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The Unlimited Potential of Microbial Rhodopsins as Optical Tools

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ABSTRACT:

Microbial rhodopsins, a photoactive membrane protein family, serve as fundamental tools for optogenetics, an innovative technology to control biological activities with light. Microbial rhodopsins are widely distributed in nature and have a wide variety of biological functions. Regardless of many different types of microbial rhodopsins known, only few of them have so far been used in optogenetics for controlling neural activity to understand neural networks. The efforts of our group have been aimed at identifying and characterizing novel rhodopsins from nature and also at engineering novel variant rhodopsins by rational design. Based on the molecular and functional characteristics of those novel rhodopsins, we have proposed new rhodopsin-based optogenetics tools to control not only neural activities but also ”non-neural” activities. In this perspective, we introduce the achievements and summarize future challenges in creating optogenetics tools using rhodopsins. The implementation of optogenetics deep inside an *in-vivo* brain is the well-known challenge for existing rhodopsins. As a perspective to address this challenge, we introduce innovative optical illumination techniques using wavefront shaping that can reinforce low light sensitivity of the rhodopsins and realize deep-brain optogenetics. The applications of our optogenetics tools could be extended to manipulate non-neural biological activities such as gene expression, apoptosis, energy production and muscle contraction. We also discuss the potentially unlimited biotechnological applications of microbial rhodopsins in the future such as in photovoltaic devices and in drug delivery systems. We believe that advances in the field will greatly expand the potential uses of microbial rhodopsins as optical tools.

Biological responses are composed of various cellular activities in which complicated chemical processes of biological molecules (e.g. proteins, nucleotides, peptides and ions) are precisely regulated. To understand such biological responses, researchers have analyzed cellular activities and chemical processes of molecules using biochemical and biophysical methods. In the 21st century, as an innovative way to break through that limitation, an optical technique termed "optogenetics" has emerged. Optogenetics allows us to have control over the biological activities of living cells and organisms by genetically expressing photoactive proteins.[1-4] As classical methods to actively control biological activities, chemical administration, physical stimulation and genetic modification have been utilized. However, those methods are partially invasive even to non-targeted cells. In contrast, the light stimulation used in optogenetics is non-invasive and offers high spatial- and time- resolutions. Therefore, optogenetics has become a focus of interest as a promising technique to non-invasively control biological activities.[5] As optogenetics tools, various photoactive proteins, such as microbial rhodopsins, animal opsins and their chimeric proteins (i.e. optoXRs), and flavoproteins, have been used (Table 1).[6-16] Actually, microbial rhodopsins have been used for optical control of neural activities by ion transportation[5, 17] whereas animal opsins and optoXRs can optically control G protein signaling cascade and neural activities.[18, 19] Moreover, flavoproteins have been used for optical regulation of gene expression, cAMP production and kinase activation in the cells.[20]

Table 1. Optogenetic application of photoactive proteins.

Protein family	Photoactive protein	Optogenetic application	Reference
Animal opsin	Melanopsin	• G protein signaling	13
	OptoXR	• Neural activation • Neural silencing	14
Flavoprotein	Light-oxygen-voltage-sensing protein	• Control of cell motility	15
	Photoactivatable adenylyl cyclase	• cAMP production	16
Microbial rhodopsin	PR	• Voltage imaging	32
	AR3	• Voltage imaging • Neural silencing	9, 12, 33, 43, 49
	TR	• Neural silencing	12
	NpHR	• Neural silencing	7, 8
	KR2	• Neural silencing	10, 40
	GtACR1 and GtACR2	• Neural silencing	11, 46, 47, 51, 53
	CrChR2 and related variants	• Neural activation	3, 6, 27-30
	ASR	• Regulation of gene expression	69

Microbial rhodopsins are seven-transmembrane photoactive proteins containing vitamin A aldehyde retinal as a chromophore (Fig. 1A).[21, 22] Exceptionally, enzyme rhodopsins, which exhibit guanylate cyclase and cyclic nucleotide phosphodiesterase activities, have one extra helix upstream of the seven-transmembrane helices.[23, 24] The retinal covalently binds to the apoprotein opsin via a protonated Schiff base linkage with a conserved Lys residue located in the last helix. The *trans-cis* photoisomerization of the retinal triggers sequential conformational changes of the opsin during which the biological function is induced. After the sequential reactions, microbial rhodopsins return to the initial state by heat absorption (Δ in Figure 1B), which is called as photocycle.[21] The cyclic reaction allows microbial rhodopsins to repeatedly exhibit their functions

