JPP JOURNAL OF PROTEINS AND PROTEOMICS
2(2), July-December 2011, pp. 115-123

FUNCTIONAL DIVERSITY AND OPTOGENETIC POTENTIALS OF MICROBIAL RHODOPSINS

Mayanka Awasthi and Suneel Kateriya

Department of Biochemistry, University of Delhi South Campus, Benito Juarez Road, New Delhi – 110021, India

Abstract: Microbial or type-1 rhodopsins are light sensitive proteins that utilize all-trans retinal as chromophore. Microbial rhodopsins are present in archaea, eubacteria and eukaryotes. Their broad and patchy distribution among the three domains of life is attributed to the lateral gene transfer mechanism of evolution. Microbial rhodopsins function as sensory rhodopsins, light-gated ion pumps and light-activated ion channels in nature. In this review, we present functional diversity and optogenetics applications of microbial rhodopsins.

Key words: Microbial rhodopsin; Retinal; Neural activity; Phototransduction; Channelrhodopsin; Optogenetics.

1. Introduction

Light is an important abiotic factor in the ecosystem. Animals utilize it for vision and photoperiodism, whereas plants use it for several purposes like photomorphogenesis, photomovement, photoperiodism and photosynthesis (Briggs and Olney, 2001; Nagatani, 2002; Stone *et al.*, 2005). Photomovement is an interesting phenomenon, which involves phototaxis and photophobic responses. In phototaxis, organism perceives light and moves towards (positive phototaxis) or away from light (negative phototaxis) and in photophobic movement (photoshock) the organism stops abruptly upon perception of intense light and changes its direction of movement to avoid photodamage. In general, five sensory photoreceptors (rhodopsin, phototropin, cryptochrome, phytochrome and BLUF) are important to achieve photophysiological responses (Hegemann, 2008). These photoreceptors are comprised of two components, the protein moiety and chromophore. There are several informative reviews published that gives latest update on each of these photoreceptors (Hegemann, 2008; Kateriya *et al.*, 2004). Understanding the mechanism involved in photoperception and the downstream signaling leading to physiological responses has captivated the interest of the scientists over the last few decades. Rhodopsins, which serve as the major photoreceptor in many organisms, from unicellular to multicellular animals, must be widely and carefully studied. Here, we review the functional diversity and biotechnological applications of microbial rhodopsins in detail.

Rhodopsins belong to G-protein coupled receptor family of proteins containing bundles of seven transmembrane helices named H1 to H7, which are involved in forming an internal retinal binding pocket (Lanyi, 2004). In all rhodopsins, chromophore (retinal) is bound to lysine residue of the seventh transmembrane helix of opsin via a Schiff base linkage. Illumination of the rhodopsins with suitable light energy leads to isomerization of the bound retinal in the ground state, which in turn activates downstream signaling components for photophysiological responses (Spudich *et al.*, 2000). Rhodopsins are classified into two groups (microbial and animal rhodopsins) based on the nature of the bound chromophore in the ground (dark) state. Microbial

Corresponding Author: **Suneel Kateriya**
E-mail: skateriya@south.du.ac.in

rhodopsins bind to all-trans retinal (Chu Kung *et al.*, 1975) in ground state (dark state) and are distributed in all domains of life to execute diverse functions. These rhodopsins evolved separately in different organisms to perform specialized photophysiological functions (Marsh and Griffiths, 2005; Sharma *et al.*, 2007; Zhang and Yokoyama, 1997). They function as light-gated ion pumps (Bacteriorhodopsin and Halorhodopsin), sensory receptors (sensory rhodopsin1 and 2) and light-gated ion channel proteins (Channelrhodopsin1 and 2) in nature, reviewed by Klare *et al.* (Klare *et al.*, 2008). In some organisms (e.g. algae, archeae), microbial rhodopsins mediate photobehavioral responses (Govorunova *et al.*, 2004; Hegemann, 2008). Animal rhodopsins bind cis-retinal in dark state and are mainly present in invertebrates and vertebrates (Drachev *et al.*, 1980).

Recently, detailed molecular characterization of novel MRs (Channelrhodopsins) with light-gated ion channel activity and their application as optogentics tools (Nagel *et al.*, 2005; Nagel *et al.*, 2002; Nagel *et al.*, 2003) have sparked interest to identify newer novel microbial rhodopsins with diverse functions and ion selectivity. This review article focuses on the molecular nature and functional diversity of the microbial rhodopsins and illuminates their biotechnological potential as optogenetics tool.

