Doctoral Thesis

Two-dimensional crystallization of the mouse serotonin 5-HT3A receptor

Author(s):
Rheinberger, Jan Steffen Harald Claudius Urs

Publication Date:
2016

Permanent Link:
https://doi.org/10.3929/ethz-a-010610848

Rights / License:
In Copyright - Non-Commercial Use Permitted
Two-dimensional crystallization of the mouse serotonin 5-HT$_{3A}$ receptor

A thesis submitted to attain the degree of

DOCTOR OF SCIENCE of ETH ZURICH

(Dr. sc. ETH Zurich)

Presented by

Jan Steffen Harald Claudius Urs Rheinberger

Dipl. Chem., Albert-Ludwigs-University Freiburg im Breisgau

born 17.06.1986

citizen of Germany

accepted on the recommendation of

Prof. Dr. Gebhard F.X. Schertler
Prof. Dr. Henning Stahlberg
Prof. Dr. Samuel Zeeman
Dr. Xiaodan Li

2016
Table of Contents

Summary ........................................................................................................................................ 2
Zusammenfassung ......................................................................................................................... 4
List of publications ......................................................................................................................... 7
1. 2D crystallization of the mouse serotonin 5-HT3A receptor ...................................................... 8
   1.1. Introduction .......................................................................................................................... 8
       1.1.1. Neurons and Synapses ................................................................................................. 8
       1.1.2. Serotonin 5-HT3 receptor – a pentameric ligand-gated ion channel ....................... 9
       1.1.3. Electron crystallography and single particle electron microscopy of membrane proteins ......................................................................................................................... 13
           1.1.3.1. Electron crystallography ....................................................................................... 13
           1.1.3.2. Single particle analysis ......................................................................................... 15
           1.1.3.3. Electron microscopy grid preparation ............................................................... 17
           1.1.3.4. Improvements in the electron microscopy field ................................................... 18
       1.1.4. Aim of the work .......................................................................................................... 19
   1.2. Optimizing 2D crystallization of mouse serotonin 5HT3A receptor .................................. 20
       1.2.1. Introduction ................................................................................................................. 20
       1.2.2. Materials and Methods ............................................................................................. 21
           1.2.2.1. Purification of wild-type and protease treated mouse serotonin 5-HT3A receptor ................................................................................................................................. 21
           1.2.2.2. Detergent stability assay ....................................................................................... 22
           1.2.2.3. Lipid preparation for 2D crystallization ............................................................... 22
           1.2.2.4. 2D crystallization of serotonin 5-HT3A receptor ............................................... 23
           1.2.2.5. Decoration of 2D crystals with nanobodies ......................................................... 23
           1.2.2.6. Cryo-electron microscopy sample preparation .................................................. 23
           1.2.2.7. Image processing of 2D crystals and single particle analysis ............................ 24
           1.2.2.8. Analytical size exclusion chromatography of nanobody binding .................... 24
           1.2.2.9. Limited proteolysis assay of 5-HT3A receptor-nanobody complex ............... 25
           1.2.2.10. Carbon film preparation and grid coating ......................................................... 25
       1.2.3. Results ......................................................................................................................... 27
           1.2.3.1. Wild-type and truncated 5-HT3A receptor purification and homogeneity analysis ................................................................................................................................. 27
           1.2.3.2. Influence of detergents on protein homogeneity and stability against proteases ................................................................. 29
           1.2.3.3. Chemical nature of lipids in 2D crystallization .................................................. 31
1.2.3.4. Influence of protein modification in 2D crystallization..............................32
1.2.3.5. Lipid-to-protein ratio, lipid mixtures and buffer conditions in 2D crystallization...........................................................................................................32
1.2.3.6. Nanobody decoration of 2D crystals influence protein packing........35
1.2.3.7. Effects of nanobody binding to solubilized 5-HT3A receptor........37
1.2.3.8. Proteolytic behavior of the 5-HT3A receptor-nanobody complex......41
1.2.4. Discussion ........................................................................................................43
1.3. Outlook ..................................................................................................................47

2. 2D and 3D crystallization of rhodopsin and rhodopsin-G protein complex for free-electron laser experiments.........................................................48
2.1. Introduction ..........................................................................................................48
2.1.1. Structure and light activation of rhodopsin .....................................................48
2.1.2. Rhodopsin-G-protein complex ......................................................................48
2.1.3. Pump-Probe experiments at the free-electron laser ....................................50
2.1.4. Aim of the work ..............................................................................................51
2.2. 3D crystallization of native bovine rhodopsin for FEL experiments ...........52
2.2.1. Introduction .....................................................................................................52
2.2.2. Methods ..........................................................................................................53
2.2.2.1. Purification of native bovine rhodopsin .....................................................53
2.2.2.2. Batch crystallization of native bovine rhodopsin ......................................54
2.2.2.3. Electron microscopy analysis of 3D nano-crystals ..................................54
2.2.2.4. Electron microscopy data collection ..........................................................54
2.2.3. Results ...........................................................................................................55
2.2.3.1. Purification of native bovine rhodopsin .....................................................55
2.2.3.2. 3D batch crystallization sample preparation and XFEL measurements of native bovine rhodopsin .................................................................55
2.2.3.3. Electron microscopy analysis of nanometer-sized crystals ....................57
2.2.4. Discussion .......................................................................................................58

2.3. 2D crystallization of rhodopsin-transducin complex ..................................59
2.3.1. Introduction .....................................................................................................59
2.3.2. Methods ..........................................................................................................60
2.3.2.1. Preparation of 1D4-sepharose .................................................................60
2.3.2.2. Transducin (Gt) purification .................................................................60
2.3.2.3. Rhodopsin-transducin complex formation and purification ....................61
2.3.2.4. Rhodopsin-transducin complex stability assay .......................................62
2.3.2.5. Purification of rhodopsin (M257Y) mutant ........................................ 62
2.3.2.6. Lipid preparation for 2D crystallization ........................................... 63
2.3.2.7. Two-dimensional crystallization of rhodopsin (M257Y) .................... 63
2.3.2.8. Image processing of 2D crystals ..................................................... 64
2.3.2.9. UV/Vis analysis of crystals .............................................................. 64
2.3.2.10. Complex formation with G-protein on 2D crystals ......................... 64

2.3.3. Results ................................................................................................. 65
2.3.3.1. Purification of rhodopsin (M257Y) transducin complex .................... 65
2.3.3.2. Stability of rhodopsin (M257Y) transducin complex ........................ 65
2.3.3.3. Purification and 2D crystallization of rhodopsin (M257Y) ............... 67
2.3.3.4. Complex formation on 2D crystals of rhodopsin (M257Y) ............... 70

2.3.4. Discussion ............................................................................................ 71

2.4. Outlook .................................................................................................... 72

Reference ........................................................................................................ 73
Acknowledgements .......................................................................................... 82
Summary

The serotonin 5-HT$_3$ receptor is an important component of neuronal signal transduction and belongs to the pentameric ligand-gated ion channels (pLGIC). In the past ten years several structures of protein family members were solved and the underlying mechanism of ligand binding and channel opening started to be revealed. The x-ray structure of the homopentameric mouse serotonin 5-HT$_{3A}$ receptor showed for the first time the intracellular domain of mammalian pLGIC member providing some explanation on the function of this domain. However, how pLGICs get activated is still unknown.

There are two major parts of my thesis. In the first part, I have investigated the formation of two-dimensional (2D) crystals of mouse serotonin 5-HT$_{3A}$ receptor for structural studies in native-like lipid environment. With a systematic approach I was able to find initial conditions for reconstituting the receptor at high density into lipid bilayers using only a few-hundred microgram of the purified protein. By analyzing the influence of major components involved in the crystallization process - protein sample, lipid property and lipid-to-protein ratio, detergent, buffer condition - I obtained ordered 2D crystals of the receptor. The most important component affecting the 2D crystallization is the protein. Using limited proteolysis of wild-type receptor, protein variants with different flexible parts could be generated. The tandem-protease treatment with trypsin and chymotrypsin provided the best results in terms of generating the first 2D crystals. The improvement of diffraction quality could only be achieved by adding conformational nanobodies. Different conformational nanobodies demonstrated significant effects on crystal packing as well as receptor stability and sample homogeneity. Receptors together with various conformational nanobodies will be very interesting to investigate receptor conformations using cryo electron microscopy single particle analysis.

In the second part of my thesis, I summarize 2D and 3D crystallization experiments of rhodopsin and rhodopsin-G protein complex for XFEL studies. I have participated in 3D crystal sample preparation of native bovine rhodopsin for XFEL experiments at LCLS, Stanford, USA, resulting in nanometer-sized rhodopsin crystals diffracting to 4-5 Å at the XFEL. This is an important step towards pump-probe experiments investigating the structural changes of rhodopsin in the activation process. Although the structure of rhodopsin was the first to be solved of a prototype G protein coupled receptor (GPCR), the rhodopsin-transducin complex was not crystallizable, due to instability of the complex in detergent solution. Using a constitutively active rhodopsin mutant, I have tried to obtain 2D crystals of the rhodopsin-
transducin complex following two paths: 1) crystallization of purified complex and 2) adding of G-protein to 2D crystals of rhodopsin. To my knowledge, I obtained for the first time crystalline arrays of a recombinantly expressed rhodopsin or GPCR in general and could prove binding of transducin after light activation, which was not possible for 2D crystals of native bovine rhodopsin. Crystal quality and incompleteness of complex formation prevented further investigations.
Zusammenfassung

Der Serotoninrezeptor 5-HT₃ ist ein wichtiger Bestandteil der neuronalen Signalübertragung und gehört zur Proteinfamilie der pentameren ligandenabhängigen Ionenkanälen. In den vergangenen zehn Jahren wurden mehrere Strukturen von Mitgliedern dieser Proteinfamilie gelöst, was zum Verständnis des zugrundeliegenden Mechanismus der Ligandenbindung sowie der Öffnung der Kanals beigetragen hat. Die Struktur des mäusischen homopentameren Serotoninreceptors 5-HT₃A zeigte erstmals die intrazelluläre Domäne eines Säugetierrezeptor und gab erste Aufschlüsse über deren Funktion. Die Aktivierung der pentameren Rezeptoren ist jedoch weiterhin unbekannt.


Im zweite Teil meiner Arbeit werden die Ergebnisse aus 2D und 3D Kristallisationexperimenten von Rhodopsin und des Rhodopsin-G-Protein Komplexes für Messungen am XFEL, LCLS, Stanford, USA, behandelt. Meine Beteiligung an der Vorbeitung von 3D Kristallen des nativen Rinderrhodopsins resultierte in Nanokristallen, die am XFEL mit einer Auflösung von bis zu 4-5 Å streuten. Dies war ein wichtiger Schritt in Richtung der strukturellen Untersuchung des Aktivierungsprozesses von Rhodopsin mittels
List of publications

During my work I was involved in different projects resulting in publications. My contribution in these projects is mentioned in the related section.

Rheinberger J., Hassaine G., Chami M., Stahlberg H., Vogel H. & Li X. (submitted) Two-dimensional crystallization of the mouse serotonin 5-HT$_{3A}$ receptor.


1. 2D crystallization of the mouse serotonin 5-HT$_{3A}$ receptor

1.1. Introduction

1.1.1. Neurons and Synapses

The central and peripheral nervous system is an important part of vertebrates and is also present in invertebrates in reduced complexity. In vertebrates the nervous system connects the brain with the rest of the body through a network of joined neuronal cells transporting signals from the brain to all parts of the body, for example to certain muscles inducing their contraction, but also the other way around delivering information, like pain or sensory stimulus, to the brain for processing. Each neuron has the fundamental task of receiving signals from the previous cell, propagating it and transmitting it to the following cell. The area of contact between the two cells is known as synapse (Fig. 1.1A). At this interface electrical signal generated by depolarization of the plasma membrane are in most cases transformed in a biochemical signal and delivered to the following neuron, where it can again lead to membrane depolarization. With an action potential propagating along the axon the membrane depolarization reaching the presynaptic site induces opening of voltage-gated calcium channels leading to influx of Ca$^{2+}$ ions. Increased concentration of Ca$^{2+}$ in axon terminal triggers fusion of primed synaptic vesicles with the presynaptic cell membrane via the SNARE protein complex. These vesicles are filled with neurotransmitter like serotonin or acetylcholine releasing them into the synaptic cleft between the two neurons. The neurotransmitter molecules are recognized by highly selective ligand-gated ion channels located in the postsynaptic plasma membrane leading to the opening of these channels. Depending on the type of ion channel, opening induces membrane depolarization (cation channels) which can again lead to propagation of the signal or has an inhibitory effect by stabilizing the membrane polarization (chloride channels) making it more difficult to induce a new action potential. To allow a high rate of signal transduction the neurotransmitters are rapidly removed from the synaptic cleft by enzymatic degradation or reuptake of the presynaptic neuron terminal.

1.1.2. Serotonin 5-HT₃ receptor – a pentameric ligand-gated ion channel

Neurotransmitters released at the presynaptic membrane are recognized by highly specific ligand-gated ion channels in the postsynaptic membrane inducing excitatory or inhibitory postsynaptic potentials (PSP). My work focuses on the serotonin 5-HT₃ receptor, a member of the pentameric ligand-gated ion channels (pLGICs), responsible for the fast conversion of chemical signals into electrical signals.

Human pLGICs are classified by their triggering neurotransmitter and ion selectivity. The nicotinic acetylcholine receptor (nAChR) and the serotonin receptor 3 (5-HT₃R) are cation (Na⁺, K⁺, Ca²⁺) selective receptor generating a excitatory PSP, while the γ-aminobutyric acid receptor A (GABA_AR) and the glycine receptor (GlyR) have a selectivity for Cl⁻ ions inducing an inhibitory PSP. pLGICs participate in signal transduction of the central and peripheral nervous system (CNS and PNS) and are linked to different neurological disorders like Alzheimer's disease, Parkinson's disease, Tourette’s syndrome, epilepsy, myasthenia gravis, schizophrenia, anxiety, depression as well as alcohol dependence and smoking addiction [1–5]. This makes them potent targets in drug development with marketed drugs already targeting pLGICs [1]. In addition, the functions of pLGICs are influenced by numerous chemical reagents such as nicotine, alcohol, anesthetics and benzodiazepines [3]. Furthermore pLGICs are not only present in mammals, but also in all other metazoans, for example in invertebrates (e.g. insects and worms) or fish (e.g. Torpedo fish) [6], and interestingly homologs can also be found in a few bacteria species [7], indicating their functional importance during evolution.

pLGIC are built from five subunits forming a channel by a five-fold symmetrical or semi-symmetrical arrangement (Fig. 1.1C und 1.1D). Each type of receptor has a set of different subunits [8] resulting in a variety of channels with slightly changed overall structure. 5-HT₃R can be built from five different subunits with unique encoding genes (5-HT₃A/B/C/D/E [9]) of which 5-HT₃A is the only one forming homopentameric channels while the other subunits can only have heteropentameric arrangement in combination with 5-HT₃A [10,11]. The first quaternary structure of this five-fold arrangement came from the nicotinic acetylcholine receptor (nAChR) of Torpedo marmorata using electron microscopy images in 1985 [12]. In 2005, a refined 3D model of the closed channel conformation of nAChR from 342 electron microscopy images of helical tubes yielded 4 Å resolution. This model provided structural information on the ligand-binding and intracellular domains of nAChR [13,14].
Figure 1.1: Overview of neuronal signal transduction and related pentameric ligand-gated ion channels. (A) Schematic image of a synapse, the interaction area between two neuronal cells [15] (B) Summary of all structurally characterized pLGICs, sorted by the year of their first published structure. From left to right: nAChR (2BG9), ELIC (2VL0), GLIC (3EHZ), GluCl (3RHW), GABA\(_A\) (4COF), 5-HT\(_{3A}\) (4PIR) and GlyR (3JAE). The receptors show high structural similarity in the extracellular ligand binding domain (ECD) and the transmembrane pore domain (TMD), the intracellular domain (ICD) is only resolved in nAChR and 5-HT\(_{3A}\).
X-ray structure of 5-HT3A showing the arrangement of subunits forming the receptor in complex with nanobodies (top view: (C), side view (D)) and (E) the tertiary structure of one subunit as well as the location of the different domans (adapted from [16]). (F) The ligand binding pocket is located between two subunits in the extracellular domain. The colored loops are involved in ligand binding with the yellow loop responsible for the induced conformational changes leading to the activation (adapted from [16]). (G) The transmembrane helices are arranged on three concentric rings: inner ring M2, middle ring M1 and M3 and on the outer ring M4 (adapted from [1]).