every light stimulation, which is advantageous to optogenetics in contrast to vertebrate visual opsins (i.e. vertebrate rhodopsin and cone visual pigments) in which the retinal is dissociated after photoreaction and the additional retinal is required to regenerate the pigments.[21, 25] Recent progress in genomics and bioinformatics revealed that microbial rhodopsins are widely distributed in all three biological domains, archaea, bacteria and eukarya, and have diverse functions such as outward proton pumps, inward chloride pumps, outward sodium pumps, inward proton pumps, cation channels, anion channels, phototaxis sensors, transcriptional regulators and enzymes (Fig. 1B).[21, 22] Rhodopsins have also been discovered in viruses.[26] The work by Nagel et al. molecularly and functionally characterized a light-gated cation channel with demonstration of light-induced depolarization of vertebrate cells (i.e. *Xenopus* oocytes and HEK293 cells), and then the authors named this new protein Channelrhodopsin-2.[3] As a first demonstration of optogenetics controlling neural activities, Deisseroth and coworkers utilized a channelrhodopsin with which genetically-modified neurons were successfully depolarized.[6] Around the same time, other groups controlled neural activities with Channelrhodopsin-2,[27-30] provided by the authors of ref. 3 [27-29] or synthesized according to ref. 3.[30] Since then, neuroscience researchers have started to regulate the neural activities of specific neurons using microbial rhodopsin-based optogenetics tools to investigate effects on biological activities, such as memory, learning, motion, sleep and awakening.[5, 17, 31] In addition, a few of microbial rhodopsins, such as Archaerhodopsin-3 (AR3) and proteorhodopsin (PR), have been utilized as the genetically encoded voltage indicators since they exhibit voltage-dependent fluorescence changes in the cells (Table 1).[32, 33] Through numerous optogenetic analyses combined with optical observations, the causality between biological functions and chemical processes in living cells has been clearly elucidated in the neuroscience field. However, regardless of many different types of microbial rhodopsins known, only few of them have so far

been used as optogenetics tools in the neuroscience field, which limits the expansion of application using rhodopsin-based optogenetics tools (e.g. deep tissue illumination, non-neural optogenetics, and tuning and enhancing photochemistry) in the life science field. In this perspective, we introduce the achievements and summarize future challenges to produce rhodopsin-based optogenetics tools.

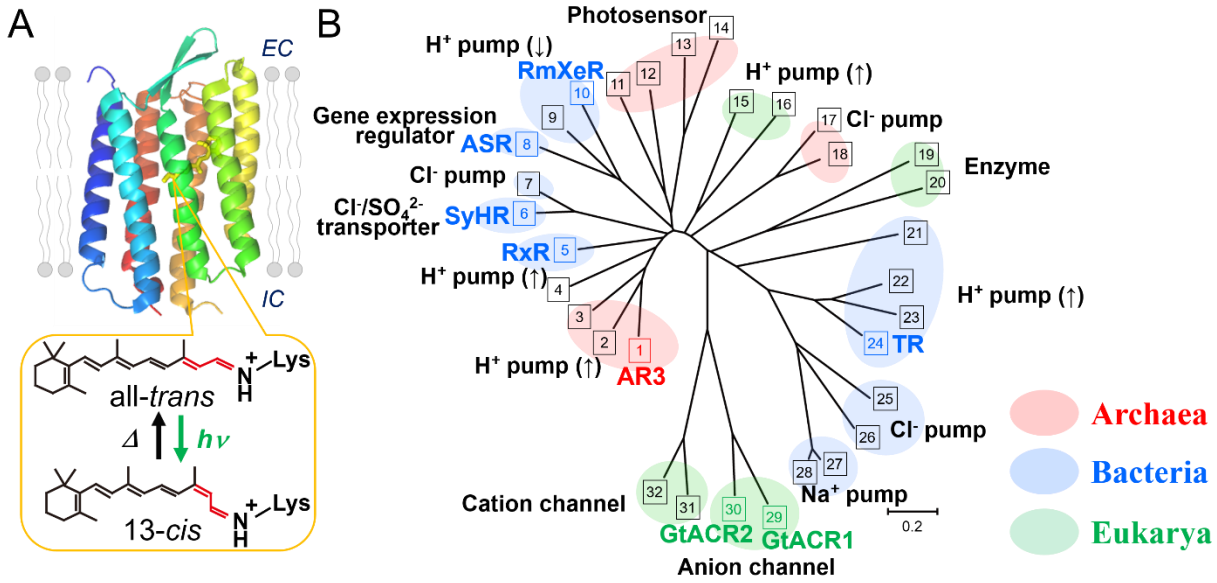

Figure 1. Introduction of diverse microbial rhodopsins.

