Doctoral Thesis

Structural insights in the molecular causes of congenital stationary night blindness

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Structural insights in the molecular causes of congenital stationary night blindness

A thesis submitted to attain the degree of

Doctor of Sciences of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2014
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{280 \text{ nm}}$</td>
<td>Absorption at 280 nm</td>
</tr>
<tr>
<td>$A_{500 \text{ nm}}$</td>
<td>Absorption at 500 nm</td>
</tr>
<tr>
<td>ADRP</td>
<td>Autosomal dominant retinitis pigmentosa</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BE</td>
<td>Brain extract</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-Bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BME</td>
<td>Beta-mercaptoethanol</td>
</tr>
<tr>
<td>$C_8E_4$</td>
<td>Tetraethylene glycol octylether</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNBr</td>
<td>Cynogen bromide</td>
</tr>
<tr>
<td>CPM</td>
<td>7-Diethylamino-3-(4’-Maleimidylphenyl)-4-Methylcoumarin</td>
</tr>
<tr>
<td>COOT</td>
<td>Crystallographic object oriented toolkit</td>
</tr>
<tr>
<td>CSNB</td>
<td>Congenital stationary night blindness</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxy-terminal</td>
</tr>
<tr>
<td>DDM</td>
<td>n-dodecyl-β-D- maltopyranoside</td>
</tr>
<tr>
<td>DM</td>
<td>n-decyl- β -D-maltopyranoside</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotheritol</td>
</tr>
<tr>
<td>EBV</td>
<td>Epsteiner Barr virus</td>
</tr>
<tr>
<td>EL</td>
<td>Extracellular loop</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraaceticacid</td>
</tr>
<tr>
<td>$Em$</td>
<td>Emission</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>Ex</td>
<td>Excitation</td>
</tr>
<tr>
<td>$\varepsilon_{280 \text{ nm}}$</td>
<td>Extinction coefficient at 280 nm</td>
</tr>
<tr>
<td>$\varepsilon_{500 \text{ nm}}$</td>
<td>Extinction coefficient at 500 nm</td>
</tr>
<tr>
<td>For</td>
<td>Forward</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>G418</td>
<td>Geneticin</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanidine diphosphate</td>
</tr>
<tr>
<td>GnTI-</td>
<td>N-acetylglucosaminyltransferase 1- negative</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G protein-coupled receptors</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanidine triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanidine triphosphate-gamma-sulfate</td>
</tr>
<tr>
<td>HEK293 S</td>
<td>Human embryonic kidney 293 suspension</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HTG</td>
<td>Heptylthiogluicoside</td>
</tr>
<tr>
<td>IL</td>
<td>Intracellular loop</td>
</tr>
<tr>
<td>LDAO</td>
<td>n-dodecyl-N,N-dimethylamine-N-oxide</td>
</tr>
<tr>
<td>MR</td>
<td>Molecular replacement</td>
</tr>
<tr>
<td>NBCS</td>
<td>Newborn calf serum</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue Tetrazolium</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonyl phenoxypolyethoxylethanol</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Amino-terminal</td>
</tr>
<tr>
<td>OD&lt;sub&gt;280&lt;/sub&gt;</td>
<td>Optical density at the wavelength of 280 nm</td>
</tr>
<tr>
<td>OD&lt;sub&gt;500&lt;/sub&gt;</td>
<td>Optical density at the wavelength of 500 nm</td>
</tr>
<tr>
<td>OG</td>
<td>Octylglucoside</td>
</tr>
<tr>
<td>PBS</td>
<td>Phospho buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PEM</td>
<td>Protein expression medium</td>
</tr>
<tr>
<td>Pen</td>
<td>Penicillin</td>
</tr>
<tr>
<td>Phenix</td>
<td>Python based hierarchical environment of integrated crystallography</td>
</tr>
<tr>
<td>Rev</td>
<td>Reverse</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Strep</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>$T_{m50}$</td>
<td>Temperature at which half of the protein is unfolded</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tetr</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>XDS</td>
<td>X-ray detector software</td>
</tr>
</tbody>
</table>
Summary

G protein-coupled receptors (GPCRs) are the largest family of membrane proteins in the human genome, mediating signal transduction in response to a variety of signals. Defective signaling by these integral membrane proteins causes a number of acquired and inherited diseases. Many of these maladies are associated with single point mutations present across the GPCR sequence. The dim light photoreceptor rhodopsin is no exception. More than 150 point mutations are known in rhodopsin to cause a group of vision impeding diseases. The majority of these mutations result in severe retinal degeneration called retinitis pigmentosa (RP). Early stage RP is characterized by night blindness that slowly progresses with age towards impairment of day light vision until patients are completely blind at mid to old age.

Four mutations are known to cause congenital stationary night blindness (CSNB) and rod dysfunctions similar to the early stages of RP, but without progressive impairment of day vision. Despite considerable progress in the characterization of rhodopsin, the structural basis of how single mutations can translate into a pathologic phenotype is still elusive.

This thesis presents crystal structures of CSNB causing G90D(s-s) and T94I(s-s) rhodopsin mutants in the light activated conformation. Overall the determined structures are similar to light activated metarhodopsin II and the structural impact of G90D(s-s) and T94I(s-s) is limited to the ligand binding pocket. The charged G90D introduced a salt bridge with K296 that interfered with covalent ligand binding, whereas neutral T94I had the opposite effect and further stabilized binding of the ligand in the active metarhodopsin II conformation. Differences in the biochemistry and their impact on the binding pocket of the light activated structures and the G90D(s-s) opsin state indicate that changes to the active state are not the common denominator between the two investigated CSNB mutations. To further envisage the common denominator for CSNB, we employed biochemical and molecular dynamic studies on models of the G90D(s-s) and T94I(s-s) ground state. Based on our comparative study on G90D(s-s) and T94I(s-s), it seems most likely that CSNB mutants alter the ground state. The structural, biochemical and computational studies presented in the thesis provide a comprehensive insight into the mechanism of CSNB. Structures of light activated CSNB mutants, and particularly T94I(s-s) due to its high resolution, provide key insights into the subtle interactions in the retinal binding pocket of rhodopsin and may provide clues for future pharmacological intervention to alleviate RP.
In the context of GPCR research, this thesis describes the first crystal structures of the disease causing constitutively activating mutations as a cause of human disease, also found in other GPCRs.
Zusammenfassung


Im Kontext der GPCR Forschung beschreibt diese Dissertation zum ersten Mal die Kristallstrukturen von Krankheitshervorrufenden, konstitutiv-aktiven Mutationen, welche auch in anderen GPCRs vorkommen.
Publications

• **Singhal A**, Guo Y, Matkovic M, Dawson R, Schertler G, Deupi X, Yan E, Standfuss J; Role of T94I rhodopsin mutant in congenital stationary night blindness, (in preparation)


• Vishnivetskiy SA, Ostermaier MK, **Singhal A**, Panneels V, Homan KT, Glukhova A, Sligar SG, Tesmer JJ, Schertler GF, Standfuss J, Gurevich VV.et al; Constitutively active rhodopsin mutants causing night blindness are effectively phosphorylated by GRKs but differ in arrestin-1 binding, *Cell Signal* 10.1016 (2013)


Chapter 1 Introduction

1.1 G protein-coupled receptors: An overview

Guanosine nucleotide binding protein-coupled receptors (GPCRs) represent the single largest family of membrane surface receptors responsible for signal transduction. In the human genome approximately 800 GPCR genes are known (ref: IHGS). GPCRs are found in all strata of eukaryotic phyla including, yeast (1), plants (2) and animals. In terms of physiological function, the GPCR family of proteins is very diverse responding to diverse ligands such as nucleotides, pheromones, peptides, lipids, amines as well as sensory stimuli such as light. Their ability to initiate and modulate cellular reactions in response to the extracellular molecules makes them key players in human physiology. GPCRs have been evolved to fulfill a variety of physiological functions including neurotransmission, smell, taste, vision, embryogenesis, chemotaxis, oncogenesis, cell growth, differentiation and function of exocrine and endocrine glands. The remarkable physiological diversity is complemented by a spatial distribution into specific tissues, for example: rhodopsin is present in the vertebrate eye and mediates the conversion of electromagnetic signals into a cellular signal; beta adrenergic receptors are present in the muscle, liver, adipose tissue and mediate changes in fuel metabolism; chemokine receptors are present on immune cells like neutrophils or lymphocytes and mediate cellular changes in response to antigen or inflammation.

All GPCRs share a seven hydrophobic transmembrane helical (TM) core fitting to the width of the plasma membrane (3,4) (Figure 1.1). Accordingly, GPCRs are also called serpentine receptors, heptahelical receptors, 7TMs and 7 transmembrane receptors. The seven TMs are connected by three extracellular loops (EL1-3) and three intracellular loops (IL1-3) with the N-terminus located on the extracellular and the C-terminus on the intracellular side of the membrane. Most GPCRs also have an intracellular short amphipathic helix 8. The extracellular part of GPCRs can be glycosylated and is involved in ligand interactions. Two conserved cysteines in the extracellular loop form a disulfide bond further stabilizing the receptor structure. The intracellular region takes part in mediating interaction with G proteins (5-7), G protein kinases (GRKs) (8,9), arrestins (10) (11,12) and other effector molecules, recruited by arrestin.
GPCRs vary highly in sequence length, which varies from 289 (Mas related GPCR) to 6300 (VLGR1) (14). The difference in chain length is due to the enormous variability of the extracellular region (15) The extracellular region plays a key role in ligand recognition and variability in signal transduction across different GPCR families (16). The variability of the intracellular region is much lower as this region couples G protein and other downstream effector molecules in a mechanism largely conserved among GPCRs. Signal transduction in GPCRs can be compared to a doorbell system where the extracellular signal is transmitted to the interior of the cell without transfer of the signaling molecule itself. In kind to a doorbell, GPCRs initiate a variety of signal responses depending upon the kind of signaling molecule. To fulfill their signaling function, GPCRs exist in equilibrium between active and inactive conformations. Binding of ligands shifts the equilibrium via several low affinity intermediate towards a high affinity ligand-receptor complex that allows coupling of the G protein. This active signaling complex further interacts with other downstream effector molecules such as channels, transporters and enzymes bringing the cell response in a cascade dependent manner. Subsequently, GPCR kinases (GRKs) phosphorylates the receptor and recruits arrestin leading to receptor desensitization and internalization (17) (Figure 1.2).
Figure 1.2 Signal transduction cascade in GPCRs: Schematic overview of the agonist induced activation in class A GPCRs. All the intermediates and steps of activation correspond to the GPCR rhodopsin (Image is taken from Structural insights into agonist-induced activation of GPCRs (17)).

Many GPCRs such as the ghrelin receptor (18), melanocotropin4 receptor (19), or the histamineH4 receptor (20) exhibit various levels of ligand independent G protein signaling. The endogenous constitutive activity is an interplay of alternative splicing, RNA editing, polymorphism within species, variations among species and coupling to various G proteins, all suggesting the existence of multiple regulatory switches to fine-tune GPCR basal activities (21-30).

This basal or constitutive activity can be increased by single point mutations. For example, many receptors display single nuclear polymorphism that gives rise to amino acid substitutions and theoretically can increase basal activity of the receptor. Such single point mutations are known as constitutively active mutants (CAMs) and seem to alter equilibrium between the inactive and active state of the receptor, driving the equilibrium towards the G protein signaling state of the receptor even in the absence of the activating ligand (31,32). CAMs can be further activated by addition of ligands. Many CAMs increase basal activity by disruption of intramolecular interactions in the inactive state, giving rise to a partially active conformation (33). Thus, CAMs can serve as remarkable templates for unraveling the constraining intramolecular interactions that keep the receptor in a non-signaling state. Malfunction of GPCRs has been implicated in several diseases; neurodegeneration, cancer metastasis, hyperthyroidism, heart ailments, hypocalcaemia, diabetes insipidus, visual defects (34) (Table 1.1) Many of these defects can be attributed to single point mutations present
across the GPCR sequence. Many disease causing mutations increase constitutive activity and usually result in the gain-of-function associated with the mutated GPCR. In turn, this eventually has deleterious effects and inhibits the associated cell function. The effect of these CAMs on the associated cell function varies among GPCRs. Despite considerable progress in the structural characterization of GPCRs, the structural basis of how single point mutations can translate into a pathological outcome is still elusive. Structural characterization of medically interesting CAMs would facilitate our understanding of disease mechanisms as well facilitate structure-based drug design.
<table>
<thead>
<tr>
<th>Family</th>
<th>GPCR</th>
<th>OMIM</th>
<th>Inheritance</th>
<th>Phenotype</th>
<th>CAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Rhodopsin</td>
<td>180380</td>
<td>Autosomal dominant</td>
<td>Congenital stationary night blindness</td>
<td>G90D\textsuperscript{2,57}, T94I\textsuperscript{2,61}, A292E\textsuperscript{7,39}, A295V\textsuperscript{7,42}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Autosomal dominant</td>
<td>Retinitis pigmentosa</td>
<td>T17M, P23H, K296E\textsuperscript{7,43}</td>
</tr>
<tr>
<td>A</td>
<td>TSHR</td>
<td>603372</td>
<td>Autosomal dominant</td>
<td>Non-autoimmune hyperthyroidism</td>
<td>G431S\textsuperscript{1,49}, M463V\textsuperscript{2,53}, S505R\textsuperscript{3,36}, V509A\textsuperscript{3,40}, A623V\textsuperscript{6,34}</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Somatic</td>
<td>Familial male - limited precocious puberty</td>
<td>L368D\textsuperscript{1,41}, A373V\textsuperscript{1,46}, M398T\textsuperscript{2,43}, L457R\textsuperscript{3,43}, I542L\textsuperscript{5,54}, S564G\textsuperscript{6,30}, A568V\textsuperscript{6,34}</td>
</tr>
<tr>
<td>A</td>
<td>LHCGR</td>
<td>152790</td>
<td>Autosomal dominant</td>
<td>Spontaneous ovarian hyperstimulation syndrome</td>
<td>T449A/I\textsuperscript{3,32}, D443N\textsuperscript{3,26}, I545T\textsuperscript{5,54}</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Somatic</td>
<td>FSH independent spermatogenesis</td>
<td>D567G\textsuperscript{6,30}</td>
</tr>
<tr>
<td>A</td>
<td>FSHR</td>
<td>136435</td>
<td>Autosomal dominant</td>
<td>Jansen’s metaphyseal chondrodysplasia</td>
<td>H223R\textsuperscript{2,46}, T410R/P\textsuperscript{6,39}, I458R\textsuperscript{7,52}</td>
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<tr>
<td>B</td>
<td>PTHR1</td>
<td>168468</td>
<td>Autosomal dominant</td>
<td>Autosomal dominant hypocalcaemia</td>
<td>A843E\textsuperscript{7,38}</td>
</tr>
<tr>
<td>C</td>
<td>CaSR</td>
<td>601199</td>
<td>Autosomal dominant</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1 Examples for pathophysiological conditions associated with constitutively active point mutations across GPCRs. OMIM: Online Mendelian Inheritance Database; TSHR: Thyroid stimulating hormone receptor; LHCGR: Luteinizing hormone chorigonadotropin receptor; FSHR: Follicle stimulating hormone receptor; PTHR1: Parathyroid hormone receptor 1; CaSR: Calcium sensing receptor; CAM: Constitutively active mutations.(35). (superscripts denote the Ballesteros-Weinstein general GPCR numbering (36))

1.2 Classification of GPCRs

Based on sequence homology eukaryotic GPCRs can be classified into 6 classes (A-F or 1-6) (37). However, many of the members of class E and F are not found in humans. Human GPCRs are, therefore, often classified using the GRAFS classification system introduced by
Fredriksson in 2005 based on the sequence and domain similarity (38). Accordingly, GPCRs can be broadly classified into five families: glutamate (G with 15 members), rhodopsin (R with 701 members), adhesion (A with 24 members), frizzled/taste2 (F with 24 members), and secretin (S with 15 members) having the acronym GRAFS. Out of the five families, the rhodopsin family is the largest and has several salient features. These include the presence of the NSxxNPxxY motif in TM7, the DRY or D(E)RY(F) motif at the interface of TM3 and IL2 and palmitoylation at one or more sites of the C-terminal tail or IL3. With few exceptions, such as the lutenizing hormone or follicle-stimulating hormone receptor where ligands bind the N-terminal domain, ligands bind within the cavity at the extracellular side of rhodopsin type GPCRs (39). The rhodopsin family can be further subdivided into 4 major groups: alpha, beta, gamma and delta. The rod visual pigment (RHO) belongs to the opsin receptor cluster, classified under alpha group of the rhodopsin family. The opsin receptor cluster also consists of short-wave sensitive opsin (OPN1SW), medium-wave sensitive opsin (OPN1MW) and long-wave sensitive opsin (OPN1LW). Members of the opsin cluster are the principal photosensors of our visual sense.

