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Review

Of ion pumps, sensors and channels — Perspectives on microbial rhodopsins between science and history ☆

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ABSTRACT

We present a historical overview of research on microbial rhodopsins ranging from the 1960s to the present date. Bacteriorhodopsin (BR), the first identified microbial rhodopsin, was discovered in the context of cell and membrane biology and shown to be an outward directed proton transporter. In the 1970s, BR had a big impact on membrane structural research and bioenergetics, that made it to a model for membrane proteins and established it as a probe for the introduction of various biophysical techniques that are widely used today. Halorhodopsin (HR), which supports BR physiologically by transporting negatively charged Cl^- into the cell, is researched within the microbial rhodopsin community since the late 1970s. A few years earlier, the observation of phototactic responses in halobacteria initiated research on what are known today as sensory rhodopsins (SR). The discovery of the light-driven ion channel, channelrhodopsin (ChR), serving as photoreceptors for behavioral responses in green alga has complemented inquiries into this photoreceptor family. Comparing the discovery stories, we show that these followed quite different patterns, albeit the objects of research being very similar. The stories of microbial rhodopsins present a comprehensive perspective on what can nowadays be considered one of nature's paradigms for interactions between organisms and light. Moreover, they illustrate the unfolding of this paradigm within the broader conceptual and instrumental framework of the molecular life sciences. This article is part of a Special Issue entitled: Retinal proteins — You can teach an old dog new tricks.

1. Introduction

In the following, we present three case histories on the discovery and development of research on bacteriorhodopsin, sensory rhodopsins and channelrhodopsin, ranging from the 1960s to the present date (see Box 1 for a brief description of halorhodopsin discovery). These stories not only provide detailed insight into how these today well-known objects of research were shaped at the crossroads of different research fields, but also demonstrate different modes of discovery in the molecular life sciences. Moreover, they allow insight into conceptual and instrumental developments of these sciences in recent decades.

As for the large number of publications in each field, our references cannot remain but incomplete. Therefore, interested readers are referred to specific reviews or historical accounts on each of the topics.

2. Bacteriorhodopsin

2.1. How BR became an object of research (c. 1965–1977)

When electron microscopist Walther Stoeckenius started a project on the membrane structure of the halophilic microbe *Halobacterium* in the late 1960s, he certainly did not have in mind that these studies, through a number of interactions with biochemistry and biophysics, would lead to the formation of a novel influential research field.[1] At a time when the molecular architecture of membranes was still controversial, Stoeckenius had become interested in *Halobacterium*, as the organism was suspected to possess a so-called "subunit membrane". The latter, composed of discrete lipoprotein particles, was considered by some as an alternative to the various bilayer models. Stoeckenius, then working in George Palade's lab at Rockefeller University, showed that a subunit membrane for *Halobacterium* was not supported by his data. He kept working on the organism, now with

Abbreviations: BR, bacteriorhodopsin; ChR, channelrhodopsin; HR, halorhodopsin; PM, purple membrane; SR, sensorhodopsin

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[1] A more detailed account of early BR research is presented in [8]. This paper also contains extensive references of original literature. For Stoeckenius' recollections, see W. Stoeckenius, From membrane structure to bacteriorhodopsin, J. Membrane Biol. 139 (1994) pp. 139–148.

a focus on what were thought to be intracellular "membranes", e.g. those of gas vacuoles. Stoeckenius was not the only researcher interested in using *Halobacterium* to study membrane organization. In 1967, Colin W.F. McClare published on the subject, and the spectroscopic data recorded on a "purple pellet" prepared from cell envelopes could be easily explained by the presence of bacteriorhodopsin in light of later results [1,2]. For a proper understanding of the historical development, however, one should note that back then, the organism's light reactions, retinal proteins or 'ion pumps' were neither a subject of Stoeckenius' early work, nor of McClare's or others'.

When Stoeckenius received a tenured position at the University of San Francisco Medical School, he took the *Halobacterium* membrane project to California and put two young researchers to work on it. The biophysicist, Allen E. Blaurock, had studied retinal photoreceptor membranes in Maurice Wilkin's department at King's College, London, and continued X-ray diffraction and other structural investigations. The biochemist, Dieter Oesterhelt, was on a sabbatical leave after he had finished a thesis in enzymology with Nobel laureate Feodor Lynen in Munich. Initially, Stoeckenius had assigned him the task to de- and re-assemble the halobacterial membrane by varying the salt concentration of the medium. This idea, which may sound simplistic in hindsight, was based on the rationale that lowering of the salt concentration was known to cause membrane disintegration. In fact, this observation had been seen as evidence for a subunit membrane, held together by non-covalent bonds.

When preparing membrane fractions from *Halobacterium*, Oesterhelt also obtained the so-called "purple fraction" or "purple pellet", which had been noted already in the earlier papers, but treated somewhat marginally, more as a contamination on the way to gas vacuole preparation. Oesterhelt observed that on the addition of certain organic solvents, such as acetone or ether, which would separate lipid from protein, the purple fraction turned yellow in the test tube. Few years later, this effect was found to be reversible in the presence of salt and ether, which allowed examining photoreactions of the purple material in the test tube by absorption spectroscopy [3]. Allen Blaurock, who analyzed the prepared purple membrane fraction, observed that it displayed a high degree of molecular order [4]. When a contamination by salt crystals had been ruled out, and it became clear that a membrane protein was responsible both for the crystalline structure within the membrane, as well as the color effect, this protein moved into the focus of attention.

To make a long story short, through a contingent analogy, the idea got ground that this protein might contain retinal. In what would become the founding papers of the bacteriorhodopsin field, Blaurock, Oesterhelt and Stoeckenius argued that the purple membrane (PM) of *Halobacterium* contained a retinal protein in a hexagonal crystalline lattice [5,6].

Although (or maybe because) the project had moved far away from the initial goals, its results fell on fertile grounds. Around 1970, molecular biology had entered what Gunther Stent described as its "academic phase", and many protagonists were looking for new topics [7]. Max Delbrück, for example, was enthusiastic about the purple membrane, as he had been working on a fungal photoreceptor for years [8]. Generally, the acceptance of Singer and Nicolson's "fluid mosaic model" of membranes initiated a transfer of enzymological knowledge to the study of "integral membrane proteins", which were now considered culprits for membrane transport phenomena (on the history of membrane research, see [13]).

Both Stoeckenius and Oesterhelt, the latter had returned to the University of Munich, continued to work on the purple membrane. In 1973, they published simple pH measurements with intact cells as well as spectroscopic and physiological studies, arguing that the function of this "new photoreceptor membrane" was that of a light-dependent proton-transporter, or a "proton pump" [9].

The find of an easily purified, functional membrane protein led to its rapid adoption in bioenergetics. In the middle of the controversy of oxidative phosphorylation, reconstitution of BR with the mitochondrial ATPase in lipid vesicles provided good evidence for Peter Mitchell's "chemiosmotic mechanism", according to which a proton gradient alone was sufficient for ATP formation in the respiratory chain [10]. In the context of bioenergetics and visual rhodopsin research, BR had also been taken up by Soviet researchers, among them Yuri A. Ovchinnikov and Vladimir Skulachev, under the umbrella of a broad research framework funded by the USSR Academy of Sciences [11].

Finally, the exceptional structural properties of the PM had found the attention of Richard Henderson at Cambridge's famed Laboratory of Molecular Biology. He and Nigel Unwin developed a Fourier transform electron microscopic method with unstained specimen of the PM. By averaging Fourier transforms of numerous micrographs taken from different angles, the team was able to construct an electron density map of the PM at a resolution of 6.5 Å [12]. The first structural model of an integral membrane protein, published in *Nature* in 1975, revealed that BR's seven transmembrane helices spanned the membrane from the inside to the outside, thereby providing something like a "pore" rather than the rotating or diffusing carriers that had been surmised to accomplish membrane transport before [13].

