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

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# Chapter 7

# Conclusions and perspectives

## Conclusions

### *Saccharomyces cerevisiae* system

In **Chapter 2** we summarized the principles and developments in yeast research applied to 11 different G protein-coupled receptors (GPCRs), and highlighted and generalized the experimental findings of GPCR function in the *Saccharomyces cerevisiae (S. cerevisiae)* system since the year 2005. These yeast systems are very useful platforms in GPCR research providing insight into GPCR activation and signaling, and facilitate agonist and antagonist identification. There are many advantages of using a yeast assay system, as it is a cheap, safe and stable; it is also convenient for rapid feasibility and optimization studies. Moreover, it offers "null" background when studying human GPCRs.

Here, we summarize the principles of how we developed 'a single-GPCR-one-G protein yeast system' (Fig. 1). The whole process can be divided into two parts: mutants construction and characterization. There are many concepts and sources for selecting mutants, such as from modeling prediction (**Chapter 3**), and sequence motifs or specific areas known to be important in other receptors (**Chapters 4** and **5**). For the characterization of mutants in this thesis we focused on three differences between the mutant and the wild-type receptor: agonist efficacy, antagonist binding and expression level. Our overall aim was to find important residues involved in or critical for G protein coupling and receptor activation. Not only did we study different wild-type receptors and mutants, but also in the context of a "library" of yeast MMY strains containing one G protein subtype. The yeast strains used in this thesis were thus 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 $G_{\alpha}$ subunit exchanged for the corresponding sequence stretch in the yeast G protein. A tyrosine residue (Y) at the C-terminus of the $G_{\alpha}$ subunit proved vital for controlling the activation of GPCRs, which was discussed in **Chapter 3**. In this way we determined G protein coupling profiles of one single GPCR (whether wild-type or mutant). This

approach allowed us to compare the G protein coupling of different receptors (within or outside subfamilies) and to identify key residues or motifs critical for specific G protein coupling or control switching between different G protein pathways.

The wild-type $hA_{2B}$ receptor (**Chapter 3**) was promiscuous in its G protein coupling preference and activated all yeast strains, although to a different extent. In the presence of nicotinic acid the wild-type $hHCA_2$ receptor activated almost all G protein pathways except $G_{\alpha q}$ (MMY14) and $G_{\alpha s}$ (MMY28), which we did not use further as the empty plasmid (i.e. without receptor present) showed a high constitutive activity. The $hHCA_2$ receptor was more promiscuous in its G protein coupling preference than the $hHCA_3$ receptor, which only activated $G_{\alpha i1}$ (MMY23) and $G_{\alpha i3}$ (MMY24) pathways (**Chapter 5**).

## The importance of residues in motifs: DRY and NPxxY(x)$_{5,6}$F; Helix 8 and C terminus

At first, we made a small number of mutants to establish and develop the entire assay. Computer predictions derived from crystal structures or homology models proved an efficient way to select mutants for deciphering their role in receptor-G protein coupling or in receptor activation. In **Chapter 3**, we used a $hA_{2B}$ Receptor/$G_s$ protein homology model, which was generated from the crystal structure of the $\beta_2$ adrenergic receptor in contact with the $G_{\alpha s}$ protein (PDB: 3SN6) to predict amino acids of the $A_{2B}$ receptor interacting with the $G_{\alpha s}$ subunit. $R103^{3.50}$, $I107^{3.54}$, $L213^{IL3}$, $S235^{6.36}$ and $L236^{6.37}$ were selected for mutation into alanine as representatives of 16 residues that were shown to interact with the $G_{\alpha s}$ subunit from the homology model prediction. We learned that mutation of a single residue can lead to receptor silence and thus be critical for G protein coupling and receptor activation. $R103^{3.50}$ and $I107^{3.54}$ were shown to be important in G protein coupling and the activation of the $hA_{2B}$ receptor, because the mutant $R103^{3.50}$A failed to couple to all G protein pathways and $I107^{3.54}$A failed to couple to almost all G protein pathways, whereas $L213^{IL3}$ turned out to be more important in G protein inactivation. $S235^{6.36}$A showed the most divergent