2. Distribution and Functional Diversity of Microbial Rhodopsins in Nature

2.1. Microbial Rhodopsins in Archaea

Prokaryotes (archaea and bacteria) constitute the most primitive form of living organisms that survived the harsh environmental conditions and occupied almost every ecological niche. An archaea like *Halobacterium salinarum* is halophilic bacteria that usually live in high salt (upto 5M NaCl) concentrations. It possess four types of microbial rhodopsins (Bacteriorhodopsin, halorhodopsin and sensory rhodopsins) (Oesterhelt and Stoeckenius, 1971). These different rhodopsins show different mechanisms of action and help the bacteria to survive in high salt conditions. BR is a light activated proton pump that allows the translocation of protons from cytoplasm to extracellular region thus establishing an electrochemical potential. This electrochemical gradient across the membrane is utilized for ATP synthesis (Kataoka *et al.*, 1994) that provides energy for the survival of the organism. HR is a dual light activated pump that uses the energy of yellow light to move chloride, halide and nitrate ions into the cell to maintain osmotic balance inside and outside of the cell (Ogurusu *et al.*, 1981). *S*ensory rhodopsins, SRI and SRII, are responsible for phototaxis and photophobic responses, respectively. SRII is constitutively synthesized in the cell, while SRI, BR and HR are expressed mainly under low oxygen tension (Mukohata and Kaji, 1981a, b). SR II enables bacteria to seek dark (away from blue light) when oxygen supply is plenty thus preventing cells from photo-oxidative damage. SRI guides cell to yellow red light spectrum, which is optimal for BR and HR activity (Spudich and Bogomolni, 1984). SRI is also responsible for photostop activity, upon UV light exposure. As in eubacteria chemotaxis, analogous transducers (Histidine Kinase) activate a response regulator that in turn switches rotation of the flagella motor (target response). This type of biochemical signaling between sensory and effector systems is known as a two-component system (Oprian, 2003). The BR, HR and SRs display conserved secondary structures within the TMHs 3-7 (Landau *et al.*, 2003). Upon light absorption, all four microbial rhodopsins undergo photocycle. All of these light-induced thermal reactions have to be reversible because once a cycle is completed the protein has to regain its ground state. The detail characterizations of microbial type rhodopsins in archaea left no doubt that these proteins are immensely important for the survival of these organisms.

2.2. Microbial Rhodopsins in Eubacteria

It has emerged from experimental evidence that the MRs have spread beyond the borders of archaea taxa in nature (Losi *et al.*, 2000; Spudich *et al.*, 2000). Genes with clear sequence similarities to archaeaal rhodopsins were characterized in other domains of life as well. MR sequences were found in some non-cultivated proteobacteria (Eubacteria) that encoded a protein called proteorhodopsin, a transport rhodopsin that function as a light-driven proton pump (Beja *et*

al., 2000). Proteorhodopsin was the first microbial type rhodopsin to be identified in the eubacteria. PR has an absorption maximum at 520nm (Beja *et al.*, 2000). The primary proton donor D96 of BR (of *H.salinarium*) shows conservative exchange with E108 in PR. Primary proton acceptor D85 of BR is conserved in PR, at position D97. Residues (R82, D212 and K216) that are important for counterion of the protonated Schiff base of BR are also conserved in PR (R94, D227, and D231) (Beja *et al.*, 2000). The photocycle of PR is also similar to that of BR. However, the physiological importance of PR in proteobacteria is yet to be established. Several other eubacteria (cyanobacterium), which are also known as blue green algae (e.g. *cyanothece, anabena* etc) uses microbial type rhodopsins (Sineshchekov *et al.*, 2005a). The rhodopsin domain of anabaena sensory rhodopsin is similar to that of haloarcheal sensory rhodopsins but possess a different hydrophilic C-terminus (Sineshchekov *et al.*, 2005b). It is interesting to note that ASR utilizes both *all-trans* and *13-cis* configuration retinal and shows different absorption maxima of 550 nm and 537 nm, respectively (Vogeley *et al.*, 2004). Furthermore, ASR C-terminus might interact with some cytoplasmic soluble protein that is very different from the haloarcheal transducer protein (Htrs) and named as putative anabena sensory rhodopsin transducer by Vogely and Lueke (Vogeley and Luecke, 2006). ASR undergoes a photocycle similar to that of SRII from *H. salinarum* (Vogeley *et al.*, 2007). Currently, it is required to assign physiological functions to these microbial type rhodopsins of eubacterial origin using functional genomics approaches.

2.3. Microbial Rhodopsins in Eukaryotes

It was speculated that MRs might be confined to prokaryotes only. Recently, however, MRs have been reported in a few eukaryotes, fungi (Bergo *et al.*, 2002) and dinoflagellates (*Pyrocystis lunula*, Cryptomonad algae, *Guillardia theta*) (Sineshchekov *et al.*, 2005a). Presence of MRs in higher plants is still debatable. Several green algae (e.g. C*hlamydomonas, Volvox*, *Oterococcus)* belonging to the viridiplantae group also harbours multiple MRs (Braun and Hegemann, 1999; Foster *et al.*, 1984; Hegemann, 2008; Spudich, 2006). These green algae are unicellular, multicellular or colonial and show both phototaxis and photophobic responses. MRs from algal systems is characterized in detail and is found to be important for optogenetics applications.

2.3.1. Microbial Rhodopsins in Algae

Members of chlorophycae, *Chlamydomonas, Volvox* etc (green algae) possess the most primitive form of eye. Unicellular *C. reinhardtii* is the most prominent model system for basic research to answer many fundamental questions of photobiology, cell and molecular biology. This alga is only 8 to 10 µm in size, possesses a cell wall, chloroplast, an eyespot (stigma) that perceives light, and two anterior flagella. The eyespot takes up approximately one percent of the cell surface and is about 1µm in diameter. An electron microscopic study revealed that the eyespot consists of one to several layers of carotenoid rich lipid globules stacked in the thylakoid membrane (Foster and Smyth, 1980). Special structural arrangement of the eyespot enables the organism to perceive maximum incident light when the eyespot is facing towards the light source. *Chlamydomonas* rotate in counterclockwise direction following a helical path and during rotation scans the environment for optimal light for survival and growth. *Chlamydomonas* shows photomotility (phototaxis and photophobic) responses, which help organism to optimize photosynthesis and avoid photo-damage due to excessive light. The organism responds differently to different light intensities. At low light intensity, the flagellar beating favors positive phototaxis whereas intense light flashes results in photophobic responses. Light induced action spectroscopy analysis suggested the involvement of rhodopsin like photoreceptor in photobehavioral response of *C. reinhardtii* (Foster *et al.*, 1988). The role of rhodopsin as a photoreceptor in *C. reinhardtii* was confirmed by the observation that supply of exogenous retinal restored the photobehavioral responses in blind cells (retinal synthesis disrupted; FN806 strain). HPLC analysis of the retinoid extracted from *Chlamydomonas* cells verified the presence of *all-trans* retinal in *Chlamydomonas* (Hegemann *et al.*, 1991). It has also been established that the photomotility responses