In short, a good decade after Stoeckenius had taken up Halobacterium, BR research had become a burgeoning field of what one could call "*molecular membrane biology*", with an output that quickly reached a hundred papers per year after 1975. BR research had ties with established actors, institutions and fields such as enzymology, bioenergetics or structural biology. However, it also became an arena for a younger generation of molecular life scientists in the 1970s, and BR itself a prototype for what one could call a "molecular gaze" on membrane processes. The general concept of BR as a seven helix transmembrane protein with a retinal cofactor that accomplishes proton transport upon illumination was accepted in the second half of the 1970s, and many immediate follow-up questions were addressed. How exactly was the photoisomerization of retinal coupled to proton transfer? Where was the retinal moiety located within the protein? Did BR undergo a photocycle similar to what was known from animal rhodopsins? Was one BR molecule functional, or was oligomerization needed, or how could BR function be related to the concentration or the electrical term of the membrane potential? Two reviews presented a comprehensive overview on both the answers to these questions, as well as open issues. Thus, the late 1970s can be considered as a sensible end of the early phase of BR research ([14,15, see [16]]).

2.2. BR as a model system of membrane research and a technical object (1980–1990s)

Roughly by the turn of the decade the field began to take a new turn. From an object of immediate scientific interest, BR increasingly developed into a model system to study membrane transport and protein structure, as well as into a technical object. In the history of the molecular life sciences, such developments have also been described for e.g. phage, viruses or DNA modifying enzymes. Whereas in the early phase of molecular biology, these were genuine objects of inquiry, they became tools for research with the beginning of recombinant DNA or models to understand the biology of more complex systems after 1970 [7,17]. A good example for a very early use of BR as a technical object in a wider sense (not necessarily linked to biotechnology as an economic activity) is provided by Racker and Stoeckenius' 1974 experiments. Reconstituted in a lipid vesicle, BR figured as a mechanistically understandable component of an organism, which could be pieced together with other proteins and lipids in a functional, chimeric arrangement. This latter then served for a more detailed examination of metabolic processes [18]. Uses of BR as a "module", an organismic component to be manipulated and transferred between different experimental contexts thus provided one facet of its development towards a

technical object. Yet, from the 1980s BR also became a model system. As the use of manifold biophysical and biochemical techniques were pioneered with BR (e.g. FTIR spectroscopy, solid state NMR, ultra fast spectroscopy, see below), it became a probe to establish methods that would afterwards be used more widely in the molecular life sciences, and a model to understand membrane transport and light-energy-conversion in general. Finally, one should not forget that the BR community formed a global social arena that assembled researchers from different disciplines around one scientific object. Many of these researchers would then continue their work on e.g. SR, HR, ChR or visual rhodopsins, or cross-fertilize with other fields such as metagenomics ([19], see Conclusion section).

In the following, we present some examples that illustrate these developments in the context of understanding details of BR's structure–function-relationships.

As to the high number of papers in this field after the mid-1970s, this part has to remain less focused on individual persons than before, and the examples cited cannot remain but partial.

2.3. Novel means for structure–function-studies: the advent of molecular genetics in BR research

Roughly the first decade of BR research was carried out without any knowledge or methods of molecular genetics. As the field rose in the wake of molecular biology, this is a curious situation, and worth a thought experiment from today's perspective. Imagine the limitations of working on a membrane protein without any knowledge about the organism's genes, or techniques of recombinant DNA.

To be sure, BR researchers had not been the first to work with *Halobacterium*. Yet, those microbiologists dealing with halophiles had not been involved in post-War development of bacterial genetics either (see e.g. [19,20]). Thus, the transfer of molecular genetic methods to *Halobacterium* occurred only around 1980, a key figure being H. Gobind Khorana of the MIT, renowned for his works on the problem of the genetic code in the 1960s, or for *in vitro* syntheses of functional genetic elements.[2] Retrospectively, Khorana explained his shift from molecular genetics to membranes, and especially BR, with his hope that this "experimental system" might ultimately bring him towards neurobiology and signal transduction, thus underlining BR's role as a model [21].[3]

In the case of BR, the DNA work was preceded by the analysis of the protein's primary sequence. Following Fred Sanger's and Per Edman's methods of iterative modification and degradation of amino acids, protein sequencing had become a routine activity in the 1970s [7]. Membrane proteins, however, proved recalcitrant to these methods due to their hydrophobicity. The task was tackled in the late 1970s both by Khorana's group and a Soviet team, led by academician and high rank functionary, Yuri A. Ovchinnikov, and by Nadik Abdullaev, from the Shemyakin-Institute of Bioorganic Chemistry at Moscow. The Soviet sequence was published first, including a beautiful model of BR's membrane integration, yet the paper lacked extensive methodical information [22]. The American sequence, that had been established in collaboration with mass spectrometry pioneer Klaus Biemann, also at MIT, came out shortly afterwards, displaying several differences [23].

Using a cDNA approach, Khorana's group then "fished", subcloned and sequenced the BR gene by help of a radioactive RNA probe in 1981 [24]. In the following years, the methodic arsenal of recombinant DNA became used to scrutinize BR structure and function. Khorana's group established a 'synthetic BR gene' in a cassette, that allowed to exchange DNA fragments through restriction digest, the construction of truncated BR variants and the introduction of site specific mutations in order to characterize functional amino acid residues [21]. Thus, the molecular structure of BR could be manipulated on a different scale, up to the point that fragments were used to re-assemble a functional protein. The protein's transmembrane domains were systematically screened for function by mutations or for their relative spatial orientation by cross-linking. In a sense, BR became akin to a substance in an organic chemistry lab, which could be synthesized, modified and studied under all kinds of different conditions. For that reason, BR pioneered a nowadays ubiquitous way of studying membrane proteins.

However, when looking back roughly thirty years, one should not forget what techniques researchers did not have in hand at the time. PCR, for example, was only invented in 1984, but also protocols for re-introducing recombinant DNA back into *Halobacterium* were unknown. Genetic strategies that were applied to *E. coli* since the 1950s, became only available after 1990, e.g. through phage transduction or polyethylene-glycol assisted transformation, using shuttle vectors displaying specific resistance markers [20].

The way methodical limitations of molecular genetics have affected mutation studies of BR function can be illustrated by comparing a number of papers from the 1980s, in which several aspartic acid residues were analyzed, the protonation state of which was known to change during BR's photocycle from Fourier transform infrared studies (FTIR) ([25,26], see Section 2.4).

Khorana's group introduced site-specific mutations of the aspartates *in vitro* [27]. As BR expressed heterologously in *E. coli* was not folded correctly, the purified protein had to be refolded in the presence of retinal, and reconstituted in proteoliposomes [21]. Studies from Oesterhelt's department at the MPI of Biochemistry at Martinsried took another point of departure for analyses of these aspartate residues. BR genes from phenotypically detected phototrophic-negative mutants were screened for point mutations [28,29]. From both approaches, aspartic acid residues 85 and 96 could be confirmed as crucial to BR's proton transport. Whereas Asp-85 is considered the proton acceptor from the Schiff base, Asp-96 serves to re-protonate this latter. Comparing these mutation-based studies to spectroscopic approaches such as FTIR, one should also note that whereas the former may have disturbed protein structure to an unknown degree, the latter were performed on a protein close to the native state. The two approaches were combined by measuring FTIR spectra of BR variants with mutated aspartate residues [28,30].

The transformation of BR into a "technical object" is epitomized in plans and engineering attempts to use the purple membrane in photosensitive films, e.g. for optical data storage. Whereas such schemes seem to have surfaced already in the early to mid-1980s in several countries, the availability of mutants with modified photocycles, such as BR D96N, intensified interest in the early 1990s [19,31].

2.4. Light, ion gradients and molecular dynamics — BR as a probe for spectroscopy and electrophysiology

In the late 1970s, it was accepted that BR underwent a photocycle upon illumination, leading from the dark state BR, absorbing at 568 nm, through the K (590 nm), M (412 nm) and N states back to the dark state [32,33].

Resonance Raman (RR-) spectroscopy lent itself to address the issue of how the states of the photocycle could be mapped on surmised steps of proton transfer across the membrane, as this method allowed to detect differing protonation states of certain chemical groups. Already in 1974, Aaron Lewis, in cooperation with Stoeckenius' group, had shown that the retinylidene lysine of the Schiff base in BR was protonated in the 568 nm state, whereas the proton was absent in the 412 nm intermediate [34]. Improving RR-spectroscopy through low temperature measurements as well as a spatial separation of the

[2] For brevity's sake, Carl Woese's postulate of the Archaea as a taxonomic group separate to Bacteria have to be omitted. As Woese used rRNA sequencing for his analyses, archaeal research from this tradition also contributed to the adaptation of molecular genetic methods to study halophiles in the 1980s. See e.g. A. Oren, Halophilic microorganisms and their environments. Dordrecht: Kluwer Academic Publishers; 2002.

