REVIEW

New approaches towards the understanding of integral membrane proteins: A structural perspective on G protein-coupled receptors

Reinhard Grisshammer*

Department of Health and Human Services, Membrane Protein Structure Function Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Rockville, Maryland 20852

Received 8 May 2017; Accepted 15 May 2017

DOI: 10.1002/pro.3200

Published online 25 May 2017 proteinscience.org

Abstract: Three-dimensional structure determination of integral membrane proteins has advanced in unprecedented detail our understanding of mechanistic events of how ion channels, transporters, receptors, and enzymes function. This exciting progress required a tremendous amount of methods development, as exemplified here with G protein-coupled receptors (GPCRs): Optimizing the production of GPCRs in recombinant hosts; increasing the probability of crystal formation using high-affinity ligands, nanobodies, and minimal G proteins for co-crystallization, thus stabilizing receptors into one conformation; using the T4 lysozyme technology and other fusion partners to promote crystal contacts; advancing crystallization methods including the development of novel detergents, and miniaturization and automation of the lipidic cubic phase crystallization method; the concept of conformational thermostabilization of GPCRs; and developing microfocus X-ray synchrotron technologies to analyze small GPCR crystals. However, despite immense progress to explain how GPCRs function, many receptors pose intractable hurdles to structure determination at this time. Three emerging methods, serial femtosecond crystallography, micro electron diffraction, and single particle electron cryo-microscopy, hold promise to overcome current limitations in structural membrane biology.

Keywords: integral membrane protein; G protein-coupled receptor; three-dimensional structure determination; lipidic cubic phase (LCP); serial femtosecond crystallography (SFX); micro electron diffraction (MicroED); single particle electron cryo-microscopy (cryo-EM)

Introduction

Determining three-dimensional structures of integral membrane proteins was largely deemed impossible until the 1970s. In 1975, a seminal paper by Richard Henderson and Nigel Unwin¹ presented the first structural information for an integral membrane protein, a

7 Å resolution structural model of bacteriorhodopsin, a light-driven proton pump from the archaebacterium *Halobacterium salinarum*. This structure was obtained from two-dimensional crystals imaged by room temperature electron microscopy, but it took another 15 years of methods development before an atomic model of bacteriorhodopsin was published in 1990, which was the first structure of any protein determined by electron microscopy.² In the intervening

*Correspondence to: Reinhard Grisshammer; E-mail: rkgriss@helix.nih.gov

Figure 1. Milestones of GPCR structure determination: PDB codes are given in parentheses. (A) Rhodopsin (1F88), (B) β_2 -adrenergic receptor (2RH1), (C) β_2 AR with nanobody (3P0G), (D) β_2 AR with heterotrimeric G protein (3SN6), (E) Rhodopsin with arrestin (4ZWJ), (F) Adenosine A_{2a} receptor with mini G protein (5G53), (G) Calcitonin receptor (CTR) with heterotrimeric G protein (5UZ7). Structures shown in panels A–F were solved by X-ray crystallography, whereas the structure shown in panel G was solved by single particle cryo-EM. The T4 lysozyme fusion partner facilitating crystal formation is indicated as T4L. Individual protein chains of receptor complexes are labeled and shown in different colors. Publication years are given at bottom of figure.

period, the first structure of an integral membrane protein, a bacterial photosynthetic reaction center, was determined by X-ray crystallography,³ and in 1988, Hartmut Michel, Johann Deisenhofer, and Robert Huber were awarded the Nobel Prize in Chemistry for this work. These accomplishments launched the era of membrane protein structural biology.

During the last three decades, breathtaking progress has been made in elucidating how membrane proteins function through determining their three-dimensional structures and utilizing information from nonstructural experimental approaches. For this to happen, strategies, reagents, and tools for recombinant expression, purification, stabilization, crystallization and data collection needed to be developed⁴ because many membrane protein targets, especially eukaryotic membrane proteins, are not abundant naturally and are unstable in detergent solution owing to their conformational flexibility. This review summarizes concepts that have advanced the field of membrane protein structure determination, using GPCRs as an example, and discusses emerging avenues of investigation and new technological developments with the potential to overcome current limitations in membrane protein structural biology.

G protein-coupled receptors

GPCRs are integral membrane proteins that reside in the plasma membrane of eukaryotic cells. They are central to transmitting signals from the extracellular milieu to the inside of the cell. Ligand binding on the extracellular surface of GPCRs leads to conformational changes within the receptor, triggering the activation of G proteins and the interaction with kinases and arrestin molecules in the cytosol.⁵ With more than 800 members in the human genome,

GPCRs constitute the largest protein family involved in signal transduction, and are major targets for drug development.

GPCRs exist in an equilibrium between inactive, nonsignaling states, and active states that can interact with signaling partners within the cell. In contrast to rhodopsin, many GPCRs exhibit basal activity that has been correlated with structural instability.⁶ Ligands range from inverse agonists, which inhibit basal activity, to full agonists, which maximally activate the receptor. The conformational dynamics of GPCRs provide the basis for the complexity and fine-tuning of hormone signaling, but also render GPCRs difficult to crystallize because of their conformational heterogeneity and instability.

In the year 2000, the first crystal structure was determined of a GPCR, the visual pigment rhodopsin.⁷ The second crystal structure, the β_2 -adrenergic receptor (β_2 AR), was published only in 2007.^{8–10} 10 years later, crystal structures of 38 unique GPCRs have been reported.¹¹ Extraordinary progress towards understanding the activation mechanism of GPCRs was achieved by the crystallization of receptors in an active state, coupled to a cytoplasmic heterotrimeric G protein,¹² an engineered mini G protein,¹³ G protein mimicking nanobodies (Nbs),^{14–17} or arrestin¹⁸ (Fig. 1). This exciting progress required a tremendous amount of methods development, with the most notable achievements described below. The foundation of today's successes was the decades of research by groups throughout the world who were convinced that structures of GPCRs will be solved through sufficient effort and the development of novel approaches. Indeed, it took two decades of hard work after the cloning of the respective cDNAs^{19–23} before the structures of the β_2 AR,^{8–10} the β_1 -adrenergic receptor

(β_1 AR),²⁴ the adenosine A_{2a} receptor (A_{2a} R),^{25–28} and the neurotensin receptor (NTSR1)^{29–32} were determined.

What are the challenges in solving membrane protein structures?

This section addresses key considerations for membrane protein crystallization and serves as a guide to navigate through the different aspects discussed in this review. Hurdles for obtaining high-resolution structures may present themselves at virtually every step, from recombinant expression to crystal formation and diffraction data acquisition.

Despite the miniaturization of the actual crystallization process by robotics, the target GPCR needs to be produced in yeast, insect cells, or mammalian cells at milligram scale. These production methods are preferred because only very few GPCRs can be expressed in functional form in bacteria.^{29,33–35} Owing to space limitations, the reader is referred to reviews that cover the topic of recombinant expression of membrane proteins in detail [e.g., Refs. (36–38)].

GPCRs are highly dynamic molecules and their inherent flexibility is the underlying cause for their often-observed biochemical instability. Successful structure determination requires that detergent-solubilized GPCRs are maintained in a biologically relevant form, as aggregated or denatured protein will not form crystals. Bovine rhodopsin was the first GPCR to be crystallized,^{7,39} reflecting its high stability in many detergents as long as the receptor was kept in its inactive, dark state. However, most other GPCRs are labile in the presence of detergent⁴⁰ owing to their highly dynamic properties. Remedies to stabilize receptors include addition of a high-affinity ligand to stabilize a particular signaling conformation thus reducing conformational flexibility and increasing receptor stability;⁴¹ conformational thermostabilization of GPCRs⁴² as a means to lock the receptor in a particular ligand-dependent state; or complexation with signaling mimetics or partners such as nanobodies,⁴³ mini G proteins^{44,45} or G protein¹² to improve stability. Continued efforts to develop novel mild detergents such as bicyclic alkylmaltosides,⁴⁶ facial amphiphiles,⁴⁷ and maltose-neopentylglycols^{48,49} have been instrumental in receptor stabilization and concomitant formation of well-ordered crystals.¹²

Protein crystals are materials in which molecules are arranged in a highly ordered microscopic structure characterized by symmetry. Thus, any flexible entity of a receptor, such as poorly structured termini and loops, or dynamic features of the transmembrane core, counteract the growth of highly diffracting crystals. Unstructured receptor regions have been removed by protein engineering in many receptor constructs optimized for crystallization. Replacement of loops by crystallization modules such as T4 lysozyme (T4L) has restricted the movement of adjacent transmembrane

helices.¹⁰ Conformational thermostabilization⁴² and co-crystallization with conformation-specific antibodies,^{9,50,51} nanobodies,^{14–17} or G protein^{12,13} have resulted in more homogenous protein preparations, and diffracting crystals.