In the following years x-ray structures of the bacterial homolog cation-selective ion channel from *Erwinia chrysanthemi* (ELIC) [17,18] and *Gloeobacter violaceus* (GLIC) [19–21], the invertebrate anion-selective glutamate-gated ion channel from *Caenorhabditis elegans* (GluCl) [22,23] (in mammals glutamate-gated ion channels are tetrameric receptors located in the brain, like the AMPA receptor from rat [24,25]) as well as the human type-A γ-aminobutyric acid receptor (GABA<sub>A</sub>R) [26] and the mouse serotonin 5-HT<sub>3A</sub> receptor [16] plus the recent single particle structures of the zebrafish glycine receptor (GlyR) [27] have been solved at different conformational states (Fig. 1.1B). All structures share the same quaternary arrangement and each subunit consists of the extracellular ligand binding domain (ECD), the transmembrane pore domain (TMD). In addition, eukaryotic pLGIC have an intracellular domain (ICD) as demonstrated in nAChR and 5-HT<sub>3A</sub> (Fig. 1.1E). In the following paragraphs, I will compare the structures of nAChR and 5-HT<sub>3A</sub> discussing the general structure of pLGIC.

The ECD of the mouse serotonin 5-HT<sub>3A</sub> receptor has a N-terminal helix followed by ten β-strands forming an inner (β1, β2, β3, β5, β6, β8) and an outer (β4, β7, β9, β10) antiparallel β-sheet and containing three glycosylation sites [28]. The β6 and β7 are connected by a disulfide bridge containing loop, which is the hallmark of the Cys-loop receptor family within the pLGIC (so far only found in metazoans). The Cys-loop has thirteen residues between the two cysteines, which are highly conserved within the members. Located close to the TMD, the Cys-loop is involved in propagation of conformational changes from the ECD to the TMD upon ligand binding, together with other loops in the receptor [29]. The ligand binding pocket is located at the interface of the ECD between two subunits generating an electronegative cleft (Fig 1.1.F). From the β10 the ECD is connected through a short linker with the TMD.

The TMD consists of four transmembrane spanning α-helices (M1, M2, M3, M4). In the receptor these helices are located on three concentric rings around the pore (Fig 1.1G). The inner ring consists of five tightly packed M2 forming the ion channel pathway. The middle ring is made of M1 and M3, stabilizing the inner ring. The outer ring is formed by five M4
helices determining the extracellular radius of the receptor. In addition, the TMD has binding sites for allosteric modulators, which affect the ion conduction kinetic [30,31].

The transmembrane helices M3 and M4 of the TMD are connected through the ICD. The ICD consists of two short helical elements, a membrane associated helix (MA) and a short α-helix (MX). MA and MX are helical extension of M4 and M3 respectively and connected by a presumably unstructured loop missing in the structure of 5-HT3A and could not be resolved in the structures of nAChR. The ICDs are only present in vertebrate pLGICs. They are involved in trafficking and clustering of receptors at the postsynaptic membrane [32,33] modulating gating kinetics [34] and channel conductance in 5-HT3A [35,36].

Ion selectivity is determined by the charge distribution of the amino acids along the ion pathway formed by M2 helices. Already at the lower part of the ECDs of nAChR and 5-HT3A, predominantly negative charged amino acids forming a “welcome” environment for cations. Residues on the TMD helices M2 are clustered in two parts: the upper part with hydrophobic residues serving as a gate and the lower part with mainly negatively charged residues controlling cations permeation and selectivity [37,38].

Upon binding of the agonist, the ECD rapidly undergoes conformational changes by rearrangements of the loop between β9 and β10 (C-loop) towards the ligand-binding pocket leading to the stepwise isomerization of the subunit interface. These changes in the EDC propagate to the interface with the TMD where the β1-β2-loop and Cys-loop induce an outward motion of the loop connection M2 and M3 that results in tilting of M2. Finally the conformational changes in M2 lead to the opening of the channel pore allowing the ions to pass. The release of ions at the ICD is still not understood, but mutation studies [39] supported the hypothesis that ions are released through a solvent exposed channel in the upper part of the MA helices [1].

The recently published structure of mouse serotonin 5-HT3A receptor was an important step towards the understanding of mechanistic details of pLGICs [16]. For the first time the ICD of a mammalian receptor was resolved with high resolution which was removed in the human GABA_A receptor published at the same time [26]. It is important to mention that the crystal structure of 5-HT3A was obtained as a complex with a conformational nanobody [40] locking the protein in this specific conformation and creating new crystal contacts improving the crystallization process. This approach was also successfully used in the crystallization of the GluCl from C. elegans [22]. The first structural information of 5-HT3A without
conformational nanobody was the low resolution density map obtained by sub tomogram averaging of densely packed receptors in vesicles mimicking the native environment using cryo electron microscopy (cryo EM) [41]. The EM density map and 3D structure agree well, but low resolution prevents a more detailed analysis of the structural differences of the receptor with and without nanobody.

As structural knowledge about 5-HT3A is further increasing there is still no high resolution structure of the apo-protein, a ligand bound state or of the wild-type receptor with intact intracellular loop. With the known lipid binding motive of pLGICs [42] the structural determination of 5-HT3A in a lipid environment could give further insight into the activation mechanism and the influence of lipid on ligand binding. I want to address the question of ligand-binding in the lipid environment using the 2D crystallization approach.

If not otherwise stated, the information provided in this chapter was obtained from [1,16] and “Molecular Biology of the Cell” by B. Alberts et al, Garland Science, 6th Edition, Chapter 11, pages 629-633.

1.1.3. Electron crystallography and single particle electron microscopy of membrane proteins

Electron microscopy (EM) is a visualizing technique covering cell organelle arrangements with micrometer scale down to single protein structures at near-atomic resolution (angstrom range) and can help biologists to address structure-related questions. In the following paragraphs I will focus on electron crystallography and single particle analysis; two cryo electron microscopy methods used to study near-atomic resolution structures of membrane proteins [43–45].

1.1.3.1. Electron crystallography

In contrast to detergent-based and potentially detergent-compromised single particle reconstruction, electron crystallography utilizes the crystalline order of membrane proteins reconstituted into lipid bilayers, known as two-dimensional (2D) crystals, and enables structural characterization of membrane proteins in a native-like environment [46,47]. The lipid bilayer provides the lateral pressure and the matching hydrophobicity [48–50] which is required by integral membrane proteins for their structure and function [51,52]. In addition, many membrane proteins are too small to be analyzed by single particle methods with the current technical state. The first three-dimensional (3D) density map calculated from 2D
crystals was obtained from bacteriorhodopsin at a resolution of 7 Å [53]. The best resolution obtained by electron crystallography so far was 1.9 Å from aquaporin 2D crystals using electron diffraction [45]. 2D crystallization as well as single particle analysis starts with purified proteins followed by addition of detergent-solubilized lipids generating a protein-lipid-detergent mixture [54]. Then the detergent is removed mostly by dialysis (slow) against a detergent-free buffer [55] reconstituting the protein in a lipid bilayer (summarized in Fig. 1.2A). Other methods to lower the detergent concentration below its critical micelle concentration (CMC) inducing reconstitution and crystallization are addition of bio-beads (fast) [56] or cyclodextrin [57] and dilution [58]. Reconstitution and crystallization generate a variety of shapes of the resulting lipid bilayer: sheets, tubes and vesicles [54]. From all published conditions of successful 2D crystallization trials, sheets with a single lipid bilayer have the highest rate of high-resolution structures followed by tubular arrangements and vesicles [59] (Fig. 1.2B). Stacked sheets are only useful if the layers are perfectly aligned to each other, like for aquaporin where the stacking is induced by the protein and depends on the surrounding lipids [52].

In 2D crystallization, there are limited parameters determining the outcome of the reconstitution. The protein, which is the most important factor for all structural determination methods, should be as pure and homogeneous as possible [60]. Lipid properties and lipid-to-protein ratio have a huge impact on crystallization, but finding the right lipid still requires a lot of screening [61] although some trends have been published analyzing conditions of all successful 2D crystallizations [59]. The detergent is less important in the crystal formation compared to the other parameters as it is removed during crystallization [60]. However, detergents have a significant impact on protein stability, protein activity and sample homogeneity as well as on the speed of removal and affect size and packing of the proteoliposomes [62]. The buffer condition used for reconstitution influence crystal formation. pH, salt type and concentration of the buffer have to be evaluated and in some cases low amount of organic solvents and divalent ions can improve reconstitution [60].

Images of tilted and non-tilted crystals are acquired under low dose condition [63] during data collection and processed to obtain a 3D reconstruction. Processing in this work was carried out with the graphical 2dx software package that is based on further developed MRC scripts [64]. From the Fourier transform of electron micrographs the reciprocal lattice parameters can be determined allowing extraction of amplitudes and phases of each lattice point, an important advantage of electron microscopy compared to x-ray crystallography. 2D crystals consist of a
thin lipid bilayer and can easily bend, creating lattice distortion. In combination with crystal mosaicty this reduces the data quality. To still extract the best possible structural information small imperfections can be computationally corrected to generate a better image of the crystal. The procedure is called crystal unbending and is based on the cross-correlation of Fourier filtered reference with the whole crystal. Comparison of the determined distribution of unit cells in the crystals with their position in the ideal lattice allows identification of the divergent regions and to correct them. If the distortions are large these areas can be excluded from the processing by applying a mask. After correcting for the contrast transfer function, the mathematical description of image modifications in the EM due to microscopy aberrations, amplitude and phases can be extracted with their corresponding index (h, k) from the improved Fourier transform. The extracted information from non-tilted images allows the calculation of a 2D projection map (summarized in Fig. 1.2C). For a 3D density map the information of many crystals with different tilt angles have to be combined in order to fill the lattice lines in Fourier space, thus obtaining amplitude and phase information in three dimensions that allow the calculation of the 3D model.

Unless indicated otherwise all information about image processing of 2D crystals were obtained from [61].

1.1.3.2. Single particle analysis

Single particle analysis of electron microscopy images is a structural determination method of proteins without a crystalline specimen. The first structure solved by single particle methods was a icosahedral virus [65,66]. The implementation of direct electron detectors enabled determination of multiple structures reaching near-atomic or atomic resolution [43,67–70] with the structure of the 170 kDa γ-secretase as smallest cryo-EM structure at atomic resolution [44]. Well-characterized high-quality protein samples and cryo-conditions are key parameters leading to the success of the structure determination. Separation of different conformational states during image processing is possible to a certain extent [71]. However, it cannot circumvent the biochemical purification [72]. Determination of sample purity and homogeneity using SDS-PAGE and size exclusion chromatography is not sufficient and further biochemical and biophysical characterizations are needed to obtain the best protein sample. Electron microscopy can be used to assess sample quality [73]. Removal of flexible parts without disturbing protein function [74] and fixation of certain conformation with ligands as well as detergents and lipid for membrane proteins [69], can reduce the intrinsic
heterogeneity. In addition, the exchange of detergents with amphipols could improve protein stability and increase homogeneity of membrane proteins [75].

Figure 1.2: Electron crystallography and single particle analysis. (A) Schematic representation of the workflow in 2D crystallography. Membranes containing the protein of interest are solubilized by addition of detergent. After purification of the membrane protein, solubilized lipids are added and the protein is reconstituted into a lipid bilayer by removal of the detergent from the protein-lipid-detergent mixture. Under the right conditions reconstitutions produce 2D crystals (adapted from [54]). (B) Schematic overview of different types of 2D crystals arising from reconstitution of membrane proteins. Top to bottom: single bilayer sheets, tubes, stacked sheets and vesicles (adapted from [54]). (C) Image processing workflow in electron crystallography starting with an electron micrograph of a 2D crystal. After determining the lattice parameters in the Fourier transform (FT) small crystal defects can be removed to improve the results. At the end, amplitudes and phases can be directly obtained from the image (adapted from [61]). (D) Schematic overview of the different steps in single particle analysis. Particles are selected from electron micrographs and extracted. After alignment of all particles with respect to each other, they are classified according to their similarity. The class averages allow the selection of good particles used for 3D reconstruction (adapted from [76]).
Data collection and 3D reconstruction in single particle analysis requires the recording of many images of homogeneous and well distributed protein particles at low dose conditions to avoid radiation damage [63]. Particles can then be selected and extracted using one of numerous programs available. Assuming a random distribution of protein orientations on the grid, all necessary information for reconstruction is directly observable. Particle images are brought into the same relative orientation by rotation and translation and classified according to similar views of the object [76]. Removal of “bad” particles can be achieved by only selecting particles of relevant class averages (summarized in Fig. 1.2D). Building a 3D model from 2D projections relies on the exact determination of orientation of each particle using low resolution templates [73]. In this work I used two GUI-based single particle processing tools: EMAN 2 [77] to determine the particle coordinates and RELION [78] for extracting particle images and their 2D classification by the reference free Bayesian approach [79].

1.1.3.3. Electron microscopy grid preparation

Depending on the questions asked, different sample preparation techniques are available. In negative staining, the specimen is embedded in a layer of dried heavy metal salt solution, mostly uranyl acetate (UA) or uranyl formate (UF), increasing the contrast of the images [80,81], due to stronger electron scattering properties of the heavy-metal stain atom compared to the lighter atoms of the biological sample [82,83]. Staining of the sample enables grids to be handled at room temperature allowing obtaining information within a short time. Drawbacks of this sample preparation method is the limited resolution obtainable, due to dehydration of the protein and grain size of the heavy-metal salt crystals (in most cases maximally 20 Å resolution [84]). Negative staining is mostly used to check sample quality, helping to improve purification conditions. Low resolution class averages can provide valuable insights addressing the biological function and arrangement of multi-domain complexes [24,85–88]. In 2D crystallization negative stain electron microscopy is used to assess results of reconstitution optimization trials before collecting high resolution data [60].

To obtain high resolution information the specimen has to be kept in a hydrated state while under vacuum as mandated by the electron microscope. By plunge freezing the specimen grid in liquid ethane the sample is embedded in a thin film of vitrified water avoiding the formation of ice crystals and ensuring the sample being fully hydrated [89]. Operating the electron microscope at liquid nitrogen temperature allows collection of data for all techniques described. In cryo electron microscopy the image contrast is reduced compared to negative
stain. Finding optimal cryo-condition is very time consuming and is one of the technical key parameter for high-resolution data collection.

A mixture of both preparations is cryo negative stain electron microscopy combining contrast enhancement of negative stain with sample hydration of cryo preparation and was successfully used for well-characterized membrane protein complexes [90,91].

1.1.3.4. Improvements in the electron microscopy field

In recent years, multiple improvements in data collection and processing algorithms pushed the EM to the front of atomic-resolution structure determination: Introduction of direct electron detectors improved image quality, allowing for collection of higher resolution data [92,93]. The movie mode of the cameras allowed for 1) beam induced drift correction increasing image quality [94,95] and 2) dose fractionation improving image processing results by radiation-damage weighting of each frame [96]. Automation of data collection and image processing [97] accelerates the EM structure determination process from traditionally months or years to currently days or weeks. All technical improvements of EM increased the chances for getting atomic resolution of membrane proteins assuming no issues on the sample quality and cryo preparation.
1.1.4. Aim of the work

There is no structural information of 5-HT\textsubscript{3A} in the native environment of the membrane. This work focuses on addressing this problem using 2D crystallization of the receptor in the lipid bilayer by modification of the receptor and addition of a conformational nanobody.