[3] This edited collection contains many important publications of Khorana's group.

pump and probe laser beams, Richard Mathies' group at Berkeley was able to obtain information on BR's early photoproduct, the K state. It was shown that after the *cis-trans* isomerization of retinal, the protein's chromophore was present in a distorted conformation [35].

In the course of the 1980s, Fourier transform infrared (FTIR) spectroscopy became used to scrutinize changes of protonation state in greater detail. Among others, Laura Eisenstein from Urbana-Champaign and Koji Nakanishi's group at Columbia pioneered the technique by showing e.g. the chromophore distortion, which was known from RR analyses [36]. After carboxyl groups had been found as suspected sites of action in the light-induced proton pumping mechanism, BR with specific ^{13}C labeled amino acids was used to reveal these as four aspartic acid residues [25,26]. As has been discussed above, two of these were later identified by mutation studies as the proton acceptor Asp-85 and Asp-96, active in re-protonation (see Section 2.2). In the late 1980s, the ^{13}C labeling technique was also used for magic angle sample spinning NMR studies of BR, providing further insight into protonation states of aspartic acid residues (e.g. [37]).

Among the plethora of biophysical methods applied to BR, revealing ever more intricate glimpses of structural changes along its photocycle, one could also mention neutron diffraction with deuterated samples, addressing helical movements in the chromophore's vicinity [38]. Finally, site-specific mutagenesis allowed to specifically attach thiol-reactive spin labels to the protein. Here a collaboration between Khorana and the electron spin resonance (ESR) group of Wayne Hubbell at the University of California at Los Angeles allowed to probe the microenvironment of labeled positions with respect to mobility, accessibility to water, oxygen etc. [39]. As in many other cases, these studies preceded the use of the spin-labeling technique to study eukaryotic rhodopsins.

Yet, for an understanding of BR's functionality, electrophysiological studies on black-lipid membranes were very important and insightful as well. These studies started in the mid-seventies [40–42], confirming and detailing the light-activated proton pumping function of BR. As above, one of the big advantages of bacteriorhodopsin was its ready availability and its unusual stability at room temperature which was necessary for long term experiments. This situation changed with the tremendous success of gene technology, when proteins could be investigated without the need of protein purification, simply by expressing a protein in the cellular system of choice. Even though a lot was already known about BR in the 1990s, the exact voltage dependence of proton pumping remained unclear. Georg Nagel and Ernst Bamberg decided to study BR in the membrane of an animal cell, the oocyte of *Xenopus laevis*. This host system allowed the exact determination of the voltage dependence of light-activated proton pumping in a wide voltage range [43]. Later, the Cl^--pump halorhodopsin, channelrhodopsin and the phototaxis-mediating sensory rhodopsins were also studied successfully in oocytes (see Section 4.2, [44]. However, the functionality of individual residues along the proton or chloride transport pathways with regard to pump efficiency and strength at high electrochemical load is still unresolved.

If one asks as to why BR occupied a pioneering position for the use of molecular biophysical methods, several factors need to be mentioned. First and foremost, as for the biochemical studies of the 1970s, BR provided the rare example of a stable membrane protein that could be purified in large quantities. Second, the fact that it could be activated by light, rather than by pipetting in substrates as in the case of other membrane transporters, meant that intermediates of its functional cycle could be controlled precisely by specific illumination. Finally, when techniques for selective manipulation within the protein through mutagenesis or isotope labeling were available, it became possible to pinpoint spectroscopic changes both to specific sites of the protein and to steps in the photocycle. Thus, a model mapping the dynamics of BR both in time and in space became conceivable — ultimately to be imagined as a cartoon or movie. Regarding such models, however, we need to take into account the development of structural research.

2.5. BR and methods to membrane protein structure

The path to further advances in BR structure turned out more crooked than one might think with the rapid successes until 1975 in mind. Therefore, in order to follow the development of BR structural research, it might again be helpful to remind the reader of what was *not* known in the late 1970s. First, the resolution of Henderson and Unwin's electron crystallographic model allowed to assign BR's helices, but not their amino acid residues. The position of the retinal co-factor was unknown as well. Second, and trivially, only after the amino acid sequence of the protein was known could scientists start to map the primary structure on the spatial model of BR in the membrane. A modeling approach fit the known transmembrane stretches into the seven helical elements of the 1975 structure on the basis of matches with connecting linkers [45].

In Oesterhelt's group, then at the University of Würzburg, post-doc Hartmut Michel was studying membrane bioenergetics of BR, when he observed accidental crystal formation of BR. This led him to attempts to obtain BR crystals of sufficient quality for structure determination by X-ray crystallography. These studies were carried out first in collaboration with Richard Henderson at Cambridge, and later with Robert Huber's department at the MPI of Biochemistry [46]. Although BR proved recalcitrant to structure determination by X-ray crystallography at the time, the protocols developed to crystallize BR in various detergents were crucial for the first successful X-ray structure determination of a membrane protein complex, the photosynthetic reaction center of *Rhodopseudomonas viridis* [47,48].

Meanwhile, the electron microscopic approach to BR structure was carried forward after 1984 in cooperation between Richard Henderson and the electron microscopy department at the Fritz-Haber-Institut of the MPG at Berlin. Using a Helium-cooled cryo-objective lens, and relying on the instrumental expertise of Ernst Ruska's former group, the project gave rise to the first significantly improved structural model of BR in 1990, 15 years after the Henderson and Unwin's seminal publication. With a near-atomic resolution down to 3.5 Å in the direction parallel to the membrane plane, the authors could resolve many molecular features, among others the β-ionone ring of the retinal chromophore (Fig. 1). They also located the crucial residues Asp-85 and Asp-96 on the proton's pathway from the intracellular toward the Schiff base and the extracellular surface. The data from cryo-electron microscopy were also used in ensuing X-ray structure studies of the 1990s ([49], see also [50]).

The problems of structure determination by X-ray crystallography were only resolved in the late 1990s, when so-called lipidic cubic phases were introduced as a novel approach for the crystallization of membrane proteins. Ehud Landau and Jürg Rosenbusch from the Biozentrum Basel used BR to demonstrate that well-ordered 3D crystals could be obtained under these conditions [51]. The advantage of this method was that the curved, continuous lipid bilayers of the cubic phases allowed both diffusion of the protein and the formation of crystal contacts. As the X-ray data of BR in a cubic lipid phase showed the same crystal lattice as for native PM, it was concluded that the approach could be used to obtain so-called type I crystals of membrane proteins, which are formed by stacking of membraneous 2D crystals [52]. Before, the majority of membrane proteins had been crystallized as so-called type II crystals in the presence of detergents, with polar surface parts protruding out of the micelle forming the crystal contacts.

On the basis of this new approach, the first high-resolution X-ray structure of BR was obtained [53]. Later, however, it became clear that these structures had been affected by crystal twinning, which required a refinement of the interpretation [54]. Another crystallization approach, based on heterogeneous nucleation (leading to a form of type II crystals), allowed to analyze the trimeric structure of BR as well as lipid–protein-interactions [55].