Crystallogenesis requires that protein molecules engage in crystal lattice contacts. As for water-soluble proteins, polar interactions contribute to the stabilization of crystals of membrane proteins.⁵² The polar surfaces of many GPCRs are small, and using stabilizing, large detergents such as dodecyl-maltoside, results in occlusion of the hydrophilic receptor regions essential to form crystal contacts. Small detergents such as octyl-glucoside are in theory better suited for crystallization⁵² but are often highly denaturing precluding their use for most GPCR targets. Two approaches for promoting receptor crystal formation have evolved: Increasing the hydrophilic area by engineered fusion proteins such as T4L,^{10,53} antibodies,^{9,50,51} or nanobodies,⁴³ so that mild detergents with a large micelle can be used; or thermostabilizing receptors to make them withstand harsh, short-chain detergents.⁴²

Fusion partners and antibody fragments

Obtaining high-resolution structures of GPCRs other than rhodopsin has been challenging because of their inherent structural instability. In addition, many GPCRs contain relatively small hydrophilic loops, and the scarcity of hydrophilic regions available to form potential crystal contacts is a major impediment to obtaining diffraction quality GPCR crystals. Replacement of inner loop 3 (IL3) with T4L, a well-folded protein that adds a polar surface and restricts the movement of the adjacent transmembrane helices, proved critical for obtaining the well-ordered crystals of the β_2 AR.¹⁰ An expanded set of fusion partners for crystallizing GPCRs has subsequently been developed, most notably a thermostabilized apocytochrome b₅₆₂ (BRIL),⁵³ rubredoxin,⁵³ and the catalytic domain of *Pyrococcus abyssi* glycogen synthase (PGS).⁵⁴ These fusion partners have been used in various ways to replace IL3 (β_2 AR-T4L,¹⁰ chemokine receptor CCR5-rubredoxin,⁵⁵ orexin receptor OX2-PGS⁵⁴), or IL2 (metabotropic glutamate receptor mGlu5-T4L),⁵⁶ or positioned at the receptor N-terminus (T4L- β_2 AR,^{12,57} BRIL-nociceptin/orphanin FQ receptor⁵⁸).

Antibody fragment-mediated crystallization⁵⁹ has also been successful through reducing the conformational dynamics of the receptor and extending the hydrophilic surface area available to make crystal contacts. Receptors crystallized in the presence of mouse monoclonal antibody fragments include β_2 AR bound to Fab5, which recognizes a three-dimensional epitope on the intracellular side of the β_2 AR,^{9,50} and A_{2a} R bound to Fab2838, which binds to the intracellular surface of A_{2a} R where it acts as an allosteric antagonist.⁵¹

Nanobodies and mini G proteins

The formation of highly ordered 3D crystals necessitates that the receptor is preferentially in one particular conformation. This is achieved by the use of high-affinity inverse agonist or full agonist ligands, and in the case of active-state receptors, by complexation with a signaling partner or mimetic. To date, only one structure of a GPCR-arrestin complex (rhodopsin-arrestin¹⁸) and only one structure of a GPCR-G protein complex (β_2 AR bound to the adenylate cyclase stimulating G protein G_s ¹²) have been reported, providing the first insight into the arrangement of those complexes. In addition, receptors have been crystallized in an active state coupled to peptide fragments of the $G\alpha$ subunit^{60,61} or arrestin.⁶² Crystallizing GPCR-arrestin and GPCR-G protein complexes remains very challenging, but the development of G protein mimicking nanobodies⁶³ and most recently minimal G proteins⁴⁴ facilitated crystallization of several active-state receptors (β_2 AR-Nb80,¹⁶ β_2 AR-Nb6B9,¹⁷ muscarinic receptor M_2 R-Nb9-8,¹⁵ μ -opioid receptor-Nb39,¹⁴ A_{2A} R-mini G_s ¹³).

Nanobodies⁴³ are the recombinant variable domains of camelid heavy-chain antibodies with full antigen-binding capability, and are selected from peripheral blood lymphocytes of an immunized llama through combinatorial methods such as phage display, yeast display, or ribosome display.^{43,63} Nanobodies have evolved as alternatives to conventional antibodies because of their elongated shape and convex antigen binding site, allowing access to cavities on proteins that are difficult to access by conventional antibodies. Nanobodies have been instrumental in obtaining structures of conformationally stabilized active-state GPCRs.

Mini G proteins are engineered, small soluble proteins composed of a truncated form of the GTPase domain of the G protein, including point mutations to stabilize the protein in the absence of $G\beta\gamma$ and in the presence of detergent. Three truncations eliminate the switch III region, part of the N terminus, and the α -helical domain of the $G\alpha$ protein.⁴⁴ Mini G proteins efficiently couple to GPCRs in the absence of $G\beta\gamma$ subunits.⁴⁴ A growing number of mini G proteins provide now the tools to study GPCRs in their active conformation and facilitate complex formation of GPCRs for crystallization experiments.⁴⁵

In surfo and in meso crystallization

Common and successful approaches to membrane protein crystallization are methods that make use of detergent-solubilized membrane protein preparations in crystallization trials (*in surfo*: vapor diffusion, dialysis, microbatch) [see Ref. (64)]. While protein-protein interactions dominate the crystallization of soluble proteins, both detergent-detergent and protein-detergent interactions are additional

considerations for the membrane protein crystallization, and choosing the right detergent has been key to successful membrane protein crystallization efforts. GPCRs (except rhodopsin) show poor stability in detergent solutions, especially in harsh short-chain detergents, preferred for crystal contacts to occur. Conformational thermostabilization is one possible approach to make the protein more tolerant to short-chain detergents, and this approach has been successfully used to determine GPCR structures *in surfo*.^{29,42,65} Alternatively, receptors have been prepared in mild, stabilizing detergents such as dodecyl-maltoside or neopentylglycol detergents. However, the hydrophilic regions of the membrane protein, essential to form crystal contacts, are then often occluded by the detergent micelle, preventing crystal growth. Extending the hydrophilic surface area by using a monoclonal F_{ab} antibody fragment⁵¹ or replacing IL3 with BRIL⁶⁶ allowed the structures of antagonist-bound A_{2A} R to be determined from crystals grown by vapor diffusion and using large-micelle detergent.

Lipidic cubic phase (LCP) is a membrane-mimetic matrix suitable for stabilization and crystallization of membrane proteins in a lipidic environment (*in meso* crystallogenesis).⁶⁴ This methodology was introduced by Ehud Landau and Jürg Rosenbusch back in 1996,⁶⁷ and optimized for miniaturization and automation by Martin Caffrey and Vadim Cherezov.⁶⁴ The success of using LCP for crystallization can be attributed to the stabilizing membrane-like environment of LCP and type I crystal packing, where crystal contacts are formed between both polar and nonpolar parts of the protein, contributing to better order and diffraction. LCP crystallization accommodates membrane proteins prepared in mild long-chain detergents. Almost all *in meso* GPCR structures have utilized receptors modified by fusion partners, although no fusion partner was used for the chemokine CCR9 receptor⁶⁸ and the high-resolution structure of β_1 AR.⁶⁹ LCP crystallization is not limited to GPCRs, but structures of other membrane proteins, both α -helical and β -barrel, including single and multisubunit proteins, have been reported [see Ref. (70)]. Crystals grown *in meso* are often small, and the development of microfocus X-ray synchrotron technologies such as the minibeam⁷¹ combined with rasterization capabilities⁷² and the development of fast detector technologies have been indispensable for data collection.

