Activation of G protein-coupled receptors : the role of extracellular loops in adenosine receptors

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CHAPTER 1

GENERAL INTRODUCTION

G PROTEIN-COUPLED RECEPTORS (GPCRS)

G protein-coupled receptors (GPCRs) form one of the largest protein families known. In humans, already over 800 members have been identified. When we take into account all the different variants, for example differences in mRNA splicing, this number is even considerably larger. GPCRs are involved in crucial signal transduction pathways, including vital processes such as reproduction and immunological responses [1]. Many drugs that are currently on the market (~ 40%) are directed against members of this immense superfamily for the treatment of a wide variety of diseases. Unfortunately, we still do not know exactly what happens between the event of drug binding and the intracellular response that eventually results in the drug's effect on the body. Increasing our knowledge of GPCRs would greatly aid in the design of new drugs with increased selectivity, thereby potentially decreasing the occurrence of side-effects. For this reason, research groups all over the world are intensively studying these receptors, and initiatives are formed to fast improve our knowledge. All of these efforts are paying off; in 2010 alone over 12,000 research papers discussing GPCRs were published (keywords: GPCR, G protein-coupled receptor, 7TM receptor, seven transmembrane receptor; www.pubmed.com). We even have access to a handful of high resolution crystal structures now that give us a good view of what these receptors actually look like [2,3,4] (see also Chapter 2 of this thesis).

The GPCR superfamily consists of five main classes, of which class A (or rhodopsin-like) GPCRs by far form the largest subfamily [5]. They all have a similar structure, with an extracellular N-terminus, seven transmembrane helices connected by three extracellular and three intracellular loops (IL1-3), and an intracellular C-terminus. However, when looking more closely at the crystal structures, we do notice many differences between class A family members. Especially the lesser conserved regions of the receptor, and in particular the extracellular loops, can adopt many different structural poses (see Chapter 2).

ADENOSINE RECEPTORS

The adenosine receptors (ARs) form a small subfamily within the class A GPCRs. Four subtypes of adenosine receptors are known (A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R), all of which are ubiquitously expressed in the human body [6]. The endogenous ligand for this subfamily is adenosine, a nucleoside composed of an adenine ring attached to a ribose moiety via a β-N_9-glycosidic bond (**Figure 1**). Extracellular adenosine originates from the breakdown of ATP by 5'-ectonucleotidases and is then quickly metabolized by adenosine kinase to form AMP or by adenosine deaminase to form inosine. Under normal conditions, extracellular adenosine concentrations are in the nM-μM range. In response to metabolic stress and cell damage, e.g. in conditions of ischaemia, hypoxia, inflammation and trauma, adenosine accumulates in the extracellular space [7]. This stress signal subsequently leads to the activation of adenosine receptors, generating a range of tissue responses mainly focused on organ protection [8].

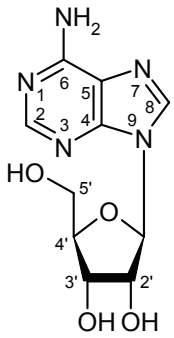

Figure 1. Chemical structure of adenosine

Although all four subtypes respond to adenosine, they do so at different adenosine concentrations and they are coupled to different intracellular signaling pathways. The A_1R and the A_3R subtypes mainly signal through G_i proteins mediating the inhibition of adenylyl cyclase, which leads to decreased levels of cAMP in the cell. The $A_{2A}R$ and $A_{2B}R$ cause an increase in intracellular cAMP levels by coupling mainly to G_s proteins resulting in the activation of adenylyl cyclase [9].

In this thesis, two subtypes of adenosine receptors are the main focus of our studies: the A_1R and the $A_{2B}R$. The A_1R is considered to be the high affinity receptor of the subfamily. The $A_{2B}R$ has the lowest affinity for the endogenous ligand adenosine. Only when adenosine levels increase to high micromolar concentrations in response to metabolic stress, the $A_{2B}R$ can be activated [7]. This only occurs in pathological conditions and subsequently leads to the activation of the immune system (**Figure 2**). For this reason, the adenosine A_{2B} receptor ($A_{2B}R$) is an interesting drug target and it has been implicated in asthma [10], chronic obstructive pulmonary disease (COPD) [11], and other inflammatory diseases [8]. Ligands for the A_1R are also of great interest for the pharmaceutical industry and a number of A_1R agonists and

