Universiteit Leiden
The Netherlands

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

Peeters, M.C.

Citation
Peeters, M. C. (2011, November 17). *Activation of G protein-coupled receptors : the role of extracellular loops in adenosine receptors*. Retrieved from https://hdl.handle.net/1887/18092

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/18092

Note: To cite this publication please use the final published version (if applicable).

CHAPTER 7

GENERAL DISCUSSION, FUTURE PERSPECTIVES AND CONCLUSION

The research described in this thesis has provided new insights in the activation mechanism of class A GPCRs and in particular of adenosine receptors. By a variety of mutagenesis approaches and the use of a robust yeast reporter gene system, we identified several regions and amino acid positions that contribute to both agonist responses and constitutive activity of the human adenosine A_1 receptor and the human adenosine A_{2B} receptor. These results reveal new and surprising roles of the extracellular loops in the activation mechanism, greatly contributing to our notion of receptor activation.

In this chapter, I will compare and discuss the knowledge gathered in the separate chapters, providing a view of the results in a broader context.

CONSTITUTIVELY ACTIVE MUTANTS (CAMS) IN THE A_{2B}R

The level of constitutive or basal activity a receptor displays depends on the equilibrium between the inactive (R) and the active state (R*) and the energy required to transition between the two as was originally postulated in the two-state-receptor model [2]. Where it is essential for some signaling pathways to remain largely inactive until a specific time point, e.g. after hormone secretion (for the thyroid stimulating hormone receptor (TSHR)), other GPCRs need to exhibit a high level of activation to constantly keep the signaling pathway 'on', as is the case for the viral chemokine receptors but also for some neurotransmitter GPCRs like the opioid and cannabinoid receptors [3,4,5]. It is believed that all GPCRs, including orphan receptors, show some level of constitutive activity and that this basal activity is essential in normal receptor function [6]. Since constitutive activity is in fact the first step in the receptor activation mechanism, studying mutations that result in an increase or decrease in spontaneous activity can reveal much on how the receptor is activated and which residues are involved in this mechanism.

In Chapter 4, the results of a random mutagenesis study were described where we screened for constitutively active and gain-of-function mutations of the adenosine A_{2B} receptor. The mutated fragment used in this study encompassed transmembrane domains 4 and 5 (TM4, TM5) and the second extracellular loop (EL2), obtained by the two DNA restriction sites KpnI and BglII (**Figure 1**). Previously in our laboratory, the fragment upstream of the KpnI restriction site and the fragment downstream of the BglII restriction site were also submitted to a random mutagenesis screen. Many

of the mutations identified from these screens were published by Beukers et al. in 2004 [7]. The two mutated residues in the first extracellular loop (EL1) that were discussed in Chapter 3 were originally identified from the screen performed on the first part of the receptor, involving the N-terminus, transmembrane domains 1, 2, and 3 (TM1, TM2, TM3), the first and second intracellular loops (IL1, IL2) and the first extracellular loop (EL1) (**Figure 1**).

Now that we have finally screened the full receptor, we can assemble the various findings into a more general picture of which residues are involved in constitutive activity and which positions have a main function in silencing the $A_{2B}R$ in its basal state.