(A) Crystal structure of bacteriorhodopsin (BR) from the archaeon *Halobacterium salinarum* (PDB code; 1C3W) as the most-studied microbial rhodopsin.[34] EC and IC indicate extracellular and intracellular sides, respectively. The retinal covalently binds to the apoprotein opsin and its isomerization from the all-*trans* to the 13-*cis* form is triggered by light (*hν*). (B) Phylogenetic tree of representative microbial rhodopsin amino acid sequences. Rhodopsins from bacteria, eukarya and archaea are indicated by ellipses filled with blue, green and red, respectively. Rhodopsins used as optogenetics tools in our research are indicated by bold characters.: Archaerhodopsin-3 (AR3), anabaena sensory rhodopsin (ASR), *Guillardia theta* anion channelrhodopsin-1 and -2 (GtACR1 and GtACR2, respectively), *Rubricoccus marinus* xenorhodopsin (RmXeR), *Rubrobacter*

xylanophilus rhodopsin (RxR), Synechocystis halorhodopsin (SyHR), and Thermophilic rhodopsin (TR). All the sequences used to construct the phylogenetic tree are listed in Supplementary Table S1. Up arrow and down arrow represent the direction of ion transportation, i.e. outward direction from IC to EC and inward direction from EC to IC, respectively.

Optical control of neural activities

After the first demonstration of optogenetics controlling neural activities using *Chlamydomonas reinhardtii* channelrhodopsins-2 (CrChR2),[6, 27-29] various natural and engineered channelrhodopsin variants with characteristic properties (e.g., fast and slow channel closing, and blue- and red-shifted spectral sensitivities) have been discovered from nature and produced by mutational analysis as neural activation tools.[35-39] As a neural silencing tool, an inward chloride pump, NpHR, was firstly demonstrated to induce hyperpolarization responses in light-dependent manner.[7, 8] After that, several ion-pumping rhodopsins and their variants (e.g. an archaeal outward proton pumping rhodopsin, AR3 and an outward sodium pump, KR2) were demonstrated as neural silencing tools.[9, 10, 40] We also have actively explored and characterized novel microbial rhodopsins from nature,[22] and we have succeeded in modifying their functions and properties as novel variant molecules.[41] By understanding and exploiting the functions and characteristics of rhodopsins, we have been proposing new optogenetics tools (Fig. 2). We term this strategy composed of 'Exploration', 'Modification' and 'Application' as a bottom-up approach. In 2012, we identified an outward proton pumping rhodopsin named Thermophilic rhodopsin (TR) from an extremely thermophilic bacterium *Thermus thermophilus*,[42] which has an extremely-high thermal stability among rhodopsins. Therefore, we expected that the high stability of TR would allow it to be

functionally expressed without denaturation and misfolding in animal cells. To confirm this, we attempted to use TR as a neural silencing tool in living *C. elegans* (Fig. 2, upper and lower panels).[12] TR was well-expressed in neurons as we expected and could indeed cause locomotion paralysis by silencing neural activities. A similar result has been obtained with AR3.[43] Next, we used ion channeling rhodopsins as neural silencing tools. In general, upon illumination, ion pumping rhodopsins transport only one ion during each photocycle whereas ion channeling rhodopsins transport numerous ions, resulting in highly efficient hyperpolarization responses in neurons. In addition, proton pumping rhodopsins such as AR3 move a proton across the cell membrane and their long-term activation may decrease the extracellular pH, which would induce undesired secondary effects in neurons, suggesting that other substrate ions (i.e. Cl^- and Na^+) are favorable for neural silencing. In 2015, Spudich and coworkers discovered two light-gated anion channelrhodopsins (GtACR1 and GtACR2) from the cryptophyte algae *Guillardia theta* and demonstrated that GtACRs selectively conduct monovalent anions such as Cl^- and generate a larger photocurrent (approximately 1000-fold) than AR3 in mammalian cells.[11] Therefore, GtACRs are expected to be much more efficient silencing tools without pH changes as a secondary effect.[44, 45] We used GtACR1 and GtACR2 as neural silencing tools in neurons of *C. elegans* and mice.[46, 47] GtACR1 and GtACR2 could cause locomotion paralysis at around 1 $\mu W/cm^2$ light intensity, which is three orders of magnitude smaller than the light intensity required by AR3 (1 mW/cm^2).[46] In mouse neurons, GtACR2 also showed efficient and temporally-stable neural silencing effects.[47] In addition, GtACRs are less harmful to worms under long-term illumination.[46] Those studies demonstrated that GtACR1 and GtACR2 work as hypersensitive and harmless neural silencing tools in most neural regions (Fig. 2, upper and lower panels). Note that, for specific regions such