1.3 Visual System
Vision is a multistep process to convert electromagnetic signals to neuronal signals. Light is reflected or emitted from an object of interest and traverses through pupil, lens, vitreous humor and reaches the retina that senses light (Figure 1.3). Within the retina the signal is converted to an electrical signal that can travel through the optic nerve towards the brain. As such the retina can be seen as a biological detector, similar to the light sensing chip in a digital camera but far more sophisticated and complex. The retina is formed from a highly organized collection of sensory neurons called photoreceptor cells (40). Photoreceptor cells with bipolar neurons are specialized for photo transduction. The two main types of photoreceptor are: 1) cone cells discriminate red, green and blue colors, but are less sensitive to light and 2) rod cells sense dim light without color distinction. Each retina has 6-7×10^6 cone cells, more densely packed in the center of the retina. In comparison, rod cells are more abundant with 1-2×10^8 cells distributed throughout the retina.
**Figure 1.3 Perception of light in the vertebrate eye:** A. The lens converge light on the retina, which contains a highly organized collection of photoreceptor cells called rods and cones responsible for perception of night and color vision, respectively. The rods and cones form synapses with interconnecting neurons and eventually transmit the electrical signals to the brain via the optic nerve. B. Vertebrate retina rotated at 90 degrees. The rods are shown in black and the cones are shown in red, green and blue. Image taken from R. Tzekov, L. Strin and S. Kaushal. “Protein Misfolding and Retinal Degeneration”. In: Cold Spring Harbor Perspectives in Biology 3.11 (2011), a007492

Each rod cell has a long and slender cell body with two distinct cellular compartments: the outer segment, which is a modified cilium having stacks of 1000-2000 disc membranes and the inner segment, having mitochondria for supplying ATP molecules (Figure 1.4). The rhodopsin/dim light receptor/rod visual pigment is present in the rod-shaped outer segment (ROS) of the retina. ROS membranes are the only nerve membranes containing an excitable component linked to the chromophore (41).

**Figure 1.4 The rod photoreceptor cell:** The rod outer segment (ROS) is stacked with membranous discs containing the photoreceptor rhodopsin and the inner segment with mitochondria, nucleus and other organelles. Reprinted from Canadian light source; URL: http://www.lightsource.ca/education/bert_fox.php
1.4 Rhodopsin: The dim light receptor

Rhodopsin is the molecular species that initiates the cascade of biochemical reactions in response to dim light. It is a 348 amino acid holoprotein (Mr 40 kDa) consisting of the apoprotein opsin and the ligand 11-cis-retinal covalently bound by an imine bond to Lys296 forming a Schiff base (42-44) (Figure 1.5).

![Diagram of rhodopsin holoprotein formation]

**Figure 1.5 Formation of a rhodopsin holoprotein:** 11-cis-retinal binds to Lys 296 in opsin to form a Schiff base.

Rhodopsin is a member of the GPCR family of proteins and signals via interaction with G protein, arrestin and kinases like other GPCRs. The main difference is that it is activated by light induced isomerization of a prebound ligand instead of by a diffusible ligand. The sequence of molecular events during the visual signal transduction (Figure 1.6) can be broadly divided into two phases: photon induced biochemical reactions in the ROS and recovery/adaptation phase during dark adaptation (45,46).

Dark state rhodopsin has very low activity. To achieve this exceptionally high level of regulation, covalent binding of 11-cis-retinal further reduces the already very low activity of the apoprotein opsin. 11-cis-retinal thus acts as a classical inverse agonist that locks the receptor into an inactive conformation. Activation of rhodopsin involves a continuum of short lived intermediate states that get populated during the activation cycle (Figure 1.6). Upon absorption of a photon, 11-cis-retinal isomerizes to all-trans-retinal. The conformational changes in the ligand are followed by conformational changes in the ligand binding pocket and ultimately drives the formation of the metarhodopsin II state that couples to the heterotrimeric G protein transducin (Gtαβγ). In the activated receptor bound state, an exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) occurs in the G-alpha subunit. Subsequently, the G protein heterotrimer dissociates into G-alpha and G-beta-gamma. The G-alpha subunit interacts with the cGMP phosphodiesterase (PDE) present on the disc membrane, which converts cGMP to the inactive 5’GMP. Activated PDE reduces the amount of cGMP by degrading several molecules of cGMP to biologically inactive 5’ GMP.
in the ROS. Lowering of the cGMP concentration closes cGMP gated ion channels, preventing the influx of Na\(^+\) and Ca\(^{2+}\) and as a result the ROS membrane gets hyperpolarized. Hyperpolarized membrane generates the initial stimulus, which is then passed as an electrical signal through the optic nerve to the brain.

The recovery/adaptation phase begins shortly after the illumination of rod cells and shuts off the photosensory system. The alpha subunit of the transducin has an intrinsic GTPase activity that hydrolyzes GTP and causes re-association of the G-alpha with the G-beta-gamma subunits within milliseconds after a decrease in light intensity. This, in turn, inhibits the PDE activity due to release of its inhibitory subunit from G-alpha. Low concentration Ca\(^{2+}\) triggers guanyl cyclase to convert GTP to cGMP, resuming back the cytosolic concentration of the cGMP to the dark state. Meanwhile, the rhodopsin kinases phosphorylates several Thr and Ser at the C-terminal of rhodopsin and recruits visual arrestin, preventing further interaction between activated rhodopsin and transducin. Recoverin regulates the activity of rhodopsin kinases and inhibits them in response to high concentration of Ca\(^{2+}\). Slowly, all-trans-retinal leaves the binding pocket and rhodopsin is regenerated with 11-cis-retinal, ready for the next cycle of photo transduction.

**Figure 1.6 Molecular consequences of light absorption by the photoreceptor rod cell:** The top panel shows the excitation phase of the rhodopsin cycle and the bottom panel depicts the recovery/adaptation phase of the rhodopsin cycle. Adapted from Lehninger: Principles of Biochemistry, Volume 4.
1.5 Rhodopsin structural studies

Rhodopsin as a dim light sensor has been a focus of research for more than 130 years. Recognition of rhodopsin as a member of the GPCR super family (47) has further enhanced the interest in this receptor. Although a plethora of electrophysiological and single photon experiments along with biophysical, biochemical and cellular studies provided insights into the process of dim light vision, it was not until 1993 that the first projection structure of rhodopsin was solved using 2D electron-microscopy (48) and, 2 years later, a low resolution 3D structure was determined from tilts of 2D crystals (49). It took another 5 years of technology development and optimization to obtain the first high resolution crystal structure by X-ray crystallography (50). Only in 2007 it became possible to solve crystal structures from recombinantly produced rhodopsin (51), opening the way for the structural characterization of rhodopsin mutations.

Studies of rhodopsin greatly influenced our understanding of GPCR structure and function. Until the first high resolution structure of the β2-adrenergic receptor (β2AR) was determined (52,53) in 2007, only crystal structures of bovine rhodopsin (13,50,54) were available to study GPCRs on a molecular level. Studies of rhodopsin were facilitated through several features including a) high natural abundance of rhodopsin in the retina b) the ability to study rhodopsin by simple spectroscopic methods and to trigger activation by light (Figure 1.7), c) availability of recombinant expression systems to study the impact of mutations (HEK293, insect and COS cells) d) tetracycline inducible cell lines to overcome over-expression induced cell cytotoxicity, e) HEK293GnTI- cell lines to obtain homogenous glycosylation to improve chances for a successful crystallization f) identification of biochemically neutral, thermo stabilizing disulfide bond that increases the stability of the receptor by 10 °C in comparison to the wild type native rhodopsin (55).

These factors expedited crystallographic studies and made it possible to obtain crystal structures of inactive rhodopsin (13,50,51) as well as various early intermediate states (56) from both native and recombinant sources. However, understanding the activation mechanism requires the active conformation of the receptor. The 2D crystal structure of metarhodopsin I (57,58) revealed that the activation is initiated by a series of small conformational changes in the retinal ligand and the conformation of some of the key residues, which then later translates to the bigger conformational changes associated with the subsequent stages of the activation process. Thus, suggesting the presence of certain molecular switches that get turned on during receptor activation. While the structures of
batho, lumi and metarhodopsin I intermediates provided the first glimpse in the multistep finely tuned signal transduction process, the crystal structure of metarhodopsin II (59,60), post metarhodopsin II (61) and active opsin state (62) provided the first structural insights into fully activated GPCRs. Together with its unique accessibility for biophysical methods, such as NMR (63), fluorescence (64), FTIR (65), UV (66), Raman (67), DEER (68), and EPR spectroscopy (69), single molecule experiments (70), the rhodopsin crystal structures served as an ideal model for understanding the function of other members from the extensive GPCR family.

Due to its low conformational heterogeneity, rhodopsin provides detailed information about the structural changes associated with GPCR activation. Other agonist bound active state GPCR crystal structures (71-74) as well as the structure of the β2-adrenergic receptor (β2AR)-G protein complex (75) suggest a conserved mechanism of activation. Thus, implying the usability of rhodopsin structural studies for answering many key questions that yet need to be addressed: What is the molecular basis of coupling to different downstream effector molecules?, To what extent do water molecules, ions, lipids and cholesterol affect the structure and function of GPCRs (76)?, What is the effect and molecular basis of constitutive activity shown by CAMs in GPCRs?, What is the molecular basis of genetic diseases caused by single point mutations in GPCRs? The answers to these questions will not only facilitate a better understanding of the structural determinants of GPCR function, but also help design new therapeutic approaches to their treatment.

1.6 Rhodopsin constitutively active mutants (CAMs): As structural determinants of GPCR activation and basal activity

Intriguingly, a multitude of point mutations across the rhodopsin sequence can lead to constitutive activity. Many of these rhodopsin CAMs are conserved across class A GPCRs and are determinants for activation as well as ligand binding. Many of the rhodopsin CAMs are biochemically as well as spectroscopically, very well characterized and can be expressed in a minimally engineered, near native system. Thus, rhodopsin CAMs can be beneficial structural tools to understand structural activity relationships and the quasi-continuum conformational states across class A GPCRs.

CAMs in rhodopsin can be grouped in two classes based on their position in the receptor: those that are present in the retinal binding pocket like the E113Q mutation (77) and those that are present in the ionic lock region and G protein binding site like M257Y (78). Crystal structures of two CAMs i.e. E113Q and M257Y of rhodopsin in combination with dynamic
studies such as DEER and FTIR spectroscopy provide a well-illustrated example for the use of CAMs to understand the GPCR activation mechanism (60,61). Based on the comparison of the inactive rhodopsin ground state (13) and light activated structures of E113Q and M257Y metarhodopsin II, the transformation of the rhodopsin from inactive to active metarhodopsin II involves, a) opening of the interhelical salt bridge between E113$^{3.28}$-Schiff base, followed by proton transfer from protonated Schiff base to E113$^{3.28}$, which is a critical protonation switch that regulates the conformational transition from inactive to active metarhodopsin II conformation and acts as a primary activation switch b) ligand i.e. retinal induced changes in the conformation of W265$^{6.48}$ c) global arrangement of 7TM helical bundle d) activation of conserved residues near the G protein binding site. Successful case studies of rhodopsin CAMs provide an ideal base for the structural characterization of CAMs in other GPCRs that bind a diverse plethora of ligands providing further templates for the identification of molecular signatures of GPCR activation. Moreover, many CAMs are implicated in malfunctioning of GPCRs. Thus, their structural characterization can facilitate understanding of the disease mechanism and hopefully will help in better drug design.

1.7 Disease variants of rhodopsin
Over 150 point mutations are known in rhodopsin (79-81). Rhodopsin associated diseases can be classified into 2 groups: retinitis pigmentosa (RP) and congenital stationary night blindness (CSNB). The majority of mutations cause RP and only four point mutations are known to cause CSNB (82-85) (Table 1.1). RP associated mutations are found all over the receptor sequence, whereas CSNB associated mutations are present in the ligand binding pocket of the receptor and are spatially unique (Figure 1.7)
Retinitis pigmentosa is a degenerative eye disease and is inherited in autosomal dominant, autosomal recessive or X-linked form (87-89). RP is a progressive disease, characterized by night blindness, degeneration of photoreceptor cells, blurring of vision, tunnel vision, and eventual complete blindness. Tunnel vision in RP patients is associated with the spatial distribution of rods and cone cells, as the disease starts from peripherally located rod cells of the retina and eventually progresses towards the centrally located cone cells (Figure 1.8).

CSNB is genetically heterogeneous with an autosomal dominant inheritance. It is a milder form of RP characterized by congenital night blindness without retinal degeneration. The CSNB affected person has a normal vision during daytime throughout their life and are less inhibited in their normal day to day activity in comparison to RP patients.
Figure 1.8 Vision of retinitis pigmentosa affected patients: Top panel: Progressive tunnel vision in the RP affected individual (3rd and 4th from left) in comparison to the panoptic vision in normal person (1st from left) and CSNB affected individual (2nd from left) during the day. Middle panel: The night vision of both CSNB (2nd from left) and RP (3rd and 4th from left) affected individual is impaired in comparison to the normal person (1st from left). Bottom panel: The visual appearance of the retina in healthy persons (1st from left), CSNB affected individual (2nd from left) and RP patients (3rd and 4th from left). The retina of the RP affected persons is characterized by the presence of plaques and degenerated blood capillaries with increasing severity over time. The very early stages of RP are difficult to distinguish from CSNB. Retinal images taken from the URL: http://www.gfmer.ch/genetic_diseases_v2/gendis_deta-il_list.php?cat3=1084.

1.8 Molecular causes of rhodopsin mediated blindness

RP mutants in rhodopsin are associated with defects at various levels of receptor biosynthesis and activity. The majority of them is characterized by misfolding, trafficking problems, reduced thermal stability of the receptor, constitutive activity and its inability to bind retinal (90-93). It is still unclear how the disease progresses from rods to cones to cause degeneration of the whole retina. Several studies suggest that rod secreted epidermal growth and cone stabilizing factors are necessary to maintain healthy cones (94). With the death of the rods cells, eventually cones would die due to the lack of these survival factors. In contrast, CSNB mutants fold properly and express on the rod outer segment membrane surface. The CSNB phenotype is further associated with constant basal activation at low gain as shown by mouse and Xenopus studies (95-98). All CSNB mutations are constitutively active and it has been suggested that the disease phenotype arises by a number of mechanisms. An increased rate of thermal isomerization (99), constitutive activity of opsin (98), significant decrease in the amount of photo bleachable pigment (95) or the presence of a perturbed ground state (97). Since both RP and CSNB are associated with point mutations in
the rod photoreceptor rhodopsin, understanding the structural impact of those mutations on different intermediates of the rhodopsin activation cycle may pave the way for a better understanding of the molecular causes of RP and CSNB. Many of the RP and CSNB mutants are CAMs and increase the basal activity of the receptor by perturbing the molecular switches that keep the receptor in non-signaling ground state. Most of the RP associated CAMs also have attributes like lower thermal stability and misfolding propensity, making it hard to characterize them structurally. However, CSNB associated CAMs are stable and fold well and are ideal targets to probe the structural determinants of rhodopsin mediated diseases. In addition, CSNB associated rhodopsin CAMs are well characterized in vitro as well as in vivo. Together with structural studies, CAMs could potentially serve as a template to probe the molecular mechanisms of CAM related GPCR maladies.