antagonists have reached the clinical phase, and are or were under investigation for the treatment of peripheral nerve injury, hypertriglyceridemia in diabetes, heart failure, and kidney dysfunction [12]. Also, its high expression in the central nervous system (CNS) and the neuroprotective effects of activation has implicated the A_1R as a promising drug target in neurological disorders such as in Huntington's and Alzheimer disease [9,13,14]. This receptor has been studied in more detail compared to the $A_{2B}R$, and many more selective ligands have been identified, including allosteric ligands. Allosteric modulators are compounds that are able to bind the receptor at a site distinct from the orthosteric site where the endogenous ligand binds. These allosteric binding sites are thought to be less conserved than the endogenous binding site and can therefore provide more selectivity for a single receptor subtype.

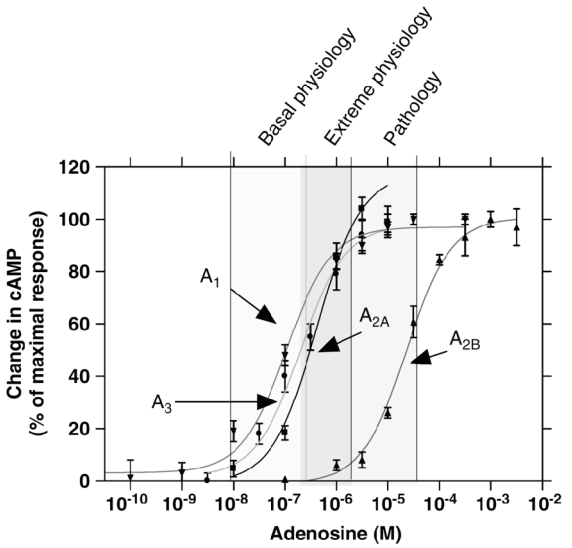

Figure 2. Activation profiles of the different adenosine receptor subtypes. The adenosine A_{2B} receptor is only activated when adenosine levels reach pathological concentrations. Figure was originally published in *Fredholm, B.B., Cell Death Differ 14 (2007).*

ADENOSINE RECEPTOR STRUCTURE

In 2008, the crystal structure of the adenosine A_{2A} receptor was published (**Figure 3**) [15]. At that point, it was only the third human GPCR of which the structure was elucidated. Just one year previously, the structures of the human β_1- and β_2-adrenergic receptors were published [16,17,18]. Even though the $A_{2A}R$ was greatly modified to enable the crystallization process, the structure was a great contribution to the adenosine receptor research field. Now we were able to explain mutational and pharmacological data in a more relevant three-dimensional fashion. In the beginning of 2011 another high resolution $A_{2A}R$ crystal structure was elucidated: the receptor in an active conformation with the agonist UK-432097 bound to it, followed quickly by two other active structures bound to NECA and adenosine, respectively [19,20].

With these structures another big leap was taken. By comparing the inactive and active structures, we can learn to understand the transitions the receptor goes through during the activation process better. However, there are some considerations that need to be taken into account: (1) the receptors were heavily modified to increase stability, with the T4-lysosyme fused to the third intracellular loop and a deleted C-terminal tail (ZM241385 and UK-432097 structures [15,19]) or with several thermo-stabilizing mutations (NECA and adenosine structures [20]) (2) the active structures provide single views of possibly multiple active conformations, providing a limited view on the activation mechanism, and (3) crystal packing may have had an effect on the structure, especially on the intra- and extracellular regions of the receptor. Nonetheless, all four structures provide the framework for a better understanding of adenosine receptor activation. These should be supplemented with combined mutagenesis and functional studies for appreciating the dynamics of this activation process, and that is exactly what we aimed for in this thesis.