In Chapter 1.2 I investigated 5-HT\textsubscript{3A} by electron crystallography to find the optimal conditions for reconstitution and 2D crystal formation. In a systematic approach, I tested the major parameters in 2D crystallization: protein, lipid, lipid-to-protein ratio, detergent, buffer conditions. After evaluation of the impact of each parameter on the 2D crystallization, I ranked each based on the outcome. It turned out that modification of 5-HT\textsubscript{3A} protein samples is the key determinant for obtaining diffraction 2D crystals. Further addition of conformational nanobodies resulted in tremendous improvements of crystal diffraction quality.

The presented protocol is easy to use and can be applied to any membrane protein. With a minimal amount of purified protein (few hundred micrograms), everyone can determine the viability of a project.
1.2. Optimizing 2D crystallization of mouse serotonin 5HT$_3$A receptor

1.2.1. Introduction

The serotonin 5-HT$_3$A receptor is an important protein involved in signal transduction of the central and peripheral nervous system. Structural information, like the recent x-ray structure of the mouse 5-HT$_3$A receptor [16], helps to build models for understanding the mechanism of receptor activation. The x-ray structure was solved from crystals of 5-HT$_3$A in complex with a conformational nanobody (VHH15), which acted as inhibitor shown by electrophysiology measurements and radioligand binding competition assays [16]. By comparison with structures of other homologous pentameric ligand-gate ion channels (nAChR: closed (2BG9) [13], open 4AQ9) [14]; GLIC: closed (3TLS) [98], resting (4NPQ) [21], open (3EHZ/3EAM) [19,20]; ELIC: closed (2VL0) [17]; GluCl: closed/resting (4TVN) [23], open (3RHW) [22]; GABA$_A$: desensitized (4COF) [26]; GlyR: closed (3JAD), modulated (3JAF), open (3JAE) [27]) the expected closed state of 5-HT$_3$A could not be clearly assigned. The minimal pore diameter of the transmembrane region of 5-HT$_3$A was between the one of open or closed channels while the backbone conformation was comparable to an open channel [16]. The discrepancy between inhibitory nanobody and supposedly open pore conformation was the starting point for my work. To avoid the compromising effect of detergent and to work in an environment closer to physiological conditions the receptor was reconstituted and crystallized into a lipid bilayer generating a native-like surrounding and providing the lateral pressure on the protein. In this work, the best condition for reconstitution and crystallization of 5-HT$_3$A was evaluated by systematic variation of the main parameters of 2D crystallization: protein, chemical nature of lipid, lipid-to-protein ratio, detergent and buffer condition. As a result, a simple approach on 2D crystallization was explored to test the viability of a project using minimal amount of the target protein.

A major breakthrough in the crystallization of 5-HT$_3$A was the modification the receptor and the addition of nanobodies, with the different nanobodies demonstrating different influences on the 2D crystal diffraction quality. Further investigation revealed effects of the nanobodies on sample homogeneity of the solubilized 5-HT$_3$A receptor and on its stability against proteases.
1.2.2. Materials and Methods

1.2.2.1. Purification of wild-type and protease treated mouse serotonin 5-HT\textsubscript{3A} receptor

The mouse 5-HT\textsubscript{3A} receptor was expressed heterologously in HEK293 TRex cells (received from collaborators: Prof. Horst Vogel, EPFL and Dr. Ghérici Hassaine, Theranyx, Marseille). The purification was adapted from previous publications \cite{16,99}.

Cell pellets were thawed overnight at 4 °C, diluted in 10 ml/g cells with membrane preparation buffer (10 mM HEPES pH 7.5, 1 mM EDTA, Roche complete protease inhibitor) and homogenized by Douncing. Homogenized cells were kept on ice and disrupted using an Ultra-Turrax for 30 s at each speed and 1 min on highest speed with 30 s break in between each step to avoid heating of the sample. The plasma membranes were collected by ultracentrifugation (235000 x g, 1.5 h, 4 °C, Ti45 rotor). The plasma membrane pellet was resuspended and homogenized in solubilization buffer (50 mM HEPES, 500 mM NaCl). After solubilization with 1% C\textsubscript{12}E\textsubscript{9} (Polyoxyethylene-(9)-dodecyl ether) for 2 h rotating at 4 °C with a final volume of 25 ml solution per 1 g of membrane the non-solubilized material was removed by ultracentrifugation (370000 x g, 1.5 h, 4°C, Ti70 rotor). The supernatant was filtered through a 0.45 μm pore-size filter and applied to a 1 ml Streptactin column (StrepTrap HP, GE Healthcare) equilibrated in running buffer (50 mM Tris pH 7.4, 125 mM NaCl, 0.01% C\textsubscript{12}E\textsubscript{9}). After washing the column for 20-25 column volumes with running buffer the protein was eluted with running buffer containing 5 mM d-desthiobiotin (IBA GmbH). The main fraction eluted around 1.6 ml was concentrated to 2-3 mg/ml receptor using a 100 kDa MWCO concentrator (Vivaspin, GE Healthcare). The resulting protein preparation was referred as wild-type receptor, still containing an N-terminal double Twin-Strep-tag on each protein subunit.

For limited proteolysis the wild-type receptor was treated with trypsin in a ratio of 1 μg protease per 80 μg of protein \cite{16,99}. Thereafter, the sample was concentrated to ~300 μl and applied on a Superdex200 10/300 size-exclusion chromatography column equilibrated in 50 mM Tris pH 7.4, 125 mM NaCl, 0.01% C\textsubscript{12}E\textsubscript{9}. The elution peak at 10.5 ml was pooled and concentrated again to 2-3 mg/ml receptor.

In an additional step the limited proteolyzed receptor was further treated with chymotrypsin under the same condition as for trypsin. The protein was then directly used in crystallization
trials. Furthermore deglycosylation with PNGase-F was tested using previously found conditions (5 units/µg of protein, incubated for 2 h at 37 °C) [16,99].

For checking the sample quality by electron microscopy, fresh receptor samples were applied on glow discharged (5 mA, 15 s, Cressington carbon coater 208carbon with glow discharge unit) carbon coated copper grids (3 mm, 400 meshes) and stained with 0.75% uranyl formate [80] with some minor changes. 3 µl of diluted protein sample was incubated for 1 min on the grid. Without blotting, the grid was washed on four drops of 50 µl deionized water with blotting after each drop. The sample on the grid was stained on a 50 µl drop of 0.75% uranyl formate twice, for one second and after blotting for 20 sec. The excessive stain was removed and the grid dried.

1.2.2.2. **Detergent stability assay**

Purified wild type and trypsin truncated 5-HT₃A receptor were diluted with buffers containing 1.5-3 x CMC of new detergent and thus lowering the concentration of C₁₂E₉ below its CMC. After incubating the sample for 1 h on ice, the influence of the detergent exchange was analyzed. The diluted samples were negatively stained for electron microscopy analysis as mentioned above (1.2.2.1). In addition, the wild-type samples were incubated with trypsin using the condition from the purification (1.2.2.1). The digestion pattern was evaluated by SDS-PAGE.

1.2.2.3. **Lipid preparation for 2D crystallization**

All steps below were conducted with Hamilton syringes and in glass vials with chloroform resistant lids. All glassware was cleaned with chloroform and dried before use. Lipids (purchased from Avanti Polar Lipids) were transferred into glass vials, either by weighting the powder or using the Hamilton syringe for chloroform solutions. In case of a solution, chloroform was evaporated under a constant stream of argon, followed by 3 h drying under vacuum to remove residual solvent. Dried lipids were dissolved in 0.02% C₁₂E₉ at a final concentration of 4 mg/ml by vortexing, forming a homogeneous suspension.
1.2.2.4. 2D crystallization of serotonin 5-HT3A receptor

Purified 5-HT3A receptor in C12E9 (1.2.2.1) and the lipid detergent stock solution (1.2.2.3) were mixed at different lipid-to-protein ratios (LPR, w/w) and diluted to a protein concentration of 1 mg/ml with crystallization buffer (50 mM HEPES 7.5, 125 mM NaCl, 3 mM NaN₃, 0.02% C12E9). The final sample volume was 30 µl. The mixture was kept at 4 °C overnight creating protein-lipid-detergent mixed micelles.

The final mixture was dialyzed at room temperature for 2 weeks against 50 ml detergent free crystallization buffer using a 100 kDa MWCO membrane (Vivaspin 500 concentrators, GE Healthcare) until the solution became turbid and the crystal suspension was stored at 4°C until further usage.

For analysis of the crystallization by electron microscopy 3 µl crystallization suspension was applied on a glow discharged (5 mA, 15 sec, Cressington carbon coater 208carbon with glow discharge unit) carbon coated copper grid (3 mm, 400 mesh). After 1 min incubation the excessive liquid was removed by blotting and the sample was stained with 3 µl of 2% uranyl acetate for 1 min without additional wash steps. The stain was blotted and the grid was air dried.

1.2.2.5. Decoration of 2D crystals with nanobodies

Crystal suspension was mixed with different nanobodies (expressed and purified by Dr. Ghérici Hassaine, Theranyx, Marseille) in a molar ratio of 5:1 nanobody:channel. After 2 h incubation on ice, the crystals were pelleted by centrifugation (10000 x g, 5 min, 22 °C, Eppendorf centrifuge 5424) and the supernatant was removed. Crystals were washed twice by resuspension in dialysis buffer (50 mM HEPES 7.5, 125 mM NaCl, 3 mM NaN₃) followed by centrifugation (10000 x g, 5 min, 22 °C, Eppendorf centrifuge 5424) and removal of the supernatant. Finally the crystals were resuspended in dialysis buffer and analyzed by negative stain electron microscopy as mentioned above (1.2.2.4) and SDS-PAGE.

1.2.2.6. Cryo-electron microscopy sample preparation

Promising results from analysis of negatively stained 2D crystals were selected for cryo electron microscopy. Three microliters of crystals suspension was applied to glow discharged (5 mA, 15 s, Cressington carbon coater 208carbon with glow discharge unit) coated copper
grids (400 mesh) that were either coated with a flat layer of carbon film or had a preformed holy carbon layer (Quantifoil, R2/1) which then was also coated with a carbon film to generate a continuous flat surface. After a short incubation time of about 1 min the samples were plunge frozen in liquid ethane with a Vitrobot (FEI) or a Cryoplunge 3 (Gatan) at 22°C. The humidity was between 80% (Cryoplunge 3) and 100% (Vitrobot) and the blotting time was two to four seconds. For Cryoplunge 3, a blotting force of 3 bar was used while at the Vitrobot, the internal blotting force scale was set from 1 to 3. The grids were then analyzed by cryo electron microscopy on a Phillips CM200FEG and on a JEOL2200FS.

1.2.2.7. **Image processing of 2D crystals and single particle analysis**

Images of negatively stained 2D crystal samples were acquired at 74000 fold magnification (JEOL2200FS, 200kV), cryo electron microscopy images at 50000 fold magnification (Philips CM200 FEG, 200 kV), both on a CCD camera (TVIPS F416, 4k x 4k). Images showing a diffraction pattern in the Fourier transform were processed with the 2dx software package [61,64], following the standard procedures and protocols.

For negatively stained particles, picking was done with e2boxer from EMAN2 using a box size of 128 pixels (1.89 Å/pixel) [77]. The resulting particle coordinates were transferred into RELION and the particle images were extracted [79]. For 2D classification 100-200 classes were generated distributing the particles based on their similarity using the Bayesian approach in RELION.

1.2.2.8. **Analytical size exclusion chromatography of nanobody binding**

Purified trypsin treated 5-HT3A receptor (1.2.2.1) was mixed with different nanobodies in a molar ratio of 10:1 nanobody:channel and adjusted with running buffer (50 mM Tris pH 7.4, 125 mM NaCl, 0.02% C12E9) to a final concentration of 0.5 mg/ml receptor. The mixture was incubated on ice for 1 h and 10 µl equal to 5 µg of receptor were injected on an analytical size exclusion chromatography column (Superose 6 3.2/300, equilibrated in running buffer). Samples with only receptor or nanobodies at the same concentration were used as control. Selected peak fractions were analyzed by SDS-PAGE using silver staining to visualize the protein bands and electron microscopy of negative stained samples (0.75% uranyl formate) following the protocol mentioned above (1.2.2.1).
1.2.2.9. Limited proteolysis assay of 5-HT$_{3A}$ receptor-nanobody complex

Purified wild-type and trypsin-treated 5-HT$_{3A}$ receptor (1.2.2.1.) were mixed with different nanobodies in a molar ration of 1:10 and incubated for 1 h on ice. After adding trypsin to the wild-type receptor or chymotrypsin to trypsin-treated receptor, the mixtures (both proteases: 100 µg protease/8 mg protein) were incubated for 2 h at 30 °C and analyzed by SDS-PAGE.

1.2.2.10. Carbon film preparation and grid coating

Freshly cleaved mica sheets were placed in the carbon coater (Balzers Union MED 010) and after the vacuum reached 8 x 10$^{-6}$ mbar, carbon was evaporated for 1 s from a carbon thread using high current. Grids were submerged onto filter paper in a water tank and the carbon film was slid off the mica by lowering it slowly into the water leaving the carbon film floating on the water surface. Lowering the water level the carbon film could be placed on the grids. After drying the grids were ready to use.
1.2.3. Results

1.2.3.1. Wild-type and truncated 5-HT$_{3A}$ receptor purification and homogeneity analysis

The recent report on the high resolution structure of the 5HT$_{3A}$ receptor showed that receptor treated with trypsin yielded 3D crystals which diffracted to higher resolution than those obtained from wild-type receptor [16]. Following the published purification strategy, I was able to obtain purified wild-type and truncated receptor. The wild-type receptor showed a typical elution profile for this type of affinity chromatography (Fig. 1.3A). SDS-PAGE (Fig. 1.3E) indicated a pure sample with the receptor monomer at around 60 kDa as well as the expected impurity of the binding immunoglobulin protein (BIP, also called GRP-78 or HSPA5 [100]) at 75 kDa [99]. Size exclusion chromatography after limited proteolysis yielded a symmetric main peak suggesting a homogenous sample, but with a small shoulder towards smaller retention volumes stemming most likely from non-proteolyzed receptor (Fig. 1.3C). Analyzing the fraction of the main peak by SDS-PAGE (Fig. 1.3E) revealed the same digestion pattern with the large N-terminal fragment (37 kDa) and the MA-M4 helix fragment (12 kDa) already seen before [16]. In addition I added another truncation step with chymotrypsin on top of the trypsin treatment to increase the variety of our protein samples. The protein band of the large N-terminal fragment shifted slightly to smaller molecular weight on SDS-PAGE (Fig. 1.3E) and was not split into two or more fragments meaning removal of either N- or C-terminal parts. Changes to the MA-M4 helix fragment were not apparent from the SDS-PAGE.

I compared all three samples for their purity and homogeneity by negative stain electron microscopy (wild-type: Fig. 1.3B, trypsin treated: Fig. 1.3D, chymotrypsin treated: Fig. 1.3F). Judging from the micrographs, all samples showed a pure and homogeneous population of separated single receptor top and side views, reflecting the shapes as inferred from the 3D structure and previously conducted single particle analysis [16]. Single particle 2D classification of the trypsin-treated receptor (Fig. 1.3G) confirmed this conclusion showing various projections of 5-HT$_{3A}$. The treatment with chymotrypsin resulted in similar particle shapes as the trypsin-treated receptor thus making it a useful additional protein sample to try in 2D crystallization. In addition, protease-treated receptor was observed to give images with better contrast compared to non-treated one, probably due to removal of the strep tag.
Figure 1.3: Purification of the wild-type and protease treated 5-HT$_{3A}$ receptor. (A) Elution profile of the wild-type receptor purification with StrepTrap HP column resulted in a single peak. (B) Analysis of the peak fraction by negative stain electron microscopy revealed a homogenous protein population. (C) Wild-type receptor was treated with trypsin and purified on a Superdex200 10/300 column resulting in a main peak with a small shoulder towards higher molecular weight. (D) Sample homogeneity was confirmed by negative stain electron microscopy. (E) An additional protein sample was generated by further treatment of the trypsinized receptor sample with chymotrypsin. Analysis by SDS-PAGE revealed the purity of all samples and the stability of the receptor against proteases ((1) wild-type, (2) trypsin treatment, (3) chymotrypsin treatment). (F) The treatment with chymotrypsin did not change the protein shape judging from the negative stain electron microscopy analysis. (G) Class averages of trypsin-treated receptor showing the expected side and top views. (scale bar in (B), (D), (F): 50 nm, box size in (G): 242 Å).