1.9 Novel approaches for the cure of retinitis pigmentosa

Currently there is no effective treatment for rhodopsin mediated CSNB and RP. Research directions include retinal transplants or gene silencing but are limited either by the invasive and expensive nature of the strategy or autosomal dominant nature of the disease itself (100,101). The other possibility is to slow disease progression by pharmacological rescue. In this strategy the amount of misfolded receptor is reduced by small molecules that could potentially assist in folding and stabilizes the mutated receptor (102-105). Small molecules assisting in folding are often called pharmacological chaperones and commonly resemble analogs of the natural ligand. The non-invasive and inexpensive nature of this strategy along with the possibility to deliver small molecules orally in non-toxic concentrations holds great promise to slow the progress or even cure RP. Studies have shown a disease slowing effect of vitamin A (retinyl palmitate) but the cytotoxic effects and light sensitivity reduces their continuous usability (106-108). Some unrelated compounds like valproic acid and safranal seem to have a positive effect in reducing the retinal degeneration, but their nonspecific nature is an issue (109,110). A better understanding of the disease mechanism and the structural perturbation associated with RP/CSNB mutants would help in designing better and more specific drug molecules. Structures of rhodopsin RP mutants in the presence of the agonist retinal or several small molecule analogs, would facilitate structure-based drug design and would allow improving synthetic compounds that specifically stabilize dysfunctional rhodopsin.
1.10 Aim of thesis

When this work was started, crystal structures of ground state (13,50), an active opsin (111) and the metarhodopsin II state (59,61) had been solved. The primary goal of the project was to investigate the structural impact of rhodopsin mutants causing CSNB or RP. In order to study disease causing point mutations, rhodopsin isolated from native ROS membranes is not a workable option and thus a recombinant expression system (112) had to be established at the Paul Scherrer Institute (PSI). In order to understand the effect of point mutations on the overall conformation of rhodopsin, we decided to determine the structure of various states of rhodopsin including, ground state, opsin state and the active metarhodopsin II state. We anticipated that a comparison with wild type rhodopsin structures would provide an insight into the molecular causes of rhodopsin-mediated blindness. I was particularly interested in point mutations in the TM2 close to the retinal binding pocket. Position 90 is of special interest as it is the only position where mutations can cause either RP, in case of the G90V substitution, or CSNB, in case of G90D. To further understand the impact of charged and uncharged mutations we choose T94I one helix turn downstream of G90D, as another example of a CSNB causing mutation. The structure of the two CSNB causing mutations G90D and T94I was important to understand both the cause of CSNB and progression toward the RP phenotype. An important future question is whether crystal structures of the stationary CSNB mutations can provide molecular clues on how small molecular drugs may alleviate RP progression.

In this thesis, the experiments to achieve the above mentioned goals are described. The initial chapter describes the experimental methods used for obtaining and analyzing crystal structures of G90D and T94I rhodopsin along with methods used for initial screening of crystallizable protein constructs and their large scale expression. In the next section, I will present a detailed analysis and discussion of the obtained crystal structures and their implication for the rhodopsin activation and disease mechanism in the light of known biochemical and structural data. The thesis is then concluded with future prospects resulting from my work.
Chapter 2 Materials and methods

2.1 Site-directed mutagenesis and sub-cloning of the opsin gene

Mutations were inserted by site-directed mutagenesis into a synthetic bovine opsin gene containing the opsin stabilizing mutation N2C/D282C (55) in a pcDNA3.1 vector. Forward and reverse primers for the polymerase chain reaction (PCR) containing a short overlapping region and the mutated codon of interest were designed using the program vector NTI (Table 1.1).

<table>
<thead>
<tr>
<th>5'-3' primer sequence</th>
<th>Point mutation</th>
</tr>
</thead>
</table>
| For 5'-ACAAGATGGGCGTGTTGCGCAG-3'  
Rev 5'-ACGCCCATCTTTGTGAGAAAGGAACG-3' | T17M |
| For 5'-GCAGCCATTTCGAGGGGCGCAG-3'  
Rev 5'-TCGAAATGGCTGCGTACGACGCGCCGTC-3' | P23H |
| For 5'-TCGGTGATTCACCATCACCACCTC-3'  
Rev 5'-GTGAAATTCACCGAAGACCATGAGATC-3' | G90D |
| For 5'-CCACCATTCTCTACCTCTCCATGGG-3'  
Rev 5'-TAGAGATGGGTGGAAGCCACCGAAG-3' | T94I |

Table 2.1 Primers used for site-directed mutagenesis of bovine rhodopsin in pcDNA3.1.

Amplification of the opsin constructs with point mutation was done using a PCR step-down protocol (113) in 96 well format with Guanidine-Cytosine (GC) rich and High Fidelity (HF) (Finzymes) DNA polymerase. After the PCR was completed, the methylated template DNA was digested with DpnI (Thermo Scientific) at 37 °C for overnight. All opsin constructs were then transformed into E.coli Mach1 cells and plated on LB-agar with 100 µg/ml amp for overnight incubation at 37 °C. Three amp positive colonies were sent for sequencing to confirm the introduced mutation. For N-terminal point mutations, the CMV promoter and EBV rev primers provided by Microsynth were used as forward and reverse sequencing primer. For the C-terminal point mutations internal forward primers were designed using Vector NTI and the EBV rev primer was used as a reverse sequencing primer. Plasmid DNAs were extracted using the Miniprep kit (Sigma-Aldrich) and verified by sequencing using dideoxynucleotide-sequencing method (GATC) and analyzed using Vector NTI and ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

After the initial mutagenesis, constructs were sub cloned into the pACMV-tetO vector for tetracycline inducible expression in HEK293S GnTI- TetR cells (112,114) (Figure 2.1). KpnI and NotI restriction sites were used to sub-clone the opsin construct from pcDNA3.1 into the
pACMV-tetO vector. Complete digestion was achieved by a 6 hour sequential incubation with KpnI (Fermentas) and NotI (Thermo Scientific) in a 10 µl reaction volume. Digested samples were separated using electrophoresis in a 0.7% agarose gel. The gel band with corresponding gene of interest was cut and frozen at -20 °C and then the frozen gel band was squeezed to obtain both the opsin and the pACMV-tetO cassette. The amount of purified DNA was quantified by its absorption at 260 nm. (Nanodrop 2000, Thermo SCIENTIFIC). The mutated opsin gene and the pACMV-tetO vector were mixed in a 5:1 molar ratio and ligated using T4 DNA Ligase (Thermo Scientific) enzyme before transfection in Mach1 cells. Plasmids were isolated and used for transient transfection and the creation of stable cell lines.
Figure 2.1 Construction of the pACMV-tetO-opsin vector: Vector was constructed with the point mutation in the opsin gene for tetracycline inducible opsin expression using site-directed mutagenesis and sub-cloning.

2.2 Cell culture and transient expression

Expression of opsin mutants was checked by transient expression in HEK293S GnTI- TetR cells grown as adhesion cultures at 37 °C incubator enriched with 5% CO₂. The expression medium (Dulbecco's modified Eagle's medium (DMEM), Bioconcept) contained 10% fetal bovine serum (FBS), 292 µg/ml glutamine (Invitrogen) and 1:100 dilution of penicillin/streptomycin (10000 U/ml Gibco). For transfection, cells were splitted, distributed into 10 cm Petri dishes and grown for 16-20 hour until they reached 70-80% confluence. 30 µg pcDNA3.1 or pACMV-tetO plasmid was added together with 60 µg polyethyleneimine.
(PEI) into 5 ml DMEM media with 1% FBS and incubated at room temperature for 15 minutes. After removing the old medium, cells were incubated with 5 ml DNA-PEI mixture for 1 hour at 37 °C and 5% CO₂. The DNA-PEI mixture was later supplemented with fresh DMEM medium with 10% FBS and antibiotics. The cells were harvested after 48 hours, using trypsin/EDTA to remove cells from the Petri dish. The cells were pelleted at 4,000×g for 10 minutes, washed with PBS and stored at -80 °C after flash freezing in liquid nitrogen.

In order to check the expression, cells were homogenized in PBS (pH 7.4) containing protease inhibitor cocktail (complete protease inhibitor cocktail tablet, Roche) and 1.25% DM. Unsolubilized cell debris was removed using centrifugation (12,000×g, 30 minutes, 4 °C). Cell lysates were then loaded on a 12% SDS gel for western blot analysis.

2.3 Western blot
For western blot, 12% SDS polyacrylamide gel was run. The protein bands were then transferred to nitrocellulose membrane (Amersham Biosciences) using a semi-dry electroblotting technique. The whole process of transfer involves placing nitrocellulose membrane on the top of the gel, in between a stack of TBS buffer (Tris-buffered saline) soaked Whatmann filter papers. Before placing the membrane over the gel, it is activated in methanol for 3 minutes and then equilibrated in TBS buffer for 5 minutes. Electroblotting was done for 3 hours at 50-55 mA. The membrane was then washed with TBS/Tween (TBST) and then subsequently with TBS for 10 minutes. Later, the membrane was blocked with milk powder (5% in TBST) for 1 hour at room temperature to avoid nonspecific antibody binding. After washing with TBS again, the membrane was incubated with primary antibody (1D4) at 1:1000 dilutions (1 µl/ml) for 1 hour. The membrane was again washed twice with TBS and incubated with secondary antibody at 1:10000 dilutions (0.1 µl/ml) for an hour. A reaction mixture of alkaline phosphatase substrate (5 ml), NBT (50 µl) and BCIP (50 µl) were added to the blot that gave a colored product visible on the blot (115). Once the bands appear on the blot the reaction was stopped by putting the blot in water.

2.4 Confocal microscopy
The cellular expression and localization of the opsin constructs were checked using confocal microscopy. HEK293S GnTI- TetR cells were grown on 14 mm glass cover slips up to 80-90% confluency and transfected with pcDNA3.1 containing the target opsin construct using lipofectamine (Life Technologies). Cells were grown for 2 days fixed with 37% formaldehyde for 20 minutes, followed by extensive washing with PBS. The cover slips were
then transferred from the large Petri dish to small paraffin pieces with the cells facing up. Cells were permeabilized using 1% NP40 at room temperature for 10 minutes and incubated with primary antibody (1D4) (University of British Columbia) at 1:1000 dilutions (1 µl/ml) for 2 hours. The cells were washed with PBS and incubated with secondary antibody (anti-goat) at 1:10000 dilutions (0.1 µl/ml) for 2 hours. Samples were washed again with PBS and embedded in geluatol (15% geluatol, 33% glycerol and 0.01% sodium azide). For image acquisition an Olympus IX81 epifluorescence microscope was used.

2.5 Selection of stably expressing HEK293S GnTI- cell lines

While the transient expression is a fast way to check the expression of rhodopsin mutants, the production of the amounts of protein needed for structural analysis would require large quantities of DNA. As an alternative we created HEK293S GnTI- TetO cell lines that incorporate the target gene randomly into the genome and, as an additional advantage, allow to induce protein expression by the addition of tetracycline (114).

For the selection of stable cell lines, HEK293S cells are grown as 10 cm adhesion cultures in DMEM medium enriched with 10% FBS but without additional antibiotics. Once the cells reached a confluency of 80-90% they were transfected with the pACMV-tetO vector containing the opsin gene using lipofectamine 2000. After 20 minutes incubation, cells were split 1:10 in DMEM medium containing 5 µg/ml blasticidin, penicillin and streptomycin and redistributed onto 10 cm Petri dishes. After 2-5 days the medium was replaced with DMEM medium containing 10% FBS, blasticidin (5 µg/ml), penicillin/ streptomycin (100 µg/ml) and G418 (200 µg/ml) plus 20% conditioned medium. Conditioned medium is prepared by growing the parent cell line to near confluency and then replacing the medium with fresh complete medium. During this period cells condition the medium by secreting cytokines and several other conditioning factors. Every 2-3 days the medium was changed until after 10-14 days individual patches of cells became visible. These surviving cells were resistant to blasticidin and G418 and must thus have incorporated both the Tet repressor and the pACMV-tetO vector containing the mutated opsin gene. For freezing, an appropriate number of cells were pelleted at 800 rpm (Sorvall GLC 2B General Lab rotor centrifuge) for 10 minutes, the supernatant was removed and cells resuspended at 5×10^6 cells per milliliter in ice cold DMEM supplemented with 20% FBS and 10% DMSO. Finally, cells were frozen using a cryo-freezing rack and stored in liquid nitrogen for later usage.
2.6 Small scale expression tests
Various suspension medium; custom made DMEM (Bioconcept), PEM (Invitrogen), CD293 (Invitrogen) and free style (Invitrogen) medium were tested for maximum attained cell density in 2 l suspension culture. Cells were grown in different medium in an incubator with implemented shaker (37 °C, 8% CO₂ and 125 rpm).

2.7 Large scale expression
Large scale expression of the stable HEK293S GnTI- TetO cell lines was done in a 20 l wavebag bioreactor (GE Healthcare). Stably transfected cells were first grown as adherent culture in 15 cm Petri dishes in DMEM/10% NBCS until a suspension culture with a cell density of 0.5–1×10⁶ cells per ml could be started in a 250 ml flat-bottomed plastic flask (Vitaris). Cells in suspension culture were grown in PEM medium supplemented with L-Glutamine (4 mM) and 10% NBCS in an incubator with implemented shaker (37 °C, 8% CO₂ and 125 rpm). Under these conditions cells grew with a doubling time of around 24 hours until a cell density of up to 3×10⁶ cells per ml was reached. Cells were further expanded into 2 l flat-bottomed plastic flasks (Vitaris) until enough cells for seeding of the wavebag culture were grown. Cells were transferred to the wavebag and diluted with fresh medium to a 10 l suspension culture with 0.7-0.8×10⁶ cells per ml. The temperature was kept at 37 °C and the bag supplemented with a constant flow of 8% CO₂ in air while rocking at 16 rpm with a 6° inclination. After 2-3 days cells reached a density of 3×10⁶ cells per ml and were induced by adding 2 µg/ml tetracycline and 5 mM sodium butyrate in 1 l of fresh medium. Cells were harvested after 72 hours of induction at a cell density of 3-4×10⁶ per milliliter and centrifuged down (3220×g, 15 minutes). Cells were washed with PBS, frozen in liquid nitrogen and stored at -80 °C. Each 10 l wavebag culture yielded 120-150 g of cell pellet.

2.8 Rhodopsin purification
For protein purification HEK293S GnTI- TetO cells were solubilized in detergent and the mutated rhodopsin purified using affinity chromatography essentially as described (61). For each protein purification experiment, 30 g of wet cell pellet was suspended in 250 ml of PBS buffer (pH 7.4) containing protease inhibitor tablets (complete protease inhibitor cocktail tablet, Roche). The cells were homogenized using a mechanical cell disruptor (Ultra-Turrex T25 at 11,000×g) with 30 sec on/off pulse for 5 times. The cells were then solubilized by adding DM (Anatrace, sol grade, CMC=0.087% w/v) to the final concentration of 1.25% and rotating in Ti45 tubes for 1 hour at 4 °C. The solubilized cells were spun down at 40,000 rpm
(Ti45 rotor) in the preparative ultra-centrifuge (Beckman Coulter) at 4 °C for 1 hour to remove nuclei and other insoluble material. The supernatant was then incubated with 5 ml of CNBr activated sepharose resin (Amersham Biosciences) coupled to 1D4 antibody (antibody against C-terminal of the rhodopsin, assuming 1 ml of resin binds to 1 mg of the protein) and rotated for at least 2 hours at 4 °C. The supernatant was poured into a 50 ml biorad column and the resin was washed using 100 ml PBS (pH 7.4) with 0.125% DM. Ground state rhodopsin was reconstituted with 50 µM 11-cis-retinal while rotating the columns overnight at 4 °C. Following the addition of 11-cis-retinal, all subsequent steps were performed under dim light to prevent retinal isomerization. The resin was washed with 100 ml PBS (pH 7.4) with 0.125% DM followed by 200 ml HEPES (pH 7.0) with 0.125% DM. For protein elution, the washing buffer was supplemented with 80 µM elution peptide (TETSQVAPA (Peptide 2.0 Inc)) to compete for binding of the rhodopsin C-terminus. Elution was performed in batch mode by rotating the column 45 minutes with 12.5 ml of elution buffer. This elution step was repeated 4 times until the majority of the protein had been eluted. The eluted rhodopsin was concentrated to 4-5 mg/ml using a 15 ml centrifugal concentrator (amicon ultra 30 kDa cut off, Merck) at 3220×g and 4 °C.

2.9 UV-VIS spectroscopy
Protein concentration was determined spectroscopically using either the 500 nm absorption maxima of ground state rhodopsin or, in case of the apoprotein opsins, by protein absorption at 280 nm. The ratio between 280 nm and 500 nm was also used as a quality indicator to determine how much of the present protein had been reconstituted with 11-cis-retinal. Absorption spectra were taken at room temperature with a Cary 50 Bio UV-Visible spectrometer (Varian) in a cuvette with 1 cm path length. The spectrum was recorded from 250 nm to 650 nm after baseline correction. The following molar extinction coefficient were assumed: rhodopsin $\varepsilon_{280} = 65,000 \text{ M}^{-1}\text{cm}^{-1}$ and $\varepsilon_{500} = 40,000 \text{ M}^{-1}\text{cm}^{-1}$ (44) (with $A_{280}$ nm/$A_{500}$ nm ratio of $\approx 1.6$). For the active state metarhodopsin II, the spectrum was recorded after illumination of the sample for 5 minutes with orange light with a 10 W fiber-optic light equipped with $> 515$ nm long pass filter (GG 495, Schott, Elmsford, NY) to prevent free retinal isomerization. The absorption coefficients of the mutants were identical to the wild type rhodopsin.
2.10 Thermal stability assay
Thermo-shift assays were performed in a Carry Eclipse fluorimeter (Varian) equipped with multisample holder. Thermal denaturation was followed using the thiol specific maleimide fluorochrome CPM (N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide) basically as described (116).
For each measurement, 5 µg of rhodopsin obtained by 1D4 immunoaffinity purification as described above was diluted into 120 µl ice-cold buffer (100 mM NaCl, 10 mM HEPES pH 7.5 and 0.125% DM). Immediately before the measurement, CPM (3 mg/ml in DMSO) was diluted 1:30 into buffer and 10 µl of this mixture added to the reaction mix. The increase in fluorescence (Ex: 387 nm, Em: 463 nm) was followed while ramping temperature from 20 °C to 90 °C at 2 °C/min. In samples incubated with free 11-cis or all-trans-retinal the excitation wavelength was shifted to 420 nm to minimize overlap with the CPM dye.
All resulting melting curves were fitted using a sigmoidal Boltzmann equation using non-linear least square fitting in the statistical software Prism. If necessary, data were truncated before fitting as at higher temperatures the protein aggregated interfering with the fluorescence signal. Every sample was analyzed individually and R^2 >0.998 was taken as a parameter for the quality of the each measurement.