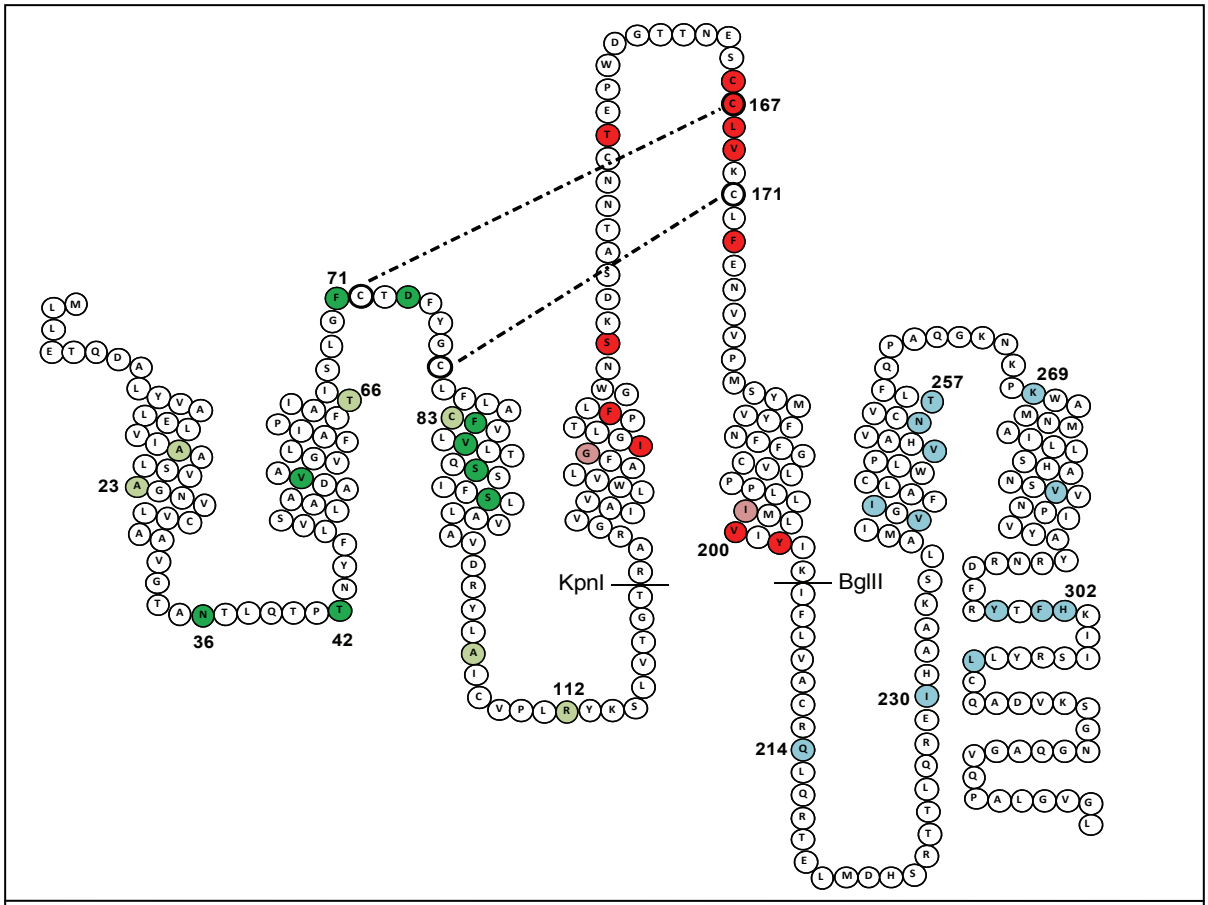


Figure 1. Snake plot of the adenosine $A_{2B}R$. Dotted lines indicate disulfide bridges. The disulfide bridge conserved in many class A GPCRs links C78 and C171. The non-conserved second disulfide bridge between EL1 and EL2, as present in the crystal structure of the adenosine $A_{2A}R$ (PDB: 3EML) and proposed to exist in the $A_{2B}R$ as well, links C72 and C167. The restriction sites *Kpn*I and *Bgl*II that were used to obtain the three fragments for random mutagenesis are in the second and third intracellular loop. Residues that were found mutated in the three separate CAM screens performed on the $A_{2B}R$ are shown in color. Bright red and green residues were found mutated in single and double mutant receptors; light green, red, and blue residues were identified in mutant receptors that contained more than 2 amino acid changes.

From the random mutagenesis screen performed on the first part of the receptor, 12 interesting mutant receptors were identified, 10 of which contained only one or two amino acid changes in the protein. As was discussed in Chapter 4, the screen of the middle fragment of the $A_{2B}R$ rendered 12 different mutant receptors. Of this selection, only one receptor contained three amino acid changes (G135A$^{4.55}$/I197L$^{5.53}$ /Y202N$^{5.58}$), the remaining mutant receptors were single or double mutants. The results of the screen performed on the last part of the receptor showed a different view. Firstly, only three mutant receptors could by identified that displayed constitutive activity. Secondly, all three mutant receptors contained multiple amino acid changes, one receptor even consisted of 7 mutations: Q214L^{IL3}/I230N$^{6.31}$ /V240M$^{6.41}$/V250M$^{6.51}$/N254Y$^{6.55}$/T257S$^{6.58}$/K269stop$^{7.32}$. In **Figure 1**, all the residues identified in the three separate screens are indicated in color; a brighter shade represents residues identified in single or double mutants and the lighter shade represents the residues identified in mutant receptors with more than three amino acid changes. Besides the clusters in TM4 , EL2, and TM5 described in Chapter 4, we now also observe similar series of residues exist in TM3 and TM6.

The relatively low amount of CAMs identified in the $A_{2B}R$ is consistent with the apparent necessity of the receptor to remain silent until high levels of adenosine are reached in stress events [8]. Activation of the receptor has been implicated in autoimmune diseases and inflammatory disorders, such as asthma [9].

Even though the technical set-up in all three screens was chosen such that both constitutively active mutants (CAMs) and gain-of-function mutants would be selected, all the identified mutant receptors displayed an increase in constitutive activity. The levels of constitutive activity of the mutant receptors ranged from a 1.5-fold change (F141L$^{4.61}$, N36D^{IL1}) compared to wild type receptor to an immense increase of 38-fold for mutant receptor G135A$^{4.55}$/I197L$^{5.53}$/Y202N$^{5.58}$.

A comparison between the inactive and active crystal structures of the $A_{2A}R$ suggest that the domains TM6 and TM7 undergo the largest conformational movement upon receptor activation [10,11,12] (**Figure 2**). These domains are clearly important in the activation mechanism and contain several switches needed for G protein activation in many class A GPCRs, such as W6.48 and Y7.53 [13,14]. In our CAM screen, we did identify a number of residues in TM6 that can result in an increase in both constitutive activity and potency when mutated.

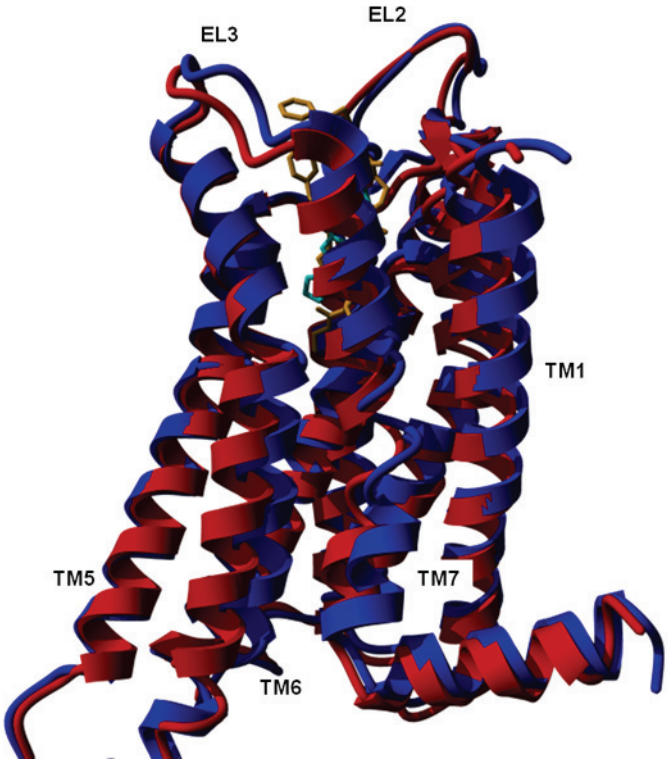


Figure 2. Overlay of the inactive (shown in blue, with the bound antagonist in cyan (PDB:3EML)) and the active structure (shown in red, with the bound agonist in orange (PDB:3QAK)) of the adenosine A_{2A} receptor. The largest movements are observed in TM6 that rotates outwards, and TM7 that shows an inwards movement. The figure was created by the software program YASARA [1].

