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

Liu, R.

Chapter 4

Scanning mutagenesis in a yeast system delineates the role of the NPxxY(x)$_{5,6}$F motif and helix 8 of the adenosine A_{2B} receptor in G protein coupling

This chapter is based upon:

Rongfang Liu, Dennis Nahon, Beau le Roy, Eelke B. Lenselink, Adriaan P. IJzerman

Biochemical Pharmacology ***2015*** *95: 290-300*

Abstract

The adenosine receptor subfamily includes four subtypes: the A_1, A_{2A}, A_{2B} and A_3 receptors, which all belong to the superfamily of G protein-coupled receptors (GPCRs). The adenosine A_{2B} receptor is the least investigated of the adenosine receptors, and the molecular mechanisms of its activation have hardly been explored. We used a single-GPCR-one-G protein yeast screening method in combination with mutagenesis studies, molecular modeling and bio-informatics to investigate the importance of the different amino acid residues of the NPxxY$(x)_6$F motif and helix 8 in the human adenosine A_{2B} receptor ($hA_{2B}R$) activation. A scanning mutagenesis protocol was employed, yielding 11 single mutations and one double mutation of the NPxxY$(x)_6$F motif and 16 single mutations of helix 8. The amino acid residues P287$^{7.50}$, Y290$^{7.53}$, R293$^{7.56}$ and I304$^{8.57}$ were found to be essential, since mutation of these amino acid residues to alanine led to a complete loss of function. Western blot analysis showed that mutant receptor R293$^{7.56}$A was not expressed, whereas the other proteins were. Amino acid residues that are also important in receptor activation are: N286$^{7.49}$, V289$^{7.52}$, Y292$^{7.55}$, N294$^{8.47}$, F297$^{8.50}$, R298$^{8.51}$, H302$^{8.55}$ and R307$^{8.60}$. The mutation Y290$^{7.53}$F lost 50% of efficacy, while F297$^{8.50}$A behaved similar to wild-type receptor. The double mutation, Y290$^{7.53}$F/F297$^{8.50}$Y, lost around 70% of efficacy and displayed a lower potency for the reference agonist 5′-(*N*-ethylcarboxamido)adenosine (NECA). This study provides new insight into the molecular interplay and impact of TM7 and helix 8 for hA_{2B} receptor activation, which may be extrapolated to other adenosine receptors and possibly to other GPCRs.

Introduction

The G protein-coupled receptors (GPCRs) form a membrane-bound protein superfamily of seven transmembrane (TM) receptors that transmit extracellular signaling to the intracellular environment[1]. All GPCRs have seven transmembrane spanning α-helices, an intracellular C-terminus with an additional α-helix ('helix 8'), an extracellular N-terminus, and 3 intra- and 3 extracellular loops. When a GPCR is activated by an extracellular stimulus (e.g., a hormone/neurotransmitter or a synthetic agonist), a change in receptor conformation is induced, leading to subsequent activation of a heterotrimeric G protein[2].

The NPxxY$(x)_{5,6}$F stretch of amino acids plays an important role in G protein activation[3], which includes the highly conserved NPxxY motif in TM7 across the class A (rhodopsin-like) GPCR family, that is linked to the intracellular helix 8, especially the residue phenylalanine (F) therein (5 or 6 residues after the NPxxY motif)[4]. Helix 8 located at the start of the carboxyl (C)-terminus of GPCRs is gaining recognition for its importance in GPCR function. Previous research has shown that key residues of helix 8 play a vital role in receptor activation, such as in the M1 muscarinic acetylcholine receptor[5], leukotriene B4 receptor[6], cannabinoid receptor 1[7], the type 1 angiotensin receptor[8] and the human β_1-adrenergic receptor[9]. However, there has been no study focusing on this helix 8 in the context of activation of adenosine receptors. All four adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) are ubiquitously expressed in the human body[10] and can target different intracellular signaling pathways by responding to the same endogenous ligand adenosine. The A_{2B} receptor has the lowest affinity for adenosine[11] and has been less investigated than the other adenosine receptors. Several studies have shown that blocking A_{2B} receptor signaling reduces experimental autoimmune encephalomyelitis[12] and inhibits growth of prostate cancer cells[13], breast tumors[14] and bladder tumors[15, 16]. On the other hand, activation of the A_{2B} receptor protects against trauma-hemorrhagic shock-induced lung injury[17], CHX-induced apoptosis[13] and also vascular injury[18]. Thus, understanding the molecular mechanisms of hA_{2B} receptor activation is of

considerable relevance for drug design and disease prevention. In this context it would be of substantial interest to determine whether the same tyrosine ($Y^{7.53}$ of TM7)–phenylalanine ($F^{8.50}$) interaction exists in the hA_{2B} receptor, as e.g., apparent in the angiotensin II type 1 receptor[8], and also to find which key residues of helix 8 are involved in hA_{2B} receptor activation.

In this study, a single-GPCR-one-G protein system in *Saccharomyces cerevisiae*[19, 20] was examined. It has been used for studying GPCR activation, such as for the adenosine A_1 receptor[21] and the adenosine A_{2B} receptor by ourselves[22], and, recently, for the glucagon-like peptide-1 receptor[23] among many other GPCRs. To investigate the importance of the different amino acid residues of the $NPxxY(x)_6F$ motif and helix 8 in hA_{2B} receptor activation, an alanine-scanning mutagenesis approach was applied to each residue of the $NPxxY(x)_6F$ motif and of helix 8 (Fig. 1). We identified the function of each residue of the $NPxxY(x)_6F$ motif and whole helix 8 in G protein coupling and receptor activation, which may be extrapolated to other adenosine receptors and GPCRs.

Materials and methods

Materials

The *S. cerevisiae* expression vectors, the pDT-PGK and pDT-PGK_hA_{2B} receptor plasmids and the MMY24 strain were kindly provided by Dr. Simon Dowell (GSK, Stevenage, UK). Six mutations, $N286^{7.49}A$, $N286^{7.49}Q$, $N286^{7.49}R$, $Y290^{7.53}F$, $Y290^{7.53}N$ and $N294^{8.47}I$ had been generated before at Leiden University, The Netherlands. The QuikChange® II Site-Directed Mutagenesis Kit was purchased from Stratagene (Amsterdam, the Netherlands). The QIAprep midi plasmid purification kits were purchased from QIAGEN (Amsterdam, the Netherlands). NECA and 3-Amino-[1,2,4]-triazole (3-AT) were both purchased from Sigma–Aldrich (Zwijndrecht, the Netherlands). The Hybond-ECL membranes and the ECL Western blotting analysis system were purchased from GE Healthcare (Eindhoven, the Netherlands). The antibody used was directed against the C-terminal region of the hA_{2B} receptor and was kindly provided by Dr. I. Feoktistov (Vanderbilt University, Nashville, TN, USA) and goat anti-

rabbit IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Rat anti-yeast α-tubulin monoclonal antibody was used as a reference protein (GTX76511, GeneTex, Irvine, CA, USA) with goat anti-rat IgG-HRP antibody (sc-2032, Santa Cruz Biotechnology, Heidelberg, Germany) as the second antibody. Adenosine deaminase was purchased from Roche (Almere, The Netherlands). Polyethylenimine (PEI) was purchased from Sigma (Zwijndrecht, the Netherlands).

Generation of point mutations

To investigate the importance of different amino acid residues of the NPxxY$(x)_6$F motif and of helix 8 (Fig. 1) in hA_{2B} receptor activation, a scanning mutagenesis was performed, yielding 11 single mutations and one double mutation of the NPxxY$(x)_6$F motif and 16 single mutations of helix 8. The DNA primers of the mutations of the hA_{2B} receptor (uniprot: P29275) were designed by the QuikChange Primer Design Program of Agilent Technologies (Santa Clara, CA, USA) and contained a single substitution resulting in a codon change for the desired amino acid substitution. The reverse primer sequence of each mutation was the reverse complement of the forward primer. These primers and their complements were synthesized (Eurogentec, Maastricht, the Netherlands) and then used to generate mutation plasmids according to the QuikChange method from Stratagene/Agilent Technologies. The mutagenic reaction contained 45 ng of the constructed pDT-PGK_hA_{2B} plasmid as dsDNA template, 10 μM of each primer, 1 μl of dNTP mix, 2.5 μl of 10 × reaction buffer and 2.5 U *PfuUltra* HF DNA polymerase. The following thermal cycling parameters were used in the PCR apparatus (T100™ Thermal Cycler, Bio-Rad, Hercules, CA, USA): 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 10 min. The number of mutagenic PCR cycles was set to 22. Methylated or hemimethylated non-mutated plasmid DNA was removed by adding 5 U *Dpn* I restriction enzyme for 2 h at 37 °C. The mutated DNA products were transformed into XL-1 Blue supercompetent cells and other details were according to the manual of the QuikChange® II Site-Directed Mutagenesis Kit. The mutations were confirmed by DNA sequencing (LGTC, Leiden, the Netherlands).

Transformation in a *S. cerevisiae* strain (MMY24)

Each mutation plasmid was transformed according to the Lithium-Acetate procedure[24] into an engineered *S. cerevisiae* yeast strain, MMY24, expressing one specific Gpa1p/$G_{\alpha i3}$ chimeric G protein. The yeast strain was derived from the MMY11 strain and further adapted to communicate with mammalian GPCRs. Hereto the last five amino acid residues of C-terminus of Gpa1p/$G_{\alpha i3}$ chimera had been replaced with the same-length sequence from mammalian $G_{\alpha i3}$ protein[19]. The genotype of the MMY24 strain is: **MATa** *his3 leu2 trp1 ura3 can1 gpa1_::G_i3 far1 ::ura3 sst2_::ura3 Fus1::FUS1-HIS3 LEU2::FUS1-lacZ ste2_::G418R* and the sequence of these last 5 C-terminal amino acid residues of Gpa1p/$G_{\alpha i3}$ chimera is ECGLY^{COOH}.

