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The activation mechanisms of G protein-coupled receptors : the case of the adenosine A2B and HCA2/3 receptors

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Chapter 1

General introduction

G protein-coupled receptors (GPCRs)

The G protein-coupled receptors (GPCRs) are a superfamily of seven transmembrane receptors (7TM) that communicate extracellular signals to the internal environment. Examples of extracellular signals that can be transmitted are: ions, photons, small organic molecules and proteins. Activation of GPCRs can induce signalling via multiple distinct pathways, such as β-arrestin or/and G protein pathways[1]. As the name implies receptors are coupled to so-called G proteins. These are heterotrimeric protein complexes comprised of three subunits, the α-, β- and γ-subunit, which together act like a switch and amplifier involved in cellular signal transduction (Fig. 1). GPCRs control many cellular and physiological responses in mammals, such as cell growth and differentiation, energy metabolism, cardiovascular function, neurotransmission and immune responses[2]. There are approximately 800 different GPCRs in the human genome. GPCRs are the target for around 30-40% of clinically prescribed drugs and for 25% of the top 100 in sales[3, 4]. GPCRs are divided into 6 different families according to their signature of conserved residues and ligand interaction: Class A, rhodopsin-like; Class B, secretin-like, Class C, glutamate; Class D, adhesion, Class E, frizzled-taste-2 receptors and Class F, other 7TM proteins[2, 5].

GPCR activation

GPCRs all have a similar structure: an extracellular N-terminus, seven transmembrane helices connected via three intracellular and three extracellular loops (TM1-7, IL1-3 and EL1-3), helix 8, and an intracellular C-terminus. Most structural studies have been performed on Class A (rhodopsin-like receptors), the largest and most known subfamily. Previous research identified important conserved residues and a shared common mechanism of receptor activation among different receptor families. The (D/E)RY motif is located at the interface between TM3 and IL2, which is involved in the well-studied mechanism of action that breaks the ionic lock/salt bridge between $R^{3.50}$ and $E^{6.30}$ after activation[7] (The superscript numbers of these residues are based on

Ballesteros-Weinstein numbering[8]). This mechanism is present in many Class A receptors, such as rhodopsin[9], β_2–adrenergic receptor[10], serotonin 2A receptor (5-hydroxytryptamine 2A, $5HT_{2A}$)[11, 12]. However, the ionic lock is not a key activation microswitch in many other GPCRs[7]. The NPxxY motif located in TM7 is another essential motif, which connects ligand binding with intracellular helix 8 and G protein activation[5]. Most Class A, rhodopsin-like, receptors have the NPxxY$(x)_{5,6}$F motif, which is located at the junction between TM7 and the connecting cytosolic helix 8, such as in rhodopsin itself[9, 13], mammalian melanin-concentrating hormone receptor 1[14], the type 1 angiotensin receptor[15], β_1–adrenergic receptor[16], α_{2B}-adrenergic receptor[17] and the A_1 adenosine receptor[18]. The important interaction between TM7, helix 8 can transfer the signal from outside ligand binding to inside G protein, such as in protease-activated receptor 1 has TM7-helix 8-IL1 activation mechanism[19], which may be conserved in other Class A. However, each receptor is unique and it binds its own outside ligands and triggers different downstream signalling pathways, some of which may be shared with other receptors. In a recent review Moreira et al. reported about the structural features of the G protein/GPCR interaction. Most key regions for GPCR/G protein coupling are located at the interface between transmembrane helices and intracellular loops, which are represented by a red rectangle (Fig. 2). It should be mentioned that even mutation of a single residue may lead to receptor silence and thus be critical for G protein coupling and receptor activation[20, 21].