as presynaptic terminal in the hippocampus where the intracellular Cl^- concentration is lower than the extracellular one,[48] GtACRs would not be the efficient silencing tool.

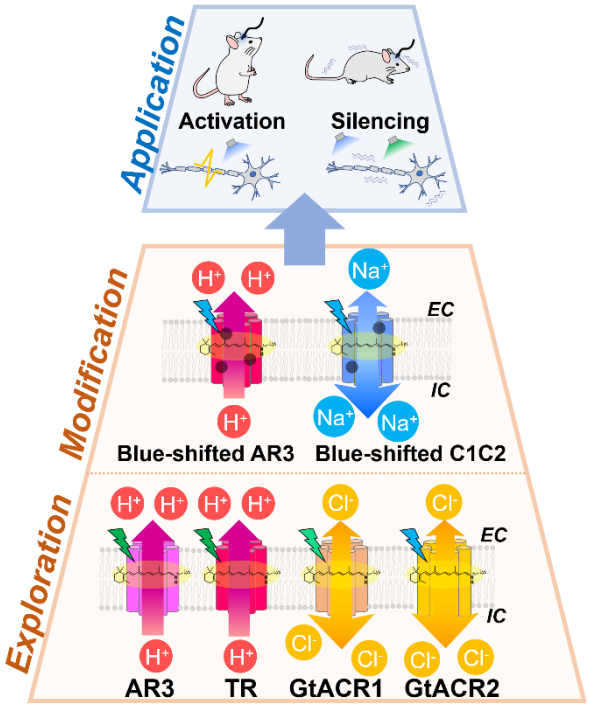

Figure 2. Optical control of neural activities.

Schematic of bottom-up approach to produce optogenetics tools to control neural activity. Bottom-up approach consists of three parts; 'Exploration', 'Modification' and 'Application'. Among the rhodopsins prepared by the 'Exploration' and 'Modification' steps in this bottom-up approach, appropriate rhodopsins for neural control are chosen in the 'Application' step. EC and IC indicate extracellular and intracellular sides, respectively.

By exploiting our ability to produce novel variants of rhodopsins with characteristic functions and properties by rationally introducing mutations, we have been using those mutant rhodopsins at optogenetics tools. As a first example, we introduce here a blue-shifted AR3 mutant.[49] Archaeal proton pumping rhodopsins such as AR3 generally absorb green and yellow

light (550 - 570 nm). We succeeded in producing a ~50 nm blue-shifted AR3 mutant by introducing several mutations around the retinal chromophore while maintaining its robust proton pumping activity (Fig. 2, middle panel). Therefore, this AR3 mutant should be useful as a blue light sensitive neural silencing tool. Using the same strategy, we also produced a blue-shifted mutant of a chimera of cation channelrhodopsins, C1C2 (Fig. 2, middle panel).[50] In fact, we could depolarize mouse neurons expressing that mutant with 445 nm blue light (Fig. 2, upper panel).

Summarizing the above studies, many achievements of our research group on the exploration and modification of novel rhodopsins has expanded the potential of rhodopsins to precisely control neural activities for optogenetics (Table 1). However, when one wants to use these novel rhodopsins in *in-vivo* brain of rodent, an optical fiber needs to be inserted into the region of interest. The reason is that the light sensitivity of the rhodopsins is not sufficient enough currently and the use of the fiber that can efficiently deliver lights reinforces such the low sensitivity. The insertion of the optical fiber is very invasive to the brain and therefore the implementation of optogenetics analysis without the fiber in the intact brain has been highly desired. In the following subsection, as a future perspective for deep-brain optogenetics without an optical fiber, we will briefly shift our focus from microbial rhodopsins to state-of-the-art optical illumination techniques.