of *Chlamydomonas* is controlled via calcium ion flux through channel proteins present in the cell membrane. Suction pipette recordings of this calcium current suggested that two distinct light regulated inward current called the photoreceptors current control the light regulated behaviour of the cell. Light induced calcium currents bring about the change in the beating pattern of the flagella (Beck and Uhl, 1994). This current is localized in the eyespot and flagellar region of the microalga. Primary electrical event is the photoreceptor current, also known as P-current; when this photoreceptor current reaches the critical value it gives rise to flagellar current components, FF and FS (fast and slow) flagellar currents, respectively (Holland *et al.*, 1997).

Opsin was biochemically characterized by tritium (H^3) labeling experiment; tritium labeled retinal added to retinal deficient mutant restored their phototactic behavior. Membrane fraction prepared from such cells showed the presence of 30kDa protein as tritium labeled retinal protein (Beckmann and Hegemann, 1991) and was named as Chlamyopsin1. Chlamyopsin2 was a second alternative translational product of primary Cop mRNA. Chlamyopsins were localized in the eyespot region of the cell and their expression were developmentally regulated. PTGS study confirmed that the abundant proteins present in the eyespot, viz., Cop1 and Cop2 do not serve as photoreceptor for the phototaxis and photophobic responses (Fuhrmann *et al.*, 2001). This led to the identification of the novel microbial rhodopsins, channelrhodopsin1 and channelrhodopsin2. The opsin region of channelrhodopsin comprises seven putative transmembrane segments. It includes a retinal binding motif LDxxxKxxW where K296 in ChR1 and K257 in ChR2 serve as the retinal binding site (Nagel *et al.*, 2002; Nagel *et al.*, 2003). ChR1 and 2 are also referred to as ACOP1 and ACOP2, respectively. ACOP1 is localized in the eyespot region of *Chlamydomonas* cells (Suzuki *et al.*, 2003). These proteins are also called as CSRA and CSRB, respectively, by some authors (Sineshchekov *et al.*, 2002). Double stranded RNA interference (RNAi) technology selectively suppresses expression of the genes CSRA or CSRB in *Chlamydomonas* (Sineshchekov *et al.*, 2002). Photocurrent measurement from RNAi transformants showed that these two rhodopsins are responsible for the light generated currents associated with phototaxis. Action spectra of CSRA peak between 470 and 500 nm. CSRA enriched cells generate a fast photocurrent that saturates at high light intensities. CSRB enriched cells exhibit a slow photocurrent that saturates at much lower light intensities than CSRA photocurrent (Sineshchekov *et al.*, 2002). Superposition of these currents gives rise to composite signals that are characteristic for the phototaxis receptors of the green flagellated algae.

Expression of full length or rhodopsin domain of ChR1 in *Xenopus* oocytes showed photoactive channel activity and selectivity for protons (H+) (Nagel *et al.*, 2002). Similarly, functional expression of ChR2 in both *Xenopus laevis* oocytes and mammalian cells showed ChR2 to be a light-gated cation channel (Nagel *et al.*, 2003). Photoconductance measurement of ChR2 revealed that it is permeable to several monovalent cations and some divalent cations like Ba^{2+} and Ca^{2+}. ChR2 desensitizes in continuous light to smaller steady-state conductance. Extracellular H+ and negative membrane potential accelerates recovery from this desensitized state whereas intracellular H+ decelerates the closing of ChR2 ion channel. The photocurrent of ChR2 is much larger than that of ChR1. Therefore, ChR2 showed greater potential to serve as a tool to depolarize cells or to maintain ion flux simply by illumination (Nagel *et al.*, 2003). Several other MR sequences with homology to prokaryotic transducer proteins were also found in the *Chlamydomonas* genome and were named as Cop5, Cop6 and Cop7 (Kateriya *et al.*, 2004). All of them showed higher homology with sensory rhodopsins than other MRs. Cop5 and Cop7 possess a RR sequence downstream of the transducer (Histidine Kianse) region while Cop-5 additionally harbors a cyclase domain after the RR region. Recently, it has been shown that cAMP plays an important role in phototaxis responses of *Chlamydomonas* and author had suggested that opsin coupled cyclase(s) might be the responsible candidate for this response. Photobehavioral studies with chlamyopsin(s) gene knockout mutants of *Chlamydomonas* are required to establish functional importance of multiple MRs in the system.