2.11 Rhodopsin purification for biochemical analysis of ground state rhodopsin
Cell pellets of G90D(s-s), T94I(s-s) and WT(s-s) rhodopsin was solubilized in 50 mM Tris, 100 mM NaCl, 1 mM CaCl_2, 1% w/v DDM, 0.1 mM PMSF, pH 6.8 for 3 hours at 4 °C. The solubilized cells were centrifuged and regenerated with excess 11-cis-retinal (7.5 µM) for 2 hours at 4 °C. The protein was purified using the 1D4 antibody coupled to Sepharose beads. The resin was washed three times with 50 mM Tris, 100 mM NaCl, 0.1% DDM pH 6.8 and three times with 50 mM sodium phosphate, 0.1% DDM pH 6.5. The protein was eluted in 50 mM sodium phosphate, 0.1% DDM pH 6.5 supplemented with 0.18 mg/ml elution peptide (TETSQVAPA).

2.12 Thermal decay
Thermal decay of rhodopsin ground state was monitored using UV-VIS spectroscopy (117). All the measurements were performed in the dark with a Shimadzu UV-2450 spectrophotometer. 2 ml of the buffer (50 mM Tris, 100 mM NaCl, 0.1% DDM, pH 6.5) was equilibrated in a water-jacketed cuvette at 55 °C. The temperature was monitored using thermo couple placed in the cuvette holder. At t=0, ice-cold, concentrated rhodopsin solution
(20 µl) was added to the equilibrated buffer to obtain a final concentration of 1-3 µM. The temperature change upon addition was small due to the high dilution factor and could be neglected. UV-Visible spectra were taken at various time points. The decrease in $A_{500/480}$ was fitted in a single exponential decay curve with time in Prism to obtain half-lives. After each measurement, the sample was immediately frozen in a glass vial precooled by dry ice and stored at 4 °C to quench any thermal process. Later, these samples were divided into two equal halves and were used for measuring the rate of Schiff base hydrolysis and thermal isomerization.

2.13 Thermal isomerization
The rate of thermal isomerization was measured as a function of the rate of retinal isomerization at different time point during the thermal decay process (117,118). The first half of the samples stored after measuring the rate of thermal decay were incubated with hydroxylamine at 4 °C for half an hour to break the Schiff base linkage between retinal and the opsin protein. Organic solvents (methanol, hexane and dichloromethane) were used to denature the opsin protein and subsequently the retinal was extracted in the form of retinaloximes. The various isomeric forms of retinaloximes obtained at different time points during thermal decay process were analyzed using reverse phase chromatography. Samples were injected into a Beckmann analytical silica column (25 cm x 4.6 mm inner diameter, 5 µm particle size) that was connected to an HPLC system (Beckmann Coulter SYSTEM GOLD 125 Solvent Module®). A mobile phase of hexane supplemented with 8% diethyl ether and 0.33% ethanol was used for the run. UV absorption at 360 nm was used for detection (Beckmann Coulter SYSTEM GOLD® 168 detector).

2.14 Schiff base hydrolysis
The rate of Schiff base hydrolysis was measured at various time points using an acid denaturation assay (118-121). The second part of the samples stored at different time points during the thermal decay experiment were denatured using 4 µl of 1 M HCl. The pH of the reaction mixture was measured and confirmed to be around 1-2. The UV-Visible spectrum of each sample was taken. $OD_{440}$ characterizes the intact protonated Schiff base and the $OD_{380}$ characterizes the free retinal after hydrolysis of the Schiff base. The fitted intensity at $OD_{440}$ was plotted in a single exponential decay curve as a function of time in Prism to obtain the half-life for Schiff base hydrolysis.
2.15 Reconstitution of rhodopsin into nanodiscs
Both *in vitro* phosphorylation of the mutants or WT rhodopsin and binding of the arrestin to the mutants or WT rhodopsin were measured after reconstitution of the rhodopsin in nanodiscs (122). For the preparation of nanodiscs, palmitoyloleoylphosphatidyleholine (POPC) and palmitoyloleoylphosphatidylglycerol (POPG), which self-assemble into lipid particles (123), were mixed at a 3:2 molar ratio and dried under nitrogen. Remaining chloroform was removed by 3 hour incubation in vacuum provided by a SpeedVac system (Savant). Dried lipids were solubilized in buffer A (20 mM HEPES, 100 mM NaCl and 1 mM EDTA, pH 8) containing 2.4% DM to a concentration of 24 mM by sonication [2”pulse-4”pause during 3 min @ampl.28%] and freeze/thaw cycles until the solution was liquid and transparent at 4 °C. Under the dim red light, a maximum of 10 μM purified mutant or wild type rhodopsin and 8 mM lipids, 0.8% DM, 100 μM MSP1E3D1 (124) were gently mixed with one volume of equilibrated Bio-Beads and rocked at 4 °C overnight. Size exclusion chromatography on a Superdex S200 HR10/30 in buffer A was used to purify nanodiscs containing rhodopsin. Reconstituted rhodospin was quantified by absorbance at 500 nm (40,600 M⁻¹cm⁻¹).

2.16 *In vitro* phosphorylation assay
Rhodopsin was phosphorylated in kinase buffer (20 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 10 μg/ml BSA, 0.5 mM ATP) by GRK1 (2.5 μM), purified as described (125,126) for 30 min at room temperature under constant illumination (incandescent light). Analytical assays were done in 10 μl reaction volume in the presence of [γ-³²P] ATP to obtain final specific activity 1,100–1,500 cpm/pmol. An equal volume of SDS sample buffer was added to stop the reaction. Samples were run on electrophoresis in a 4% stacking and 10% running gel. Gels were stained using Coomassie Blue, de-stained with water, dried and exposed to X-ray film for 1-3 hours. Rhodopsin bands were excised and then quantified using a scintillation counter. Preparative reactions were performed in a volume of 120 μl (a 10 μl aliquot was added to [γ-³²P] ATP, incubated in parallel) and used to determine phosphorylation stoichiometry. Regeneration of the phosphorylated opsin was done with 3 molar excess of 11-cis-retinal at room temperature for 2 hours.

2.17 Arrestin binding to rhodopsin in nanodiscs
The bovine arrestin-1 with 34 leucines was labeled using [³H] leucine and [¹⁴C] leucine with a specific activity of the mix 1.5-3 Ci/mmol leucine, yielding a specific activity 51-102 Ci/mmol (113-226 dpm/fmol) of bovine arrestin-1.
Gel-filtration on 2 ml Sephadex G-75 column was used to separate unincorporated labeled leucine from the translated labeled arrestin-1. Arrestin-1 was incubated with 7.5 pmol (0.3 µg) of different functional forms of rhodopsin in nanodiscs in 50 µl of 50 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 1.5 mM dithiothreitol, 100 mM potassium acetate for 5 min at 30 °C under room light (Rh*, P-Rh*, and P-Ops) or in the dark (Rh and P-Rh). The samples were immediately cooled on ice and rhodopsin-bound and free arrestin-1 was separated on 2 ml Sephadex G-100 columns equilibrated with 10 mM Tris-HCl, pH 7.5, 100 mM NaCl (underdim light for Rh and P-Rh samples) and quantified using liquid scintillation. Nonspecific binding was determined in the presence of equal amount of empty nanodiscs and subtracted.

2.18 Crystallization

2.18.1 Gel filtration
The bigger micelle size of DM is unsuitable for crystallization of rhodopsin. Size exclusion chromatography (SEC) was used to exchange the detergent prior to crystallization to 1% OG (CMC=0.7% w/v). OG has a higher CMC and small micelle size in comparison to DM and is suitable for the crystallization of rhodopsin (61).

For SEC, a Superdex 200 10/300 GL gel filtration column (GE healthcare) was equilibrated with 10 mM sodium acetate buffer (pH 5.0) containing 1% OG and 100 mM NaCl for approximately 2 column volumes. 500 µl of the concentrated protein from affinity chromatography was injected in a 1 ml injection loop and loaded on the column. The column connected to an HPLC system (Äkta prime) with the flow rate of 0.1 ml/min and fractions of 0.5 ml were collected. The gel filtration was performed in the dim light condition at 4 °C. The UV-visible spectra of fractions from the peak at 14 ml were taken to control the quality and quantity of the eluted protein. Fractions containing protein from the major elution peak were pooled and concentrated to 8-10 mg/ml using a 5 ml 30 kDa cut off centrifugal concentrator (amicon ultra Merck) at 3220×g (4 °C).

2.18.2 Crystallization set up
Crystallization of rhodopsin mutants was done by vapor diffusion in hanging drops using 24 well plates (VDX, Hampton Research) and 22 mm siliconized cover slips (1 ounce, Hampton Research) under dim light conditions. Various crystallization setups were used for crystallizing various states of rhodopsin. All crystallization trials were set up at 4 °C in vapor
diffusion against the reservoir solution, combining the effects of salt, various lipid ratios and pH on the protein.

For crystallization, the protein at concentration of 5 mg/ml or 7.5 mg/ml was mixed with dried brain lipid extract (Avanti Polar Lipids, 25 mg/ml in chloroform, 1 w/w) in various protein to lipid ratios (w/w) to the total volume of 30 µl. Furthermore, additives were added depending on the state of the rhodopsin as shown in the table below (Table 2.2).

<table>
<thead>
<tr>
<th>Rhodopsin State</th>
<th>Additive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opsin</td>
<td>none</td>
</tr>
<tr>
<td>Metarhodopsin II with peptide</td>
<td>all-trans-retinal (10 µM) + 3 mg/ml Gα C-terminal peptide {GαCT1 (ILENLKDCGLF)/ GαCT2 (ILENLKDVGFL)}</td>
</tr>
<tr>
<td>Metarhodopsin II without peptide</td>
<td>all-trans-retinal (10 µM)</td>
</tr>
</tbody>
</table>

Table 2.2 Additives used for crystallizing various states of the rhodopsin.

For crystallization of metarhodopsin II, the brain extract and all-trans-retinal were dried overnight in a desiccator covered with aluminium foil at room temperature. The mutant protein was mixed with dried brain extract and all-trans-retinal and after incubated for 30 minutes at 4 °C. Prior to crystallization, the protein was selectively light activated for 5 minutes using a > 475 nm long pass filter to prevent exposure of free retinal and metarhodopsin II. The protein was spun down for 1 min at 12,000×g at 4 °C. In the final crystallization phase, the rhodopsin was pipetted against a 1 ml reservoir solution containing 2.5-3.5 M (NH₄)₂SO₄, 100 mM NaOAc (pH 4.0-6.0) in the dark. The rim of the wells in the VDX plates was greased with industrial grease (Dow corning® vacuum grease, Hampton Research). Reservoir solutions were pipetted at room temperature, cooled and stored at 4 °C. The same steps were followed for the crystallization of the opsin except that all-trans-retinal and the light-activation steps were skipped.

The protein to reservoir ratio in the hanging drop was 1:1 in the final drop volume of 2 µl. For crystallization of rhodopsin and metarhodopsin II, plates were examined regularly using a stereomicroscope (Leica MZ 75) supplemented with red glass filter in dim light at 4 °C. Setups with opsin could be examined without the red glass filter in normal light. The crystals were cryo-protected in 10% (w/v) trehalose by adding the cryo-protectant solution directly to the drop. The crystals were then harvested using nylon loops of variable sizes mounted on the magnetic wand and flash frozen in liquid nitrogen.
2.19 Crystal structure determination

2.19.1 Data collection
Diffraction quality of obtained rhodopsin crystals was accessed using synchrotron radiation at the Swiss Light Source (SLS) beamlines X06SA-PXI or X06DA-PXIII with high-resolution diffractometer.

All data sets except one were collected using a fine slicing, low-dose/high redundancy data collection strategy. In this strategy data were collected for a range of 360° with oscillation angle of 0.05° or 0.1°

2.19.2 Data processing and structural validation
The data were processed using XDS (127), CCP4 suite (128), PHENIX (129), and COOT (130). The data were integrated using XDS and brought onto the same scale using XSCALE and converted to the CCP4 usable format using XDSCONV. For highly redundant data $R_{merge}$ values do not accurately reflect data quality. Instead, $I/\sigma I$, $R_{pim}$ and CC (1/2) were used to define the resolution cut off (131). The phases were obtained by molecular replacement using the structural model of metarhodospin II (PDB ID: 4A4M (60)) and PHASER (132). The obtained structural models were then refined using iterative cycles of model building in COOT and refinement (rigid body, energy minimization, simulated annealing, individual B-factor refinement) using PHENIX. Geometric restraints for the ligand and heteroatoms were prepared using the PRODRG2 server (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg). TM2 and 7 were removed to decrease model bias and were refined and built using PHENIX and COOT respectively.

The following four criteria were used to add water molecules to the model: the presence of clear positive difference peaks in the $F_o-F_c$ electron density map calculated after simulated annealing without water molecules and contoured at 2.5σ; two or more hydrogen bonds to the protein or other water molecules; clear electron density of the water molecule in the $2F_o-F_c$ electron density map contoured at 1σ; and a B-factor cut off within 20% of the average. Ramachandran analysis was performed using Procheck (133). Overall validation of the structure was done using MolProbity (http://molprobity.biochem.duke.edu)

2.20 Molecular dynamics simulations
For the simulations of dark, inactive state wild-type rhodopsin, we used the structure of bovine rhodopsin in a trigonal crystal form (PDB ID: 1GZM (13)). The dark state of the rhodopsin mutant G90D(s-s) and T94I(s-s) was modeled using 1GZM structure with an
additional stabilizing disulfide bond as a template, and substituting residue Gly90 and Thr94 to Asp and Ile using the backbone-dependent rotamer library (134) implemented in PyMOL (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC), and choosing the Asp and Ile rotamer that minimized steric clashes with the surrounding residues. We added crystallographic waters, resolved in the 1GZM ground state structure. Cysteines 322 and 323 were palmitoylated. Glu, Asp, Arg and Lys residues were set as charged, except Glu122$^{3.37}$ and Asp83$^{2.50}$ (135). We also set up additional models where Glu113 was considered neutral. Topology and parameter definitions for palmitoyl-cysteine and retinal bound via protonated Schiff-base link to lysine (136-142) were obtained from the parameter/topology repository of NAMD.

The models of WT(s-s) and mutant rhodopsin were embedded in a solvated and pre-equilibrated lipid bilayer consisting of approx. 300 molecules of 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphatidylcholine (POPC) and approximately 22000 water molecules. Sodium and chloride ions were added to a concentration of 0.15 M NaCl, and then additional ions were added to achieve charge neutrality. The system measured roughly 75 x 75 x 90 Å$^3$, with a total of approximately 59000 atoms. These systems were equilibrated as follows: first a short (0.5 ns) simulation was performed in which only the lipid tails were allowed to move, in order to induce the appropriate disorder of a fluid-like bilayer. Then, the geometry of the entire system was optimized by 1000 steps of energy minimization, followed by two equilibration steps with the protein constrained (0.5 ns) and without constraints (0.5 ns). In order to estimate the structural stability of the retinal binding site, the equilibrated structures were subjected to 15 ns of simulated annealing, where the temperature was raised from 300 K to 450 K in 7.5 ns, and then lowered to 300 K in 7.5 ns. Simulations were carried out using NAMD 2.8 (143,144) at constant pressure (1 atm), and using a time step of 2 fs.
Chapter 3 Results

3.1 Expression of disease causing rhodopsin mutants

Representative for the 150 disease mutations known for rhodopsin, I choose two mutations (G90D, T94I) causing the mild CSNB phenotype and two mutations (T17M, P23H) representing the severe RP phenotype for initial expression tests. All four mutations were combined with the thermo stabilizing N2C/D282C background (indicated as (s-s) throughout this thesis) to increase expression levels and simplify purification. Introduction of this background further removed one of the two glycosylation sites, reducing carbohydrate heterogeneity and potentially increasing crystallizability of the protein. In the initial testing phase Hek293S GnTI- TetR cells were transfected with pcDNA3.1 expression vectors and the mutant rhodopsin expressed for 48 hours. Cells were harvested, solubilized in 1.25% DM and the solubilisate cleared by centrifugation. All mutants expressed at sufficient levels, enough for western plot analysis.

To access folding propensity and cellular expression of different rhodopsin mutants, we analyzed their expression on the plasma membrane of HEK293S GnTI- cells by confocal microscopy (Figure 3.1). Both CSNB mutants i.e. G90D(s-s) and T94I(s-s) were predominantly localized in the plasma membrane, implying normal trafficking and folding of the protein. However, RP mutants i.e. T17M(s-s) and P23H(s-s) were predominantly localized in the cytoplasmic compartment with impaired trafficking to the plasma membrane in agreement with previous reports (80,90,145).
Figure 3.1 Distribution of various opsin mutants on the cell surface of HEK293S GnTI- cell: HEK293 cells were transiently transfected with pcDNA3.1 vector containing various opsin mutants, then fixed and stained with 1D4 primary antibody and fluorescently labeled goat anti-mouse as secondary antibody.

After verifying both in vitro and cellular expression, stable cell lines were generated for each rhodopsin mutant using geneticin (G418) as a selectable marker. The stable cell lines were created for each rhodopsin mutant and were stored in liquid nitrogen. The opsin mutant T17M(s-s) and P23H(s-s) were excluded from further structural work due to their low expression and misfolding propensity and only G90D(s-s) and T94I(s-s) were further structurally characterized due to their WT like expression and their propensity to fold well.

3.2 Small scale medium optimization

A prerequisite for any crystallization project is the availability of milligram amounts of purified protein. To establish the expression of rhodopsin mutants at a larger scale, I used stable cell lines expressing constitutively active M257Y(s-s) opsin. This mutant opsin was an attractive choice for the following reasons. First, expression of M257Y(s-s) in a wavebag bioreactor had already been established in the Schertler group at the Laboratory of Molecular Biology and its reestablishment at the Paul Scherrer Institute would serve as a positive control and proof of principle for other CSNB mutants that might behave differently. Second, the use of M257Y(s-s) opsin has allowed me to establish large-scale expression already before stable cell lines expressing rhodopsin disease mutants were available. As a first step I tested cell growth and reachable cell density in a series of four commercially available expression media. In DMEM, CD293 and Free Style medium cells reached a maximal density of around $2 \times 10^6$ cells per ml in 250 ml flat-bottomed plastic flasks (Figure 3.2). In contrast, cells in PEM medium reached a cell density of around $3.5 \times 10^6$ cells per ml, suggesting PEM medium as the best choice among the tested media.
Figure 3.2 Comparison of the maximum cell density in the suspension medium with various compositions: HEK293GnTI-TetO cells with opsin mutant M257Y(s-s) were used for screening with respect to the maximum cell density. Average maximum cell density and standard deviation were calculated over two independent measurements.

3.3 Large scale expression

For scaling up to 10 l cell culture, a 20 l wave bag bioreactor was used. The initial culture was started in 8 l culture volume (in 20 l bag) with 2 l of inoculum to a starting cell density of 0.7-0.8×10^6 cells per ml. Cells were allowed to grow to 3×10^6 before induction with tetracycline and sodium butyrate. The ideal time for protein harvesting was 72 hours, post induction, as the cell density started to decrease due to exhaustion of nutrition in the medium or because of toxic effects of tetracycline induced overexpression. The total time from starting of the initial suspension culture to cell harvesting was 10-12 days (Figure 3.3). The step-wise dilution technique used in large scale wave bag bioreactor bypasses the need for transfers, as would be the case using 2 l suspension flasks. Subsequently, the large scale production of CSNB causing rhodopsin mutant T94I(s-s) and G90D(s-s) and stabilized WT(s-s) was also done using the 20 l wave bioreactor. The obtained cell mass of HEK293S for WT(s-s), G90D(s-s) and T94I(s-s) was comparable to that for the M257Y(s-s) and ranged from approximately 120-150 g of wet cells from a 10 l wave bag bioreactor culture. The amount of cell mass is equivalent to the cell mass obtained from approximately 1200, 15 cm adherent cell culture plates and expression in suspension culture, thus leads to a significant reduction of time and costs in the production of rhodopsin.
3.4 Extraction and purification of CSNB causing mutant rhodopsin

Purification of rhodopsin WT(s-s) and mutants for crystallization experiments was achieved using a two-step purification procedure; 1D4 affinity chromatography followed by size exclusion chromatography (SEC) (Figure 3.4 & 3.5). For both G90D(s-s) and T94I(s-s), SDS-PAGE and SEC profile of the ligand free opsin state were identical to the 11-cis-retinal reconstituted ground state, with the only difference that the small shoulder peak of free 11-cis-retinal was absent (Figure 3.4). The band observed on SDS-PAGE is approximately 37 kDa, corresponding to the molecular weight of rhodopsin. Typically, 3-5 mg of purified WT(s-s) opsin was obtained from 40-50 g of HEK293 cells of WT(s-s) (Figure 3.4) whereas a similar amount of cells resulted in 2-3 mg of purified G90D(s-s) and T94I(s-s) rhodopsin (Figure 3.5).
Figure 3.4 SDS-PAGE and SEC profile of WT(s-s) opsin state at 4 °C: WT(s-s) opsin was purified from HEK293 in 0.125% DM. SEC in 10 mM Sodium acetate pH 5.0, 100 mM NaCl, 1% OG. The chromatogram shows symmetric mono-disperse peak. SDS-PAGE showing final purity of the purified protein after affinity chromatography and gel filtration. Lane 1, Molecular marker; lane 2, the wild type(s-s) after 1D4 elution.

Figure 3.5 SDS-PAGE and SEC profile of 11-cis-retinal reconstituted ground state rhodopsin mutants at 4 °C: G90D(s-s) (upper panel) and T94I(s-s) (lower panel) were purified from HEK293 in 0.125% DM. A) SDS-PAGE showing final purity of the purified protein after affinity chromatography. Lane 1a-1c/1a-1d, different elutions of the protein. B) SEC in 10 mM Sodium acetate pH 5.0, 100 mM NaCl, 1% OG. The chromatogram of both G90D(s-s) and T94I(s-s) show symmetric mono-disperse peak with a small shoulder of free 11-cis-retinal following the major protein peak.

The UV-Visible spectroscopy was used to determine the concentration of purified protein (Figure 3.6). Rhodopsin has a characteristic absorption at 500 nm resulting from the bound 11-cis-retinal chromophore. The ratio between protein absorption at 280 nm and chromophore absorption at 500 nm is a reliable indicator for the fraction of reconstituted
protein, analogous to a radio-ligand binding experiment commonly used to probe functionality of GPCRs. Purified WT(s-s) rhodopsin yielded $A_{280}/A_{500}$ ratios of 1.6-1.7, while G90D(s-s) and T94I(s-s) had $A_{280}/A_{500}$ ratios of 2.2-2.2 and 1.8-2.0, respectively (Figure 3.7 A). Although the fraction was lower, both mutants were thus able to bind 11-cis-retinal and reconstitute into functional proteins. The retinal binding pocket was, however, perturbed to some extent as indicated by the blue shift to 482 nm in case of G90D(s-s) and 478 nm in case of T94I(s-s). Upon isomerization of 11-cis-retinal by light both G90D(s-s) and T94I(s-s) peaks shifted to 380 nm characteristic for the G protein activating metarhodopsin II state (Figure 3.7 B).

3.5 Biochemical characterization of CSNB mutants

To investigate the effect of G90D(s-s) and T94I(s-s) on protein integrity as well as on the binding of arrestin, a series of biochemical experiments were conducted.
3.5.1 Thermal stability measurement

Fluorescence based thermal stability assays were conducted to access the impact of G90D(s-s) and T94I(s-s) mutations on protein stability. The thermal stability of G90D(s-s) and T94I(s-s) were compared to that of the WT(s-s) both in the presence and absence of 11-cis and all-trans-retinal. In the presence of 11-cis-retinal the transition temperature of WT(s-s) increased by 10 °C from 53 °C to 63 °C. Unlike WT(s-s), the effect of 11-cis-retinal on the melting temperature of G90D(s-s) and T94I(s-s) was less pronounced. The melting temperature of T94I(s-s) increased from 49 °C to 53 °C upon incubation with 11-cis-retinal, whereas the effect on G90D(s-s) opsins was insignificant in comparison to the WT(s-s) opsins. Interestingly, the melting temperature of G90D(s-s) opsins was with 58 °C, 5 °C higher than that of WT(s-s) opsins, whereas that of T94I(s-s) opsins was 4 °C lower. The melting temperature of G90D(s-s) both in the presence of 11-cis-retinal and all-trans-retinal was similar to that in absence of retinals suggesting that the majority of G90D(s-s) didn’t bind retinal and remain in the opsin form. The increase in the melting temperature of T94I(s-s) in the presence of all-trans-retinal was insignificant. These results indicated that the G90D(s-s) opsin is more stable than WT(s-s) and both G90D(s-s) and T94I(s-s) do perturb WT protein integrity. This suggests that the stability of the G90D(s-s) and T94I(s-s) ground state is lower in comparison to the WT(s-s), in agreement with their perturbed absorption spectra.(Figure 3.8).

![Figure 3.8 Effect of CSNB mutants G90D(s-s) and T94I(s-s) on the thermal stability of the protein](image)

**Figure 3.8 Effect of CSNB mutants G90D(s-s) and T94I(s-s) on the thermal stability of the protein:** The mean melting temperature \(T_{m,0}\) was measured for WT(s-s), G90D(s-s) and T94I(s-s) in the presence or absence of retinal isomers in a fluorescence based thermal stability assay. Average melting temperature standard deviation was calculated over four independent measurements (86).
3.5.2 Thermal decay of CSNB mutants

The rate of thermal decay was monitored by taking UV-Visible spectra at various times of 55 °C incubation for G90D(s-s), T94I(s-s) and WT(s-s) rhodopsin (Figure 3.9A-C). With increase in time, there is a decrease in $A_{500}/A_{480}$ with concomitant increase in absorption at 380 nm. The decrease in 500 nm for WT(s-s) or 480 nm for CSNB mutants correspond to the thermal decay of the rhodopsin ground state with time. The increase in the 380 nm can be attributed to either free retinal or to the formation of all-trans-retinal bound metarhodopsin II. The decrease in $A_{500}/A_{480}$ was plotted as a function of time and yielded half-lives of 1043±75 min, 21.9±1.4 min and 424±31 min for the WT(s-s), T94I(s-s) and G90D(s-s), respectively (Figure 3.9D). The T94I(s-s) ground state decays 47 times faster in comparison to the WT(s-s) whereas the decay rate for G90D(s-s) was only 2 times faster, suggesting T94I(s-s) ground state to be less stable in agreement with the thermo-shift measurements.

3.5.3 Thermal isomerization of retinal

Retinal was extracted at different time points of thermal incubation to determine the ratio between different retinal isomers by HPLC analysis. A total of four peaks was observed in the chromatogram corresponding to 11-cis (the major peak at $t=0.1$ min) and all-trans-retinal (major peak in the last trace), which form the major peaks and some minor peaks of 13-cis-15-syn and 11-cis-15-anti (Figure 3.9E-F). The decrease in the 11-cis-retinal peak concurs with the increase in all-trans-retinal peak, indicating temperature induced isomerization of retinal. The fraction of 11-cis was plotted as a function of time. Fitting to a single exponential decay yielded a decay time of 1136 ± 27 min for WT(s-s), 58.6 ± 6.4 min for T94I(s-s) and 503 ± 93 min for G90D(s-s), suggesting thermal isomerization of 11-cis-retinal in T94I(s-s) to be 19 times and in G90D(s-s) to be 2 times faster compared to the WT(s-s) (Figure 3.9G).

3.5.4 Hydrolysis of the Schiff base

The kinetic of Schiff base hydrolysis was measured by acid denaturation of the ground state (Figure 3.9 I-K). Acid denaturation resulted in the formation of a 440 nm peak, originating from retinal bound to the protein by a protonated Schiff base. Hydrolysis of the Schiff base and the concurrent appearance of free retinal is indicated by absorption at 380 nm. The decrease in the 440 nm peak was plotted as a function of time in a single exponential decay curve, yielding a decay rate of 797 ± 25 min for WT(s-s), 22.2 ± 1.3 min for T94I(s-s) and 406 ± 91 min for G90D(s-s) (Figure 3.9L). The Schiff base hydrolysis, which is 35 times
faster in T94I(s-s) as compared to the WT(s-s), also confirms the low stability of the T94I(s-s) ground state.

Figure 3.9 Kinetic characterization of rhodopsin decay: Thermal decay of the ground state rhodopsin: A) WT(s-s), B) G90D(s-s) and C) T94I(s-s) rhodopsin. D) Absorption maximum plotted as a function of time and fitted to a single exponential decay. Acid denaturation experiment of ground state: E) WT(s-s), F) G90D(s-s) and G) T94I(s-s) rhodopsin. H) OD440 is plotted as a function of time and fitted to a single exponential decay. HPLC traces for retinal extraction experiment for ground state: I) WT(s-s), J) G90D(s-s) and K) T94I(s-s) rhodopsin, L) Ratio of 11-cis-retinal is plotted as a function of time and fitted to a single exponential decay. Experiments in collaboration with Ying Guo and Elsa Yan, Yale University.

3.5.5 Phosphorylation and arrestin binding

To probe their ability to be phosphorylated by G protein-coupled receptor kinase 1 (GRK1) and to subsequently bind arrestin-1, we reconstituted rhodopsin variants into the native like environment of high density lipoprotein particles (HDL) also known as nanodiscs (147). The achieved phosphorylation levels, for the two CSNB mutants G90D(s-s) and T94I(s-s), as well as stabilized rhodopsin, and the constitutively active mutant M257Y(s-s) as controls, are shown in Figure 3.10. The level of phosphorylation reached 2-3 phosphates per rhodopsin in case of all samples. Intriguingly, G90D(s-s) and T94I(s-s) showed slightly higher levels of phosphorylation with 2.84 and 2.74 respectively. M257Y(s-s) reached the lowest level of phosphorylation with 1.72 phosphates per rhodopsin.
Figure 3.10 Phosphorylation levels G90D(s-s) and T94I(s-s) in nanodiscs: Phosphorylation levels of light activated WT(s-s), G90D(s-s), T94I(s-s) and M257Y(s-s) rhodopsin. (122). Experiment in collaboration with Serjey Vishnivetskiy and Seva Gurevich group, Vanderbilt University.

Binding of arrestin-1 to phosphorylated rhodopsin in nanodiscs was measured using a direct binding assay (148) with radiolabeled arrestin-1 produced in cell-free translation (149). As expected arrestin-1 did not bind to phosphorylated rhodopsin in the dark (P-Rh), nor did it bind light-activated but unphosphorylated rhodopsin (Rh*). On the other side clear binding was observed with rhodopsin both light-activated and phosphorylated (P-Rh*). Interestingly arrestin-1 also bound the phosphorylated apoprotein opsin (P-opsin) to a similar extent as the fully activated P-Rho*. All three constitutively active mutants showed a similar picture with slightly increased binding to the non preferred forms P-Rh and Rh* in case of M257Y(s-s) rhodopsin and Rh* in case of G90D(s-s) rhodopsin. Surprisingly, binding of arrestin-1 to G90D(s-s) phosphorylated opsin was reduced by 70% unlike the other two constitutively active T94I(s-s) and M257Y(s-s) mutants, suggesting less binding of arrestin-1 is not a CAM associated phenomenon and is specific for G90D(s-s) opsin (Figure 3.11). It also suggests that reduced binding to P-opsin is not common for the two CSNB mutants.
Figure 3.11 Arrestin binding in nano discs to WT(s-s) rhodopsin and three constitutively active mutants: Interaction of arrestin-1 with different forms of rhodopsin (P-Rh: phosphorylated rhodopsin, P-Rh*: light activated phosphorylated rhodopsin, Rh: unphosphorylated rhodopsin; Rh*: light activated unphosphorylated rhodopsin; P-opsin: phosphorylated opsin) was accessed using direct binding assay. The mean and S.D. were determined from four experiments (122). Experiment in collaboration with Serjey Vishnivetskiy and Seva Gurevich, Vanderbilt University.