It is probably fair to say that by the turn of the millennium, roughly thirty years after BR had been isolated, a fairly comprehensive and

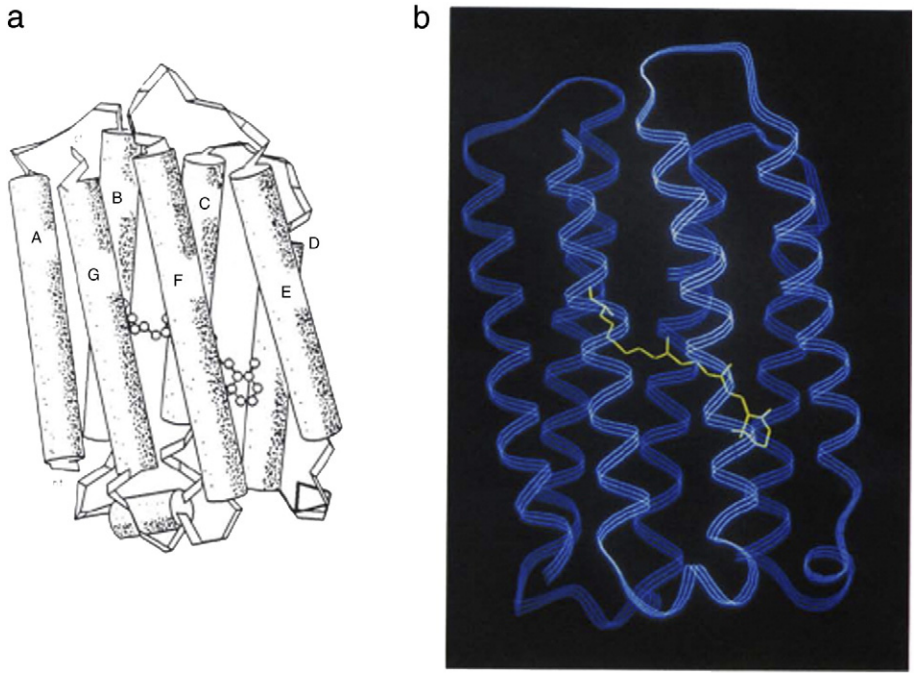

Fig. 1. Cryo-EM model of BR, 1990 (see Section 2.5 for details). From R. Henderson, J.M. Baldwin, T.A. Ceska, F. Zemlin, E. Beckmann, K.H. Downing, Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy, Journal of Molecular Biology, Volume 213, Issue 4, 20 June 1990, 899–929, Fig. 14. Reproduced with permission.

accepted model of how this "molecular pump" coupled the absorption of light energy to proton transport across the membrane existed. This might also be reflected in the fact that yearly publication activity indexed with keywords "BR" or "purple membrane" has begun to fall after peaking in 1997, in contrast to growth or permanence in the two and a half decades before (as determined by ISI Web of Knowledge, February 2013).

The essence of this "mechanistic narrative" of BR function is summarized briefly in a review of Janos K. Lanyi as follows:

> "The wealth of spectroscopic data, together with the information from low resolution electron diffraction maps, pointed to a well-defined transport mechanism. There was general agreement over the main steps (…). After photoisomerization of the retinal from all-*trans* to 13-*cis*, 15-*anti*, the Schiff base proton is transferred to Asp-85 located on the extracellular side, and a proton is then released to the bulk from a site near the surface. (…) Reprotonation of the Schiff base is from Asp-96 located on the cytoplasmic side, aided by tilts of the cytoplasmic ends of helices F and G that were thought to result in increased hydration of this region. Reprotonation of the Schiff base through a proposed chain of water molecules is followed by reprotonation of Asp-96 from the cytoplasmic surface and reisomerization of the retinal to all-*trans*. Finally, transfer of a proton from Asp-85 to the vacant proton release site completes the cycle." [56].

This does naturally not imply that everything about BR and its role in membrane physiology is clear. The simple observation, for example, that upon illumination of intact cells the acidification of the medium (the physiological hallmark of BR-mediated proton export) is preceded by a brief increase of pH (alcalinization) has found competing explanations, but no experimental resolution (see e.g. [14])

3. Sensory rhodopsins

Living species have to respond towards light in order to seek favorable conditions or to avoid harmful radiation. Consequently, Nature has developed appropriate photoreceptors which mediate the relationship between the incoming light and the corresponding cellular answer. For example, phototropins that mediate phototropism in higher plants contain a FMN chromophore, while archaeal sensory rhodopsins utilize *all-trans* retinal as light receptor. The downstream signal transduction chains include inter alia G-protein or two-component systems related pathways. One of the well-studied systems is the phototaxis signaling chain found in Archaea which shows distinct homologies to the chemotactic signaling network of enteric bacteria [57–59].

First observations on phototactic behavior of bacteria have already been made in the late nineteenth century by Theodor Wilhelm Engelmann. Using bacteria from the Rhine River (probably *Chromatium* species) he observed their accumulation at spectral regions in the infrared and between 510 nm and 570 nm. These results certainly showed for the first time taxis behavior of photosynthetic bacteria. T.W. Engelmann's scientific work and life has recently been appreciated by G. Drews [60]. Experiments on light and wavelength dependent orientation of bacteria were only resumed some 60 years later when R.K. Clayton (1953) studied phototaxis of *Rhodospirillum rubrum* [61]. Another two decades later studies on phototaxis were taken up on halophilic bacteria. The new interest was stimulated by the above mentioned finding that the archeon *Halobacterium salinarum* contains a rhodopsin–like pigment, namely bacteriorhodopsin. It soon became evident that these phototrophic microbes could serve as a model system to study phototaxis because signaling was not hampered by other complex sensory pathways (reviewed in [62]).

3.1. Discovery of sensory rhodopsins

In 1975 Eilo Hildebrand and Norbert Dencher published a paper in which they spectroscopically identified two photosystems, PS 565 and PS 370, in *H. salinarum* [63]. They concluded from their data that PS 565 enables the bacteria to assemble in a spectral region optimal for the function of the light driven proton pump bacteriorhodopsin. On the other hand at 370 nm an avoidance response of the bacteria was observed, preventing them from potentially damaging UV-light. By

microscopically observing single bacteria of the *H. salinarum* mutant strain R1 at different wavelengths, Hildebrand and Dencher [63] obtained action spectra proving the existence of two photosystems. It seemed that PS 565 and PS 370 were independent from each other [64], but it was evident that both photosystems required retinal [64–66].

In further studies John and Elena Spudich showed in 1982 that mutants lacking both ion pumps bacteriorhodopsin and halorhodopsin (see Box 1 for a short description of the halorhodopsin discovery) still possess normal color-discriminating phototaxis, indicating that at least one individual photoreceptor must be present [67]. At the same time Roberto Bogomolni (who had worked on BR in Stoeckenius's lab since the early 70s) and John Spudich identified a rhodopsin-like pigment in *H. salinarum*, which they named sR (slow rhodopsin-like pigment). The photostationary state of sR absorbed around 585 nm and 373 nm [68]. Based on only one photoreceptor, the same authors proposed subsequently a mechanism of color discrimination of *H. salinarum* [69]. The quintessence of the model is that the photophilic response is triggered by absorption of one photon at the long wavelength maximum of 587 nm. On the other hand the repellent signal is generated in a two photon process by producing an intermediate absorbing at 373 nm (S_{373}) through background illumination with red light. The second photon excites S_{373}, thereby triggering the photophobic response. This model of a photoreceptor displaying dual functionality is now well accepted. In the same paper, the authors renamed slow rhodopsin as "sensory rhodopsin" (SR) in order to emphasize its sensory function. The discrepancy between these spectroscopic data and the physiological results of Hildebrand and Dencher [63] was explained by the presence of bacteriorhodpsin and halorhodopsin in the experimental set up of the latter authors. The much higher sensitivity of the photophobic response was attributed to downstream amplification steps [69].

Box 1
Halorhodopsin.

An ion pump with inverted transport activity was first reported by A. Matsuno-Yagi and Y. Mukohata. [147]. The authors, working with a differently pigmented (red) strain of *Halobacterium*, postulated the "presence of a bacteriorhodopsin different from that in the purple membrane" (ibid., p. 237). After a subsequent link of this protein to the phenomenon of light-dependent sodium export in *Halobacterium* cells[148] and thus its function as a Na^+-pump, the new retinal protein named halorhodopsin (HR) was finally recognized as a chloride importer by Brigitte Schobert and Janos K. Lanyi [149]. The spectroscopic properties of HR are quite similar to those of BR with the exception of a missing M-intermediate [150]. Complementary to BR whose absorption maximum can be bathochromically shifted by 20 nm by acidification of the Schiff base counter ion, in HR from *H. salinarium* the absorption is shifted from 565 to 578 nm by removal of Cl^- and even to 600 nm in *N. pharaonis* (NpHR) [151]. A crystal structure of HR revealed that the Cl^--binding site is close to the protonated Schiff base, where a Thr residue replaces the corresponding Asp in BR (Asp85) [152]. Due to the superb expression of NpHR in neurons, this variant (together with BR) is now widely used for cellular hyperpolarization and inactivation in the neurosciences (see Section 4 on channelrhodopsins).
The mechanism of chloride transport has been in the focus of quite a number of publications (see [153,154] for reviews). This intriguing question was related to the different modes of actions of ion pumps and sensors although their structures were strikingly similar. A consensus model was put forward explaining the different outputs by an isomerization/switch/transfer (IST) model [155].

