression of adenosine A_{2A}^{CFP-N}, A_{2A}^{WT} and A_{2A}^{YFP} receptors by confocal microscopy. 48 hours after transfection, HEK 293 cells were washed, fixed, permeabilized and processed for immunostaining. In the case of the $A_{2A}R^{WT}$, since it is not linked to a fluorescent molecule, it was incubated with the mouse anti-$A_{2A}R$ monoclonal antibody and detected using Cy5-conjugated donkey anti-mouse IgG antibody. Due to the properties of Compact Laser Scanning Confocal Microscope, both fluorescence and differential interference contrast microscopy (**DIC**) imaging can be acquired simultaneously.

In this immunofluorescence assay, alike to what was done in the prior experiment, a sample with empty vector pcDNA3.1 was tested as a negative control. It was verified that there is no

fluorescence background – all the fluorescence is specific from the constructions (data not shown).

Concerning the detection of the wild-type receptor, since it does not have any fluorescence of its own, indirect immunofluorescence was performed by labeling the receptor with a specific primary antibody, monoclonal mouse anti-$A_{2A}R$, followed by secondary fluorescent-antibody detection, using Cy5-conjugated donkey anti-mouse IgG antibody. According to the images obtained, it seems that the receptor $A_{2A}{}^{WT}$ is distributed both in the plasma membrane and in the cytosol.
Regarding $A_{2A}{}^{YFP}$ and $A_{2A}{}^{CFP\text{-}N}$ receptors, both are mostly present in the plasma membrane, although the distribution of the former also extends to the interior of the cell, similarly to the $A_{2A}R^{WT}$. These differences are probably due to the fact that the $A_{2A}R^{CFP\text{-}N}$ construct has a peptide signal that came from the PTHR used for cloning, which is responsible for making almost every synthesized receptor to be delivered to the plasma membrane – this signal sequence is not present in the $A_{2A}{}^{YFP}$.

3.2.2 FUNCTION OF THE RECEPTOR CONSTRUCT| Acute activation of $A_{2A}R^{CFP\text{-}N}$ construct by adenosine resulted in robust cAMP accumulation above basal levels.

To validate the functionality of the $A_{2A}R^{CFP\text{-}N}$ construct, we tested its signalling capability. As mentioned above, A_{2A}Rs are coupled to Gα proteins, thus activating adenylate cyclase and increasing the intracellular levels of cAMP. In HEK-293 cells transiently expressing the construct under study, agonist challenge (adenosine 10μM) induced a similar increase in cAMP accumulation when compared to the $A_{2A}R^{WT}$ transfected cells (figure 18).

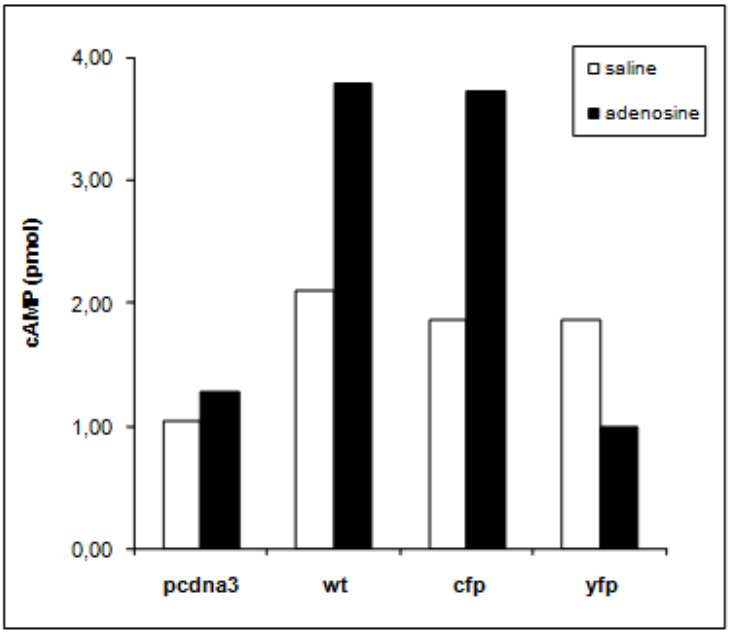


Figure 18 - cAMP response to adenosine 10µM through the empty vector pcDNA3.1 (control), $A_{2A}R^{WT}$, $A_{2A}R^{CFP}$ and $A_{2A}R^{YFP}$ expressed in HEK-293 cells.

After agonist challenge, levels of cAMP accumulation were determined. The basal level of cAMP was approximately 1 pmol, while the maximal level was achieved with adenosine treatment and reached around 4 pmol.

Once again, cells transfected with the empty vector pcDNA3.1 were used as a control – as expected, upon agonist stimulation there was no significative change in the levels of cAMP. In addition, considering that these control values correspond to the basal levels of cAMP, when taking into account the levels of this second messanger accumulation when receptors only receive saline solution, it seems that all adenosine receptors in study have some level of constitutive activity, a phenomenon well documented for several GPCRs (Alewijnse et al., 1997; Seifert et al., 2002). Since these assays were performed in the presence of the phosphodiesterase inhibitor Zardaverine, therefore preventing the inactivation of the intracellular cAMP, the increases in this second messenger were concluded to be through activation of adenylate cyclase.