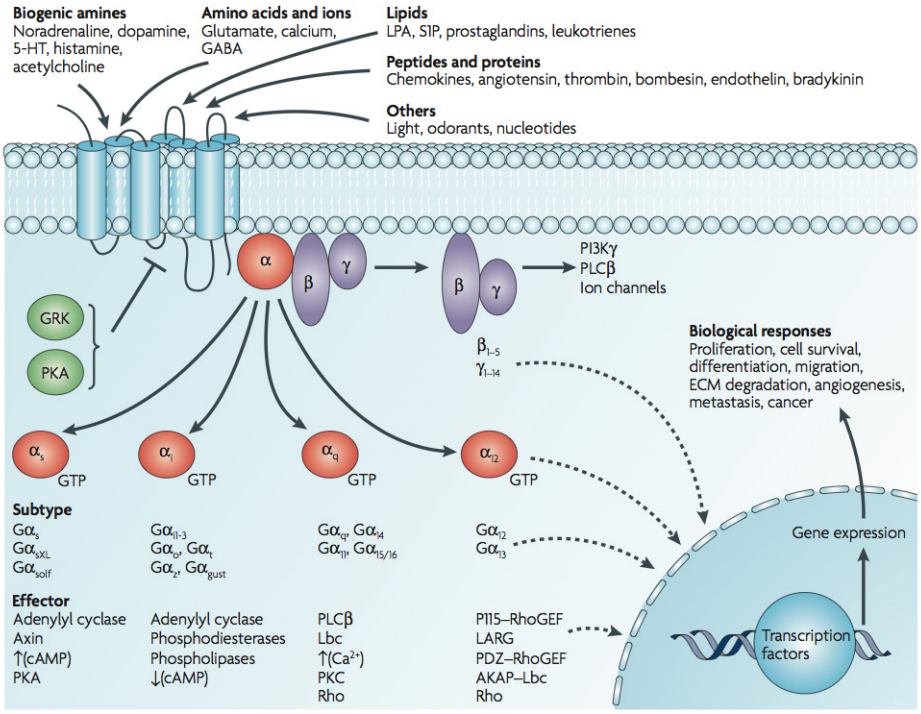

Fig. 1. Activation and signalling pathways of GPCRs and their respective G proteins[6]. Reproduced with permission.

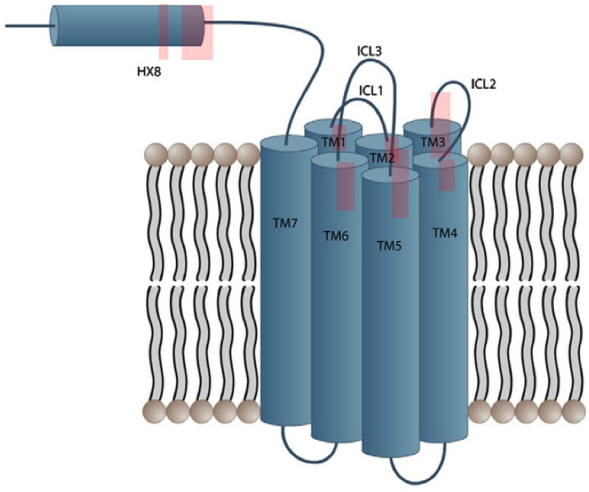

Fig. 2. Schematic representation of a G protein-coupled receptor. Key regions for GPCR/G protein coupling are represented by red rectangles (different sizes are related to the dimension of the interacting region)[22]. Please, note the influence of the 'rhodopsin community' in which it is common to draw the extracellular region upside down. Reproduced with permission.

G protein and G protein selectivity

With an agonist in the binding pocket of the GPCR the receptor is in the active state by stabilizing an agonist-preferring structural conformation. This change in conformation in turn changes the conformation of the G protein allowing it to be 'switched on' by exchanging guanosine-5'-diphosphate (GDP) for guanosine-5'-triphosphate (GTP) at the G_α subunit. This G_α subunit subsequently dissociates from the $\beta\gamma$-complex and interacts with an effector protein in the cell (Fig. 1). G proteins can be divided based on the G_α subunit and its sequence identity; there are 4 different families of G_α proteins: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha 12}$, and $G_{\alpha q}$[23] (Fig. 1). Effector proteins can be many, ranging from kinases to ion channels, which upon activation either increase or decrease the amount of secondary messengers associated with it (Fig. 1). Second messengers like cAMP or calcium ions (Ca^{2+}) induce physiological changes like proliferation, secretion or apoptosis. Hydrolysis of the GTP bound to the α-subunit to GDP deactivates the G_α subunit and leads to re-association with the $\beta\gamma$-complex, upon which the effector protein reverts back to its original state[6].