3.6 Crystallization of CSNB mutants

All crystallization experiments were done exclusively at 4 °C by vapor diffusion in a hanging drop set up. Equal volumes of concentrated protein and reservoir solution (1+1 µl) were mixed and equilibrated against reservoir solution containing ammonium sulfate as a precipitant solution in a 24 well hanging drop set up. For opsin and light-activated rhodopsin, various concentrations of ammonium sulfate versus pH were screened and for ground state crystallization in addition to ammonium sulfate, various concentrations of lithium sulfate were tested based on previously described conditions (13,51,54,61). Crystallization was done for various states of G90D(s-s) and T94I(s-s) rhodopsin including opsin, light-activated and ground states. Crystallization of light-activated G90D(s-s) and T94I(s-s) rhodopsin was done in the presence and absence of Gα peptide. Crystals were found for light-activated G90D(s-s) and T94I(s-s) with and without Gα peptide at 2.5-3 M ammonium sulfate concentration and pH 4.5-6, after 3-4 days (Figure 3.12). Furthermore, crystals of WT(s-s) and G90D(s-s) apoprotein opsin were also obtained under a similar condition. In spite of extensive screening
around G90D(s-s) opsin crystallization condition, T94I(s-s) opsin could not be obtained. No crystals were obtained for G90D(s-s) and T94I(s-s) ground state.

Figure 3.12 Crystallization of the WT(s-s) opsin and CSNB mutants, G90D(s-s) and T94I(s-s): Crystal of the WT(s-s) opsin A: under normal light microscope. B: under cross polarizer. Well diffracting crystal of the light activated C: G90D(s-s); D: T94I(s-s). Crystals of WT(s-s) opsin and both mutants grew at 5-7.5 mg/ml protein concentration at low pH in the presence of ammonium sulfate as precipitant (60,61) and were frozen with 10% trehalose as a cryoprotectant. Light activated crystals display the yellow color characteristic for all-trans-retinal.

3.7 Crystallographic Data collection

Data was collected for G90D(s-s) opsin and for light activated G90D(s-s) and T94I(s-s) both in the presence and absence of modified C-terminal Gα peptide. The peptide resembles the C-terminus of the Gα subunit that specifically binds the active metarhodopsin II conformation (150). GαCT1 used for light-activated G90D(s-s) has a single amino acid (K341L) substitution that increases the affinity by two orders of magnitude in comparison to native Gα peptide (151). For T94I(s-s), GαCT2 peptide was used, which is a derivative of high affinity GαCT1 with an additional single amino acid (C347V) substitution and was shown to improve crystal diffraction quality (59). Crystals of G90D(s-s)-GαCT1 (Data set ID GD28), G90D(s-s) (Data set ID GD17) and G90D(s-s) opsin (Data set ID OG22) allowed data collection to 3.9 Å, 3.3 Å and 2.9 Å, in agreement with increased stability on adding ligand and the ability of C-terminal Gα peptides to bind and stabilize the active state.
Crystals of light activated T94I(s-s) bound with GaCT2 (Data set ID AS120) diffracted to 2.8 Å (Figure 3.13). Surprisingly, light activated T94I(s-s) crystals grown without peptide (Data set ID TA10) diffracted to 2.3 Å. Improvement in the resolution of T94I(s-s) is partly due to technical advances in detector and optics and improvement in crystal handling and freezing with time, but the major difference was in the data collection strategy. For data collection from T94I(s-s) GaCT2 crystals, an oscillation angle of 0.5° was used, whereas for T94I(s-s) without peptide 0.1° oscillations were used. T94I(s-s) without peptide data set was collected at the SLS beamline PXI-X06SA using a Pilatus detector. Pilatus is a silicon hybrid pixel X-ray detector that operates in a single photon (152) mode. Hybrid-pixel detectors like Pilatus have several advantages over conventional CCD detectors including a fast readout time, a high resolution of closely spaced reflections, a high dynamic range and absence of readout noise (153), well suited for using a fine slicing strategy. Mosaicity of T94I rhodopsin crystals was very low, equally suited for a fine slicing strategy. Furthermore, the fine slicing strategy is also advantageous for membrane proteins that usually exhibit poor diffraction and higher background scattering (154,155). We think, thus, using fine slicing data collection strategy in combination with a Pilatus detector resulted in substantially improved scaling statistics for T94I(s-s) rhodopsin crystals.

Most of the crystals of WT(s-s) opsin were diffracting less than 5 Å. The best crystal (Dataset ID OW12) diffracted to 2.5 Å and complete data set was collected from a single crystal using fine slicing strategy at PXI-X06SA beamline with Pilatus detector.
Figure 3.13 Diffraction image of the CSNB mutant T94I(s-s): Single frame of T94I(s-s) with oscillation of 0.5°. Diffraction images collected with the focused X-ray beam at PXI-X06SA beamline with the Pilatus detector at Swiss Light Source (SLS).
Table 3.1: Data collection and refinement statistics for light activated G90D(s-s) with/without \( \alpha \)CT peptide (PDB ID: 4bey and 4bez) and G90D(s-s) opsin (86).

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\( ^{a} \) highest resolution shell is shown in parenthesis
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**Refinement**

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\(^a\) highest resolution shell is shown in parenthesis.

**Table 3.2:** Data collection and refinement statistics for T94I metarhodopsin II with/without GαCT peptide. (Singhal et al, in preparation)

### 3.8 Crystal Structures of CSNB causing G90D(s-s) and T94I(s-s) rhodopsin

Crystal structures of light activated G90D(s-s) and T94I(s-s) both in the presence and absence of GαCT peptide and constitutively active G90D(s-s) opsin state were solved by molecular replacement with our previous structure of light activated metarhodopsin II (60). It should be noted that the low resolution obtained for G90D(s-s) opsin did not allow accurate refinement of an atomic model. However, no obvious alterations of the protein backbone could be detected.

All four structures consist of 1-326 amino acids of the apoprotein, lacking 22 C-terminal amino acids that were not resolved in the corresponding electron density maps, probably because of high flexibility. Furthermore, each model has a thermal stabilizing (N2C/D282C) disulfide bond, retinal, one acetate molecule bound near a proposed retinal exit channel (156),
an oligosaccharyl chain at N15, several waters and two n-octyl-β-D-glucopranoside molecules. The model also contains one molecule of palmitic acid covalently bound to C323, a second palmitic residue at residue C322 is only included in T94I(s-s) without a GaCT2 peptide model due to lack of clear electron density in the lower resolution models. The overall structure shows canonical seven TM helices connected by cytoplasmic and extracellular loops (CL1-CL3 and EL1-EL3), followed by a short amphipathic helix H8. Overall structures differ significantly from the ground state (PDB ID: 1GZM (13)) and resemble that of constitutively active M257Y(s-s) (60) metarhodopsin II and to metarhodopsin II obtained by back soaking of the all-trans-retinal (59). The observed root mean square deviation (rmsd) values for (G90D(s-s)-GaCT1/Opsin*), (G90D(s-s)/Opsin*), (T94I(s-s)/Opsin*) and (T94I(s-s)-GaCT2/Opsin*) are 0.6 Å, 0.53 Å, 0.13 Å and 0.23 Å. Both G90D(s-s) and T94I(s-s) show an outward movement of TM6 by 5 Å with respect to the ground state, a movement critical for G protein activation (68). The presence or absence of the GaCT1/GaCT2 peptide did not cause rearrangement of the overall receptor structure (Figure 3.14). This is in agreement with biochemical and spectroscopic characterizations of G90D(s-s) and T94I(s-s) that showed both mutants essentially fold well and are capable of light induced G protein activation (146). Both CSNB mutations do not cause larger alterations of the overall architecture, but introduced more subtle changes in the corresponding retinal binding pocket.
Figure 3.14 Overall architecture of the CSNB mutants G90D(s-s) and T94I(s-s) crystal structures: Comparison of the light activated G90D(s-s) and T94I(s-s) structures with metarhodopsin II obtained by light activation of M257Y(s-s) rhodopsin (60). All-trans-retinal (yellow), cis-retinal isomers (orange), site of retinal attachment K296 (slate blue), counter ion E113 (slate blue), CAMs (green) and GaCT1/GaCT2 peptide (cyan) are shown as spheres. Palmitoylation and glycosylation shown as sticks. The table shows the root mean square deviations (rmsd) of Ca atoms between structures of G90D(s-s) (PDB ID: 4BEY (86)), T94I(s-s) (Singhal et al, in preparation) and M257Y(s-s) (PDB ID: 4A4M (60)) with apoprotein opsin without GaCT peptide (PDB ID: 3CAP (62)). In addition, the rmsd values are also calculated for amino acids in the retinal binding pocket (defined with a distance cut off of 4 Å in the 4A4M metarhodopsin II structure).

Some residual electron density was observed in the ligand binding pocket of light activated G90D(s-s) suggesting that some retinal was retained in G90D(s-s) (Figure 3.15 A). However, the density for the K296 was not continuous with electron density of the retinal indicating that retinal is not covalently bound to the apoprotein opsin. The observed electron density in the ligand binding site of G90D(s-s) represents a heterogeneous population of noncovalently bound retinal, similar to the retinal moiety observed in E113Q(s-s) rhodopsin (61). In
comparison with E113Q(s-s), a more pronounced contribution of 13-cis-retinal in G90D(s-s) is noticeable. Furthermore, we observed a continuous electron density for D90$^{2.57}$-K296$^{7.43}$ that is due to the formation of a salt bridge between the carboxyl group of D90$^{2.57}$ and ε-amino group of K296$^{7.43}$. Initially, we thought that bulkier hydrophobic isoleucine side chain would behave similar to an Asp side chain of G90D(s-s) and would directly interfere with retinal binding. In stark contrast to G90D(s-s), the retinal binding pocket in T94I(s-s) however, resembles that of the light activated M257Y(s-s) structure. A strong electron density is observed connecting the all-trans-retinal to the side chain of K296$^{7.43}$ indicating covalently bound all-trans-retinal as a characteristic of the metarhodopsin II state (Figure 3.15B). Moreover, –CH$_3$ group of the bulkier isoleucine side chain is at a distance of 3.6 Å from Cε atom of the K296$^{7.43}$ side chain, stabilizing the Schiff base through direct van der Waals contact.

Figure 3.15 Electron density in the retinal binding pocket of G90D(s-s) and T94I(s-s): Electron density ($2F_o-F_c$ contoured at 1.5/1.8 sigma for G90D(s-s) and T94I(s-s), blue and $F_o-F_c$ contoured at 3.5 sigma, green) of the retinal-binding pocket calculated after simulated annealing refinement with omitted G90D(s-s) and T94I(s-s) side chain. The obtained difference map shows a clear difference peak for the introduced G90D(s-s) and T94I(s-s) mutation. A) A clear positive density for retained retinal in the binding pocket of G90D(s-s) and formation of a salt bridge between D90$^{2.57}$-K296$^{7.43}$ (86) B) The structure of T94I(s-s) resembles clean metarhodopsin II with intact Schiff base and retinal in all-trans conformation (Singhal et al, in preparation).

3.9 Molecular dynamics simulation of G90D(s-s) and T94I(s-s) ground state

As an alternative to the crystal structure of G90D(s-s) and T94I(s-s) ground state, we have employed molecular dynamics simulation to study the impact of the two CSNB mutations. We put particular emphasis on the interaction between the protonated Schiff base and its counterion E113$^{3.28}$ as this interaction is close to all four CSNB mutations and its release is one of the critical steps in rhodopsin activation. As a starting point for the simulations, we embedded the crystal structure of ground state rhodopsin (1GZM, (13)) into a lipid bilayer consisting of 300 lipid molecules. The system was further solvated with 22000 water molecules in addition to crystallographically determined intramolecular waters. The ligand binding pocket (defined as all residues having atoms within 6 Å of retinal) of WT(s-s)
rhodopsin remained remarkably stable with a final rmsd of 1.2 Å² and a distance between the protonated Schiff base and the counterion of 1.9 Å (Figure 3.16) indicating a closed activation switch. While preparing the G90D(s-s) initial model we protonated E113^{3.28} as was concluded from a previous FTIR spectroscopic study (157). The final rmsd for G90D(s-s) was 1.9 Å², slightly higher than observed for WT(s-s) rhodopsin. Importantly, during the simulation, the D90^{2.57} side chain was positioned between the counterion E113^{3.28} and the Schiff base. This increased the distance between E113^{3.28} and the Schiff base nitrogen to 7.5 Å, suggesting an opening of this critical activation switch. In case of T94I(s-s) rhodopsin we had no information on the protonation state of E113^{3.28}. Thus we simulated the T94I(s-s) ground state with both deprotonated E113^{3.28} as in WT rhodopsin and a protonated E113^{3.28} similar to the other CSNB mutant G90D(s-s). The observed final rmsd for T94I(s-s) with both protonated and deprotonated E113^{3.28} was 1.6 Å². Unlike G90D(s-s), the simulated annealing of T94I(s-s) without E113^{3.28} protonation did not show a complete opening of the E113^{3.28} - Schiff base activation switch, however the distance between E113^{3.28} and Schiff base increased to 2.4 Å. On the other hand, when we protonated E113^{3.28}, the I94^{2.61} side chain rested in a similar position between E113^{3.28} and the Schiff base as in our previous simulation using the G90D(s-s) mutation. The distance between the SB and –OE1 of E113^{3.28} increased to 6.8 Å, consistent with an open activation switch. Thus, both G90D(s-s) and T94I(s-s) with protonated E113^{3.28} have a very similar effect on the rhodopsin ground state, whereas the impact of I94^{2.61} with deprotonated E113^{3.28} was less pronounced.
Figure 3.16 Accelerated molecular dynamic simulation of the ground state: WT (PDB ID: 1GZM (13)) with additional thermo stabilizing disulfide bond, G90D(s-s), T94I(s-s) and T94I(s-s) with protonated E113\textsuperscript{3,28}. In the absence of the G90D(s-s) and T94I(s-s) ground state structure, both aspartate and isoleucine were placed in the WT(s-s) (PDB ID: 1GZM (13)) using a favorable rotamer without introducing major clashes with the rest of the protein. However, both isoleucine and aspartate in the ground state structures introduced clashes with several amino acids and were therefore optimized using energy minimization before simulation. WT(s-s) simulation was done with the protonated Schiff base while in the G90D(s-s) system, both E113\textsuperscript{3,28} and Schiff base were protonated (157). The protonated state of E113\textsuperscript{3,28} was unknown in T94I(s-s), therefore simulation were done for both deprotonated and protonated E113\textsuperscript{3,28}. T94I(s-s) ground state conformation with deprotonated E113\textsuperscript{3,28} was similar to that of the WT and was stable, whereas the ground state of G90D(s-s) and T94I(s-s) with protonated E113\textsuperscript{3,28} was unstable with a concomitant movement of E113\textsuperscript{3,28} residue away from the Schiff base, thereby, causing movement of TM3. Molecular dynamics simulation in collaboration with Milos Matkovic and Xavier Deupi, PSI, Villigen.
Chapter 4 Discussion

4.1 Structural insights into the ligand binding pocket of metarhodopsin II

The photoactivateable ligand of rhodopsin is 11-cis-retinal, covalently attached via a protonated Schiff base bound to K296 in TM7. After light induced isomerization of 11-cis-retinal to all-trans-retinal and formation of the metarhodopsin II state, the Schiff base is hydrolyzed leading to the formation of the inactive apoprotein opsin. Schiff base hydrolysis is thus a physiologically important process for the deactivation of rhodopsin.

Schiff base hydrolysis is a two-step process that is initiated by base catalyzed water and proceeds through the formation of protonated carbinolamine intermediate (158). According to the proposed mechanism, attack of base catalyzed water present in the vicinity of a point charge (aspartate or glutamate) leads to the formation of a carbinolamine intermediate, followed by the uptake of proton by –NZ of K296 from E181EL2. The suggested hypothesis is also consistent with observed changes in the carboxylate protonation state shown by FTIR spectroscopy (159-161). Finally deprotonation of the carbinol occurs that breaks the linkage to form a retinal aldehyde (162). Until recently the absence of a high resolution metarhodopsin II structure made it difficult to decipher the precise mechanism of water mediated Schiff base hydrolysis.

Previously determined metarhodopsin II structures revealed a potential H-bonding network that exists in the vicinity of the Schiff base and extends from E181EL2 to Y268 (59). A further water molecule connects the main chain carbonyl of E181EL2, the side chain of S186EL2 and –NH groups of S186EL2 and C185EL2 and constrains the structural fold of EL2. This network is further extended to T94I and E113 and is shown to be important for stabilizing the metarhodopsin II and, especially, one in the vicinity of the retinal binding pocket is critical in determining both integrity and rate of Schiff base hydrolysis (163).