1.2.3.2. Influence of detergents on protein homogeneity and stability against proteases

The influence of detergent on the ligand binding active state of 5-HT$_{3A}$ receptor has been reported previously showing that C$_{12}$E$_9$ is the best detergent to keep the protein active in solution [101]. Due to the low CMC and therefore long-time dialysis of C$_{12}$E$_9$, I tested additional detergents often used in 2D crystallography [59,102]: DDM, DM, LDAO, OG.

Exchange of detergent had an impact on the homogeneity of the sample as visualized by electron microscopy. Exchange to DDM (Fig. 1.4B) or DM (Fig. 1.4C) had minimal effect on particle size and shape showing homogeneous sample distributions comparable to C$_{12}$E$_9$ (Fig. 1.4A). The short chain detergents LDAO (Fig. 1.4D) and OG (Fig. 1.4E) on the other hand influenced the shape of the receptor and led to aggregation, especially in OG.

Judging from the electron microscopy images, DDM and DM could be useful detergents for the 2D crystallization of 5-HT$_{3A}$. To validate their potential as well as the negative influence of LDAO and OG, susceptibility to trypsin was tested (Fig 1.4F). As expected, the short chain detergents LDAO and OG were not able to stabilize 5-HT$_{3A}$ and most of the receptor was digested. DM yielded better results, keeping more receptor molecules intact. DDM showed a similar digestion pattern as the standard in C$_{12}$E$_9$ making it a potential candidate for crystallization. Based on these results I assumed that shorter chain detergents formed micelles too small to shield the protein from proteolytic degradation or that they induce a conformational change in the channel making it more accessible to trypsin.
Figure 1.4: Effect of detergent on homogeneity and protease stability of 5-HT$_{3A}$. Negative stain electron microscopy images of trypsin-treated 5-HT$_{3A}$ receptors in different detergents revealing their effect on sample homogeneity: (A) C$_{12}$E$_9$, (B) DDM and (C) DM showed homogeneous particle distribution with minimal differences in shape. (D) LDAO changed the protein shape and (E) in OG protein clusters indicative of aggregation can be observed. (F) By SDS-PAGE similar tendency were visible for protease treatment of wild-type receptor with trypsin in different detergents: (1) wild-type; protease treatment in (2) C$_{12}$E$_9$, (3) DDM, (4) DM, (5) LDAO, (6) OG. (scale bar: 100 nm).

In summary C$_{12}$E$_9$ and DDM were the detergents best suited to keep the receptor stable and homogeneous, allowing the use of proteases for limited proteolysis. As DDM results in reduced ligand binding activity and as it can hardly be rescued by detergent exchange [101], I continued using C$_{12}$E$_9$ for the reconstitution and crystallization.

1.2.3.3. Chemical nature of lipids in 2D crystallization

In 2D crystallization the chemical nature of the lipids is an important parameter besides the protein and the detergent. It influences the state of the resulting bilayer, the protein-protein and protein-lipid interaction and affects the protein function [42]. By systematically analyzing the effect of the three major lipid chemical properties: nature of the head group, alkyl chain length and double bonds in the alkyl chain (Fig. 1.5A), the best lipid environment for the reconstitution and crystallization of 5-HT$_{3A}$ was found. The study was carried out with the wild-type receptor since in the beginning the yield of truncated receptors was too low. Throughout the first trials, the lipid-to-protein ratio was kept constant at LPR = 0.25.

Reconstitution trials resulted in rather sharp boundaries for the lipid properties. Lipids with unsaturated eighteen carbon alkyl chains resulted in reconstitutions with high receptor densities. Shorter alkyl chains and/or the absence of double bonds lead to aggregation of the receptor or nearly empty vesicles with isolated receptor molecules. From the tested head groups the zwitter-ionic phosphatidylcholine (Fig. 1.5B) and phosphatidylethanolamine (Fig. 1.5C) allowed the receptor to be reconstituted into the lipid bilayer. Comparison between the two head groups showed that using phosphatidylcholine resulted in larger protein lipid patches (DOPC = 60 – 160 nm, DOPE = 30 – 45 nm). The negatively charged phosphatidylglycerol head group was not successful in reconstituting the receptor.

In summary, the best lipid for reconstitution of 5-HT$_{3A}$ was DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine, 18:1, Δ9-cis) with two unsaturated eighteen carbon atom alkyl chains and a
phosphatidylcholine head group. In the following experiments DOPC was used as standard lipid for reconstitution and crystallization of the mouse serotonin 5-HT$_{3A}$ receptor.

1.2.3.4. Influence of protein modification in 2D crystallization

Using the best lipid condition for reconstitution of the 5-HT$_{3A}$ receptor I investigated the influence of the different protein modifications generated by limited proteolysis on reconstitution and crystallization. As mentioned above (1.2.3.1) all protein modifications were homogeneous and pure judging from size-exclusion chromatography, negative stain electron microscopy and SDS-PAGE.

Comparison of the wild-type to the two limited proteolysis modifications confirmed the effect of protein modification, as in the 3D crystallization. Reconstitution of trypsinized receptor (Fig. 1.5D) resulted in larger (100-225 nm) and better packing compared to that of wild-type receptor (60-160 nm, Fig. 1.5B) resulting in first order diffraction spots in the Fourier transform (FT). Further treatment with chymotrypsin increased the size and quality of crystalline patches of 5-HT$_{3A}$ (275-500 nm, Fig. 1.5E). The improvement in quality was confirmed by the increased number of spots appearing in the FT (Fig. 1.5F), however the powder-ring in the second diffraction order is smearing. Reasons for this could be heterogeneous packing, the random rotational alignment of the 5-fold receptors to each other or conformational flexibility of the extracellular domain.

After trypsinizing the receptor, I used PNGase-F to remove the sugars from the receptor. Deglycosylation was successfully used in the 3D crystallization. However, deglycosylation did not improve reconstitution and 2D crystallization in comparison to the trypsin treated receptor.

In summary the protein modification by limited proteolysis had the most positive impact on the 2D crystallization of 5-HT$_{3A}$, while deglycosylation had no impact.

1.2.3.5. Lipid-to-protein ratio, lipid mixtures and buffer conditions in 2D crystallization

After obtaining vesicles with crystalline packed receptors, the next step was to improve the 2D crystals size and packing to get samples for higher resolution data collection. One decisive parameter is the amount of lipid available for the 2D crystallization. By changing the ratio...
between lipid and protein (LPR) in the crystallization mixture before dialysis, effects of the LPR on the reconstitution and crystallization of 5-HT$_3$A could be observed. From LPR 0.2 to 0.5, size and protein packing of formed vesicle did not change significantly as shown in the images above. When the LPR was smaller than 0.2, vesicles became smaller and the remaining receptors formed aggregates. While LPR bigger than 0.5, resulted in a lot of empty vesicles and the packing of the receptors got loose and the size of vesicles decreased. LPR at 0.25 produced more vesicles with big size and densely packed receptors.

The effect of the lipid mixture was tested for hydrophobic matching of the receptor and the packing among receptors in the lipid bilayer at LPR 0.25. After testing different combinations, a DOPC/DOPG (4:1, w/w) mixture was found to increase the size of the vesicles (600-750 nm) and to preserve the crystalline packing of the receptors (Fig. 1.5G) resulting in similar FT patterns as before (Fig. 1.5H). The information obtained was sufficient for image processing to yield in a projection map with two doughnut-shaped proteins per unit cell ($a = 109.9\pm0.8$ Å, $b = 108.9\pm1.4$ Å, $\gamma = 90.6\pm1.3^\circ$) (Fig. 1.5I). The shape of the densities matches the known top view from the 3D structure of 5-HT$_3$A. Their relative intensities stem most likely from the different amounts of stain deposited on the two membrane sides, suggesting a head-to-tail orientation of the neighboring receptors [103]. The head-to-tail packing is supported by the fact that differences between neighboring receptors could not be detected when the stain was thicker. The final proof of the packing has to be confirmed by structure determination or electron tomography.

pH and ionic strength can influences vesicle size and receptor packing. Between pH 7.0 and pH 9.0, reconstitutions were comparable with pH 7.5, which was used in all conditions mentioned above. pH-values below or above this range resulted in loss of protein packing and reduced vesicle size. The influence of the ionic strength was insignificant. Changing salt concentration and type of salt as well as adding divalent ions like Mg$^{2+}$ or Ca$^{2+}$ only had a minor influence on the patch size but never improved the crystalline packing. Also the use of different ligands could not influence the packing of the receptor. The detergent exchange from C$_{12}$E$_9$ to DDM was tested at this stage. The purification in DDM gave similar results as shown in Figure 1.4 and the reconstitution yielded tightly packed protein. In comparison to C$_{12}$E$_9$, there were no crystalline patches in the vesicles.
## Chemical structure of lipid head group

<table>
<thead>
<tr>
<th>Chain length (number of carbon atoms)</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidylethanolamine</th>
<th>Phosphatidylglycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine (14:0, DMPC)</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (14:0, DMPE)</td>
<td>1,2-dimyristoyl-sn-glycero-3-phospho(1’-rac-glycerol) (14:0, DMPG)</td>
</tr>
<tr>
<td>16</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16:0, DPPC)</td>
<td>1-palmitoyl-2-stearoyl-sn-glycero-3-phosphocholine (16:0, DPPE)</td>
<td>1-palmitoyl-sn-glycero-3-phospho(1’-rac-glycerol) (16:0, POPG)</td>
</tr>
<tr>
<td>18</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1, DOPE)</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (18:1, DOPE)</td>
<td>1,2-dioleoyl-sn-glycero-3-phospho(1’-rac-glycerol) (18:1, POPG)</td>
</tr>
</tbody>
</table>

## Images

**A** Chemical structure of lipid head group

**B**

**C**

**D**

**E**

**F**

**G**

**H**

**I**

Scale bar: 50 nm
Figure 1.5: Lipid condition and influence of protein sample on the two-dimensional crystallization of 5-HT$_{3A}$ using negative stain electron microscopy. (A) Chemical nature of lipids used in initial reconstitution trials with different head group, alkyl chain length and degree of saturation. (B) DOPC was the best lipid resulting in small vesicles with tightly packed receptors (100-225 nm). (C) Vesicles formed from DOPE were smaller (60-160 nm) compared to DOPC vesicles. (D) Trypsinization improved packing and amount of vesicles. (E) Chymotrypsin treatment of trypsinized receptor resulted in larger vesicles (275-500 nm). (F) Lipid condition could be further improved by the mixture DOPC:DOPG 4:1 (w/w) resulting even larger 2D crystals (600-750 nm). (G) The lipid mixture had no effect on the crystal packing and the corresponding FT showed the similar pattern as in (F). (I) Resulting projection map with 2x2 unit cells indicated an up-side-down orientation of two receptor molecules per unit cell ($a = 109.9\pm0.8$ Å, $b = 108.9\pm1.4$ Å, $\gamma = 90.6\pm1.3^\circ$). All reconstitution experiments were done with LPR = 0.25 (scale bar: 100 nm)

1.2.3.6. Nanobody decoration of 2D crystals influence protein packing

Nanobodies have been shown to be beneficial in determination of x-ray crystallography and cryo electron microscopy structures. They introduce new crystals contacts resulting in better protein packing and stabilize the protein in single conformations, thus generating more homogeneous samples. Transferring this knowledge on the 2D crystallization of 5-HT$_{3A}$, two methods were tested using five different conformational nanobodies: I) crystallization of pre-formed receptor-nanobody complexes, II) complex formation on formed 2D crystals.

Co-crystallization of 5-HT$_{3A}$ with nanobodies resulted in smaller, less ordered crystals compared to 5-HT$_{3A}$ alone. This could be due to the larger size of the complex generating spatial obstacles that prevent the packing of the protein. Binding of nanobodies to pre-formed 2D crystals on the other hand improved in most cases protein packing. I tested five different nanobodies (VHH4, VHH5, VHH7, VHH12, VHH15) and verified their influence on crystals by comparing the corresponding FT. Except for nanobody VHH5 (Fig. 1.6C) that reduced diffraction spot quality compared to non-decorated crystals (Fig. 1.6A), all other nanobodies had a positive effect on crystal packing. Nanobodies VHH4 (Fig. 1.6B), VHH12 (Fig. 1.6E) and VHH15 (Fig. 1.6F) produced sharper higher order spots allowing detection of separated diffraction spots. VHH7 yielded the best diffraction quality (Fig. 1.6D) showing hardly any blurring of the diffraction spots. Binding of nanobodies was confirmed by SDS-PAGE (Fig. 1.6G).
**Figure 1.6: Influence of different nanobodies on the 2D crystal packing.** Fourier transforms of negative stain electron microscopy images of 5-HT\textsubscript{3A} 2D crystals decorated with different nanobodies. Compared to the naked 2D crystal (A) adding nanobodies improved the packing in most cases (B) VHH4, (E) VHH12, (F) VHH15 with VHH7 (D) the best, resulting in the sharpest spots. Only VHH5 (C) did not show an enhancement in the FT but more likely a degradation of the crystal quality. (G) SDS-PAGE of 5-HT\textsubscript{3A} nanobody complex formation on 2D crystals: (S) supernatant after incubation of 2D crystals with excessive amount of nanobody, (W1/W2) supernatant of the two wash steps (2D). Sample used for electron microscopy showing the presence of all nanobodies.

The improvement of protein packing in 2D crystals by complex formation with nanobody VHH7 allowed data collection by cryo electron microscopy (Fig. 1.7A and Fig. 1.7B) and was used in future experiments. Comparison of 2D projection maps of receptors with (Fig. 1.7C) and without nanobody (Fig. 1.5I) revealed rearrangements of the receptor molecule in the crystals. Along a zig-zag pattern, receptors with alternating orientation moved closer together. Based on the projection map (space group P1\textsubscript{2}\textsubscript{1}a/b suggested by ALLSPACE) a 3D model of the protein packing could be generated using the 3D x-ray structure with a P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} space group [16] assuming similar binding sites of nanobody VHH7 and nanobody VHH15. This can explain the rearrangements of the receptor molecule by interaction of one nanobody with the intracellular domain of the following neighboring receptor (Fig. 1.7D). Despite the symmetry in the unit cell, a full decoration of the receptor is not possible due to steric hindrance of the nanobodies bound to receptors facing the same direction (Fig 1.7E). The low resolution (about 30 Å, estimated from the FT) might be a result of the non-uniform binding of the nanobodies generating a heterogeneous orientation of the receptors. Otherwise it is possible that the conditions for the crystallization are still not optimal and further optimization might be helpful to improve the crystal quality.

**1.2.3.7. Effects of nanobody binding to solubilized 5-HT\textsubscript{3A} receptor**

To follow up previously described the results, binding and effects on detergent solubilized trypsin treated receptor were analyzed. The analytical size exclusion chromatography profiles of the different receptor-nanobody-complexes showed very similar retention volumes compared to 5-HT\textsubscript{3A} (Fig. 1.8A). The peak of most complexes even came at slightly later volumes, probably due to changes of protein shape. Only nanobody VHH15 resulted in a shift to the void volume of the column suggesting aggregation of the receptor. The profile of 5-HT\textsubscript{3A}-VHH5 on the other hand showed an asymmetrical peak pointing towards potential sample heterogeneity induced by the nanobody.
Figure 1.7: Cryo electron microscopy of 2D crystals of 5-HT$_3$A with nanobody VHH7. (A) Cryo-Electron microscopy image of 2D-crystals decorated with nanobody VHH7. (B) Corresponding FT showed sharp spots up to 30 Å. (C) 2D projection maps of protein density indicates formation of new crystal contacts through the nanobody ($a = 109.7 \pm 1.2$ Å, $b = 107.8 \pm 1.5$ Å, $\gamma = 89.9 \pm 1.1^\circ$, suggested symmetry P12$_1$a/b). (D) Generating a 3D model with a P2$_1$2$_1$2$_1$ space group using the published x-ray structure with VHH15 fitted on the 2D projection map might explain the zig-zag motif of receptor with alternating orientation moving closer together. Receptors are connected by the interaction of one nanobody with the following receptors intercellular domain. (E) In the same model the potential spatial problem between receptors facing the same direction with full nanobody decoration was visible. (scale bar: 100 nm).

To prove binding of the nanobodies the fraction of the main peaks were further analyzed by SDS-PAGE using silver staining to visualize the protein bands (Fig. 1.8B). All samples showed the presence of the receptor (37 kDa) as well as the nanobodies (12-15 kDa) confirming the binding of each nanobody.
All peak fractions were additionally analyzed using negative stain electron microscopy. The resulting images agreed in most cases with the results from the size exclusion chromatography. Nanobody VHH15 created oligomeric protein clusters instead of aggregating it (Fig. 1.8H), which explains the peak shift. These protein clusters showed mostly top views and the protein itself still looks intact. Nanobody VHH5 formed small but more aggregate-like patches resulting in a more heterogeneous sample (Fig. 1.8E) as indicated in the size exclusion chromatography. The images of nanobodies VHH4 (Fig. 1.8D), VHH7 (Fig. 1.8F) and VHH12 (Fig. 1.8G) showed a homogeneous distribution of the receptor complexes. For the 5-HT\textsubscript{3A}-VHH7 complex binding of the nanobody could be detected by eye with star-shaped particles of receptor top views in the electron micrographs (Fig. 1.9A). To validate the presence of nanobody, the extracted particles (Fig. 1.9B) were used for 2D classification. Comparing the classification of trypsin treated receptor (Fig. 1.3G) with the nanobody receptor complex classes (Fig. 1.9C) revealed five additional densities per receptor top view. The symmetrical arrangements of the extra density appearing without applying any symmetry operation during classification were clear indications for the nanobody. In the side views the extra densities were not visible.

By statistically analyzing acquired images of the different receptor-nanobody-complexes in terms of number of particles and ratio between top and side views an additional effect of the nanobodies became observable (Fig 1.9 D). In comparison to 5-HT\textsubscript{3A} mostly showing receptor top views the introduction of nanobodies generated more side views resulting in a more even ratio. This might be due to the change in protein shape allowing for different interaction with the carbon film despite the disturbance of the detergent micelle. Between the nanobodies the effect on the ratio was different. Nanobody VHH5 and VHH7 showed a majority of top views, while nanobody VHH4 had an equal distribution. Interestingly, nanobody VHH12 turns the ratio in opposite direction showing more receptor side view then top views of 5-HT\textsubscript{3A}. Transferability of these results to cryo single particle analysis with holey carbon film was not tested. The influence of nanobody VHH15 could not be analyzed due to its oligomerization effect.
**Figure 1.8: Effects of nanobody on the 5-HT\textsubscript{3A} receptor.** (A) Analytical size exclusion chromatography (Superose6 3.2/300) profiles of 5-HT\textsubscript{3A} with and without nanobodies. The peak position slightly shifted to later volume due to the changed shape of the complex compared to the receptor alone except nanobody VHH15 forming receptor aggregates. (B) Binding of all nanobodies to the receptor was confirmed by silver stained SDS-PAGE of the main peak fractions. The effect of the nanobody on receptor homogeneity was analyzed by negative stain electron microscopy. Nanobody VHH4 (D), VHH7 (F) and VHH12 (G) generate homogeneous complex sample, while nanobody VHH5 (E) induces the aggregation of the protein. (H) In comparison nanobody VHH15 induces clustering of the receptor molecules, but their shapes indicate intact protein. (scale bar 100 nm)

1.2.3.8. **Proteolytic behavior of the 5-HT\textsubscript{3A} receptor-nanobody complex**

Limited proteolysis in the presence and absence of the nanobody provides information on nanobody protection on the receptor. Trypsin treatment of wild-type receptor (Fig. 1.9E) as well as limited proteolysis of trypsin-treated receptor with chymotrypsin (Fig. 1.9F) with or without nanobody revealed differences of the nanobodies. These differences were consistent between both proteases. VHH4, VHH7, VHH12 and VHH15, revealing protein clusters by electron microscopy (Fig. 1.8H), stabilized the receptor against the proteases resulting in stronger protein bands on the SDS-PAGE (37 kDa) compared to 5-HT\textsubscript{3A}. Receptor heterogeneity induced by nanobody VHH5 reflected in weak and smeared protein bands weaker than 5-HT\textsubscript{3A} alone. The proteolysis experiments on the receptor-nanobody complex showed the protection effect of nanobody on the receptor and indicated the similar binding site of the nanobody on the receptor. Cryo single particle analysis could help to determine the exact binding site revealing the mechanism of receptor stabilization by nanobodies.
<table>
<thead>
<tr>
<th>Sample</th>
<th>No. Particle</th>
<th>No. Top views</th>
<th>No. Side views</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT$_3$A</td>
<td>332</td>
<td>292 (88%)</td>
<td>40 (12%)</td>
</tr>
<tr>
<td>5-HT$_3$A-VHH4</td>
<td>524</td>
<td>264 (49%)</td>
<td>278 (51%)</td>
</tr>
<tr>
<td>5-HT$_3$A-VHH5</td>
<td>324</td>
<td>224 (69%)</td>
<td>100 (31%)</td>
</tr>
<tr>
<td>5-HT$_3$A-VHH7</td>
<td>604</td>
<td>416 (69%)</td>
<td>188 (31%)</td>
</tr>
<tr>
<td>5-HT$_3$A-VHH12</td>
<td>507</td>
<td>207 (41%)</td>
<td>300 (59%)</td>
</tr>
<tr>
<td>5-HT$_3$A-VHH15</td>
<td>Aggregated</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.9: Further analysis of the receptor nanobody complex. (A) Enlarged electron microscopy image of 5-HT$_{3A}$-VHH7 complex. Red circles indicate star shaped particles showing the complex formation. (B) The star-shape was also visible in the selection of extracted particles with top and side views of the receptor. (C) Representative classes from 2D classification revealed extra densities in the top view. The five-fold symmetrical arrangement of the densities reflects the full decoration of 5-HT$_{3A}$. (D) Statistical analysis of the ratio between top and side views of the different 5-HT$_{3A}$ nanobody complexes showed a shift of the preferential complex orientation on the grids and might be useful in cryo electron microscopy. Protease stability of different 5-HT$_{3A}$ nanobody complexes analyzed by SDS-PAGE: (E) Wild type receptor (WT) treatment with trypsin, (F) trypsin treated receptor proteolyzed with chymotrypsin, both showing identical tendencies. Complexation with nanobody VHH4 (2), VHH7 (4), VHH12 (5) and VHH15 (6) stabilized the receptor showing increased band intensity compared to the receptor alone (1). Nanobody VHH5 (3) has a negative influence on the protein resulting in the degradation of the receptor. (scale bar 50 nm)

1.2.4. Discussion

For the structural determination of membrane proteins by electron crystallography a simple protocol to test the viability of a project and to produce high quality 2D crystals has to be established. Here I used the structurally well characterized mouse serotonin 5-HT$_{3A}$ receptor to generate an easy-to-apply protocol covering all major parameters in the crystallization. Beside detergent, lipid properties and the lipid-to-protein ratio, which are also required, the most important parameter is the protein itself. As in determination of the 3D x-ray structure of 5-HT$_{3A}$ [16], limited proteolysis and the use of conformational antibodies proved to be important variables to obtain well-packed 2D crystals.

In addition to the wild-type and the trypsinized protein, known to have different behavior in forming 3D crystals, I further introduced a limited proteolysis step with chymotrypsin generating a whole set of protein modifications for testing their properties to reconstitute and form 2D crystals. Purification of all three modifications resulted in pure and homogeneous receptor, but the yield of truncated receptors compared to the wild-type receptor was considerably lower. Even though the yield of all modifications increased over time, the initial conditions for reconstitution were generated using the wild-type receptor.

In 2D crystallization the detergent has to keep the receptor in a stable, homogeneous and active state, but determines the rate of detergent removal and reconstitution into the lipid bilayer and influences the size of the membrane patches and protein packing [62]. C$_{12}$E$_{9}$ used in the 3D crystallization [101] was the only detergent supporting the formation of 2D crystals of 5-HT$_{3A}$ while DDM allowed the reconstitution of the receptor.

The nature of the lipids generating the bilayer in 2D crystals is of central importance. Lipids interact with the protein and determine the properties of the whole bilayer. The three variable
features of lipid molecules are the head group with different charge distributions, the alkyl chain length and degree of unsaturation determining the thickness and flexibility of the lipid bilayer. In case of the 5-HT$_{3A}$ receptor DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine (18:1, ∆9-cis)) with the zwitter-ionic phosphatidylecholine head group and two long unsaturated alkyl chains was the best lipid for reconstitution and 2D crystallization. Using a mixture (4:1 molar ratio) of DOPC and DOPG (1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (18:1, ∆9-cis)), which has a negatively charged head group, the size of the 2D crystals patches could be increased, but the protein packing was not affected. The LPR was for 5-HT$_{3A}$ not as important as for many other membrane proteins with a rather large range from LPR 0.2 to LPR 0.5 resulting in 2D crystals with similar size and packing. Below or above this range the protein packing gets lost and the size of the membrane patches is reduced.

The most important parameter for the 2D crystallization of 5-HT$_{3A}$, judging from this work, is the protein. The result of the reconstitution was significantly different depending on the protein modification by limited proteolysis. With wild-type receptor, only small but tightly packed vesicles could be generated. For finding initial conditions with limited amount of protein, this was sufficient. The large polar purification tag, in this case two Twin-Strep-tags per subunit, possibly interferes with the protein-protein interaction in the vesicles preventing formation of crystalline arrays. This is supported by the removal of the tag using limited proteolysis with trypsin improving the protein packing and generating first order diffraction spots. The protease treatment also removes unstructured and flexible part of the intracellular loop, which was especially helpful for the 3D crystallization. Further removal of flexible parts with chymotrypsin could improve the protein packing and resulted in larger patches with a crystalline array of 5-HT$_{3A}$. These crystals still only provided low resolution information with one or two orders of spot in the Fourier transform. Interestingly, higher resolution information could not be resolved in sharp spots but was smeared like a powder pattern, resulting from heterogeneous protein packing or non-uniform crystal contacts due to the five-fold symmetry receptor within each unit cell. Conformational flexibility in the extracellular domain of the receptor could be another reason the low resolution of the 2D crystals, supported by the positive effect of conformational nanobodies fixating a specific receptor conformation, reducing the flexibility and increasing the diffraction quality. Nanobodies could also provide new crystal contacts stabilizing protein arrangements in the crystals.
As for the 3D crystallization of 5-HT$_{3A}$, the introduction of nanobodies was also beneficial in the 2D crystallization. From different conformational nanobodies one was striking in terms of improving the crystals. Interestingly it was not the same nanobody helping to reach 3.5 Å in x-ray crystallography. VHH7 most likely introduces additional protein interaction sites, stabilizing head-to-tail arrangement of neighboring receptors. This reduced the sample heterogeneity resulting in sharper spots without any powder pattern in the FT. But the low resolution of about 30 Å prevents a more detailed statement about the arrangement of the receptor and nanobody in the crystal. An overlay of the 3D x-ray structure with the 2D-projection illustrated potential spatial problems for a full decoration of 5-HT$_{3A}$ with nanobody VHH7, assuming similar binding position than nanobody VHH15, which could generate non-uniform binding of nanobodies. Steric hindrances shown in the 3D model of the receptor packing could also be an explanation for the failed crystallization trials with pre-formed receptor-nanobody complex.

Nanobodies also have, in addition, positive effects on the solubilized 5-HT$_{3A}$ receptor. The receptor-nanobody complexes showed increased stability against proteases, likely due to stabilization of a specific receptor conformation reducing the flexibility and shielding the receptor from the protease. These receptor-nanobody complexes are potent candidates for single particle analysis due to reduced sample heterogeneity. A more equal distribution of receptor orientations on the grid, shown by preliminary investigations using negative staining electron microscopy, can be useful for the single particle analysis. This might be due to interactions with the carbon film, but if these results could be transferred to cryo-electron microscopy using holey carbon film grids this could facilitate the 3D reconstruction using single particle analysis. The ratio between top and side views differs between the different nanobodies suggesting multiple binding positions. VHH7 visualized the presence of the nanobody showing 2D classes averages with five extra densities per receptor suggesting full decoration. Interestingly nanobody VHH15, the key in x-ray crystallography of 5-HT$_{3A}$, aggregates the receptor. It seems that the nanobody is not only stabilizing the protein in one conformation but might also induce nucleation in the crystallization and is involved in all crystal contacts [16]. Single particle structures of the receptor-nanobody complexes in combination with cell biological or electrophysiological data on ion conductance could improve our knowledge about the serotonin 5-HT3A receptor and pLGIC in general.

In summary, I was able to obtain diffracting 2D crystals of 5-HT$_{3A}$ receptor using a systematical approach. By testing the different parameters, I identified the protein as the
major player in the crystallization. Beside limited proteolysis and conformational nanobody one could also optimize the protein by thoughtful construct design. Knowing the influence of the important parameters in the 2D crystallization of membrane proteins can help to verify the viability of a project. For 5-HT$_3$A, its five-fold symmetry makes the whole system more challenging. The receptor-nanobody-complexes are interesting targets for structural studies as they are locked in one conformation and the different nanobodies have various binding sites. This could expand the structural knowledge about 5-HT$_3$A leading to a better understanding of the function and activation mechanism of the receptor.
1.3. Outlook

In 2D crystallization of 5-HT_{3A}, conformational nanobodies marked a major improvement in receptor packing. Complete decoration could not solve symmetry related issues and might not be possible due to steric hindrance. To avoid these problems careful titration of nanobody to receptor crystals could lead to further improvement of the protein packing and obtainable resolution if nanobodies occupy preferential binding position on crystallized receptors. Thoughtful screening to find better crystallization conditions could further improve the protein packing leading to higher resolution. Heteropentameric receptors like A_{3}B_{2}-5-HT_{3} [104] would be helpful to circumvent symmetry related issues, especially if nanobodies, found for the homopentameric receptor, binds specifically at the interface between two A-subunits. Resulting structural information would be beneficial for understanding function and ion conductance modulation of serotonin 5-HT_{3} receptors.

Besides 2D crystallization, cryo single particle analysis of the homopentameric serotonin 5-HT_{3A} receptor in complex with nanobodies could help to obtain atomic resolution structures. Nanobodies described in this work stabilized the receptor and influenced distribution of protein orientations on grids. The stabilization is needed to obtain the high resolution structures. Cell-based activity assay and electrophysiological measurement can determine the effect, either activating or inhibiting, of the different nanobodies on the receptor allowing the biological classification of resulting structures. This could improve the knowledge about the activation process of pentameric ligand-gated ion channels.

Furthermore the structure of the full-length wild-type serotonin 5-HT_{3A} receptor could help to understand the influence of intracellular domains in ion conductance. In combination with structures of agonists and antagonists bound to the receptor this could generate a whole set of information improving our knowledge about the pentameric ligand-gated ion channels.
2. 2D and 3D crystallization of rhodopsin and rhodopsin-G protein complex for free-electron laser experiments

2.1. Introduction

2.1.1. Structure and light activation of rhodopsin

Rhodopsin is the visual pigment involved in our dim light vision. It belongs to the G protein coupled receptor (GPCR) protein family, a very important class of proteins involved in numerous processes in the signal transduction through the cell membrane. With over 800 members GPCRs are the largest family of eukaryotic membrane proteins interacting with extracellular signals like hormones, neurotransmitter and light in the case of rhodopsin [105,106]. The first structure of a GPCR was of native bovine rhodopsin solved by electron crystallography revealing the typical seven transmembrane helices topology [107]. In the following years numerous additional structures of rhodopsin were solved by x-ray crystallography (review [108]) and electron crystallography [109,110]. Compared to other GPCRs rhodopsin is a special case having its ligand covalently bound to the protein. The apo-protein opsin and the ligand retinal forming a Schiff base at Lys296 [111]. In the dark state retinal is in the 11-cis conformation acting as an inverse agonist locking the protein in the inactive state [111–113]. Upon light activation 11-cis retinal isomerizes to all-trans retinal inducing conformational changes in the receptor (Fig. 2.1A). The activation process is spectroscopically well characterized [110,114–116] (Fig. 2.1C) and some intermediate state structures are available [110,117–119]. However, to understand the whole process the characterization of all states would be necessary.

2.1.1. Rhodopsin-G protein complex

The final state in the activation cascade of rhodopsin before desensitization and regeneration is the Meta-II state. The conformational changes in the protein induce by isomerization of retinal from 11-cis to all-trans allow the binding of transducin (Gt) the most abundant G-protein in the rod outer segment membranes. Upon binding, transducin gets activated allowing the release of bound GDP and recruitment of GTP resulting in the dissociation of the G-protein in α- and βγ-subunits. The Gα-subunit activates a cyclic nucleotide phosphodiesterase inducing hydrolysis of cGMP and leads to closure of the related ligand-gated ion channels. The resulting hyperpolarization of the membrane stops release of
neurotransmitters from the synaptic terminus of the rod cell stopping the inhibition of the following neurons and resulting in a neuronal signal.

These information were obtained “Molecular Biology of the Cell” by B. Alberts et al, Garland Science, 6th Edition, Chapter 15, pages 844, 845, 848, 849 and [120–122].

Figure 2.1: Light activation process of rhodopsin: (A) Schematic image depicting the signal process and desensitization of rhodopsin and other GPCRs. After light activation rhodopsin undergoes conformational changes leading to the binding and activation of the G-protein (adapted from [123]). (B) 3D structure of the β2-adrenergic receptor Gs complex (3SN6) visualizes the arrangement of the subunits (GPCR: orange, Gα: blue, Gβ: purple, Gγ: green, nanobody: cyan, T4L: yellow). (C) Overview of the intermediate states in the activation of rhodopsin with the spectroscopic properties and time scale of conversion. (D) Schematic setup for pump-probe experiments of 2D and 3D crystals at Free electron lasers (adapted from [116]).
The rhodopsin-transducin complex is an important step in signal transduction in dim light vision and structural information can help to understand underlying molecular mechanisms as well as expand the knowledge of GPCR signaling in general. During past years different groups were able to successfully obtain a stable complex using various approaches combining native or recombinant expressed rhodopsin with native transducin or exchanging the α-subunit with Ga₁ [124–127]. One question always arising in these studies was the stoichiometry of rhodopsin and transducin. While there were some debates about the ratio most studies came to the same conclusion, a 1:1 ratio. Despite the known sample preparation protocol no structure of the rhodopsin-transducin complex could be solved so far. But there are different structures of rhodopsin and opsin the Meta-II state stabilized by the C-terminal peptide of the Ga₁-subunit [128–130]. They give insights in the structural rearrangement in rhodopsin and initial indication for the interaction with the G-protein, but they cannot answer all questions. The first structure of a GPCR in complex with a G-protein was the β₂-adrenergic receptor Gs complex [131] revealing the interaction between the proteins and activation mechanism (Fig 2.1B). The structure of a rhodopsin G-protein complex would still be beneficial for understanding the molecular mechanism in the vision process and the interaction of GPCRs and G-proteins in general.

2.1.2. Pump-Probe experiments at the free-electron laser

Free-electron lasers (FEL) are new x-ray sources influencing the structural determination of proteins in the past 5 years and resulting in numerous solved structures [132,133]. These linear accelerators are able to generate bright x-ray pulses with duration in the range of femtoseconds [134]. Due to the high brilliance the x-ray pulse destroys the samples after the interaction, but the short pulse length allows operating in the “diffraction before destroy” regime obtaining the structural information before the destruction of the sample [135]. To take advantage of the high pulse frequency of FELs a constant supply of fresh sample and removal of the destruction products has to be ensured. Depending on the type of sample different methods were developed. As 2D crystals require a flat surface, the use of solid supports allowed continuous hits on fresh sample areas by transversal movement [136,137]. While solid supports can also be used for 3D crystals [138] in most cases liquid jets are used generating a constant flow of nano- and micron-sized crystals, further classified into fast aqueous [139] and slow lipidic-cubic phase based jets [140], depending on the crystallization technic used to obtain the 3D crystals. Combination of all snapshots containing diffraction
pattern of single crystals generates complete datasets for structure determination [141,142].
The constant supply of fresh sample allows time dependent structural studies of molecular processes. By variation of the delay between activation of the protein and detection of the diffraction pattern from the x-ray laser pulse different intermediate states can be analyzed (Fig 2.1D). The ideal samples for these pump-probe experiments are light triggered protein like rhodopsin, bacteriorhodopsin or photosystem I and II [116,143–145]. The proteins can be activated by laser pulse and afterwards diffraction data generated by the x-ray pulse is collected.

2.1.3. Aim of the work
G-Protein coupled receptors (GPCR) are important proteins involved in many cellular processes. With more than 800 members in humans they are the largest class of membrane protein transducing extracellular signals like hormones, neurotransmitter and light. In the pharmaceutical industry they are popular drug targets with over 30% of all marketed drugs targeting GPCR [146]. Structural information regarding GPCRs and signaling mechanism are of great interest for further drug developments.

In this part of the thesis I want to obtain structural information about the activation and signaling mechanism on rhodopsin, the visual pigment in the rod out segment membranes. Rhodopsin has a unique property with is covalently bound ligand activating the receptor after light induction. The spectroscopically well characterized activation includes multiple intermediate states with missing structural information which can be address by pump probe experiments at a free electron laser (FEL)

In Chapter 2.2 I summarized my participation in time-resolved serial femtosecond crystallography experiments targeting the protein activation mechanism. The 3D crystallization led to first promising medium resolution diffraction patterns at the FEL.

After the activation of rhodopsin, the signal is forwarded into the cytoplasm. At the interface between membrane and solution occurs the signal transduction through the rhodopsin-transducin complex. In Chapter 2.3 I tested strategies for structural investigations of this transition state at native-like conditions using electron crystallography.
2.2.3D crystallization of native bovine rhodopsin for FEL experiments

2.2.1. Introduction

The light activation process of rhodopsin is complex and structural information is available for a few points of the cascade only [110,118,119,129,130]. Using pump-probe experiments at XFEL enables dynamic structural studies of rhodopsin after light induction helping to understand the activation mechanism.

This chapter is about my participation in experiments conducted at the Linac Coherent Light Source (LCLS) at SLAC National Accelerator Laboratory, USA, resulting in multiple publications [116,147] (one more is submitted and under review). I was involved in crystal and sample preparation for liquid jet injection at the XFEL resulting in a virtual powder pattern with 4-5 Å resolution. In the following I will describe my part in the project and the experimental outcome.
2.2.2. Methods

2.2.2.1. Purification of native bovine rhodopsin

All steps were conducted under red light or in the dark as described before [148][149]. 200 frozen dark adapted bovine retinas (J. A. & W. L. Lawson Co., Lincoln, Nebraska, USA) were thawed at 24 °C in a water bath after adding 100 ml 35% sucrose (w/w) in ROS buffer (10 mM Tris pH 7.4, 150 mM KCl, 2 mM MgCl$_2$, 1 mM EDTA, 2 mM β-mercaptoethanol, 0.1 mM PMFS, Roche complete protease inhibitor (1 tablet/100ml buffer)). The cells were disrupted by vigorous shaking for 2 min in a glass flask. After centrifugation (3000 x g, 30 min, 4 °C, F15-6x100y rotor) the supernatant was collected on ice. The pellets were resuspended with 15 ml 35% sucrose (w/w) in ROS buffer per centrifugation tube by vigorous shaking and the cell debris again was removed by centrifugation (3000 x g, 30 min, 4 °C, F15-6x100y rotor). The combined supernatants were diluted with ROS buffer to a final volume of 450 ml and mixed by shaking. The membranes were collected by centrifugation (22800 x g, 30 min, 4 °C, F15-6x100y rotor) and resuspended in ROS buffer to a final volume of 120 ml. The membrane suspension was applied on a sucrose gradient (bottom to top: 8 ml 35% sucrose (w/w) in ROS buffer, 8 ml 25.7% sucrose (w/w) in ROS buffer, 20 ml membrane suspension) followed by ultracentrifugation (72100 x g, 30 min, 4 °C, SW28 rotor). The ROS membranes were collected at the interface between both sucrose layers and diluted 1:1 with ROS buffers. After centrifugation (22800 x g, 30 min, 4 °C, F15-6x100y rotor) and removing the supernatant the membranes were resuspended in the same amount of ROS buffer and centrifuged (22800 x g, 30 min, 4 °C, F15-6x100y rotor) again. The supernatant was removed and the extracted ROS membrane pellets were stored at -80 °C until further use.

For 3D crystallization the affinity purification was scaled up three times and performed at room temperature [147].

ROS membrane pellet (equal to 150 retinas) was resuspended in 85.5 ml ConA buffer (50 mM NaOAc pH 6.0, 150 mM NaCl, 3 mM MgCl$_2$, 2 mM MnCl$_2$, 3 mM CaCl$_2$, 1 mM EDTA) and homogenized by Douncing. Protease inhibitor (Roche complete, 1 tablet/100 ml) and LDAO were added to a final concentration of 1%. The ROS membranes were solubilized for 1h stirring at room temperature. The insoluble parts were removed by ultracentrifugation (126000 x g, 1h, 4 °C, Ti70 rotor) and the supernatant was collected. The supernatant was applied at room temperature on a ConA sepharose column (90 ml resin) equilibrated in ConA-A buffer (ConA buffer, 0.1% LDAO). Afterwards the column was washed with 7
column volumes ConA-A buffer and rhodopsin was eluted with ConA-B buffer (ConA buffer, 0.1% LDAO, 200 mM methyl-α-D-mannopyranoside). Fractions with ratio $A_{280}/A_{500} < 2.1$ and protein concentration $> 0.2$ mg/ml determined by UV/Vis spectroscopy (Shimadzu UV-2401PC) were concentrated for 3D crystallization to 11-15 mg/ml (Amicon, 30 kDa, 3000 x g, 4 °C). The concentration was determined by UV/Vis spectrometry (Shimadzu UV-2401PC; $1 \text{Abs}_{500} = 1$ mg/ml rhodopsin). The sample was stored at -80 °C until further use. After 5 times usage the Con A-sepharose column was repacked with fresh resin.

2.2.2. Batch crystallization of native bovine rhodopsin

Purified rhodopsin in the dark state was mixed with precipitation solution (ratio 1:2) in micro insert vials (8004-HP-H/i3m, Infochroma AG) and incubated over night at 18°C. Crystal quality was analyzed by power diffraction, SONICC (second-order nonlinear imaging of chiral crystals) imaging and infra-red microscopy.

2.2.2.3. Electron microscopy analysis of 3D nano-crystals

Five microliters of crystals suspension was applied under dim light condition on glow discharged (5 mA, 15 sec, Cressington carbon coater 208carbon with glow discharge unit) carbon coated copper grids (400 mesh) with a flat layer of carbon film and incubated for 30 sec. Excessive liquid was removed by blotting and grids were stained with 5 μl 2% uranyl acetate solution for 1 min. After blotting the grid to remove excessive staining solution the grids were air-dried.

For cryo preparation the applied crystal solution was diluted 1:1 with PEG-free crystallization buffer immediately before plunge-freezing in liquid ethane with one-sided blotting using a prototype plunge freezer from MRC built for freezing rhodopsin 2D crystals. Since the prototype has no humidity detector it was estimated to be similar to the Cryoplunge 3 which is normally around 80%. The blotting time were varied between 20 sec and 70 sec.

2.2.2.4. Electron microscopy data collection

Negative stain images of 3D crystals were acquired at 25000 to 100000 fold magnification on a CCD camera (TVIPS F416, 4k x 4k) at an acceleration voltage of 200 kV (JEOL2200FS).
2.2.3. Results

2.2.3.1. Purification of native bovine rhodopsin

To obtain suitable 3D crystals of native bovine rhodopsin for pump probe experiments at free electron lasers (FELs) we followed the published protocols leading to the 2.65 Å 3D crystal structure [148]. The ConA affinity chromatography resulted in a similar elution profile with its asymmetric peak typical for this kind of affinity resins (Fig. 2.2A). The UV/Vis spectrum of the sample had the typical peaks at 280 nm for aromatic amino acids and at 500 nm for bound 11-cis retinal with a ratio of A280/A500 of about 1.80 (Fig. 2.2C). Compared to the theoretical ratio of 1.56 [150] of purified rhodopsin the discrepancy can be attributed to presence of natural co-purified lipids needed for crystallization as well as co-purified apo-protein opsin. This was supported by SDS-PAGE showing a pure sample with only one prominent protein band at the expected molecular weight of rhodopsin (Fig. 2.2B).

At this point the sample was handed over to collaborators for further purification and successive 3D crystallization experiments.

2.2.3.2. 3D batch crystallization sample preparation and XFEL measurements of native bovine rhodopsin

Initial crystallization conditions were found by collaborators using vapor diffusion setups. Conditions with crystals were transferred into the batch crystallization. High salt conditions were avoided because of the potential damage to the detector during measurement by hitting a salt crystal with the bright XFEL beam. Afterwards conditions were further optimized to obtain the best diffraction quality detectable by powder diffraction.

At the FEL beam time at the LCLS, I was involved in the on-site crystal preparation to exclude crystal degradation during transport. With the established protocols we were able to obtain crystal using batch crystallization and analyzed them using SONICC. The analysis allowed us to select only good preparations with crystals at high density but not clustering or large crystals. Comparison between both crystal preparations, transported and prepared on-site, did not show any differences in sample quality, providing us with useful information for further measurements.

In the further beam time I helped in sample preparation for the fast liquid jet dealing repeatedly problems clogging of the tubing during measurements, potentially due to larger
crystals within the suspension. The hit rate of crystals within the repetition frequency of the FEL was about 1%, and in the end we were able to obtain a virtual powder pattern merging 2186 diffraction patterns of individual crystals extending to 4-5 Å resolution (Fig. 3 of [147]).

Figure 2.2: Purification, 3D crystallization on native bovine rhodopsin. (A) Elution profile of native bovine rhodopsin from the ConA sepharose affinity column. (B) SDS-PAGE of pooled and concentrated peak fractions showed a singular protein band with the molecular weight of rhodopsin. (C) UV/Vis spectroscopy analysis of peak fractions with the typical absorption for rhodopsin in the dark state at 280 nm (protein) and 500 nm (bound 11-cis retinal). The ratio $A_{280}/A_{500}$ was around 1.80 (1.56 for pure protein [150]) attributed to the presents of lipids and apo-protein opsin. (D) Electron microscopy overview showed long clustered needles ($\Omega = 125$ nm, length up to 2 µm). (E) Magnified view on a single needle crystals showed protein packing. (F) Corresponding FT showed low resolution diffraction spot forming a crystal lattice. (scale bar: (D) 200 nm, (E) 100 nm)
2.2.3.3. Electron microscopy analysis of nanometer-sized crystals

3D crystals used at the FEL experiments were further analyzed by negative stain electron microscopy to determine their size and shape. EM images showed long but thin needles with a diameter of about 125 nm and a length of up to 2 µm. The crystals cluster on the grid (Fig. 2.2D). Related to the FEL experiment, the clustering of the crystals could lead to sedimentation during the measurement and account for the low hit rate. The needle shape of the crystals might be the reason for the anisotropic distribution of resolution in the virtual powder pattern by preferred orientations of the crystals within the liquid stream. The clogging of the liquid jet was due to larger crystals. These crystals might be removed during grid preparation or are too thick to be imaged by EM. The FT (Fig. 2.2F, comparable to other protein crystals [151]) of a single crystals (Fig. 2.2E) showing low resolution diffraction spots proved, that the transfer of the rhodopsin nano-crystals onto EM grid electron microscopy is possible. The low resolution diffraction is expected from the negative staining conditions.