Even though the GPCRs are a very diverse family all of them bind to a much smaller number of G proteins. There are 21 G_α, 6 G_β, and 12 G_γ subunits in human cells[24]. Previous research has shown one GPCR can activate multiple G protein pathways, while at the same time different GPCRs can activate the same G protein pathways[25]. This dictates that the interface between G proteins and GPCRs has interactions that determine which G proteins can, and which G proteins cannot interact with a given receptor. Even though the mechanisms and dynamics of interaction between GPCRs and their G proteins are poorly understood, important regions of G_α subunits for GPCR-G_α protein binding and selectivity include the α4-helix and α4-β6 loop[26, 27], the N-terminus[28] and C-terminus of G_α subunits[29, 30]. Of those regions, the C-terminus of the G_α subunit is most intimately involved in binding to the receptor. This was already proved by available crystal structures[31, 32] and Kling et al. reported three residues at the C-terminus of $G_{\alpha s}$ are in close contact with at least 5 amino acids of the β_2–adrenergic receptor, based on molecular dynamics calculations[33]. Even though

the G_α subunit has most of the interactions with the receptor it is not the only G protein subunit that confers specificity. Both the G_β[34] and G_γ[35] subunits are also able to specify by which GPCRs they are activated. Many interactions have been identified both on the G protein side and on the receptor side, however, the exact nature of the connections is still unclear[17]. Hence it is very important to discover key residues or motifs within GPCRs or G proteins that can switch on/off G protein/GPCR activation, knowledge that eventually may be useful for drug discovery.

Adenosine A_{2B} receptor

All the adenosine receptors (P1 Purinergic receptors) are ubiquitously expressed in the human body[36]. They belong to the Class A subfamily of GPCRs and their endogenous ligand is adenosine. Adenosine is a purine nucleoside, which consists of an adenine ring bound to a ribose sugar group via a β-N_9-glycosidic bond. Normal levels of extracellular adenosine are well below 1 μM in unstressed cells. During inflammation and ischemia these levels can increase to upwards of 100 μM. For example, patients suffering from sepsis, which is associated with inflammation, have adenosine levels of about 8 μM[37]. Adenosine has been found to be involved in tissue protection and regeneration of injured tissue in a number of ways through the adenosine receptors[38]. The adenosine receptors include four subtypes: A_1, A_{2A}, A_{2B} and A_3 receptors, which have attracted much attention as therapeutic targets in recent years. They can target different intracellular signalling pathways by responding to the same endogenous ligand adenosine. The A_1 and A_3 adenosine receptors (which share 49% sequence identity) inhibit cAMP production while the A_{2A} and A_{2B} receptors (sharing 59% sequence identity) stimulate the production of cAMP.

The A_{2B} receptor has the lowest affinity for adenosine[39] and has been less investigated than other adenosine receptors. The A_{2B} receptor is coupled to the G_s and G_q proteins (Fig. 3A)[40], although the preference is more towards the G_s pathway[41]. The G_s subunit can open a calcium channel either directly by binding to it or indirectly through stimulating cAMP production by activating

protein kinase A (PKA), which is also capable of opening the calcium channel[42]. The G_q pathway can bind to phosphatidylinositol-specific phospholipase C (PI-PLC), leading to the production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates the mobilization of calcium from intracellular storage sites. DAG activates protein kinase C (PKC).