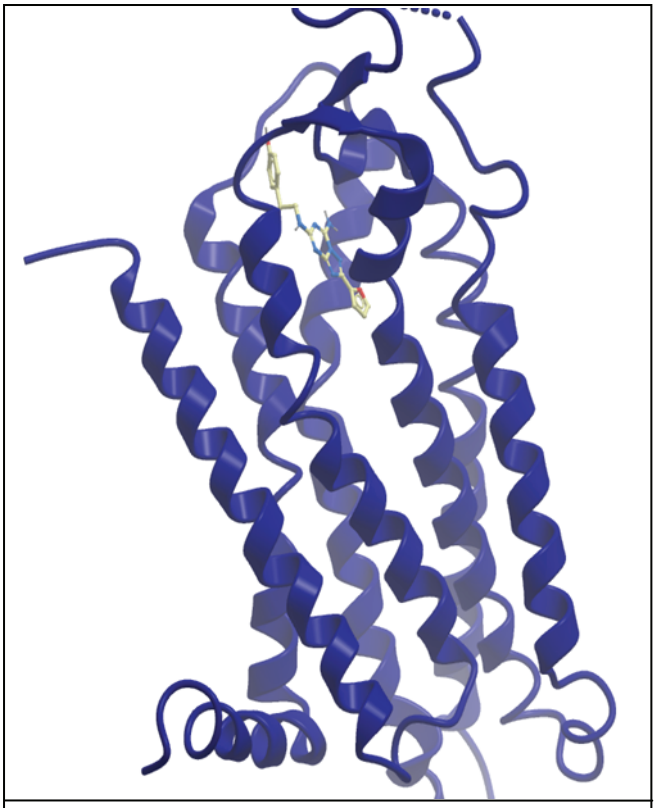

Figure 3. Crystal structure of the human adenosine A_{2A} receptor (in blue ribbon) bound to the antagonist ZM241385 (in ball & stick representation).

MUTAGENESIS

Mutagenesis is a powerful tool when examining GPCR function and activation. By changing single residues or even entire receptor domains followed by functional pharmacological studies, we can greatly improve our insight in how activation occurs upon ligand binding, but also which regions are specifically involved. Especially since the structural information is relatively limited in GPCRs, we still greatly depend on mutagenic data.

Mutagenic techniques range from approaches purely based on rational, such as site-directed mutagenesis where a single residue in the protein is targeted and changed to a specific alternative residue, to highly unbiased approaches like random mutagenesis where mutations are left to occur by chance [1].

Site-directed mutagenesis is the most used technique, the rationale for which mainly originates from computational analyses and/or structure-activity-relationship studies of receptor ligands indicating an important role of the residue in the protein [21,22].

A variation to this technique that is slightly less specific is site-saturation mutagenesis in which a single residue is changed into every other (naturally) occurring amino acid (see Chapter 3) [23]. This method provides insight into the structure-function-relationship of the residue of interest and the role of its side chain in binding and activation. Another approach is scanning analysis; this technique is mostly chosen when there are indications of the importance of a protein region, but not which exact amino acid would be involved (see Chapter 6). In this approach consecutive residues are replaced by one type of amino acid, for example alanine or cysteine. Alanine is often chosen as the replacing amino acid due to its small size and lack of reactive functional groups. It also has no or minor influence on the protein backbone, contrary to the more flexible glycine [24]. The advantage of cysteine replacements is that these residues are highly reactive and can form disulfide bridges with other cysteines. This property can be used to examine ligand-dependant conformational changes without the need to purify the protein in a disulfide-cross linking approach [25]. Another application can be in a cysteine-accessibility study using a cysteine-reactive biotin probe. Here, the ability of biotinylated mutant receptors to react with a steptavidin-HRP-conjugated antibody is used to examine differences in accessibility of the residue, providing insight in the conformational state of the receptor [26]. A less detailed, but just as informative method is the creation of chimeric receptors. Domains of one receptor are swapped with the corresponding domains of another (often related) receptor to investigate effects of selectivity, signaling or to identify the ligand binding site [27].

The most unbiased approach in mutagenic studies is random mutagenesis, where mutations are randomly introduced in the gene encoding (parts of) the receptor (see Chapters 3, 4, and 5). This can be achieved by UV irradiation, chemical methods like alkylation and deamination, or by error prone PCR. The first two approaches can be very successful, but it is difficult to control the frequency in which the mutations are

introduced. The third technique is based on manipulating the PCR reaction by changing the ratio between Mg^{2+} and Mn^{2+} ions, compromising the fidelity of the DNA polymerase. Furthermore, an excess of one of the nucleotides is added to force errors to occur. By choosing the conditions carefully, the frequency in which the mutations are introduced can be fine-tuned. Both low frequency random mutagenesis and saturation random mutagenesis have been applied in studying GPCR function [28,29]. This method was first described in 1995 [30] and since then has been optimized to use larger fragments in this technique and even commercial kits that are able to aid in the introduction of random mutations in whole gene sequences have become available [31]. In coupling a screening assay to a random mutagenesis approach, residues that show a phenotype of interest when mutated can be easily identified. In this way, information about how the receptor is activated or inactivated can be obtained as well as the role specific residues play in this process. One convenient screening platform is the *S. cerevisiae* model that when modified can function as a reporter system with growth as an easy read-out [32].