Our high resolution 2.4 Å T94I(s-s) metarhodopsin II structure represents a clean metarhodopsin II state and resolves four more water molecules in the retinal binding pocket with one of them in the close proximity to the Schiff base (Figure 4.1). It seems unlikely that these water molecules are T94I(s-s) metarhodopsin II specific as the protein environment did not change between the two structures (59,60). Furthermore, the presence of these water molecules is cross verified with the native metarhodopsin II structure (59) that also show positive difference electron density peaks but were not placed due to low resolution. The
newly resolved 3 water molecules form a continuous H-bonding network extending from Y192\textsuperscript{EL2} to Y189\textsuperscript{EL2}. These water molecules are present between main chain carbonyl of I179\textsuperscript{EL2} and the side chain of Y192\textsuperscript{EL2}, further extending to the side chains of Y191\textsuperscript{EL2} and E181\textsuperscript{EL2} via water molecule and finally extending to the main chain carbonyl of I189\textsuperscript{EL2} and E181\textsuperscript{EL2}, stabilizing both the \(\beta\)-sheets in EL2. Our interpretation is further supported by the effect of Y191\textsuperscript{EL2} and I189\textsuperscript{EL2} mutants on the decay kinetics of metarhodopsin II decay (164-166). We thus think the extended H-bonding network in the retinal binding pocket in the vicinity of EL2 helps in stabilizing metarhodopsin II.

![Potential H-bonding network in retinal binding pocket of the active metarhodopsin II](image)

**Figure 4.1 Potential H-bonding network in retinal binding pocket of the active metarhodopsin II:** The H-bonding network in the retinal binding pocket of metarhodopsin II obtained by back soaking of all-trans-retinal and T94I(s-s) metarhodopsin II (Singhal et al, in preparation) The water molecules that are refined in the crystal structure are shown as red spheres and potential water molecules not shown in the PDB file (PDB ID: 3PQR) are shown as orange. The Schiff base in T94I(s-s) is linked via two molecules to side chains of E113\textsuperscript{3.28}, S186\textsuperscript{EL2} and E181\textsuperscript{EL2}.

But what about the water molecule present in the vicinity of Schiff base, which is bound to E113\textsuperscript{3.28} and is at an H-bonding distance of 3.3 Å from the retinal Schiff base. Unlike previously resolved water molecules, this water molecule lies just above the plane of the Schiff base and shows correct geometrical orientation to initiate a base catalyzed attack on the Schiff base (Figure 4.2) and may thus be involved in Schiff base hydrolysis. The proposed implication of this water molecule in Schiff base hydrolysis is further supported by the biochemical and mutagenesis studies on E113Q rhodopsin (163). E113Q is one of the few mutants known that reduces the rate of hydrolysis in metarhodopsin II. However, currently the orientation of the carbinol intermediate in the retinal binding pocket is uncertain and its determination would need further investigation to understand the finer mechanistic details of protonation of \(-NZ\) of K296\textsuperscript{7.43} and deprotonation of \(-OH\) of carbinol intermediate before breaking off the zwitter ion to form inactive apoprotein and free all-trans-retinal.
Figure 4.2 Schiff base hydrolysis in metarhodopsin II: Retinal binding pocket with E113$^{3.28}$, E181$^{EL2}$, T94$^{2.61}$ and S186$^{EL2}$ shown as sticks, water molecules are shown as red spheres, newly resolved water molecule based on T94I$^{2.61}$ structure shown as orange and water molecule after participation in the hydrolysis shown as light orange A) Base catalyzed attack of water molecule linked to E113$^{2.28}$ on the Schiff base; B) Formation of tetragonal carbinolamine intermediate.

Furthermore, previous biochemical studies on T94I shows that rate of ground state hydrolysis is faster, but on the contrary, the rate of metarhodopsin II hydrolysis is slower than WT (146). T94I along with E113Q and S186A are the only three mutants known to reduce the rate of hydrolysis in metarhodopsin II. Comparison of the H-bonding network in the vicinity of Schiff base in previously solved metarhodopsin II (59) and T94I(s-s) metarhodopsin II shows a difference of a water molecule between T94$^{2.61}$, E113$^{3.28}$ and S186$^{EL2}$ that is found missing in T94I(s-s) (Figure 4.3A and B). Likely, this is because the T94I mutation removed one hydrogen bonding partner of the water molecule. The missing water may have an implication in weakening the H-bonding strength of E113$^{3.28}$ and probably affects proton transfer during metarhodopsin II hydrolysis. To further investigate the effect of bulkier, hydrophobic I94$^{2.61}$, the accessible surface area of the Schiff base was compared with that of WT metarhodopsin II. Indeed, there is a 30% decrease in the accessibility of Ce and NZ of K296$^{7.43}$ that can be attributed to van der Waals interaction with I94$^{2.61}$ (Figure 4.3B). It seems most likely that I94$^{2.61}$ either affects the accessibility of the Schiff base or hinders the formation of the protonated carbinolamine intermediate during Schiff base hydrolysis. The above explanation agrees well with the accelerated decay observed for T94A metarhodopsin II (167). Therefore, impairment of the H-bonding network in close proximity of the critical residue, E113$^{3.28}$ and increased steric hindrance in the vicinity of Schiff base are responsible for the increased metarhodopsin II lifetime in T94I rhodopsin.
Figure 4.3 Comparison of retinal binding pocket of native and T94I(s-s) metarhodopsin II: A) metarhodopsin II (PDB ID:3PQR (59)) and B) T94I(s-s) (Singhal et al, in preparation)

4.2 Impact of G90D and T94I on retinal Schiff base integrity in the active state

Both T94I and, one helix turn down towards the intracellular side, G90D forms part of the ligand binding pocket close to the retinal Schiff base. As described above, the T94I mutation affects rhodopsin deactivation that requires the hydrolysis of the Schiff base in metarhodopsin II to release all-trans-retinal. Surprisingly, the effect of the G90D mutation on the retinal binding pocket is strikingly different from T94I as G90D has a very short lived metarhodopsin II (146). Unlike T94I, G90D interferes with the cycle of rhodopsin regeneration by introducing a charged side chain in the vicinity of the retinal attachment site. The observed shift of the absorption peak to 482 nm in 11-cis-retinal regenerated G90D(s-s) ground state is in accordance with previous studies (85,157) and indicates perturbation in the protonated Schiff base environment. We also observed an increased $A_{280}/A_{482}$ ratio to 2-2.2 that indicates an alteration in the rate of retinal binding G90D(s-s). The measured slower rate of retinal binding (146) and increased hydroxylamine accessibility for the protonated Schiff base in G90D (157,168,169) supports our structural findings. No similar effect on the process of retinal regeneration and the rate of retinal uptake were found in T94I (146). The observed heterogeneity of retinal in the binding pocket can be attributed to the close proximity of the introduced D902.57 side chain to E1133.28, which is 3.6 Å between the two carboxyl groups. The charged carboxyl group of E1133.28 is critical for rhodopsin function as it acts as a counterion (170,171) for both maintenance and hydrolysis of the retinal Schiff base, depending upon the conformation. Moreover, E1133.28 is also important for keeping rhodopsin in an inactive non-signaling state through a stabilizing salt bridge with protonated Schiff base (172). The important role of E1133.28 in keeping the basal activity of rhodopsin very low is also evident in opsin, where it forms a similar salt bridge with the K2967.43 (173). The weakening of the E1133.28-K2967.43 salt bridge due to interference by D902.57 side chain is one explanation for the observed constitutive activity in G90D2.57. Indeed, our structural
observation supports the previously formed hypothesis and provides more detailed insight for the effect of D90$^{2.57}$ side chain in the retinal binding pocket that indicates the formation of the salt bridge between the carboxyl group of D90$^{2.57}$ and the ε-amino group of K296$^{7.43}$ (Figure 4.3B). The observed 80 times slower rate of 11-cis-retinal uptake and increased thermal stability of G90D(s-s) opsins (Figure 3.8) are also consistent with our structural findings. Intriguingly, the observed heterogeneity of noncovalently bound retinal as well as the introduced salt bridge has no influence on the overall conformation of the ligand binding pocket in G90D(s-s) similar to that in E113Q(s-s) structure (Figure 4.3A).

Despite of the nearly identical overall conformation, the spatial effect of acidic and neutral point mutation in the retinal binding pocket is evident. The neutral I94$^{2.61}$ further stabilized binding of all-trans-retinal in the active metarhodopsin II conformation whereas the charged D90$^{2.57}$ residue had the opposite effect and introduced a salt bridge with K296$^{7.43}$ that interfered with covalent ligand binding of the all-trans-retinal.

![Figure 4.4 Comparison of retinal binding pocket of light activated E113Q(s-s) and G90D(s-s) rhodopsin structures: A) E113Q(s-s) (PDB ID: 1X72 (61)), B) G90D(s-s) (PDB ID: 4BEY (86)).](image)

**4.3 Structural impact of G90D or T94I mutation on the ground state**

Our X-ray crystallographic data provide interesting insights into the effect of CSNB mutations on the rhodopsin metarhodopsin II state. Despite extensive efforts we were, however, not able to crystallize the inactive conformation of either G90D(s-s) or T94I(s-s). Likely, this is because of the lower stability (Figure 3.8) and faster decay of the ground state in both mutants (Figure 3.9). Spin labeling experiments on G90D rhodopsin (173) and a higher rate of hydroxylamine reactivity as compared to WT rhodopsin (96) further indicate conformational heterogeneity in the ground state of rhodopsin CSNB mutants. The ground state of WT rhodopsin has exceptionally low basal activity and low conformational flexibility, further stabilized by salt bridge interactions between the protonated Schiff base and E113$^{3.28}$. Transition from inactive ground state to metarhodopsin II involves both deprotonation of the Schiff base and the concomitant opening of the salt bridge with E113$^{3.28}$. 

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All four known CSNB mutations are in close proximity to this E113\textsuperscript{3.28}-Schiff base activation switch and already early on, it had been recognized that the G90D (85,174) and T94I mutations (146,175) interfere with the E113\textsuperscript{3.28} counterion. To study how charged and neutral CSNB mutations may influence this crucial interaction we employed molecular dynamic simulations. The observed difference between final rmsd values of WT(s-s), G90D(s-s) and T94I(s-s) is not significant and don’t point towards major rearrangement of the binding pocket. The striking difference is the movement of the counterion E113\textsuperscript{3.28} away from the Schiff base in G90D(s-s), suggesting an opening of the primary activation switch and displacement of the counterion from E113\textsuperscript{3.28} to D90\textsuperscript{2.57} in the G90D(s-s) rhodopsin mutant. These results are in agreement with our biochemical analysis and prior studies showing increased hydroxylamine reactivity (96,169), structural rearrangements (173) and lower stability (86,168) of the G90D(s-s) dark state. We observed a similar opening of the E113\textsuperscript{3.28}-Schiff base activation switch and movement of TM3 in T94I(s-s) with protonated E113\textsuperscript{3.28}. However, in T94I(s-s) with deprotonated E113\textsuperscript{3.28}, we only saw an increase in the distance between K296\textsuperscript{7.43} and E113\textsuperscript{3.28} but not a full opening of the activation switch. This raises the question about the protonation state of E113\textsuperscript{3.28} in T94I(s-s). The absorption maxima of G90D(s-s) or T94I(s-s) rhodopsin are very similar with 482 nm and 478 nm respectively. This large shift with respect to the 500 nm observed for WT(s-s) rhodopsin suggests that both mutations introduce similar changes in the vicinity of the retinal Schiff base and its counterion. It should be noted that in G90D(s-s) movement of E113\textsuperscript{3.28} is due to steric clashes of the D90\textsuperscript{2.57} side chain with TM3. Moreover, the observed movement is not due to the change in the protonation state of counterion E113\textsuperscript{3.28} or loss of counterion as the position of D90\textsuperscript{2.57} in the final simulation is such that it can easily displace the role of E113\textsuperscript{3.28} as counterion. The implied role of D90\textsuperscript{2.57} as counterion is supported by the FTIR study on G90D that also indicates retinal binding pocket to be neutral in G90D (157). This is further consistent with the biochemical studies done in E113Q and E113Q/G90D double mutant (146). The absorption maximum of E113Q ground state rhodopsin shifts to 380 nm, consistent with the loss of counterion E113\textsuperscript{3.28} due to mutation. Further, the addition of the G90D mutation on the E113Q mutant shifts the ground state spectrum back to 472 nm, indicating the partial restoration of the WT like phenotype in the E113Q/G90D double mutant. The observed absorption maximum in the E113Q/G90D double mutant is similar to G90D(s-s) and supports our structural explanation for the opening of the E113\textsuperscript{3.28}-Schiff base activation switch and displacement of D90\textsuperscript{2.57} as counterion. The above stated role of D90\textsuperscript{2.57} as counterion in G90D is in stark contrast to the biochemical study of the E113Q/T94I double
mutant. Unlike the E113Q/G90D double mutant, the E113Q/T94I double mutant has an absorption maximum of 380 nm only, and was not at all able to restore a WT like phenotype. However, E113Q/T94D double mutant behaved similar to the E113Q/G90D double mutant and was able to restore WT like spectral behavior, indicating that position 94 is close to the Schiff base and can have an effect on the E113$^{3.28}$-Schiff base activation switch depending on the nature of the substitution (146). Therefore, based on the inability of T94I to displace the counterion in the E113Q mutant, we suggest that T94I(s-s) ground state most likely has E113$^{3.28}$-Schiff base activation switch very similar to WT rhodopsin with the E113$^{3.28}$ residue deprotonated. The observed opening of E113$^{3.28}$-Schiff base activation switch and movement of E113$^{3.28}$ seems specific to G90D(s-s) only. Then what could be the effect of the T94I mutation on the E113$^{3.28}$-Schiff base activation switch? The titration studies of E113Q/T94I double mutant shows that T94$^{2.61}$ aids in stabilization of a positive charge on the Schiff base of the E113Q$^{3.28}$ residue (146). Although the opening of the E113$^{3.28}$-Schiff base is not observed in the T94I(s-s) ground state, however, the hydrophobic I94$^{2.61}$ will most likely weaken the strength of the ionic bond, consistent with an increase in the E113$^{3.28}$-Schiff base distance from 1.9 Å to 2.4 Å in our molecular dynamic simulations. Furthermore, we observe an increase in the distance between I94$^{2.61}$ and F212$^{5.47}$ in the TM5 by 2 Å, pointing towards conformational flexibility in the binding pocket. The ground state of G90D(s-s) or T94I(s-s) thus, will be similar in a way that both have a weaker stabilization of the protonated Schiff base either due to the switching of the counterion from E113$^{3.28}$ to D90$^{2.57}$ or due to the presence of a bulkier hydrophobic I94$^{2.61}$ side chain in the vicinity of the E113$^{3.28}$-Schiff base activation switch. Our interpretation for the weakening of E113$^{3.28}$-Schiff base activation switch in G90D(s-s) and T94I(s-s) ground state is also consistent with our biochemical studies that showed a higher rate of thermal decay and Schiff base hydrolysis for both mutants.

4.4 Relevance to GPCR biased signaling

In GPCR biology, it is generally assumed that there is a direct link from ligand binding to specific changes in the receptor conformation, binding of effector proteins and biological response. Many GPCRs interact with more than one type of G protein, and some preferentially interact with arrestin, a concept known as “biased signaling” or “functional selectivity”. Here, we present an example of how the single point mutation G90D in a GPCR can interfere with these mechanisms by increasing constitutive G protein activation (85) and reducing binding of arrestin, particularly to the unliganded opsin form of the receptor. What
could be the structural mechanism for such a functional bias? The G90D mutation is located in a strong helix distortion in TM2 introduced by two adjacent glycines at positions 89\textsuperscript{2.56} and 90\textsuperscript{2.57}. These two glycines are highly conserved among visual pigments, whereas in other GPCRs, proline residues produce similar kinks that shape the ligand-binding pocket and are involved in the signal transduction mechanism (176). For instance, a P82A\textsuperscript{2.57} (equivalent to G90\textsuperscript{2.57} in rhodopsin) mutation in the angiotensin II type 1 receptor does not alter affinity for the activating peptide angiotensin II but severely reduces signal transduction (177), likely by modifying the kink in TM2 and influencing interactions with TM3 and TM7 as suggested by the authors based on homology modeling of the receptor structure. Similarly, recent structures of the \(\beta1\) adrenergic receptor show that arrestin-biased ligands make additional contacts to TM2, TM3, and TM7 compared to other structurally characterized \(\beta\)AR inverse agonists, which may promote G protein-independent signaling (178). A recent NMR spectroscopic characterization of the \(\beta2\)-adrenergic receptor demonstrated that arrestin-biased agonists predominately affect the conformational state of TM7 promoting a different protein conformation than agonists favoring G protein activation (179). As Class A GPCRs are believed to share common activation mechanisms (17,180), the unnatural D90\textsuperscript{2.57}-K296\textsuperscript{7.43} salt bridge in rhodopsin is likely limiting the conformational flexibility of the receptor by linking TM2 and TM7/H8, thereby reducing arrestin binding and biasing the receptor in favor of G protein coupling. In comparison the CSNB mutant A292E, which based on our structural comparison forms a salt bridge between different parts of TM7 (Fig. 5), does not prevent arrestin-1 binding (181). The relevance of the introduced structural link between TM2 and TM7 is in agreement with recent pump probe experiments suggesting a role of TM7/TM8 dynamics in arrestin binding (182). Most importantly, these data suggests that the conformation of G protein bound opsin is different from arrestin bound opsin.