Differently from what happened with the control, both $A_{2A}R^{WT}$ and $A_{2A}R^{CFP}$ present a clear and similar enhance of cAMP accumulation after agonist stimulation, thus indicating that the N-terminal tagged $A_{2A}R$ is functional – apparently, having the CFP protein in this

extracelular terminal poses no impediment to the full functionality of the $A_{2A}R^{CFP}$ construction.

Surprisingly enough, the $A_{2A}R^{YFP}$, a construction used in all previous experiments as a comparison model to verify if the recently cloned $A_{2A}R^{CFP}$ was correctly expressed, showed a much diminished cAMP accumulation upon adenosine challenge. One possibility that could explain such a reduction of function relative to the basal levels, would be a change in the G protein coupling, like a switch from a Gα to a Gi.

3.3 LIVE-CELL IMAGING: FRET ASSAYS|

3.3.1 cAMP FRET| Acute stimulation of HEK-293 cells transfected with both cAMP sensor and $A_{2A}R^{CFP}$ induced a rapid and significant cAMP production.

Construct functionality was also analyzed by means of intramolecular FRET. Although a direct quantification of the second messenger in study is not accomplishable with this method, it has a number of advantages, starting with the absence of radioactivity. Also, it allows the monitoring of intracellular cAMP levels in a *single, intact, living* cell, which confers to the extracted information good spatial and temporal resolution.

Therefore, with the intention of recording the $A_{2A}R^{N\text{-}CFP}$-mediated cAMP synthesis upon agonist stimulation, HEK-293 cells were transfected with both intramolecular FRET cAMP sensor $Epac1^{CFP/YFP}$ (Nikolaev et al., 2004) and the construct $A_{2A}R^{CFP}$. Stimulation of these cells with 10µM adenosine induced an acute cAMP production, in conformity to the observations from the [3H]-cAMP assay – cAMP FRET results can be found in Annex (figure 19).

When CFP excitation occurs, some of the energy is transferred to YFP, meaning that intramolecular FRET of cAMP sensor is occurring - CFP excitation leads to YFP emission, which can be observed in the figure 19 by a change in the math ratio (fluorescence emission at 480 nm / fluorescence emission at 535 nm). The reason why no major changes are visible on the CFP channel is because in this experiment its signal was saturated. Despite of that, obtained results may be used to validate the correct functioning of the receptor.

Upon agonist (adenosine 10µM) stimulation, there is a sudden alteration of the emission levels of the fluorescent proteins, as well as a substantial change in the math ratio. This is due to the fact that receptor activation leads to the stimulation of the intracellular signaling cascade which consequently results in the production of cAMP. This second messenger binds to the cAMP sensor, in a binding domain fused between CFP and YFP, inducing a conformational change. Specifically, an increase in distance between the fluorophores occurs, so now CFP is not transferring energy to YFP – the ratio CFP/YFP diminishes and a decrease in FRET is measured (figure 20).

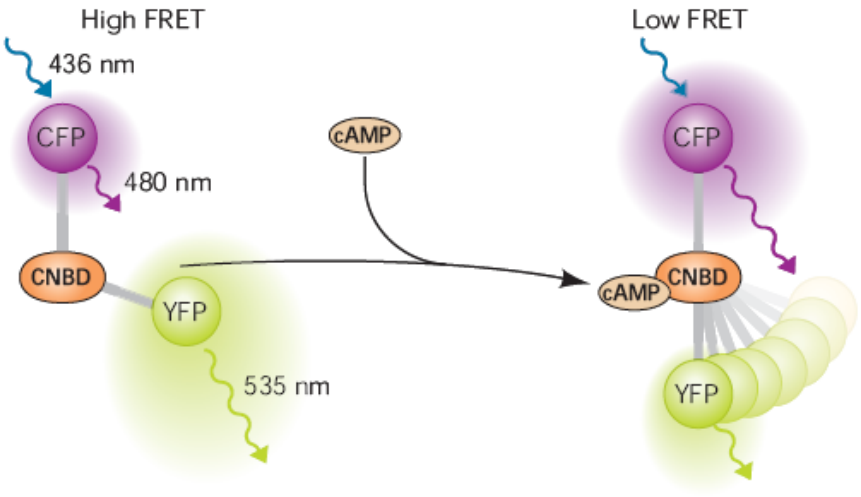


Figure 20 – Scheme of what happens at the level of cAMP sensor when cell is challenged with agonist. The cAMP sensor is comprised of a single cyclic nucleotide binding domain (CNBD) fused between CFP and YFP. When cAMP is produced and released in the cell, it induces a conformational change leading to an increase in distance between the fluorophores, which is measured as a decrease in FRET. (Taken from Nikolaev et al., 2006)

A control using the PTHR, a receptor known to be well expressed at the cell surface and functionally active, capable of using the cAMP signaling pathway, was also performed – as expected, the addition of adenosine had no effect on the fluorescence intensity of YFP due to the absence of FRET between the $A_{2A}R$ agonist and the PTHR (results available in Annex, figure 21), confirming that the FRET recorded in the anterior experiment was due to the specific ligation of adenosine to $A_{2A}R^{N\text{-}CFP}$.