CONSTITUTIVE ACTIVITY

Constitutive activity, or basal activity, is the basal level of signaling a receptor displays without a ligand present. In a simple scheme an equilibrium exists between an inactive (R) and active conformation (R*) [33]. The fraction of R* in the total receptor population as well as the energy needed to transition between the two states determines the level of constitutive activity. This activation state is essential in maintaining physiological function and many pathogenic mutations have been reported that disturb the equilibrium causing an increase in activation (Constitutively Active Mutants or CAMs) or a decrease in basal activity (Constitutively Inactive Mutants or CIMs) [34,35,36]. These mutations have not only increased our knowledge on the pathophysiology in which GPCRs play a role, but also advanced our insight in the structure-function relationship of GPCRs.

The α_{1B}-adrenergic receptor (α_{1B}AR) was the first GPCR in which point mutations were shown to trigger receptor activation [23]. A conservative substitution (A293L) in the cytosolic extension of TM6 of the α_{1B}AR resulted in its constitutive activity. In the absence of an agonist, cells expressing the mutated receptor exhibited higher basal

levels of inositol phosphates compared to cells expressing the wild type α_{1B}AR. Since then, an impressive number of CAMs have been identified located in practically every domain of the receptors [37] (www.gpcr.org/7tm).This indicates that activation of a GPCR can be triggered by manipulations of the receptor structure in many different regions whose function cannot be obviously linked to agonist binding or G protein interaction [34]. Identifying specific regions and/or residues that can shift the equilibrium between the active and inactive conformation when mutated can be a strong tool in elucidating triggers involved in the activation mechanism. Several screening methods have been applied in search for these mutations [29,31,38].

S. CEREVISIAE AS A MODEL SYSTEM

GPCRs are among the oldest devices devoted to signal transduction and can be found in all eukaryotes. They are present in large numbers in vertebrates but are also expressed in plants, yeast, and protozoa [39,40].

In *S. cerevisiae*, three endogenous GPCRs have been identified: the Ste2 and Ste3 receptors and the Grp1 receptor [41]. Gpr1 is a glucose and sucrose sensing receptor that couples to the G protein Gpa2 leading to increased levels of intracellular cAMP. The subsequent events lead to a large remodeling of the yeast metabolism that results in an increase in yeast growth rate (**Figure 4A**) [42,43].

S. cerevisiae can stably exist as either haploid or diploid. The haploid cell types MAT**a** and MATα are to mate to form a diploid cell. Both cell types express mating specific proteins such as the **a**-factor pheromone and the α-factor receptor (Ste2) in MAT**a**-cells, and the α-factor and the **a**-factor receptor (Ste3) in MATα-cells [41,44]. Activation of the Ste2 or Ste3 receptor results in the activation of the MAP kinase pathway through the G protein Gpa1 following activation of nuclear proteins that control transcription, cell polarity, and progression through the cell cycle (**Figure 4B**) [44]. *S. cerevisiae* is an attractive expression system to study GPCRs and until now, more than 50 GPCRs have been functionally expressed in various yeast strains. Besides the presence of a full functional GPCR signaling machinery and mammalian-like post-translational modification, yeast is relatively easy to genetically manipulate, has a well characterized physiology and is inexpensive [44,45].