Also, we identified another prominent switch in receptor activation: Y5.58 (Y197 in the A_{2A}R) at the bottom of TM5. The aromatic side chain displays a large rotameric shift upon activation. While in the inactive structure bound to ZM241385 the conserved $Y197^{5.58}$ is located in between TM3 and TM6, in the agonist-bound forms this residue moves outward allowing TM5 to shift toward TM6. As a result, the intracellular ends of TM5 and TM6 move closer together in the active structures compared to the inactive structure, enabling access of the G protein [10,11,12]. Besides Y5.58, that has been suggested to be involved in disturbing the ionic lock between TM3 and TM6, we did not identify any mutations at the proposed "general" GPCR activation switches such as the DRY-motif at the bottom of TM3 and the toggle-switch residue W6.48 [14].

Another important residue in our mutational analysis was F173 in EL2; mutation to a leucine yielded a large increase in both constitutive activity and potency for the agonist NECA. The corresponding position in the adenosine A_{2A} receptor (F168) is involved in both the binding of the A_{2A}R antagonist ZM241385 and the agonists UK-432097, NECA, and adenosine that were co-crystallized in the published X-ray structures [10,11,12]. Mutant receptor $Q214L^{IL3}$/$I230N^{6.31}$/$V240M^{6.41}$/$V250M^{6.51}$ /$N254Y^{6.55}$/$T257S^{6.58}$/$K269stop^{7.32}$ that was identified in the BglII-stop screen also contained three residues involved in agonist binding: $V250^{6.51}$, $N254^{6.55}$, $T257^{6.58}$ [7,12]. Besides these four residues, all positions identified from the CAM screens are located at distance from the adenosine binding site. This proves that (constitutive) activity of the receptor is not just determined by the ligand binding site or the

intracellular region where the G protein binds to the protein, but that many other regions of the receptor contribute to the activation mechanism. When a mutation disturbs these regions, e.g. by inducing more flexibility, the energy levels necessary for the transition between activation states are lowered, leading to an increased active receptor population reflected by a higher potency for the agonist and increased basal activation.

So far, only a limited number of natural variants have been identified in the $A_{2B}R$ that result in an amino acid change. In the GPCR Natural Variants database, mutations A35V$^{1.60}$, L96F$^{3.43}$, G137R$^{4.57}$, and K316R$^{C\text{-term}}$ are described [15]. None of these residues were identified in the random mutagenesis screens of the $A_{2B}R$.

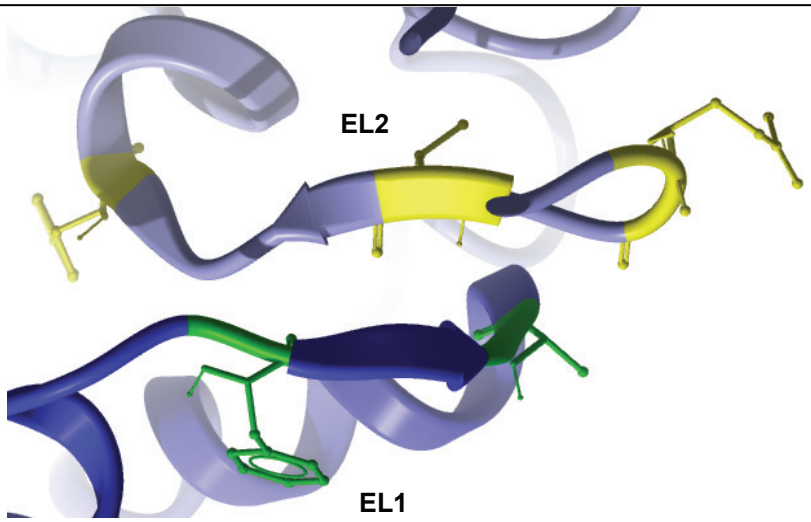
CAMs IN THE EXTRACELLULAR LOOPS

In both the random mutagenesis screen of the first fragment (ATG-KpnI) and the screen of the second fragment (TM4-EL2-TM5) of the $A_{2B}R$, CAMs were identified in the extracellular region (**Figure 1**). These mutations were not found in combination with mutations in the transmembrane domains, suggesting that the influence of EL2 on receptor activation is at a different level than that of the transmembrane domains.

No CAMs were identified in EL3, even though in the $A_{2A}R$ crystal structure this loop also seems to influence the shape of the ligand binding pocket of both ZM241385 and UK-432097 [10,11]. The structures of the $A_{2A}R$ bound to the much smaller NECA and adenosine do not show this involvement of EL3, indicating that its role is unique for larger ligands such as ZM241385 and UK-432097 [12].

The mutated residues identified in EL2 that cause constitutive activity, are located in a small cluster in a cysteine-rich region of the loop (see also Chapter 4). One of these cysteines (C167 in the $A_{2B}R$) is potentially involved in a non-conserved disulfide bridge with C72 in EL1 (indicated with a dotted line in **Figure 1**). The equivalent positions in the adenosine $A_{2A}R$ are able to form such an additional disulfide bridge as seen from the crystal structures of this receptor [10,11,12]. In these structures another structural feature connects EL1 and EL2; that is an anti-parallel β-sheet (shown with opposite arrows in **Figure 3**). When comparing the inactive structure of the $A_{2A}R$ bound to ZM241385 with the active structures bound to NECA and adenosine, we observe a movement of this region of ca. 2.5 Å parallel to the ligand

binding pocket, implicating the anti-parallel β-sheet in receptor activation. The two mutated residues identified in EL1 of the $A_{2B}R$, F71 and D74, are on either side of C72 and are located within the β-strand in EL1 (Chapter 3). In **Figure 3**, we have mapped our $A_{2B}R$ mutations onto the corresponding positions in the crystal structure of the $A_{2A}R$ according to the sequence alignment. This 3D perspective shows that residues F71 and D74 in EL1 further extend the CAM cluster identified in EL2.




```
EL1:
A2AR    66 ISTGFCAAC 74
A2BR    67 ISLGFCTDF 75

EL2:
A2AR   143 WN-------NCGQPKEGKNHSQGCGEGQVACLFEDVV 172
A2BR   144 WNSKDSATNNCTEPWDGTTNESCC---LVKCLFENVV 177
```

Figure 3. Top view of the adenosine $A_{2A}R$ structure showing the corresponding positions of the CAMs of the $A_{2B}R$ in EL1 (green) and EL2 (yellow) according to the multiple sequence alignment. The figure was created with Molsoft ICM version 3.6-1 h.
In the alignments of EL1 and EL2 of the $A_{2A}R$ and the $A_{2B}R$, the mutated positions of the $A_{2B}R$ are shown in red.

Also, the constitutively active mutants are centered around the anti-parallel β-sheet. Not only does this further confirm our hypothesis that a structural feature linking EL1 to EL2 exists in the $A_{2B}R$ as seen in the $A_{2A}R$ structure, but it also indicates an essential role for the anti-parallel β-sheet and the non-conserved disulfide bridge in $A_{2B}R$ activation. When these residues are mutated, they influence the interaction between the loops, hereby allowing the receptor to display an increase in constitutive activity. However, when the structural conformation is compromised too much, for instance by mutating the hydrophobic and large phenylalanine at position 71 to the small and hydrophilic glycine, activation is completely lost (Chapter 3).

CAMs vs CIMs

In Chapter 4, a random mutagenesis screen of the $A_{2B}R$ was described containing mutations in the fragment TM4-EL2-TM5, where mutant receptors were selected that displayed constitutive activity (CAMs) or increased potency to the agonist NECA. In Chapter 5, we made use of the same mutagenic library, but selected for a different phenotype, namely constitutively **in**active mutant receptors (CIMs). To our knowledge, we are the first to examine a GPCR for both activating and inactivating mutations using an unbiased random mutagenesis approach. We used the same random mutagenesis library and the same host yeast strain (MMY24) without any additional modifications, which allows for a direct comparison of CAMs and CIMs. This was unique too, as we were able to design a screening method using only slight changes in the selection procedure to discriminate between different activation phenotypes.

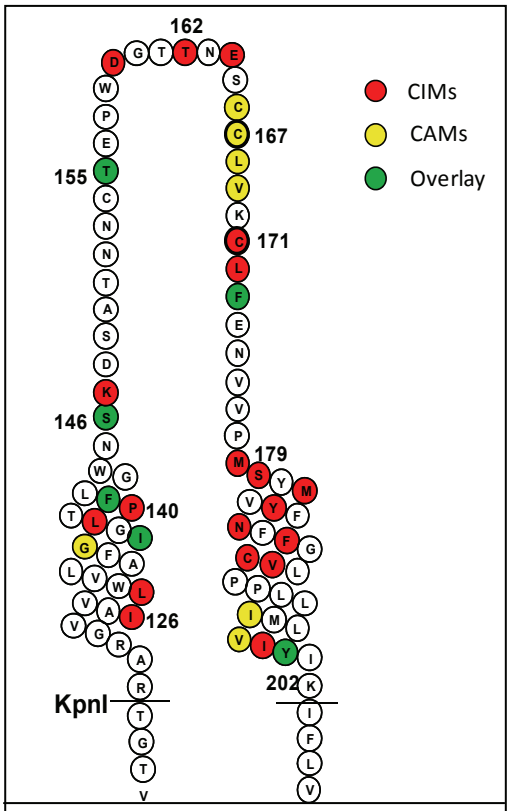


Figure 4. Snake plot view of the $A_{2B}R$ fragment (TM4-EL2-TM5) used in both the CIM screen and the CAM screen. In red all the residues identified from the CIM screen are indicated, in yellow the CAM residues are shown and the green residues were found in both screens.

The first observed difference between the CAM and the CIM screen are the number of mutated receptors identified. In the CAM screen we identified 11 different single and double mutant receptors and one triple mutant that fulfilled the requirements of the selection procedure. The CIM screen rendered a larger amount of mutant receptors, of which we chose the 22 single and double mutants to investigate further. Also, where in the CAM screen we did not identify any combinations of mutated residues in EL2 and either of the two transmembrane domains, several of those were found in the CIM screen. Especially mutations in TM5 were often combined with a mutation in EL2 within one mutant receptor. This was the case in six different mutant receptors, four of which contained a mutation of C190$^{5.46}$, indicating an important role for this residue in receptor activation. In the inactive structure of the adenosine $A_{2A}R$, the corresponding position C185$^{5.46}$ is involved in a

Van der Waals interaction with residue I135$^{4.56}$. This latter residue was also identified in our CIM screen: I136L$^{4.56}$. Also, a hydrogen bond exists between the side chain of Q89$^{3.37}$ with the backbone of C185$^{5.46}$ [10]. In the active structures, a rotameric shift of the side chain of C185$^{5.46}$ occurs which in turn causes the movement of V186$^{5.47}$ and ultimately a shift of H250$^{6.52}$ by 2 Å into the ligand binding pocket. As a result, the hydrogen bond between Q89$^{3.37}$ and C185$^{5.46}$ is broken [11,12]. The observed shift of C185$^{5.46}$ is more pronounced in the structures where NECA and adenosine are bound compared to the UK-432097 bound structure. It needs to be noted though that in the structures with NECA and adenosine, the stabilizing mutation Q89A$^{3.37}$ was introduced [12].

In **Figure 4**, all the positions identified in both screens are indicated, with CAMs in red and CIMs in yellow. In Chapter 4, we noted 3 distinct clusters of the CAMs identified from the screen. The clusters at the top of TM4 and the bottom of TM5 seem also susceptible for inactivating mutations and three of the residues in these regions were found in both screens (I136$^{4.56}$, F141$^{4.61}$, and Y202$^{5.58}$). It has to be noted though, that the mutation of Y202 in the CAM (Y202S$^{5.58}$) is the same as in the CIM screen (F141S$^{4.61}$/ Y202S$^{5.58}$). However, in the CIM mutant the mutated residue is found in combination with a mutation of the phenylalanine at position 141$^{4.61}$ that is therefore presumably the responsible mutation for the observed phenotype, i.e. a 10-fold decrease in constitutive activity and a complete loss of the ability to be activated by the agonist NECA (Chapter 5). In the crystal structures of the A_{2A}R, a shift of the side chain of this amino acid (a methionine in A_{2A}R) is observed upon activation, which might facilitate the movement of EL2 (*vide infra*).

The third CAM cluster in EL2 remains a full CAM cluster and no mutations were identified in this region that resulted in a decrease in agonist potency. Besides a specific CAM region, we also identified a typical CIM region at the top of TM5, in which only residues were shown as mutated to cause a decrease in receptor activation. Of all CIM receptors identified, only mutant M182L$^{5.38}$/N186D$^{5.42}$ is likely involved in agonist binding, as the corresponding positions in the A_{2A}R directly interact with adenosine in the crystal structure [12]. The cytoplasmic half of TM5 undergoes a inward movement towards TM6 upon activation resulting from a 4 Å shift of C185$^{5.46}$ that is accompanied by a rotameric switch of the tyrosine at position 5.58 (Y202 in the A_{2B}R). The upper part of TM5 remains overall in position when the

receptor is in the active or inactive state [11,12]. Maintaining the position of the top half of TM5 might be essential to facilitate the movements of the bottom half of TM5 and TM6 and TM7. This could be the main reason that mutations in this region result in decreased receptor activation or even complete loss of the signaling ability. The vasopressin V2 receptor is perhaps the best known receptor in which inactivating mutations are responsible to cause disease. Over 100 missense mutations have been identified that are involved in diabetes insipidus and also here, many mutated residues are located at the top of TM5, indicating that this region might be an important area in other class A GPCRs as well [16].

As mentioned above, several of the residues identified from the activation screen were found again in the inactivating screen (indicated in green in **Figure 4**). Position F141$^{4.61}$ for instance, was found mutated to a tyrosine and a serine in the CIM screen, but caused a large increase in NECA potency when mutated into a leucine. An isoleucine at position 136$^{4.56}$ was found to decrease NECA potency when mutated to a leucine, however, when mutated to a threonine it resulted in an increased potency for NECA. This emphasizes that even subtle changes can lead to large effects in receptor function that are not necessarily caused by a direct intervention with the ligand binding site or G protein coupling.

EXTRACELLULAR LOOPS…HOW DIFFERENT CAN THEY BE?

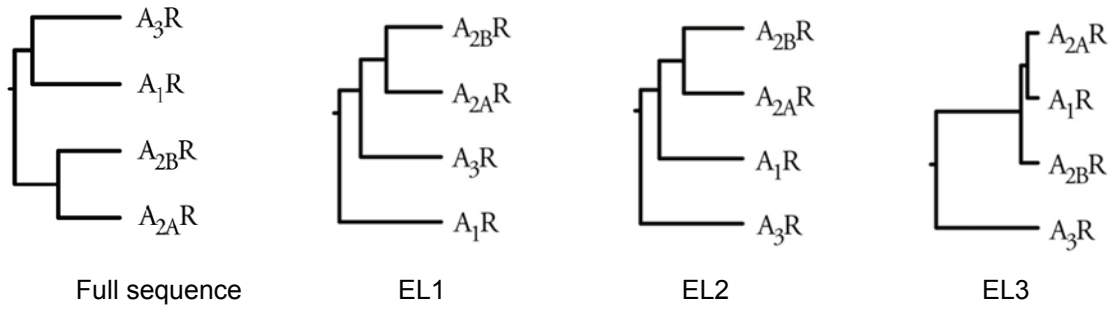
In Chapter 2, an overview was given of the extracellular loops as seen in the crystal structures of the five class A GPCRs that were available in 2010: rhodopsin, β_1-adrenergic receptor, β_2-adrenergic receptor, adenosine A_{2A} receptor, and the chemokine receptor CXCR4 [10,17,18,19,20]. A striking outcome of this comparison was that the extracellular loops show large differences in structure. Especially EL2 was completely different in each known receptor structure. Since the publication of this review, the crystal structures of two new receptors were elucidated: the dopamine D3 receptor and the histamine H1 receptor [21,22]. These structures showed yet again a different structural organization of the extracellular loops. The dopamine D3 receptor structure is perhaps most similar to the adenosine A_{2A} receptor structure, with EL2 bending towards EL1 and an additional disulfide bridge being present within EL3. However, no other non-conserved disulfide bridges are observed and EL3 seems to be more distant from the other two extracellular loops,

bending strongly outwards and not participating in the ligand binding pocket. Also, no specific structural features in EL2 such as the anti-parallel β-sheet in the $A_{2A}R$ or a helical structure in the β_1-adrenergic and β_2-adrenergic receptor are present in the dopamine D3 receptor structure. Interestingly, the newest crystal structures of the $A_{2A}R$ with NECA and adenosine co-crystallized now also appear to contain a helical structure in EL2, where in both the ZM241385 and the UK-432097 bound crystals, no structure could be determined [10,11,12].

With now seven known class A GPCR structures and seven different extracellular conformations, we can only expect to see many more differences in structural arrangements in crystal structures that will be elucidated in the future. So, how different can the extracellular loops be? Will they also differ within subfamilies that show high sequence similarity and are able to be activated by the same endogenous ligands? The mutational analysis of the extracellular loops discussed in this thesis and especially the results shown in Chapter 6 would indicate that this might actually be the case. An indication for this is readily provided in the sequence analysis shown in Figure 8 of Chapter 6. In this analysis, the sequences of the loops of all four adenosine receptors were aligned and distance trees were formed based on the alignment score. When we create such a tree for the alignments of the whole protein sequences, the distance tree is as previously reported in literature, with the $A_{2A}R$ and $A_{2B}R$ subtypes and the A_1R and A_3R subtypes grouped together [23]. **Figure 5** shows the trees of the whole protein alignment and of the alignments of the three extracellular loops.

All three extracellular loops show a different distance tree compared to the tree formed based on the whole protein sequence. Both in EL1 and EL2, the $A_{2A}R$ and $A_{2B}R$ are still closest related, however, the A_1R is not grouped together with the A_3R anymore. In Chapters 3, 4, and 5, we have discussed the mutation data of the $A_{2B}R$ in view of the only available crystal structure of the adenosine subfamily, the $A_{2A}R$. The $A_{2A}R$ and the $A_{2B}R$ are the closest related family members within the subfamily, and also their extracellular loops are closely related (**Figure 5**). However, it is most likely that these two receptors also differ in structure at the extracellular surface. For instance, not all the disulfide bridges formed in the $A_{2A}R$ are possible in the $A_{2B}R$ and the length of both EL2 and EL3 differs as well. Using the $A_{2A}R$ crystal structure to explain mutations in EL2 in other adenosine subtypes may be problematic, as is demonstrated by the results of the alanine scan on EL2 of the A_1R described in

Chapter 6. Where we identified a “hot spot” for CAMs in EL2 of the adenosine $A_{2B}R$ that is likely to act as a negative regulator for receptor activation, EL2 in the A_1R appears to have a more activating role. Almost all alanine conversions induced in the loop resulted in a decreased receptor activation profile. A similar difference in function of the second extracellular loop appears to be present within the muscarinic acetylcholine receptors (MR). Mutagenesis studies on the M_1R and M_2R resulted in several EL2 mutations that showed increased activity, while a random mutagenesis screen on the M_3R yielded many inactivating mutations [24,25,26].



	Full sequence				EL1				EL2				EL3			
	A_1R	$A_{2A}R$	$A_{2B}R$	A_3R	A_1R	$A_{2A}R$	$A_{2B}R$	A_3R	A_1R	$A_{2A}R$	$A_{2B}R$	A_3R	A_1R	$A_{2A}R$	$A_{2B}R$	A_3R
A_1R	0.0	0.9	1.0	0.8	0.0	2.5	1.2	1.5	0.0	2.0	1.7	2.3	0.0	0.8	1.1	36
$A_{2A}R$	0.9	0.0	0.6	1.2	2.5	0.0	0.8	2.4	2.0	0.0	1.3	3.2	0.8	0.0	3.0	5.9
$A_{2B}R$	1.0	0.6	0.0	1.2	1.6	0.8	0.0	0.9	1.7	1.3	0.0	1.9	1.1	3.0	0.0	12
A_3R	0.8	1.1	1.2	0.0	1.5	2.4	0.9	0.0	2.3	3.2	1.9	0.0	36	5.9	12	0.0

Figure 5. Loop similarity analysis. Multiple sequence alignments were used to create distance trees, representing sequence similarity of the whole protein and the separate loops of the four adenosine receptor subtypes. The distance tree created for the whole protein sequences and the trees corresponding to the similarity of each of the three extracellular loops are shown. Below the distance trees the alignment score tables are shown on which the distance trees were based. The alignment scores were calculated using the Phylip package.

FUTURE PERSPECTIVES

Thermostability and CIMs

Since the release of the first crystal structure of a human class A GPCR in 2007, that of the β_2 adrenergic receptor, many more structures have followed [10,11,12,17,18,19,20,21,22,27,28,29,30]. However, this does not mean that we have solved all the challenges involved in crystallizing membrane proteins. All the receptors crystallized so far (except for opsin) have been greatly modified to obtain the high quality crystals needed to elucidate their structures. The two main successful approaches so far include: i) insertion of a T4 lysozyme at the intracellular part of the receptor and ii) introducing mutations that increase the stability of the receptor outside of the cell membrane [31]. These “bio-stable” mutations are numerous and often involve mutations of residues known to be important in receptor activation and structure. Also, the search for such stabilizing mutations is laborious and involves manually scanning the entire receptor sequence.

Applying an unbiased screening method, like the yeast growth screens discussed in this thesis, can contribute greatly to this particular challenge. Since receptors with a decreased activation profile tend to be more stable than wild type or constitutively active receptors [32,33], the newly developed CIM screen would be particularly useful (Chapter 5). By using a random mutagenesis approach where mutations are introduced in low frequency in combination with the described screening method, interesting mutations can be identified in an unbiased fashion that would otherwise not have been found easily in more biased approaches such as a typical alanine scan. In short: let nature do the work instead of the scientist. As shown in Chapter 5, the mutant receptors that are identified using this method contain limited receptor changes that are mostly outside of the ligand binding and G protein coupling regions and are often still able to be activated by an agonist, indicating that the receptors are still very much functional.

We could take this even one step further and also employ yeast screening to directly identify mutant receptors that are more stable when isolated from their membrane environment. Increasing the thermal stability of a receptor has proved to be beneficial in keeping the isolated receptor stable enough for crystallization purposes [34,35]. Again, it is very laborious to identify mutations that improve thermal stability. So far, the receptor has to be (partially) isolated to confirm that the designed mutant receptor

indeed increases stability. A screening method that quickly identifies mutations to possessing this phenotype would dramatically reduce the efforts needed to reach the actual crystallization stage. One method would be to submit the yeast cells after transformation with a mutagenic library to a heat shock that will destroy the wild type GPCR of interest. Only yeast cells containing mutant receptors that can resist this heat shock are able to grow on the selection plates. The major challenge in this procedure is to develop a heat shock protocol in which the wild type GPCR is greatly compromised, but that the host is able to endure. Efforts to develop and optimize such a screen are currently undertaken in our laboratory and the preliminary results are promising.

"Full-family" Crystallization

Earlier in this chapter, I raised the following question: How different in structure can the extracellular loops be? To get an answer to at least part of this question more class A GPCR structures would need to be elucidated, ideally of all members belonging to one subfamily. The adenosine receptor subfamily would be an exceptionally good candidate. Even though the subfamily consists of only four subtypes, they all respond differently to the endogenous ligand adenosine and even signal differently upon activation by this agonist. Also, all four receptors now have their own designed set of ligands, both orthosteric and allosteric (for A_1R and A_3R), that are highly selective for that subtype [36]. Furthermore, their extracellular domains are highly divergent in sequence and also in length in the case of the second and third extracellular loops. Especially the A_3R seems to be quite distant from the other three subtypes, even when compared to the A_1R that appears closely related at first sight (**Figure 5**). Crystallizing this particular adenosine receptor would be of great interest, not in the least since a number of allosteric ligands have been identified for this receptor [36,37]. So far, no GPCR structures have been elucidated yet with an allosteric modulator bound. And while mutagenesis studies and the use of bivalent ligands have given us clues to where allosteric binding sites might be located, an exact binding pocket has yet to be determined [26,38] (also Chapter 6).

Besides the adenosine subtypes, the class A GPCR superfamily contains more small receptor subfamilies that despite high sequence similarity displays different activation profiles. The muscarinic acetylcholine receptors (MRs), for instance, exist in five different subtypes of which the M_1R, the M_3R and the M_5R couple to G_q proteins

while the subtypes M_2R and M_4R transmit their signal through G_i proteins [39]. Much mutagenesis data is available for this receptor subfamily, including information that suggests specific residues that could be involved in the allosteric binding site [26,40,41,42].

Also the family of histamine receptors would be an attractive candidate for full-family crystallization. This subfamily consists of four family members, coupling either to G_q, G_s, or G_i proteins resulting in a wide variety of responses. Their involvement in immune regulation has made them interesting drug targets [43]. One of the family members, the histamine H_1 receptor, has recently been crystallized by Shimamura et al. [22]. Gaining thorough structural insights would help to explain the mechanisms through which drugs initiate their desirable effects and adverse reactions.

Ligand directed signaling and activation states

In recent years, it has become clear that receptors do not follow a linear sequential process of activation. Different ligands are able to stabilize different conformational states of the receptor, potentially resulting in different intracellular signaling that does not necessarily include coupling to a G protein [44,45]. Several “biased ligands” that show a preference for one particular signaling pathway have been identified for various GPCRs, including 5-HT_2 serotonin receptors, adrenergic receptors, dopamine receptors and opioid receptors [46]. Biased signaling has also been observed for the adenosine receptor subfamily [47]. It is more than likely that in the activation of these different signaling pathways, different activation mechanisms and different receptor residues are involved. Furthermore, ligands that cause activation of the same pathway as the endogenous ligand could stabilize the active conformations differently. An indication of this has been presented in Chapters 3 and 4, in which we subjected a selection of mutant receptors to the non-ribose agonist BAY60-6583. This structurally distinct ligand showed no changes in activation response compared to NECA on mutations present in EL1, whereas BAY60-6583’s potency was affected by mutations in EL2. Therefore, it would be of great interest to investigate the effect of the mutations described in this thesis on a wide variety of ligands, including the endogenous agonist adenosine. In the studies on the $A_{2B}R$ described in this thesis, we made use of the more potent adenosine derivative NECA as it is more convenient in functional in vitro studies considering the low affinity and the metabolic instability of adenosine. The only difference between NECA and adenosine is an altered 5’

position on the ribose moiety, however, this addition leads to a substantial increase in potency for the A_{2B} receptor [48]. It would be highly informative to investigate which residues might be involved in this improvement in potency. This might also provide insights in the origin of the low affinity the A_{2B}R has for adenosine compared to the other subtypes.

Post-translational modifications

In this thesis, we did not go into detail concerning a particular topic in protein research; the post-translational modifications a protein can undergo. Nonetheless, these modifications can also contribute to receptor activation. EL2 is often the site for glycosylation; over 32% of class A GPCRs possess at least one consensus N-glycosylation site in the second extracellular loop [49]. Glycosylation may play a role in cell surface expression rather than ligand binding or activation as shown for the vasopressin 1a receptor [50]. The adenosine receptors all contain at least one possible glycosylation site. In the adenosine A_{2B} receptor these are $N153^{EL2}$ and $N163^{EL2}$; neither positions have been identified in the screens described in this thesis (Chapters 4 and 5). In the adenosine A_1 receptor a possible site for glycosylation is located at residue $N159^{EL2}$ (http://www.gpcr.org/7tm/). Mutating this residue to an alanine resulted in a 4-fold decrease in potency for the agonist NECA but this mutant receptor could still reach maximal activation levels (Chapter 6).

OVERALL CONCLUSION

The research described in this thesis demonstrates intricate details of GPCR activation. Through evolution, GPCRs have evolved in many different fine-tuned subtypes, all with their own sets of ligands, levels of constitutive activity and transmitted signals. Even though all 800 subtypes of GPCRs in humans look alike, not two receptors behave the same. Originally, it was thought that mainly two domains determine the activation profile of each receptor, i.e. the ligand binding site and the domain responsible for G protein coupling. That this view is over-simplified is clearly demonstrated in this thesis, especially regarding the extracellular loops. With each receptor structure that has been elucidated so far, a different extracellular structural arrangement is seen. Together with the experimental data that has become

available over the last decade, including the results presented here, we conclude that the extracellular domains are certainly not just anchors to maintain the receptor in the membrane but active participants in the activation mechanism defining each unique receptor.