Two alternative crystallization techniques, in which protein is crystallized from a lipid membrane environment, are the bicelle crystallization method^{9,73} and crystallization by vesicle fusion.⁷⁴ These methods have had some successes with a variety of membrane proteins.

Conformational thermostabilization

GPCRs are highly dynamic molecules with low thermal barriers between the inactive and active signaling

states, displaying in many cases basal activity. Furthermore, GPCRs show poor stability in detergent solution. Therefore, conformational flexibility and instability, once extracted from the membrane, impede the growth of well-ordered crystals.⁴² Two strategies have been used to address these shortcomings: Use of high-affinity ligands, with slow off-rates to allow full occupancy during the crystallization process, lock the wild-type receptor into a single conformation, which increases the receptor stability in detergent solution and therefore the probability of crystal formation. Alternatively, improving the thermostability of the GPCR itself makes the protein more tolerant to short-chain detergents, preferred in vapor diffusion crystallization experiments. The latter approach allows co-crystallization with low-affinity ligands, because the enhanced stability is encoded within the receptor protein itself.⁴² To isolate thermostabilizing mutations in a receptor, mutations are introduced throughout the receptors by either systematic scanning mutagenesis [see Refs. (65,75)] or random mutagenesis as part of an evolution-based strategy.^{76,77} In systematic scanning mutagenesis, the most stabilizing single mutations are then combined to generate a mutant receptor with an augmented melting temperature. The combination of mutagenesis with a specific selection strategy was used to stabilize agonist^{78,79} and antagonist^{65,78} conformations of GPCRs.

Crystal structures of agonist-bound GPCRs have relied on the use of either exceptionally high-affinity agonists or receptor stabilization by mutagenesis. However, many endogenous ligands bind with low affinity and are structurally not tractable in complex with a receptor. Using directed evolution, Ring and colleagues¹⁷ used yeast surface display together with a conformationally specific selection strategy to engineer a high-affinity nanobody that minimizes receptor heterogeneity and stabilizes the active state of the β_2 AR to determine the crystal structure of the activated receptor bound to the endogenous agonist adrenaline.

***In situ* data collection**

Membrane protein crystals are often tiny and fragile, and thus pose challenges for handling such as mounting the crystals or soaking in cryoprotectants. Recently published methods on *in situ* room temperature data collection have made a major contribution to address these issues. Axford *et al.*⁸⁰ mounted an entire 96-well sitting-drop crystallization plate at a synchrotron beam line and successfully collected a data set from many membrane protein crystals. Huang and colleagues⁸¹ implemented an *in situ* approach with *in meso* crystallization, termed the “*in meso in situ* serial crystallography” (IMISX) method. Both methodologies pave new avenues to data collection from crystals that are too fragile and

too small to mount without losing diffraction quality through mechanical shear-induced stress.

Techniques that complement structural work

The focus of this review is on approaches yielding high resolution structural information. By nature, crystal structures provide static rather than dynamic views and typically capture the lowest energy state within an ensemble of conformations that may differ from other signaling conformations of membrane-inserted receptors, not observed in crystal structures. Therefore, techniques that complement structural work are essential to understand how proteins function. For example, molecular dynamics simulations sample interactions of a membrane protein with lipid and partner proteins, and their internal dynamics;^{30,82,83} single molecule spectroscopy and super-resolution imaging allows the study of macromolecular function;⁸⁴ fluorescence spectroscopy probes conformational changes within a receptor,^{85,86} and native mass spectrometry has been used to characterize noncovalent protein–protein and protein–ligand complexes and lipid interactions.⁸⁷ Nuclear magnetic resonance (NMR) techniques and site-directed electron paramagnetic resonance (EPR) spectroscopy^{88–90} are powerful tools for examining intramolecular dynamics and distance distributions between domains of a protein.⁹¹

Nuclear magnetic resonance (NMR)

Two main methods have been employed to study the structure and dynamics of membrane proteins, namely solution NMR and solid-state NMR. Solution NMR methods require that the sample undergoes fast rotational diffusion and this therefore poses limitations on the size of the target membrane protein and its preparation (detergent-solubilized and lipid micelles, small bicelles, nanodiscs).⁹² Solid-state NMR methods have principally no size limitations because they are used on static samples, or samples rotated at high speed, and thus allow membrane protein samples to be prepared in lipid bilayers mimicking the protein’s natural environment. However, solid-state NMR methods still meet technical barriers, because solid-state NMR spectra are complex, leading to spectral crowding of uniformly labeled samples of large proteins, restricting the size of structures that can be determined.⁹²

Solving the complete structure of a membrane protein has been very challenging and has been impaired by substantial difficulties of sample preparation of sufficient quantities of isotopically ¹⁵N- and ¹³C-labeled protein, limited thermal stability, and sample heterogeneity. Nevertheless, the backbone assignment of the archaeal seven-helix transmembrane receptor sensory rhodopsin II⁹³ has been accomplished by solution NMR. Furthermore, the backbone structure of a phospholipid bilayer embedded GPCR,

the chemokine receptor CXCR1, has been reported by using solid-state NMR techniques.⁹⁴ For this, CXCR1 was expressed and isotopically labeled in *E. coli* as inclusion bodies, purified, and refolded into DMPC proteoliposomes.

In contrast, selective labeling of residues with ¹⁹F, ¹³C, or ¹⁵N has been used to provide evidence for ligand- and G protein-dependent conformational states and energy landscapes of GPCRs, and their local dynamics by solution NMR methods.⁹¹ Chemical conjugation with isotope-labeled compounds, either alkylation of cysteine residues or dimethylation of lysine residues, has been used to gather site-specific information and to avoid presently intractable spectral overlap of a uniformly labeled sample. Chemical labeling is limited by solvent accessibility and the efficiency of the chemical reactions. Despite this limitation, chemical labeling has the advantage that highly sensitive nuclei, such as ¹⁹F, can be incorporated, thus leading to excellent signal/noise ratios. Selective isotopic labeling during protein biosynthesis accesses any labeling sites, even those in the hydrophobic environment of the membrane protein. Site-specific labeling is an excellent tool for fingerprinting the range of potential states of a receptor, but a major challenge remains of relating these observations to the structural changes that give rise to these phenomena.

¹⁹F NMR probes have been introduced into detergent-purified receptors^{91,95,96} at naturally occurring cysteine residues or at a selected position mutated to a cysteine⁹⁶ and provide exquisite sensitivity to solvent exposure or side chain packing. To monitor a unique receptor environment associated with specific conformational changes requires use of minimal cysteine receptor versions,⁹¹ which have to be thoroughly tested for ligand binding and G protein activation to assure integrity of the mutated receptor. Specific labeling of methyl groups, such as ¹³CH₃-methionines, provide ideal NMR probes for high molecular weight proteins because the length and flexibility of the methionine side chain compensates for the slow tumbling, improving the resolution and sensitivity of the spectra. Methionine residues are dispersed throughout the receptor and many are found in the transmembrane region, not accessible for study by fluorine NMR, which requires the addition of small-molecule probes to surface-exposed cysteines.⁹⁷ To study specific receptor regions, methionines that face the exterior of the receptor may be mutated thus simplifying the spectrum and eliminating signals from ¹³CH₃-methionine residues that do not undergo structural changes when comparing active and inactive structures.⁹⁷ The labeling of methionine residues is achieved by growing for example insect cells in methionine-deficient media in the presence of ¹³C methyl-labeled methionine. Alternatively, ¹³C labels

can be introduced into detergent-purified GPCRs by reductive methylation of, for example lysine residues, with ¹³C-formaldehyde.⁹⁸ The above NMR methods have provided data of functional relevance on selected side-chains. A recent investigation by Isogai and colleagues⁹⁹ studied receptor motions followed at backbone sites by selective labeling with ¹⁵N-valine, accomplished by growing insect cells in medium devoid of unlabeled valine in the presence of ¹⁵N-valine.⁹⁹

In elegant work, Smith and colleagues¹⁰⁰ have characterized the activation triggers of the visual pigment rhodopsin using solid-state NMR approaches. The opsin is produced in HEK293S cells and reconstituted with retinal. The information is obtained through measurements of chemical shift and dipolar couplings. This does not yield complete 3D protein structural information because of the expense of uniform ¹³C-, ¹⁵N-labeling of proteins using eukaryotic cell lines. Rather, selective labeling of specific amino acid types or different ligands allows the structural features of the ligand or protein of interest to be studied in exquisite detail. For this, stable tetracycline-inducible suspension HEK293S cell lines containing the opsin gene and its mutants were used to express rhodopsin¹⁰¹ in medium supplemented with specific ¹³C-labeled amino acids.