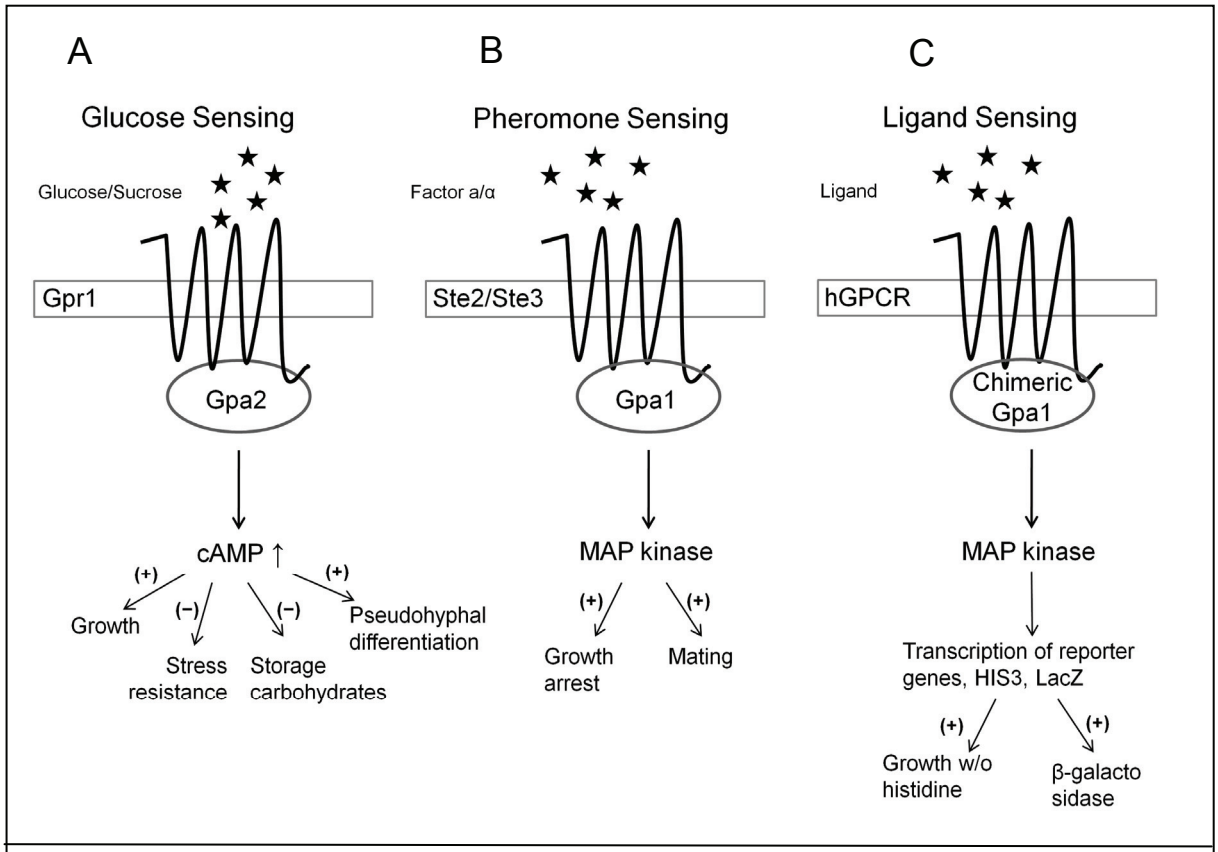

Figure 4. Schematic overview of GPCR signaling in *S. cerevisiae*. (*A*) Signaling pathway of the glucose sensing GPCR Gpr1. (*B*) Signaling pathway of the Mating GPCRs Ste2 and Ste3. (*C*) Signaling pathway in the engineered yeast strain MMY24 expressing a human GPCR.

The general approach is the conversion of a *S. cerevisiae* strain to function as a reporter gene assay [1,44,46]. For this purpose, the pheromone signaling pathway through the Gpa1 G protein is high jacked (**Figure 4**). In order for a human GPCR to take control of the signaling pathway, the endogenous G protein Gpa1 had to be "humanized". This has been accomplished by exchanging the C-terminal end of the yeast Gα protein by the mammalian Gα protein sequence, resulting in a chimeric G protein that is now able to couple to human GPCRs as well as activate the yeast pheromone pathway [32,47]. Activation of the expressed receptor activates the MAP kinase pathway in the same way as the pheromone response and subsequently induces the *FUS1* promoter that leads to the transcription of reporter genes. Several reporter genes can be used for the activation read-out. For instance, the *FUS1-HIS3* reporter gene provides yeast cells the ability to grow on histidine deficient medium upon activation [32]. Similarly, the *FUS1-Hph* reporter gene allows yeast cells containing an activated receptor to grow on hygromycin-containing medium [48]. A reverse growth method is the use of the reporter gene *FUS1-Can1*. Here, an active receptor results in the expression of the Can1 channel that can transport the toxin

canavanine and leads to cell death. This system has been specifically designed to screen for and investigate inactivating mutations in GPCRs [49]. A different non-growth approach is the *FUS1-LacZ* reporter gene that increases the production of the enzyme β-galactosidase when the GPCR is activated. This provides a colorimetric read-out by using chlorophenolred-β-D-galactosidase (CPRG) or *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrates for the enzyme. Contrary to the growth read-outs, this method only requires a short response time [32,44].