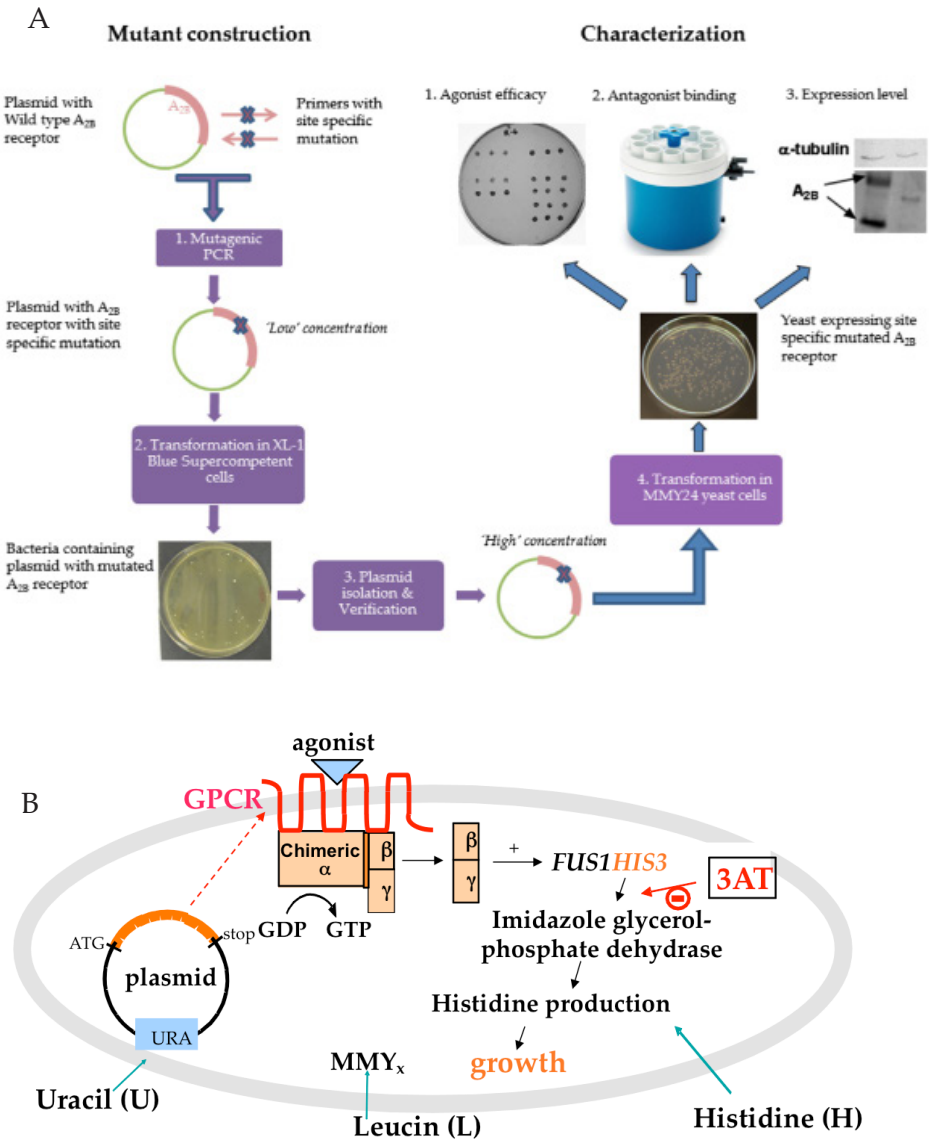


**Fig. 1.** (A) A schematic overview of "a single-GPCR-one-G protein yeast system" with the experimental set-up used during the research described in **Chapter 3** of this thesis. (B) Schematic drawing of GPCR activation in genetically modified yeast strains[1]. When heterologously expressed mammalian receptors are activated by agonists, β and γ subunits of the yeast G protein disassemble from the α subunit and activate the *FUS1* promoter which in turn activates the transcription of reporter genes *HIS3* which enables yeast cells to synthesize histidine allowing them to grow in histidine-deficient medium. Deficient selection markers medium: MMY strains: YNB-L; one MMY strain with the plasmid: YNB-UL; and functional assay of the GPCR activation (e.g. agonist efficacy): YNB-ULH.

G protein coupling profile, whereas L236$^{6.37}$A decreased receptor activation in all G protein pathways, although to a different extent.

