The mechanism of G protein coupled receptor activation: the serotonin receptors

Jessica Sallander

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DIRECTORS:

Dr. Leonardo Pardo, Laboratory of Computational Medicine, UAB

Dr. Mercedes Campillo, Laboratory of Computational Medicine, UAB

TUTOR:

Dr. Ferran Sanz, CEXS Department - UPF

UNIVERSITAT POMPEU FABRA

Till Adam och Henrik

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Abstract

One of the main questions in G protein coupled receptors (GPCRs) molecular pharmacology is to understand the structural arrangements of the seven transmembrane (TM) helices that occur to stabilize either the ground state (Rg) or different active states (R*) of the receptors. In order to understand the mechanism that shift the equilibrium of the ensemble to an active R* state models of the inactive and the active state of three serotonin receptors (5-HT_4, 5-HT_6, and 5-HT_7) were built based on the latest information from X-ray crystallography. The resulting models were mainly used to understand the interaction between a ligand and its receptor and the mechanism of action. With the help of pharmacological and chemical data these models and complexes were improved and evaluated. These findings may prove valuable for structural based drug discovery efforts and facilitate the design of more effective and selective pharmaceuticals.

Resumen

Una de las principales cuestiones en farmacología molecular de los GPCR es entender los mecanismos estructurales de las siete hélices transmembrana (TM) que se producen para estabilizar ya sea Rg o los diferentes estados R*. Para entender el mecanismo que cambia el equilibrio del conjunto a un estado activo R* se construyeron tres de los receptores de la serotonina (5-HT_4, 5-HT_6, y 5 HT_7) sobre la base de su información más reciente de cristalografía de rayos X. Dando lugar a dos modelos de cada receptor: una inactiva y otra activa. Los modelos, mejorados y evaluados con la ayuda de datos farmacológicos y químicos se utilizaron principalmente para comprender la interacción entre un ligando y su receptor y su mecanismo de acción. Estos hallazgos estructurales pueden a su vez resultar útiles para el diseño de nuevos fármacos más eficaces y selectivos.

Preface

G protein-coupled receptors (GPCRs) exist in equilibrium of many different conformational states. GPCRs adopt inactive and active states. Activating ligands binding at the extracellular part of the receptor or constitutively active mutations stabilize active conformations that increase cytoplasmic G protein binding. Agonists or constitutively active mutations disrupt intramolecular interactions that stabilize inactive conformations. In contrast, inverse agonists reinforce the constraints that keep the receptor in inactive conformations. One of the main questions in GPCR molecular pharmacology is to understand the structural arrangements of the 7 transmembrane (7TM) helices that occur to stabilize either the inactive or different active states.

All the crystal structures of the inactive state have permitted to get an understanding of the non-covalent interactions between side chains that maintain GPCRs in the inactive conformation. Understanding the mechanisms that shift the equilibrium of the ensemble to the active conformations have now also been possible due to the release of some X-ray crystallography structures in the active state. However, it is still very challenging.

First, we will present the homology models of serotonin receptors 4, 6 and 7. These were based on a combination of the latest information about GPCR structure with data from chemical synthesis, site-directed mutagenesis, and biophysical experiments. Then the mechanisms by which binding of ligands might trigger a certain signal have been investigated. Furthermore, we introduce our findings of the identification of regions and ligand-receptor interactions associated with selectivity, which could be exploited for the design of new improved compounds.

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Abbreviations

3D	three dimensinal
5-HT	Serotonin, 5-Hydroxytryptamine
5-HT_{1A}	Subtype 1A serotonin receptor
5-HT_{4}	Subtype 4 serotonin receptor
5-HT_{6}	Subtype 6 serotonin receptor
5-HT_{7}	Subtype 7 serotonin receptor
7TM	7 transmembrane helices
bRho	bovine rhodopsin
cAMP	cyclic AMP, cyclic adenosine monophosphate
CNS	central nervous system
EL	Extracellular loop
GPCR	G protein-coupled receptor
hAA2AR	human adenosine receptor
hB2AR	human beta 2-adrenergic receptor
hCXCR4	human CXCR4 chemokine receptor
hDRD3	human dopamine D3 receptor
IL	intracellular loop
MD	molecular dynamics
PC	phosphatidyl–cholines
sRho	squid rhodopsin
tB1AR	turkey beta 1-adrenergic receptor
TM	transmembrane

List of publications

Articles:

- Benzimidazole derivatives as new serotonin 5-HT6 receptor antagonists. Molecular mechanisms of receptor inactivation.

  de la Fuente T, Martín-Fontecha M, **Sallander J**, Benhamú B, Campillo M, Medina RA, Pellissier LP, Claeysen S, Dumuis A, Pardo L, López-Rodríguez ML.
  J. Med. Chem. 2010 Feb 11;53(3):1357-69

- Synthesis of new serotonin 5-HT7 receptor ligands. Determinants of 5-HT7/5-HT1A receptor selectivity.

  Medina RA*, **Sallander J***, Benhamú B, Porras E, Campillo M, Pardo L, López-Rodríguez ML.
  J. Med. Chem. 2009 Apr 23;52(8):2384-92

- Conformational toggle switches implicated in basal constitutive and agonist-induced activated states of 5-hydroxytryptamine-4 receptors.

  Pellissier LP*, **Sallander J***, Campillo M, Gaven F, Queffeulou E, Pillot M, Dumuis A, Claeysen S, Bockaert J, Pardo L.
  Mol Pharmacol. 2009 Apr;75(4):982-90*

* equally contributed to this work, orden alphabetically

Life is not measured by the number of breaths we take but by the moments that take our breath

(Unknown)

1. INTRODUCTION

1.1 Seven transmembrane receptors

1.1.1 The cell membrane

A cell membrane surrounds all living cells and acts as a physical barrier to separate the interior of a cell from the outside world. The cell membrane is selectively permeable to ions and organic molecules and controls the movement of substances in and out of cells.

Biological membranes consist of organized assemblies of lipids and proteins, which are involved in a variety of cellular processes such as cell adhesion, ion conductivity and cell signaling. The current knowledge of how they are structured is still scarce due to the difficulties associated to the experimental techniques required to investigate their properties. However, the current vision of membranes is that lipids organize a matrix where proteins are distributed in regions of biased composition with varying protein environment[1].

Membranes consist of a complex mixture of lipids, where different proteins both, integral and peripheral are embedded. Protein content varies greatly among the different kinds of membranes, ranging typically between 15-75% depending on the functions that they must carry out[2]. Furthermore, lipid composition changes from one membrane to another, due to enormous structural diversity found, that can be associated with the differential roles and properties of each membrane or region. The most widely found lipids consists of a structure of a fatty acid linked by an ester bond to an alcohol such as glycerol or cholesterol, or through amide bonds to a sphingoid base or to other amines. Most lipids have a highly polar head group and two hydrocarbon tails. In a typical membrane, approximately half of the lipids are phospholipids. Other mayor components following in importance are sphingolipids, glycolipids and cholesterol[3]. Cholesterol may be involved in modulating membrane protein function by two different mechanisms: i) in modifying bulk membrane properties such as fluidity and curvature or ii) by direct interactions with the membrane protein in question[4]. The lipids are arranged in a bilayer with their polar, hydrophilic heads facing

outwards, and their nonpolar, hydrophobic fatty acid tails facing each other in the middle of the bilayer. However, since the two sides of the membrane bilayer must deal with different surroundings, the two sides of the membrane typically exhibit an asymmetric composition[5].

Phosphatidyl–cholines (PC) is the most abundant type of lipid in animal cells, and bilayers constituted by these lipids are among the most widely studied model membrane systems. PC consists of a glycerol backbone bound to two fatty acid chains named sn-1 and -2 and a phosphate group attached to choline [6].

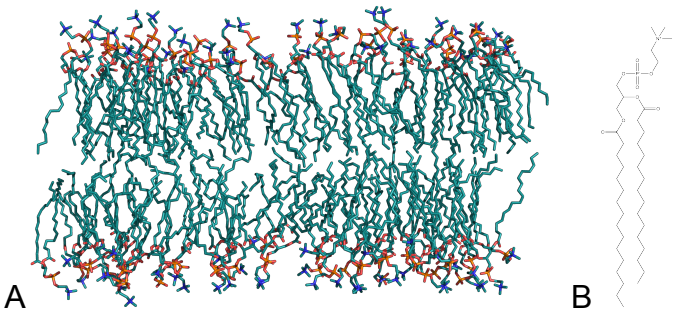


Figure 1 A) Structure of a thin slice of a DPPC lipid bilayer. The atoms are colored by atom type; hydrogen atoms are not present. B) 2D structure of one DPPC lipid.

1.1.2 Membrane proteins

Membrane proteins spanning through the cell membrane are often divided into two groups: integral and peripheral, based on the strength of their association with the membrane. Specifically, the former are permanently attached either to the lipid bilayer or to integral proteins. Integral proteins are divided into transmembrane and monotopic proteins. While the former span along the entire membrane, the latter are permanently attached to the membrane from only one side. Membrane function is mediated in a large extent by integral membrane proteins, which are often organized as assemblies of polypeptide segments interacting with the lipid bilayer and serving as channels, receptor and energy transducers. Accordingly, they constitute biological machines involved in essential biological process like ion and molecular transport across the membrane, cell communication and signaling. Therefore, their study is a field of enormous interest.

1.1.3 The GPCR family

G protein coupled receptors (GPCRs) are the largest family of membrane proteins, serving as key components of signal transduction pathways across cell membranes and as important drug targets. It's estimated that 30-50% of current drug targets are GPCRs[7-8]. Interesting is that they make up just about 3% of genes in the human genome[9], which proportionally makes very few but fairly important genes in the human genome that are predicted to encode GPCRs. GPCRs are activated by diverse ligands, including odorants, fatty acids, peptides and neurotransmitters. GPCRs contain seven membrane-spanning α-helical segments separated by alternating intracellular and extracellular loop regions, leaving the N-terminus in the extracellular milieu and the C-terminus in the cytoplasm (Figure 2). They are commonly divided into five families[10] based on their sequence and structural similarity: rhodopsin (family A, the largest and most diverse family of GPCRs); secretin (family B); glutamate (family C); adhesion; and frizzeled/taste2.

The rhodopsin family is by far the largest, accounting for about 84% of all receptors. Notably, the rhodopsin family comprises the numerous olfactory receptors, which on their own, account for about 48% of all GPCRs[11].

1.1.4 Crystal structures

Knowledge of the three-dimensional (3D) structure of a protein is of utmost importance for drug discovery, as it serves as a basis to understand the functions of a protein at a molecular level. In the field of structural biology one uses several techniques to determine the structure of a protein (X-ray crystallography, NMR spectroscopy, and dual polarization interferometry).

Crystal structures of an increasing number of therapeutic targets are becoming available. Structures with a ligand bound to the protein can help in the optimization process to improve drug-like properties. Not only providing ideas on how to improve binding affinity or selectivity, but also showing where the compound can be

modified in attempting to modulate physico-chemical properties and biological efficacy.

It's been over 50 years since the very first crystal structure of a protein was determined (myoglobin[12]). Since then many protein structures have been determined, and lately because of advances in technology and technique the number of crystal structures have rapidly increased. They can all be found in the current public database; the protein data bank (the PDB, http://www.rcsb.org).

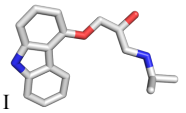
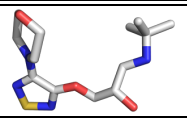
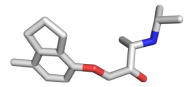
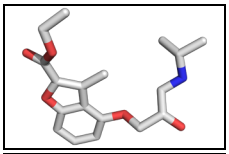
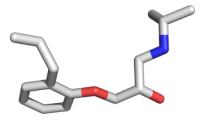
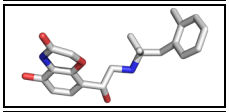
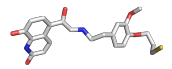
The pioneering structures of bovine rhodopsin[13] that was revealed in 2000, was a breakthrough for the computational models of family A proteins. It unveiled for the first time the topology of a GPCR at the atomic level. Therefore, up until recently the atom-level understanding of GPCRs has been based on rhodopsin in its inactive state. However, with just one reference structure one did not know if it was ideal representative for drawing generalized conclusions about the other family members. Lately, the available crystallographic structures of family A GPCRs have been expanded [14-24, 124]. There are many lists put together on this subject, and here the list made by P. Nollert[127] have been expanded.

	"inactive"	Pdb code	"active"	Pdb code
β2-adrenergic receptor	hB2AR[16] (human)	2RH1	hB2AR*[25] (human)	3PDS
β1-adrenergic receptor	tB1AR[19] (turkey)	2VT4	tB1AR*[26] (turkey)	2Y00-2Y04
Adenosine receptor	hAA2AR[18] (human)	3EML	hAA2AR*[23] (human)	3QAK
Rhodopsin receptor	sRho[125] (squid)	2Z73	-	-
Rhodopsin receptor	bRho[27] (bovine)	1U19	Rho*[28] MetaII*[24] Ops*[17; 20]	2X72, 3PXO, 3PQR 3CAP, 3DQB
Dopamine D3 receptor	hDRD3[29] (human)	3PBL	-	-
CXCR4 Chemokine Receptor	hCXCR4[30] (human)	3ODU, 3OE0, 3OE8, 3OE9, 3OE6	-	-

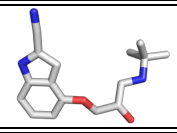
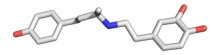
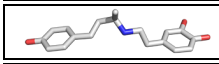
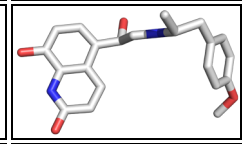
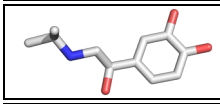
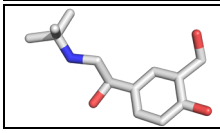
Tabla 1. Crystal structures and their abbreviations used in this study.

The new structural data has broadened the knowledge of the conserved and variable features and dynamic properties of GPCRs, providing additional templates for homology modeling. The first receptors were all crystallized with an inverse agonist or antagonist and therefore represent inactive conformations. However, recently several receptors representing active states have been crystallized, providing important information about the structural changes associated with activations of GPCRs. In the following tables a summary of the crystal structures and its bound ligands is presented.

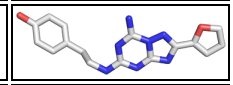
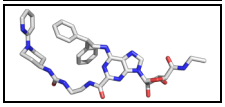
β2 Adrenergic Receptor

Structure Notes	PDB	Reso-lution [Å]	Ligand Structure	Reference
b2AR365-Fab5 complex	2R4S, 2R4R	3.4 3.4		Rasmussen et al. **2007**[15]
Complex with Carazolol ligand and bound Cholesterol; T4 lysozyme fusion in 3rd intracellular loop	2RH1	2.4	 I	Cherezov et al. **2007**[16]
T4 lysozyme fusion in 3rd intracellular loop, bound cholesterol	3D4S	2.8		Hanson et al. **2008**[31]
methylated receptor	3KJ6	3.4		Bokoch et al. **2010**[32]
T4 lysozyme fusion in 3rd intracellular loop, bound cholesterol, with mutations, inverse agonist ICI 118,551	3NY8	2.84		Wacker et al. **2010**[21]
T4 lysozyme fusion in 3rd intracellular loop, bound cholesterol, with mutations, inverse agonist Timolol	3NY9	2.48		Wacker et al. **2010**[21]
T4 lysozyme fusion in 3rd intracellular loop, bound cholesterol, mutations, antagonist alprenolol	3NYA	3.16		Wacker et al. **2010**[21]
With a nanobody	3POG	3.50		Rasmussen et al. **2011**[22]
In complex with a covalently bound irreversible agonist BI-167107	3PDS	3.50		Rosenbaum et al. **2011**[25]

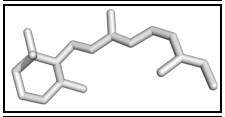
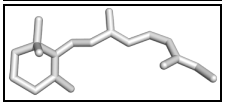
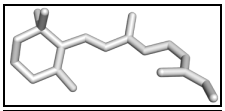
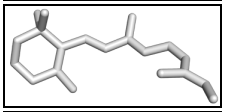
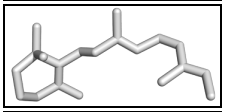
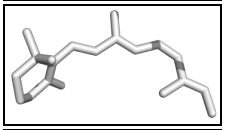
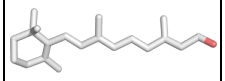
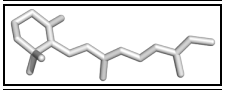
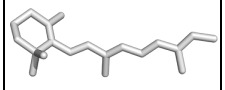
β1 Adrenergic Receptor

Structure Notes	PDB	Reso-lution [Å]	Ligand structure	Reference
With stabilizing mutations and bound cyanopindolol	2VT4	2.7		Warne T. et al. **2008**[19]
With stabilizing mutations and bound partial agonist dobutamine	2Y00	2.5		Warne et al **2011**[26]
With mutations and bound partial agonist dobutamine	2Y01	2.6		Warne et al **2011**[26]
With stabilizing mutations and bound agonist carmoterol	2Y02	2.6		Warne et al **2011**[26]
With stabilizing mutations and bound agonist isoprenaline	2Y03	2.85		Warne et al **2011**[26]
With stabilizing mutations and bound partial agonist salbutamol	2Y04	3.05		Warne et al **2011**[26]

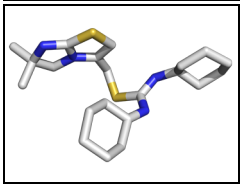
Adenosine Receptor

Structure Notes	PDB	Reso-lution [Å]	Ligand structure	Reference
Bound antagonist ZM241385	3EML	2.6		Jaakola et al.[18]
Bound agonist UK-432097	3QAK	2.71		Xu et al.[23]

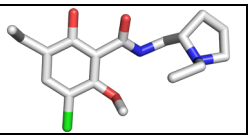
Rhodopsin

Structure Notes	PDB	Resolution [Å]	Ligand structure	Reference
first experimental GPCR structures, bound retinal	1F88, 1HZX	2.8		Palczewski K. et al, **2000** [13] Teller DC. Et al [33]
shows functional water molecules, bound retinal	1L9H	2.6		Okada et al, **2002**[34]
focus on the retinal conformation	1U19	2.2		Okada et al, **2004**[27]
Stabilized with mutations	2J4Y	3.4		Standfuss et al, **2007**[35]
photoactivated and ground state, with retinal bound	2I35, 2I36, 2I37	3.8 4.1 4.15		Salom et al, **2006**[14]
Retinal removed: Opsin	3CAP	2.9		Park et al, **2008**[17]
activated form of Ops*-GalphaCT peptide complex, no ligand bound	3DQB	3.2		Scheerer et al, **2008**[20]
Squid Rhodopsin with retinal bound	2Z73	2.5		Murakami et al **2008**[125]
constitutively mutant rhodopsin complex with a peptide derived from the carboxy terminus of the a-subunit of the G protein transducin. Retinal bound.	2X72	3.0		Standfuss et al **2011**[28]
Metarhodopsin II with retinal	3PXO	3.0		Choe et al **2011**[24]
Metarhodopsin II in complex with a C-terminal peptide derived from the Galpha subunit of transducin	3PQR	2.85		Choe et al **2011**[24]

CXCR4 Chemokine Receptor

Structure Notes	PDB	Reso-lution [Å]	Ligand structure	Reference
In complex with small molecule antagonist IT1t and cyclic peptide antagonist CVX15, T4 lysozyme insertion in 3rd intracellular loop, stabilizing mutations	3ODU, 3OE0, 3OE8, 3OE9, 3OE6	2.5 Å 2.9 Å 3.1 Å 3.1 Å 3.2 Å		Wu et al, 2010[30]

Dopamine D3 Receptor

Structure Notes	PDB	Reso-lution [Å]	Ligand structure	Reference
D3 dopamine receptor, T4 lysozyme insertion in 3rd intracellular loop, in complex with Eticlopride,	3PBL	2.9 Å		Chien et al, 2010[29]

1.1.5 Three dimensional structure of GPCRs

Structurally GPCRs are characterized by an extracellular N-terminus, followed by seven transmembrane (7TM) □-helices (TM1 to TM7) connected by three intracellular (IL1 to IL3) and three extracellular loops (EL1 to EL3), and finally an intracellular C-terminus (Figure 2).

Even though the sequence similarity among GPCRs is low, they contain residues that are highly conserved. These highly conserved residues are more important for setting the structure than their sequence identity. The numbering scheme used throughout this thesis was based on this conservation. Ballesteros and Weinstein designed a scheme in 1995, which allows for easy comparison among residues in the 7TM segments of different receptors. This scheme assigns the most conserved residue in a TM number 50, then the numbers decreases towards N-terminus and increases towards C-terminus.

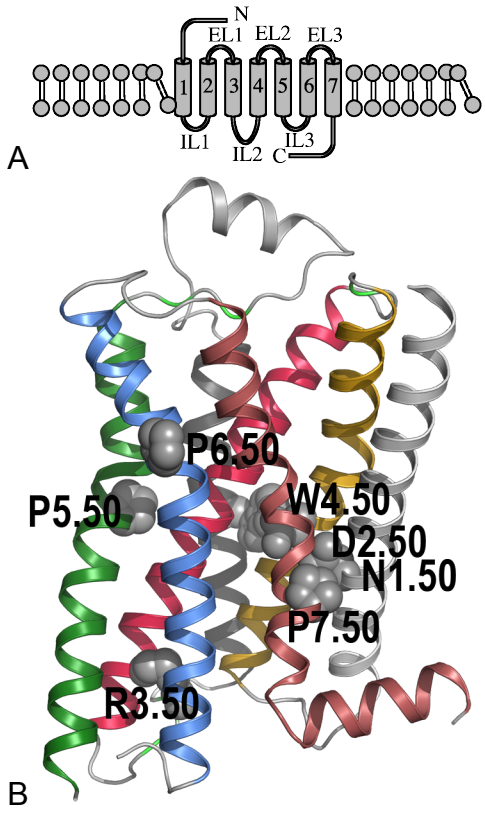


Figure 2. A) A general topology model of G protein-coupled receptor families with extracellular N-terminus, 7 TM, intracellular C-terminus and the EL1-3 and IL1-3. B) human b2-adrenergic receptor (hB2AR) with the most conserved residues shown in grey spheres. The color code of the helices is TMs 1 in grey, 2 in yellow, 3 in red, 4 in dark gray, 5 in green, 6 in blue, and 7 in salmon pink.

Protein structure and function

All proteins are polymers of amino acids with a size of nanoparticles (1-100 nm). Protein amino acids are combined into a single polypeptide chain in a condensation reaction. The protein synthesis reaction is carried out in the ribosomes in a process known as translation. In this process, the amino acids are incorporated into the protein according to the mRNA template. The protein then folds into its secondary structure. It has been shown that the main driving force for folding water-soluble globular protein molecules is to pack hydrophobic side chains into the interior of the molecule, thus creating a hydrophobic core and a

hydrophilic surface[36]. However, the main chain that is highly polar must also fold into the interior, and be neutralized by hydrogen bond formations. Such secondary structures are usually of two types: α-helices or β-sheets.

Ideal α-helix

Normally an ideal α-helix is interpreted as follows: it has approximately 3.6 residues per turn, which has a twist angle of about 100º (360/3.6); a closed helical segment, with < 3.6 residues per turn, possesses a twist >100º; whereas an open helical segment with > 3.6 residues per turn, possesses a twist of <100. The opened and the closed turn leads to a change in the orientation of the α-helices, sometimes dramatically and sometimes just slightly.

Side chains

There are 20 amino acids with different physical and chemical properties that make up a protein. The side chain is the part of an amino acid's chemistry that differentiates it from other amino acids. The atoms along the side chain are named following the Greek alphabet. The dihedral angles around the bonds between these atoms are named χ_1, χ_2, χ_3, etc. Side chains tend to adopt different staggered conformations called gauche- (*g*-), trans (*t*), and gauche+ (*g*+), which corresponds to rotation angles of 60°, 180°, and -60°, respectively, around the sp3-sp3 bonds.

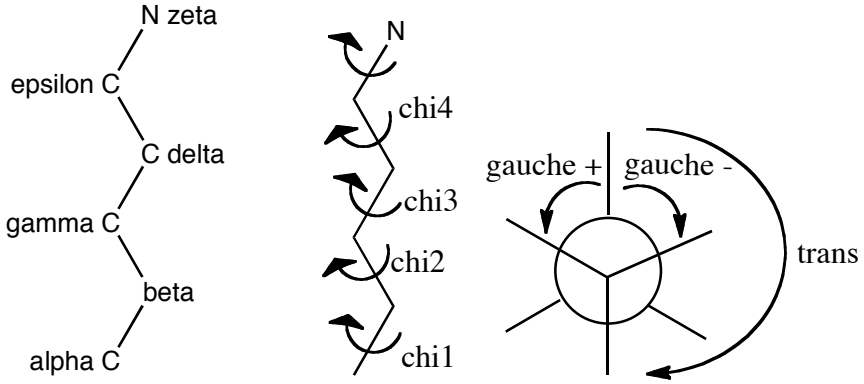


Figure 3. Schematic representation of Lysine side chain: A) The side chains atoms are named in the Greek alphabet. B) The side chain torsion angles are named chi1, chi2, etc. C) The chi angle is subject to certain restrictions, which arise from steric hindrance between side chain atoms and the main chain. The different conformations of the side chain as a function of chi are referred to as gauche+, trans and gauche -.

Pro – kink

Each α-amino acid consists of a backbone part that is preset in all the amino acid types, and a side chain that is unique to each type of residue. An exception from this rule is proline (pro), where a bond to the side chain replaces the hydrogen atom. Pro residues are normally observed in TM helices where they induce a significant distortion named pro-kink[37]. This bend of the TM is caused in order to avoid a steric clash between the pyrrolidine ring of pro and the carbonyl oxygen of the residue in the preceding turn[38]. This induces a bend angle of approximately 20º in the helical structure [39, 40].

Gly-bend and gly-gly bulge/torsion

Glycine does not have a side chain and, it provides high flexibility to the polypeptide chain. By other words, it may adopt torsion angles, which are normally not allowed for other amino acids. That is why glycines are often found in loop regions, where the polypeptide chain makes a sharp turn. This is also the reason for the high conservation of glycine residues, since turns are important for the preservation of the particular fold of the protein structure.

Serine, threonine and cysteine residues in α-helices

Ser, Thr, and Cys residues have short polar side chains with a strong hydrogen bond potential. In these residues, the *gauche* - (g-), *gauche* + (g+), and *trans* (t) staggered side chain conformations are strongly preferred relative to the eclipse conformations[41]. The conformations of these amino acids differ because they either hydrogen bond the backbone or causing a steric clash with the backbone. The short and β-branched side chain of threonine are limited to the g+ (85% of the side chains) and g- (15%) whereas the t conformation is unfavorable because of the steric clash of the side chain methyl group with the backbone carbonyl at the i-3 position[42]. In contrast, serine can adopt either the g+ (52%), g- (20%), or t (28%) rotamer conformation. While cysteine can adopt g+ (71%) or t (29%), the g- conformation is not possible, due to a steric clash between the S_γ atom and the carbonyl at the i-3 position.

Disulphide bridge

It is a covalent bond, found in proteins that are formed between the thiol groups of cysteine residues. This bond is important for stabilizing and folding proteins.

These are important features that make up the 3D structure of a protein.

1.1.6 Comparison of inactive crystal structures

Even though the sequence similarity is low, the overall structure of the above (1.1.4) presented crystal structures are very conserved. The orientation and length of the helices vary some and the greatest differences are not so surprisingly observed in the intracellular and extracellular loops. In addition, which might be considered minor differences but may have a great impact on ligand binding for example are the changes in the orientations of side chains. The study of common features among the TM by Worth et al[131] has here been expanded.

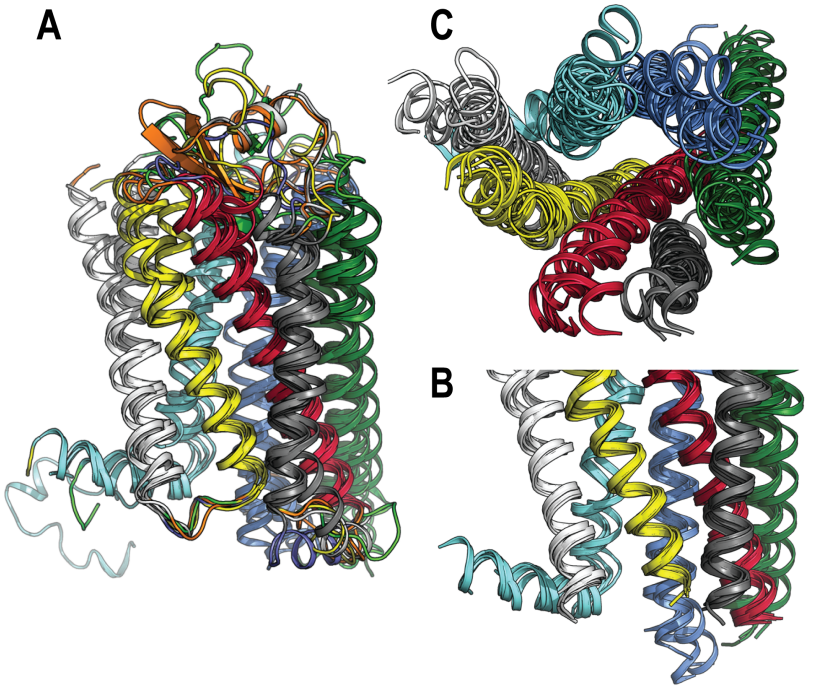


Figure 4. A) Comparison of the currently available crystal structures of GPCRs in the inactive conformation: bRho, the tB1AR, hB2AR, hAA2AR, hDRD3, CXCR4 receptors. Clearly, the structure of the cytoplasmic part is highly conserved (Figure 1B), with the exception of CXCR4 that contains very different TM4 and TM7-Hx8 domains. This structural conservation correlates with the fact that most conserved residues are clustered in the central and intracellular regions of the receptor [43]. In contrast, there is a low degree of sequence conservation among different GPCRs at their extracellular domains. Accordingly, the structure of the extracellular part of TM helices is more divergent (Figure 1C). B) The structure of the cytoplasmic part is highly conserved, with the exception of CXCR4 that contains very different TM4 and TM7-Hx8 domains (not shown). C, The structure of the extracellular part of the TM helices is more divergent. The color code of the helices is TMs 1 in white, 2 in yellow, 3 in red, 4 in gray, 5 in green, 6 in dark blue, and 7 in light blue.

		bRho	sRho	hB2Ar	tB1AR	hAA2AR	hDRD3	hCXCR4
TM1	Pro distortion	√						
	Gly-Gly bulge		√					
	Helix shift towards the central axis	√	√			√	√	√
TM2	Pro distortion		√	√	√	√	√	√
	Gly-Gly distortion	√						
	Bulge due to insertion		√					
EL1	Disulphide bridge to EL2					√		
	Beta-strand (indicated by above disulphide bridge)					√		
TM3	Conserved Cys forms disulphide bridge to EL2	√	√	√	√	√	√	√
	Second disulphide bridge to EL2					√		
	Gly-(gly)-bend	√	√					
	Bend caused by disulphide bridge between TM3 and EL2					√		
IL2	Helical				√	√	√	
	Tyr forming h-bond to Asp in DRY-motif				√	√	√	
TM4	Pro distortion	√	√	√	√	√	√	√
	Bulge due to insertion			√	√			
	Gly bend							√
	Intracellular helix extension							√
	Distortion							√
EL2	Disulphide bridge to TM3	√	√	√	√	√	√	√
	Beta-sheet	√	√					√
	Alpha helix			√	√			
	Beta-strand					√		
	Intra EL2 disulphide bridge			√	√			
	Disulphide bridge to EL1					√		
	Second disulphide bridge to TM3					√		
TM5	Extracellular helix extension							√
	Pro kink	√	√	√	√	√	√	√
	Intracellular helix extension		√					
IL3	Partial structure			√	√	√	√	√
TM6	Pro kink	√	√	√	√	√	√	√
	Intracellular helix extension		√					
EL3	Disulpide bridge to TM6					√		
TM7	Extracellular helix extension							√
	Pro distortion	√	√	√	√	√	√	√
	Disulphide bridge to N-term							√

Table 1. Structural features observed in GPCR crystal structure

TM1
It is only Rho that has got a Pro in position 1.48. Pro1.48 causes a slight kink with a bend angle of 9º[44]. However, the sRho[125] causes a similar kink because of a Gly-Gly bulge. Even though, neither of hAA2AR, hCXCR4, hDRD3 contain either a pro nor a Gly-Gly bulge the extracellular ends of the receptors are also shifted towards the central axis of the receptor compared with hB2AR and tB1AR which does not. By causing this shift towards the central axis it packs the helices closer together.

The highly conserved asparagine Asn1.50, probably influences the conformation of GPCRs in TM1. It participates in an extensively hydrogen-bonded network with Asp2.50 in TM2 also involving several water molecules[45].

TM2
The TM2 runs parallel to TM3 from the cytoplasmic side of the transmembrane bundle and then just before the first extracellular loop the TM2 bends towards TM1, and leans away from TM3 due to a Gly-Gly bulge in the bRho and due to Pro2.59 and an insertion in sRho. The same Pro2.59 also provokes a shift towards the central axis in hB2AR, tB1AR and hDDRD3, the shift is even greater in the hAA2AR, probably because the TM1 is more closely packed than in hB2AR.

EL1
In the hB2AR and tB1AR, there is a Trp3.28 that seems to be engaged in aromatic-aromatic interaction with a Trp in the middle of EL1. This restrains the EL1, and keeps EL1 pulled down to the helical bundle.

TM3
A conserved disulphide bridge between TM3 and EL2 is observed in all the structures. The ionic lock[13-46] between TM3 and TM6 is only present in the bRho and hDRD3. The disruption of the ionic lock in the hB2AR might be due to the incorporation of a sulfate ion that is forming an ionic bond with Arg3.50.

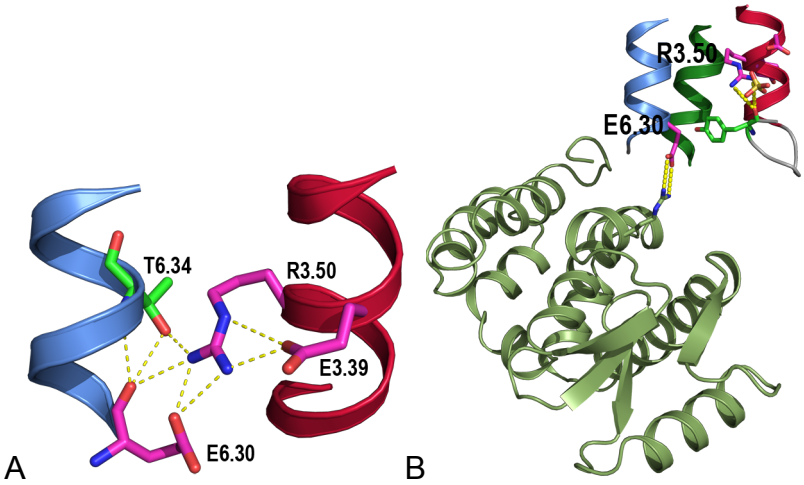


Figure 5. The ionic lock in: A) bRho and B) hB2AR

IL2

The intracellular loop 2 is helical in hA2AAR, tB1AR and the hDRD3 but is coil-like in the other receptors. These three receptors contain a Tyr in the loop that forms a hydrogen bond with the Asp3.49 in the DRY-motif. The hA2AAR and tB1AR also have an Arg4.41 stabilizing the alpha helical conformation by interacting with the backbone of IL2. This probably helps stabilizing the loops in a helical formation; another contributing factor is the length of the loop.

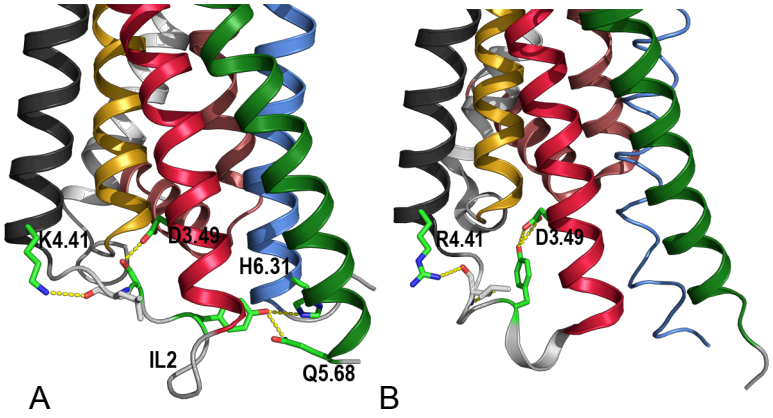


Figure 6. IL2 in A) hB2AR and B) AA2AR

TM4
A pro kink was observed in all crystal structures. The highly conserved Trp4.50 seems to be interacting with a partner in TM2 and probably serving to help stabilizing packing among these helices. In the crystal structure of hB2AR[31], there were cholesterols found to interact with the protein. Cholesterols were interacting between TM2, TM3 and TM4, this site seems to be conserved across multiple members of class A GPCR (cholesterols at this site were also observed in other crystal structures[16,21,31]), thus more proteins can be expected to contain cholesterol at this site.

EL2
A conserved disulfide bridge to TM3 is observed in all the structures. The hA2AAR contains many disulfide bridges, and they seem to stabilize a short helical segment of EL2, but the tip of EL2 is highly flexible and not observed in the electron density maps. In s- and b-Rho this extracellular loop forms a β-strand and hovers very low over the binding pocket, closing its access from the extracellular side, in the hB2AR, tB1AR, AA2AR and hDRD3 have a completely different topology that renders the pocket open and readily accessible by the ligands and all except the dopamine receptor include a short alpha-helical segment. Comparing the rhodopsin receptors with the adrenergic and adenosine receptors, one see that the topology is unique and has probably something to do with ligand selectivity and ligand binding.

TM5
A pro kink was observed in all the structures. There are several residues that seem to be important for ligand interaction and ligand selectivity (for example in positions 5.42 and 5.46). Then there are many Phe, which either interacts with ligands and/or stabilize an interaction with the TM6.

IL3
In the bRho the very conserved Y5.58, may be involved in stabilizing the inactive conformation of IL3, keeping it pulled upward toward helices 5 and 6 through hydrogen-bonding and/or π–π stacking interactions.

TM6

A pro kink was observed in all the structures. A conserved water molecule is observed in the sRho, tB1AR, hAA2AR, CXCR4, hB2AR that is found in close contact to this Pro6.50. It probably forms an important architectural element in formation of the bend in TM6.

EL3

The hA2AAR contains an intraloop disulfide bridge, were its function might be to structurally constrain this receptor to form multiple ligand binding interactions[4].

TM7

A pro distortion was observed in all the structures. A recent simulation of rhodopsin suggested that cholesterol-protein interactions might modulate the kink angles of TM7[47]. Close to the NPxxY-motif there are important structural waters observed in the crystal structures: hB2AR, bRho, sRho, hAA2AR, tB1AR, CXCR4.

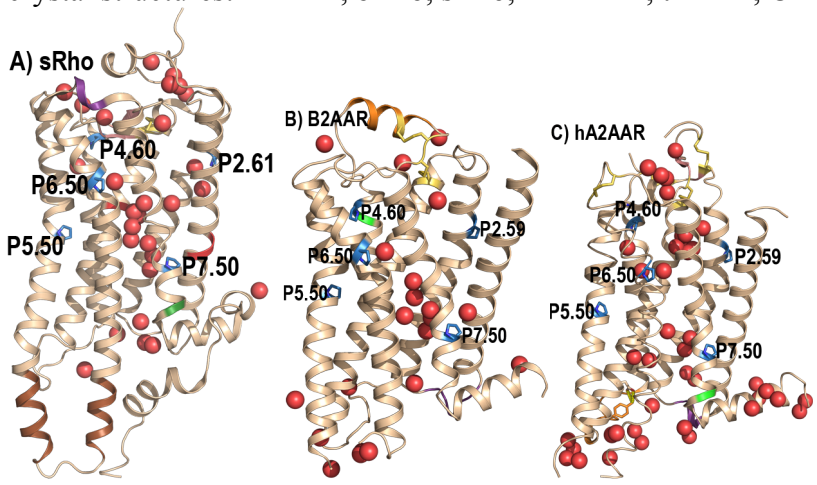


Figure 7. Structural features identified as seen in **¡Error! No se encuentra el origen de la referencia.**. A) bRho B) hB2AR C) hAA2AR. Water shown as red spheres. Pro distortions shown in blue, insertions green, Gly distortion in red. Disulphide bridges are shown in yellow. A loop formed as an alpha helix are shown in orange and 3_{10} in purple. Extensions of the TM is shown in Brown.

1.1.7 GPCR activation

G-proteins are comprised of a Gα subunit and dimeric Gβγ subunit. In total there are now approximately 20 known Gα, 6 Gβ and 11 Gγ subunits[48]. These are grouped into four subclasses: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$[49]. Interesting is that G-protein-mediated signaling, activation of GPCRs also leads to biochemical events that do not involve G-proteins[50]. GPCRs should therefore more correctly be called seven transmembrane receptors.

The activation of a receptor can be divided into two parts. The first part is how the cognate ligand or a synthetic ligand is recognized by the extracellular part of the receptor. The second part is the process that propagates the signal from ligand binding site to the amino acids of the cytoplasmic side of the TM bundle. This conformational change facilitates an interaction between an intracellular domain of the receptor and the heterotrimeric GTP-binding protein (G-proteins)[51]. In the inactive state, the receptor is coupled to G-protein that has GDP bound within its Gα subunit. The G-protein is activated and dissociates from the receptor into the active GTP bound Gα subunit and the Gβγ dimeric and interacts with intracellular effectors to produce the biochemical signals that are measured following receptor activation[52].

The activated Gα-GTP subunit and the Gβγ dimeric subunit can then engage a variety of enzymatic effectors within the cell. The signaling is terminated when an intrinsic GTPase hydrolyzes the GTP bound to the Gα subunit. The Gα-GDP complex then re-associates with the Gβγ subunit, and this heterotrimer can bind to the unliganded ground-state receptor again, and awaits further activation/inactivation cycles[52].

The classical view was that agonists activated receptors to produce a single signal or perhaps multiple signals but with comparable efficacy. More recently, it has become apparent that different ligands can have different degrees of efficacy in different signaling pathways. It is now believed that when agonists with different molecular structures bind to the receptors, they induce and stabilize different ligand-receptor conformations. In addition, induces different signaling pathways in the proteins and may also ultimately activate different cellular responses.

1.1.8 Active structures of GPCRs

A more clear understanding of the mechanisms that shift the equilibrium of the ensemble to the "active" conformations has been possible thanks to the recent crystal structures of ops*, metaII, brho*, hB2AR*, tB1AR* and hAA2AR*.

		Rho*	Ops*	MetaII	hB2AR*	B1AR*	hAA2AR*
TM1	Pro distortion		√	√			
	Helix shift towards the central axis	√	√	√			√
TM2	Pro distortion				√	√	√
	Gly-Gly distortion	√	√	√			
EL1	Disulphide bridge to EL2						√
	Beta-strand (indicated by above disulphide bridge)						√
TM3	Conserved Cys fors disulphide bridge to EL2	√	√	√	√	√	√
	Second disulphide bridge to EL2						√
	Bend caused by disulphide bridge between TM3 and EL2						√
IL2	Helical					√	√
	Tyr forming h-bond to Asp in DRY-motif					√	√
TM4	Pro distortion	√	√	√	√	√	√
	Bulge due to insertion				√	√	
	Gly bend						
EL2	Disulphide bridge to TM3	√	√	√	√	√	√
	Beta-sheet	√	√	√			
	Alpha helix				√	√	
	Beta-strand						√
	Intra EL2 disulphide bridge				√	√	
	Disulphide bridge to EL1						√
	Second disulphide bridge to TM3						√
TM5	Pro kink	√	√	√	√	√	√
	Helix extension	√	√	√			
IL3	Partial structure				√		√
TM6	Pro kink	√	√	√	√	√	√
	Helix extension	√	√	√			
EL3	Disulpide bridge to TM6						√
	3_{10} helix						
TM7	Pro kink	√	√	√	√	√	√
	Disulphide bridge to TM1-Loop						

Table 2. Structural features observed in the active GPCR crystal structures.

Comparison of this "active" ops*, metaII and rho*, with the structure of "inactive" bRho leads to the conclusion that during the process of GPCR activation the TM3 rotates clockwise (viewed from the intracellular side), the intracellular part of TM6 tilts outwards by 6-7 Å, TM5 approaches TM6, and Arg3.50 within the DRY-motif in TM3 adopts an extended conformation pointing towards the protein core[17,53]. The salt bridge between

Arg3.50/Glu6.30 is broken. Arg3.50 switches conformation and is stabilized by Asp3.49 and other local polar residues. Which leads to a disruption at the bottom of TM6, where the hydrogen bond between Tyr5.58 and TM6 is disrupted, allowing it to move towards residue 5.62, which affects the conformation of IL3, and the alpha subunit of the G protein can enter.

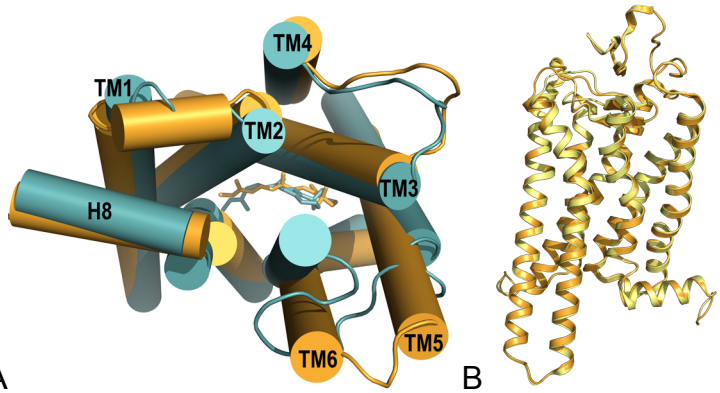


Figure 8. A) Superposition of rhodopsin in light teal and Meta II in orange viewed from the cytoplasmic surface. Helices are shown as cylinders and some loops have been removed for clarity. The TM5 and especially the TM6 have moved upon receptor activation. Very tiny in the middle are the retinals, which have twisted upon receptor activation. B) Superposition of Meta II in orange and opsin in yellow. The position of the carbon alpha is almost identical, just some loops that vary.

Structural changes observed comparing hB2AR with hB2AR* at the cytoplasmic domains involve an outward movement of TM6 and inward movement of TM3 and TM7[126]. However, these are relatively small compared to the changes observed in rhodopsin described above. In addition, comparing hAA2AR and hAA2AR* one also observe a movement of TM5, an outward tilt and rotation of the cytoplasmic half of TM6, and an axial shift of TM3. The seesaw movement of TM7 and a shift of EL3 are according to the author[23] likely specific to A2AAR and its ligand.

Active states are also accomplished by the rearrangement of side chains forming different networks of interactions between helices, often named microswitches[54]. For example the non-conserved side chain at position 3.36 has been suggested to act as a toggle switch simultaneously with W6.48 [55-57] see paper 3. In this mechanism, the side chain at position 3.36 moves away from TM 6 and reorients towards TM 7 while W6.48 breaks its water-mediated hydrogen

bond interactions with TM 7 and reorients towards TM 5. These switches are provoked by the ligand-encoded extracellular signal propagated at the binding site into intracellular microdomains known to be important in receptor activation.

1.1.9 Ligand interaction

The binding site crevice is located between the extracellular parts of TMs 3, 5, 6, and 7 and EL 2 for bRho, tB1AR (Figure 8C) and hB2AR (Figure 8B), hDRD3 (Figure 8F), and AA2AR (Figure 8D). In contrast, IT1t binds CXCR4 through TMs 2, 3, and 7 and EL 2 (Figure 8E). Another difference found is the hAA2A where the ligand is binding the receptor in an extended conformation and perpendicular to the plane of the membrane (Figure 8D). In all these crystal structures, EL 2 defines the binding site crevice as has been previously proposed by a study using site-directed mutagenesis and applying the cysteine-substituted accessibility method (SCAM)[58]. However, EL 2 is highly variable in length, amino acid content, and structure among available crystal structures[59]. EL 2 of rhodopsin, formed by two β-strands, buries the binding site from the extracellular environment (Figure 8A), whereas EL 2 of hCXCR4, also formed by two β-strands, fully exposes the binding site to the extracellular environment (Figure 8E). In contrast, a helical segment forms EL 2 of the tB1AR and hB2AR (Figures 8B and 8C). This a-helix is probably not conserved in the other members of the biogenic amine receptor family, as it was not found in the structure of the hDRD3 (Figure 8F). Each receptor subfamily has probably developed a specific EL 2 to adjust the structural characteristics of its cognate ligands. EL 2 plays a key role for the selective affinity of a drug for a given receptor, and, thus, it is highly relevant for structure-based drug design.

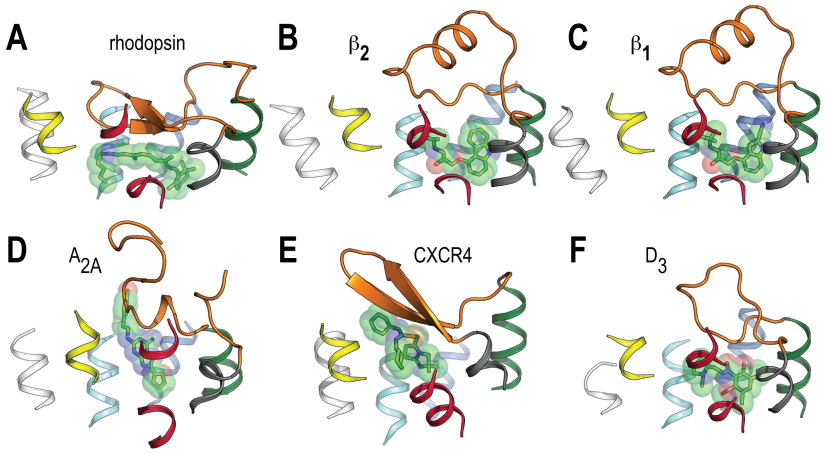


Figure 9. Detailed view of the binding site crevice for bRho (A), hB2AR (B), tB1AR (C) hAA2A (D), CXCR4 (E), and hDRD3 (F). Extracellular loops are not shown with the exception of EL 2 in orange. Ligands are shown as spheres. The color code of the helices is TMs 1 in white, 2 in yellow, 3 in red, 4 in gray, 5 in green, 6 in dark blue, and 7 in light blue.

Comparing the ligand-binding pocket of the partial inverse agonist carazolol bound hB2AR with the active state agonist BI-167107 bound receptor. The greatest difference between the inactive and active structures in the ligand-binding site is an inward bulge of TM5 centered around Ser5.46, and there are a smaller inward movements of TM6 and TM7. The agonist BI-167107 and the inverse agonist carazolol binding interaction are very similar. The major difference is the interactions between the heterocycle of the agonist and Ser5.46, Ser5.42 and Asn6.55. In the inactive structure there is just one polar interaction with Ser5.42 and the carazolol heterocycle. The Tyr7.43 is in the inactive state stabilizing the receptor by interacting with Asp3.32. There is no change seen in the side chain rotamer of Trp6.48, but its position slightly shifts[22].

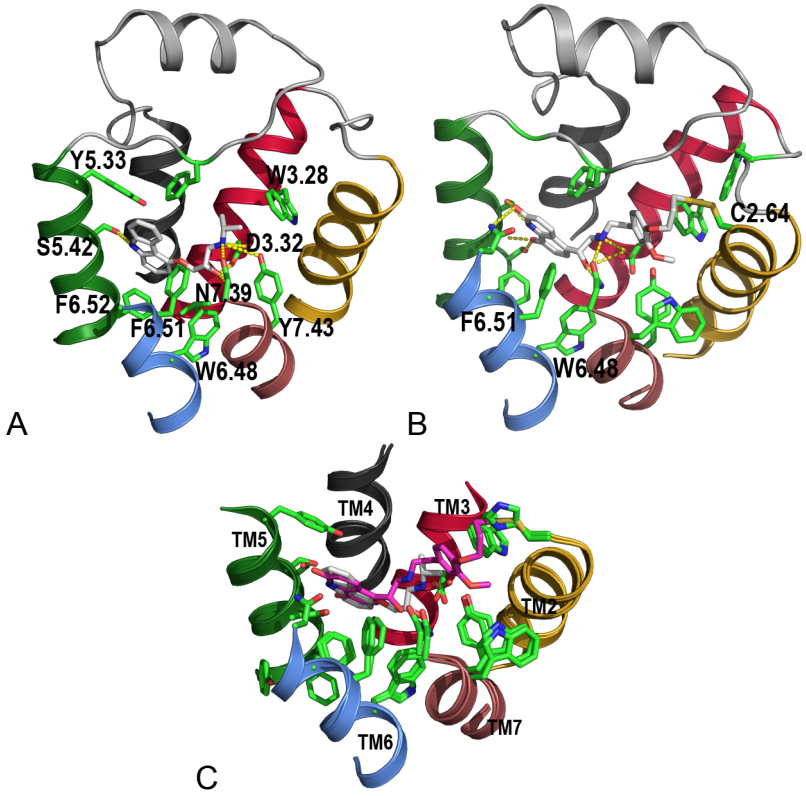


Figure 10. A) Ligand binding pocket of hB2AR. Carazolol is a partial inverse agonist with a binding affinity that slightly reduces the basal activity of the receptor. Carazolol interacts with Phe6.51, and Phe6.52. B) Binding pocket of the activated hB2AR by BI-167107, which is a full agonist. The carbonyl oxygen, amine and the hydroxyl groups on the heterocycle of BI-167107 are interacting with Ser5.42, Ser5.46 and Asn6.55, and Tyr7.35. C) Superposition of hB2AR* on hB2AR, Carzolol in white and BI-16707 in pink. The color code of the helices is TMs 2 in yellow, 3 in red, 4 in gray, 5 in green, and 6 in blue.

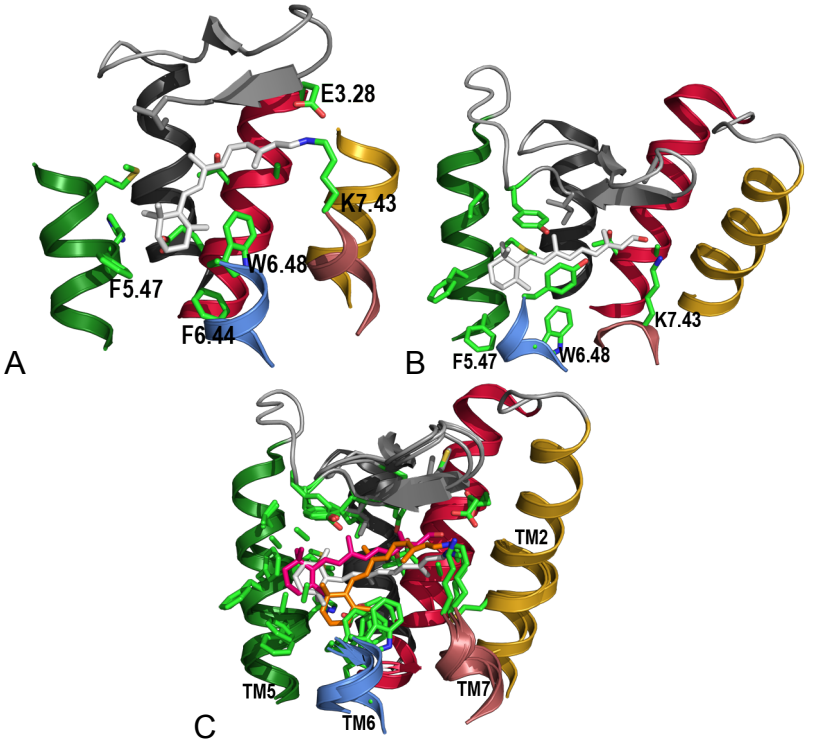


Figure 11. A) Binding pocket of bRho. 11-*cis*-retinal is a full inverse agonist covalently bound to K7.43. ß-ionone ring of retinal extends deep into the binding pocket, where it interacts with highly conserved Trp6.48, this might be why it is an inverse agonist. B) The translocation of the ß-ionone ring of retinal seems to lead to a rotation of TM6, which is the critical conformational change on activation. C) Superposition of bRho (retinal in white), metaII (retinal in orange), and Rho* (retinal in pink). The translocation of the ß-ionone ring leaves room for the move of Trp6.48. The color code of the helices is TMs 2 in yellow, 3 in red, 4 in gray, 5 in green, and 6 in blue.

In the tB1AR* full agonist forms hydrogen bonds with two conserved serine residues in TM5, namely Ser5.42 and Ser5.46. However partial agonists only interact with Ser5.42. The difference between the active and inactive tB1AR concentrating on the binding pocket is that Ser5.42 and Ser5.46 undergoes a rotamer conformational change when the receptor is binds to an agonist.

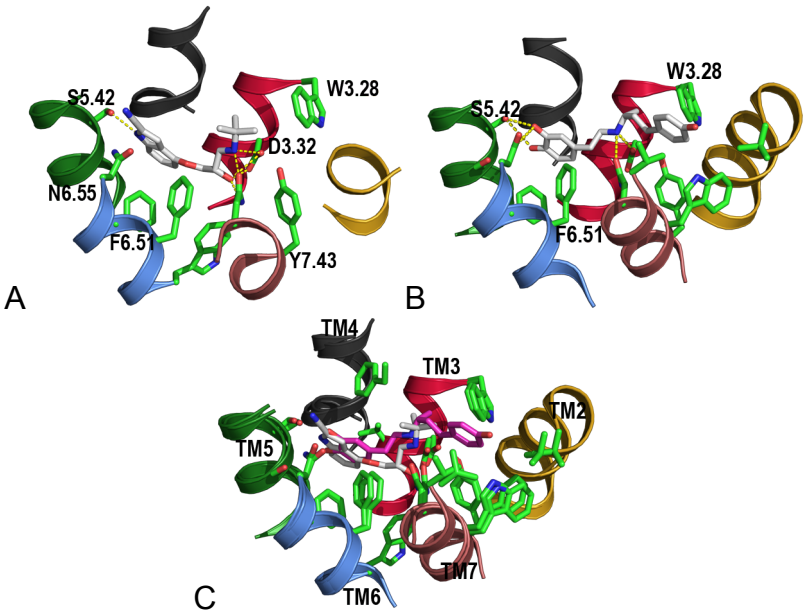


Figure 12. A) inactive and in B) active tB1AR. The secondary amine and β –hydroxyl groups forms potential hydrogen bonds with Asp3.32 and Asn7.39, and the meta-hydroxyl forms a hydrogen bond with Asn6.55. C) superposition of tB1AR* (carmoterol in pink) on tB1AR (cyanopindolol in white). The color code of the helices is TMs 2 in yellow, 3 in red, 4 in gray, 5 in green, and 6 in blue.

1.2 Serotonin receptors

Serotonin receptors are typical group A rhodopsin-like G protein-coupled receptors in that they are predicted to possess seven transmembrane spanning helices, three intracellular and three extracellular loops, an extracellular amino terminus a an intracellular carboxy terminus. The serotonin receptor family is larger than any other GPCR neurotransmitter receptors: 13 distinct genes encoding for receptors in this class. In addition, there is one ligand-gated ion channel; the 5-HT_3 receptor. The true structures of these receptors remain unknown, although thinking of how many crystal structures that have been released lately provides promise for the solution of the structures of the serotonin receptors in the near future. Functionally, at the extracellular part it binds a ligand, especially the endogenous ligand serotonin, which passes on a signal.

Serotonin, also known as 5-hydroxytryptamine, is classically classified as a biogenic monoamine. It contains an ethylamine moiety linked to an aromatic ring system. Serotonin is synthesized in serotonergic cells from the aromatic amino acid tryptophan by the successive action of tryptophan hydroxylase and aromatic amino acid decarboxylase (Figure 13).

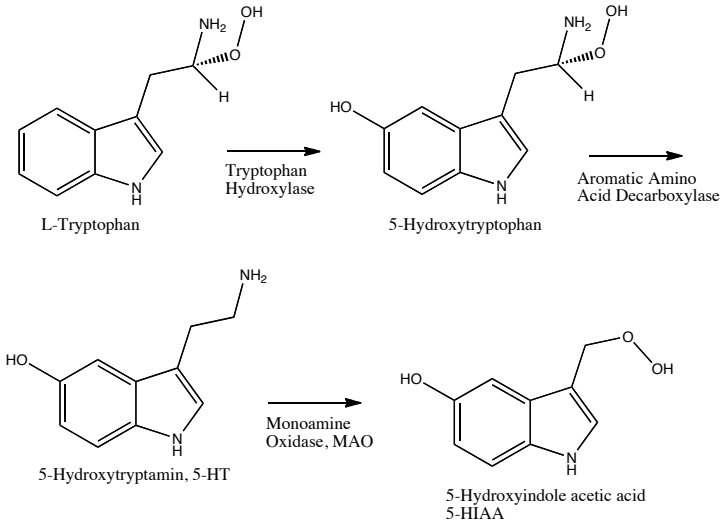


Figure 13. 5-HT is biosynthetically derived by two enzymatic steps: Ring hydroxylation of the essential amino acid tryptophan hydroxylase, the rate limiting step[60], and then side chain decarboxylation by aromatic amino acid decarboxylase.

In the brain, serotonin is produced within axon terminals, where it is released in response to an action potential and then diffuses across the synapse to activate postsynaptic receptors.

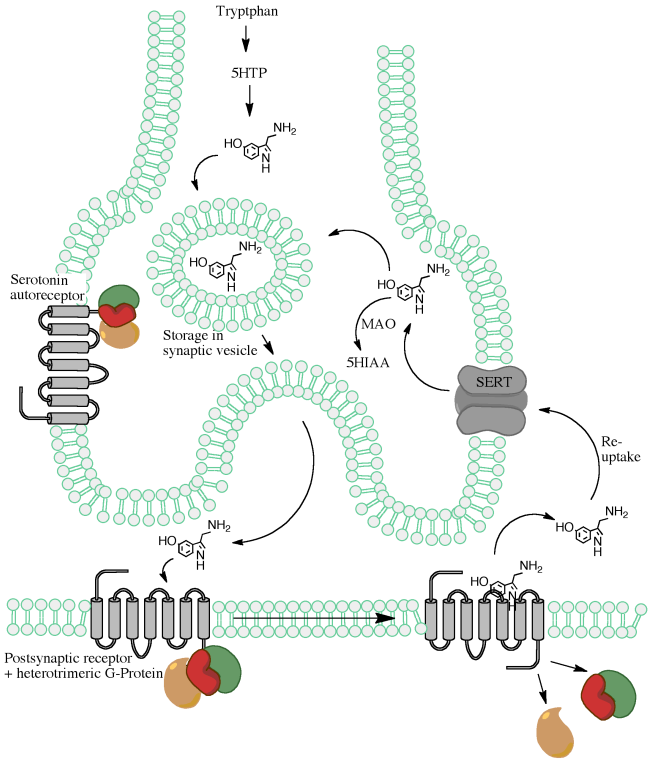


Figure 14. Model of a serotonergic synapse. After the synthesis, serotonin is packaged into vesicles. An influx of calcium, and fusion of the vesicle with the terminal membrane occurs after an axon potential reaches the terminal region, and provokes a membrane depolarization. Serotonin is released into the synaptic space, where it diffuses across to activate postsynaptic receptors, initiating the signal cascades within the cell. When the job of serotonin is finished it is extracted from the synapse by the serotonin reuptake protein (SERT). The SERT pumps the free serotonin back into the neuron terminal, where it is repackaged in the vesicles, to repeat the cycle. Serotonin that is free in the cytoplasm and not stored in vesicles is deaminated by monoamine oxidase in the mitochondrial membrane to produce the biologically inert metabolite 5-hydroxyindole-3acetic acid (5-HIAA).

GPCRs as a protein family are believed to have evolved about 1.2 billion years ago. Significantly, serotonin receptors appears to be among the oldest receptors within the rhodopsin-like family, and predates the evolution of muscarinic, dopaminergic, and adrenergic receptor systems[61-62].

As a result of this long evolutionary history, serotonin plays a variety of roles in normal physiology. Most of the serotonin in mammals is found within the gut, produced principally by enterchromaffin cells. It is also stored within blood platelets. The serotonin in the central nervous system has proven to have a

number of varied and extremely important functions. In mammalian species, serotonin in the brain arises from specialized groups of cell bodies known as the raphe nuclei, located in the brainstem reticular formation[63].

There are six classes of G protein coupled 5-HT receptors, namely 5-HT_1 - 5-HT_7 (with the exception of the 5-HT_3 receptor, which is a ligand gated ion channel). These classes are further subdivided as follows. The 5-HT_1-receptor class contains the 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptor subclasses. The 5-HT_2-receptor class contains the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. The 5-HT_5-receptor class contains the 5-HT_{5A}, and 5-HT_{5B} receptor subclasses. The 5-HT_4, 5-HT_6 and 5-HT_7 classed do not contain subclasses of receptors and sequence diversity in these classes is provided mainly by alternative mRNA splicing[64].

a) 5-HT_4

The receptor was discovered in mouse colliculi neuronal cells and in the guinea pig ileum in the end of the eighties (Dimuis et al, 1989) and early nineties (Hoyer et al, 1994). It is expressed both in the central nervous system (CNS) and in the periphery. Within the CNS the human 5-HT_4 receptor can be found in the striato-nigral system, notably in the caudate nucleus, lenticular nucleus (putamen and globus pallidus) and the substanitia nigra, hippocampus and the frontal cortex[65-66].

The 5-HT_4 receptor is involved in a variety of pathological disorders such as irritable bowl syndrome, gastroparesis, cardiac atrial arrhythmia, dysfunction of the urinary tract, and memory deficit.

b) 5-HT_6

The human 5-HT_6 receptor was cloned in 1996[67] as a gene codifying a polypeptide chain of 440 amino acids[67-68] that is positively coupled to the adenenylate cyclase[69-70] cascade via the G_s protein.

The high affinity of several antipsychotics and antidepressant agents[67-71] boosted the first studies exploring the 5-HT_6 receptor potential for the treatment of schizophrenia and bipolar affective

disorder. However, contradictory results were obtained about the role of the receptor in these therapeutic indications. Later on, 5-HT_6 receptor function was associated with the control of cholinergic neurotransmission[72-73], which prompted much interest into the possible implication of the receptor in cognitive impairment[74] (memory and learning) related the neurological diseases such as Alzheimer's. In the last five years, it has been shown that selective 5-HT_6 receptor has emerged as a promising target for the treatment of obesity and related metabolic syndrome[74-75], a disease with an increasing global prevalence and high unmet clinical need. There is much evidence that the human 5-HT_6 receptor is involved in the pathogenesis of CNS diseases[74-76] related to cognitive or eating disorders.

It is known to be expressed almost exclusively in the CNS[71, 77-79] and mainly in the olfactory tubercle, striatum, nucleus accumbens, cortex, olfactory tubercle and hippocampus[78, 80-81], it is possible that new therapeutic agents targeting this receptor might have relatively few peripheral side effects.

c) 5-HT_7

The 5-HT_7 receptor was also recently discovered[82-83], it was first cloned in 1993[69, 77] and is positively coupled to adenylyl cyclase via Gs protein.

Recent distribution studies in the brain have revealed a high abundance of 5-HT_7 receptor protein in hippocampus, thalamus, hypothalamus and cerebral cortex[84]. In the hypothalamus, it is mainly expressed in the suprachiasmatic nucleus, which functions as a circadian clock. It is thus a potential target for sleep disturbances[85], such as jet lag, and for related psychiatric disorders such as mental fatigue or depression[86]. The presence in the hypothalamus also correlates with its involvement in thermoregulation and endocrine function[82]. In addition, the significant density of the 5-HT_7 receptor in the hippocampus accounts for its role in learning and memory[87]. The 5-HT_7 receptor subtype has also been found in smooth muscle cells and in blood vessels of the skull and other peripheral tissues[88-89], so it is suggested as a putative target for migraine[90] and irritable bowel syndrome[91] treatments.

All together, many important functional roles for the 5-HT$_7$ receptor have been reported in various pathophysiological processes. However, there have not been many 5-HT$_7$ receptor ligands in clinical development programs, and current and future research should determine whether a ligand of this receptor is suitable as a therapeutic agent.

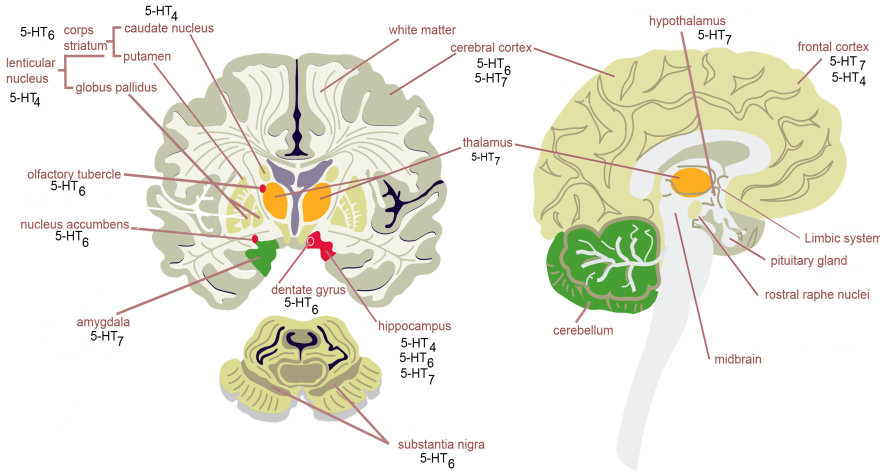


Figure 15. The distribution of the receptors: 5-HT$_4$, 5-HT$_6$ and 5-HT$_7$ in the brain.

1.4 Serotonin receptor activation

The serotonin receptors are activated by fairly small compounds, like their endogenous ligand serotonin. For serotonin to activate a receptor (Figure 14) it first needs to be synthesized. After the synthesis serotonin is packed into vesicles to avoid being deaminated. When this vesicle reaches the terminal region it is depolarized. This depolarization at the serotonergic axon terminals causes an influx of calcium ions and fusion of serotonin-containing vesicles with the cell membrane (Figure 14). The serotonin is released and diffuses across the cell synaptic space, where it interacts with receptors located on the postsynaptic membrane. Presynaptic autoreceptors can also respond to the presence of serotonin and regulate synthesis and release within the presynaptic axon terminal. The serotonin is cleared from the synapse by a specialized reuptake protein, comprised by a bundle of 12 membrane-spanning α-helices. Once inside, serotonin can be

repackaged into vesicles for rerelease. Monoamine oxidase is located in the mitochondrial outer membrane deaminates any transmitter molecules that are not stored in vesicles.

Binding of serotonin to one of its receptors leads to activation of heterotrimeric GTP-binding proteins (G-proteins) within the cell that are coupled to the intracellular loops and C-terminus of the GPCR. In addition, activation of $G\alpha_s$, which is the case of 5-HT_4, 5-HT_6, and 5-HT_7 receptors, leads to stimulation of adenylyl cyclases, resulting in the conversion of ATP to cyclic AMP (cAMP).

5-HT_4	G_s, G_{12}, G_{13}, G_q, G_i[52]
5-HT_6	G_s
5-HT_7	G_s, G_{12}

1.5 Serotonin receptor oligomerization

GPCRs were for a long time considered as monomeric proteins, and oligomerization of GPCR has been intensely debated, but is now widely accepted. In a number of studies they have revealed dimerization/oligomerization of GPCRs [92-100]. Although, it is quite clear that oligomers do exist, many studies indicate that class A GPCRs do not need to dimerize to transduce signals [101-107]. Their physiological role is not completely understood, but it has been proposed to play roles in post-translational processing and trafficking to the plasma membrane, and in generating functional diversity through hetero-oligomerization[108] and of cell surface targeting of GPCRs[109]. Some GPCRs seem to form stable oligomeric complexes, while other spend most of their time wandering around alone. However, the signal might be different when in complex or alone, for example shifting from G-protein to B-arrestin coupling[109].

There is now evidence that serotonin receptors form dimers in both cell culture and endogenous systems [110-115].

Although, the physiological significance of GPCR oligomerization is not entirely understood, research in this field is rapidly growing

2. METHODS

2. METHODS

In this section some of the bioinformatics tools used within this thesis are presented.

2.1 Homology modeling of GPCRs

Rhodopsin has served for years as the base for the construction of 3D models of the other members of the superfamily with unknown 3D structure through an *in silico* technique known as homology modeling. This method is based on assuming that the template and target proteins will have reasonable structural similarity as long as they have similar origin[116-117]. Meaning that through evolution, proteins substantially conserved their 3D structure, even to a higher degree than their amino acid sequence. As a result, homologous proteins, proteins that descend from a common ancestor, closely resemble each other from the 3D perspective. However, for about 7 years there was nothing that confirmed that this was true for the family A of GPCRs until the release of the hB2AR (and then followed by several other receptors: tB1AR, hAA2AR, hDRD3). These data confirmed the hypothesized structural homology of family A GPCRs, revealing a very good overlap of the helical bundles of the crystallized receptors. Not so surprisingly, more pronounced differences were instead noted at the level of the extracellular and intracellular loops that connects the TMs. Among these, of particular pharmaceutical relevance is the second extracellular loop (EL2), which connects the extracellular ends of the forth and fifth TMs (TM4 and TM5) and for many receptors has been suggested to be implicated in ligand binding[118]. The loops and TM were therefore modeled in a different way.

The modeling process was carried out in six steps:

1) *Identification of known templates related to the protein to be modeled.*
Which template/s to use is becoming more and more difficult because of all the new crystal structures? One should take into account, the overall sequence identity, and the sequence identity at the binding pocket, structural similarity to ligands, homologous

proteins, and resolution of the crystal structure.

2*) Alignment of the protein sequences with the template*
Usually the sequence identity is low so an automatic sequence alignment could become problematic. However, since the family A GPCRs shows a large number of conserved sequence patterns, the alignment can be reliable even though the sequence identity is low. At least one amino acid is remarkably conserved in each transmembrane: N in TM1 (98%), D in TM2 (92%), R in TM3 (97%), W in TM4 (96%), P in TM5 (77%), P in TM6 (98%), and P in TM7 (96%).

3) *Building of the model*
Only the structure of the transmembrane bundle is likely to be conserved in the class A of GPCRs. Therefore, the sequence of the transmembrane region of the target receptor is superimposed on the template. The superimposition is done using the amino acid sequence alignment between template and the target GPCR, and the highly conserved motifs in each transmembrane are used as anchors.

4) *Optimization of the model*
The positions of the sidechains are optimized using an energy minimization protocol, which will lead the system to the nearest energy minimum, basically through the optimization of van der Waals and electrostatic interactions.

5) *Modeling of the loops*
It has been demonstrated that the extracellular loops, specifically EL2, also interact with low-weight ligands (not just high-molecular-weight peptidic ligands). Furthermore, several site-directed mutagenesis studies have highlighted the effects of mutations in EL2 on agonist and antagonist binding, reinforcing the importance of this region for ligand binding and structural integrity[119]. The accurate modeling of the loops is therefore quite important for understanding ligand recognition and functional attributes of the structure, as well as for dockings. If the structures of membrane proteins are similar comparative modeling is a potential strategy. It is a true challenge to model loops but can be modeled with reasonable confidence if they bear similarity in length with the template. In addition, if it has important anchor points like

disulphide bridges. If not, it would be necessary to use fragment-search based or *ab initio* based methods[120] for predicting these loop conformations.

6) *Validation of the model.*
The model is now checked manually in order to be sure that some key interactions are kept. In addition, experimental studies are taken into account, to adjust the side chain conformations.

2.3 Similarities/ differences to existing crystal structures

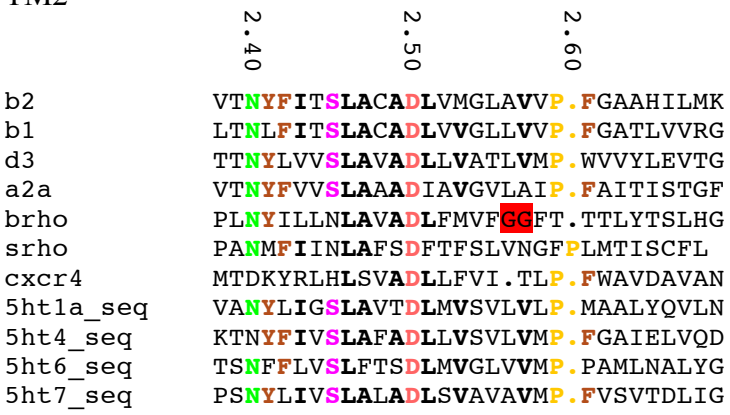
In the validation of the homology models, differences between the serotonin receptors 4, 6 and 7 in comparison to some family A crystal structures was of special interest.

TM1

```
                          1.40          1.50          1.60
b2              VVGMGIVMSLIVLAIVFGNVLVITAIAKFERLQTVTNYF
b1              EAGMSLLMALVVLLIVAGNVLVIAAIGSTQRLQTLTNLF
d3                 YALSYCALILAIVFGNGLVCMAVLKERALQTTTNYL
a2a             SSVYITVELAIAVLAILGNVLVCWAVWLNSNLQNVTNYF
brho            FSMLAAYMFLLIMLGFPINFLTLYVTVQHKKLRTPLNYI
srho            YYSLGIFIGICGIIGCGGNGIVIYLFTKTKSLQTPANMF
cxcr4                TIYSIIFLTGIVGNGLVILVMGYQKKLRSMTDKY
5ht1a_seq       QVITSLLLGTLIFCAVLGNACVVAAIALERSLQNVANYL
5ht4_seq        KVVLLTFLSTVILMAILGNLLVMVAVCWDRQLRKIKTNY
5ht6_seq        SGWVAAALCVVIALTAAANSLLIALICTQPALRNTSNFF
5ht7_seq        KVVIGSILTLITLLTIAGNCLVVISVCFVKKLRQPSNYL
```

The 5-HT_4, $5HT_6$ and 5-HT_7 does not contain a Pro1.48 nor a Gly-Gly bulge, however it probably prefers having the helices more closely packed together as in hAA2AR, hCXCR4, hDRD3, sRho, and bRho. Moreover, there are several conserved water molecules in the region close to Asn1.50, which are probably also conserved in the 5-HT_4, $5HT_6$ and 5-HT_7.

TM2



There are several Pro residues in TM2 in GPCRs, probably introducing structural changes. About 36% of the family A GPCRs contain a Pro residue at position 2.59, which induces a bend of the TM2 in the direction of TM1 and the central axis. This is probably the case in the 5-HT_4, $5HT_6$ and 5-HT_7 receptors because they all contain a Pro in this position.

TM2 and TM3

In bRho there is an interaction between TM2 and TM3 through the Gly2.57 and Leu3.27, which probably fixes this position and does not make it bend towards the central axis. However, in for example the hDRD3 we have first of all a Val in position 2.57 and then another bulky Val in position 3.27. It makes it impossible for the hDRD3 to form this interaction and we see a bend towards the central axis. The 5-HT_4, $5HT_6$ and 5-HT_7 also contains Val in positions 2.57 and 3.27, and are likely to bend in a similar way as in the hDRD3.

TM3

```
                      3.30          3.40          3.50
b2              FWCEFWTSIDVLCVTASIETLCVIAVDRYFAIT
b1              FLCELWTSLDVLCVTASIETLCVIAIDRYLAIT
d3              ICCDVFVTLDVMMCTASIWNLCAISIDRYTAVV
a2a             HGCLFIACFVLVLTQSSIFSLLAIAIDRYIAIR
brho            TGCNLEGFFATLGGEIALWSLVVLAIERYVVVC
srho            AACKVYGFIGGIFGFMSIMTMAMISIDRYNVIG
cxcr4           FLCKAVHVIYTVNLYSSVWILAFISLDRYLAIV
5ht1a_seq       VTCDLFIALDVLCCTSSILHLCAIALDRYWAIT
5ht4_seq        VFCLVRTSLDVLLTTASIFHLCCISLDRYYAIC
5ht6_seq        GLCLLWTAFDVMCCSASILNLCLISLDRYLLIL
5ht7_seq        FFCNVFIAMDVMCCTASIMTLCVISIDRYLGIT
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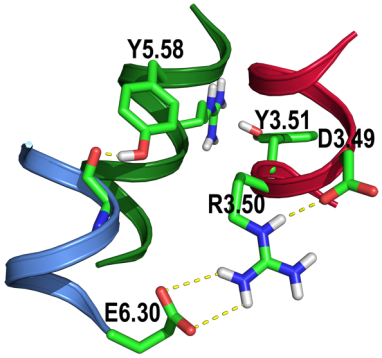

Figure 16. The ERY-motif in bRho.

It is very likely that in the 5-HT_4, $5HT_6$ and 5-HT_7 receptors, Y5.58 is in the inactive state interacting with the backbone in TM6. R3.50 is holding TM2, TM3 and TM6 tightly together by interacting with E6.30 and D3.49, a similar interaction as seen in the bRho. The $5HT_6$ contains a Trp in position 3.28 and in EL1 just like the hB2AR, and will probably pull down the EL1 and stabilize the association at the tops of TM2 and TM3. The 5-HT_7 contains a Phe in position 3.28, like the hDRD3 and will probably not pull down the EL1. The 5-HT_4 does not contain an aromatic in position 3.28 but an Arg, that most likely will stabilize the tops of TM2 and TM3 by interacting with Glu2.64.

IL2

The Arg4.41 and Tyr in loop IL2 seem to be important for stabilizing the alpha shaped helix of internal loop 2. In the 5-HT_4, and $5HT_6$ receptors we have these two residues so an alpha shaped helix is very likely. However, in the 5-HT_7 the Arg4.41 is missing just like in the hB2AR and the IL2 in the 5-HT_7 receptor is more likely to follow the shape of the hB2AR IL2.

```
TM4                4.40          4.50          4.60

b2                 KNKARVIILMVWIVSGLTSFLPIQM
b1                 RARAKVIICTVWAISALVSFLPIMM
d3                 CRRVALMITAVWVLAFAVS.CPLLFG
a2a                GTRAKGIIAICWVLSFAIGL.TPML
brho               ENHAIMGVAFTWVMALACAA.PPLVG
srho               HRRAFIMIIFVWLWSVLWAI.GPIFG
cxcr4              LLAEKVVYVGVWIPALLLT.IPDFI
5ht1a_seq          PRRAAALISLTWLIGFLIS.IPPMLG
5ht4_seq           PLRIALMLGGCWVIPTFISFLPIMQ
5ht6_seq           PLRALALVLGAWSLAALASFLPLLL
5ht7_seq           GKCMAKMILSVWLLSASIT.LPPLFG
```

All the crystal structures contain a Pro-kink in TM4. In the 5-HT_7 receptor two Pro residues can be found at positions 4.59 and 4.60 just like in the bRho, and a bend angle of about 35^{o44} can be expected, it is higher than the average Pro kink induced angle of about 20º. They all contain the conserved Trp4.50, which together whit Ser2.45, probably stabilize packing between the TM2 and TM4.

```
TM5                5.40          5.50          5.60

b2                 QAYAIASSIVSFYVPLVIMVFVYSRVFQEAKRQL
b1                 RAYAIASSIISFYIPLLIMIFVALRVYREAKEQ
d3                 PDFVIYSSVVSFYLPFGVTVLVYARIYVVLKQR
a2a                NYMVYFNFFACVLVPLLLMLGVYLRIFLAARRQL
brho               ESFVIYMFVVHFIIPLIVIFFCYGQLVFTVKEAA
srho               RSNILCMFILGFFGPILIIFFCYFNIVMSVSN
cxcr4              VVFQFQHIMVGLILPGIVILSCYCIIISKLSHS
5ht1a_seq          HGYTIYSTFGAFYIPLLLMLVLYGRIFRAARFRI
5ht4_seq           KPYAITCSVVAFYIPFLLMVLAYYRIYVTAKEHA
5ht6_seq           LPFVLVASGLTFFLPSGAICFTYCRILLAARKQA
5ht7_seq           FGYTIYSTAVAFYIPMSVMLFMYYQIYKAARKSA
```

The Pro5.50 is conserved to 77% in family A of GPCRs, and it is conserved in the 5-HT_4, $5HT_6$ and 5-HT_7 receptors. It induces a little bit smaller Pro-kink angles compared to the average angle of about 20º. This is because the positions 5.42 and 5.43 are apart of the binding site and they would be incorrectly oriented towards the lipid environment if TM5 were modeled as a regular Pro-kink helix[122].

TM6

	6.30	6.40	6.50	6.60
b2	KEHKALKTLGIIMGTFTLCWLPFFIVNIVHVI			
b1	REHKALKTLGIIMGVFTLCWLPFFLVNIVNVF			
d3	REKKATQMVAIVLGAFIVCWLPFFLTHVLNTH			
a2a	KEVHAAKSLAIIVGLFALCWLPLHIINCFTFF			
brho	AEKEVTRMVIIMVIAFLICWLPYAGVAFYIFT			
srho	AEMRLAKISIVIVSQFLLSWSPYAVVALLAQF			
cxcr4	QKRKALKTTVILILAFFACWLPYYIGISIDSF			
5ht1a_seq	RERKTVKTLGIIMGTFILCWLPFFIVALVLPF			
5ht4_seq	TETKAAKTLCIIMGCFCLCWAPFFVTNIVDPF			
5ht6_seq	KALKASLTLGILLGMFFVTWLPFFVANIVQAV			
5ht7_seq	REQKAATTLGIIVGAFTVCWLPFFLLSTARPF			

In TM6 the most conserved residue is a Pro, which pronounces a great kink in the TM bundle, it is also opened with more than 3.6 residues per turn. Because it is observed in all the crystal structures it is very likely that this kink is present in the 5-HT_4, $5HT_6$ and 5-HT_7 receptors. The $5HT_6$ receptor contains the DRY motif but is lacking the Glu6.30, instead it has an Ala in this position. This means the 5-HT_6 receptor lacks the ionic lock that is so conserved and that seems to be disrupted upon receptor activation. Not so surprisingly, in both human[123] and mice[68] this receptor have shown to have strong constitutive activity.

TM7

```
                        7.40        7.50
b2              KEVYILLNWIGYVNSGFNPLIYCR
b1              DWLFVAFNWLGYANSAMNPIIYCR
d3              PELYSATTWLGYVNSALNPVIYTT
a2a             LWLMYLAIVLSHTNSVVNPFIYAY
brho            PIFMTIPAFFAKTSAVYNPVIYIM
srho            PYAAQLPVMFAKASAIHNPMIYSV
cxcr4           HKWISITEALAFFHCCLNPILYAF
5ht1a_seq       TLLGAIINWLGYSNSLLNPVIYAY
5ht4_seq        GQVWTAFLWLGYINSGLNPFLYAF
5ht6_seq        PGLFDVLTWLGYCNSTMNPIIYPL
5ht7_seq        LWVERTFLWLGYANSLINPFIYAF
```

In helix 7 there is a Pro that is conserved to 96% in the family A of GPCRs. This Pro7.50 induces a kink in the helix, which is lower than the average Pro-kink induced angle of 20º. In the cytoplasmic end of TM7 next to Pro7.50 one can find the highly conserved residues N7.49 (77%), and Y7.53 (92%), which together form the NPxxY motif in the rhodopsin family of GPCRs[43].

EL3

Just like hDRD3 and hAA2AR the 5-HT_6 and 5-HT_7 receptors contains two Cys that most likely forms a disulphide bridge.

All this information was used in the validation of the models. Next step in the process was to study how ligands interact with these receptors. Residues in the binding pocket were especially studied and differences and similarities between the receptors helped to understand selectivity.

2.4 Serotonin ligand interaction

Like many other family A GPCRs, the 5-HT_4, 5HT_6 and 5-HT_7 receptors bind their cognate ligand serotonin through a conserved aspartic acid residue, Asp3.32. Then it seems like the indole ring of 5-HT expands toward TM5 and TM6 to hydrogen bond the key 5.43 and 6.55 side chains. However, it is always difficult to predict the exact binding site for a small, flexible ligand like serotonin. To predict bigger and bulkier structures as seen in papers 1-3 is usually an easier challenge.

2.2 Principles of molecular dynamics

To study the "jiggelings and wiggelings" (Richard Feynman) of atoms in the proteins either alone or in complex with a ligand molecular dynamics (MD) was carried out.

There are a number of steps when doing MD[121]:

1. Reading the coordinate set from the protein system.
2. Velocities to each atom are assigned, usually from a Boltzmann distribution at a given temperature.
3. Calculate forces. The force on each atom is calculated as the negative gradient of the potential energy function

$$F_i = -\frac{\partial E}{\partial \vec{x}_i} \qquad i=1, ..., N$$

where $\mathbf{F}_i$ is the force on the atom i^{th} and N - number of atoms. To calculate the potential energy (E) of the system it requires a force field. A force field is built up from two distinct components; a set of equations used to generate the potential energies and the parameters used in these equations. The force field computes the total energy as the sum of all the contributions over all the atoms in the system (bond elongation, angle and dihedral deformation, and non-bonded interactions) with the following general form:

$$E = \sum_{bonds} V^{str} + \sum_{angles} V^{bend} + \sum_{torsions} V^{tors} + \sum_{LJ} V^{LJ} + \sum_{coulomb} V^{coul}$$

There are different force fields used for simulating biological systems. The exact term describing the potential energy varies with each particular force field. The force field used depends on the system.

4. Successive coordinates and velocities are obtained by integrating the Newton's equation for the motion in each coordinate direction. In one dimension, the equation can be written as:

$$\frac{F_{xi}}{m_i} = \frac{\partial^2 x_i}{\partial t^2}$$

where m_i and x_i are the mass and position of each atom,

respectively and F_{xi} is the derivative of the potential according to a force field equation that is described in step 3. The result is a trajectory that shows how atomic positions and velocities evolve with time according to the influence of the remaining atoms in the system. Due to the large number of particles interacting with each other, the integration is performed numerically most commonly using the leap-frog algorithm (Van Gunsteren 1990). The integration step is limited to the fastest motion in the system. Therefore, for atomistic simulations the step size is usually 1 fs, or 2 fs if restraining bond lengths, generally done using SHAKE (Van Gunsteren 1977), which fix the vibrations of the fastest atoms (e.g. hydrogens) into place and LINCS (Hess 1997) algorithms.

3. OBJECTIVES

3. Objective

The main objective of this thesis is to improve the understanding of structure-function relationships of family A GPCRs, and in particular the serotonin 5-HT_4, 5-HT_6 and 5-HT_7 receptors. We aim to understand the structural determinants and changes associated with ligand binding, receptor activation and G protein coupling, and how the knowledge of these structural elements can be translated into predictive tools for the selection and design of molecules acting on receptors with a therapeutic potential.

Detailed objectives

1. We will use molecular modeling and computational simulation techniques to develop structural models of the 5-HT_4, 5-HT_6, and 5-HT_7 receptors built on the known crystal structure of inactive and active receptors. Experimental results derived from different receptors will be used to progressively improve the robustness of the structural models, and their predictive character.

2. These molecular models will raise hypothesis regarding the structural elements of both, the ligand and the receptor, involved in the binding of native ligands, antagonists or agonists. These hypotheses will be tested by our collaborators with molecular biological experiments and with new compounds developed and prepared by the organic chemists.

3. These molecular models will also raise hypothesis regarding the structural rearrangements associated with receptor activation and G protein coupling. These hypotheses will be tested by mutagenesis and functional assays.

4. RESULTS AND DISCUSSION

After the background and methodological introduction presented in the previous sections, here we will describe and discuss the results obtained in this thesis. A more detailed description of all outcome and the methods applied can be found in the publications attached in the next section.

Objective 1

> *We will use molecular modeling and computational simulation techniques to develop structural models of the 5-HT_4, 5-HT_6, and 5-HT_7 receptors built on the known crystal structure of inactive and active receptors. Experimental results derived from different receptors will be used to progressively improve the robustness of the structural models, and their predictive character.*

The first objective of this thesis was to generate 3D homology models of the 5-HT_4, 5-HT_6 and 5-HT_7 receptors. The homology modeling of all these receptors was carried out using the crystal structure of the hB2AR and ops*, which contains several distinctive features of the presumed active state, as templates. The different receptor sequences were aligned ensuring a perfect alignment of the highly conserved residues of the family A GPCRs. 3D models were then built using SCWRL[129] for the transmembrane part and MODELLER[130] for extracellular and intracellular loops. Subsequently, the obtained initial coordinates were optimized using AMBER[128]. Importantly, computational models of both inactive, basically based on the inactive conformation of hB2AR, and active, based on a combination of hB2AR and ops*, serotonin receptors were obtained.

We further validated our models through the construction of complexes with different ligands. Initial docking of the ligand was performed by interactive computer graphics using experimental data obtained in this work or by others as a guide. In a second step, the binding modes were refined using molecular dynamics simulations of the ligand-receptor complexes using AMBER. Details of this part of work are described in **publications 1, 2, and 3**.

Objective 2.

> *These molecular models will raise hypothesis regarding the structural elements of both, the ligand and the receptor, involved in the binding of native ligands, antagonists or agonists. These hypotheses will be tested by our collaborators with molecular biological experiments and with new compounds developed and prepared by the organic chemists.*

The computational simulations of the ligand receptor complexes are used to get a better understanding, at the molecular level, of the structure-affinity relationships and structure-selectivity relationships of a series of compounds synthetized for the receptor. Results of this work can be seen in **papers 1 and 2**.

In publication 1, a hB2AR-based homology model of the 5-HT_6 receptor was used to predict the mode of binding of antagonist SB-258585 and new synthesized ligands **1-20** at the group of Dr. López-Rodríguez at the Universidad Complutense de Madrid. These models served to design Ala substitution at Cys3.36, Ser5.43, Trp6.48, Phe6.52, and Asn6.55, to characterize the amino acid residues of the 5-HT_6 receptor involved in ligand binding. Substitution of Trp6.48, Phe6.52, or Asn6.55 by Ala, at the group of Dr. Claeysen at the Institut de Génomique Fonctionnelle, fully impedes compound **4** to block 5-HT-induced activation. Thus, we propose that Asp3.32 in TM3 anchors the protonated piperazine ring, the benzimidazole ring expands parallel to EL 2 to hydrogen bond Asn6.55 in TM 6, and the aromatic ring is placed between TMs 3 and 5 in CH2-containing compounds and between TMs 3 and 6 in CO-containing compounds.

In publication 2, a hB2AR-based homology model of the 5-HT_7 receptor was used to study the structural features of a series of compounds, synthetized at the group of Dr. López-Rodríguez at the Universidad Complutense de Madrid, responsible for the 5-HT_7/5-HT_{1A} receptor affinity and selectivity.
These types of studies provide the basis for the design and development of new ligands with predetermined pharmacological properties.

Objective 3.

> *These molecular models will also raise hypothesis regarding the structural rearrangements associated with receptor activation and G protein coupling. These hypotheses will be tested by mutagenesis and functional assays.*

Comparison of the structure of "active" opsin with the structure of "inactive" rhodopsin leads to the conclusion that during the process of GPCR activation the intracellular part of TM 6 tilts outwards by 6-7 Å, TM 5 nears TM 6, and Arg3.50 within the (D/E)RY motif in TM 3 adopts an extended conformation pointing towards the protein core, to interact with the highly conserved Tyr5.58 in TM 5 and Tyr7.53 of the NPxxY motif in TM 7. However, the mechanism by which binding of the extracellular ligand triggers these conformational rearrangements near the G-protein binding domain is not fully understood. In paper 3, we have shown for the 5-HT_4 receptor that these active states are accomplished by the rearrangement of side chains forming different networks of interactions between helices, often named microswitches. Using site-directed mutagenesis, performed at the group of Jöel Bockaert at the Institut de Génomique Fonctionnelle, and molecular modeling approaches we have shown the conformational arrangements at the ligand binding site occuring during stabilization of the receptor "active" induced by 5-HT, benzamides, and BIMU8.

5. PUBLICATIONS

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PUBLICATION 1

Benzimidazole derivatives as new serotonin 5-HT$_6$ receptor antagonists. Molecularmechanisms of receptor inactivation.

de la Fuente T, Martín-Fontecha M, Sallander J, Benhamú B, Campillo M, Medina RA, Pellissier LP, Claeysen S, Dumuis A, Pardo L, López-Rodríguez ML.

J. Med. Chem. 2010 Feb 11;53(3):1357-69

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PUBLICATION 2

Synthesis of new serotonin 5-HT7 receptor ligands. Determinants of 5-HT7/5-HT1A receptor selectivity.

Medina RA*, Sallander J*, Benhamú B, Porras E, Campillo M, Pardo L, López-Rodríguez ML.

J. Med. Chem. 2009 Apr 23;52(8):2384-92

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PUBLICATION 3

Conformational toggle switches implicated in basal constitutive and agonist-induced activated states of 5-hydroxytryptamine-4 receptors.

Pellissier LP*, Sallander J*, Campillo M, Gaven F, Queffeulou E, Pillot M, Dumuis A, Claeysen S, Bockaert J, Pardo L.

Mol Pharmacol. 2009 Apr;75(4):982-90

6. CONCLUSIONS

6. CONCLUSIONS

Serotonin 5-HT$_4$ receptor

1. We have shown that activation of the 5-HT$_4$ receptor involve different conformational toggle switches. Activation of WT 5-HT$_4$ receptor upon ligand binding is likely to be the result of a simultaneous double toggle switch. One is the conformational change of Trp6.48 from the inactive *g*+ (pointing towards TM7) to the active *t* (TM5) conformation, which is accompanied by the conformational transition of Thr3.36 from the inactive *g*- to the (TM6) to the active *g*+ (TM7) conformation. Both coordinated switches are necessary to go from silent Rg to the R*basal.

2. Different conformational arrangements occur during stabilization of R*basal, R*-5-HT, R*-benzamides, and R*-BIMU8.

    a) Thr3.36 in the 5-HT$_4$ receptor is fully required in the stabilization of the R*-benzamides [(S)-zacopride, cisapride and RS 67333]. We conclude that benzamides trigger activation by forming a specific hydrogen bond between the carbonylic oxygen of the ligand and the active *g*+ rotamer of Thr3.36.

    b) In the stabilization of benzimidazolone ligands such as BIMU8, we propose that R*-BIMU8 state is primarily reached after a direct interaction between the carbonylic oxygen of the ligand and Trp6.48.

    c) Finally, the natural agonist serotonin seems to activate the 5-HT$_4$ receptor by different activation pathways.

Serotonin 5-HT$_7$ receptor

3. We have analyzed the different structural elements of the ligands that influence their 5-HT$_7$/5-HT$_{1a}$ receptor binding affinity and selectivity. The major structural elements are:

a) Decreasing the distance between the protonated nitrogen and the hydrogen bond acceptor, forcing the ligand to bind Ser6.55, increases selectivity for the 5-HT_7 receptor.

b) Polar substitutions at the hydrophobic regions increases selectivity for the 5-HT_7 receptor due to their interaction with Arg7.36.

c) Increasing the size of hydrophobic regions, leading to a clash with Val2.61 in the 5-HT_7 receptor and favoring the interaction with Tyr2.64 in the 5-HT_{1a} receptor, decreases selectivity for the 5-HT_7 receptor.

Serotonin 5-HT_6 receptor

4. Site-directed mutagenesis and a homology model of the 5-HT_6 receptor were used to predict the mode of binding of antagonist SB-258585 and the new synthesized ligands. The protonated piperazine ring anchors Asp3.32 in TM3, whereas the benzimidazole ring is situated parallel to EL2, to hydrogen bond Asn6.55 in TM6. In CH2-containing compounds the Ar system is located between TM3 and TM5, to interact with Val3.33 and Ala5.42, whereas in CO-containing compounds, the Ar aromatic moiety is located between TM3 and TM6, interacting with Val3.33, Trp6.48 and Phe6.52.

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