The yeast strain used in this thesis for the screening approaches as well as the pharmacological studies of the $A_{2B}R$ and A_1R is the MMY24 *S. cerevisiae* strain created by Andrew Brown and Simon Dowell at GlaxoSmithKline [32]. This strain was derived from the MMY11 strain described by Olesnicky and coworkers [47]. The MMY24 yeast strain contains a chimeric Gpa1 G protein of which the last 5 amino acids are from a mammalian $G\alpha_i$ protein. This modification allows both $A_{2B}R$ and A_1R to couple to the yeast pheromone pathway and activate transcription of the reporter genes HIS3 and LacZ that were also incorporated into the genome.

AIM AND OUTLINE OF THIS THESIS

The aim of the work presented in this thesis was to gain insight in the activation mechanism of class A GPCRs, more specifically of the adenosine receptors, and how the extracellular loops are involved in this process. This research was part of the project “GPCR forum for established targets” of Top Institute Pharma (D1-105). This Dutch public-private partnership strives to join forces of industry and academia to speed up the drug research process. At the start of this research project, little was known about the role of the highly variable extracellular loops in receptor activation. The general perception was that one overall activation mechanism should exist among class A GPCRs and that mainly the ligand binding site and the (intracellular) region that couples to the G protein were involved in determining how intracellular signaling pathways are activated. This view changed dramatically over the last years, due to mutagenesis studies and to the elucidation of several high resolution crystal structures of class A GPCRs mentioned before that all show different structural conformations of the extracellular loops.

In **Chapter 2**, we demonstrate the importance of the extracellular loops in receptor activation in a review of the current literature on this subject in which we made use of the structures available at that time.

In the investigation of the activation mechanism of the $A_{2B}R$ and the A_1R, we made use of a wide variety of mutagenesis techniques, combined with screening and pharmacological validation in the expression system *S. cerevisiae*. This approach has proven to be highly successful as we were able to identify several regions and specific residues in the $A_{2B}R$ and the A_1R that are essential for normal receptor function. The results presented in this thesis will greatly help increasing the knowledge on how adenosine receptors are being activated through ligand binding as well as how they maintain their basal or constitutive activation state.

In **Chapter 3**, we reveal an essential role for the first extracellular loop in the adenosine A_{2B} receptor. In particular, two residues, a phenylalanine and an aspartic acid at positions 71 and 74 respectively, proved to be vital in maintaining the tertiary structure of the extracellular domain that is crucial for receptor activation and constitutive activity.

In **Chapter 4**, we describe a random mutagenesis screen in which we selected constitutively active mutant receptors in a fragment of the $A_{2B}R$ involving the transmembrane domains 4 and 5 and the second extracellular loop. Three specific clusters were identified that presumably are responsible for silencing the receptor in its basal state.

In **Chapter 5**, we introduce a new screening method using our MMY24 yeast strain. This screening method makes it possible to select constitutively inactive mutant receptors (CIMs). Applying this method to the adenosine A_{2B} receptor revealed many residues involved in maintaining the equilibrium that exists between the inactive and active conformation.

Chapter 6 discusses another subtype of adenosine receptors, the adenosine A_1 receptor. A mutagenic alanine scanning study on the second and third extracellular loop of this receptor showed a particularly important role for EL2 in receptor activation and even allosteric modulation. This role is opposite to the role seen in the $A_{2B}R$, acting more as a positive rather than a negative regulator of activation.

Chapter 7 will bring the discussions together, comparing the activating and inactivating regions identified in Chapter 4 and 5 and its structural implications as well as reflecting on the clearly different roles of EL2 in receptor activation within the

adenosine receptor subfamily. Also, future perspectives that emerge from the results of this thesis will be presented.

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