The A_{2B} receptor is widely expressed in many cell types and tissues[40] and plays many roles in different organs and pathologies[43] (Fig. 3B). Previous studies have described that inhibition of A_{2B} receptor signalling reduces experimental autoimmune encephalomyelitis, of relevance for the treatment of multiple sclerosis[44], and inhibits growth of prostate cancer cells[45], bladder tumors[46, 47] and breast tumors[48], and reduces obesity or insulin resistance[49]. On the other hand, activation of A_{2B} receptor signalling protects against trauma-hemorrhagic shock-induced lung injury[50], CHX-induced apoptosis[45], cardiac diseases[51], and also vascular injury[30, 52]. Likewise, it has been found that activation of the A_{2B} receptor plays a role in suppression of inflammation during tissue hypoxia[53], whereas antagonism might inhibit growth of lung tumors[54] and also hyperlipidemia[55]. Therefore, the A_{2B} receptor is a promising drug target for the treatment of kidney failure, inflammatory diseases, type 2 diabetes, asthma, ischemic injuries, diarrhea, and central nervous system disorders[56-60]. To understand the activation mechanism of the A_{2B} receptor is therefore very relevant for drug development at the A_{2B} receptor.

Human HCA_2 and HCA_3 receptors

The hydroxycarboxylic acid (HCA) receptors also belong to Class A subfamily of GPCRs and consist of three members HCA_1, HCA_2 and HCA_3, which function as metabolic sensors that are activated by intermediates of energy metabolism. Their endogenous ligands are hydroxycarboxylic acids: 2-hydroxy-propanoic acid (lactate), 3-hydroxybutyric acid and 3-hydroxy-octanoic acid for HCA_1, HCA_2 and HCA_3, respectively[61, 62] and they were deorphanized in 2008 (HCA_1, GPR81)[63, 64], 2005 (HCA_2, GPR109A/HM74A, high affinity nicotinic acid receptor)[65] and 2009 (HCA_3, GPR109B/HM74, low affinity nicotinic acid

receptor)[66], respectively. The HCA_1 receptor is the most divergent member with approximately 50% amino acid sequence identity with both HCA_2 and HCA_3. The HCA_2 and HCA_3 receptors are highly homologous with 95% sequence identity[62]. The main difference between the HCA_2 and HCA_3 receptors is that HCA_2 has a shorter C-terminus than HCA_3 that carries 24 amino acid residues more at its C-terminus. Although the two receptors have high overall sequence homology, there are also 17 different amino acid residues in other parts of the two receptors that mainly cluster around ECL1 and ECL2[62, 67].

The HCA_2 receptor has been found only in mammals, whereas the HCA_3 receptor is only expressed in higher primates, which indicate that this receptor is the result of a recent gene duplication[68]. They all are predominantly expressed in adipocytes, where they mediate antilipolytic effects through coupling to the $G_{\alpha i}$ protein pathway[69, 70]. An overview of cellular functions regulated by HCA receptors is shown in Figure 4[68]. Activation of the HCA_2 receptor can have therapeutic benefits, such as an anti-dyslipidemic effect[71], anti-inflammatory effect[72-74], neuroprotective effect[75, 76] and on energy metabolism[77]. For the past 50 years nicotinic acid has been used to treat patients who suffer from cardiovascular disease, dyslipidemia and progression of atherosclerosis[78,79]. However next to this beneficial anti-dyslipidemic effect, nicotinic acid also induces a HCA_2 receptor-mediated side effect of severe flushing, resulting in low patient compliance[80]. Earlier research was focused on deorphanizing and characterizing the HCA receptors, with a main emphasis of the pharmacological role of the HCA_2 receptor activated by nicotinic acid. That sparked the interest in the development of novel agonists with higher affinity and selectivity, and other therapeutic modalities such as partial agonists, allosteric agonists and positive allosteric modulators[70, 81]. Interestingly, so far no antagonists have been discovered for the HCA receptors. For the research presented in this thesis, we focused on two aspects, i) the screening of high affinity agonists with long residence time and ii) the G protein activation/G protein selectivity profile of the HCA_2 and HCA_3 receptors.