The high PEG concentration in the precipitation solution resulting in high viscosity made transfer of crystals into cryo conditions for electron microscopy more difficult. Plunge freezing the crystal suspension without further treatment resulted in a too thick layer of ice preventing the electrons to pass through. Dilution with PEG free crystallization buffer resulted in dissolution of the crystals.
2.2.4. Discussion

Native bovine rhodopsin is an interesting target for pump-probe experiments at free electron laser (FEL) with its cascade of intermediate states in the activation process. During my thesis, I was participating in experimental measurements at the free electron laser (FEL) in Stanford. The Goal of the experiment was the structural investigation of the activation process of rhodopsin by time resolved pump-probe experiment using a liquid jet. I was involved in preparation of 3D crystals of native bovine rhodopsin in dark state necessary for this type of experiment [147]. More precise, I was supporting my colleagues in the purification and on-site crystallization helping to realize this beam time.

Despite the best possible preparation before this beam time optimizing crystal samples as much as possible with the available analysis tool (SONICC, powder diffraction, IR microscopy) we had to cope with multiple problems. Especially larger crystals and cluster crystals clogging the tubing, preferential orientation of the crystals in the jet and low hit rate limited information gain of these experiments. At the end, about 2200 crystal hits generated a virtual powder pattern reaching to 4-5 Å resolution. To make a reliable statement about the conformational changes occurring during light activation a minimum resolution of 2.5 Å would be necessary. Later electron microscopy analysis, already proven its strength in 3D nano-crystallization [151–154], allowed the characterization of the sample confirming the clustering of the crystals by negative staining. The samples contained long, thin needles (2 µm, Ø = 125 nm) explaining the preferential orientation in the jet. A more detailed analysis with cryo electron microscopy was not possible due to the high concentration of PEG in the precipitation solution and dissolution of the crystals after dilution.

In further FEL experiments the fast liquid jet was exchanged to a slower jet using bacteriorhodopsin crystallized in lipidic cubic phase (LCP) by colleagues of our laboratory. The LCP jet was more stable and resulted in a higher hit rate. With better diffracting bacteriorhodopsin crystals we were able conducted the first pump probe experiment and solve the structures of multiple intermediate states of bacteriorhodopsin (publication submitted and under review).
2.3. 2D crystallization of rhodopsin-transducin complex

2.3.1. Introduction

The complex between GPCR and G-protein is an important part of the signal transduction process and marks the point where the signal is distributed to the effector proteins within the cell. The structure of the β2-adrenergic receptor-Gs protein complex [131] showed for the first time structural details of the interaction between both proteins and the activation of the G-protein. To expand the structural knowledge of the receptor G-protein interaction and activation mechanism additional structural information on a variety of complexes is needed. With the rhodopsin transducin (Gt protein) complex I not only use a different well characterized receptor but also the G-protein has a different subunit composition helping to better understand the signal transduction process. Using electron crystallography with the protein in lipid bilayer generating a native-like environment the resulting structure should resemble a more native conformation compared to the detergent-based x-ray crystallography. In this part of the work I describe the process of obtaining 2D crystals of constitutively active rhodopsin mutant in complex with the native G-protein transducin.
2.3.2. Methods

2.3.2.1. Preparation of 1D4-sepharose

For preparation of the affinity resin the published protocols were applied [155]. In the following a short outline is presented. Starting from CNBr-activated sepharose 4B (GE healthcare), the resin was resuspended in 1 mM HCl (50 ml/g resin) and stirred for 30 min at 22 °C. The activated resin was transfer into an open column (Econo-column, Bio-Rad) and washed with 1 mM HCl (25 ml/g resin) followed by a wash with buffer A (100 mM NaHCO₃ pH 8.5, 500 mM NaCl; 50 ml/g resin). After the resin was transferred into to a glass bottle, 1D4 antibody (14 mg/g resin) was added and the mixture was incubated on a roller for 16 h at 4 °C. The resin was then washed with 200 mM Glycine pH 8.0 (50 ml/g resin) and incubated in additional glycine buffer (7.5 ml/g resin) for 2 h at 22 °C. Afterwards the resin was washed in buffer A (50 ml/g resin), buffer B (100 mM NaOAc pH 4.0, 500 mM NaCl; 20 ml/g resin) and buffer A (50 ml/g resin) followed by PBS (20 ml/g resin). The resin was resuspended in PBS with a final volume of 5 times the resin volume and stored at 4 °C until further use.

2.3.2.2. Transducin (G₄) purification

Transducin was purified as described before [156]. Briefly, frozen bovine retinas extracted in the dark (J. A. & W. L. Lawson Co., Lincoln, Nebraska, USA) were thawed at 24 °C for 2 h exposing them to light. After adding 47% sucrose (w/w) in 20 mM Tris pH 7.4, 1 mM CaCl₂, 2 mM DTT (75 ml/50 retinas) the retinas were broken by passing them multiple times through a syringe. By centrifugation (42000 x g, 21 min, 4°C, Ti45 rotor) cell debris were separated from rod outer segment (ROS) membranes in the supernatant. The supernatant was diluted with buffer A (20 mM Tris pH 7.4, 1 mM CaCl₂, 2 mM DTT; 100 ml/50 retinas) and the ROS membranes were collected by centrifugation (19600 x g, 26 min, 4 °C, Ti45 rotor). The membrane pellet was resuspended in buffer A (35 ml/50 retinas) and applied on a sugar gradient (per tube (bottom to top): 8 ml 30% sucrose (w/w) in 20 mM Tris pH 7.4, 1 mM CaCl₂, 2 mM DTT, 10 ml 25% sucrose (w/w) 20 mM Tris pH 7.4, 1 mM CaCl₂, 2 mM DTT, 15 ml membrane suspension). After ultracentrifugation (131000 x g, 26 min, 4 °C, SW28 rotor) the orange membrane band was collected at the interface between 25% and 30% sucrose and diluted with buffer A (80 ml/50 retinas). The ROS membranes were wash once with buffer B (10 mM Tris pH 7.4, 100 mM NaCl, 5 mMCl₂, 2 mM DTT; 50 ml/50 retinas) and twice with buffer C (10 mM Tris pH 7.4, 0.1 mM EDTA, 2 mM DTT; 50 ml/50 retinas) using centrifugation (42000 x g, 21 min, 4°C, Ti45 rotor). At the last wash step the
membranes were collected by ultracentrifugation (72700 x g, 26 min, 4°C, Ti45 rotor), resuspended in buffer C (22 ml/50 retinas), transferred in Ti70 tubes and GTP was added (0.25 ml 4 mM GTP/tube) to release transducin. After 30 min incubation on a roller at 4 °C and ultracentrifugation (370000 x g, 30 min, 4 °C, Ti70 rotor) the supernatant was collected and filtered through a 0.22 µm filter. The filtrate was dialysis against buffer D (10 mM Tris pH 7.4, 2 mM MgCl2, 50% glycerol, 1 mM DTT; 500 ml/50 retinas) and the buffer was changed three times every half day. The transducin was collected and stored at -20 °C until further use. The concentration was measured by nanodrop and Bradford assay.

2.3.2.3. Rhodopsin-transducin complex formation and purification

Expression, purification and complex formation of the rhodopsin/transducin complex was described before [127,157]. Constitutively active mutant of the apo-protein opsin (N2C, M257Y, D282C) [130] was expressed in HEK293 GnTI- cells by collaborators (Roche). Here I describe the purification protocol as it was performed during this work.

The cells were thawed at 24 °C, resuspended in cold PBS plus 2 mM PMSF to a final volume of 5 ml/g cell and disrupted using an Ultra-Turrax (4 x 1 min, slowest speed) on ice. For solubilization DDM was added to a final concentration of 1% and the mixture was stirred for 2 h at 4 °C. Insoluble parts were removed by ultracentrifugation (205000 x g, 40 min, 4 °C, Ti45 rotor) and the supernatant was diluted with PBS to 10 ml/g cell. The diluted supernatant was mixed with 1D4-resin (2.3.2.1) (0.5 ml suspension/g cell or 0.1 ml/g cell resin) and stirred for 4 h at 4°C. The mixture was transferred to open column (Econo-column, Bio-Rad) and the resin was washed with buffer A (10 mM Tris pH 7.4, 100 mM CaCl2, 0.1 mM MgCl2, 0.02% DDM; 30 column volumes). From this point on every step was carried out in the dark or with red light only (wave length larger than 700 nm). The resin was resuspended in 10 CV buffer A, 11-cis retinal was added (final concentration 10 µM) and the suspension was incubated on a roller for 16 h at 4 °C. Afterwards the resin was washed with buffer A (30 column volumes) and resuspended in buffer A (10 column volumes). The suspension was left at 22 °C for 30 min and then mixed with G-protein (2.3.2.2) (2 mg/column volume) and apyrase (0.25 U/column volume). The complex formation was induced by light activation for 15 min (515 nm filter) keeping the resin cooled followed by 1 h incubation at 4 °C on a roller. After the resin was washed with buffer B (10 mM HEPES pH 7.5, 100 mM NaCl, 0.02% DDM; 30 column volume) the complex was eluted by incubation of the resin with buffer C (10 mM HEPES pH 7.5, 100 mM NaCl, 0.02% DDM, 80 mM 1D4 elution peptide; 6 column
volume) for 3 h followed by a second incubation with buffer C (3 column volume) for 2 h, both at 4 °C. The elution fraction were concentrated to 1 ml (Vivaspin20, 100 kDa, 3000 x g, 4 °C) and applied on a size exclusion column (Superdex200 10/600) equilibrated in buffer D (10 mM HEPES pH 7.5, 100 mM NaCl, 10% glycerol, 0.02% DDM, 2 mM β-mercaptoethanol). The peak at around 20.7 ml was collected and concentrated to around 3-10 mg/ml (Vivaspin20, 100 kDa, 3000 x g, 4 °C). The concentration was measured by UV/Vis spectroscopy (Shimadzu UV-2401PC; 1 Abs₃₈₀ = 3.2 mg/ml complex) the complex was stored at -80 °C.

2.3.2.4. Rhodopsin-transducin complex stability assay

Purified complex in DDM and DM were mix with different lipids (Avanti Polar Lipids) at a ratio of 4:1 (w/w) and diluted with running buffer (10 mM HEPES, 100 mM NaCl, 10% glycerol, 2 mM β-mercaptoethanol, 0.02% DDM or 0.15% DM) to a final concentration of 0.2 mg/ml. The mixtures were incubated at different temperatures (4 °C, 10 °C, 20 °C) for various times (1 day, 8 days, 15 days) in the dark. The samples were analyzed by size exclusion chromatography injecting 80 µl onto a Superdex200 PC3.2/300 equilibrated in running buffer. The purified complex without lipid additives served as reference.

2.3.2.5. Purification of rhodopsin (M257Y) mutant

Expression and purification of constitutively active mutant M257Y of rhodopsin was described before [130]. The apo-protein opsin (N2C, M257Y, D282C) was expressed in HEK293 GnTI- cells by collaborators (Roche) [127]. Here I describe the purification protocol as it was performed during this work.

The cells were thawed at 24 °C, resuspended in cold PBS plus protease inhibitor to a final volume of 10 ml/g cell and disrupted using an Ultra-Turrax (5 x 30 min, slowest speed) on ice. For solubilization DM was added to a final concentration of 1.25% and the mixture was stirred for 1 h at 4 °C. Insoluble parts were removed by ultracentrifugation (205000 x g, 40 min, 4 °C, Ti45 rotor) and supernatant was mixed with 1D4-resin (2.3.2.1) (0.5 ml suspension/g cell or 0.1 ml/g cell resin) and stirred for 4 h at 4°C. The mixture was transferred to open column (Econo-column, Bio-Rad) and the resin was washed with buffer A (PBS, 0.125% DM; 40 column volumes). From this point on every step was carried out in the dark or with red light only (wave length larger than 700 nm). The resin was resuspended in 4
column volume buffer A, 9-cis retinal was added (final concentration 50 µM) and the suspension was incubated on a roller for overnight at 4 °C. Afterwards the resin was washed with buffer A (40 column volumes) and buffer B (10 mM HEPES pH 7.0, 0.125% DM; 40 column volumes). Rhodopsin was eluted by incubating the resin with buffer C (10 mM HEPES pH 7.0, 0.125% DM, 80 mM 1D4 elution peptide; 4 column volume) for 45 min at 4 °C and repeating it four times. The elution fraction containing protein were concentrated to 500 µl (Amicon, 30 kDa, 4000 x g, 4 °C) and applied on a size exclusion column (Superdex200 10/300) equilibrated in buffer D (10 mM NaOAc pH 5.0, 100 mM NaCl, 1% OG). The peak at around 13.9 ml was collected and concentrated to around 5-10mg/ml (Amicon, 30 kDa, 3000 x g, 4 °C). The concentration was measured by UV/Vis spectroscopy (Shimadzu UV-2401PC; 1 Abs485 = 1 mg/ml rhodopsin) the protein was stored at -80 °C.

2.3.2.6. Lipid preparation for 2D crystallization
All steps below were conducted with Hamilton syringes and in glass round-bottom flask. Before use all glass ware was cleaned by rinsing with chloroform and dried. Lipids (Avanti Polar Lipids) were transferred into the glass, either by weighting the powder or using the Hamilton syringe for chloroform solutions. In case of a solution chloroform was evaporated under a constant stream of argon, followed by 3h drying under vacuum to remove residual solvent. Afterwards the dried lipids were dissolved in 20% OG at a final concentration of 10 mg/ml by stirring overnight, forming a clear solution.

2.3.2.7. Two-dimensional crystallization of rhodopsin (M257Y)
Purified rhodopsin (M257Y, N2C, D282C) in OG and different lipid detergent stock solution were mixed at different lipid-to-protein ratios (LPR, w/w) and diluted to a protein concentration of 1-2 mg/ml with detergent free purification buffers (10 mM NaOAc pH 5.0, 100 mM NaCl) The detergent concentration was kept above the CMC at all time and adjusted to 1.5% OG in the final mixture. The final sample volume was 20 µl and the mixture was kept at 4 °C overnight creating protein-lipid-detergent mixed micelles.

The final mixture was dialyzed in the dark at different temperatures for 10 days against various detergent free crystallization buffers using a 10 kDa MWCO micro slide-A-lyzer (Pierce, Thermo Scientific) until the solution became turbid. Until further usage the crystal suspensions were stored at 4°C.
For analysis of the crystallization by electron microscopy 3 µl crystallization suspension was applied on a glow discharged (5 mA, 15 sec, Cressington carbon coater 208carbon with glow discharge unit) carbon coated copper grid (3 mm, 400 mesh). After 1 min incubation the excessive liquid was removed by blotting and the sample was stained with 3 µl of 2% uranyl acetate for 1 min without additional wash steps. The stain was blotted and the grid was air dried.

2.3.2.8. Image processing of 2D crystals
Images of negatively stained samples were acquired at 74000 times magnification (JEOL2200FS, 200kV) on a CCD camera (TVIPS F416, 4k x 4k). Images showing a diffraction pattern in the Fourier transform were processed with the 2dx software package [61,64], following the standard procedures and protocols.