3. Homology and Phylogenetic Analysis of the Microbial Rhodopsins

Microbial rhodopsins or type 1 rhodopsins were first discovered in halophillic archaea. Series of vertical and less frequent horizontal gene transfer helped in the distribution of these genes in few eubacteria and in some eukaryotic species. MRs were evolved to perform diverse physiological functions in the different domains of life (Ruiz-Gonzalez and Marin, 2004). Diverse functions performed by these rhodopsins include light activated ion channel and pump activity and their function as a sensory receptor for phototaxis and photophobic responses. Multiple sequence alignment of characterized rhodopsin sequences originating from different organisms belonging to archaeaa (SR and BR), *Cryptomonas, Cyanothecae and Anabena* and few algal rhodopsins shows the relative positions of the seven transmembrane helices (**Fig. 1**). Important amino acids critically required for the rhodopsin function are also conserved (shown in coloured boxes; **Fig. 1**). These conserved amino acid residues are mainly present in the region of retinal binding pocket. Asp85 and Asp96 are the key amino acids in BR, which are required for its light regulated proton pumping activity. Proton transfer in BR first occurs from Schiff base linkage to Asp85. Conservative exchange of Glu162 and Glu123 is seen in channelrhodopsin 1 and 2 respectively (**Fig. 1**). Asp96 plays role in the reprotonation of the Schiff base, which is replaced by aromatic (Phe and Tyr) residues in sensory rhodopsins (SRI and SRII) and Ser in anabena sensory rhodopsin. Because of the presence of aromatic amino acid (Phe and Tyr) with hydrophobic side chain or non-polar residue (Ser), optimal proton transfer required for the pump activity is interrupted and these rhodopsins function as sensory rhodopsins. MR in eukaryotic species like *Anabena, Cryptomonad* and *Cyanothecae* are not functionally characterized but homology and structural analysis suggests that they could function as sensory rhodopsins. Evolutionary analyses of these rhodopsins represent that eukaryotic microbial sensory rhodopsins like ASRs originated from prokaryotic sensory rhodopsins as a result of horizontal gene transfer. ASR is evolved to form a separate clade but shows close relationship with other SRs (**Fig. 2**). It is surprising to note that ChRs are more homologous to sensory rhodopsin than light-gated pump proteins (**Fig. 2**). Preliminary rectangular cladogram obtained by phylogenetic analysis of these rhodopsins by neighbor joining method shown in **Fig. 2** depicts anticipated evolutionary relationship among functionally diverse MRs. It would be interesting to elucidate their evolutionary aspects as well.

4. Application of Microbial Rhodopsin for the Development of Optogenetic Tools

Until recently electrical stimulation technique was the only known tool to study electrically excitable cells (skeletal cell, smooth muscle cell, neural cell, myocardiac cells etc.) (Gelsema *et al.*, 1989; McCreery and Agnew, 1983; Ryan *et al.*, 1990).

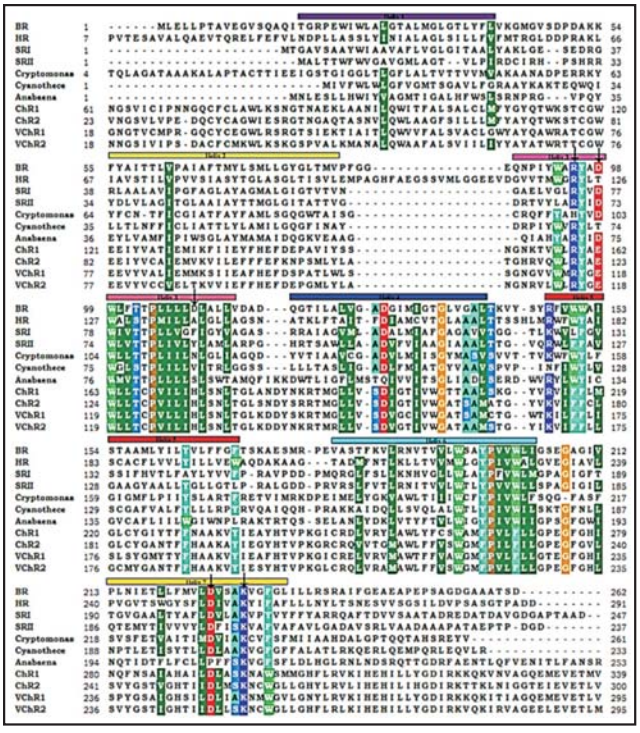

Figure 1: **Amino acid sequence comparision of type 1 rhodopsins present in different organisms (archaeaa, eubacteria and eukaryotes). Bars indicate the putative position of the helices from 1 to 7 (according to Spudich et al. 2000). Arrows indicates the positions of conserved amino acids required for rhodopsin function. Amino acids residues conserved in at least 80% of the sequences are highlighted in different color bars. Abbreviations: BR (Bacteriorhodopsin from Halobacterium salinarum), HR (halorhodopsin from Natronobacterium pharaonis), SRI and SRII (sensory rhodopsin I and II from Halobacterium salinarum, respectively), ChR1 and ChR2 (Chlamydomonas channelrhodopsin I and II respectively), VChR1 and VChR2 (Volvox channelrhodopsin I and II respectively)**

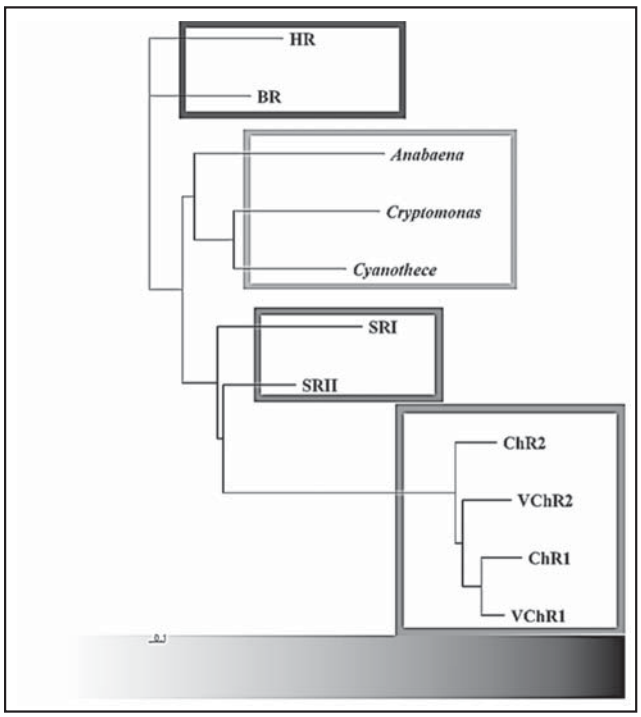

Figure 2: **Phylogenetic relationship among the trans-membrane domain of type 1 rhodopsins present in 11 species from different strata of life, represented in the form of rectangular phylogram. HR (Halorhodopsin) and BR (Bacteriorhodopsin) are chloride and proton pump respectively. Membrane spanning domain of *Anabena*, *Cyanothece* and *Cryptomonas* (eukaryotes) work as a sensory rhodopsin. SRI (sensory rhodopsin-I) and SRII (sensory rhodopsin-II) are the sensory photoreceptors. ChR1 and ChR2 (*Chlamydomonas* channelrhodopsins), VChR1 and VChR2 (*Volvox* channelrhodopsins) are light activated ion channel proteins. Scale represents 0.1 estimated amino acid substitutions per site**

However, this method is invasive in nature and shows limited spatial resolution in heterogeneous tissues. Scientists have proposed that photostimulation can provide an alternative method to electrode stimulation in such tissues. Richard Fork had used an intense beam of blue light to evoke action potentials in Aplysia ganglion (Fork, 1971). Later, chemically inactivated caged messengers were applied and the active compound released by a bright flash of blue/UV-light. These methods, however, had severe limitations, because intense blue or UV light damages the cell. Photostimulation (optogenetics) can provide a better alternative tool to simulate and control electrical activity of electrically excitable (Arenkiel *et al.*, 2007; Douglass *et al.*, 2008; Wang *et al.*, 2007; Zhang *et al.*, 2006) cells. It provides a non-invasive way to regulate the activity of these cells with high precision and efficiency. Under physiological conditions, ChR2-rhodopsin domain is able to depolarize *Xenopus* oocytes by illumination with blue light. ChR1 is unable to modulate neuronal excitability because it permeates lesser number of protons through channel at physiological pH. ChR2 on the other hand has a property of very rapid opening and closing of channel, which could control membrane potential on millisecond time scale. Neuroscientists applied truncated ChR2 to control intracellular ion fluxes and/or membrane potential of animal cells, simply by illumination (Liewald *et al.*, 2008; Nagel *et al.*, 2005; Zhang *et al.*, 2008; Zhang *et al.*, 2007). Engineered ChR2 showed improved spectral properties and kinetics for controlling neuronal activities (Lin *et al.*, 2009). New variants of ChRs are utilized by neuroscientist for the better understanding of the cause of some neurodegenerative disorders. Expression of ChR2 in the muscle cells of nematode C.*elegans* showed light regulated muscular contraction (Nagel *et al.*, 2005). Engineered halorhodopsin (NpHR) has also been applied as optogenetic tool to switch off neural activity by illumination with orange yellow light. It has higher extracellular Cl^- affinity and stability and has excitation maxima near 580nm. ChR2 can bring about the depolarization of cells simply by illumination with blue light (Nagel *et al.*, 2003). NpHR acts as a counterpart to ChR2, as it suppresses the action potential evoked by ChR2 and silencing the neurons (Zhang *et al.*, 2007). The light regulated properties of ChR2 and NpHR are exploited by expressing both in the targeted neuronal cells (Han and Boyden, 2007). Spinal neurons present in and around the phrenic motor pool were transfected with expression constructs of ChR2, and the photostimulation of ChR2 showed restoration of respiratory motor function after spinal cord injury in adult animals (Alilain *et al.*, 2008). MR based optogenetics approach has also been utilized for the treatment of neurological and psychiatric disorders, including parkinson's disease and major depression (Gradinaru *et al.*, 2009). ChR2 gene based strategies for curing retinitis pigmentosa abnormality was performed in mice; ChR2 expressing retinal ganglion cells directly respond to light thereby imparting vision evoked

potential (Tomita *et al.*, 2009). Recently, optical control of heart was achieved by expressing ChR2 in myocardiac cells of mice, which generated the action potential and ionic currents similar to natural cardiac cycle. Mice with ChR2 in the myocardiocites could be paced *in vivo* by giving regulated light pulses. ChR2 expression could be used to noninvasively control and regulate the beating frequency of the heart in condition like atrial fibrillation. This technique provides the way to achieve prolonged depolarization and to study the functioning of heart cell transplants.

5. Future Prospects of Optogenetics

Optogenetics being the most exciting area of research provides the best platform to know the behavioural responses controlled by brain. Various studies in the model organisms including flies, rats, mice, monkeys and worms, clearly demonstrated that optogenetics would eventually be applied in clinical applications. It involves the targeting of light sensitive molecules to neural cells, thus converting them into light sensitive cells. Such studies are to date restricted to animal model organisms but not in humans. Human brain is much more complicated and has even more complicated immune system than these model organisms. Thus, the use of light sensitive molecules like channelrhodopsins in humans to reveal the theories formulating human brain could be another big challenge. As the life span of humans is much more than mouse or other model systems, the stable and functional expression of channelrhodopsins must be improved in leaps and bounds to be effectively applicable in the human context (Zhang *et al.*, 2006). However, with the targeted delivery of light sensitive molecules in animals a new ray of hope has dawned to fight the deadly diseases like Parkinson's, retinitis pigmentosa, myocardial infarction etc. in a much reliable and non-invasive way.

Acknowledgments

We thank Dr. Sindhu K.V for her scientific advice and critical discussion. We are thankful to DBT, Government of India for financial assistance to SK (BT/PR9090/BRB/10/540/2007) for the support of basic research project. UGC, Government of India is duly acknowledged for research fellowship to MA.

Abbreviations

LGT, lateral gene transfer; GPCR, G-protein coupled receptor; TMH, transmembrane helices; MR, microbial rhodopsins; ChRs, channelrhodopsins; BR, bacteriorhodopsin; HR, halorhodopsin; SR, sensory rhodopsins; RR, response regulator; PR, proteorhodopsin; ASR, anabaena sensory rhodopsin; Cop1, chlamyopsin1; Cop2, chlamyopsin2; PTGS, post transcriptional gene silencing; ChR1, channelrhodopsin1; ChR2, channelrhodopsin2.

References

[1] Alilain, W. J., Li, X., Horn, K. P., Dhingra, R., Dick, T. E., Herlitze, S., and Silver, J. (2008), Light-induced Rescue of Breathing after Spinal Cord Injury. *J Neurosci* **28**, 11862-11870.

[2] Arenkiel, B. R., Peca, J., Davison, I. G., Feliciano, C., Deisseroth, K., Augustine, G. J., Ehlers, M. D., and Feng, G. (2007), In vivo light-induced activation of neural circuitry in transgenic mice expressing channelrhodopsin-2. Neuron **54**, 205-218.

[3] Beck, C., and Uhl, R. (1994), On the localization of voltage-sensitive calcium channels in the flagella of Chlamydomonas reinhardtii. *J Cell Biol* **125**, 1119-1125.

[4] Beckmann, M., and Hegemann, P. (1991), In vitro identification of rhodopsin in the green alga Chlamydomonas. *Biochemistry* **30**, 3692-3697.

[5] Beja, O., Aravind, L., Koonin, E. V., Suzuki, M. T., Hadd, A., Nguyen, L. P., Jovanovich, S. B., Gates, C. M., Feldman, R. A., Spudich, J. L., *et al.* (2000), Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* **289**, 1902-1906.

[6] Bergo, V., Spudich, E. N., Spudich, J. L., and Rothschild, K. J. (2002), A Fourier transform infrared study of Neurospora rhodopsin: similarities with archaeal rhodopsins. *Photochem Photobiol* **76**, 341-349.

[7] Braun, F. J., and Hegemann, P. (1999), Two light-activated conductances in the eye of the green alga Volvox carteri. *Biophys J* **76**, 1668-1678.

[8] Briggs, W. R., and Olney, M. A. (2001), Photoreceptors in plant photomorphogenesis to date. Five phytochromes, two cryptochromes, one phototropin, and one superchrome. *Plant Physiol* **125**, 85-88.

[9] Chu Kung, M., DeVault, D., Hess, B., and Oesterhelt, D. (1975), Photolysis of bacterial rhodopsin. *Biophys J* **15**, 907-911.

[10] Douglass, A. D., Kraves, S., Deisseroth, K., Schier, A. F., and Engert, F. (2008), Escape behavior elicited by single, channelrhodopsin-2-evoked spikes in zebrafish somatosensory neurons. *Curr Biol* **18**, 1133-1137.

[11] Drachev, L. A., Kalamkarov, G. R., Kaulen, A. D., Ostrovsky, M. A., and Skulachev, V. P. (1980), Animal rhodopsin as a photogenerator of an electric potential that increases photoreceptor membrane permeability. *FEBS Lett* **119**, 125-131.

[12] Fork, R. L. (1971), Laser stimulation of nerve cells in Aplysia. *Science* **171**, 907-908.

[13] Foster, K. W., Saranak, J., Patel, N., Zarilli, G., Okabe, M., Kline, T., and Nakanishi, K. (1984), A rhodopsin is the functional photoreceptor for phototaxis in the unicellular eukaryote Chlamydomonas. *Nature* **311**, 756-759.

[14] Foster, K. W., Saranak, J., and Zarrilli, G. (1988), Autoregulation of rhodopsin synthesis in Chlamydomonas reinhardtii. *Proc Natl Acad Sci U S A* **85**, 6379-6383.

[15] Foster, K. W., and Smyth, R. D. (1980), Light Antennas in phototactic algae. *Microbiol Rev* **44**, 572-630.

[16] Fuhrmann, M., Stahlberg, A., Govorunova, E., Rank, S., and Hegemann, P. (2001), The abundant retinal protein of the Chlamydomonas eye is not the photoreceptor for phototaxis and photophobic responses. *J Cell Sci* **114**, 3857-3863.

[17] Gelsema, A. J., Agarwal, S. K., and Calaresu, F. R. (1989), Cardiovascular responses and changes in neural activity in the rostral ventrolateral medulla elicited by electrical stimulation of the amygdala of the rat. *J Auton Nerv Syst* **27**, 91-100.

[18] Govorunova, E. G., Jung, K.H., Sineshchekov, O. A., and Spudich, J. L. (2004), Chlamydomonas sensory rhodopsins A and B: cellular content and role in photophobic responses. *Biophys J* **86**, 2342-2349.

[19] Gradinaru, V., Mogri, M., Thompson, K. R., Henderson, J. M., and Deisseroth, K. (2009), Optical deconstruction of parkinsonian neural circuitry. *Science* **324**, 354-359.

[20] Han, X., and Boyden, E. S. (2007), Multiple-color optical activation, silencing, and desynchronization of neural activity, with single-spike temporal resolution. PLoS One *2*, e299.

[21] Hegemann, P. (2008), Algal sensory photoreceptors. *Annu Rev Plant Biol* **59**, 167-189.

[22] Hegemann, P., Gartner, W., and Uhl, R. (1991), All-trans retinal constitutes the functional chromophore in Chlamydomonas rhodopsin. *Biophys J* **60**, 1477-1489.

[23] Holland, E. M., Harz, H., Uhl, R., and Hegemann, P. (1997), Control of phobic behavioral responses by rhodopsin-induced photocurrents in Chlamydomonas. *Biophys J*. **73**, 1395-1401.

[24] Kataoka, M., Kamikubo, H., Tokunaga, F., Brown, L. S., Yamazaki, Y., Maeda, A., Sheves, M., Needleman, R., and Lanyi, J. K. (1994), Energy coupling in an ion pump. The reprotonation switch of bacteriorhodopsin. *J Mol Biol* **243**, 621-638.

[25] Kateriya, S., Nagel, G., Bamberg, E., and Hegemann, P. (2004), "Vision" in single-celled algae. *News Physiol Sci* **19**, 133-137.

[26] Klare, J. P., Chizhov, I., and Engelhard, M. (2008), Microbial rhodopsins: scaffolds for ion pumps, channels, and sensors. *Results Probl Cell Differ* **45**, 73-122.

[27] Landau, E. M., Pebay-Peyroula, E., and Neutze, R. (2003), Structural and mechanistic insight from high resolution structures of archaeal rhodopsins. *FEBS Lett* **555**, 51-56.

[28] Lanyi, J. K. (2004), Bacteriorhodopsin. *Annu Rev Physiol* **66**, 665-688.

[29] Liewald, J. F., Brauner, M., Stephens, G. J., Bouhours, M., Schultheis, C., Zhen, M., and Gottschalk, A. (2008), Optogenetic analysis of synaptic function. *Nat Methods* **5**, 895-902.

[30] Lin, J. Y., Lin, M. Z., Steinbach, P., and Tsien, R. Y. (2009), Characterization of engineered channelrhodopsin variants with improved properties and kinetics. *Biophys J* **96**, 1803-1814.

[31] Losi, A., Wegener, A. A., Engelhard, M., Gartner, W., and Braslavsky, S. E. (2000), Aspartate 75 mutation in sensory rhodopsin II from Natronobacterium pharaonis does not influence the production of the K-like intermediate, but strongly affects its relaxation pathway. *Biophys J* **78**, 2581-2589.

[32] Marsh, L., and Griffiths, C. S. (2005), Protein structural influences in rhodopsin evolution. *Mol Biol Evol* **22**, 894-904.

[33] McCreery, D. B., and Agnew, W. F. (1983), Changes in extracellular potassium and calcium concentration and neural activity during prolonged electrical stimulation of the cat cerebral cortex at defined charge densities. *Exp Neurol* **79**, 371-396.

[34] Mukohata, Y., and Kaji, Y. (1981a), Light-induced ATP synthesis dependent on halorhodopsin-pH regulation. *Arch Biochem Biophys* **208**, 615-617.

[35] Mukohata, Y., and Kaji, Y. (1981b), Light-induced membrane-potential increase, ATP synthesis, and proton uptake in Halobacterium halobium, R1mR catalyzed by halorhodopsin: Effects of N,N′-dicyclohexylcarbodiimide, triphenyltin chloride, and 3,5-di-tert-butyl-4-hydroxybenzylidenemalononitrile (SF6847). *Arch Biochem Biophys* **206**, 72-76.

[36] Nagatani, A. (2002), [Plant photoreceptors and their signal transduction]. Tanpakushitsu Kakusan Koso *47*, 1700-1704.

[37] Nagel, G., Brauner, M., Liewald, J. F., Adeishvili, N., Bamberg, E., and Gottschalk, A. (2005), Light activation of channelrhodopsin-2 in excitable cells of Caenorhabditis elegans triggers rapid behavioral responses. *Curr Biol* **15**, 2279-2284.

[38] Nagel, G., Ollig, D., Fuhrmann, M., Kateriya, S., Musti, A. M., Bamberg, E., and Hegemann, P. (2002), Channelrhodopsin-1: a light-gated proton channel in green algae. *Science* **296**, 2395-2398.

[39] Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., and Bamberg, E. (2003), Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci U S A *100*, 13940-13945.

[40] Oesterhelt, D., and Stoeckenius, W. (1971), Rhodopsin-like protein from the purple membrane of Halobacterium halobium. *Nat New Biol* **233**, 149-152.

[41] Ogurusu, T., Maeda, A., Sasaki, N., and Yoshizawa, T. (1981), Light-induced reaction of halorhodopsin prepared under low salt conditions. *J Biochem* **90**, 1267-1273.

[42] Oprian, D. D. (2003), Phototaxis, chemotaxis and the missing link. *Trends Biochem Sci* **28**, 167-169.

[43] Ruiz-Gonzalez, M. X., and Marin, I. (2004), New insights into the evolutionary history of type 1 rhodopsins. *J Mol Evol* **58**, 348-358.

[44] Ryan, A. F., Miller, J. M., Wang, Z. X., and Woolf, N. K. (1990), Spatial distribution of neural activity evoked by electrical stimulation of the cochlea. *Hear Res* **50**, 57-70.

[45] Sharma, A. K., Walsh, D. A., Bapteste, E., Rodriguez-Valera, F., Ford Doolittle, W., and Papke, R. T. (2007), Evolution of rhodopsin ion pumps in haloarchaea. *BMC Evol Biol* **7**, 79.

[46] Sineshchekov, O. A., Govorunova, E. G., Jung, K .H., Zauner, S., Maier, U. G., and Spudich, J. L. (2005a), Rhodopsin-mediated photoreception in cryptophyte flagellates. *Biophys J* **89**, 4310-4319.

[47] Sineshchekov, O. A., Jung, K. H., and Spudich, J. L. (2002), Two rhodopsins mediate phototaxis to low- and high-intensity light in Chlamydomonas reinhardtii. Proc Natl Acad Sci U S A **99**, 8689-8694.

[48] Sineshchekov, O. A., Trivedi, V. D., Sasaki, J., and Spudich, J. L. (2005b), Photochromicity of Anabaena sensory rhodopsin, an atypical microbial receptor with a cis-retinal light-adapted form. *J Biol Chem* **280**, 14663-14668.

[49] Spudich, J. L. (2006), The multitalented microbial sensory rhodopsins. *Trends Microbiol* **14**, 480-487.

[50] Spudich, J. L., and Bogomolni, R. A. (1984), Mechanism of colour discrimination by a bacterial sensory rhodopsin. *Nature* **312**, 509-513.

[51] Spudich, J. L., Yang, C. S., Jung, K. H., and Spudich, E. N. (2000), Retinylidene proteins: structures and functions from archaea to humans. *Annu Rev Cell Dev Biol* **16**, 365-392.

[52] Stone, B. B., Esmon, C. A., and Liscum, E. (2005), Phototropins, other photoreceptors, and associated signaling: the lead and supporting cast in the control of plant movement responses. *Curr Top Dev Biol* **66**, 215-238.

[53] Suzuki, T., Yamasaki, K., Fujita, S., Oda, K., Iseki, M., Yoshida, K., Watanabe, M., Daiyasu, H., Toh, H., Asamizu, E., *et al.* (2003), Archaeal-type rhodopsins in Chlamydomonas: model structure and intracellular localization. *Biochem Biophys Res Commun* **301**, 711-717.

[54] Tomita, H., Sugano, E., Isago, H., and Tamai, M. (2009). Channelrhodopsins provide a breakthrough insight into strategies for curing blindness. *J Genet* **88**, 409-415.

[55] Vogeley, L., and Luecke, H. (2006), Crystallization, X-ray diffraction analysis and SIRAS/molecular-replacenent phasing of three crystal forms of Anabaena sensory rhodopsin transducer. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **62**, 388-391.

[56] Vogeley, L., Sineshchekov, O. A., Trivedi, V. D., Sasaki, J., Spudich, J. L., and Luecke, H. (2004), Anabaena sensory rhodopsin: a photochromic color sensor at 2.0 A. Science **306**, 1390-1393.

[57] Vogeley, L., Trivedi, V. D., Sineshchekov, O. A., Spudich, E. N., Spudich, J. L., and Luecke, H. (2007), Crystal structure of the Anabaena sensory rhodopsin transducer. *J Mol Biol* **367**, 741-751.

[58] Wang, H., Peca, J., Matsuzaki, M., Matsuzaki, K., Noguchi, J., Qiu, L., Wang, D., Zhang, F., Boyden, E., Deisseroth, K., *et al.* (2007), High-speed mapping of synaptic connectivity using photostimulation in Channelrhodopsin-2 transgenic mice. *Proc Natl Acad Sci U S A* **104**, 8143-8148.

[59] Zhang, F., Prigge, M., Beyriere, F., Tsunoda, S.P., Mattis, J., Yizhar, O., Hegemann, P., and Deisseroth, K. (2008), Red-shifted optogenetic excitation: a tool for fast neural control derived from Volvox carteri. *Nat Neurosci* **11**, 631-633.

[60] Zhang, F., Wang, L.P., Boyden, E.S., and Deisseroth, K. (2006), Channelrhodopsin-2 and optical control of excitable cells. *Nat Methods* **3**, 785-792.

[61] Zhang, F., Wang, L.P., Brauner, M., Liewald, J.F., Kay, K., Watzke, N., Wood, P.G., Bamberg, E., Nagel, G., Gottschalk, A., *et al.* (2007), Multimodal fast optical interrogation of neural circuitry. *Nature* **446**, 633-639.

[62] Zhang, H., and Yokoyama, S. (1997), Molecular evolution of the rhodopsin gene of marine lamprey, Petromyzon marinus. *Gene* **191**, 1-6.