REFERENCES

[1] Krieger, E., Koraimann, G., Vriend, G., *Proteins* (2002) 47:393-402.
[2] Leff, P., *Trends Pharmacol Sci* (1995) 16:89-97.
[3] Kobilka, B.K., Deupi, X., *Trends Pharmacol Sci* (2007) 28:397-406.
[4] Seifert, R., Wenzel-Seifert, K., *Naunyn Schmiedebergs Arch Pharmacol* (2002) 366:381-416.
[5] Vischer, H.F., Hulshof, J.W., de Esch, I.J., Smit, M.J., Leurs, R., *Ernst Schering Found Symp Proc* (2006) 187-209.
[6] Bond, R.A., IJzerman, A.P., *Trends Pharmacol Sci* (2006) 27:92-96.
[7] Beukers, M., van Oppenraaij, J., van der Hoorn, P., Blad, C., den Dulk, H., Brouwer, J., IJzerman, A., *Mol Pharmacol.* (2004) 65:702-710.
[8] Fredholm, B.B., *Cell Death Differ* (2007) 14:1315-1323.
[9] Wilson, C.N., *Br J Pharmacol* (2008) 155:475-486.
[10] Jaakola, V.P., Griffith, M.T., Hanson, M.A., Cherezov, V., Chien, E.Y., Lane, J.R., IJzerman, A.P., Stevens, R.C., *Science* (2008) 322:1211-1217.
[11] Xu, F., Wu, H., Katritch, V., Han, G.W., Jacobson, K.A., Gao, Z.G., Cherezov, V., Stevens, R.C., *Science* (2011) 332(6027):322-7.
[12] Lebon, G., Warne, T., Edwards, P.C., Bennett, K., Langmead, C.J., Leslie, A.G., Tate, C.G., *Nature* (2011) 474:521-525.
[13] Nygaard, R., Frimurer, T.M., Holst, B., Rosenkilde, M.M., Schwartz, T.W., *Trends Pharmacol Sci* (2009) 30:249-259.
[14] Ahuja, S., Smith, S.O., *Trends Pharmacol Sci* (2009) 30:494-502.
[15] Kazius, J., Wurdinger, K., van Iterson, M., Kok, J., Back, T., IJzerman, A.P., *Hum Mutat* (2008) 29:39-44.
[16] Schoneberg, T., Schulz, A., Biebermann, H., Hermsdorf, T., Rompler, H., Sangkuhl, K., *Pharmacol Ther* (2004) 104:173-206.
[17] Wu, B., et al., *Science* (2010) 330:1066-1071.
[18] Palczewski, K., et al., *Science* (2000) 289:739-745.
[19] Warne, T., Serrano-Vega, M.J., Baker, J.G., Moukhametzianov, R., Edwards, P.C., Henderson, R., Leslie, A.G., Tate, C.G., Schertler, G.F., *Nature* (2008) 454:486-491.
[20] Rosenbaum, D.M., et al., *Science* (2007) 318:1266-1273.
[21] Chien, E.Y., et al., *Science* (2010) 330:1091-1095.
[22] Shimamura, T., et al., *Nature* (2011) 475:65-70.
[23] Fredholm, B.B., IJzerman, A.P., Jacobson, K.A., Klotz, K.N., Linden, J., *Pharmacol Rev* (2001) 53:527-552.
[24] Goodwin, J.A., Hulme, E.C., Langmead, C.J., Tehan, B.G., *Mol Pharmacol* (2007) 72:1484-1496.
[25] Scarselli, M., Li, B., Kim, S., Wess, J., *J Biol Chem.* (2007) 282:7385-7396.
[26] Avlani, V.A., Gregory, K.J., Morton, C.J., Parker, M.W., Sexton, P.M., Christopoulos, A., *J Biol Chem* (2007) 282:25677-25686.
[27] Murakami, M., Kouyama, T., *Nature* (2008) 453:363-367.
[28] Cherezov, V., et al., *Science* (2007) 318(5854):1258-65.
[29] Rasmussen, S.G., et al., *Nature* (2011) 469:175-180.
[30] Rasmussen, S.G., et al., *Nature* (2007) 450:383-387.
[31] Jaakola, V.P., IJzerman, A.P., *Curr Opin Struct Biol* (2010) 20:1-14.
[32] Kobilka, B., Schertler, G.F., *Trends Pharmacol Sci* (2008) 29:79-83.
[33] Gether, U., Ballesteros, J.A., Seifert, R., Sanders-Bush, E., Weinstein, H., Kobilka, B.K., *J Biol Chem* (1997) 272:2587-2590.
[34] Serrano-Vega, M.J., Magnani, F., Shibata, Y., Tate, C.G., *Proc Natl Acad Sci U S A* (2008) 105:877-882.

[35] Standfuss, J., Xie, G., Edwards, P.C., Burghammer, M., Oprian, D.D., Schertler, G.F., *J Mol Biol* (2007) 372:1179-1188.
[36] Fredholm, B.B., IJzerman, A.P., Jacobson, K.A., Linden, J., Muller, C.E., *Pharmacol Rev* (2011) 63:1-34.
[37] Muller, C.E., *Curr Top Med Chem* (2003) 3:445-462.
[38] Narlawar, R., Lane, J.R., Doddareddy, M., Lin, J., Brussee, J., IJzerman, A.P., *J Med Chem* (2010) 53:3028-3037.
[39] Brown, D.A., *J Mol Neurosci* (2010) 41:340-346.
[40] Gregory, K.J., Hall, N.E., Tobin, A.B., Sexton, P.M., Christopoulos, A., *J Biol Chem* (2010) 285:7459-7474.
[41] Jager, D., et al., *J Biol Chem* (2007) 282:34968-34976.
[42] Nawaratne, V., Leach, K., Felder, C.C., Sexton, P.M., Christopoulos, A., *J Biol Chem* (2010) 285:19012-19021.
[43] Hill, S.J., et al., Histamine receptors, IUPHAR database (IUPHAR-DB), 2008.
[44] Kenakin, T., *Trends Pharmacol Sci* (2007) 28:407-415.
[45] Zheng, H., Loh, H.H., Law, P.Y., *IUBMB Life* (2010) 62:112-119.
[46] Urban, J.D., et al., *J Pharmacol Exp Ther* (2007) 320:1-13.
[47] Verzijl, D., Ijzerman, A.P., *Purinergic Signal* (2011).
[48] de Zwart, M., Link, R., von Frijtag Drabbe Kunzel, J.K., Cristalli, G., Jacobson, K.A., Townsend-Nicholson, A., AP, I.J., *Nucleosides Nucleotides* (1998) 17:969-985.
[49] Conner, M., Hawtin, S.R., Simms, J., Wootten, D.L., Lawson, Z., Conner, A.C., Parslow, R.A., Wheatley, M., *J Biol Chem* (2007) 282:17405-17412.
[50] Hawtin, S.R., Davies, A.R., Matthews, G., Wheatley, M., *Biochem J* (2001) 357:73-81.