In 1985, a Japanese team from Hokkaido University under the guidance of Y. Kobatake published a paper on a photophobic system with a maximum of the photorepellent response at around 480 nm [70]. In a subsequent paper a thorough spectroscopic analysis revealed a slow photocycle with at least three intermediates [71]. The authors named this new pigment phoborhodopsin. Similar results were subsequently obtained by Wolff et al., who used a *H. salinarum* mutant lacking all other retinylidene proteins, i.e. bacteriorhodopsin, halorhodopsin, and sensory rhodopsin [72]. Marwan and Oesterhelt published a paper with similar conclusions and named the photoreceptor P_{480} [73]. Although it was obvious that the same photoreceptor had been identified by various groups, the former authors proposed to name this pigment sensory rhodopsin II (SR-II) and to rename Spudich's and Bogomolni's sensory rhodopsin into SR-I. This nomenclature has now generally been accepted.

The next step in the elucidation of structural and functional properties of sensory rhodopsins was their purification and amino acid sequence determination which occurred long after the functional studies due to the small abundance in the cells. It is interesting to note that in the case of BR, in contrast to SR, the protein was first isolated, followed by the determination of its function (see Conclusion section).

Generally, the photoreceptors were first isolated and purified from their natural host. This allowed the determination of a partial amino acid sequence. With this information, the corresponding gene was isolated and sequenced. SR-I was purified and spectroscopically characterized by Schegk and Oesterhelt [74] in 1988, who a year later determined also the primary amino acid sequence of SR-I [75]. It turned out that SR-I possesses 14% homology to the ion pumps bacteriorhodopsin and halorhodopsin. Interestingly, an Asp residue, which mediates in bacteriorhodopsin the reprotonation of the Schiff base (D96), is replaced in SR-I by a tyrosine (Y87). This observation was originally taken as an explanation for the slow cycling properties of this pigment, although it is now clear that other sites are also involved in the timing of the photocycle [76].

The determination of the primary structure of SR-II was hampered by the fact that its structural and functional stability was quite poor under the conditions of purification, especially in the presence of detergents which are necessary to solubilize the membrane-inserted protein. In her thesis, Birgit Scharf in Martin Engelhard's group followed up an observation of Bivin and Stoeckenius [77], who described in addition to halorhodopsin an SR-II like pigment expressed in *Natronmonas pharaonis*, which they isolated from alkaline salt lakes at Wadi Natrun in Lower Egypt. Originally, these bacteria had been characterized by Soliman and Trüper [78]. Comparing the spectroscopic properties of SR-II from *H. salinarum* (HsSRII) and *N. pharaonis* (NpSRII) she concluded on the functional similarity of the two pigments [79]. It was also evident that this homolog has much higher stability under conditions of low salt and the presence of detergents. Additionally, NpSRII was also stable at wide ranges of pH and temperature. Low temperature studies on the photocycle of NpSRII by Hirayama et al. [80] and FTIR experiments [81] revealed an M-like intermediate like that had also been found for bacteriorhodopsin. The benign properties of NpSRII made it possible to determine the primary sequence [82] (the amino acid sequence of HsSRII was later determined by Zhang et al. [83]). With these primary sequences of archaeal retinal proteins at hand, the function of amino acids at position 85 and 96 was deduced: proton pumps are characterized by Asp-85 and Asp-96; chloride pumps by Thr-85 and Ala-96; and sensors by Asp-85 and Tyr-96 or Phe-96 [81]. With more sequences of microbial rhodopsins becoming available, this simple classification turned out to be not completely generalizable.

3.2. Discovery of halobacterial transducers

Research on sensory rhodopsins raised the question how the signal transmission from the receptor to the flagellar motor is organized in *H. salinarum*. Quite early it was demonstrated that methylation of membrane proteins is involved in the photo- and chemotactic behavior of *H. salinarum*, which suggested a similar signal transduction network as

in *E. coli* [84,85]. These methylation sites have been identified in a recent study [86]. A sequence determination of the gene of SRI and SRII revealed upstream of *sopI* and *sopII* loci open reading frames corresponding to halobacterial transducers of rhodopsin (Htr) [82,83,87]. Genomic sequencing revealed 18 transducers [88], which are part of a protein interaction network of a taxis signal transduction system in *H. salinarum* [89]. It turned out that Htr's consist of two transmembrane helices connected by an extracellular tight loop (in HsHtrII the loop is replaced by a (probably serine) receptor domain). The general structure of the transducer molecules is quite similar to that of the chemoreceptors. The transmembrane domain is followed by a cytoplasmic domain, which contains the sub-domaINS for signal transfer to the histidine kinase CheA and for adaptation by reversible methylation of Glu residues. The long rod-shaped cytoplasmic domains are arranged in a four-helix bundle, the X-ray structure of which has been resolved for the serine chemoreceptor [90] (for a review see [91]). Two HAMP domains (present in Histidine kinases, Adenylate cyclases, Methyl accepting proteins and Phosphatases), important structural elements involved in signal transfer, were identified proximal to the membrane domain (for an analysis of HAMP domains see [92,93].

3.3. Structure of receptor transducer complex

Two developments were important for the structural analysis of SRII and its transducer complex. The expression of retinal proteins in *E. coli* [94,95] enabled the production of large amounts of highly purified proteins. Another advancement that had already affected BR research was the introduction of crystallization in cubic phases by Landau and Rosenbusch [51] which led to the structure determination of NpSRII by two groups [96,97]. An overlay of the backbone traces of the three known structures bacteriorhodopsin, halorhodopsin and SRII (the structure of SRI has not been solved by now) discloses only very minor differences. Apparently, this scaffold serves as template for different functionalities which are triggered by similar primary photochemistry.

Using electron paramagnetic resonance (EPR), a topological model of the receptor transducer (NpSRII/NpHtrII; a shortened transducer was used in these experiments) was proposed [98], which could later be confirmed by a crystallographic study [99]. The expected dimer of the complex is formed by a crystallographic two-fold rotation axis, which is located in the middle of four transmembrane helices consisting of dimeric transmembrane helices TM1 and TM2 of the transducer. The transmembrane helices F and G of the receptor are in contact with the helices of the transducer (see Fig. 2).

The interactions between the NpSRII and NpHtrII are mainly of hydrophobic nature with important anchor points consisting of specific hydrogen bonds. Another observation was that electron densities for the cytoplasmic fragment of the transducer were missing, either due to problems of crystal packing or due to dynamic properties of this part of NpHtrII (see below).

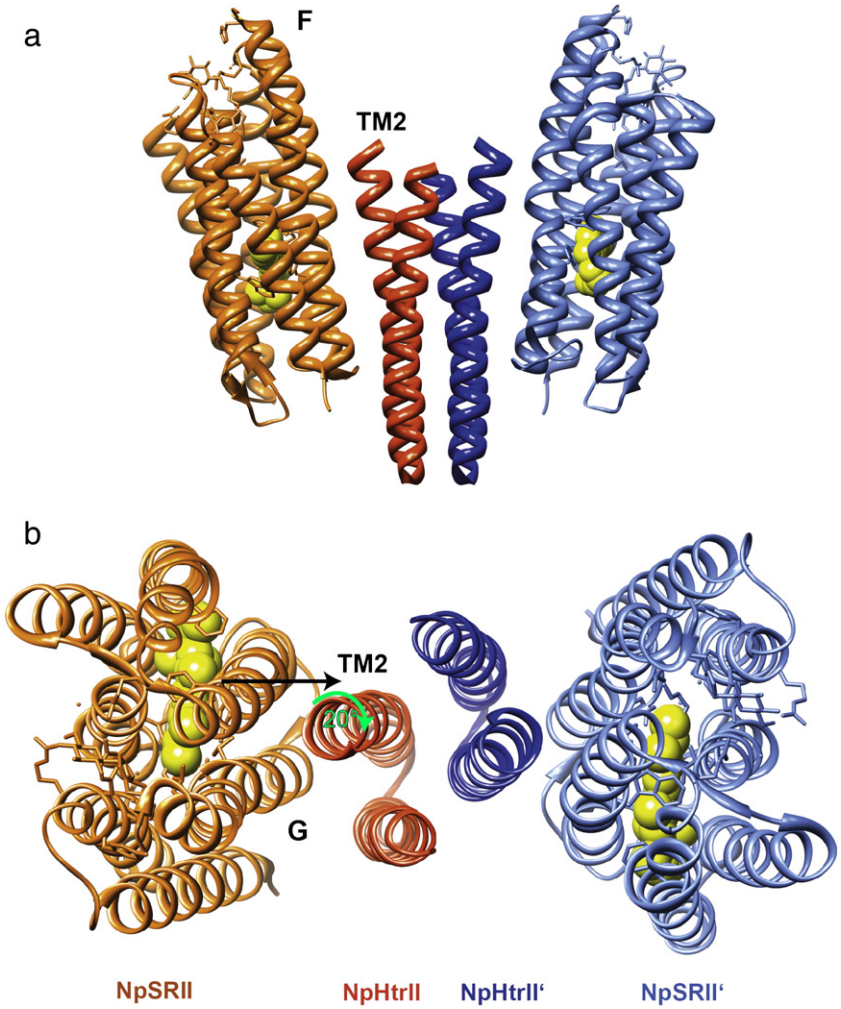

Fig. 2. Transducer activation by photoactivated NpSRII. a) Crystal structure (PDB 1H2S) of dimeric NpSRII/NpHtrII complex; view from the membrane. b) Crystal structure of dimeric NpSRII/NpHtrII complex; cytoplasmic view. The arrows indicate the flab-like movement of helix F (black arrow) and the resulting rotation of TM2 by about 20° (green arrow). Color coding: NpSRII, orange; NpHtrII, dark orange (for the shortened cytoplasmic domain no electron density was observed). NpSRII′ and NpHtrII' are depicted in blue and dark blue, respectively; retinal: yellow.

3.4. Signal transfer

The activation of the photoreceptors by light is quite similar to that of bacteriorhodopsin displaying, all the canonical intermediates. Indeed, three mutations in bacteriorhodopsin convert its function into an SRII-like phototaxis receptor with robust phototaxis responses [100]. For an understanding of signal transfer from the receptor to the transducer, a step of the photocycle which occurs between the M1 and M2 transition is relevant. Electron spin resonance (EPR) data [98,101] on selectively spin labeled samples were interpreted as a flap-like outwardly directed movement of the cytoplasmic half of helix F, similar to the motion of the corresponding helix in BR which has been demonstrated by various techniques including X-ray structural analysis [54]. This movement of helix F seems a general feature of the activation of microbial rhodopsins and GPCR's like e.g. rhodopsin [102].

The rigid body movement of helix F carries along TM2 of the transducer translating it into a rotation of about 20° [98]; a conformational change that has also been deduced from an X-ray analysis of the M-state of the receptor/transducer complex [103]. How this small signal travels almost 26 nm along the rod shaped cytoplasmic domain is still a question of debate. A crucial role is attributed to the membrane proximal HAMP domains. Wang et al. have analyzed the HAMP domain signal relay mechanism in an NpSRII/NpHtrII complex [104]. The two HAMP domains of NpHtrII display opposite conformational changes, which correspond to opposite output signals. In another work it was proposed that the relatively facile modulation of the HAMP domain dynamics exerted by environmental input provides the means for how small changes in TM2 can trigger the physiological response [105]. In any case it seems that the formation of trimers of dimers as observed for chemotaxis receptors (reviewed in [106]) play an important role in signal transfer and amplification also for archaeal photoreceptors.

4. Channelrhodopsin: "BACK TO THE BASIC"[4]

The idea to let channelrhodopsins appear as a separate chapter in the history of microbial rhodopsins is justified by the fact that the light-gated ion channel, Channelrhodopsin (ChR), displays a novel function of a microbial rhodopsin that is now used by more than 1000 research laboratories to probe neural circuits with light [107]. These applications study the molecular events during the induction of synaptic plasticity and map long-range functional connections from one side of the brain to the other, as well as the spatial location of inputs on the dendritic tree of individual neurons [108]. The many applications of ChR in research have been summarized in several recent reviews [109,110]. This article focuses on the history of ChR and presents a biophysical perspective on this remarkable class of proteins.

4.1. Ancient physiological studies

The discovery of ChR is based on work by numerous researchers who characterized the swimming behavior and light responses of motile microalgae over at least 140 years. We can only briefly review the first 100 years of photobiological research and mention but a few researchers that have built the basis on which ChR was discovered many years later. Early studies on green microalgae root back to the German naturalist Treviranus [111]. A quite detailed description of the behavioral responses of microalgae was presented in German by Andrej S. Famintzin, a scientist from St. Petersburg University [112]. The microalgae species that have been studied most in Europe by German botanists are *Euglena gracilis* and *Chlamydomonas reinhardtii*.

Chlamydomonas is an oval-shaped photosynthetic alga with an equatorial diameter of approximately 8 μm and two flagella that beat in a manner similar to a swimmer's breaststroke. *Chlamydomonas* flagella are considered the most powerful models for investigating ciliar defects in humans (ciliopathies) [113]. During helical swimming, the 1-μm orange eye advances the flagellar beating plane by approximately 23° to ensure the timing required after light absorption to transport signals from the eye to the flagella. The light sensitivity of this eye was first reported by Samuel O. Mast, a scientist at John Hopkins University [114]. Per Halldal, a Norwegian scientist at Stanford, found that behavioral responses depend on Mg^{2+} and Ca^{2+} [115]. Jeffrey A. Schmidt and Roger Eckert at Stanford correlated Ca^{2+} influx with changes in the flagellar beat frequency and showed that this response is graded over a wide range of light intensities [116].

The next important contribution came from Oleg Sineshchekov, originally from Moscow State University. He recorded electrical light responses from the green alga *Haematococcus pluvialis* and other algal species with a jelly cell wall, which are known in the medical sciences for producing the antioxidant Astaxanthine. Sineshchekov used a suction pipette technique that had been simultaneously employed by Denis Baylor to record photocurrents from bovine photoreceptor cells [117]. However, at that time information about the nature of the photoreceptor was unavailable.

Two years later, in 1980, Kenneth W. Foster, a former graduate student in physics of Max Delbrück, reanalyzed published action spectra for phototactic movement (the movement toward or away from light) and postulated that the sensory photoreceptor is rhodopsin [118]. Foster substantiated his theory by restoring behavioral light responses in blind algae through complementation with retinal and retinal analogues [119]. Despite these groundbreaking results, the field of photobiology did not truly appreciate this explanation and progress on algal rhodopsins remained slow. Years later, Hartmann Harz in Peter Hegemann's group recorded photocurrents from *Chlamydomonas* by applying Sineshchekov's technique to a *Chlamydomonas* cell wall-deficient mutant. They recorded action spectra that led them to propose that photocurrents are evoked by a rhodopsin that most likely also mediates phototaxis and phobic responses. Based on the extremely fast appearance of the photoreceptor current, the authors postulated that the photoreceptor is intimately linked to an ion channel, forming a single protein complex [120,121].

4.2. Proof of principle

After many years of diligent work, the Hegemann group was unable to purify the photoreceptors biochemically due to the instability and heterogeneity of the proteins. In 2001 in parallel, Suneel Kateriya of Hegemann's group [122] and Kwang-Hang Jung in John Spudich's group [123] identified novel DNA sequences that encode large microbial type rhodopsins. To verify the concept of a light-gated channel, P. Hegemann started to collaborate with Georg Nagel, who expressed the two rhodopsin DNAs in *Xenopus* oocytes in order to explore their function by two electrode voltage clamp measurements. Nagel was a well-known electrophysiologist, who had two decades of experience in the electrophysiology of CFTR and microbial rhodopsins. The team demonstrated that the two DNAs encoded two directly light-gated ion channels, that they named Channelrhodopsin-1 (ChR1) and Channelrhodopsin-2 (ChR2) [122,124], thereby confirming the hypothesis of direct coupling of light sensor and channel. The team also expressed ChR2 in human kidney cells and suggested that ChR2 could be used in other cells to depolarize them with light. The Spudich group performed an antisense approach and demonstrated by using an electrical population assay that both ChR1 and ChR2 are the photoreceptors triggering photocurrents in the alga [123].