The conformational and dynamic heterogeneity observed in these and other recent NMR studies provides new insights into membrane-protein function that would have been impossible to obtain through crystallography alone.

New Approaches

Despite tremendous progress to elucidate the structural biology of GPCRs, many receptors pose unsurmountable obstacles to structure determination owing to a multitude of reasons such as very low expression levels, high instability even in mild detergents, extensive post-translational modifications, formation of microcrystals not suitable for use with current synchrotron mini X-ray beams, or resistance to crystallization. Three emerging methods, serial femtosecond crystallography, micro electron diffraction, and single particle electron cryo-microscopy, hold promise in addressing some of these limitations. The first two approaches relate to micro-crystals, whereas the latter does not require crystals at all.

Serial femtosecond crystallography (SFX)

SFX¹⁰² is an emerging technique that relies on extremely bright and ultrashort pulses produced by X-ray free-electron lasers (XFELs), permitting the collection of high-resolution diffraction intensities from very small crystals at room temperature with minimal radiation damage, using the principle of “diffraction before destruction”.¹⁰³ A single diffraction pattern is collected before each crystal is destroyed by the

powerful XFEL pulse. To obtain a single data set, diffraction patterns from thousands of randomly oriented crystals need to be collected. The crystals are therefore delivered into the beam in a continuous hydrated stream, and diffraction images are recorded by a detector operating at the XFEL pulse repetition rate.¹⁰³ Technical improvements related to the delivery, detector technology, data acquisition, and analysis¹⁰⁴ have transformed macromolecular crystallography. It is now possible to determine structures using XFEL using less than a milligram of protein.¹⁰⁵ In 2011, a low-resolution electron density map from crystals of photosystem I demonstrated proof-of-principle¹⁰³ and now structures have been determined at sub-2 Å resolution.¹⁰⁶ Several structures of GPCRs at high resolution have also been determined.^{18,107–111} Most excitingly, time-resolved serial femtosecond crystallography at an X-ray free electron laser has allowed the visualization of conformational changes in bacteriorhodopsin from nanoseconds to milliseconds following photoactivation¹¹² and the light-induced structural changes for oxygen evolution by photosystem II.¹¹³ However, access to XFELs for SFX is currently very limited and not a routine approach for most membrane protein crystallographers.

Micro electron diffraction (MicroED)

MicroED¹¹⁴ is a newly developed technique that records electron diffraction patterns from very small crystals in the electron microscope. Extremely tiny 3D crystals are required as electrons cannot penetrate thick samples. Samples are vitrified, as hydration is critical. The specimen is rotated and multiple diffraction patterns are taken from a single crystal, resulting in a series of diffraction patterns that can be indexed and processed with available X-ray crystallography software. The continuous rotation of the sample requires direct electron detectors. The extremely low electron dose rate results in greatly reduced radiation damage of biological samples that are dose sensitive, but this still produces quality diffraction patterns of high resolution. Proof of principle of the MicroED approach was achieved using lysozyme, yielding a structure to 2.9 Å resolution from 3D microcrystals in cryogenic conditions.¹¹⁵ Subsequently, several protein structures have been solved using improved techniques,¹¹⁴ but the structure of a membrane protein by MicroED is yet to be determined.

Single particle electron cryo-microscopy (Cryo-EM)

To date, the majority of membrane protein structures has been determined by X-ray crystallography and by electron crystallography. These approaches necessitate the availability of well-ordered, three-dimensional, or two-dimensional crystals that are a major road block for many membrane protein targets.¹¹⁶ In contrast, single particle cryo-EM sidesteps

the requirement of well-ordered crystals for structure determination, thus providing an attractive alternative to crystallography. Major technological advances in the last few years have improved detection (direct electron detection camera), classification of images of heterogeneous sample conformations, correction for beam-induced motion blurring of images, and dose-fractionation to use the best subframes for data processing.^{117,118} This has resulted in the ‘resolution revolution’¹¹⁹ and an increasing rate of high-resolution structures deposited in the EM Database.¹²⁰ Indeed, single particle cryo-EM has already been used extensively to characterize soluble proteins in multiple conformations at high resolution¹²¹ and is being used increasingly on membrane proteins.^{122–128} The recent structure determination of hemoglobin (molecular weight 64 kDa) at 3.2 Å resolution is a milestone in cryo-EM,¹²⁹ which has had an immediate impact in the GPCR field; the identical methodology was recently used to determine the structure of the calcitonin receptor coupled to heterotrimeric G_s .¹³⁰ Further structures of GPCRs in complex with other binding partners will no doubt follow swiftly, which will result in a revolution in our understanding of the active states of GPCRs.

Conclusions

Membrane protein structure determination has advanced beyond all expectations our insight into mechanistic aspects of how they function. New arising concepts and technological developments hold promise to overcome some of the remaining experimental limitations, propelling us into an exciting time, where structures of integral membrane proteins are no longer regarded as insurmountable challenges.

ACKNOWLEDGMENTS

The research of R.G. is supported by the Intramural Research Program of the National Institutes of Health, National Institute of Neurological Disorders and Stroke. The author has no conflict of interest to declare. I thank Chris Tate (LMB-MRC) and Anirban Banerjee (NICHD, NIH) for comments on the manuscript.

REFERENCES

1. Henderson R, Unwin PN (1975) Three-dimensional model of purple membrane obtained by electron microscopy. *Nature* 257:28–32.
2. Henderson R, Baldwin JM, Ceska TA, Zemlin F, Beckmann E, Downing KH (1990) Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J Mol Biol* 213:899–929.
3. Deisenhofer J, Epp O, Miki K, Huber R, Michel H (1985) Structure of the protein subunits in the photosynthetic reaction centre of *Rhodospseudomonas viridis* at 3 Å resolution. *Nature* 318:618–624.

4. Grisshammer R (2013) Why we need many more G protein-coupled receptor structures. *Expert Rev Proteomics* 10:1–3.
5. Rosenbaum DM, Rasmussen SG, Kobilka BK (2009) The structure and function of G-protein-coupled receptors. *Nature* 459:356–363.
6. Gether U, Ballesteros JA, Seifert R, Sanders-Bush E, Weinstein H, Kobilka BK (1997) Structural instability of a constitutively active G protein-coupled receptor. Agonist-independent activation due to conformational flexibility. *J Biol Chem* 272:2587–2590.
7. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, Yamamoto M, Miyano M (2000) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* 289:739–745.
8. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* 318:1258–1265.
9. Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, Burghammer M, Ratnala VR, Sanishvili R, Fischetti RF, Schertler GF, Weis WI, Kobilka BK (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* 450:383–387.
10. Rosenbaum DM, Cherezov V, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Yao XJ, Weis WI, Stevens RC, Kobilka BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* 318:1266–1273.
11. <http://blanco.biomol.uci.edu/mpstruc/>. Accessed 30 May, 2017.
12. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK, Kobilka BK (2011) Crystal structure of the beta2 adrenergic receptor–Gs protein complex. *Nature* 477:549–555.
13. Carpenter B, Nehme R, Warne T, Leslie AG, Tate CG (2016) Structure of the adenosine A(2A) receptor bound to an engineered G protein. *Nature* 536:104–107.
14. Huang W, Manglik A, Venkatakrishnan AJ, Laeremans T, Feinberg EN, Sanborn AL, Kato HE, Livingston KE, Thorsen TS, Kling RC, Granier S, Gmeiner P, Husbands SM, Traynor JR, Weis WI, Steyaert J, Dror RO, Kobilka BK (2015) Structural insights into mu-opioid receptor activation. *Nature* 524:315–321.
15. Kruse AC, Ring AM, Manglik A, Hu J, Hu K, Eitel K, Hubner H, Pardon E, Valant C, Sexton PM, Christopoulos A, Felder CC, Gmeiner P, Steyaert J, Weis WI, Garcia KC, Wess J, Kobilka BK (2013) Activation and allosteric modulation of a muscarinic acetylcholine receptor. *Nature* 504:101–106.
16. Rasmussen SG, Choi HJ, Fung JJ, Pardon E, Casarosa P, Chae PS, Devree BT, Rosenbaum DM, Thian FS, Kobilka TS, Schnapp A, Konetzki I, Sunahara RK, Gellman SH, Pautsch A, Steyaert J, Weis WI, Kobilka BK (2011) Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature* 469:175–180.
17. Ring AM, Manglik A, Kruse AC, Enos MD, Weis WI, Garcia KC, Kobilka BK (2013) Adrenaline-activated structure of beta2-adrenoceptor stabilized by an engineered nanobody. *Nature* 502:575–579.
18. Kang Y, Zhou XE, Gao X, He Y, Liu W, Ishchenko A, Barty A, White TA, Yefanov O, Han GW, Xu Q, de Waal PW, Ke J, Tan MH, Zhang C, Moeller A, West GM, Pascal BD, Van Eps N, Caro LN, Vishnivetskiy SA, Lee RJ, Suino-Powell KM, Gu X, Pal K, Ma J, Zhi X, Boutet S, Williams GJ, Messerschmidt M, Gati C, Zatsepin NA, Wang D, James D, Basu S, Roy-Chowdhury S, Conrad CE, Coe J, Liu H, Lisova S, Kupitz C, Grotjohann I, Fromme R, Jiang Y, Tan M, Yang H, Li J, Wang M, Zheng Z, Li D, Howe N, Zhao Y, Standfuss J, Diederichs K, Dong Y, Potter CS, Carragher B, Caffrey M, Jiang H, Chapman HN, Spence JC, Fromme P, Weierstall U, Ernst OP, Katritch V, Gurevich VV, Griffin PR, Hubbell WL, Stevens RC, Cherezov V, Melcher K, Xu HE (2015) Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature* 523:561–567.
19. Dixon RA, Kobilka BK, Strader DJ, Benovic JL, Dohlman HG, Frielle T, Bolanowski MA, Bennett CD, Rands E, Diehl RE, Mumford RA, Slater EE, Sigal IS, Caron MG, Lefkowitz RJ, Strader CD (1986) Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. *Nature* 321:75–79.
20. Furlong TJ, Pierce KD, Selbie LA, Shine J (1992) Molecular characterization of a human brain adenosine A2 receptor. *Brain Res Mol Brain Res* 15:62–66.
21. Kobilka BK, Dixon RA, Frielle T, Dohlman HG, Bolanowski MA, Sigal IS, Yang-Feng TL, Francke U, Caron MG, Lefkowitz RJ (1987) cDNA for the human beta 2-adrenergic receptor: a protein with multiple membrane-spanning domains and encoded by a gene whose chromosomal location is shared with that of the receptor for platelet-derived growth factor. *Proc Natl Acad Sci USA* 84:46–50.
22. Tanaka K, Masu M, Nakanishi S (1990) Structure and functional expression of the cloned rat neurotensin receptor. *Neuron* 4:847–854.
23. Yarden Y, Rodriguez H, Wong SK, Brandt DR, May DC, Burnier J, Harkins RN, Chen EY, Ramachandran J, Ullrich A, Ross EM (1986) The avian beta-adrenergic receptor: primary structure and membrane topology. *Proc Natl Acad Sci USA* 83:6795–6799.
24. Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG, Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* 454:486–491.
25. Dore AS, Robertson N, Errey JC, Ng I, Hollenstein K, Tehan B, Hurrell E, Bennett K, Congreve M, Magnani F, Tate CG, Weir M, Marshall FH (2011) Structure of the adenosine A(2A) receptor in complex with ZM241385 and the xanthines XAC and caffeine. *Structure* 19:1283–1293.
26. Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, Ijzerman AP, Stevens RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* 322:1211–1217.
27. Lebon G, Warne T, Edwards PC, Bennett K, Langmead CJ, Leslie AG, Tate CG (2011) Agonist-bound adenosine A2A receptor structures reveal common features of GPCR activation. *Nature* 474:521–525.
28. Xu F, Wu H, Katritch V, Han GW, Jacobson KA, Gao ZG, Cherezov V, Stevens RC (2011) Structure of an agonist-bound human A2A adenosine receptor. *Science* 332:322–327.
29. Egloff P, Hillenbrand M, Klenk C, Batyuk A, Heine P, Balada S, Schlinkmann KM, Scott DJ, Schutz M, Pluckthun A (2014) Structure of signaling-competent neurotensin receptor 1 obtained by directed evolution in *Escherichia coli*. *Proc Natl Acad Sci USA* 111:E655–E662.
30. Krumm BE, Lee S, Bhattacharya S, Botos I, White CF, Du H, Vaidehi N, Grisshammer R (2016) Structure and

dynamics of a constitutively active neurotensin receptor. *Sci Rep* 6:38564.

31. Krumm BE, White JF, Shah P, Grishammer R (2015) Structural prerequisites for G-protein activation by the neurotensin receptor. *Nat Commun* 6:7895.
32. White JF, Noinaj N, Shibata Y, Love J, Kloss B, Xu F, Gvozdenovic-Jeremic J, Shah P, Shiloach J, Tate CG, Grishammer R (2012) Structure of the agonist-bound neurotensin receptor. *Nature* 490:508–513.
33. Calandra B, Tucker J, Shire D, Grishammer R (1997) Expression in *Escherichia coli* and characterisation of the human central CB1 and peripheral CB2 cannabinoid receptors. *Biotechnol Lett* 19:425–428.
34. Grishammer R, Duckworth R, Henderson R (1993) Expression of a rat neurotensin receptor in *Escherichia coli*. *Biochem J* 295:571–576.
35. Weiss HM, Grishammer R (2002) Purification and characterization of the human adenosine A(2a) receptor functionally expressed in *Escherichia coli*. *Eur J Biochem* 269:82–92.
36. Andrell J, Tate CG (2013) Overexpression of membrane proteins in mammalian cells for structural studies. *Mol Membr Biol* 30:52–63.
37. Grishammer R, Tate CG (1995) Overexpression of integral membrane proteins for structural studies. *Q Rev Biophys* 28:315–422.
38. Tate CG, Grishammer R (1996) Heterologous expression of G-protein-coupled receptors. *Trends Biotechnol* 14:426–430.
39. Li J, Edwards PC, Burghammer M, Villa C, Schertler GF (2004) Structure of bovine rhodopsin in a trigonal crystal form. *J Mol Biol* 343:1409–1438.
40. Lee S, Mao A, Bhattacharya S, Robertson N, Grishammer R, Tate CG, Vaidehi N (2016) How do short chain nonionic detergents destabilize G-protein-coupled receptors? *J Am Chem Soc* 138:15425–15433.
41. Shibata Y, White JF, Serrano-Vega MJ, Magnani F, Aloia AL, Grishammer R, Tate CG (2009) Thermostabilization of the neurotensin receptor NTS1. *J Mol Biol* 390:262–277.
42. Tate CG (2012) A crystal clear solution for determining G-protein-coupled receptor structures. *Trends Biochem Sci* 37:343–352.
43. Manglik A, Kobilka BK, Steyaert J (2017) Nanobodies to study G protein-coupled receptor structure and function. *Annu Rev Pharmacol Toxicol* 57:19–37.
44. Carpenter B, Tate CG (2016) Engineering a minimal G protein to facilitate crystallisation of G protein-coupled receptors in their active conformation. *Protein Eng Des Sel* 29:583–594.
45. Nehme R, Carpenter B, Singhal A, Strege A, Edwards PC, White CF, Du H, Grishammer R, Tate CG (2017) Mini-G proteins: novel tools for studying GPCRs in their active conformation. *PLoS One* 12:e0175642.
46. Hovers J, Potschies M, Polidori A, Pucci B, Raynal S, Bonnete F, Serrano-Vega MJ, Tate CG, Picot D, Pierre Y, Popot JL, Nehme R, Bidet M, Mus-Veteau I, Busskamp H, Jung KH, Marx A, Timmins PA, Welte W (2011) A class of mild surfactants that keep integral membrane proteins water-soluble for functional studies and crystallization. *Mol Membr Biol* 28:171–181.
47. Lee SC, Bennett BC, Hong WX, Fu Y, Baker KA, Marcoux J, Robinson CV, Ward AB, Halpert JR, Stevens RC, Stout CD, Yeager MJ, Zhang Q (2013) Steroid-based facial amphiphiles for stabilization and crystallization of membrane proteins. *Proc Natl Acad Sci USA* 110:E1203–E1211.
48. Bae HE, Mortensen JS, Ribeiro O, Du Y, Ehsan M, Kobilka BK, Loland CJ, Byrne B, Chae PS (2016) Tandem neopentyl glycol maltosides (TNMs) for membrane protein stabilisation. *Chem Commun (Camb)* 52:12104–12107.
49. Chae PS, Rasmussen SG, Rana RR, Gotfryd K, Chandra R, Goren MA, Kruse AC, Nurva S, Loland CJ, Pierre Y, Drew D, Popot JL, Picot D, Fox BG, Guan L, Gether U, Byrne B, Kobilka B, Gellman SH (2010) Maltose-neopentyl glycol (MNG) amphiphiles for solubilization, stabilization and crystallization of membrane proteins. *Nat Methods* 7:1003–1008.
50. Day PW, Rasmussen SG, Parnot C, Fung JJ, Masood A, Kobilka TS, Yao XJ, Choi HJ, Weis WI, Rohrer DK, Kobilka BK (2007) A monoclonal antibody for G protein-coupled receptor crystallography. *Nat Methods* 4:927–929.
51. Hino T, Arakawa T, Iwanari H, Yurugi-Kobayashi T, Ikeda-Suno C, Nakada-Nakura Y, Kusano-Arai O, Weyand S, Shimamura T, Nomura N, Cameron AD, Kobayashi T, Hamakubo T, Iwata S, Murata T (2012) G-protein-coupled receptor inactivation by an allosteric inverse-agonist antibody. *Nature* 482:237–240.
52. Michel H (1983) Crystallization of membrane proteins. *Trends Biochem Sci* 8:56–59.
53. Chun E, Thompson AA, Liu W, Roth CB, Griffith MT, Katritch V, Kunken J, Xu F, Cherezov V, Hanson MA, Stevens RC (2012) Fusion partner toolchest for the stabilization and crystallization of G protein-coupled receptors. *Structure* 20:967–976.
54. Yin J, Mobarec JC, Kolb P, Rosenbaum DM (2015) Crystal structure of the human OX2 orexin receptor bound to the insomnia drug suvorexant. *Nature* 519:247–250.
55. Tan Q, Zhu Y, Li J, Chen Z, Han GW, Kufareva I, Li T, Ma L, Fenalti G, Li J, Zhang W, Xie X, Yang H, Jiang H, Cherezov V, Liu H, Stevens RC, Zhao Q, Wu B (2013) Structure of the CCR5 chemokine receptor-HIV entry inhibitor maraviroc complex. *Science* 341:1387–1390.
56. Dore AS, Okrasa K, Patel JC, Serrano-Vega M, Bennett K, Cooke RM, Errey JC, Jazayeri A, Khan S, Tehan B, Weir M, Wiggin GR, Marshall FH (2014) Structure of class C GPCR metabotropic glutamate receptor 5 transmembrane domain. *Nature* 511:557–562.
57. Zou Y, Weis WI, Kobilka BK (2012) N-terminal T4 lysozyme fusion facilitates crystallization of a G protein coupled receptor. *PLoS One* 7:e46039.
58. Thompson AA, Liu W, Chun E, Katritch V, Wu H, Vardy E, Huang XP, Trapella C, Guerrini R, Calo G, Roth BL, Cherezov V, Stevens RC (2012) Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* 485:395–399.
59. Hunte C, Michel H (2002) Crystallisation of membrane proteins mediated by antibody fragments. *Curr Opin Struct Biol* 12:503–508.
60. Deupi X, Edwards P, Singhal A, Nickle B, Oprian D, Schertler G, Standfuss J (2012) Stabilized G protein binding site in the structure of constitutively active metarhodopsin-II. *Proc Natl Acad Sci USA* 109:119–124.
61. Scheerer P, Park JH, Hildebrand PW, Kim YJ, Krauss N, Choe HW, Hofmann KP, Ernst OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* 455:497–502.
62. Szczepek M, Beyriere F, Hofmann KP, Elgeti M, Kazmin R, Rose A, Bartl FJ, von Stetten D, Heck M, Sommer ME, Hildebrand PW, Scheerer P (2014) Crystal structure of a common GPCR-binding interface for G protein and arrestin. *Nat Commun* 5:4801.
63. Pardon E, Laeremans T, Triest S, Rasmussen SG, Wohlkonig A, Ruf A, Muyldermans S, Hol WG, Kobilka