4.5 Relevance to congenital stationary night blindness

Patients with CSNB mutations G90D and T94I show impairment of dim light vision resulting from massive loss of rod function without any significant rod cell death. Indeed, the structure of the retina is very similar to that of a normal healthy person and shows a normal amount of rhodopsin (99,183). Characterization of the CSNB causing G90D mutant in the animal model shows that rod cells carrying mutant rhodopsin are functionally desensitized as if under continuous activation at low gain (96-98). Based on dark current measurement in mice carrying a single copy of G90D gene, the intensity of the background light in the WT rod necessary to produce similar extent of desensitization is equivalent to 82-130 light activated
rhodopsin/rod/sec (97). Thus, the background activity of only a few out of the $10^8$ rhodopsin molecules present per rod cell is sufficient for continuous activation of the signaling cascade, leading to congenital stationary blindness. This equivalent background activity would decrease signal to noise ratio critical for dim-light sensing and as a result will impair our dim light vision. It is very clear that CSNB mutants increase the background basal activity that leads to disease phenotype, however, which molecular species is still debated.

Three mutually inclusive explanations have been proposed: 1) constitutive activity of opsin (85,98); 2) spontaneous activation due to increased rate of the thermal isomerization of retinal (99); c) continuous basal activation from a preactivated ground state with bound 11-cis-retinal (97).

In order to find a common denominator for hydrophobic and charged amino acid substitutions in TM2 causing CSNB, we employed crystallographic, biochemical and simulation studies for G90D(s-s) and T94I(s-s) rhodopsin (Figure 4.5). Although it is not proven that G90D and T94I cause CSNB by the same mechanism, we think this is likely as both have a very similar disease phenotype and are located on the adjacent turns of TM2, forming part of the retinal binding pocket. Our structures of light activated G90D(s-s) and T94I(s-s) and that of G90D(s-s) opsin show highly similar overall protein conformations. Both G90D and T94I selectively stabilize the active conformation, however, the subtle effect of aspartate and isoleucine side chain on the retinal binding pocket, is contrastingly opposite.

In G90D(s-s) the introduced aspartate side chain displaces the retinal counterion E113$^{3.28}$ and forms a salt bridge with K296$^{7.43}$, stabilizing the active conformation with some residual density for mixed retinal isomers in the retinal binding pocket (86). On the contrary, the structure of light activated T94I(s-s) obtained under nearly identical conditions showed a clean metarhodopsin II with covalently bound all-trans-retinal. In addition, both mutations have a contrasting effect on the rate of metarhodopsin II hydrolysis. The G90D(s-s) has a very short lived metarhodopsin II, whereas in T94I(s-s), the metarhodopsin II decay is much slower in agreement with previous studies (146,163,167). The differences in impact of the G90D and T94I mutations on the structure as well as biochemistry clearly excludes the light activated receptor as the common denominator for the two CSNB mutations.

All four known CSNB mutants are constitutively active in their apoprotein opsin conformation suggesting this as a potential source of the increased activity in rod cells (83). It has been shown that opsin exists in equilibrium between active and inactive conformations (65) and all the CSNB mutants shift this equilibrium towards the active conformation. The extent of this shift varies among the four known CSNB mutants, with A292E being the most
and T94I being the least constitutively active. In our structures, the G90D mutation selectively stabilizes the constitutively active opsin conformation by interfering with the E1133,28-K2967,43 salt bridge, critical for keeping basal activity of the opsin very low. Moreover, the introduced salt bridge had a stabilizing effect on the overall stability of the G90D(s-s) opsin, which increases transition temperature of G90D(s-s) opsin in comparison to the WT(s-s) (Figure 3.8). On the contrary, stability of the T94I(s-s) opsin was reduced in comparison to the WT(s-s), most likely due to absence of similar stabilizing interaction present in G90D(s-s) opsin. In addition to the aforementioned differences in the stability of G90D(s-s) and T94I(s-s) opsin, constitutive activity of the opsin conformation does not seem to be a unique feature associated with CSNB mutants, as there are many mutations in the retinal binding pocket that increase the constitutive activity of opsin (167). Moreover, these mutations exhibit much higher levels of constitutive activity in comparison to the T94I(s-s) opsin. An important mechanism to suppress the constitutive activity of the opsin is phosphorylation by GRK1 and successive binding of the arrestin-1. Disturbance with this mechanism may contribute to the increase background activity characteristic for CSNB. Indeed G90D(s-s) opsin binds arrestin-1 less efficiently, however, T94I(s-s) does not (122).

Overall, the opsin form, thus seems sufficiently different to be excluded as a common denominator between the two CSNB mutations. In vivo studies with transgenic mice carrying the G90D gene present another convincing data for excluding constitutively active opsin species to be responsible for CSNB. The transgenic mice showed a similar disease phenotype as in CSNB patients in the sense that they have light adapted rod cells and showed decreased light sensitivity. In contrast to previous studies done on COS cells (85) and transgenic frog (98), the G90D opsin mutation did not show increased basal activity in mice. Moreover, the effect of the mutant could not be reversed by regeneration with 11-cis-retinal, even though this completely suppresses the remaining constitutive activity of opsin (97). In agreement with the findings in transgenic mice, desensitization in G90D patients is not reversed even after 12 hours of dark adaptation (99). The authors of the mouse study suggested that the disease is caused by spontaneous changes in molecular conformation of the rhodopsin ground state that activates the visual cascade with low gain. The ground state of G90D and T94I rhodopsin indeed share many features indicative of a disturbed inactive conformation. Both mutants have a significantly reduced thermal stability, increased sensitivity to hydroxylamine (96,169) and increased rates of decay, Schiff base hydrolysis and retinal isomerization. Together with the blue-shifted absorption maxima, these results are indicative of changes close to the protonated Schiff base. Our molecular dynamics simulations show how G90D(s-
s) and T94I(s-s) with deprotonated E113\textsuperscript{3.28} can decrease the strength of the interhelical ionic bond in the E113\textsuperscript{3.28}-Schiff base activation switch. This activation switch, together with an extended hydrogen bonding network and covalent binding of the 11-cis-retinal inverse agonist are key determinants suppressing the basal activity of rhodopsin. Weakening of the strength of E113\textsuperscript{3.28}-Schiff base activation switch is, however, not sufficient to fully activate rhodopsin, for this at least on additional requirement is needed, uptake of a proton by Glu134\textsuperscript{3.49} of the conserved E(D)RY motif at the cytoplasmic terminus of TM3. Without this second step only very few receptors can adopt the active conformation under physiological pH. Indeed, both G90D and T94I rhodopsin do not have increased basal activity within the detection limit of in vitro G protein activation assays. The M257Y/G90D double mutant shows in vitro activity, however, individually neither M257Y nor G90D shows any dark activity (78,172). The human rod cells are however highly optimized for single photon sensitivity. Already a small increase that is hard to detect in in vitro assays, may be sufficient to cause CSNB. For this, only a very small number of the approximately 10\textsuperscript{8} rhodopsin molecules in a rod cell need to be activated at any given time to reduce dim light vision as observed in CSNB patients (96,97). Furthermore, reduced binding of arrestin in G90D(s-s) opsin might contribute to the severity of the disease similar to the loss of arrestin binding leads to night blindness in people with Oguchi disease.

![Figure 4.5 The molecular cause of the congenital stationary night blindness](image)

The question remains that whether weakening of the ionic bond strength in the E113\textsuperscript{3.28}-Schiff base switch is unique to G90D and T94I or is to be expected in the other two CSNB
mutants A292E and A295V also (Figure 4.6). The A292E mutant present in TM helix 7, just one turn up from K296\(^{7.43}\) can potentially form a salt bridge with K296\(^{7.43}\), analogous to that between D90\(^{2.57}\) and K296\(^{7.43}\), suggesting a similar effect of an acidic side chain in the binding pocket. The fourth CSNB mutant, A295V, is just one residue further from K296\(^{7.43}\). Similar to T94I(s-s) metarhodopsin II, it may induce a stabilizing effect by bridging TM6 and TM7 through van der Waals interaction with W265\(^{6.48}\), another activation switch in close contact with the retinal (13). In the dark state, however, A292E and A295V most likely would have a destabilizing effect on the E113\(^{3.28}\)-Schiff base activation switch. E292\(^{7.39}\) can potentially displace counterion E113\(^{3.28}\) in a similar way as D90\(^{2.57}\) in G90D(s-s). Unlike, A292E, the β-branched side chain of the introduced A295V mutation is in steric clash with W265\(^{6.48}\) and pushes it away that perturbs the retinal binding pocket as suggested by the 18 nm blue-shifted absorption spectrum of this mutant (83). It seems most likely that all CSNB mutants selectively stabilize active state by forming additional stabilizing interaction with K296\(^{7.43}\) and destabilize the inactive ground state by weakening the ionic bond strength of E113\(^{3.28}\)-Schiff base activation. Thus we think that for all the CSNB mutants selective destabilization of the E113\(^{3.28}\)-Schiff base activation leads to increase in the basal noise in rods, characteristic of CSNB patients.

**Figure 4.6 CSNB and RP mutants in the vicinity of the retinal binding pocket:** Comparison of the CSNB (green) and RP (blue) causing mutations of light activated rhodopsin. All the four CSNB mutants form stabilizing interaction with K296\(^{7.43}\) whereas RP mutants don’t show a similar pattern and point away from retinal binding pocket (86).
4.6 Relevance to the retinitis pigmentosa

Unlike the four known CSNB mutants, the majority of rhodopsin mutations are associated with the more severe form of vision impairing phenotype, retinitis pigmentosa. Most of the RP mutants are constitutively active (78,184), interfere with retinal binding (167), have an increased rate of thermal isomerization or ground state decay (185), features shared by CSNB mutants also. The observed biochemical similarity between RP and CSNB mutants raise a further question about the factors responsible for causing disease progression from CSNB to RP. The structural characterization of the CSNB mutants, especially G90D that can also cause RP when mutated to G90V (186), provides an insight about subtle differences in the impact of an acidic and hydrophobic mutation at the same locus. Both G90D and G90V are constitutively active (169), however, no other mutation at this position can mimic similar interactions with K2967.43 as the D902.57 side chain. Moreover, in contrast to all the CSNB mutants that generally fold well (183), most of the RP mutations reduce stability and show lower expression on the membrane surface due to their higher propensity to cause misfolding of the protein (80,106). A minimal threshold of stability is shown beyond which only little rhodopsin was transported to the rod outer segment membrane surface (187), pointing towards the stability of the protein as one of critical determinants for progression to RP. The observed rescuing potential of 11-cis-retinal, retinal analogues or engineered disulfide bond (N2C/D282C) (80,107) is most likely due to increase in the thermal stability of the RP mutants above the minimal threshold. Based on the rate of thermal decay and rate of retinal uptake studies of various RP mutants, most of the RP mutants either have a very high rate of thermal decay above the minimal threshold, or rate of decay lying below the threshold but with a very slow rate of retinal uptake that expedites the opsin degeneration. Thus, lower thermal stability of the opsin and slower rate of retinal uptake are most likely two important parameters defining progression from CSNB to RP. A better structural and biochemical understanding of the RP mutants is still needed to draw conclusions for the disease progression.
Chapter 5 Conclusion and perspectives

GPCRs are one of the most versatile, widely distributed family of membrane proteins, involved in a plethora of biological functions. Being key players in human physiology, their potential as a drug target is widely recognized. Rhodopsin is one of the best-studied GPCRs and has contributed immensely to our structural and functional understanding of this class of proteins. However, despite of several high resolution structures of rhodopsin in various active and inactive conformations, rhodopsin associated visual disorders have not yet been targeted by structure based drug design. Understanding the molecular causes of rhodopsin mediated, and particular the disease progression from CSNB to RP, seems a prerequisite for novel pharmaceutical approaches for the cure of rhodopsin mediated retina degeneration.

In this project, we investigated the common underlying mechanism of the CSNB phenotype. The key findings of the work on rhodopsin CAMs that cause CSNB are discussed here with its future implications. The crystal structures of G90D(s-s) and T94I(s-s) are the first crystal structures of GPCR rhodopsin related to the CSNB phenotype. Structural analysis of the light-activated crystal structures of G90D(s-s) and T94I(s-s) provide insight into the impact of the underlying mutation on the retinal binding pocket. Together with biochemical analysis and molecular simulation of the ground state, structural analysis of the G90D(s-s) and T94I(s-s) crystal structures point towards perturb ground state that is most likely the common denominator also for another two CSNB mutant. All the four CSNB mutants are unique in the sense that they specifically impair the E113<sup>3.28</sup>-K296<sup>7.43</sup> Schiff base activation switch in the ground state and selectively stabilize the active state of the receptor either by favoring interactions with K296<sup>7.43</sup> whereas none of the RP mutants show similar kind of interactions with K296<sup>7.43</sup>.

Most of the RP mutants are known to have reduced thermal stability that leads to misfolding of the protein (80,106). Many studies have shown that small molecular chaperones are effective for the pharmacological rescue of several RP mutants such as P23H and T17M (107,110,188). The unique stabilizing interaction with K296<sup>7.43</sup> found in our study of CSNB mutants, provides a nature’s subtle clue for designing small drug molecules to alleviate RP. Any small molecular compound that could mimic such a stabilizing interaction would potentially increase the stability of the misfolding prone RP mutants and would increase their expression in rod disc membranes. Due to its high resolution the T94I(s-s) structure could serve as a template for designing a library of potential small molecular compounds. To test such a library, a combination of highly powerful fluorescence based thermal stability
measurement and structure based screening can be used; can be effectively scaled up for high throughput screening. We have already seen the effect of 11-cis and all-trans-retinal on the thermal stability of G90D(s-s) and T94I(s-s) as a proof of principle that any compound that would potentially bind to the binding pocket will increase melting temperature of the apoprotein opsin. The fluorescence based thermal stability assay needs further optimization for screening a library of potential compounds in a high throughput manner. We were successful in determining a 2.5 Å high resolution structure of WT(s-s) opsin. In principle, these methods can now be applied to study the binding properties of such small molecular compounds through co-crystallization and soaking methods. In essence, the crystallographic results and establishing high throughput thermal stability screening are a promising strategy to identify lead compounds against RP. However, such a compound would not completely cure RP patients, but would prevent progression towards the severe day vision impairment by preventing rods and eventually cones degeneration, characteristic for the RP phenotype.

Besides providing the molecular basis of CSNB phenotype, G90D(s-s) and T94I(s-s) CAMs also provide insight into the basis of constitutive activity. On the basis of thermal stability differences, it is most likely that the difference between various rhodopsin CAMs are limited to the receptor dynamics and presumably alter the energetics and dynamics of the ligand-free receptor conformation. The concept of biased signaling and activation via coupling to more than one G protein or pathways in a ligand specific manner have raised several new questions regarding ligand recognition and specificity of GPCRs for various interacting partners. CAMs, which are presumably capable of mimicking the active like conformation, could potentially help in capturing these ligand specific various conformations as well as complexes of GPCRs with different interacting partners. CAMs that are specially located in the ligand-binding pocket are equally promising in delineating molecular signatures of ligand recognition and binding across the GPCRs. CAMs can also be used in combination with free electron laser crystallography to capture short-lived intermediate conformations that escape normal 3D crystallography due to their shorter lifetime. Therefore, structural characterization of various CAMs in combination with biophysical characterization, molecular pharmacology and bioinformatics could potentially answer many important questions underlying GPCRs pharmacology as well as facilitate better targeting of GPCRs for drug design.

The presented data on CAMs G90D(s-s) and T94I(s-s), thus, illustrate the potential of CAMs to elucidate more detailed understanding of activation mechanism and structural basis of the CAMs mediated CSNB phenotype as well as to identify potential lead compounds against RP.
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