4.3. Transfer to neuroscience, the birth of optogenetics

Based on the results of Hegemann and Nagel, several groups began working with ChRs, primarily with a truncated version of ChR2 that expresses better than full-length ChR2 or ChR1. Seminal publications

[4] Title of a great solo piano album by Ryuichi Sakamoto.

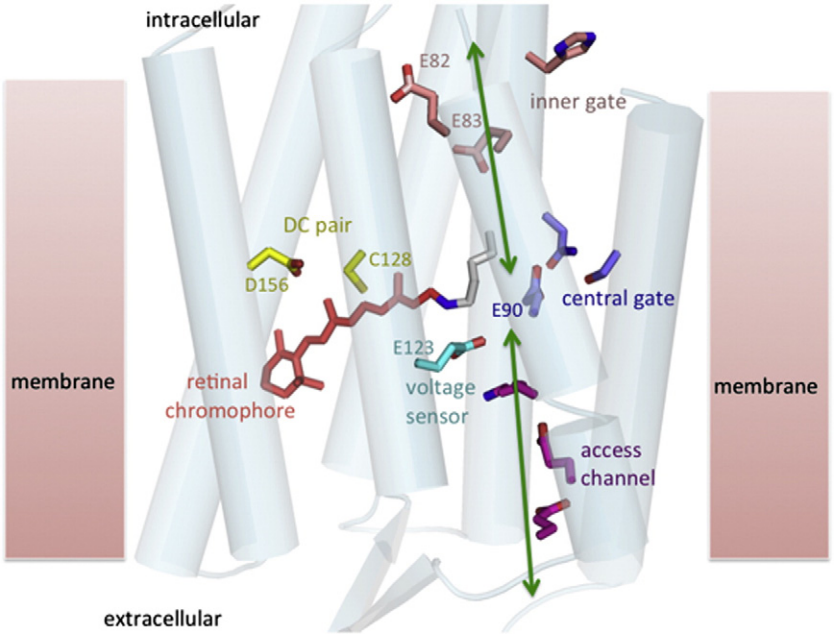

Fig. 3. Schematic of the 7TM-fragment of ChR according to Kato et al. [131] with key residues colored accordingly: voltage sensor E123 (cyan), the access channel (magenta), central gate (blue), and inner gate (orange). The retinal Schiff base is colored in pink.

appeared in 2005 and 2006 from the laboratories of Karl Deisseroth, Stefan Herlize, Hiromu Yawo, Alexander Gottschalk, and Zhuo Pan, who demonstrated the functionality of ChR2 in hippocampal neurons, PC12 cells, mouse brain slices, spines of living chicken embryos, transgenic worms (*C. elegans*), and the retina of blind mice [125–129]. These publications represent the beginning of what we call optogenetics today. In this new field, researchers express light-activated proteins in well-defined cell subpopulations of a neuronal context under the control of host cell specific promoters and activate these cells by using short light pulses. Already during earlier studies, such as those performed in the laboratory of Gero Miesenböck at Oxford, researchers were attempting to implement photosensitive actuators into host cells. However, the visual system used (i.e., *Drosophila*) was too complicated and too slow. [130]. Nevertheless, neuroscientists continued to search for approaches that use light-sensitive proteins.

4.4. Architectural design and function

ChRs are microbial rhodopsins, composed of seven trans-membrane helices that are thereby structurally similar to BR and SR, but with long C-terminal extensions involved in protein targeting and activation of secondary channels (Fig. 3). These ChR-linked channels are needed for low light responses but still await molecular discovery. Structurally, the ChR core protein (7TM fragment) is also related to mammalian rhodopsins and GPCRs, although the structure is more compact and the helices are more ordered as compared to GPCRs [131].

The light-absorbing chromophore retinal is imbedded within the hydrophobic center of the seven helices of all rhodopsins and is enclosed by helices 3 to 7. Unexpectedly, sufficient amounts of retinal are made or delivered in most neuronal cells. The affinity of the opsin-protein for retinal is in the nanomolar range but varies amongst the ChR isotypes and mutants. This property partially explains why some variants work better than others in neurons despite equivalent expression and membrane targeting [132].

What determines the color of the chromophore and therefore the wavelength used experimentally by optogeneticists? The color of all rhodopsins, especially ChR, is determined by the charge distribution along the chromophore in its ground state and the electronically excited state after light absorption. The retinal is connected to a conserved lysine via a Schiff base linkage (C N; Fig. 3) with an absorption around 370 nm. However, in nearly all rhodopsins, the absorption is shifted into the visible range of the spectrum by protonation of the retinal Schiff-Base ($RSBH^+$). This protonation of the chromophore is stabilized by a complex negatively-charged counter ion that is imprinted by two carboxylic acids (in a few cases only one) that, together with $RSBH^+$, form the *active site*.[5] An increase in distance between the negatively-charged counter ion complex and the positive NH^+ of the $RSBH^+$ further shifts ChR absorption to a longer wavelength because the charge has more "freedom" to move along the polyene chain of the chromophore. This is evident for ChR2 after mutation of the Schiff-Base counter ion residue E123 (corresponding to D85 in BR) into Thr or Ala in the ChR2-E123X mutants that both exhibit a red-shifted spectra. But, more interestingly, these two mutants revealed that E123 also serves as a voltage sensor that slows down photocycle kinetics with increasing (less negative) membrane voltage. Substitution of E123 completely eliminates voltage sensitivity, thus allowing ultra-fast action potential firing in the E123T/A ChETA mutants (widely named ChETA) [133].

The counter ion distance cannot be varied easily because it depends on the protein backbone arrangement. However, this difficulty has been partially overcome with the development of hybrids initially introduced by the Yawo group [134]. The electronic properties of the chromophore are fine-tuned by a few polar residues arranged around the retinal polyene chain; however, functionalization is not always easy. Besides light absorption, retinal functions to activate the protein and to open the ion pore. The primary ultrafast process is, as in animal visual rhodopsins, the isomerization of the retinal. The structural rearrangement of the chromophore is very minor, but the NH^+ dipole of the Schiff base switches from facing outward to inward. As a consequence, there is a massive rearrangement of the H-bonding network and three-dimensional restructuring of the protein. Details of this rearrangement are presently unknown. Additional structural information about the conducting state is required, along with an analysis of the proton transfer reactions that follow photo-isomerization of the chromophore and drive subsequent structural displacements during conversion from the dark state to the open state. This rearrangement is a multistep process, and at present only some intermediate states can be trapped with a defined spectrum. This sequence of reactions is important for the on-kinetics of the

[5] Amino acid numbering throughout this manuscript is based on ChR. However, the numbering of residues in the C1C2 hybrid X-ray structure is different.

photocurrents (i.e., on-gating). Two reaction intermediates have been assigned thus far, P500 and P380, with the latter having a deprotonated chromophore [135]. The structural changes are reversed during closure of the conducting pore and reversion to the dark state. We know that the reaction path differs from the opening path and that the kinetics of dark state recovery is many orders of magnitudes slower. In reality, the photoreactions of ChR are more complex and are not fully explored in detail. Moreover, for example, the thermal back-reaction is branched and less homogenous than the forward reactions after several rounds of photocycling and the majority of the molecules are approaching the a light-adapted dark state (closed state C2) [136]. This closed state is photo-convertible into a second conducting state (O2) that shows slightly different selectivity compared to O1. Molecular differences between C1 and C2 or O1 and O2 remain unknown. Two residues, C128 and D156, (Fig. 3) are of fundamental importance for both *on*-gating and *off*-gating, with mutation of either residue resulting in a dramatic reduction in reaction kinetics and an increase in the open state(s) lifetime (e.g., step function rhodopsins) [133,137–139].