Implementation of optogenetics deep inside the brain

Mice have deep complex brains (~5 mm) and have become one of the best-suited models to optogenetically study high-level brain functions such as memory, learning, anger and sexual behavior. However, heterogeneous structures in the brain scatters visible light severely and such

the light scattering makes the implementation of optogenetics in the deep brain impossible (Figs. 3A and 3C). In this section, we briefly explain difficulties, current strategies, and potential breakthrough towards deep-brain optogenetics based on two different illumination targets, “neural population” and “a single neuron”.

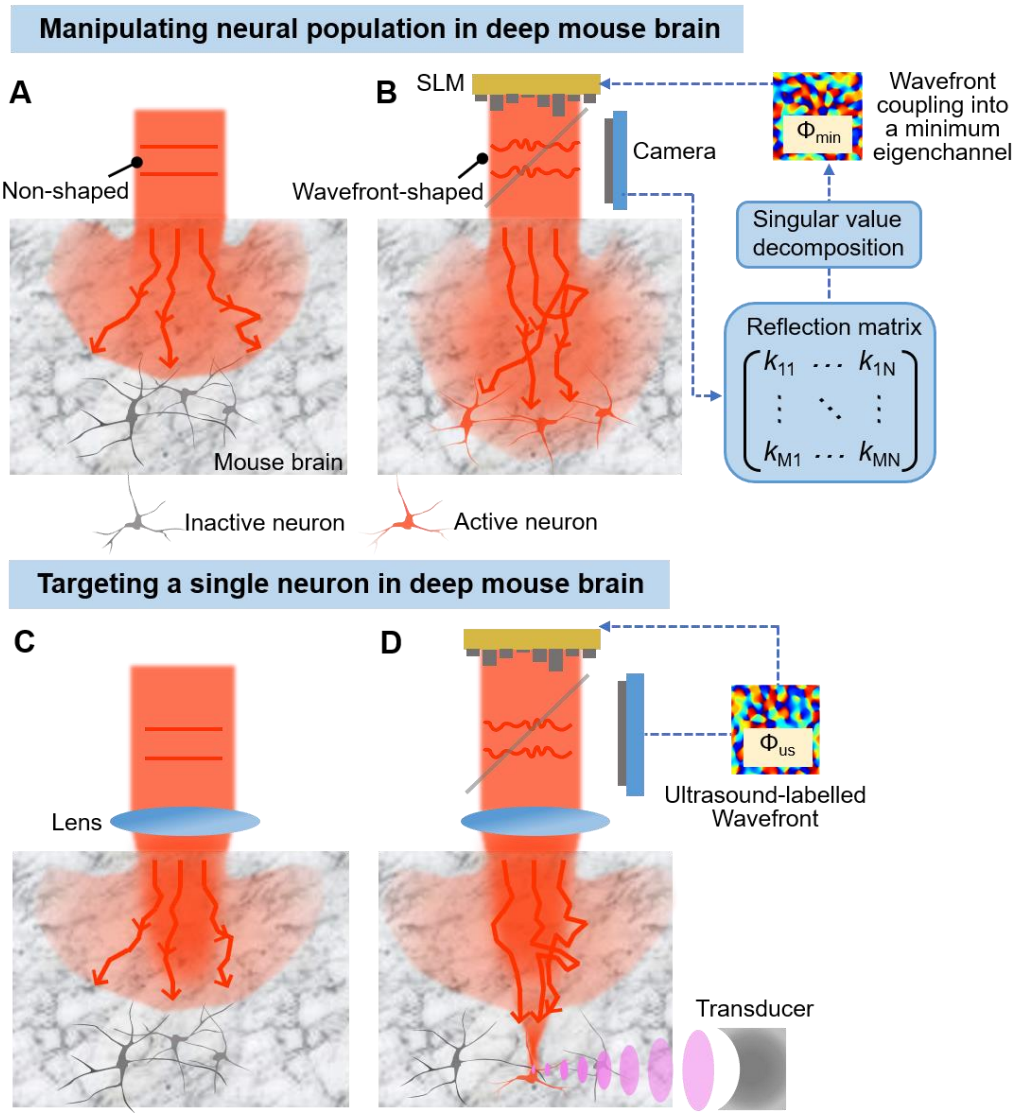

Figure 3. Optogenetics in the deep mouse brain implemented by complex wavefront shaping. (A) Non-shaped light is scattered severely within the mouse brain, preventing light from penetrating deep inside the brain. (B) Wavefront-shaped light representing a minimum eigenchannel can minimize reflected lights and can be efficiently delivered into the deep brain.