Yeast solid growth assay

To characterize the activation of the mutated receptors, concentration–growth curves were generated with a solid growth assay. To measure the signaling of GPCRs, the pheromone signaling pathway of these strains was coupled via the *FUS1* promoter to *HIS3* (imidazoleglycerol-phosphate dehydratase), an enzyme catalyzing the sixth step in histidine biosynthesis to produce histidine. 3-AT, a competitive inhibitor of imidazoleglycerol-phosphate dehydratase, was added to the assay to reduce background activity caused by endogenous histidine and constitutive activity[20]. The degree of receptor activation induced by an agonist of the GPCR was measured by the growth rate of the yeast on a histidine-deficient medium. The solid assay was performed with yeast cells from an overnight culture in YNB-UL (YNB + adenine + tryptophan + histidine, lacking uracil and leucine). These yeast cells were diluted to around 600,000 cells/ml (OD_{600} ≈ 0.02), and droplets of 1.5 µl (≈ 900 cells) were spotted on selection agar plates, YNB-ULH (YNB + adenine + tryptophan lacking uracil, leucine and histidine), containing 7 mM 3-AT and the adenosine receptor agonist NECA (concentrations ranging from 10^{-9} to 10^{-4} M). After incubation at 30 °C for 50 h, the plates were scanned and receptor activation-mediated yeast growth was quantified with Quantity One imaging software of a GS-800 Calibrated Densitometer from Bio-Rad (Hercules, CA, USA).

Preparation of yeast protein extractions and immunoblotting

Protein extractions were performed with trichloroacetic acid (TCA) according to the Clontech Yeast Protocols Handbook 2001. Yeast transformants were grown in 3 ml YAPD medium and were harvested in mid-exponential phase (adjust OD_{600} = 6, in total 1.2×10^8 cells per sample). The yeast cells were collected and washed with cold water. Subsequently the yeast cells were broken by vigorous vortexing with 200 µl of 20% TCA and 200 µl glass beads. A small hole was burnt in the bottom of the 2 ml eppendorf tubes with a needle and these tubes were placed in 1.5 ml eppendorf tubes. This double layer of tubes was centrifuged at 3,000 rpm for 2 min allowing the TCA with the broken cells, but not the glass beads, to pass through the hole from the top eppendorf tube into the bottom one. The glass beads were washed twice with 200 µl 5% TCA and spun both times at 3,000 rpm for 2 min. The tubes containing the glass beads were discarded and the tubes containing the broken cells were spun at 6,000 rpm for 2 min. The supernatant was removed as much as possible. The pellets were resuspended with 55 µl 10% SDS loading buffer (100 mM EDTA, 1 M Tris, 10% SDS, 0.5% Bromophenol blue; pH = 8) and 35 µl 1 M Tris (pH = 8.0) was added to neutralize all remaining TCA. The samples were incubated for 30 min at 37 °C and centrifuged again at 2,000 rpm for 10 min.

Each sample of 4 µl (≈ 24 µg protein) was loaded on 12.5% SDS/PAGE gel and then blotted on Hybond-ECL membranes (GE Healthcare, Eindhoven, The Netherlands) using a semi-automated electrophoresis technique (PhastSystem™, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The Hybond-ECL membranes were blocked with TBS containing 5% milk powder for 1 h and washed three times with TBST (0.05% Tween 20, TBS pH 7.6). The membranes were incubated with 1:1,250 diluted rabbit anti-human A_{2B} receptor antibody for 1 h. After thorough removal of unbound antibody from the membranes by washing three times with TBST, the membranes were incubated with 1:2,500 diluted HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h. The membranes were washed twice with TBST and once with TBS. The specific signal of the hA_{2B} receptor was probed according to the ECL Western blotting analysis system (GE Healthcare,

Eindhoven, The Netherlands) using enhanced chemiluminescence (ChemiDox XRS, Bio-Rad, Hercules, CA, USA). Tubulin as the reference household protein was analyzed simultaneously using rat anti-yeast α-tubulin monoclonal antibody at 1 µg/mL and then using goat anti-rat IgG-HRP antibody at 0.16 µg/mL as the second antibody.

Whole cell radioligand binding experiments

The whole cell radioligand binding experiments were performed as described by us before[25, 26]. Yeast cells from an overnight culture expressing the wild-type or mutated $hA_{2B}R$ were harvested from rich 3.5 ml YAPD medium by centrifuging at 2,000 × g for 5 min. The pellet of cells was washed once with 0.9% NaCl. The cells were centrifuged again using the same speed and diluted in the assay buffer (50 mM Tris–HCl pH 7.4 + 1 mM EDTA) to OD_{600} = 40 (OD_{600} = 1 ≈ 2.5 × 10^7 cells/ml) with 0.4 IU adenosine deaminase. Binding experiments were performed with 1.4 nM of the A_{2B} receptor selective antagonist [^{3}H]PSB-603, and a final cell concentration of 25 × 10^7 cells/ml in a total volume of 100 µl. Nonspecific binding (NSB) was determined by additionally adding NECA at a final concentration of 1 mM. Samples were incubated for 1 h at 25 °C keeping the cells in suspension by shaking vigorously. 1 ml of ice-cold assay buffer was added to samples to terminate incubation and the samples were harvested on a Millipore manifold with GF/B filters pre-incubated in 0.1% polyethylenimine (PEI) at a pressure of 200 mbar, to separate free from receptor-bound radioligand by washing twice with 2 ml buffer (50 mM Tris–HCl pH 7.4 + 1 mM EDTA + 0.1% BSA). The filters were transferred into mini-vials, 3.5 ml of PerkinElmer Emulsifier Safe was added, and were subsequently incubated for at least 2 h. Filter-bound radioactivity was determined as counts per minute (CPM) by scintillation spectrometry (Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences). Results were obtained from at least three independent experiments, performed in duplicate.

Data analysis

After incubation at 30 °C for 50 h, the plates of the solid growth assay were scanned and receptor mediated yeast growth was quantified with Quantity

One imaging software of the GS-800 Calibrated Densitometer from Bio-Rad (Hercules, CA, USA). After correction for the local background, the amount of yeast growth was calculated as the density (in OD/mm^2) of each spot. Results were obtained from four independent experiments, performed in triplicate. EC_{50} values and Emax values of the solid assay were calculated using the nonlinear regression package available in Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences in EC_{50} values were examined for significance by a two-tailed homoscedastic Student's *t*-test, yielding *p*-values. Fold EC_{50} is the EC_{50} value on the mutant receptor divided by the EC_{50} value on the wild-type (WT) hA_{2B} receptor.

Expression of the receptors was quantified using Quantity One imaging software from Bio-Rad after correction for the background; it was calculated as density (OD/mm^2). The α-tubulin, at approx. 130 kDa, was used as loading control and the specific hA_{2B} receptor protein bands were at 34 kDa and 55 kDa. The ratio was determined between the density of the specific bands and the density of loading control band. MMY24, carrying the wild-type hA_{2B} receptor, was set at 100% and MMY24, carrying the empty vector pDT-PGK without receptor, was set at 0%. Results were obtained from three independent experiments and all expression levels of mutant receptors were normalized by a wild-type receptor sample on the same blot.

Bio-informatics

Amino acid conservation was obtained for the $NPxxY(x)_{5,6}F$ motif and helix 8. Amino acid sequences of the adenosine receptors, nucleotide-like subfamily and class A (rhodopsin-like) family of GPCRs were collected from GPCRDB (http://www.gpcr.org/7tm/)[27]. Frequency plots displaying conservation were created using Weblogo (http://weblogo.berkeley.edu/logo.cgi)[28].

A chimera/homology model of the adenosine hA_{2B} receptor was constructed using the homology modeling tool within Maestro[29-32]. The model was based on both the β_2 adrenergic receptor/G_s complex crystal structure (PDB: 3SN6) and the crystal structure of the adenosine A_{2A} receptor co-crystallized with the agonist NECA (PDB: 2YDV). A chimera model was constructed to

ensure that the intracellular part of the homology model was in the G_s bound conformation. The model was primarily based on the adenosine A_{2A} crystal structure except for the following sequence regions which were based on the β_2 adrenergic receptor crystal structure: A31-N43, V99-I126, I203-L213, E229-G241 and V289-Y299. Moreover, residues 214–228 in ICL2 were deleted since they were (partly) missing in the crystal structures and not of interest in this study. The resulting homology model was merged with the G_s protein part of the β_2 adrenergic receptor–G_s complex crystal structure and energy-minimized. To further optimize the A_{2B}–G_s complex the side chains of the sequence regions that were based on the β_2 adrenergic receptor were re-predicted using Prime[30-32]; this was done using Monte Carlo sampling.

Results

We set out to investigate the importance of the different amino acid residues of the NPxxY$(x)_6$F motif and helix 8 in the activation of the human adenosine A_{2B} receptor. To that end we employed a scanning mutagenesis approach, yielding 11 single mutations and one double mutation of the NPxxY$(x)_6$F motif and 16 single mutations of helix 8. The amino acids in this scan have been color-coded in a so-called snake plot of the receptor (Fig. 1). We expressed the yeast plasmid pDT-PGK_hA_{2B}R and mutations in a yeast *S. cerevisiae* strain, MMY24. This particular strain was preferred over others (data not shown) as the receptor showed high affinity and efficacy of the reference agonist NECA, while it displayed low constitutive activity yielding a good window between control and agonist-stimulated receptor. When the expressed hA_{2B} receptor or mutations are activated by an agonist, the yeast pheromone signaling pathway is activated through the chimeric yeast-mammalian G protein, leading to transcription of the HIS3 reporter gene and consequently histidine production[33]. Subsequent growth of the yeast cells on a histidine-deficient agar plate was determined by measuring the absorption at a wavelength of 595 nm, which reflects the activation of the expressed receptor by the adenosine receptor agonist NECA. NECA concentration–response curves from the solid growth assay of the wild-

type and mutant receptors are shown in Fig. 2A–C and the values of Emax, EC_{50} and fold EC_{50} shift are listed in Table 1. All mutant receptors displayed negligible constitutive activity, similar to or lower than the WT receptor (data not shown).

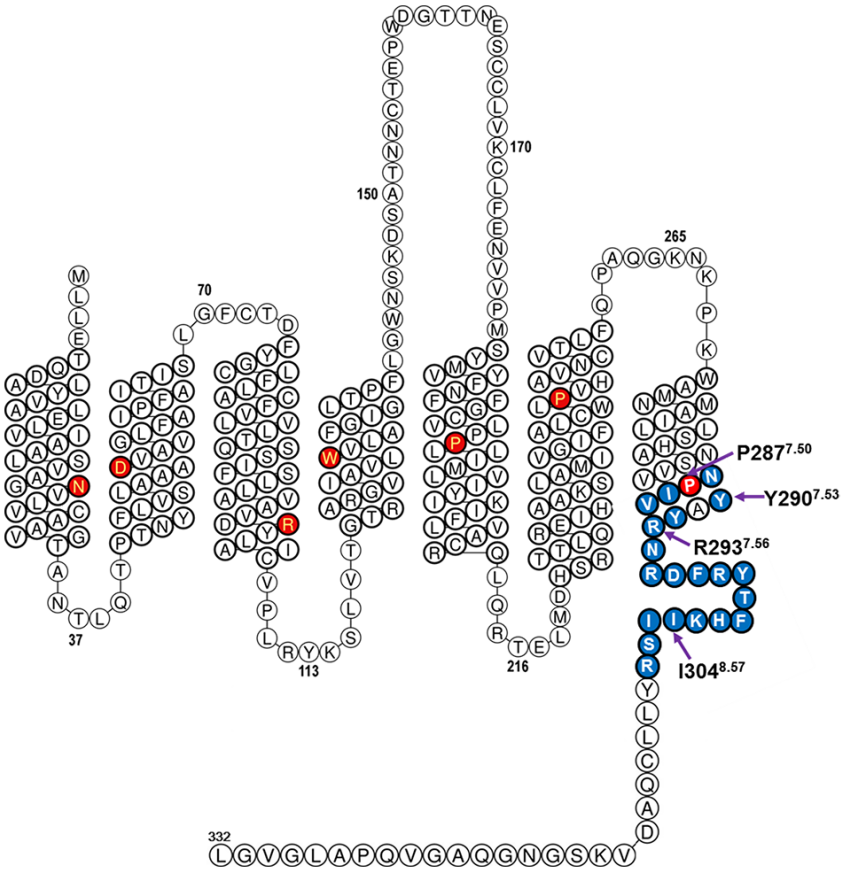

Fig. 1. Snake plot the human adenosine hA_{2B} receptor. The template was obtained from GPCRDB (http://www.gpcr.org/7tm/)[27]. Mutants were made in the $NPxxY(x)_{5,6}F$ motif and helix 8, shown as white bold letters. Four important amino acids have been labeled: $P287^{7.50}$, $Y290^{7.53}$, $R293^{7.56}$ and $I304^{8.57}$. Highly conserved residues are highlighted to indicate the Ballesteros–Weinstein numbering: $N^{1.50}$, $D^{2.50}$, $R^{3.50}$, $W^{4.50}$, $P^{5.50}$, $P^{6.50}$ and $P^{7.50}$[54].

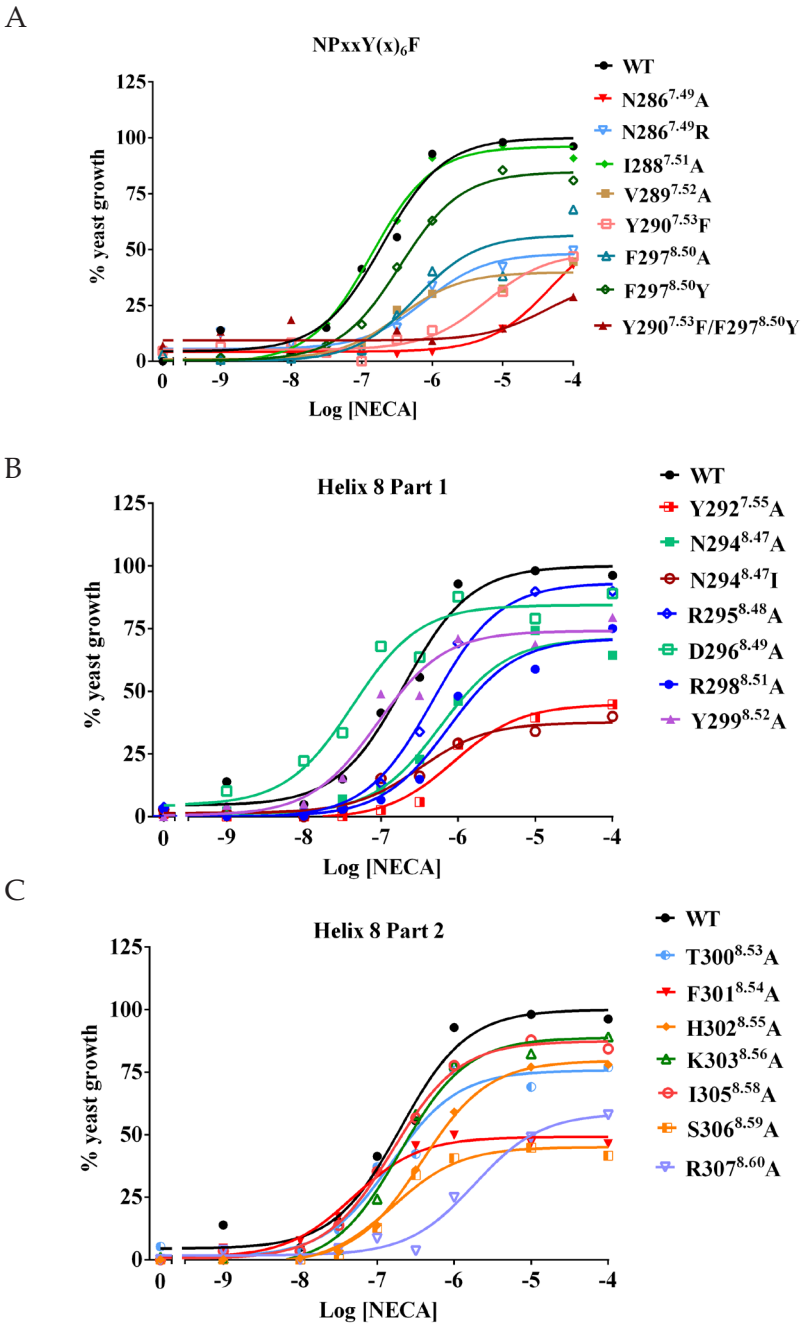

Fig. 2. Concentration–growth curves from solid assay experiments are shown for the hA_{2B} wild-type receptor and the $NPxxY(x)_6F$ mutations (A) and the hA_{2B} wild type receptor and the helix 8 mutations (B and C) responding to the adenosine receptor agonist NECA. The assay was performed on YNB-ULH agar plates. A representative experiment of a total of 4 for WT and each responsive mutation is shown.

Characterization of the function of the NPxxY(x)$_{5,6}$F motif in receptor activation

The NPxxY(x)$_{5,6}$F motif is a conserved stretch of amino acids among many subfamilies of GPCRs (Fig. 3A-C).

N286$^{7.49}$P287$^{7.50}$I288$^{7.51}$V289$^{7.52}$Y290$^{7.53}$(x)$_6$F297$^{8.50}$ is the motif present in the adenosine hA$_{2B}$ receptor.

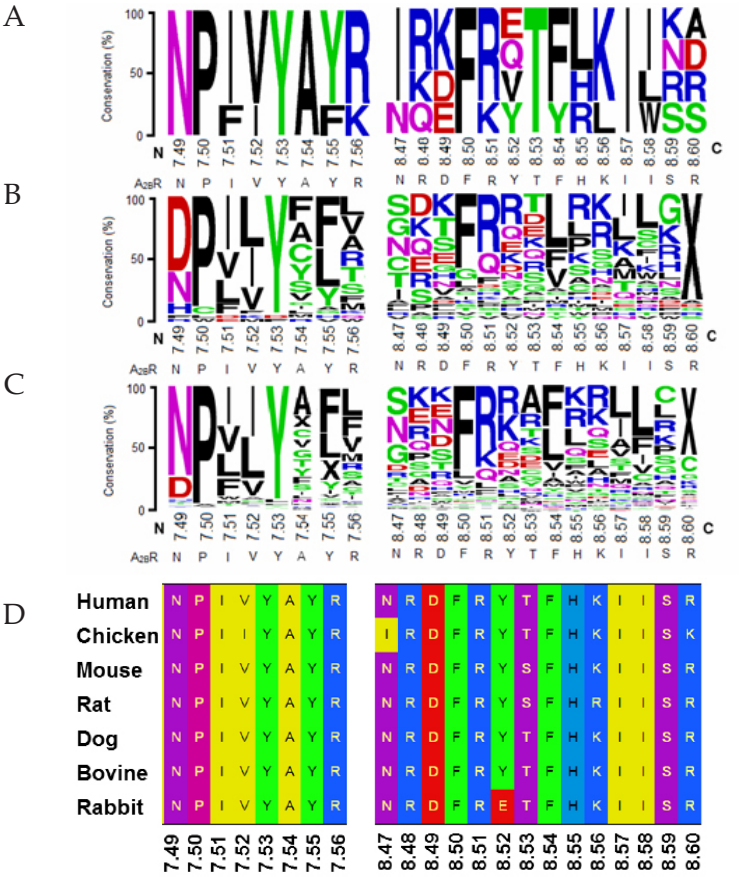

Fig. 3. Frequency plots of multiple sequence alignments for the NPxxY motif (7.49–7.56) and helix 8 (8.47–8.60) obtained from GPCRDB[27]. The size of the letters (one letter amino acid notation) corresponds to the degree of conservation across the alignment in question, with the largest letter (thus the highest degree of conservation) at the top. (A) Frequency plot of the conservation of the amino acids that were mutated in this research across the adenosine receptors; A_1, A_{2A}, A_{2B} and A_3. (B) Frequency plot of the conservation of the amino acids across the nucleotide-like subfamily (in total 32 receptors). (C) Frequency plot of the conservation of the amino acids across the class A (rhodopsin-like) family (in total 292 receptors). Gaps in the multiple sequence alignment are indicated with an X. (D) The alignment of these regions in the A_{2B} receptor between different species.

Residues of NPxxY(x)$_6$F mutated to alanine

In contrast with the wild-type hA_{2B} receptor (NECA: Emax = 100%; EC_{50} = 141 ± 79 nM), the two most conserved residues, P287$^{7.50}$ and Y290$^{7.53}$, showed a complete loss of function upon mutation to alanine; no curves could be generated in the solid growth assay, a feature similar to the empty plasmid pDT-PGK (Table 1). The efficacy values of the following three mutations, N286$^{7.49}$A, V289$^{7.52}$A and F297$^{8.50}$A were decreased to approx. 50% of the Emax value of the wild-type hA_{2B} receptor; the potency of NECA of these three mutations was significantly decreased ($p < 0.001$), although to a different extent: 160-fold, 2.7-fold and 5.7-fold, respectively (Table 1 and Fig. 2A). Only the mutation I288$^{7.51}$A did not show significant changes in both efficacy and potency of NECA, similar to the wild-type receptor (Table 1 and Fig. 2A).

Various other specific mutations

N286$^{7.49}$ was also mutated to glutamine (Q) and arginine (R) (Table 1). N286$^{7.49}$Q showed a complete loss of function and the efficacy of N286$^{7.49}$R was decreased with 42% and the potency of NECA by 5.6-fold (Fig. 2A). Y290$^{7.53}$ was also mutated to phenylalanine (F) and asparagine (N) (Table 1). The efficacy of Y290$^{7.53}$F was 63% and the potency of NECA on this mutant decreased by 30-fold (Fig. 2A). Y290$^{7.53}$N showed a complete loss of function. F297$^{8.50}$ was mutated to tyrosine (Y) and this mutation showed similar values of both efficacy and potency of NECA as the wild-type receptor. Besides these single mutations, one double mutation was made by swapping Y290$^{7.53}$ with F297$^{8.50}$, coined Y290$^{7.53}$F/F297$^{8.50}$Y (Table 1, Fig. 2A). This double mutation showed a 61% decreased efficacy and low potency of NECA, with a 129-fold reduction in EC_{50} value. The potency of NECA for the double mutation was decreased much more than expected from each single mutation.

Characterization of the function of helix 8 in receptor activation

The intracellular helix 8, close to the C-terminus of the GPCR, is increasingly recognized for its importance in G protein coupling and activation[5, 9]. To investigate the importance of the amino acid residues in this helix of the

adenosine hA_{2B} receptor, all residues were mutated one by one to alanine. Furthermore, N294$^{8.47}$ was also mutated to isoleucine (Fig. 3A), which is present in the other three adenosine receptors. F297$^{8.50}$ is part of helix 8 as well, but this residue was already discussed above, as it is often referred to in the context of the NPxxY motif.

Mutations showing a complete loss of function

R293$^{7.56}$A (Table 1), and I304$^{8.57}$A (Table 1) showed a complete loss of function; no curves were observed, similar to the empty plasmid pDT-PGK.

Mutations showing a slight increase in potency

The potency of D296$^{8.49}$A, Y299$^{8.52}$A and F301$^{8.54}$A was increased approx. 2-fold (EC_{50} from 51 nM to 87 nM), which was not significant. Y299$^{8.52}$A and F301$^{8.54}$A had partial decreases in efficacy of approx. 25% (Table 1, Fig. 2B and C), but D296$^{8.49}$A did not show a significant decrease, with 6% only, in efficacy (Table 1 and Fig. 2B).

Mutations showing a partial loss of function

There were 4 mutations of helix 8, Y292$^{7.55}$A, N294$^{8.47}$A, S306$^{8.59}$A and R307$^{8.60}$A that resulted in a partial loss of function when stimulated with NECA. Their efficacies were all decreased by more than 20%, up to 37% (R307$^{8.60}$A). These four mutations also showed a significant decrease of potency, although to a different extent: Y292$^{7.55}$A with 4.8-fold ($p < 0.001$), N294$^{8.47}$A with 3.2-fold ($p < 0.001$), S306$^{8.59}$A with 2-fold ($p = 0.015$) and R307$^{8.60}$A with 21-fold ($p < 0.001$). The specific mutation of N294$^{8.47}$I did not affect either efficacy or potency of NECA (Table 1, Fig. 2B and C).

Three other mutations of helix 8, R298$^{8.51}$A, H302$^{8.55}$A, and I305$^{8.58}$A, were slightly less affected, showing a partial loss of function when stimulated with NECA. The efficacies of these three mutations were slightly lower than wild-type, between 10% and 20% (R298$^{8.51}$A and H302$^{8.55}$A with a decrease of 19%, I305$^{8.58}$A with 13%). However, the potency of the agonist NECA was significantly reduced: R298$^{8.51}$A showed an 11-fold decrease of potency ($p < 0.001$), and

H302$^{8.55}$A had a 5.4-fold decrease of potency ($p < 0.001$); however, I305$^{8.58}$A only showed a 2.1-fold decrease of potency ($p = 0.015$) (Table 1, Fig. 2B and C).

The mutation K303$^{8.56}$A showed a significant decrease of potency of the agonist NECA (2.9-fold, $p = 0.002$), however, no significant decrease of efficacy (with 5% only) was observed (Table 1 and Fig. 2C).

Mutations with no or a slight decrease of receptor activation

Two mutations, R295$^{8.48}$A (Table 1 and Fig. 2B) and T300$^{8.53}$A (Table 1 and Fig. 2C) were more like the wild-type receptor. They showed only a 1.5-fold decrease of potency with concomitant slight decreases of efficacy around 15%.

Expression levels of wild-type receptor and all mutants

We performed a Western blot analysis to assess the expression levels of the wild-type hA_{2B} receptor, the NPxxY$(x)_6$F mutations (Fig. 4A) and the helix 8 mutations (Fig. 4B). A yeast reference protein, α-tubulin (top panel), was used as loading control, which is located at approx. 130 kDa and also appeared at the pDT-PGK MMY24 strain without receptor. The hA_{2B} receptor antibody recognized two bands (bottom panel) at 55 kDa and 34 kDa. The percentage expression level of all mutants was determined from the densities of the two specific bands and the loading control ($n = 3$, Table 1). MMY24 carrying wild-type receptor was set as 100% and MMY24 carrying the empty vector pDT-PGK without receptor was set as 0%. It should be mentioned here that these experiments reveal total receptor expression, and do not specify the location of the receptor, i.e. membrane-bound and/or intracellular.

The R293$^{7.56}$A mutant was not expressed at all, while N286$^{7.49}$A was only marginally expressed, to 15% of control. N286$^{7.49}$R,Y292$^{7.55}$A and Y299$^{8.52}$A also had significantly decreased expression levels, approx. 50% of wild-type receptor. Many mutants had expression levels not significantly different from wild-type receptor (N286$^{7.49}$Q, P287$^{7.50}$A, I288$^{7.51}$A, Y290$^{7.53}$A, Y290$^{7.53}$F, Y290$^{7.53}$N, N294$^{8.47}$A, N294$^{8.47}$I, R295$^{8.48}$A, D296$^{8.49}$A, F297$^{8.50}$A, F297$^{8.50}$Y, R298$^{8.51}$A, T300$^{8.53}$A, F301$^{8.54}$A, H302$^{8.55}$A, K303$^{8.56}$A, I304$^{8.57}$A, I305$^{8.58}$A, S306$^{8.59}$A and R307$^{8.60}$A). V289$^{7.52}$A and Y290$^{7.53}$F/F297$^{8.50}$Y showed significantly higher expression of the receptor.

[^{3}H]PSB-603 antagonist binding to wild-type receptor and all mutants

In Table 1 the percentage of specific [^{3}H]PSB-603 binding to every single mutant receptor is compared to the wild-type receptor (100%). Five mutants, N286$^{7.49}$Q, P287$^{7.50}$A, R293$^{7.56}$A, I304$^{8.57}$A and I305$^{8.58}$A lost antagonist binding completely. Four mutants, N286$^{7.49}$A, Y292$^{7.55}$A, T300$^{8.53}$A and F301$^{8.54}$A, showed slightly but not significantly higher antagonist binding than wild-type. Two mutants, R295$^{8.48}$A and Y299$^{8.52}$A, showed similar antagonist binding compared to wild-type. A majority of mutants showed significant decreases in antagonist binding, below 50% compared to wild-type receptor. They are I288$^{7.51}$A, V289$^{7.52}$A, Y290$^{7.53}$A, Y290$^{7.53}$F, Y290$^{7.53}$N, F297$^{8.50}$A, Y290$^{7.53}$F/F297$^{8.50}$Y, N294$^{8.47}$A, N294$^{8.47}$I, D296$^{8.49}$A, R298$^{8.51}$A, K303$^{8.56}$A, S306$^{8.59}$A and R307$^{8.60}$A. Three other mutants, N286$^{7.49}$R, F297$^{8.50}$Y and H302$^{8.55}$A, also showed lower antagonist binding compared to wild-type, but without significant differences.

Table 1 Characterization of the NPxxY(x)$_6$F motif and helix 8 mutations of the adenosine hA_{2B} receptor in the strain MMY24 ($G_{\alpha i3}$). Parameters of the NECA concentration-effect curves generated from four independent solid growth assays (n = 4), each performed in triplicate.

	Yeast solid assay			Radioligand binding assay	Western blot
	Emax (%)	EC_{50} (nM)	Fold EC_{50}	%[^{3}H]PSB-603 binding	% expression
WT_A_{2B}	100	141 ± 79		100 ± 11	100 ± 5
pDT-PGK	-	-	-	0	0
NPxxY(x)$_6$F					
N286$^{7.49}$A	58 ± 15	22557 ± 15293***	160	120 ± 14	15 ± 3***
N286$^{7.49}$Q	-	-	-	0	110 ± 23
N286$^{7.49}$R	58 ± 13	789 ± 671*	5.6	79 ± 16	68 ± 10*
P287$^{7.50}$A	-	-	-	0	143 ± 17
I288$^{7.51}$A	93 ± 6	193 ± 93	1.4	23 ± 9**	127 ± 10
V289$^{7.52}$A	44 ± 8	376 ± 239*	2.7	17 ± 7***	144 ± 15*
Y290$^{7.53}$A	-	-	-	17 ± 4**	93 ± 7
Y290$^{7.53}$F	63 ± 12	4197 ± 3760**	30	14 ± 9**	102 ± 9
Y290$^{7.53}$N	-	-	-	16 ± 10**	83 ± 14
F297$^{8.50}$A	46 ± 9	803 ± 188***	5.7	19 ± 8**	111 ± 5
F297$^{8.50}$Y	90 ± 10	392 ± 81***	2.8	88 ± 22	97 ± 1
Y290$^{7.53}$F/F297$^{8.50}$Y	39 ± 16	18170 ± 16357**	129	15 ± 10**	173 ± 12*
Helix 8					
Y292$^{7.55}$A	75 ± 13	679 ± 201***	4.8	122 ± 7	46 ± 14*
R293$^{7.56}$A	-	-	-	0	0
N294$^{8.47}$A	78 ± 4	449 ± 179***	3.2	24 ± 11**	122 ± 32
N294$^{8.47}$I	94 ± 11	251 ± 125	1.8	49 ± 15*	165 ± 30
R295$^{8.48}$A	86 ± 23	236 ± 151	1.7	100 ± 12	197 ± 54
D296$^{8.49}$A	94 ± 10	60 ± 31	0.4	44 ± 10**	116 ± 17
R298$^{8.51}$A	81 ± 17	1573 ± 765***	11	34 ± 12**	48 ± 18
Y299$^{8.52}$A	78 ± 11	87 ± 8	0.6	104 ± 19	54 ± 10*
T300$^{8.53}$A	83 ± 11	216 ± 63	1.5	122 ± 5	65 ± 26
F301$^{8.54}$A	73 ± 13	51 ± 8	0.4	144 ± 32	101 ± 15
H302$^{8.55}$A	81 ± 5	761 ± 321***	5.4	62 ± 13	77 ± 29
K303$^{8.56}$A	95 ± 7	403 ± 162**	2.9	45 ± 12*	99 ± 29
I304$^{8.57}$A	-	-	-	0	171 ± 36
I305$^{8.58}$A	87 ± 11	293 ± 106*	2.1	0	134 ± 31
S306$^{8.59}$A	74 ± 11	281 ± 82*	2	49 ± 14*	98 ± 27
R307$^{8.60}$A	63 ± 13	2932 ± 1776***	21	21 ± 7**	108 ± 46

Percentage maximal activity (% Emax) compared to the wild-type hA_{2B} receptor represents the intrinsic activity of the receptor. EC_{50} is the potency of the agonist NECA. The fold EC_{50} was calculated by dividing the EC_{50} of the mutant receptor by the EC_{50} of the wild-type receptor. " – " : Emax < 5%, EC_{50} and fold EC_{50} could not be determined. % [^{3}H]PSB-603 binding represents specific [^{3}H]PSB-603 binding to all mutants compared to the wild-type receptor (100%, n = 3 or 4). % expression level of all mutants was determined from the density of the two specific bands and that of the loading control (wild-type = 100%, n = 3). P-values were calculated by a two-tailed homoscedastic Student's t-test; significance is indicated as follows: *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. The superscript numbers of residues are according to Ballesteros-Weinstein numbering[54].

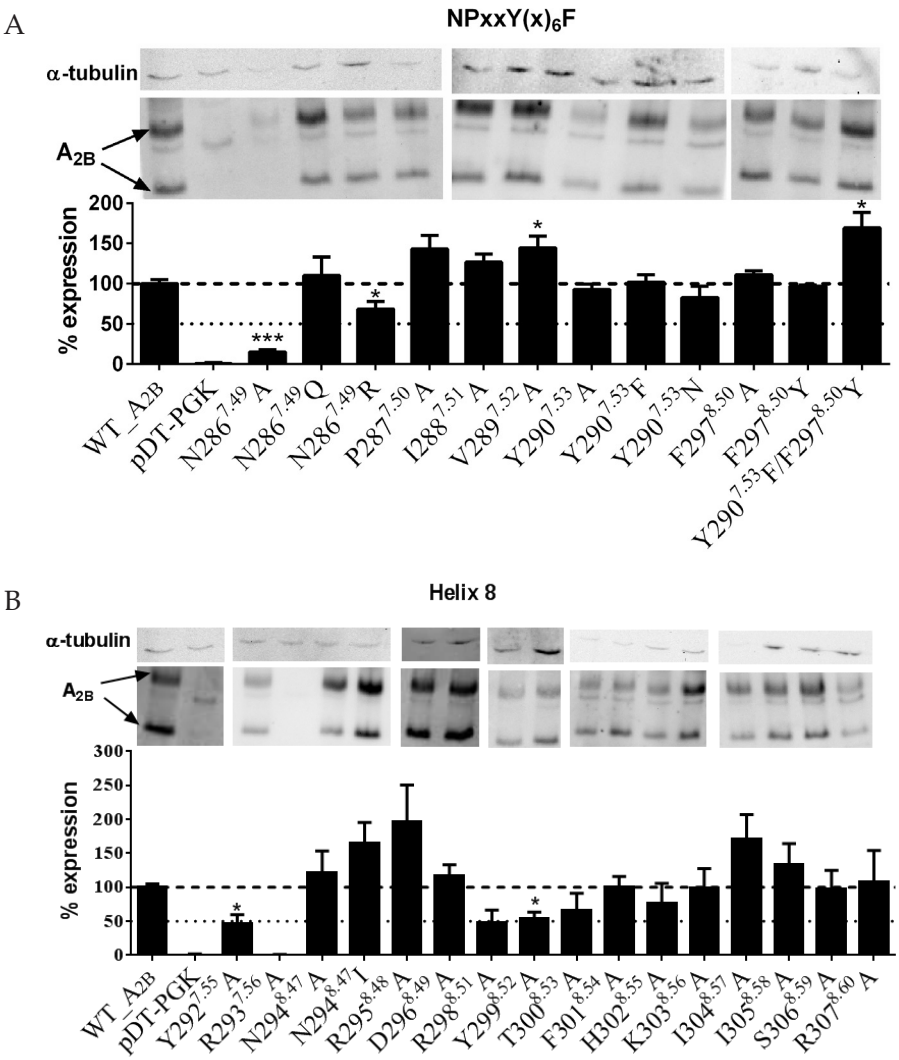

Fig. 4. Western blot analysis of the hA_{2B} wild-type receptor, pDT-PGK without any receptor, the $NPxxY(x)_6F$ mutations (A) and the helix 8 mutations (B). Yeast reference protein α-tubulin (top panel) was used as loading control, which is located at approx. 130 kDa and also appeared at pDT-PGK without receptor. The hA_{2B} receptor specific bands (bottom panel) are at 55 kDa and 34 kDa. All bands shown from a representative experiment. % expression level of all mutants in the bar graphs was determined between the density of the two specific bands and the loading control (n = 3). MMY24 carrying wild-type receptor was set as 100% and MMY24 carrying the empty vector pDT-PGK without receptor was set as 0%.

Discussion

Our previous research has shown that the single-GPCR-one-G protein yeast screening system is very well suited to study a G protein-coupled receptor and its G protein preference[26, 34]. The adenosine hA_{2B} receptor and its mutations were expressed and activated in a panel of eight yeast *S. cerevisiae* strains, covering the four major classes (corresponding to the replaced last five C-terminal residues of the mammalian G_{α} subunit): $G_{\alpha i}$, $G_{\alpha s}$, $G_{\alpha q}$ and $G_{\alpha 12}$[34]. In the current study we have identified the function of each residue of the NPxxY motif and the whole helix 8 in human A_{2B} receptor activation, as derived from the concentration–response curves of the reference agonist NECA on all mutations in a functional yeast growth assay. Firstly, our data confirmed this single-GPCR-one-G protein system is well suitable for studying GPCR activation with many advantageous features; it is cheap, fast, stable, robust and cell handling is easy. Most important, however, is that there is no (other) GPCR background and that the yeast strain has a specifically designed G protein pathway. Secondly, we found four key residues, $P287^{7.50}$, $Y290^{7.53}$, $R293^{7.56}$ and $I304^{8.57}$ that are essential in receptor activation. Thirdly, other amino acid residues were identified as quite crucial too: $N286^{7.49}$, $V289^{7.52}$, $Y292^{7.55}$, $N294^{8.47}$, $F297^{8.50}$, $R298^{8.51}$, $H302^{8.55}$ and $R307^{8.60}$. In the following paragraphs we will discuss the mechanisms of receptor activation in more detail using several sources with bio-informatics information and compare our findings with previous research. The conservation of each residue of the NPxxY motif and the whole helix 8 was compared across all adenosine receptors (Figure 3A), the nucleotide-like subfamily (Fig. 3B), and the entire class A (rhodopsin-like) family of GPCRs (Fig. 3C) and also compared within the A_{2B} receptor between different species (Fig. 3D).

NPxxY motif

The five amino acids NPxxY constitute a highly conserved motif across the rhodopsin-like GPCRs and are generally acknowledged as vital for G protein activation; the sequence motif N286$^{7.49}$P287$^{7.50}$I288$^{7.51}$V289$^{7.52}$Y290$^{7.53}$ is present in the human adenosine hA_{2B} receptor (Fig. 3C). N286$^{7.49}$, P287$^{7.50}$, and Y290$^{7.53}$ are all 100% conserved in the adenosine receptors. There are two key residues, P287$^{7.50}$ and Y290$^{7.53}$, since mutations at these positions showed a complete loss of function. N286$^{7.49}$ seems to be important too, but not as crucial as the two key residues P287$^{7.50}$ and Y290$^{7.53}$, as mutation N286$^{7.49}$A only showed a partial loss of function in both NECA efficacy and potency. However, I288$^{7.51}$A acted as the wild-type hA_{2B} receptor and V289$^{7.52}$A only showed a significant decrease in efficacy with a slight decrease in potency. These two mutants were well expressed, but showed lowered antagonist binding.

N286$^{7.49}$ and P287$^{7.50}$

When N286$^{7.49}$ was conservatively mutated to glutamine (Q), receptor activation was completely abolished. A recovery of function was observed when the asparagine was mutated to the positively charged arginine (R), which mutation showed 50% efficacy only and a 5.6-fold decrease in potency in comparison to the activity of the wild-type receptor (Table 1). The mutation to glutamine caused a complete loss of radiolabeled antagonist binding. It should be mentioned that a modest reduction in radioligand affinity may already lead to negligible binding in this assay. A receptor homology model based on the A_{2A} receptor crystal structure suggests that N$^{7.49}$ makes a stabilizing interaction with N$^{7.45}$[35]. The mutation N$^{7.49}$A in the human NK2 and CCR5 receptors showed a decrease of receptor activation as well[36, 37]. This might be explained by a possible interaction of N$^{7.49}$ with a conserved aspartate in helix 2, D52$^{2.50}$; Sealfon et al. found that swapping these two residues, D$^{2.50}$N/N$^{7.49}$D in the serotonin 5-HT_{2A} receptor restored receptor function while the D$^{2.50}$N mutation alone eliminated activation[38]. From our own results we hypothesize N286$^{7.49}$R would also be able to have this interaction with D$^{2.50}$, whereas N286$^{7.49}$Q would not keep this interaction anymore.

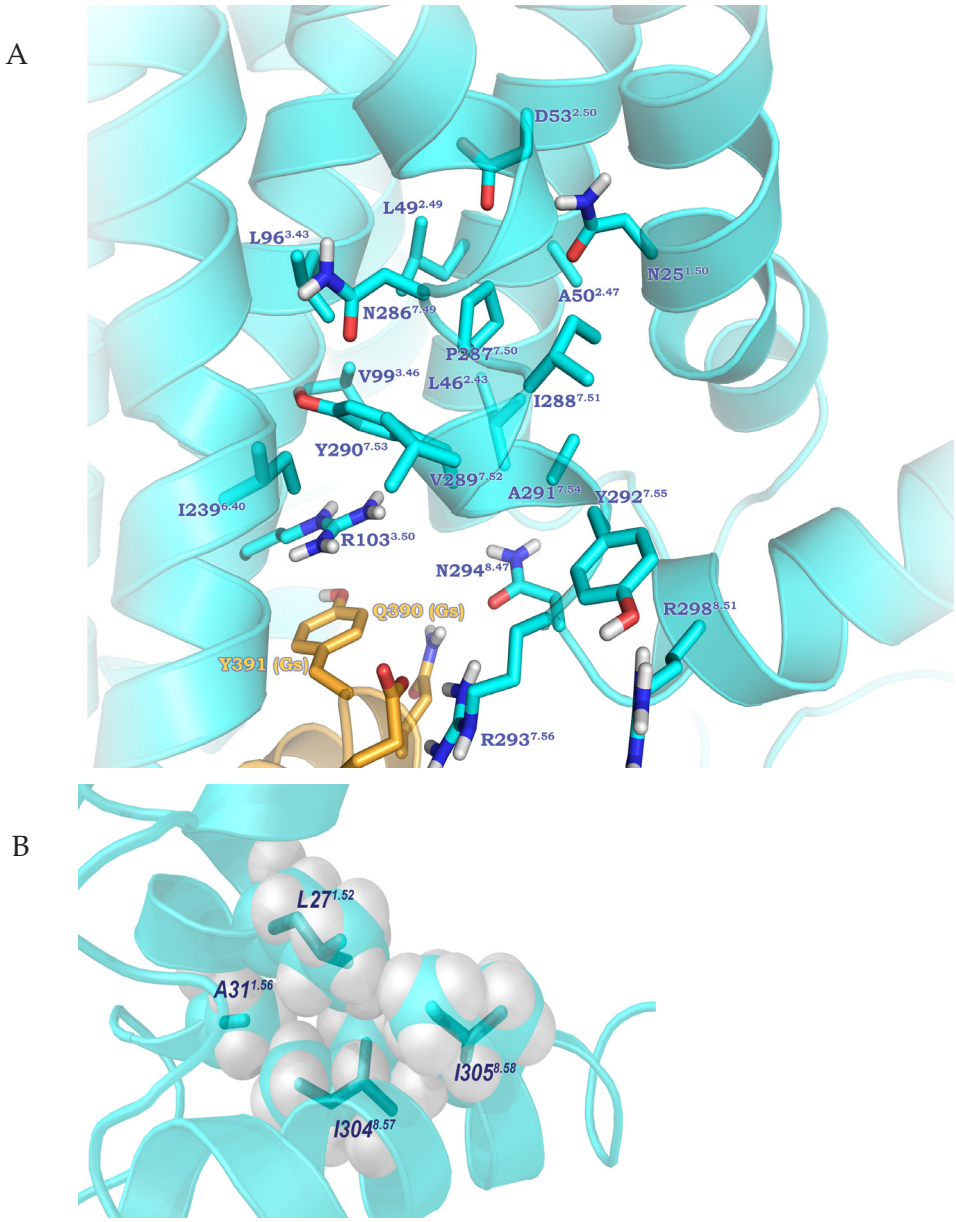

Fig. 5. The chimera/homology model of the adenosine A_{2B} receptor based on both the β_2 adrenergic receptor/G_s complex crystal structure (PDB: 3SN6) and the crystal structure of the adenosine A_{2A} receptor co-crystallized with the agonist NECA (PDB: 2YDV). (A) Residues within 4 Å of P287$^{7.50}$, Y290$^{7.53}$ and R293$^{7.56}$ are visualized. Possible interactions of residues are shown; R103$^{3.50}$ is interacting (π-cation interaction) withY391 (G_s)/Y290$^{7.53}$, R293$^{7.56}$ is interacting with E392 (G_s) and the backbone of Q390 (G_s), and N294$^{8.47}$ interacts with Q390 (G_s). (B) The interactions of loss-of-activation residue I304$^{8.57}$ and neighboring residue I305$^{8.58}$ are shown. I304$^{8.57}$ seems to be "hydrophobically locked" between residues L27$^{1.52}$ and A31$^{1.56}$, while I305$^{8.58}$ is more exposed.

The very high degree of conservation of amino acid P287$^{7.50}$ implies that it could be essential for GPCR activation, since the P287$^{7.50}$A mutation showed a complete loss of function, accompanied by a loss of antagonist binding, while receptor expression was unaffected (Table 1). Indeed, mutation of this amino acid to alanine not only abolished receptor activation in our research but also in the rat M3 muscarinic receptor[39]. However, Barak et al. found that mutation P$^{7.50}$A only reduced but not abolished agonist-mediated phosphorylation in the human β_2 adrenergic receptor; instead, they found that mutation of N$^{7.49}$A abrogated receptor phosphorylation[40]. In the rat type 1 angiotensin II receptor this was also the case: the mutation N$^{7.49}$A severely impaired receptor function, whereas P$^{7.50}$A only reduced receptor function[41]. These findings indicate that it is receptor specific whether N$^{7.49}$ or P$^{7.50}$ is essential for receptor activation; however, both seem to be important and working in close control of each other.

Y290$^{7.53}$ and F297$^{8.50}$

From Table 1 it follows that the mutations Y290$^{7.53}$A and Y290$^{7.53}$N both completely abolished activation of the receptor. The mutant Y290$^{7.53}$F showed a partial loss of function. All three mutants displayed normal receptor expression and reduced antagonist binding. This suggests that an aromatic side chain as in tyrosine and phenylalanine at this position is essential for activation. It also indicates that the hydroxyl group in tyrosine is very important in hA_{2B} receptor activation. The homology model suggests that Y290$^{7.53}$ is interacting with the conserved R103$^{3.50}$ of the DRY motif (Fig. 5A). Y$^{7.53}$ has been investigated and mutated in numerous GPCRs. The mutation Y$^{7.53}$A abolished agonist-mediated internalization, but did not influence its ability to maximally stimulate adenylyl cyclase in the human β_2 adrenergic receptor, Y$^{7.53}$F only slightly reduced this ability[42, 43]. In the human type 1 angiotensin II receptor mutation of this amino acid to either alanine or phenylalanine led to comparably and heavily impaired signaling[44]. Le Gouill et al. found that the Y$^{7.53}$A mutation in the human platelet-activating factor receptor abolished G protein coupling but not internalization, whereas the Y$^{7.53}$F mutation did not have a significant effect on either process[45]. In the murine GnRH receptor, the mutation Y$^{7.53}$A led to abrogation of inositol

phosphate production, substitution with phenylalanine resulted in normal G protein activation[46].

The mutation F297$^{8.50}$A showed a partial loss of function while the mutation F297$^{8.50}$Y behaved similarly to wild-type receptor. It has been postulated that an aromatic interaction, or pi/pi stacking, occurs between the Y290$^{7.53}$ and F297$^{8.50}$ residues, stabilizing the receptor in an inactive state[47]. However, this cannot be the only reason in view of our finding that the double mutation, Y290$^{7.53}$F/F297$^{8.50}$Y, showed a greater combined loss of function than both single mutations. Fritze et al. reported on the same double mutation in rhodopsin. The interaction between the two residues played an important role during the change from inactive to active state[47].

Key residues of helix 8: R293$^{7.56}$ and I304$^{8.57}$

Helix 8 is increasingly recognized as having a large role in assisting the receptor in G protein coupling[4]. In this study all amino acids of helix 8 in the adenosine A_{2B} receptor were mutated one by one to alanine to investigate their role in G protein activation. Two residues, R293$^{7.56}$ and I304$^{8.57}$, showed a complete loss of receptor activation. However, similar key residues had different locations in other receptors, such as in the M1 muscarinic acetylcholine receptor, where F425$^{8.50}$, R426$^{8.51}$, T428$^{8.53}$ and L432$^{8.57}$ were found important for G protein binding and signaling[5]; in the cannabinoid receptor 1 these were hydrophobic residues L404$^{8.50}$, F408$^{8.54}$, F412$^{8.58}$; in the β_1 adrenergic receptor these were D382$^{8.49}$ and R384$^{8.51}$ within a hydrophilic interface, possibly serving as a tethering site for the G protein[9]; in the type 1 angiotensin receptor Y312$^{8.53}$, F313$^{8.54}$ and L314$^{8.55}$ were identified as such[8].

R293$^{7.56}$

The mutation R293$^{7.56}$A showed a complete loss of function (Table 1), accompanied by a similar lack of receptor expression and antagonist binding. This makes it difficult to draw unambiguous conclusions. From the homology model provided (Fig. 5A) it is apparent that R293$^{7.56}$ has an interaction with the C terminal tail (QYELL^{COOH}) of the $G_{\alpha s}$ subunit, involved in activation of the adenosine hA_{2B}

receptor. However, the homology model is based on the β_2-adrenergic receptor–G_s complex crystal structure; the MMY24 cell line expresses the $G_{\alpha i3}$ subunit which has a different C terminal tail (ECGLY^{COOH}). The glutamic acid (E), with which R293$^{7.56}$ interacts, is present in both tails although at a different position, which might make this residue essential for G protein coupling. R$^{7.56}$ is highly conserved across the adenosine receptor family; the only other amino acid present is lysine, which is a large, basic, charged hydrophilic residue as well. The conservation of this amino acid across the nucleotide-like subfamily (Fig. 3B) and class A (rhodopsin-like) family (Fig. 3C) is rather low, however. This sequence element is the connection between TM7 and helix 8, which apparently is quite flexible, because it is missing in many receptors whereas some have an extra residue here (Fig. 3C). R$^{7.56}$ is also highly conserved in the A_{2B} receptor across different species (Fig. 3D). However, these results and explanations imply that the involvement of this amino acid residue R/K293$^{7.56}$ in G protein coupling may be quite specific for the adenosine receptors.

I304$^{8.57}$ and I305$^{8.58}$

The mutation I304$^{8.57}$A showed a complete loss of function, had no antagonist binding, but was very well expressed (Table 1). This I304$^{8.57}$ is present in all adenosine receptors. It has the highest rate of conservation across the nucleotide-like receptor subfamily and comes second in the entire class A (rhodopsin-like) family (Fig. 3). In contrast, mutation of L$^{8.57}$A in the human β_2 adrenergic receptor had no influence on expression, ligand binding, G protein coupling or adenylyl cyclase activity; only agonist-mediated receptor sequestration was markedly impaired[48]. Our homology model predicts that I304$^{8.57}$ is hydrophobically locked between residues L27$^{1.52}$ and A31$^{1.56}$ (Fig. 5B). This hydrophobic lock might stabilize the position of helix 8 resulting in a loss of function when this lock is broken. It is remarkable that mutation I305$^{8.58}$A, which is in close proximity of I304$^{8.57}$, showed no severe loss of function (Table 1). The reason for this might be that I305$^{8.58}$ is more exposed to the cytoplasm as opposed to I304$^{8.57}$. This isoleucine is less conserved (50%) in adenosine receptors than I304$^{8.57}$ (100%) and also less conserved in the nucleotide-like receptor subfamily and class A

(rhodopsin-like) family. The most conserved residue at both positions is leucine; however, there is a higher degree of conservation at position 8.58 (40%) than at position 8.57 (26%) (Fig. 3). This finding implies hydrophobic residues at position 8.57 and 8.58 are very common in many GPCRs, in which $I304^{8.57}$ is a key residue for hA_{2B} receptor activation.

Other residues

Five residues, $Y292^{7.55}$, $N294^{8.47}$, $R298^{8.51}$, $H302^{8.55}$ and $R307^{8.60}$ were also shown to be of high importance in receptor activation, while showing substantial receptor expression and antagonist binding. The substitution of these residues for alanine caused a significant decrease of NECA's potency ($p < 0.001$) and a reduction of its efficacy.

$R298^{8.51}$ and $R307^{8.60}$ significantly influenced the potency of NECA (EC_{50} fold > 10-fold upon mutation). $R^{8.51}$ is the second most conserved residue, next to the most conserved residue $F^{8.50}$ of helix 8 across the class A (rhodopsin-like) receptor family (Fig. 3C). However, $R307^{8.60}$ is not that conserved; this position shows different residues across the adenosine receptors, even more so across the nucleotide-like receptor subfamily and class A (rhodopsin-like) receptor family. This position is at the end of helix 8, and it appears to have very different functions in different receptors. In the adenosine A_{2A} receptor a serine is present at position 8.60; mutation $S^{8.60}A$ in the canine adenosine A_{2A} receptor showed a decrease of potency but not efficacy of adenylyl cyclase stimulation[49]. In the human dopamine D1 receptor mutation $C^{8.60}A$ had no effect on the stimulation of adenylyl cyclase[50]. Mutation $Y^{8.60}A$ in the rat type 1 angiotensin II receptor resulted in a reduction in internalization rate[51].

The neighboring residues of key amino acids $R293^{7.56}$ and $I304^{8.57}$ are very important as well, such as $Y292^{7.55}$ and $N294^{8.47}$ (neighbors of $R293^{7.56}$). Residue $Y292^{7.55}$ is highly conserved across the adenosine receptors, the other residue being the closely related phenylalanine (Fig. 3A). The tyrosine is not very conserved, but a phenylalanine is across the nucleotide-like receptor subfamily (Fig. 3B) and the class A rhodopsin-like receptor family (Fig. 3C). This may imply that an aromatic group at this position is important. Since this residue is

located in the linking sequence segment that connects TM7 and helix 8, it might be that an aromatic ring at this position is essential for a correct arrangement of these two helices with respect to each other. Secondly, it may team up with key residue R293$^{7.56}$ in receptor activation. The adenosine A_{2B} receptor carries an asparagine (N) at the 8.47 position and is unique with respect to the other adenosine receptors, which all have an isoleucine (I) at this position. Across the nucleotide-like receptor subfamily (Fig. 3B) and the class A rhodopsin-like receptor family (Fig. 3C) N$^{8.47}$ is more conserved than I$^{8.47}$ (Fig. 3B and C). However, the N$^{8.47}$I mutation did not cause significant decreases in both efficacy and potency of the agonist NECA compared to the wild-type receptor, less so than the N$^{8.47}$A mutation. These results imply that there is no apparent difference between N294$^{8.47}$ at the A_{2B} receptor or I294$^{8.47}$ in other receptors. Mutation of this amino acid in bovine rhodopsin had no effect on the GDP/GTP exchange within the G protein[52].

The conservation of H302$^{8.55}$ is low across the adenosine receptors (25%), the nucleotide-like receptor subfamily (3%) and the class A rhodopsin-like receptor family (2%). However, conservation of another basic amino acid, arginine, is the highest across the nucleotide-like receptor subfamily and the second highest across the entire Class A receptor family, in which lysine is the highest. So a charged, basic residue is common at this position. Leucine is often found as well; it is the third most conserved residue across all Class A GPCRs and accounts for half of the adenosine receptor family (Fig. 3). Sano et al. reported that L314$^{8.55}$A, together with Y312$^{8.53}$ and F313$^{8.54}$ in the cytosolic carboxyl-terminal region of the rat type 1 angiotensin II receptor is essential for coupling and activation, since mutations on these three positions resulted in a marked reduction in GTPγS effects on Ang II binding and inositol trisphosphate production[53].

In conclusion, the alanine scan of the NPxxY motif and the adjacent helix 8 of the adenosine A_{2B} receptor we performed in the present study identified a number of positions vital for receptor activation, some of which unknown at other receptors. In some instances we provided additional data by substituting alanine for other amino acids, mostly triggered by their presence in other

GPCRs. The latter approach could be brought further by, e.g., analyzing more conservative mutations in which the physicochemical nature of the substituted amino acid is taken into account. Homology modeling of the receptor and visualizing the likely interactions between amino acids in G protein and receptor provided insight into the putative molecular mechanisms for the deterioration of receptor activation.

Acknowledgements

Rongfang Liu thanks the China Scholarship Council (CSC) for her PhD scholarship. NWO provided a TOP grant to A.P. IJ. (714.011.001). Qilan Li made some of the receptor mutants. The authors are grateful to Prof C.E. Mueller of Bonn University (Germany) for the generous gift of [^{3}H]PSB-603 and to Dr. S. Dowell from GSK (Stevenage, UK) for providing the plasmids, the yeast strains and experimental protocols.

References

[1] Venkatakrishnan, A., et al. *Molecular signatures of G-protein-coupled receptors.* Nature (2013) 494: 185–194.

[2] Oldham, W.M., and Hamm, H.E. *Heterotrimeric G protein activation by G-protein-coupled receptors.* Nat. Rev. Mol. Cell Biol. (2008) 9: 60–71.

[3] O'Callaghan, K., et al. *Turning receptors on and off with intracellular pepducins: new insights into G-protein-coupled receptor drug development.* J. Biol. Chem. (2012) 287: 12787–12796.

[4] Oldham, W.M., et al. *Mechanism of the receptor-catalyzed activation of heterotrimeric G proteins.* Nat. Struct. Mol.Biol. (2006) 13: 772–777.

[5] Kaye, R.G., et al. *Helix 8 of the M1 muscarinic acetylcholine receptor: scanning mutagenesis delineates a G protein recognition site.* Mol. Pharmacol. (2011) 79: 701–709.

[6] Okuno, T., et al. *Leukotriene B4 receptor and the function of its helix 8.* J. Biol. Chem. (2005) 280: 32049–32052.

[7] Ahn, K.H., et al. *Hydrophobic residues in helix 8 of cannabinoid receptor 1 are critical for structural and functional properties.* Biochemistry (2009) 49: 502–511.

[8] Huynh, J., et al. *Role of helix 8 in G protein-coupled receptors based on structure–function studies on the type 1 angiotensin receptor.* Mol. Cell. Endocrinol. (2009) 302: 118–127.

[9] Santos, N.M.D., et al. *Characterization of the residues in helix 8 of the human β_1-adrenergic*

receptor that are involved in coupling the receptor to G proteins. J. Biol. Chem. (2006) 281: 12896–12907.

[10] Fredholm, B.B., et al. *Structure and function of adenosine receptors and their genes.* Naunyn-Schmiedeberg's Arch. Pharmacol. (2000) 362: 364–374.

[11] Fredholm, B.B., et al. *Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. Biochem. Pharmacol.* (2001) 61: 443–448.

[12] Wei, W., et al. *Blocking A_{2B} adenosine receptor alleviates pathogenesis of experimental autoimmune encephalomyelitis via inhibition of IL-6 production and Th17 differentiation.* J. Immunol. (2013) 190: 138–146.

[13] Wei, Q., et al. A. *A_{2B} adenosine receptor blockade inhibits growth of prostate cancer cells.* Purinergic Signal. (2013) 9: 271–280.

[14] Stagg, J., et al. *Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis.* Proc. Natl. Acad. Sci. U.S.A. (2010) 107: 1547–1552.

[15] Owen, S. J., Massa, H.H., and Rose'Meyer, R.B. *Loss of adenosine A_{2B} receptor mediated relaxant responses in the aged female rat bladder; effects of dietary phytoestrogens.* Naunyn-Schmiedeberg's Arch. Pharmacol. (2012) 385: 539–549.

[16] Cekic, C., et al. *Adenosine A_{2B} receptor blockade slows growth of bladder and breast tumors.* J. Immunol. (2012) 188: 198–205.

[17] Koscsó, B., et al. *Stimulation of A_{2B}adenosine receptors protects against trauma–hemorrhagic shock-induced lung injury.* Purinergic Signal. (2013) 9: 427–432.

[18] Bot, I., et al. *Adenosine A_{2B} receptor agonism inhibits neointimal lesion development after arterial injury in apolipoprotein E–deficient mice. Arterioscler.* Thromb. Vasc. Biol. (2012) 32: 2197–2205.

[19] Dowell, S.J., and Brown, A.J. *Yeast assays for G-protein-coupled receptors.* Receptors Channels (2002) 8: 343–352.

[20] Dowell, S.J., and Brown, A. J. *Yeast assays for G protein-coupled receptors.* Methods Mol. Biol. (2009) 552: 213–229.

[21] Stewart, G.D., et al. *Determination of adenosine A_1 receptor agonist and antagonist pharmacology using Saccharomyces cerevisiae: implications for ligand screening and functional selectivity.* J. Pharmacol. Exp. Ther. (2009) 331: 277–286.

[22] Peeters, M.C., et al. *Domains for activation and inactivation in G protein-coupled receptors–A mutational analysis of constitutive activity of the adenosine A_{2B} receptor.* Biochem. Pharmacol. (2014) 92: 348–357.

[23] Weston, C., et al. *Investigating G protein signaling bias at the glucagon-like peptide-1 receptor in yeast.* Br. J. Pharmacol. (2014) 171: 3651–3665.

[24] Gietz, D., et al. *Improved method for high efficiency transformation of intact yeast cells.* Nucleic Acids Res. (1992) 20: 1425.

[25] Peeters, M.C., et al. *GPCR structure and activation: an essential role for the first extracellular loop in activating the adenosine A_{2B} receptor.* FASEB J. (2011) 25: 632–643.

[26] Liu, R., et al. *A yeast screening method to decipher the interaction between the adenosine A_{2B}*

receptor and the C-terminus of different G protein α-subunits. Purinergic Signal. (2014) 10: 441–453.

[27] Isberg, V., et al. *GPCRDB: an information system for G protein-coupled receptors.* Nucleic Acids Res. (2014) 42: D422–D425.

[28] Crooks, G.E., et al. *WebLogo: a sequence logo generator. Genome Res.* (2004) 14: 1188–1190.

[29] **Schrödinger Release 2014-2**: Maestro v, (2014), Schrödinger, LLC, New York, NY.

[30] **Schrödinger Release 2014-2**: Prime v, (2014), Schrödinger, LLC, New York, NY.

[31] Jacobson, M.P., et al. *On the role of crystal packing forces in determining protein sidechain conformations.* J. Mol. Biol. (2002) 320: 597–608.

[32] Jacobson, M.P., et al. *A hierarchical approach to all-atom protein loop prediction.* Proteins: Struct. Funct. Bioinform. (2004) 55: 351–367.

[33] Peeters, M., et al. *The role of the second and third extracellular loops of the adenosine A_1 receptor in activation and allosteric modulation.* Biochem. Pharmacol. (2012) 84: 76–87.

[34] Peeters, M.C., et al. *Three "hotspots" important for adenosine A_{2B} receptor activation: a mutational analysis of transmembrane domains 4 and 5 and the second extracellular loop.* Purinergic Signal. (2012) 8: 23–38.

[35] Gutiérrez-de-Terán, H., et al. *The role of a sodium ion binding aite in the allosteric modulation of the A_{2A} adenosine G protein-coupled receptor.* Structure (2013) 21: 2175–2185.

[36] Donnelly, D., et al. *Conserved polar residues in the transmembrane domain of the human tachykinin NK2 receptor: functional roles and structural implications.* Biochem. J. (1999) 339: 55–61.

[37] Dragic, T., et al. *A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5.* Proc. Natl. Acad. Sci. U.S.A. (2000) 97: 5639–5644.

[38] Sealfon, S.C., et al. *Related contribution of specific helix 2 and 7 residues to conformational activation of the serotonin 5-HT_{2A} receptor.* J. Biol. Chem. (1995) 270: 16683–16688.

[39] Wess, J., et al. *Functional role of proline and tryptophan residues highly conserved among G protein-coupled receptors studied by mutational analysis of the m3 muscarinic receptor.* EMBO J. (1993) 12: 331.

[40] Barak, L. S., et al. *The conserved seven-transmembrane sequence $NP(X)_{2,3}Y$ of the G-protein-coupled receptor superfamily regulates multiple properties of the β_2-adrenergic receptor.* Biochemistry (1995) 34: 15407–15414.

[41] Hunyady, L., et al. *A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the type 1 angiotensin II receptor.* J. Biol. Chem. (1995) 270: 16602–16609.

[42] Gabilondo, A.M., et al. *Mutations of Tyr326 in the β_2-adrenoceptor disrupt multiple receptor functions.* Eur. J. Pharmacol. (1996) 307: 243–250.

[43] Barak, L.S., et al. *A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated β_2-adrenergic receptor sequestration.* J. Biol. Chem. (1994) 269: 2790–2795.

[44] Laporte, S.A., et al. *The tyrosine within the NPX_nY motif of the human angiotensin II type 1 receptor is involved in mediating signal transduction but is not essential for internalization.* Mol. Pharmacol. (1996) 49: 89–95.

[45] Le Gouill, C., et al. *Structural and functional requirements for agonist-induced internalization of the human platelet-activating factor receptor.* J. Biol. Chem. (1997) 272: 21289–21295.

[46] Arora, K.K., Cheng, Z., and Catt, K.J. *Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor.* Mol. Endocrinol. (1996) 10: 979–986.

[47] Fritze, O., et al. *Role of the conserved $NPxxY(x)_{5,6}F$ motif in the rhodopsin ground state and during activation.* Proc. Natl. Acad. Sci. U.S.A. (2003) 100: 2290–2295.

[48] Gabilondo, A.M., et al. *A dileucine motif in the C terminus of the β_2-adrenergic receptor is involved in receptor internalization.* Proc. Natl. Acad. Sci. U.S.A. (1997) 94: 12285–12290.

[49] Palmer, T.M., Stiles, G.L. *Identification of an A2a adenosine receptor domain specifically responsible for mediating short-term desensitization.* Biochemistry (1997) 36: 832–838.

[50] Jin, H., et al. *Elimination of palmitoylation sites in the human dopamine D_1 receptor does not affect receptor-G protein interaction.* Eur. J. Pharmacol. (1997) 324: 109–116.

[51] Thomas, W.G., et al. *Angiotensin II receptor endocytosis involves two distinct regions of the cytoplasmic tail. A role for residues on the hydrophobic face of a putative amphipathic helix.* J. Biol. Chem. (1995) 270: 22153–22159.

[52] Osawa, S., and Weiss, E.R. *The carboxyl terminus of bovine rhodopsin is not required for G protein activation.* Mol. Pharmacol. (1994) 46: 1036–1040.

[53] Sano, T., et al. *A domain for G protein coupling in carboxyl-terminal tail of rat angiotensin II receptor type 1A.* J. Biol. Chem. (1997) 272: 23631–23636.

[54] Ballesteros, J.A., and Weinstein, H. *Integrated methods for the construction of three-dimensional models and computational probing of structure–function relations in G protein-coupled receptors.* Methods Neurosci. (1995) 25: 366–428.