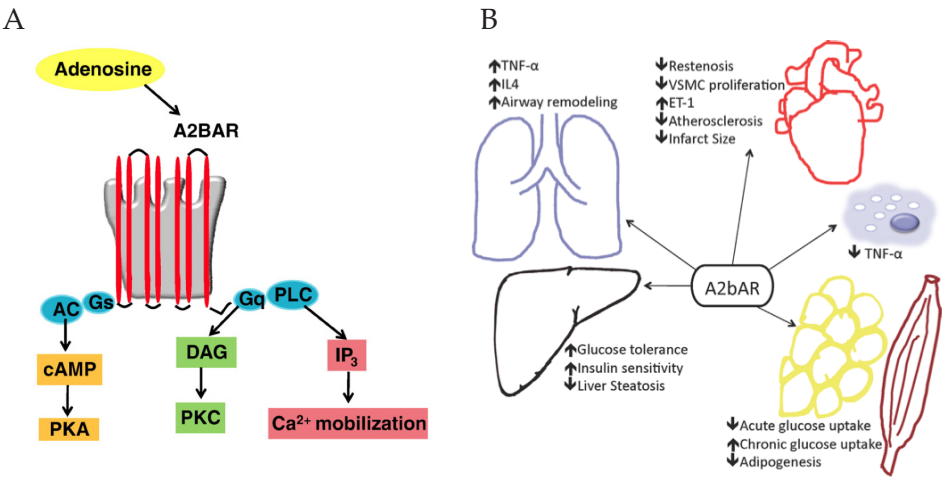

Fig. 3. (A) A schematic drawing of G protein activation and downstream signalling of the A_{2B} adenosine receptor[40]. (B) Important roles of the A_{2B} receptor in different organs and pathologies[43]. Reproduced with permission.

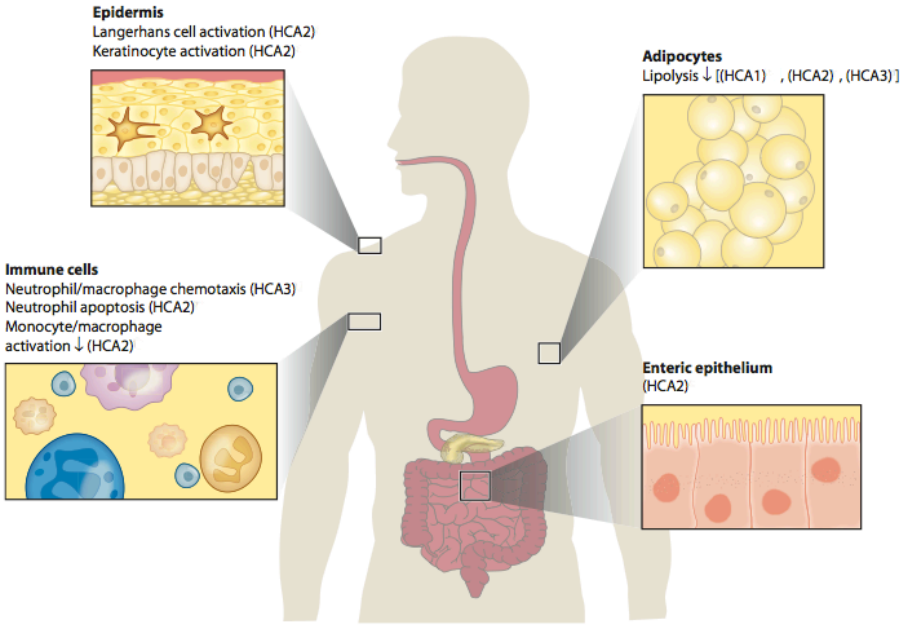

Fig. 4. Cellular functions regulated by the hydroxycarboxylic acid (HCA) receptors[68]. Reproduced with permission.

Saccharomyces cerevisiae system

Saccharomyces cerevisiae (*S. cerevisiae*) and *Pichia Pastoris* (*Pichia*) have been genetically well characterized as a model system among the many yeast species. For example, the two yeast strains are suitable 'production facilities' for membrane proteins as becomes evident from the crystal structures deposited in the PDB between 2010 and 2015[82]. The yeast system is as cheap and fast as E.coli, and which itself is 10 times cheaper and 4 times faster than insect cells and mammalian cells[83]. More than 50 GPCRs have been functionally expressed in various *S. cerevisiae* strains (more details of human GPCRs have been described in Chapter 2).

Many signal transduction pathways in *S. cerevisiae* have been discovered in the last three decades[84]. The yeast mating pheromone response pathway has been applied in GPCR research through the yeast MAP kinase pathway, which is not essential in the life of the yeast cell. In normal yeast cells the pheromone receptor (Ste2/Ste3) is bound to a heterotrimeric G protein consisting of a G_{α} (Gpa1p), G_{β} (Ste4p), and G_{γ} (Ste18p) subunit[85]. When $G_{\beta\gamma}$ is activated it signals to MAP kinase, which induces cell-cycle arrest (SST2) and mating gene transcription (Ste12p)[85]. *S. cerevisiae* has been engineered for GPCR ligand identification and for characterizing receptor pharmacology and signal transduction mechanisms[85,86]. It was found that the gene that induces cell-cycle arrest, FAR1, had to be disabled along with the negative regulator SST2 to improve the signalling characteristics of the assay[87]. Adding a reporter of Ste12p such as FUS1-HIS3 allows agonist-dependent growth on histidine-deficient medium[88] that will be used in the present thesis, while adding FUS1-LacZ would allow for quantitative readouts[89]. The biggest advantage of this system is that it provides a very clean and zero GPCR background. It has been coined 'a single-GPCR-one-G protein yeast system', because the original yeast GPCRs were knocked out. A schematic drawing of the wild-type and engineered pheromone pathway in *S. cerevisiae* is shown in Figure 5[90].

To investigate the activation mechanism of the receptor at the interface of the C terminus of the G_{α} subunit of the G protein, we expressed the yeast

plasmid with the receptor gene in eight different yeast *S. cerevisiae* strains with humanized G proteins (so called 'transplants'). The yeast strains used in this thesis are classified into five families: $G_{\alpha WT}$ (MMY12), $G_{\alpha s}$ (MMY28), $G_{\alpha i}$ (MMY23, MMY24 and MMY25), $G_{\alpha 12}$ (MMY19 and MMY20), and $G_{\alpha q}$ (MMY14, MMY16 and MMY21) corresponding to the last five C-terminal residues of the mammalian G_{α} subunit exchanged for the corresponding sequence stretch in the yeast G protein[91].

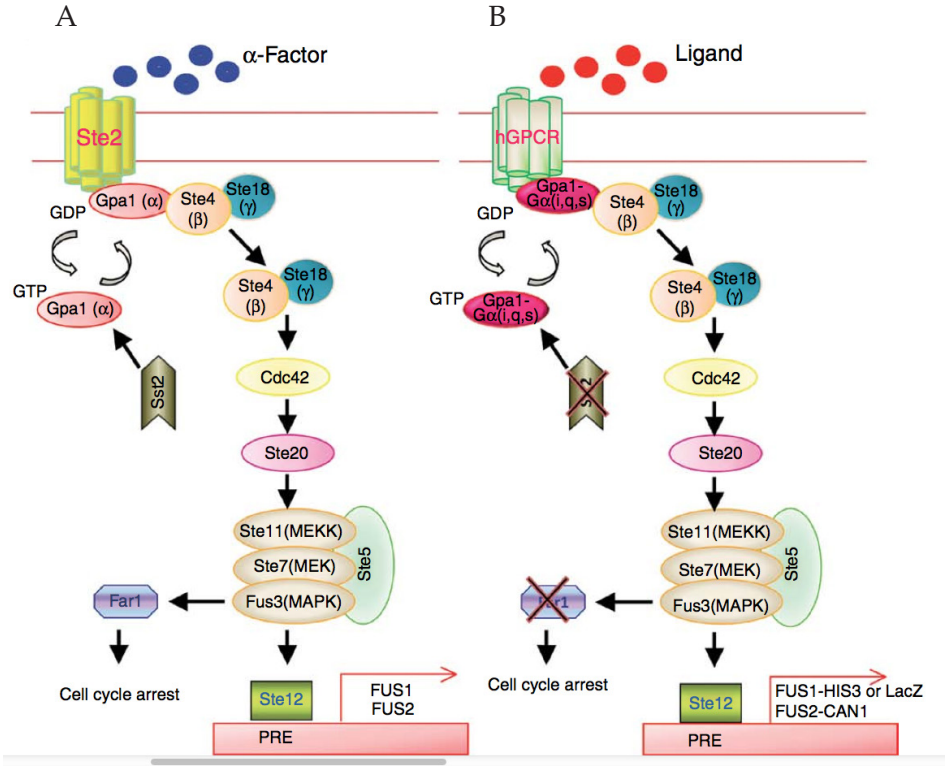

Fig.5. A schematic drawing of the wild-type and engineered pheromone pathway in *S. cerevisiae*[90]. (A) Pheromone signalling pathway mediated by yeast GPCR (Ste2) in wild-type yeast. (B) GPCR-mediated signalling pathway in an engineered yeast strain suitable for human GPCRs research. Reproduced with permission.

Aim and outline of this thesis

Identifying and elucidating the functions and activation of GPCRs will provide opportunities for novel drug discovery. In previous research it was shown that some sequence elements are important for GPCR activation, such as the DRY and NPxxY motifs. To obtain more information about receptor activation, we decided to study these and other sequence motifs to determine which parts of the receptor are key to its activation. We did so by mutating individual amino acids and meticulously checking the effect these mutations had on activation or G protein selectivity. We chose three receptors, the adenosine A_{2B} receptor and the HCA_2 and HCA_3 receptors, as paradigms. The functional assays in Chapters 3, 4 and 5 were based on the *S. cerevisiae* single-GPCR-one-G protein yeast system.

In **Chapter 2**, we summarized new developments regarding human G protein-coupled receptors in studies using *S. cerevisiae* ever since the year 2005 when we last reviewed this system. We described 11 families of GPCRs in detail including the principles and developments of each yeast system applied to these different GPCRs.

In **Chapter 3**, we predicted the residues that are important for A_{2B} receptor activation from homology modelling and we made site-directed single mutants and expressed these in yeast strains with a specific G protein pathway. This chapter focused on the above-mentioned DRY motif and on G protein selectivity.

In **Chapter 4** we studied the function of the NPxxY$(x)_{5,6}$F motif and each residue of helix 8 in receptor activation for which we used scanning mutagenesis of the two domains of the adenosine A_{2B} receptor.

In **Chapter 5**, we expressed the HCA_2 and HCA_3 wild-type receptors in yeast strains with specific G protein pathways. This chapter particularly focused on the different behaviors of these closely related receptors in G proteins coupling. The HCA_2 and HCA_3 receptors share high sequence identity but differ considerably in C-terminus length with HCA_3 having the longest tail. Hence we also made C-terminus 'swap' mutants of HCA_2 and HCA_3 to obtain insights in the function of the C terminus in G protein coupling.

Major pharmaceutical companies such as Merck, Arena and GlaxoSmithKline

have been working on the synthesis of agonists for the HCA_2 receptor in the past. Since nicotinic acid has long been the only drug on the market to raise HDL cholesterol levels, the focus of the pharmaceutical industry was on the synthesis of an HCA_2 agonist with the same desired effect, but without the flushing side effect. This resulted in a plethora of lead compounds and marketed drugs, for example Acifran and Acipimox.

In **Chapter 6** we focused on the design and synthesis of HCA_2 agonists with the additional quality of having a long residence time on the receptor, as the latter parameter may be linked to *in vivo* efficacy.

In **Chapter 7** we will summarize our findings in the present thesis and describe the advantages of the *S. cerevisiae* system and the importance of residues in conserved sequence motifs, such as DRY and NPxxY. Future perspectives for drug discovery based on our findings with respect to receptor activation and G protein coupling will conclude this thesis.

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