When the conducting state is reached after light exposure, up to 100 ions are conducted during the lifetime of the open state, which is approximately 12 ms. We can assign three regions of the channel that are of special interest for transport, the *access channel*, *central gate*, and *inner gate*. The access channel has been a major focus of research since its discovery because the cluster of glutamates in a transmembrane helix is unexpected for a rhodopsin. Mutation of one or more glutamates gradually reduced conductance but none of them is of particular importance. The heart of the channel consists of two gates, the *central gate* (S63, E90, N258), which contacts the active site, and the *inner gate* (E82, E83, H134, H265) which — if closed — borders the hydrophobic barriers that exclude water and prevent ion conductance. Conformational changes open the gates during pore formation. However, our knowledge of the conducting state(s) remains vague since structural information is lacking. The key residue of the central gate, E90, contacts $RSBH^+$. However, the mechanistic impact of this contact is unclear. Both central gate and inner gate serve as selectivity filters, and mutagenesis of the participating residues can change the ion selectivity of the channel substantially. For example, both H134R (inner gate) and E90Q (central gate) conduct more Na^+ than wildtype and produce larger currents in slightly alkaline conditions, but lower currents at acidic conditions [140,141]. The gates are a focus of intense research, with the expectation that modification of them will reveal novel ChRs with useful properties that could widen the field of optogenetic applications. Despite the huge volume of electrophysiological data, X-ray crystallography, NMR, theoretical considerations and time resolved vibrational spectroscopy must be employed to fully understand the gating and conducting processes in the future.

4.5. Perspectives

The expectations for future applications of ChR are high. However, ChR is not the universal optogenetic key even if it has shaped optogenetics significantly. ChR shows clear limitations for optogenetic uses. First, ChRs are employed by nature for gradual membrane depolarization, but not for all-or-none responses, which is the reason for their small conductance. Second, we may widen the pore by molecular engineering and attracting more water molecules, but at the cost of destabilization and thermal activation in darkness. Third, selectivity can be easily changed toward higher or exclusive H^+ conductance as found naturally in the ChR of the halotolerant alga *Dunaliella salina* [142]. Likewise, ChR is tunable toward higher selectivity for monovalent or divalent cations. However, greater selectivity for K^+ over Na^+ to be used for light-controlled hyperpolarization of host cells will be very difficult to achieve. The highly appreciated red-shifted absorption is also limited to around 630 nm due to thermal activation (dark noise) of these red light-absorbing rhodopsins. This phenomenon occurs even when synthetic retinal analogues are used as chromophores.

Despite these limitations, however, great expectations can be envisioned for the future. Engineering of ChR and other microbial rhodopsins will progress and, moreover, countless ChR variants will be discovered from the hundreds of new algal genomes sequenced. Optogeneticists will find better ways for targeting ChRs into membrane subareas, directing them into organelles, controlling expression more accurately, and guaranteeing better turnover for retinal prostheses and vision in bright light. ChRs with further red-shifted absorption and ChRs with deprotonated chromophore and ultraviolet absorption will be developed since also mammals possess UV-sensitive rhodopsins in their eyes. ChRs will be further optimized for two-photon microscopy and many novel unprecedented variants will be identified [143]. Moreover, ChRs may become commonly used analytical tools or even therapeutics for treating diseases.

5. Conclusion

Recapitulating the stories of BR, SR and ChR research, it appears striking how microbial rhodopsins have developed from what seemed an oddity of nature — a retinal-containing protein in the membrane of a rather obscure microbe, *Halobacterium* — to a paradigm for the interactions of light and life. In addition to the cases mentioned here, the natural relevance of rhodopsins became particularly obvious when metagenomic sequencing of an uncultured marine eubacterium revealed a gene with sequence homology to rhodopsins [144]. In a collaboration between the marine microbiologist Oded Béjà and the group of BR and SR-researcher John L. Spudich, the gene was heterologously expressed in *E. coli*. A classical experimental assay, similar to that used by Oesterhelt and Stoeckenius almost thirty years before [9], demonstrated retinal- and light-dependent proton transport. It is assumed that these so-called proteorhodopsins account for a significant part of oceanic phototrophy ([145]; for further information on this discovery story see also [19]).

A comparison of the BR, SR and ChR stories presented here in detail reveals that they display significant differences in the mode of research, and the way the discoveries were made. The characterization of BR resulted from *physico-chemical* analyses of membrane fractions aiming at their composition and physical structure. Thus, in 1971, Blaurock, Oesterhelt and Stoeckenius published on the properties of a specific protein from the halobacterial membrane without having any data on its *biological* role. In contrast, both the discoveries of SR and ChR started with the observation of organismic behavior, and thus biological function. When the phototaxis of *Halobacteria* or *Chlamydomonas* was analyzed by action spectroscopy, or its dependency on retinal was demonstrated, this counted as evidence for a rhodopsin-like receptor. In case of ChR, electrical measurements *in vivo* and reconstituted systems were of crucial importance. Low intensity action spectroscopy (threshold spectra), as introduced by Max Delbrück for *Phycomyces*, was the method that provided the breakthrough for the ChR case [119,120,146]. SR and ChR as purified proteins, or biochemical substances in the test tube, became only available after years of intense research, whereas this had been the starting point in the case of BR. The existence of proteorhodopsins, finally, was first hypothesized on the basis of nucleotide sequence homologies, followed by an *en bloc* import of an experimental system from BR research into a metagenomics project.

Thus, one could say that whereas the BR story followed a (bio-) chemical style of discovery that focused on the characteristics of a specific substance from a cellular preparation, the pattern of the SR and ChR discoveries followed a pattern typical for molecular biology. One could think of, for example, the analyses of nutritional mutants of the fungus *Neurospora* by George Beadle and Edward Tatum around 1940, which had led them to conclude that each enzymatic reaction in a phenotype was controlled by one gene [7].

It would certainly be of interest to analyze these different modes of discovery in the molecular life sciences more broadly. It seems that the explanatory and manipulative power of today's rhodopsin research was

built on the results from both the biochemical and the molecular biological approach. Naturally, knowledge about the role of rhodopsins in physiology, as well as that on their genetic organization, is considered essential, and in order to obtain crystal structures or to perform spectroscopic measurements, it was often necessary to prepare rhodopsins as purified material substances by overexpression. Thus, the three stories described above (and those of halorhodopsin and proteorhodopsins, which deserve full treatment elsewhere), may be different regarding the mode of discovery and the sequence of its experimental developments. Yet, over the last three decades, this research have also led to integrated uses of different techniques (crystallography/electron microscopy, magnetic and optical spectroscopy, electrophysiology), which are today applied to microbial rhodopsins. This integration was certainly fostered also by the fact that a community of microbial rhodopsin researchers has formed since 1970. As the reader will have noticed, many researchers have worked on different rhodopsins, techniques have been pioneered on one and transferred to another rhodopsin, and (not least) meetings have facilitated exchange and shaped an identity of scientists.

Moreover, one should not forget that the discoveries described here depended crucially on prior technique and knowledge about the physicochemical characteristics first of visual rhodopsin from animals' retinae, by an established scientific field, and later of BR as well. In that regard, the studies of visual purple (*Sehpurpur*) by the German physiological chemist Wilhelm Kühne in the late 19th century, and its functional and biochemical characterization by e.g. the American physiologist George Wald since the 1930s have provided a broad framework for the stories described here [8]. Considering rhodopsin research in the context of these developments, that span more than a century, one may reassess the relationship of change and continuity in science. In light of the stories we have described here (and many more), one may suspect that rhodopsin research progressed by gradual accumulation of knowledge and technique, interconnections of fields and extensions of their scope rather than through revolutionary changes.

It remains for another publication to analyze this issue and to describe microbial rhodopsin research against the background of broad developments in the life sciences, such as the integration of different research fields (e.g. membrane studies, molecular genetics, neurobiology), the impact of instrumentation and techniques (e.g. sequencing, genomics, membrane protein expression and purification) or the trend towards research carried out under the premise of biomedical or biotechnological applications. With the recent progress and promises of optogenetics in mind, however, one should not forget the decades of "basic" or "fundamental research" (if also this label needs to be qualified on a second look) on rhodopsins of organisms such as *Halobacterium* or *Chlamydomonas* that have paved the way to these developments.

What we will learn about rhodopsins in the coming years, and, presumably at least as important, what we will be able to do with them in science and beyond, naturally remains in the dark. Yet, it may well be that from the perspective of future scientists and historians of science, our vantage point of today (as much knowledge as we have amassed) appears similar to some of the beginnings mentioned in this story — an unknown substance in the test tube, a mutant organism behaving peculiarly, thus, more questions than answers. Or does it not?

Acknowledgement

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