- BK, Steyaert J (2014) A general protocol for the generation of Nanobodies for structural biology. *Nat Protoc* 9:674–693.
64. Caffrey M, Cherezov V (2009) Crystallizing membrane proteins using lipidic mesophases. *Nat Protoc* 4:706–731.
65. Serrano-Vega MJ, Magnani F, Shibata Y, Tate CG (2008) Conformational thermostabilization of the beta1-adrenergic receptor in a detergent-resistant form. *Proc Natl Acad Sci USA* 105:877–882.
66. Sun B, Bachhawat P, Chu ML, Wood M, Ceska T, Sands ZA, Mercier J, Lebon F, Kobilka TS, Kobilka BK (2017) Crystal structure of the adenosine A2A receptor bound to an antagonist reveals a potential allosteric pocket. *Proc Natl Acad Sci USA* 114:2066–2071.
67. Landau EM, Rosenbusch JP (1996) Lipidic cubic phases: a novel concept for the crystallization of membrane proteins. *Proc Natl Acad Sci USA* 93:14532–14535.
68. Oswald C, Rappas M, Kean J, Dore AS, Errey JC, Bennett K, Deflorian F, Christopher JA, Jazayeri A, Mason JS, Congreve M, Cooke RM, Marshall FH (2016) Intracellular allosteric antagonism of the CCR9 receptor. *Nature* 540:462–465.
69. Miller-Gallacher JL, Nehme R, Warne T, Edwards PC, Schertler GF, Leslie AG, Tate CG (2014) The 2.1 Å resolution structure of cyanopindolol-bound beta1-adrenoceptor identifies an intramembrane Na⁺ ion that stabilises the ligand-free receptor. *PLoS One* 9:e92727.
70. Cherezov V (2011) Lipidic cubic phase technologies for membrane protein structural studies. *Curr Opin Struct Biol* 21:559–566.
71. Riekel C, Burghammer M, Schertler G (2005) Protein crystallography microdiffraction. *Curr Opin Struct Biol* 15:556–562.
72. Cherezov V, Hanson MA, Griffith MT, Hilgart MC, Sanishvili R, Nagarajan V, Stepanov S, Fischetti RF, Kuhn P, Stevens RC (2009) Rastering strategy for screening and centring of microcrystal samples of human membrane proteins with a sub-10 microm size X-ray synchrotron beam. *J R Soc Interface* 6:S587–S597.
73. Faham S, Bowie JU (2002) Bicelle crystallization: a new method for crystallizing membrane proteins yields a monomeric bacteriorhodopsin structure. *J Mol Biol* 316:1–6.
74. Takeda K, Sato H, Hino T, Kono M, Fukuda K, Sakurai I, Okada T, Kouyama T (1998) A novel three-dimensional crystal of bacteriorhodopsin obtained by successive fusion of the vesicular assemblies. *J Mol Biol* 283:463–474.
75. Magnani F, Serrano-Vega MJ, Shibata Y, Abdul-Hussein S, Lebon G, Miller-Gallacher J, Singhal A, Strege A, Thomas JA, Tate CG (2016) A mutagenesis and screening strategy to generate optimally thermostabilized membrane proteins for structural studies. *Nat Protoc* 11:1554–1571.
76. Dodevski I, Pluckthun A (2011) Evolution of three human GPCRs for higher expression and stability. *J Mol Biol* 408:599–615.
77. Sarkar CA, Dodevski I, Kenig M, Dudli S, Mohr A, Hermans E, Pluckthun A (2008) Directed evolution of a G protein-coupled receptor for expression, stability, and binding selectivity. *Proc Natl Acad Sci USA* 105:14808–14813.
78. Magnani F, Shibata Y, Serrano-Vega MJ, Tate CG (2008) Co-evolving stability and conformational homogeneity of the human adenosine A2a receptor. *Proc Natl Acad Sci USA* 105:10744–10749.
79. Shibata Y, Gvozdenovic-Jeremic J, Love J, Kloss B, White JF, Grishammer R, Tate CG (2013) Optimising the combination of thermostabilising mutations in the neurotensin receptor for structure determination. *Biochim Biophys Acta* 1828:1293–1301.
80. Axford D, Foadi J, Hu NJ, Choudhury HG, Iwata S, Beis K, Evans G, Alguel Y (2015) Structure determination of an integral membrane protein at room temperature from crystals *in situ*. *Acta Crystallogr D Biol Crystallogr* 71:1228–1237.
81. Huang CY, Olieric V, Ma P, Panepucci E, Diederichs K, Wang M, Caffrey M (2015) In meso *in situ* serial X-ray crystallography of soluble and membrane proteins. *Acta Crystallogr D Biol Crystallogr* 71:1238–1256.
82. Chavent M, Duncan AL, Sansom MS (2016) Molecular dynamics simulations of membrane proteins and their interactions: from nanoscale to mesoscale. *Curr Opin Struct Biol* 40:8–16.
83. Dror RO, Arlow DH, Maragakis P, Mildorf TJ, Pan AC, Xu H, Borhani DW, Shaw DE (2011) Activation mechanism of the beta2-adrenergic receptor. *Proc Natl Acad Sci USA* 108:18684–18689.
84. Moerner WE (2015) Single-molecule spectroscopy, imaging, and photocontrol: foundations for super-resolution microscopy (nobel lecture). *Angew Chem Int Ed Engl* 54:8067–8093.
85. Dawaliby R, Trubbia C, Delporte C, Masureel M, Van Antwerpen P, Kobilka BK, Govaerts C (2016) Allosteric regulation of G protein-coupled receptor activity by phospholipids. *Nat Chem Biol* 12:35–39.
86. Yao XJ, Velez Ruiz G, Whorton MR, Rasmussen SG, DeVree BT, Deupi X, Sunahara RK, Kobilka B (2009) The effect of ligand efficacy on the formation and stability of a GPCR-G protein complex. *Proc Natl Acad Sci USA* 106:9501–9506.
87. Landreh M, Robinson CV (2015) A new window into the molecular physiology of membrane proteins. *J Physiol* 593:355–362.
88. Hubbell WL, Lopez CJ, Altenbach C, Yang Z (2013) Technological advances in site-directed spin labeling of proteins. *Curr Opin Struct Biol* 23:725–733.
89. Raghuraman H, Islam SM, Mukherjee S, Roux B, Perozo E (2014) Dynamics transitions at the outer vestibule of the KcsA potassium channel during gating. *Proc Natl Acad Sci USA* 111:1831–1836.
90. Verhalen B, Dastvan R, Thangapandian S, Peskova Y, Koteiche HA, Nakamoto RK, Tajkhorshid E, McHaourab HS (2017) Energy transduction and alternating access of the mammalian ABC transporter P-glycoprotein. *Nature* 543:738–741.
91. Manglik A, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, Lerch MT, Kobilka TS, Thian FS, Hubbell WL, Prosser RS, Kobilka BK (2015) Structural insights into the dynamic process of beta2-adrenergic receptor signaling. *Cell* 161:1101–1111.
92. Liang B, Tamm LK (2016) NMR as a tool to investigate the structure, dynamics and function of membrane proteins. *Nat Struct Mol Biol* 23:468–474.
93. Gautier A, Mott HR, Bostock MJ, Kirkpatrick JP, Nietlispach D (2010) Structure determination of the seven-helix transmembrane receptor sensory rhodopsin II by solution NMR spectroscopy. *Nat Struct Mol Biol* 17:768–774.
94. Park SH, Das BB, Casagrande F, Tian Y, Nothnagel HJ, Chu M, Kiefer H, Maier K, De Angelis AA, Marassi FM, Opella SJ (2012) Structure of the chemokine receptor CXCR1 in phospholipid bilayers. *Nature* 491:779–783.
95. Liu JJ, Horst R, Katritch V, Stevens RC, Wuthrich K (2012) Biased signaling pathways in beta2-adrenergic

- receptor characterized by 19F-NMR. *Science* 335:1106–1110.
96. Ye L, Van Eps N, Zimmer M, Ernst OP, Prosser RS (2016) Activation of the A2A adenosine G-protein-coupled receptor by conformational selection. *Nature* 533:265–268.
 97. Nygaard R, Zou Y, Dror RO, Mildorf TJ, Arlow DH, Manglik A, Pan AC, Liu CW, Fung JJ, Bokoch MP, Thian FS, Kobilka TS, Shaw DE, Mueller L, Prosser RS, Kobilka BK (2013) The dynamic process of beta(2)-adrenergic receptor activation. *Cell* 152:532–542.
 98. Sounier R, Mas C, Steyaert J, Laeremans T, Manglik A, Huang W, Kobilka BK, Demene H, Granier S (2015) Propagation of conformational changes during mu-opioid receptor activation. *Nature* 524:375–378.
 99. Isogai S, Deupi X, Opitz C, Heydenreich FM, Tsai CJ, Brueckner F, Schertler GF, Vepriyev DB, Grzesiek S (2016) Backbone NMR reveals allosteric signal transduction networks in the beta1-adrenergic receptor. *Nature* 530:237–241.
 100. Kimata N, Reeves PJ, Smith SO (2015) Uncovering the triggers for GPCR activation using solid-state NMR spectroscopy. *J Magn Reson* 253:111–118.
 101. Reeves PJ, Thurmond RL, Khorana HG (1996) Structure and function in rhodopsin: high level expression of a synthetic bovine opsin gene and its mutants in stable mammalian cell lines. *Proc Natl Acad Sci USA* 93:11487–11492.
 102. Schlichting I (2015) Serial femtosecond crystallography: The first five years. *IUCrJ* 2:246–255.
 103. Chapman HN, Fromme P, Barty A, White TA, Kirian RA, Aquila A, Hunter MS, Schulz J, DePonte DP, Weierstall U, Doak RB, Maia FR, Martin AV, Schlichting I, Lomb L, Coppola N, Shoeman RL, Epp SW, Hartmann R, Rolles D, Rudenko A, Foucar L, Kimmel N, Weidenspointner G, Holl P, Liang M, Barthelmess M, Caleman C, Boutet S, Bogan MJ, Krzywinski J, Bostedt C, Bajt S, Gumprecht L, Rudek B, Erk B, Schmidt C, Homke A, Reich C, Pietschner D, Struder L, Hauser G, Gorke H, Ullrich J, Herrmann S, Schaller G, Schopper F, Soltau H, Kuhnel KU, Messerschmidt M, Bozek JD, Hau-Riege SP, Frank M, Hampton CY, Sierra RG, Starodub D, Williams GJ, Hajdu J, Timneanu N, Seibert MM, Andreasson J, Røcker A, Jonsson O, Svenda M, Stern S, Nass K, Andritschke R, Schroter CD, Krasniqi F, Bott M, Schmidt KE, Wang X, Grotjohann I, Holton JM, Barends TR, Neutze R, Marchesini S, Fromme R, Schorb S, Rupp D, Adolph M, Gorkhover T, Andersson I, Hirsemann H, Potdevin G, Graafsma H, Nilsson B, Spence JC (2011) Femtosecond X-ray protein nanocrystallography. *Nature* 470:73–77.
 104. Feld GK, Frank M (2014) Enabling membrane protein structure and dynamics with X-ray free electron lasers. *Curr Opin Struct Biol* 27:69–78.
 105. Liu W, Wacker D, Wang C, Abola E, Cherezov V (2014) Femtosecond crystallography of membrane proteins in the lipidic cubic phase. *Philos Trans R Soc Lond B Biol Sci* 369:20130314.
 106. Suga M, Akita F, Hirata K, Ueno G, Murakami H, Nakajima Y, Shimizu T, Yamashita K, Yamamoto M, Ago H, Shen JR (2015) Native structure of photosystem II at 1.95 Å resolution viewed by femtosecond X-ray pulses. *Nature* 517:99–103.
 107. Batyuk A, Galli L, Ishchenko A, Han GW, Gati C, Popov PA, Lee MY, Stauch B, White TA, Barty A, Aquila A, Hunter MS, Liang M, Boutet S, Pu M, Liu ZJ, Nelson G, James D, Li C, Zhao Y, Spence JC, Liu W, Fromme P, Katritch V, Weierstall U, Stevens RC, Cherezov V (2016) Native phasing of x-ray free-electron laser data for a G protein-coupled receptor. *Sci Adv* 2:e1600292.
 108. Fenalti G, Zatssepin NA, Betti C, Giguere P, Han GW, Ishchenko A, Liu W, Guillemin K, Zhang H, James D, Wang D, Weierstall U, Spence JC, Boutet S, Messerschmidt M, Williams GJ, Gati C, Yefanov OM, White TA, Oberthuer D, Metz M, Yoon CH, Barty A, Chapman HN, Basu S, Coe J, Conrad CE, Fromme R, Fromme P, Tourwe D, Schiller PW, Roth BL, Ballet S, Katritch V, Stevens RC, Cherezov V (2015) Structural basis for bifunctional peptide recognition at human delta-opioid receptor. *Nat Struct Mol Biol* 22:265–268.
 109. Liu W, Wacker D, Gati C, Han GW, James D, Wang D, Nelson G, Weierstall U, Katritch V, Barty A, Zatssepin NA, Li D, Messerschmidt M, Boutet S, Williams GJ, Koglin JE, Seibert MM, Wang C, Shah ST, Basu S, Fromme R, Kupitz C, Rendek KN, Grotjohann I, Fromme P, Kirian RA, Beyerlein KR, White TA, Chapman HN, Caffrey M, Spence JC, Stevens RC, Cherezov V (2013) Serial femtosecond crystallography of G protein-coupled receptors. *Science* 342:1521–1524.
 110. Zhang H, Han GW, Batyuk A, Ishchenko A, White KL, Patel N, Sadybekov A, Zamylny B, Rudd MT, Hollenstein K, Tolstikova A, White TA, Hunter MS, Weierstall U, Liu W, Babaglu K, Moore EL, Katz RD, Shipman JM, Garcia-Calvo M, Sharma S, Sheth P, Soisson SM, Stevens RC, Katritch V, Cherezov V (2017) Structural basis for selectivity and diversity in angiotensin II receptors. *Nature* 544:327–332.
 111. Zhang H, Unal H, Gati C, Han GW, Liu W, Zatssepin NA, James D, Wang D, Nelson G, Weierstall U, Sawaya MR, Xu Q, Messerschmidt M, Williams GJ, Boutet S, Yefanov OM, White TA, Wang C, Ishchenko A, Tirupula KC, Desnoyer R, Coe J, Conrad CE, Fromme P, Stevens RC, Katritch V, Karnik SS, Cherezov V (2015) Structure of the angiotensin receptor revealed by serial femtosecond crystallography. *Cell* 161:833–844.
 112. Nango E, Royant A, Kubo M, Nakane T, Wickstrand C, Kimura T, Tanaka T, Tono K, Song C, Tanaka R, Arima T, Yamashita A, Kobayashi J, Hosaka T, Mizohata E, Nogly P, Sugahara M, Nam D, Nomura T, Shimamura T, Im D, Fujiwara T, Yamanaka Y, Jeon B, Nishizawa T, Oda K, Fukuda M, Andersson R, Bath P, Dods R, Davidsson J, Matsuoka S, Kawatake S, Murata M, Nureki O, Owada S, Kameshima T, Hatsui T, Joti Y, Schertler G, Yabashi M, Bondar AN, Standfuss J, Neutze R, Iwata S (2016) A three-dimensional movie of structural changes in bacteriorhodopsin. *Science* 354:1552–1557.
 113. Suga M, Akita F, Sugahara M, Kubo M, Nakajima Y, Nakane T, Yamashita K, Umena Y, Nakabayashi M, Yamane T, Nakano T, Suzuki M, Masuda T, Inoue S, Kimura T, Nomura T, Yonekura S, Yu LJ, Sakamoto T, Motomura T, Chen JH, Kato Y, Noguchi T, Tono K, Joti Y, Kameshima T, Hatsui T, Nango E, Tanaka R, Naitow H, Matsuura Y, Yamashita A, Yamamoto M, Nureki O, Yabashi M, Ishikawa T, Iwata S, Shen JR (2017) Light-induced structural changes and the site of O=O bond formation in PSII caught by XFEL. *Nature* 543:131–135.
 114. Nannenga BL, Gonen T (2016) MicroED opens a new era for biological structure determination. *Curr Opin Struct Biol* 40:128–135.

115. Shi D, Nannenga BL, Iadanza MG, Gonen T (2013) Three-dimensional electron crystallography of protein microcrystals. *Elife* 2:e01345.
116. Liao M, Cao E, Julius D, Cheng Y (2014) Single particle electron cryo-microscopy of a mammalian ion channel. *Curr Opin Struct Biol* 27:1–7.
117. Bai XC, McMullan G, Scheres SH (2015) How cryo-EM is revolutionizing structural biology. *Trends Biochem Sci* 40:49–57.
118. Fernandez-Leiro R, Scheres SH (2016) Unravelling biological macromolecules with cryo-electron microscopy. *Nature* 537:339–346.
119. Kuhlbrandt W (2014) Biochemistry. The resolution revolution. *Science* 343:1443–1444.
120. Patwardhan A (2017) Trends in the electron microscopy data bank (EMDB). *Acta Crystallogr D* 73. Doi: 10.1107/S2059798317004181.
121. Merk A, Bartesaghi A, Banerjee S, Falconieri V, Rao P, Davis MI, Pragani R, Boxer MB, Earl LA, Milne JL, Subramaniam S (2016) Breaking cryo-EM resolution barriers to facilitate drug discovery. *Cell* 165: 1698–1707.
122. Bai XC, Rajendra E, Yang G, Shi Y, Scheres SH (2015) Sampling the conformational space of the catalytic subunit of human gamma-secretase. *Elife* 4:e11182.
123. Cao E, Liao M, Cheng Y, Julius D (2013) TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature* 504:113–118.
124. Gao Y, Cao E, Julius D, Cheng Y (2016) TRPV1 structures in nanodiscs reveal mechanisms of ligand and lipid action. *Nature* 534:347–351.
125. Liao M, Cao E, Julius D, Cheng Y (2013) Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* 504:107–112.
126. Meyerson JR, Chittori S, Merk A, Rao P, Han TH, Serpe M, Mayer ML, Subramaniam S (2016) Structural basis of kainate subtype glutamate receptor desensitization. *Nature* 537:567–571.
127. Meyerson JR, Kumar J, Chittori S, Rao P, Pierson J, Bartesaghi A, Mayer ML, Subramaniam S (2014) Structural mechanism of glutamate receptor activation and desensitization. *Nature* 514:328–334.
128. Shen H, Zhou Q, Pan X, Li Z, Wu J, Yan N (2017) Structure of a eukaryotic voltage-gated sodium channel at near-atomic resolution. *Science* 355:eaal4326.
129. Khoshouei M, Radjainia M, Baumeister W, Danev R (2017) Cryo-EM structure of haemoglobin at 3.2 Å determined with the Volta phase plate. DOI: 10.1101/087841.
130. Liang YL, Khoshouei M, Radjainia M, Zhang Y, Glukhova A, Tarrasch J, Thal DM, Furness SGB, Christopoulos G, Coudrat T, Danev R, Baumeister W, Miller LJ, Christopoulos A, Kobilka BK, Wootten D, Skiniotis G, Sexton PM (2017) Phase-plate cryo-EM structure of a class B GPCR-G-protein complex. *Nature*. DOI: 10.1038/nature22327.