2.3.2.9. UV/Vis analysis of crystals
2D crystals of rhodopsin were mixed with 25 mM Glycine pH 3.5, 200 mM NaCl, 10% glycerol, 3 mM NaN₃, 1% OG diluting the sample 1:5. The insoluble parts were removed by centrifugation (21100 x g, 5 min, 4 °C, Eppendorf centrifuge 5424R) and the supernatant was analyzed by UV/Vis spectroscopy (Shimadzu UV-2401PC).

2.3.2.10. Complex formation with G-protein on 2D crystals
Two-dimensional crystals in suspension were mix with transducing (2.3.2.2) at a molar ratio of 1:5 and diluted to 0.1 mg/ml rhodopsin with complex buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT). It was assumed that all rhodopsin molecules were reconstituted during the crystallization. Apyrase was added to stabilize the complex after light activation. After 5 min light activation (515 nm filter) and incubation on ice for 30 min the crystals were pelleted by centrifugation (21100 x g, 5 min, 4 °C, Eppendorf centrifuge 5424R). The crystals were washed twice by resuspending in complex buffer and centrifugation (21100 x g, 5 min, 4 °C, Eppendorf centrifuge 5424R). The washed crystals were solubilized with 1% DDM for 30 min. As control all steps were also performed without light activation. The samples were analyzed by SDS-PAGE as well as analytical size exclusion chromatography (Tosoh SW3000 equilibrated in 25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT, 0.02% DDM).
2.3.3. Results

2.3.3.1. Purification of rhodopsin (M257Y) transducin complex

Compared to the published purification [127] the rhodopsin (M257Y)/transducin complex was purified in DDM instead of LMNG having a higher CMC and was known for its ability to generate two-dimensional crystals [59,102]. Applying a similar purification protocol I was able to purify the complex in DDM (Fig. 2.3A). The SDS-PAGE of the main peak showed the typical band pattern of rhodopsin and the α- and β-subunit of transducin (Fig. 2.3B) [127]. The small γ-subunits as well as impurities were not visible. Further analysis of the complex purity by UV/Vis spectrometry (Fig. 2.3C) revealed a ratio $A_{280}$ (protein) to $A_{380}$ (all-trans retinal) of 3.2-3.45 (Lit: $A_{280}/A_{380} = 3.5$ [127]) supporting the analysis by SDS-PAGE.

2.3.3.2. Stability of rhodopsin (M257Y) transducin complex

In two-dimensional crystallization the protein is incubated with lipids for longer time at higher temperatures until the lipid membrane has a stabilizing effect. Knowing about the low complex dissociation temperature in LMNG [127] the behavior to the complex purified in DDM was analyzed over a longer period of time at different temperatures and in presence of different lipids, simulating the conditions in the crystallization process. The samples were then analyzed by analytical size exclusion chromatography.

After incubating the complex purified in DDM for eight days and in the presence of brain total extract (porcine) the temperature showed a significant effect on the stability (Fig. 2.3D). After 8 days at 4 °C most of the complexes stayed intact, at 10 °C half of the complexes were dissociated showing a larger peak for the free component and at 20 °C no rhodopsin (M27Y)/Gt complexes were left. But even at 4 °C after 15 days only half of the initial complexes were not dissociated (Fig. 2.3E). Comparing the different lipids confirmed the theory of the positive effect of lipids on the complex stability (Fig. 2.3F). After 8 days at 10 °C all complexes mixed with lipids had a lower dissociation levels compared to the control without lipids. The level of stabilization varied between the different lipids and POPE and brain total extract showed the highest potential.
Figure 2.3: Purification and stability of rhodopsin (M257Y)/G\textsubscript{t} complex. (A) Elution profile (Superdex200 10/600) of rhodopsinM257Y/transducin complex showing a symmetrical peak. (B) Corresponding SDS-PAGE of the peak fractions revealed the typical protein band pattern of the complex. (C) UV/Vis spectrum of the peak fractions had the typical shape with absorption peaks at 280 nm (protein) and 380 nm (all-trans retinal). The ratio A280/A380 was 3.2-3.45 (lit. 3.5 [127]). Stability of rhodopsin (M257Y)/transducin complex was analyzed by analytical size exclusion chromatography (Superdex200 PC3.2/300). (D) Comparison of elution profiles of complex incubated for 8 days at different temperatures in presents of lipids (brain total extract (porcine)).
revealed the temperature sensitivity of the complex. (E) Long time incubation at 4 °C also led to complex dissociation. (F) The presence of lipids used in 2D crystallization stabilized the complex but could not prevent complex dissociation (8 days at 10 °C). (G) Elution profile (Superdex200 10/300) of rhodopsin (M257Y) showing an asymmetrical peak. (H) Corresponding SDS-PAGE of the peak fractions revealed two protein bands most likely coming from different glycosylation levels. This protein band pattern was consistent through the whole project. (I) UV/Vis spectrum of the peak fraction with typical absorption peaks at 280 nm (protein) and 485 nm (9-cis retinal). The ratio A280/A380 was 1.58 (A280/A500 (pure native rhodopsin) = 1.56 [150]).

2.3.3.3. Purification and 2D crystallization of rhodopsin (M257Y)

The detected stability issue of the complex makes 2D crystallization more difficult. Even though I tried to crystallize the complex it was never possible to get reconstitution. As a consequence the experimental approach was changed from crystallizing the complex to preforming 2D crystals of rhodopsin (M257Y) in the dark state followed by a complex formation with transducin on the crystals.

Based on an earlier publication [130] I reproduced the purification of the M257Y mutant of rhodopsin in the dark state. The size exclusion chromatography showed a singular peak which was not completely symmetrically (Fig. 2.3G). The SDS-PAGE of the peak fractions revealed two bands which continuously appear also after reconstitution (Fig. 2.3H). While the upper one is consistent with the molecular weight of rhodopsin, the lower band might be a deglycosylated version of it. The UV/Vis spectrum (Fig. 2.3I) supported this assumption as the ratio between A280 (protein) and A485 (bound 9-cis retinal) was 1.58 (A280/A500 (pure native rhodopsin) = 1.56 [150]) suggesting a relatively pure sample.

For the 2D crystallization of rhodopsin (M257Y) a different approach was chosen compared to the crystallization of 5-HT3A to find initial conditions. Instead of testing first only the influence of different lipids properties but in a systematic manner, a three dimensional screen was build testing different lipids and lipid extracts, different pH-values of the crystallization buffer and dialyzed the samples at various temperatures (Fig. 2.4A). This was possible since the amount of protein obtained in the purification was large enough. For the initial tests the lipid-to-protein was kept constant at LPR 0.2.
<table>
<thead>
<tr>
<th>A</th>
<th>Brain total extract</th>
<th>Egg PC</th>
<th>Soybean PC</th>
<th>E.coli polar</th>
<th>POPE</th>
<th>POPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 9.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

C

D

E

F
Out of the initial conditions rhodopsin was reconstituted in brain total extracted (porcine) and POPE. Temperature and pH-value of the dialysis buffer influenced the reconstitution and protein packing. The higher the temperature and the lower the pH-value the better the protein packing resulting in one condition (37 °C, POPE, pH 4.5 (25 mM NaOAc pH 4.5, 200 mM NaCl, 10% glycerol, 3 mM NaN₃) out of 96 showing diffraction spots in the Fourier transform (FT). Starting from this initial condition multiple parameters where tested, mostly changing buffer conditions. The LPR was optimized with the best reconstitution at LPR 0.15. Following the trend of the pH-values in the first screening even lower pH-values down to pH 3.0 were tested with an optimum around pH 4.0. The presence of glycine as buffer system was beneficial for protein packing. Furthermore the influence of different divalent ions and (NH₄)₂SO₄ was analyzed showing that Mg²⁺, Zn²⁺ and even NH₄⁺ improved the protein packing. Changing salt concentration (0-500 mM NaCl) or type of salt (KCl, LiCl) as well as organic solvents or reducing agents did not have a positive effect on the crystallization. For the dialysis time an optimum around 8-10 days was found. Best condition in the end was the mixture of rhodopsin (M257Y) and POPE (LPR 0.15) dialyzed at 37 °C against 25 mM Glycine pH 4.0, 200 mM NaCl, 10 mM ZnCl₂, 10% glycerol, 3 mM NaN₃. In the electron micrograph formation of small crystalline patches could be detected (Fig. 2.4C) resulting in few low resolution diffraction spots in the FT (Fig. 2.4B). UV/Vis spectrometry analysis of solubilized crystals verified the intact ground state of the protein with the characteristic peak of bound 9-cis retinal at 485 nm (Fig. 2.4D) proving that about three quarter of the initial amount of rhodopsin was reconstituted and still intact (1:5 dilution during solubilization). Over all the crystalline vehicles tend to cluster forming aggregate like structures.
2.3.3.4. Complex formation on 2D crystals of rhodopsin (M257Y)

After all trials to improve the crystals with prove of rhodopsin being reconstituted in ground state the binding of transducin was tested. With native bovine rhodopsin in 2D crystals not able to reach the Meta-II or G-protein binding state after light activation [114] it was not clear if the constitutively active mutant rhodopsin (M257Y) would be able to form the complex even though it has a stabilized Meta-II state. The binding of transducin to 2D crystals after light activation was analyzed by analytical size exclusion chromatography and SDS-PAGE of solubilized products.

Both, size exclusion chromatography (Fig. 2.4E) and SDS-PAGE (Fig. 2.4F) indicated the formation of a rhodopsin transducin complex. The light activated sample showed a small peak around the retention volume (2.8 ml) fitting to the molecular weight of the complex. In comparison the control sample was missing this peak. The same samples on the SDS-PAGE showed similar results. The protein band distribution for the complex sample corresponded to the sum of the individual components. Interestingly differences to the control sample were not as clear as for the size exclusion chromatography. The control also showed the pattern of the complex, but with less intensity for the bands of the G-protein. More detailed comparison of the protein band intensities with Figure 2.4B indicated that the complex formation was incomplete. Judging from the differences in peak height in the size exclusion chromatography a major part of rhodopsin was not in complex with transducin. This might also be result of the solubilization conditions with 1% DDM which also generated the highest peak in the size excision chromatography.
2.3.4. Discussion

The GPCR/G-protein complex is an important intermediate state in the signal transduction through the membrane as it marks the point where extracellular information activating the membrane receptor is passed on soluble intracellular signal distributor. Structural information of this intermediate state is important to further understand the activation mechanism and the design of more specific drug to target certain deceases. The structure of the β2-adrenergic receptor in complex with the Gs-protein [131] revealed for the first time the interaction between the receptor and the G-protein. To extent the knowledge about the interaction I used the rhodopsin transducin (Gt) complex, a different receptor with binding specificity for a different G-protein. In comparison to the x-ray structure β2-adrenergic receptor complex in lipidic cubic phase (LCP) I wanted to use the more native environment of 2D crystals stabilizing the complex.

The stability of the rhodopsin (M257Y) transducin complex was known to be low, especially in detergents and at temperatures used in 2D crystallization. In contrast to the β2-adrenergic receptor complex so far no conformational nanobody stabilizing the rhodopsin (M257Y) transducin complex was found. My analysis of the complex demonstrated that 2D crystallization would not be possible even though its stability could be increased by addition lipids.

The alternative approach was the complex formation on preformed 2D crystals of rhodopsin (M257Y). Thereby I was able to obtain the first, even though low resolution 2D crystals of a recombinantly expressed GPCR. The reconstituted receptors were packed in multiple small crystalline areas within a larger vesicle having random orientation to each other preventing structural determination. Nevertheless the vesicles contained intact rhodopsin able to be activated by light. I was able to showed the constitutively active mutation M257Y stabilizing the active Meta-II state of the receptor [130] can reach the G-protein binding state and bind transducin if reconstituted into lipids. This was so far not possible for 2D crystals of native bovine rhodopsin [114]. The complex formation was far from complete, which might be a result of the vesicle aggregation and, depending on the orientation of the receptors (head-to-head or head-to-tail), steric hindrances between the compared to rhodopsin larger G-proteins.
2.4. Outlook

In the pump-probe experiments 3D crystallization is currently ahead of 2D crystallization. For generating better 2D crystals purification and crystallization protocols have to be carefully analyzed and potential adapted to our laboratory equipment. In the meantime 3D crystals improved since the last beam time in June 2014 with increased resolution in powder diffractions as well as initial condition in LCP crystallization. With the upcoming beam times 3D crystals will help obtaining first pump-probe data of a mammalian GPCR.

After solving the current problems in the sample preparation for membrane protein nanocrystals crystals electron diffraction can be a powerful tool to determine the resolution of nanocrystals before beam times but can also be used to determine the structure. Especially due to lack of beam time at FELs electron diffraction is a useful alternative.

The rhodopsin transducin complex 2D crystallization led to initial crystalline sample areas of rhodopsin and allowed the complex formation but for a structural determination this is not enough. The stability of the complex is too low to be directly crystallized. Increasing the stability by conformational nanobody reducing flexibility of the Gα-subunit [87] and allowing the usage of short chain detergents, which can be removed faster by dialysis, would a major breakthrough towards 2D crystals but also 3D crystals. In the meantime the exchange of G protein from G\texti to G\texti proved to be beneficial of the stability, but only in LMNG which is not useful in 2D crystallization. The complex formation on preformed crystals of rhodopsin was far from complete. The next step would be the improvement of the 2D crystals and as mentioned in the first part protein is the most important parameter. By rational construct design a variety of protein sample could generate helping to obtain better crystals. In addition the crystallization of the active state stabilized by mutations and C-terminal Gα peptides could increase the change of complete complex formation and would be itself a new conformational state in the activation process. Currently the project is further investigated using LCP 3D crystallization as well as single particle analysis.
Reference


76


Acknowledgements

First of all, I would like to thank my supervisors Dr. Xiaodan Li and Prof. Gebhard Schertler for their support throughout my work. In particular I like to thank Dr. Xiaodan Li for the opportunity to work on such an interesting topic. Her guidance and believe in my abilities allowed me to overcome the smaller and bigger obstacles on my way. Prof. Gebhard Schertler I would like to thank for the opportunity to explore and work in the field of electron microscopy but also for enabling me to gain an inside into other technics. He helped me with various suggestions and ideas to stay on track.

Then I would like to thank Prof. Henning Stahlberg and Prof. Samuel Zeeman for their support and advice as members of my thesis committee.

Next I would like to thank all people collaborated with me supporting my work with their samples and knowledge:

- Prof. Horst Vogel’s lab at the EPFL for supply of the cell pellets with the expressed 5-HT3A receptor. Him personally for the helpful discussion and the extra point of view on the project.

- Ghérici Hassaine at Theranyx in Marseille for the supply of receptor cell pellets and purified nanobodies as well as the help in establishing the receptor purification in our lab.

- Prof. Henning Stahlberg’s lab at the University Basel for their support in the electron microscopy. Mohamed Chami for his help in cryo sample preparation and data collection at the C-CINA, Ken Goldie for introduction to the electron microscopes and Alexandra Graff for sharing her knowledge about 5-HT3 2D crystallization and single particle analysis. Henning Stahlberg for his ideas and expertise on the data analysis and interpretation of my data.
Many thanks go to all members of the LBR making the lab to a friendly and scientific excellent place. Especially Antonietta and Ulla I want to thank for keeping everything running and for their open ear for to daily problems. My lab mate Martin I would like to thank for his patience concerning the many Linux-related question and for the fruitful scientific discussions, thank you very much for making the third floor to an excellent place. Elisabeth and Takashi I want to thank for their support at the electron microscopes. Special thanks also to Martin, Christian and Daniel for the pleasant time at PSI and the joint activities beside work.

My warmest thanks go to my parents for their constant support in all situations allowing me to be where I am now. And last but not least, I would like to thank my wife Rebekka for always being there for me and supporting me at your best – I can never thank you enough for keeping everything together and making my life wonderful.

Jan