A couple of extra control experiments should have been performed as well: forskolin, a direct activator of adenylyl cyclase, should have been added to the cells, providing a positive control. On the other hand, mock transfected cells or cells expressing the empty vector pcDNA3.1 should have been stimulated as well – the lack of response would have provided the negative control for this experiment. Finaly, it would have been interesting to verify if preincubation with some selective antagonist like SCH 58261 would have prevented the increase of cAMP levels, confirming the ligand-receptor interaction specificity.

It is also important to discuss the question of the CFP signal from the $A_{2A}R^{CFP}$ construct. The observed diminishing of the ratio CFP/YFP is due not only to an increase of the YFP

signal, but it should also be due to a decrease of the CFP emission (in case its signal was not saturated) – however, it is likely that part of this signal is coming from the fluorophore of the $A_{2A}R$. To address this question a control FRET would have to be performed using the cAMP sensor and $A_{2A}R^{WT}$ – this way, the obtained FRET values could be used to calculate how much of the measured FRET in the original experiment was due to the emission of the CFP coupled to the $A_{2A}R$.

3.3.2 LIGAND-BINDING ASSAY| Fluorescent ligand CGS21680-Alexa488 of $A_{2A}R^{CFP}$ displayed significant binding for the receptor in study.

In order to begin the FRET-based ligand binding assays, one last step is needed: the $A_{2A}R^{CFP}$ ligand that will be used in future experiments needs to be tested as well. For that, HEK-293 cells transiently expressing $A_{2A}R^{CFP}$ were stimulated with an acute concentration of CGS21680-Alexa488, after which followed selective $A_{2A}R$ antagonist-induced displacement. More specifically, a single cell from the coverslip was chosen and recorded for 10 minutes while being excited at 436 nm (excitation of CFP, from $A_{2A}R^{N\text{-}CFP}$). When 2,5µM CGS21680-Alexa488 was locally added to the cell, there was a sudden raise of Alexa488 emission fluorescence, accompanied by an increase of the math ratio, which in this experiment is the fluorescence emission at 517 nm (Alexa488) / fluorescence emission at 480 nm (CFP), meaning FRET was occurring. Unsurprisingly, when selective $A_{2A}R$ antagonist SCH (10 µM) was added to the cell a couple of minutes after the cell had been stimulated with the ligand in study, it completely reversed the agonist effect on the receptor, visible by the abolishment of Alexa488 emission signal, as well as a return of the math value to basal levels (figure 22).

At a molecular level, what happened was this: upon ligation of fluorescent agonist to CFP-coupled adenosine receptor, FRET occurred between the two fluorophores as a result of their approach, caused by the conformational change of the activated receptor that brought the fluorescent molecules spatially closer (schematic representation in figure 23). Spatial and temporal resolution of such events in real time, opportunely provided by FRET microscopy techniques, is crucial to get a further understanding of the meanders of cell signaling.

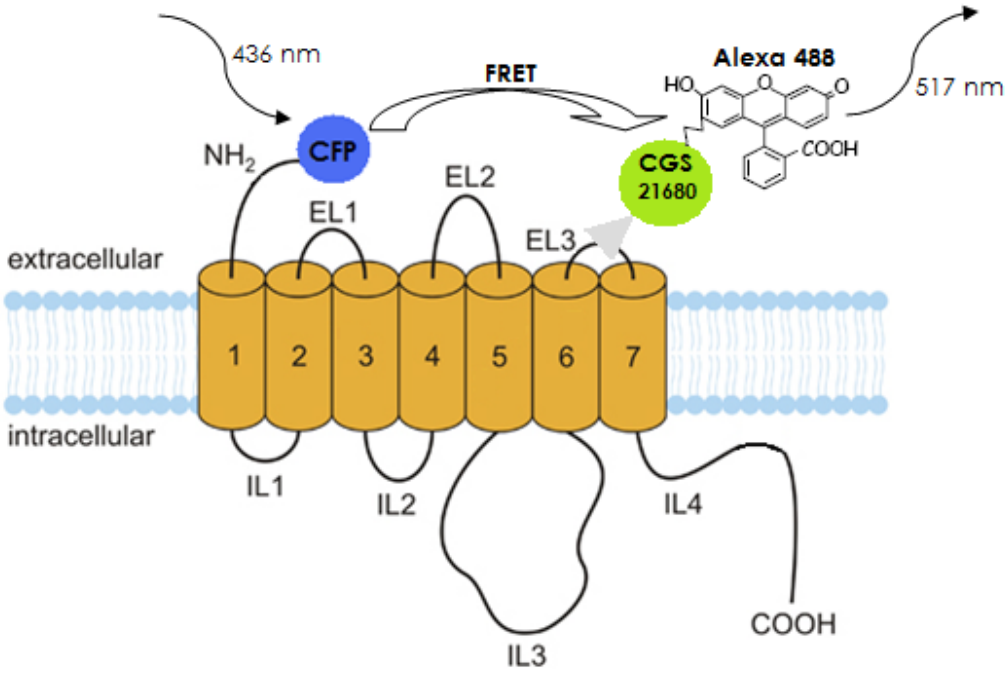


Figure 23 – Scheme of what is expected to happen in a cell transiently transfected with the construct $A_{2A}R^{N\text{-}CFP}$, upon stimulation with agonist CGS21680-Alexa488. Briefly, a selected cell is excited at 436nm which results in the excitation of CFP coupled to the adenosine receptor - at this moment, CFP is the only entity emitting fluorescence. When CGS ligand coupled to the fluorophore Alexa488 is added to the cell and interacts with the receptor, a conformational change caused by the receptor activation brings CFP and CGS21680-Alexa488 closer – close enough so that FRET can occur between the two fluorophores, which means that CFP emission excites Alexa488, which is now the principal emitter, at a different wavelength.

Again, a negative control using the PTHR was performed and, as expected, upon fluorescent agonist CGS21680-Alexa488 challenge, no changes in the levels of emitted fluorescence were detected, confirming the specificity of the ligand (result not shown).

3.3.3 CONCLUSIONS AND FUTURE DIRECTIONS| Now that a functional A_{2A} receptor tagged with a compatible fluorescent protein and the respective fluorescent agonist are available in our laboratory, it is intended to determine the binding kinetics parameters of this receptor by means of FRET techniques, taking advantage of the high spatial and temporal resolution provided by this method. Using cAMP sensors, conformational changes

produced to the receptor by its ligand may be determined and a correlation binding/receptor activation might be established by recording, at the single-cell level, sequential events occurring in real time, including the binding of agonist followed by receptor activation and cAMP response. Furthermore, by appropriately label heterotrimeric G proteins with compatible fluorophores, it is also intended to characterize the kinetics of G protein activation by means of a FRET approach, and thus to carry out a correlation between agonist binding, receptor activation (conformational change) and G protein activation (e.g. Gαi or Gαs). Also, interactions with other proteins such as mGlu5 and D_2 receptors can induce conformational changes, detected by variations in the intramolecular FRET. Within this context, transinhibition and/or transactivation phenomena between these receptors when they form part of an oligomeric complex will be particularly interesting to study.

In conclusion, in future experiments the ligand CGS21680-Alexa488 can be used as a tool for a wide number of different studies. Namely, it will be suitable to investigate the kinetics of adenosine A_{2A} receptor trafficking in living cells or, once assayed the binding patterns of this receptor, it will be possible to determine what changes occur when it oligomerizes with $mGlu5R^{YFP}$ and/or D_2R^{YFP}. The knowledge of the kinetic parameters for a named receptor expressed alone or as part of an oligomeric complex appears to be essential in order to understand its functionality in a further complex situation (e.g. the striatal spine module).

3.4 OLIGOMERIZATION STUDIES IN HEMIPARKINSONIAN RATS|

3.4.1 RELATIVE QUANTIFICATION OF GLUTAMATE, DOPAMINE AND ADENOSINE RECEPTORS IN RAT STRIATUM| By means of Western blot, relative expression levels of $A_{2A}R$, D_2R and mGlu5R were determined in hemiparkinsonian rats (figure 24), and a comparison between the left striatum homogenates (6-hydroxydopamine injured) and the right striatum homogenates (not injured), was performed.

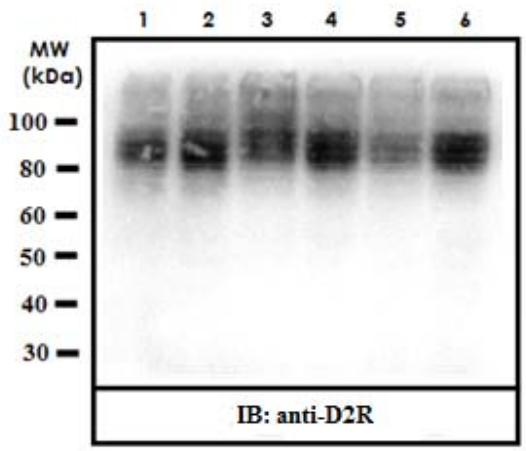


Figure 24 – Membrane extracts were prepared and 40 μg of protein from each striatum (injured and not injured) were used for immunoblot analysis in four different probes with the following antibodies: anti-$A_{2A}R$, anti-D_2R, anti-mGlu5R and anti-actinin (as a loading control). The figure is representative of independent assays performed with extracts of 3 animals – in this case it shows the immunoblot analysis with anti-D_2R antibody. Animal 1: lanes 1 (control striatum) and 2 (injured striatum); animal 2: lanes 3 (control striatum) and 4 (injured striatum); animal 3: lanes 5 (control striatum) and 6 (injured striatum).

By analyzing the representative immunoblot above, it appears that in the injured striatum, which pretends to simulate the pathological physiology of PD, D_2 receptor is more expressed relatively to the control striatum in all three animals. Similar results were obtained when the immunoblot was developed with the antibody anti-$A_{2A}R$, but no apparent difference was visible in the probe with anti-mGlu5R (results not shown). Using the Multi Gauge software, band intensity on the gels was measured and relative amounts of expressed proteins were

calculated. Values obtained were corrected with the amounts of expressed actinin, the loading control. Then the values obtained for the expressed receptors in the left striatum (injured side) were transformed in percentages considering that the control value for each receptor (100%) was the value determined in the right striatum (not injured). Results are shown in the graphic above (figure 25).

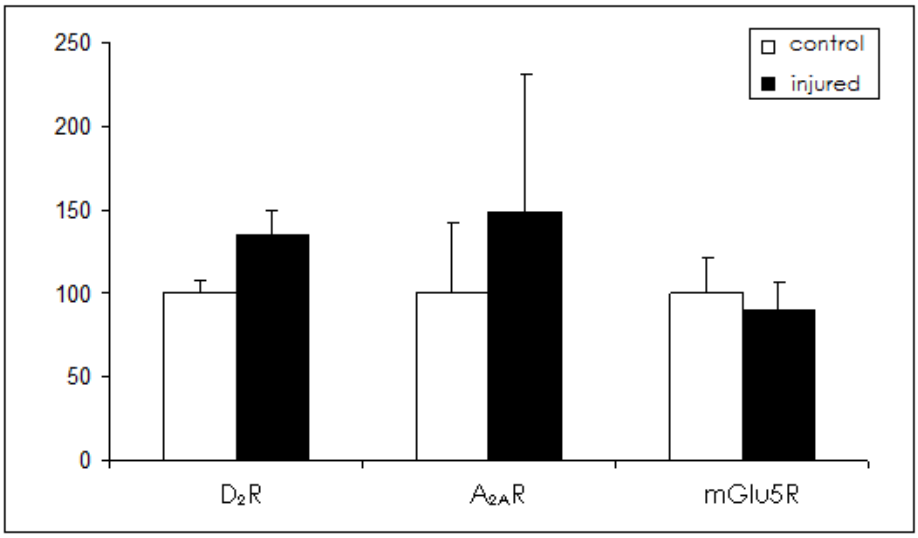


Figure 25 – Relative levels of protein expression were determined by Multi Gauge software and the obtained data processed and presented in this graphic.

Quantification of the immunoblot results, despite of the insufficient number of animals to make a statistical analysis, revealed a tendency of the D_2 and A_{2A} receptors to be more expressed in the injured striatum, while the mGlu5R appears to suffer no major alterations upon 6-hydroxydopamine treatment. These results are in line with those decribed by Hurley et al. (2000), who reported that the expression of adenosine A_{2A} receptors in the *substantia nigra pars reticulata* of brain from patients dying with PD was significantly increased when compared to *post-mortem* human brain tissue from normal subjects. Interstingly, no changes in the expression were observed in any other brain region examined. Despite of the encouraging results, such a high value of standard deviation in injured striatum cannot be disregarded, making any interpretation completely unreliable, since it is likely that when a higher number of injured rats are included in this study, the differences in the $A_{2A}R$ will not be as marked as shown in the graphic.

Regarding the D_2R, it would not be completely surprising if the receptor expression levels were actually augmented in the injured striatum, since it is plausible to think that some compensatory mechanism would be activated in order to minimize the effects of the dopaminergic neurons death. Regarding this subject, a very interesting study was performed by Gerfen et al. (1990), using the same animal model (the hemiparkinsonian rat) tested in this work. These authors observed that striatopallidal neurons show a 6-OHDA-induced elevation in their specific expression of mRNAs encoding the D_2R and enkephalin, while striatonigral neurons show a 6-OHDA-induced reduction in the expression of mRNAs encoding the D_1R and substance P. These converse results led to the conclusion that the differential effects of dopamine on striatonigral and striatopallidal neurons are mediated by their specific expression of D_1 and D_2 dopamine receptor subtypes, respectively.

3.4.2 CO-IMMUNOPRECIPITATION: PRELIMINARY RESULT| In order to fully understand the previous results and to find plausible justifications for the changes in receptors expression, in case the actual observations are confirmed by a larger number of analyzed animals, co-immunoprecipitation assays are essential.

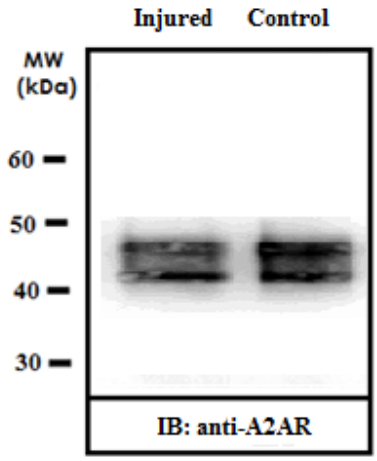


Figure 26 – Representative immunodetection of adenosine A_{2A} receptors in striatum of hemiparkinsonian rats. Total striatal membranes from control and 6-OHDA injured striatum (right and left, respectively) were processed for immunoblot using anti-$A_{2A}R$. Immunodetection was also performed with the antibodies anti-mGlu5R and anti-D_2R but a clear result was not obtained (results not shown).

In figure 26 is a representative immunoblot of membrane extracts from a hemiparkinsonian rat striatum, detected with anti-$A_{2A}R$. Although it appears that there may be differences between the expression of this receptor in the 6-OHDA injured side (left side) versus the control side, it is a very preliminary result and obviously it needs to be repeated and include a higher number of injured animals. In case these differences turnout to be confirmed and extended to mGlu5R and/or anti-D_2R, this could have a major relevance, for instance, in the stoichiometry of the mGlu5/D_2R/$A_{2A}R$ oligomeric complex, affecting its functional interactions, already described in literature (Popoli et al., 2001; Coccurello et al., 2004). Thus, the evaluation of the degree of interaction between these three receptors, through co-immunoprecipitation experiments, is of major importance since it would allow the design of a more appropriate pharmacological intervention in this motor-impaired condition.

Finally, after a clear result is obtained with this PD model, it is intended to verify if the results are reproduced in human brains from deceased PD patients.

3.4.2 CONCLUSIONS AND FUTURE DIRECTIONS| Once characterized the glutamate, dopamine and adenosine receptor expression, something that will only be achieved with a considerably higher number of hemiparkinsonian animals, extensive cross co-immunoprecipitation experiments will be carried out in order to determine the oligomerization degree of these receptors both in control and injured rats. These results will allow us to determine whether the absence of dopamine in the striatum alters the ability of these receptors to oligomerize. Interestingly, once the technique will be established for the hemiparkinsonian rat model, it is intended to do the same kind of experiments on both control and pathological (Parkinson's disease) human striatum obtained from the Brain Tissue Bank at the UB.

The detailed analysis of the potential results will determine whether the neurochemical imbalance, namely the decrease dopamine concentration as a result of the nigrostriatal dopaminergic neurons death, affects the oligomerization degree of glutamate, dopamine and adenosine receptors in the basal ganglia. This information seems to be relevant in order to design effective glutamate/dopamine/adenosine combined therapies for the treatment of Parkinson's patients.

Chapter 4

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Chapter 5

Annex

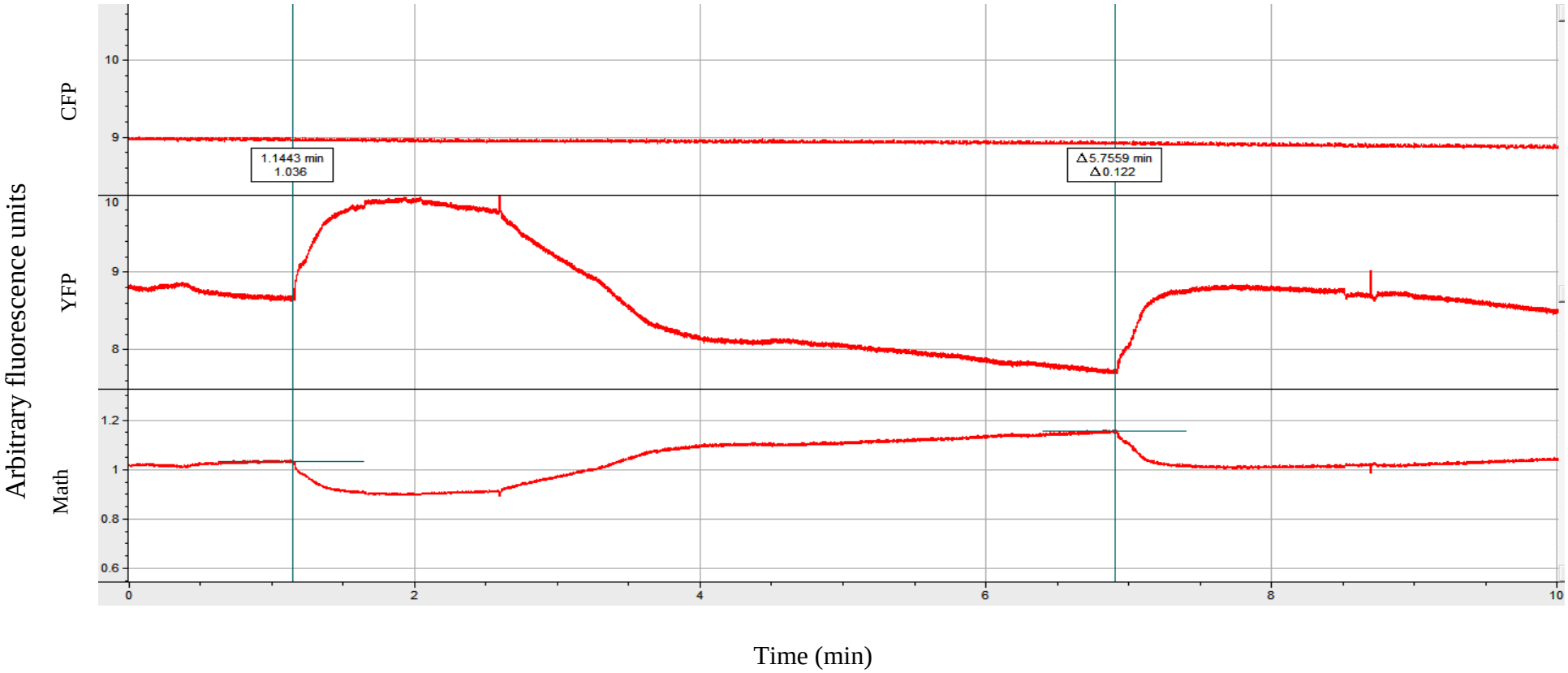

Figure 19 – FRET imaging of $A_{2A}R^{N\text{-}CFP}$-mediated cAMP signaling. HEK-293 cells were transiently transfected with $A_{2A}R^{N\text{-}CFP}$ and the cAMP sensor $Epac1^{CFP/YFP}$ in order to verify if there was cAMP synthesis in response to agonist stimulus. 10µM adenosine was used to stimulate a selected cell at two time-points (t = 1,11443min and 5,7559min after the first point) via a pipette. FRET occurs between fluorescent proteins fused to the cAMP-binding domain of cAMP sensor. The fluorescent cAMP sensor changes its conformation upon cAMP binding, which results in a decrease in the math ratio (fluorescence emission at 480 nm / fluorescence emission at 535 nm) – FRET.

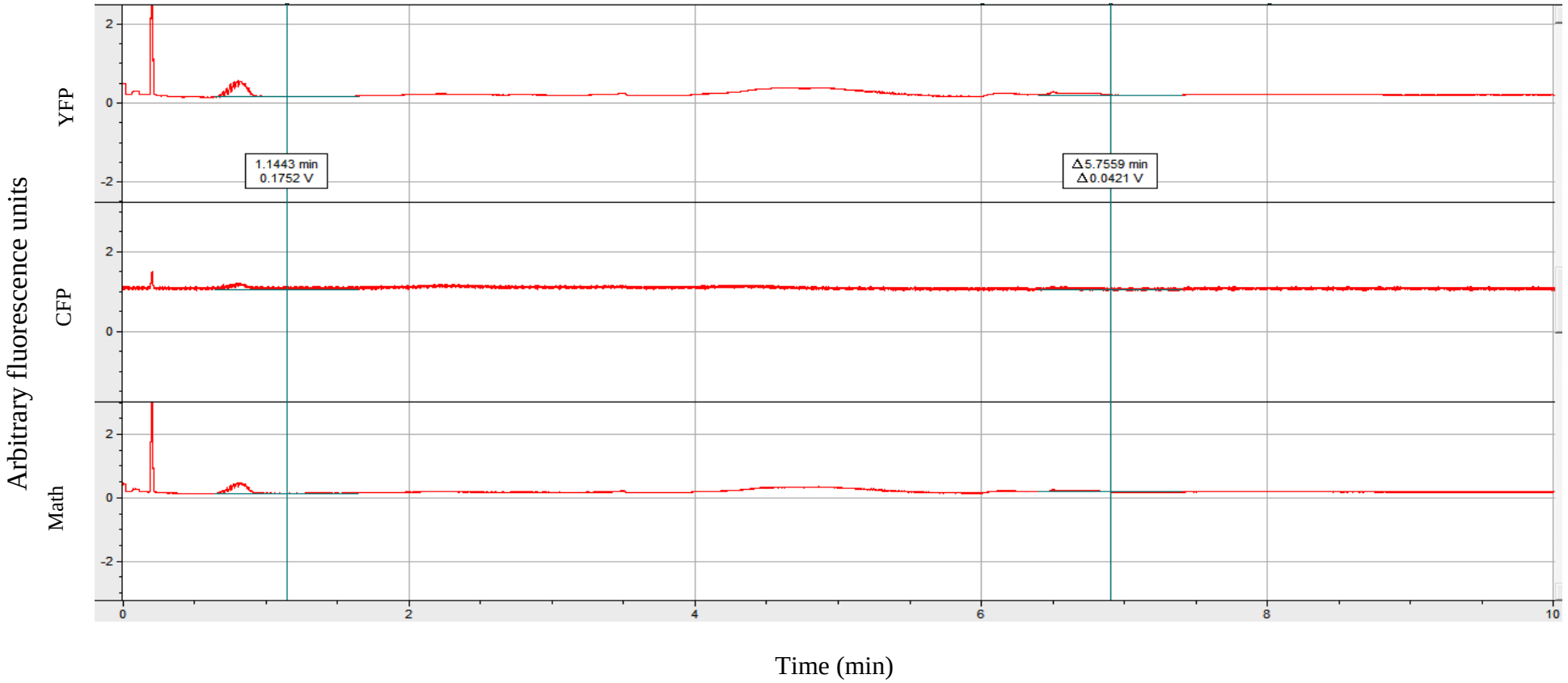

Figure 21 – HEK-293 cells were transiently transfected with receptor PTH (PTHR) and the cAMP sensor Epac1$^{CFP/YFP}$ in order to verify if there was cAMP synthesis in response to agonist stimulus. 10µM adenosine was used to stimulate a selected cell at two time-points (t = 1,11443min and 5,7559min after the first point) via a pipette. No significant alterations in the emitted fluorescences were registered.

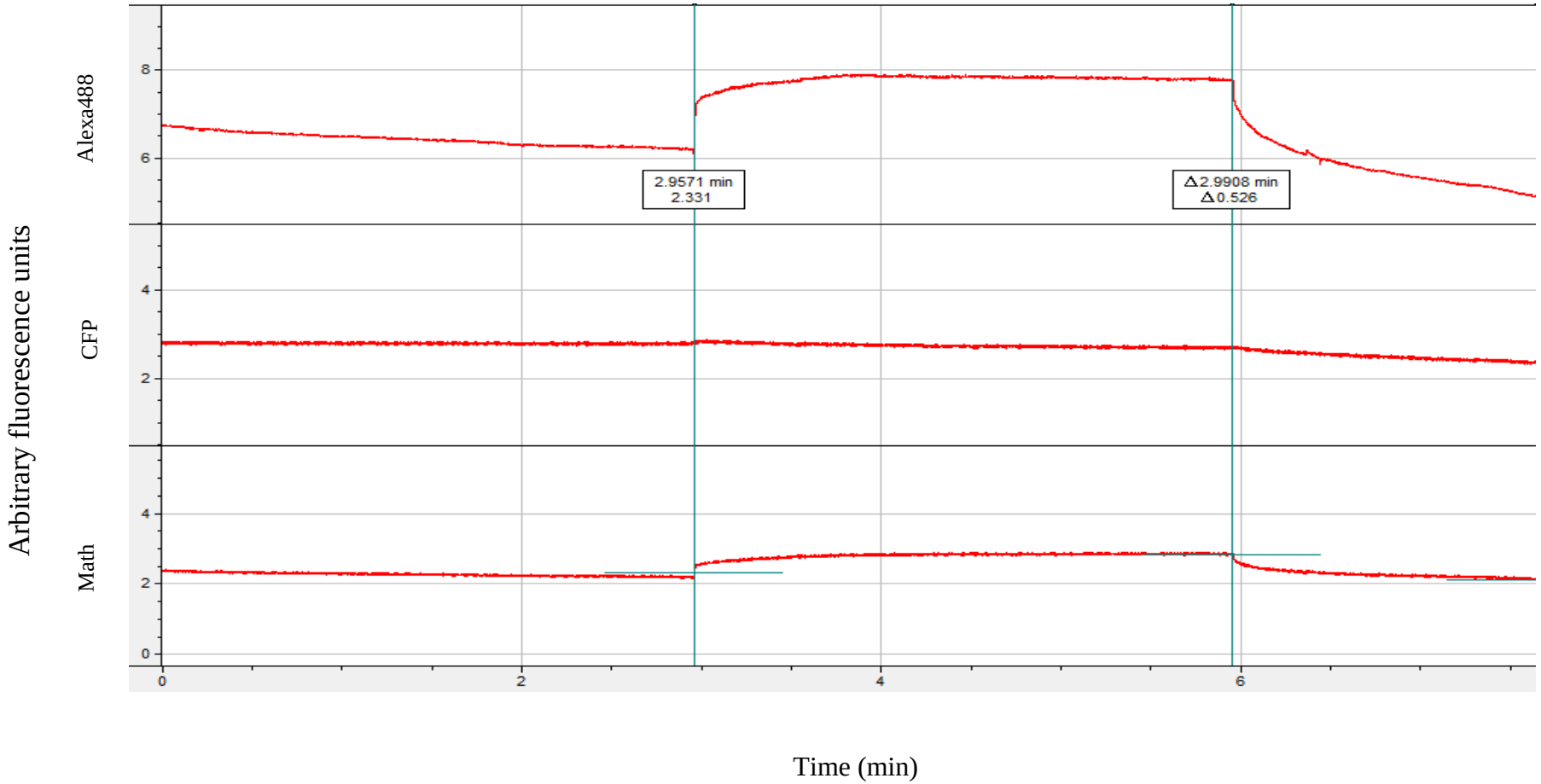

Figure 22 - HEK-293 cells were transiently transfected with receptor $A_{2A}R^{N\text{-}CFP}$. While selectively exciting at 436 nm (excitation of CFP, from $A_{2A}R^{N\text{-}CFP}$) a single cell, 2,5µM CGS21680-Alexa488 was used to stimulate the selected cell at t = 2,9571min via a pipette, and the changes caused by FRET in Alexa488 emission fluorescence (517 nm) were recorded. 2,9908min after stimulation, selective $A_{2A}R$ antagonist SCH 10µM was added, abolishing Alexa488 signal.