The wavefront Φ_{min} generating the minimum eigenchannel can be acquired from the single value decomposition of the reflection matrix for the brain. (C) Non-shaped light through a focusing lens makes no clear focus deep inside the brain due to the multiple scattering. (D) By modulating the wavefront Φ_{us} of ultrasound-labelled light, the wavefront-shaped light counteracts the effect of the multiple scattering and creates a tight focused spot deep inside the brain. The optimal wavefront Φ_{us} can be obtained by creating an ultrasound focus by a transducer and detecting the wavefront of ultrasound-labelled light.

i) Manipulating neural population deep inside brain

A correlation between specific neural population and behavioral response has been investigated by using diffused lights from the tip of an inserted optical fiber. In contrast, interrogating the entire mouse brain non-invasively without an optical fiber, a long-awaited dream for neuroscience researchers, has been still difficult to achieve. This is because the severe light scattering prevents incident light from being delivered efficiently into deep brain regions (Fig. 3A). Ion-channeling rhodopsins such as CrChR2 and GtACRs have been chosen as high light-sensitive molecular tools in the mouse brain since they generate a large photocurrent. However, even with the channeling rhodopsins, the deepest region at which the activity of the neural population can be controlled without the fiber is up to around 1 mm from the brain surface. As molecular approaches to extend that depth limitation, ion-channeling rhodopsins have been extensively engineered such that they exhibit a larger photocurrent or absorb red light maximally.[38, 39, 51] The most effective approach so far would be the injection of up-conversion nanoparticles (UCNP) within the region of interest since the UCNP allows near-infrared (NIR) light illumination that has a much lower scattering coefficient in the brain compared with visible light.[52, 53] However, all those current approaches are still not sufficient to non-invasively activate neural population within the whole mouse brain.

Here, as an emerging optical solution to tackle this challenge, we introduce a novel coherent illumination technique called 'complex wavefront shaping' (Fig. 3).[54-57] This class of technique has been one of the hottest topics in the photonics community since it can physically overcome the 'light scattering' that occurs within a disordered medium such as a biological tissue and it could potentially be a breakthrough for optical imaging and optogenetics deep inside biological tissues.[55, 56]

The wavefront shaping technique modulates the input wavefront of coherent light with a spatial light modulator (SLM) in a way to counteract multiple light scattering within a disordered medium. To determine the optimal input wavefront for overcoming the scattering, the input-output relationships of the medium, termed the transmission matrix (TM) or reflection matrix (RM), must be characterized by sequentially measuring output light fields for large number of independent input light fields (typically ~1000). One fascinating benefit for measuring the TM is its eigenvectors, termed eigenchannels. Since the eigenvalue of the TM represents the transmittance of the medium, an eigenchannel with a maximum eigenvalue will give the maximum attainable transmittance. The physical interpretation for this is that the optimal eigenchannel can cause strong constructive interference of scattered light at the rear side of the medium. Note that, when one wants to apply this method in *in-vivo* mouse brain where its rear side is optical inaccessible, the RM is used instead of the TM and its eigenchannel with a minimum eigenvalue can greatly reduce the reflectance and enhance the light transported into deep brain region. In the first demonstration using a ZnO nanoparticle layer, the eigenchannel from the TM could indeed enhance the transmitted light energy by a factor of around 4 compared with 'non-shaped' input light.[54] One might expect that such a result should also be expected in the mouse brain, in other words, the eigenchannel from the RM should help scattered light go deeper in the brain. However, the truth

is that eigenchannel-based wavefront shaping has never been available so far in the *in-vivo* mouse brain. The reason is that, while the measurement of the matrix requires several seconds to complete even with a high-speed camera, the temporal scattering response of the *in-vivo* mouse brain varies temporally in a millisecond timescale due to parameters such as the blood flow and the heartbeat.[58] Thus, the eigenchannel extracted from that measured matrix would no longer be valid. Unfortunately, no effective solutions that can overcome this time constraint has been proposed so far. Therefore, perhaps, we may need to wait for the emergence of an ultra-fast camera offering several hundred kHz frame rates. Once this technological challenge is overcome, it should be possible to apply the eigenchannel-based wavefront shaping in the optogenetics of *in-vivo* mouse brain (Fig. 3B). This novel wavefront shaping can be seen as the tool that enhances scattered and weakened light deep inside the brain, not as the tool that can solely accomplish the whole brain optogenetics. Perhaps, together with the wavefront shaping, using NIR light mediated by the UCNP may enable optogenetics in the whole mouse brain. If we can engineer rhodopsin-carotenoid complex that are NIR light-sensitive (see the section of 'