

# Contributions of H G Khorana to Understanding Transmembrane Signal Transduction

*David L Farrens and Thomas P Sakmar*

Heptahelical G protein-coupled receptors (GPCRs) are located in the cell's plasma membrane and are responsible for transmitting chemical signals across the lipid bilayer. GPCRs comprise a large family of related receptors that have evolved to bind a wide range of extracellular ligands, from biogenic amines and neuromodulatory peptides to peptide hormones and proteins, and to lipids and fatty acids, to name but a few. Recent advances in the study of structural biology of GPCRs, including reports of high-resolution crystal structures of nearly ten different receptors have transformed the field. Beginning in the mid-1980's and continuing until his retirement, H Gobind Khorana and his co-workers at MIT worked on the prototypical GPCR rhodopsin and provided an early framework of experimental technologies and discoveries which propelled the field forward and continue to have a huge impact on literally hundreds of laboratories worldwide.

## Focus on Rhodopsin

GPCRs are arguably the most important single class of pharmaceutical drug targets in the human genome, and new discoveries suggest that GPCRs will continue to be valuable drug targets in the foreseeable future. Recent reports of crystal structures of native and engineered GPCRs such as rhodopsin, opsin, A2A adenosine,  $\beta$ 2- and  $\beta$ 1-adrenergic receptors provide insights into shared ground state structures and molecular mechanism of GPCR activation. Much of the early insights concerning GPCR-mediated signal transduction came from studies of rhodopsin, a prototypical GPCR that serves as the visual pigment of the rod photoreceptor cell.

Rhodopsin resides at high concentration in the disc membrane of



(left) David L Farrens is an Associate Professor at Oregon Health & Science University in Portland, Oregon. His laboratory develops biochemical and physical methods to study GPCRs and their affiliated proteins. His research is on the visual and cannabinoid signaling systems.

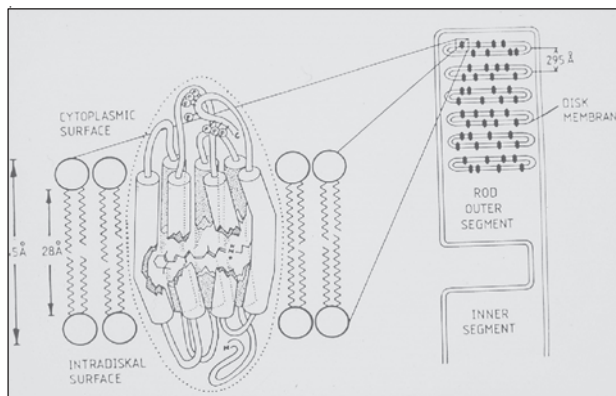
(right) Thomas P Sakmar is the Richard M. & Isabel P. Furlaud Professor at The Rockefeller University, New York. He also served as Acting President of Rockefeller. He was a medical resident at the Massachusetts General Hospital and a clinical fellow at Harvard Medical School in Boston. He carried out postdoctoral research at MIT before moving to Rockefeller in 1991.

## Keywords

Signal transduction, GPCR, Rhodopsin, structure–function, vision.

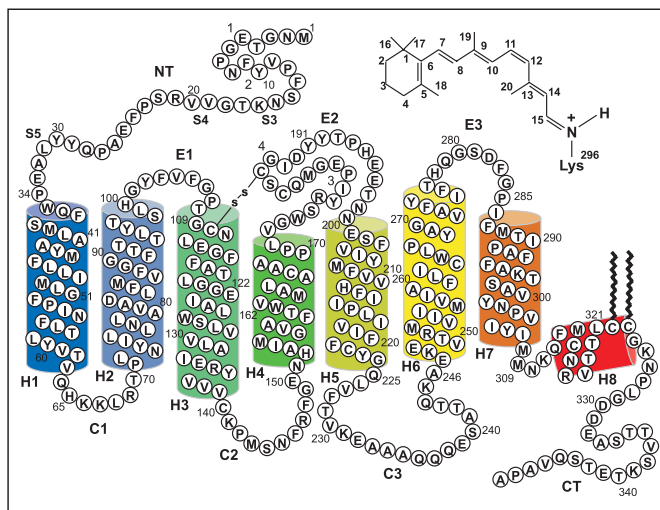


**Figure 1.** A schematic of the rod outer segment (right) showing the stack of disc membranes that contain the visual pigment rhodopsin. The membrane is expanded at left to show the arrangement of the bundle of seven transmembrane segments. Helices five and six are cut away to reveal the 11-*cis*-retinylidene chromophore. Khorana used this graphic in his early talks on rhodopsin.



the outer segment of the rod cell (*Figure 1*). In visual phototransduction, the 11-*cis*-retinylidene (vitamin A aldehyde) chromophore of rhodopsin photoisomerizes in response to photon absorption (*Figure 2*). Rhodopsin rapidly changes conformation and engages a cellular guanine-nucleotide binding regulatory protein (G protein) called transducin, which releases bound GDP. The alpha subunit of transducin then takes up GTP and is released from rhodopsin in order to engage the gamma subunit of a cGMP phosphodiesterase. The phosphodiesterase is disinhibited and cellular cGMP levels drop, which causes the closing of plasma membrane cation channels. This change in membrane cation conductance causes a relative hyperpolarization of the rod cell and a change in its rate of synaptic firing.

**Figure 2.** A secondary structure model of bovine rhodopsin. The structure of the retinylidene chromophore is depicted in the upper right. The chromophore is linked to the opsin protein to a lysine residue in helix 8 by a protonated Schiff base linkage. Over a period of more than twenty years, co-workers in Khorana's laboratory substituted essentially every amino acid residue in rhodopsin using site-directed mutagenesis.



Focusing initially on rhodopsin, Khorana's group pioneered the molecular biological approaches and biophysical methods that continue to dominate the field. The key structural features of the active site of rhodopsin and how photo-isomerization of its 11-*cis*-retinylidene chromophore causes receptor activation and subsequent visual transduction were elucidated in Khorana's laboratory in the final phase of his remarkable scientific career. The molecular elucidation of rhodopsin function was instrumental in advancing allied studies in sensory neuroscience and in the molecular genetics of a number of visual disorders, including color vision defects and autosomal dominant retinitis pigmentosa.

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As recently as the early to mid-1980s, before the advent of many useful molecular methods that we now take for granted, only genes for rhodopsin and the human cone pigments had been cloned and there was no reliable method for heterologous expression of receptors. By that time, Khorana had already committed a massive effort to the primary structural determination of bacteriorhodopsin (br), the light-driven proton pump from *Halobacterium halobium*, and his formidable laboratory team was gearing up to devise a strategy to elucidate the mechanism of proton pumping and how it was coupled to the br photocycle. The recent advent of cloning technology and the ability to produce cDNA libraries, albeit primitive by today's standards, inspired several of Khorana's post-doctoral fellows to begin work on what was thought at the time to be a related system, visual phototransduction. After all, rhodopsin is also an intrinsic membrane protein with a retinylidene chromophore and preliminary reports suggested that it also might have, like br, seven hydrophobic domains that might span the bilayer.

### **Applications of Cloning Technology and Synthetic Genes**

A small team of students and post-doctoral fellows took the initiative and set the stage for a remarkable transformation in Khorana's research program. In rapid succession, cDNA libraries were constructed from bovine retina and cloning projects (using both antibody screening and degenerate oligonucleotide



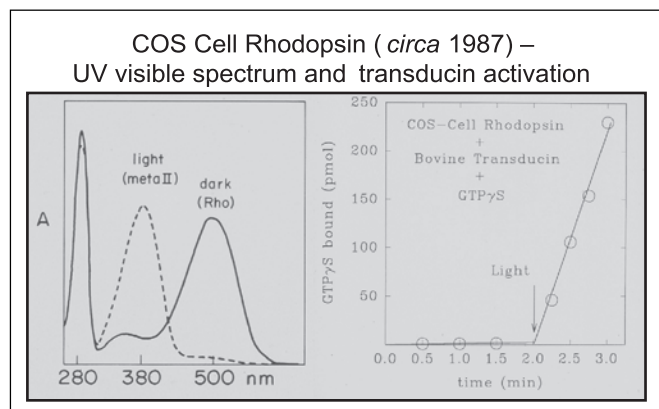
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screening and lambda gt11 and gt10) were underway to isolate cDNA for rhodopsin and the three subunits of transducin, the heterotrimeric G protein of the retinal rod outer segment. The report of the cDNA sequence of the bovine gamma subunit of transducin was one of the first reported clones for a signaling molecule and was followed by the cDNA cloning of the alpha subunit of transducin. Khorana's team was generous and open about their procedures and progress. No surprise that Khorana became a stalwart figure at early visual phototransduction meetings. When a group from Stanford Medical School reported clones for bovine and human rhodopsin, Khorana's team regrouped and rapidly engineered and synthesized a full-length gene for bovine rhodopsin using automated oligonucleotide synthesis on a solid support resin [1]. The synthesis strategy was to prepare a series of oligonucleotides that were annealed batchwise to create overlapping double stranded segments, which were then purified and ligated together to build up the full-length gene in segments. At more than one-kilobase in length, the synthetic rhodopsin gene was a tour-de-force achievement. Even more remarkable was that the methods employed were robust enough that the synthesis of a gene for the alpha subunit of transducin was achieved within just another two years.

### Expression of Rhodopsin Leads to Key Discoveries

The synthetic rhodopsin gene was designed to facilitate site-directed mutagenesis using synthetic restriction fragment replacement, which was pioneered in studies of br. But when the project was initiated, similar to the situation when Khorana began the original synthesis of tRNA genes, there was no established method to express the rhodopsin gene. Daniel D Oprea, who expressed the synthetic gene in COS-1 cells in tissue culture using a novel expression plasmid accomplished the major breakthrough [2] (*Figure 3*). Expression was detected using Western immunoblot analysis and a monoclonal antibody (mAb) called 1D4. Perhaps the most notable accomplishment, however, was the immunopurification of the expressed recombinant rhodopsin pigment in detergent solution using a 1D4 mAb immunoaffinity





**Figure 3.** A synthetic gene for bovine rhodopsin was expressed in COS cells in culture and purified. The spectrum of the recombinant material is depicted in the left panel before and after illumination. Light-dependent transducin activation was carried out as shown in the right panel. Khorana used this graphic in his early talks on rhodopsin.

resin. Over time, expression vectors and cell lines were improved, but the general method pioneered in Khorana's laboratory is still used extensively today to prepare a myriad of GPCR samples for structural studies and is considered as one of the key enabling technologies in the field.

In addition, the method to immuno-purify expressed rhodopsin facilitated a number of early mutagenesis studies of rhodopsin that would go on to have profound effects on the field of visual transduction in particular and GPCR pharmacology in general [3,4]. For example, using this method, Khorana's colleagues: 1) reported the first site-directed mutants of a GPCR that caused loss of G protein coupling and identified the highly conserved 'DRY' sequence motif (abbreviated version of a three amino acid residue stretch aspartic acid-arginine-tyrosine) as a G protein binding site, 2) elucidated the role of a highly conserved pair of cysteine residues on the extracellular surface of rhodopsin and proposed the existence of an essential disulfide bond, 3) mapped the retinal binding pocket of rhodopsin and set the stage for a series of studies elucidating the mechanism of spectral tuning by visual pigments, 4) identified the Schiff base counterion in rhodopsin, which led to an understanding of spectral tuning, activation mechanism, and the mechanism for suppression of basal 'dark noise', 5) reported the first use of n-dodecyl maltoside detergent to reconstitute active GPCRs, which is still widely used today, and 6) reported the first biochemical analysis of rhodopsin mutants that caused autosomal dominant retinitis pigmentosa.

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### Wide Reach of New Technologies

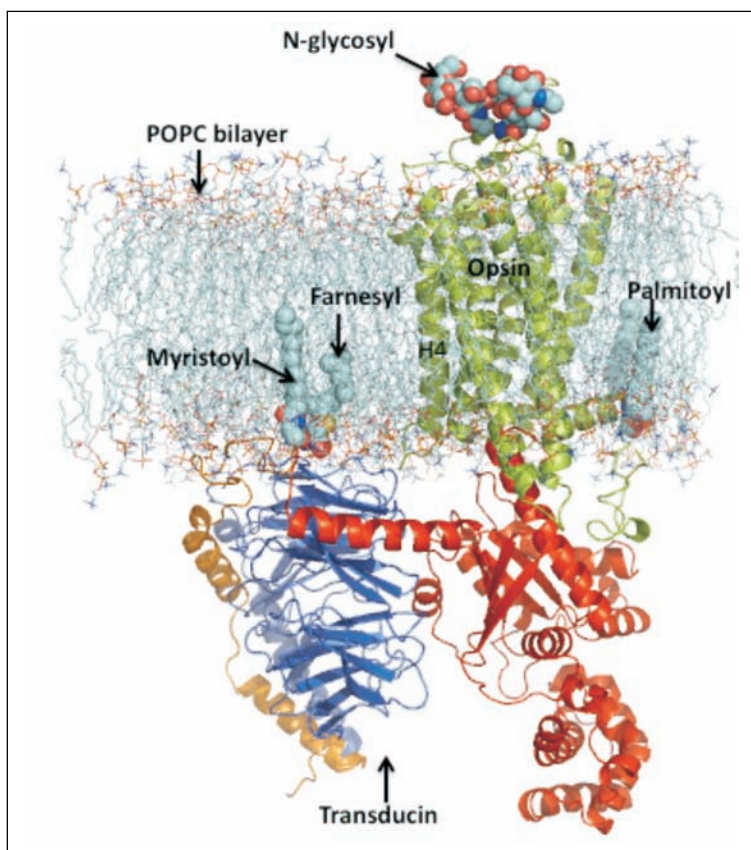
Several of Khorana's colleagues took the technologies to their own independent laboratories and established a worldwide network that studied visual pigments and a number of other GPCRs. Most importantly, as the expression systems and purification methods were refined and improved, it became feasible to contemplate biophysical studies on engineered expressed rhodopsin and other GPCR mutants. By the early 1990s, mutant visual pigments were being probed using Raman spectroscopy, Fourier-transform infrared spectroscopy, flash photolysis and rapid scanning UV-visible spectroscopy to elucidate the physico-chemical mechanism of spectral tuning and to develop the concept of 'functional microdomains' and conformational coupling of independent allosteric domains in receptor activation. Ultimately enough material was available to support a major collaborative effort with Steven O Smith, Stony Brook University to carry out detailed magic-angle-spinning solid state NMR studies of isotopically-labeled rhodopsin mutants regenerated with chemically labeled synthetic chromophores to create artificial pigments.

Another long lasting and notable collaborative effort was initiated with Wayne L Hubbell, UCLA to carry out EPR spectroscopy studies of br and then rhodopsin mutants that were chemically tagged with 'spin labels'. These collaborative studies, carried out during nearly fifteen years beginning in about 1990, were facilitated by a general methodology called 'site-directed spin labeling'. All native reactive but non-essential cysteine residues were removed from rhodopsin and then cysteines (and later pairs of cysteines) were systematically inserted into the protein and then labeled with sulfhydryl-reactive spin label tags. Large collections of mutants were studied in this way and EPR spectra were correlated with the photoactivation states of the mutants. Over time, essentially the entire cytoplasmic surface of rhodopsin was mapped in this way to provide a very accurate picture of the dynamics of receptor conformational changes that were concomitant with receptor activation.



## Helix Movement Model

Perhaps the most significant discovery relevant to the entire GPCR field came from the spin label studies and work in parallel carried out independently in other laboratories using site-directed mutagenesis, spectroscopy and reversible chemical crosslinking approaches. The ‘helix movement model’ of GPCR activation suggested that specific transmembrane helices, in particular helix 6, moved away from the seven-helix bundle upon receptor activation [6]. The change in the cytoplasmic surface conformation then facilitated proton uptake, leading to G protein binding and GDP nucleotide release. Proposed in around 1996, the helix movement model was validated by X-ray crystal structures of opsin, metarhodopsin II and then also by a number of other ligand-activated GPCR structures (*Figure 4*).



**Figure 4.** A molecular snapshot of one possible model of the complex between rhodopsin and transducin in the membrane. The extracellular surface of the receptor is oriented toward the top of the figure. The palmitoyl modifications on opsin anchor the tail of the receptor to the lipid bilayer. Likewise, myristoyl and farnesyl groups on the alpha- and gamma-subunits of transducin keep the proteins at the bilayer on the inside of the cell. Movement of rhodopsin’s transmembrane helix 6 facilitates formation of the complex. This graphic depicts Khorana’s key subject of study during the final two decades of his career.

Khorana's group contributed a formidable list of discoveries and enabled technologies that remain relevant to ongoing studies of GPCR-mediated signaling.

### Future of a Field

In the true spirit of Khorana's scientific ethos, to understand how GPCRs work with chemical precision will require the application of new technologies and ongoing interdisciplinary approaches. Computer simulations based on inputs from various experimental approaches are being used to predict and compare the stabilities of closely related structures in highly dynamic allosteric systems like GPCR signaling complexes. New experimental methods to probe signaling dynamics in native membrane environments or in reconstituted model membrane systems are being employed. And of particular note, unnatural amino acid mutagenesis based on the principle of amber codon suppression is being used to genetically encode amino acid residues with unique bioorthogonal chemical properties into expressed GPCRs. The amber codon method was of particular interest to Khorana in his waning years because it seemed to bring his remarkable career full circle, from the genetic code to structure-activity studies of expressed receptors, and also because his long-term colleague Tom RajBhandary contributed to make it work [7].

Finally, we should say that even in the last two decades of his career, Khorana's group contributed a formidable list of discoveries and enabled technologies that remain relevant to ongoing studies of GPCR-mediated signaling. Despite the phenomenal success, there was never enough time for Gobind to look back during those later years and put his work on signal transduction into proper perspective. Even in his treatise on chemical biology [8] where he reprinted his key manuscripts and put the various earlier phases of his career into perspective, he only mentions work on rhodopsin in passing. It is left for his colleagues and co-workers to carry forward his spirit of innovation, creativity and focus.

### Suggested Reading

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*Address for Correspondence*

David L Farrens  
Department of Biochemistry  
and Molecular Biology  
Oregon Health and Science  
University Portland  
OR 97239, USA  
Email: farrensd@oshu.edu

Thomas P Sakmar  
Head, Laboratory of  
Chemical Biology and Signal  
Transduction  
The Rockefeller University,  
1230 York, Ave., New York,  
NY 10065, USA  
Email:  
sakmar@rockefeller.edu