In **Chapter 4**, we continued with a bigger sequence domain to discover the function of the so-called NPxxY motif and helix 8 in adenosine $A_{2B}$ receptor activation. We characterized the functions of each residue in agonist efficacy, antagonist binding and expression level (Fig. 2). For example, amino acid residues P287$^{7.50}$, Y290$^{7.53}$, R293$^{7.56}$ and I304$^{8.57}$ were found to be critical, because their mutants (substitution with alanine) showed a complete loss of function with respect to agonist binding. The same four mutants lost antagonist binding, except for Y290$^{7.53}$A with some remaining binding. In terms of expression levels, R293$^{7.56}$A showed no expression, whereas the other three mutant proteins had expression levels comparable to the wild-type receptor. There were 8 amino acid residues that are also very important in receptor activation, but not as critical as the four residues: N286$^{7.49}$, V289$^{7.52}$, Y292$^{7.55}$, N294$^{8.47}$, F297$^{8.50}$, R298$^{8.51}$, H302$^{8.55}$ and R307$^{8.60}$, because their alanine mutants showed a partial loss of function in both NECA efficacy and potency. N286$^{7.49}$A and Y292$^{7.55}$A showed a slightly higher antagonist binding than wild-type, while other mutants showed a decrease in antagonist binding compared to wild-type receptor. N286$^{7.49}$A was only slightly expressed (15% of wild-type) and Y292$^{7.55}$A showed a significantly decreased expression level (approx. 50% of control). Many mutants were expressed similarly as the wild-type receptor (N294$^{8.47}$A, F297$^{8.50}$A, R298$^{8.51}$A, H302$^{8.55}$A, R307$^{8.60}$A). V289$^{7.52}$A had a significantly higher expression level than the wild-type receptor.

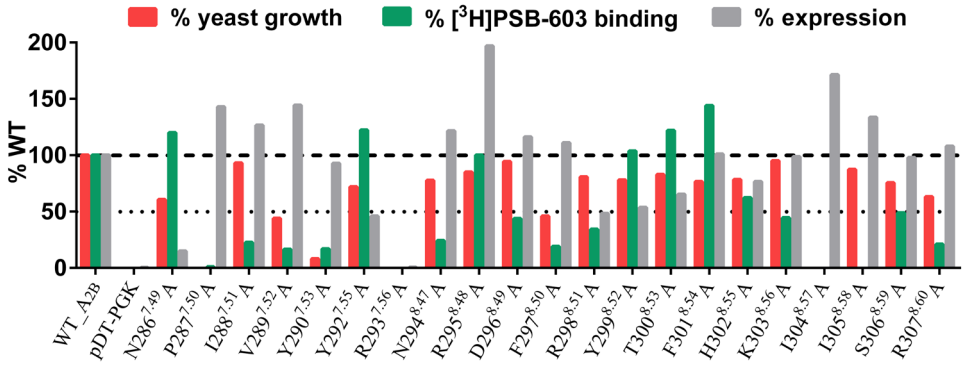


**Fig. 2.** Characterization of the NPxxY motif and helix 8 mutants of the adenosine $hA_{2B}$ receptor in the strain MMY24 ($G_{\alpha i3}$): agonist efficacy (■), antagonist binding (■), and expression level (■).

Aiming to understand the role of one single residue better we mutated such a residue into amino acids different from alanine. For example, in **Chapter 2,** the following mutants were characterized, $N286^{7.49}Q$, $N286^{7.49}R$ and $N294^{8.47}I$ to better grasp the function and role of the specific side chain of each amino acid. When we aimed to focus on the interaction between two residues, we decided to 'swap' amino acids. For example in **Chapter 2,** $Y290^{7.53}F$ and $F297^{8.50}Y$ as single mutants and the double mutant $Y290^{7.53}F/F297^{8.50}Y$ were characterized on the assumption that there exists an aromatic interaction or pi/pi stacking between the $Y290^{7.53}$ and $F297^{8.50}$ residues stabilizing the receptor in an inactive state. From our experimental data the tyrosine's hydroxyl group in $Y290^{7.53}$ appeared very important in $hA_{2B}$ receptor activation. However, the double mutant $Y290^{7.53}F/F297^{8.50}Y$ showed a greater combined loss of function than both single mutants, and did not act like the wild-type receptor. We thus conclude that the interaction between $Y290^{7.53}$ and $F297^{8.50}$ residues is more complicated than maintaining the aromatic interaction between the phenyl rings of the two side chains.

In **Chapter 5** we particularly focused on the function of the GPCR C terminus by investigating the different behaviors of the two closely related receptors $hHCA_2$ and $hHCA_3$ in G protein coupling. We found that the $hHCA_2$ receptor was promiscuous in its G protein coupling preference, much more than the $hHCA_3$ receptor. We then reasoned that, although the $HCA_2$ and $HCA_3$

receptors share high sequence identity, they differ considerably in C-terminus length with $HCA_3$ having the longest tail. Hence we constructed two mutant receptors by 'swapping' the short ($HCA_2$) and long ($HCA_3$) C-terminus. The differences in $HCA_2$ and $HCA_3$ receptor activation and G protein selectivity were not controlled, however, by their C-terminal tails, as we observed only minor differences between mutant and corresponding wild-type receptor.

## SAR and SKR studies of the $HCA_2$ receptor

Intrigued by the $HCA_2$ receptor we embarked on a medicinal chemistry project to design and study a series of agonists for this receptor subtype. It is noteworthy that in **Chapter 6** the results were not obtained from *S. cerevisiae* strains, but from human embryonic kidney (HEK293T) cells, which were transfected with the N-Flag-tagged $HCA_2$ receptor in pcDNA3.1, as this cellular background is more suitable for performing radioligand binding studies. Structure-affinity relationship (SAR) and structure-kinetics relationship (SKR) studies were combined to investigate a series of biphenyl anthranilic acid agonists (27 compounds in total) for the $HCA_2$ receptor. Twelve of them showed higher affinity than the reference agonist nicotinic acid. We discovered two compounds, the 4-OH derivative **6g** ($IC_{50}$ = 75 nM) and tetrahydro-anthranilic acid **6z** ($IC_{50}$ = 108 nM) having a long residence time profile on the receptor compared to nicotinic acid, which was exemplified by their kinetic rate index (KRI) values of 1.31 (**6g**) and 1.23 (**6z**) vs nicotinic acid (0.8).

## Perspectives

## G protein coupling profiles of GPCRs

Our findings on the DRY, $NPxxY(x)_{5,6}F$ and Helix 8 motifs of the $hA_{2B}$ receptor and on the C-terminus of the HCA receptors shed light on receptor-G protein coupling and G protein selectivity, which may help in further understanding the signaling of other GPCRs. With the yeast system at hand we can further focus

on key residues that abolish coupling to a certain G protein pathway and the ones that play important roles in the mechanisms of GPCR/G protein coupling. We can further extend our observations by performing saturation mutagenesis studies and see whether we can confirm if the same mechanisms apply to other receptors. In the future it would be very important to confirm our main findings in human cells or higher order model systems such as rodents or zebrafish.

Our yeast system with the extended G proteins library is very suitable to characterize G protein coupling profiles of other wild-type receptors, which can be very different between different receptors[2-4]. Previous research has also shown that G protein coupling of a receptor is consistent between yeast cells and mammalian cells[4]. Following the studies in this thesis it would be ideal to systematically mutate the whole receptor, yielding a complete map of an individual amino acid's function in GPCR/G protein coupling. However, this is an enormous and laborious project of low efficacy. A more effective way would be to systematically mutate only those amino acids that emerge from previous research, bioinformatics analyses, or predictions from crystal structures or homology models[5]. The activation mechanism of each receptor is most likely unique, however, there are many universal mechanisms as well. For example, Hofmann et al. reported that in rhodopsin interaction networks are formed by conserved residues, $NP^{7.50}xxY(x)_{5,6}F$, $E(D)R^{3.50}$, $Y^{5.58}(X)_7K(R)^{5.66}$, $CWxP^{6.50}$, and also functional microdomains, such as TM3-TM5 and TM5-TM6 interactions formed by $R135^{3.50}$, $E247^{6.30}$, $Y223^{5.58}$ and $K231^{5.66}$[6]. More recently, Flock et al. reported a mechanism of GPCRs and $G_\alpha$ activation in which H5 of $G_{\alpha s}$ is the critical interface element that makes residues in TM3, IL2, TM5, IL3, TM6 of $\beta_2$–adrenergic receptor interact[7]. We need to verify all these universal mechanisms and decipher the common mechanism of activation/G protein coupling between different receptors.

Another application for the yeast system with an extended G proteins library is the screening or characterization of functionally selective agonists. Different agonists may show different agonist affinity and efficacy in one G protein pathway, while they may have yet another profile of affinity and efficacy in a second G protein pathway[8, 9]. Since we have a library with agonists

of the $hHCA_2$ receptor we tested each agonist in different MMY strains with one (different) $G_\alpha$ protein. We could then analyze which part of the chemical structures of the agonists is important for activation in one specific G protein pathway (preliminary data not shown).

In conclusion, in addition to G protein coupling profiles of receptors activated by the same agonist, we can also obtain G protein coupling profiles of different agonists for the same receptor.

**Activation mechanism of Class B**

The yeast system has been used frequently in the Class A receptor subfamily, such as Serotonin (5-HT, 5-hydroxytryptmine)[10], thyroid-stimulating hormone receptor (TSHR)[11, 12], CXC chemokine receptor 1 (CXCR1)[13], also reviewed in **Chapter 2** of this thesis. However, the activation mechanism of members of the Class B family, such as adhesion receptors, has been less investigated. A single-GPCR-one-G protein yeast system is equally suitable to be applied to these receptors to decipher their activation and G protein coupling profiles. Class A and Class B subfamilies share some similar sequence motifs but are mostly completely different (Table 1[14]). There are many orphan receptors in the Class B subfamily, and hence there is no endogenous ligand known to activate the receptors. However, some of the wild-type receptors in this class show high levels of constitutive activity, and thus mutations that decrease or increase constitutive activity are telling for the importance of the original residue in receptor activation. Thus, learning how the motifs function in receptor activation/G protein coupling is very useful to discover and understand general mechanisms of activation in the Class B subfamily.

**Table 1.** Class A motifs and their class B counterparts[14].

| Region | Class | Motif | Function |
|---|---|---|---|
| IL1 | A | $K^{1.61}$KLHxxxN | structure |
| | B | $R^{1.61}$KLHxxxN | |
| TM2,3 | A | $DRY^{3.51}$ | activation |
| | B | $R^{2.39}H^{2.43}$; $E^{3.46}$ | |
| TM3 | A | $C^{3.25}$ | structure |
| | B | $C^{3.25}$ | |
| TM4 | A | $W^{4.50}$ | structure |
| | B | $W^{4.50}$ | |
| TM5 | A | $IxxL^{5.65}$ | G protein interaction |
| | B | $LXXL^{5.65}$ | |
| TM6 | A | $CWxP^{6.50}$ | activation |
| | B | P...$TY^{6.48}$ | |
| TM6 | A | $KxxK^{6.35}$ | G protein interaction |
| | B | $KxxK^{6.35}$ | |
| TM7 | A | $NPXXY^{7.53}$ | activation |
| | B | $VAVLY^{7.53}$ | |
| H8 | A | $EFxxxL^{8.54}$ | structure/restraint |
| | B | $EVxxxL^{8.54}$ | |
| TM2,3; TM7 | A | $R^{3.50}$ – $E^{6.30}$ | ionic/polar lock |
| | B | $R^{2.39}$ – $T^{6.37}$ | |

## Challenges for the $HCA_2$ receptor

In **Chapter 6** we identified two compounds, **6g** and **6z**, showing slower dissociation from the receptor than nicotinic acid. Residence time as a parameter might be a better predictor for *in vivo* efficacy and should be considered during lead optimization to potentially make the drug discovery process more efficient. Predictions from a homology model of $HCA_2$ receptor suggest important structural requirements, such as the carboxylic group forming hydrogen bonds with $R111^{3.36}$ and $R251^{6.55}$; the hydroxyl group forming a hydrogen bond with $D92^{EL1}$; and the benzene ring forming a $\pi$-$\pi$ interaction with $W93^{EL1}$ (Fig. 3). However, we failed to obtain accurate residence time values due to practical issues with the radioligand nicotinic acid. One idea would be to radiolabel other high affinity ligands of the $HCA_2$ receptor, to obtain a more precise kinetic profile of long or short residence time compounds.

The importance of the $HCA_2$ receptor has been growing, however, it is somewhat unfortunate that no antagonists have been identified yet for the $HCA_2$ receptor, which would allow a more detailed pharmacological analysis. We could use our fast and cheap yeast system to screen large compounds libraries to screen for antagonists.

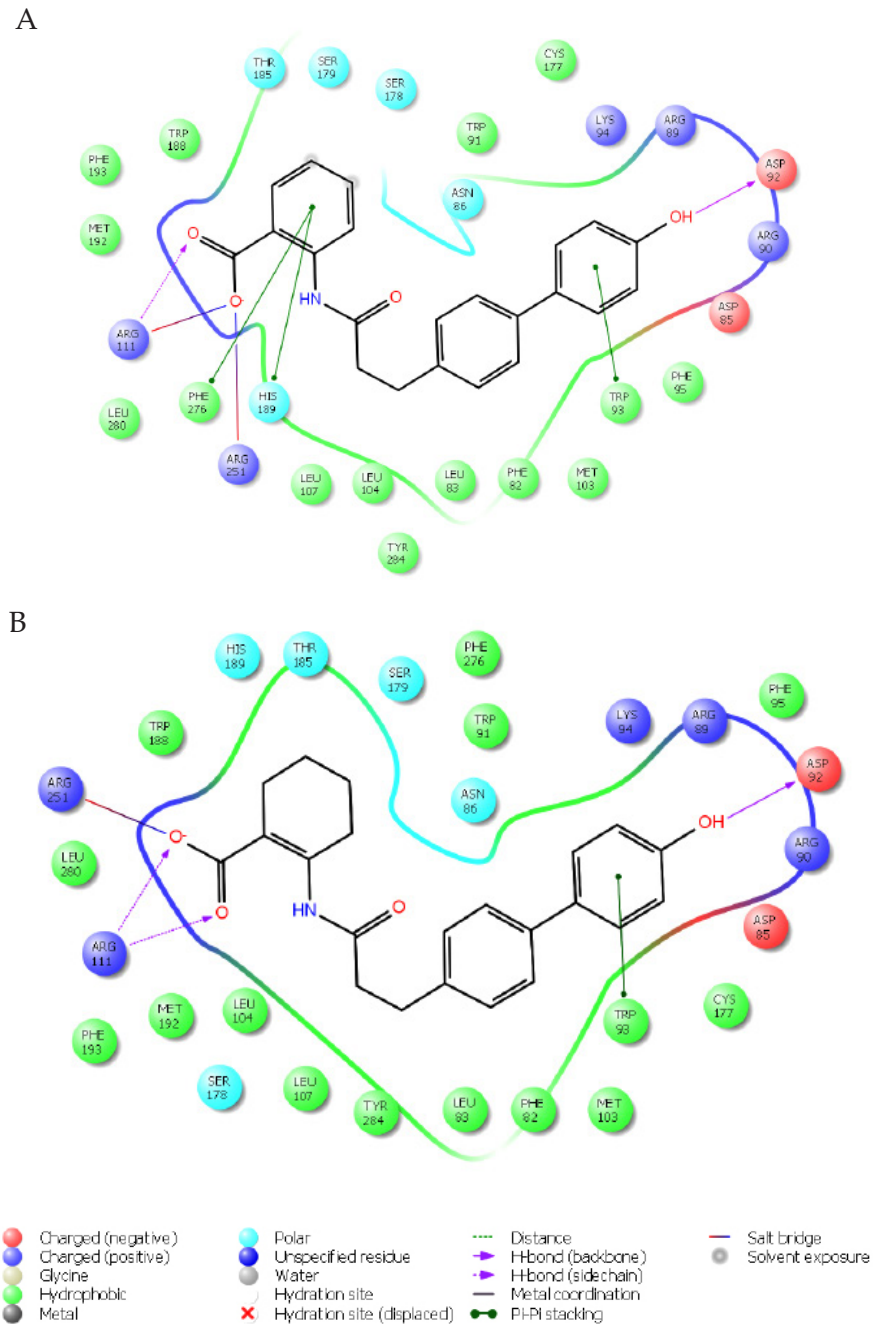


**Fig. 3.** Interaction map of **6g** (A) and **6z** (B) with surrounding residues in a composite homology model of $HCA_2$ receptor was constructed based on the structure of both the Serotonin 2b receptor co-crystalized with ergotamine (Protein Data Bank: 4IB4) and the crystal structure of the Free Fatty Acid receptor 1 bound to TAK-875 (Protein Data Bank: 4PHU)[15, 16].

## Final notes

We confirmed that a yeast system with an extended library of G proteins is very well suited for the study of GPCR activation, G protein coupling profiles, receptor-G protein binding and G protein selectivity. For example, we used a scanning mutagenesis approach of the NPxxY$(x)_{5,6}$F motif and of helix 8 of the adenosine $A_{2B}$ receptor ($A_{2B}$R), and learned among others that amino acid residues in these motifs are crucial for receptor function, since alanine mutants of these amino acid residues led to a complete loss of function. Hopefully, such findings can contribute to further drug development.

We also focused on structure-kinetics relationship (SKR) studies next to the more traditional Structure-affinity relationship (SAR) studies. We found two compounds showing longer residence times than nicotinic acid, which may provide clues for further drug discovery efforts on this receptor. All in all, the variety of methods described in this thesis provided us a detailed understanding of receptor function, suggesting that novel avenues for further drug discovery on these established targets is entirely feasible.

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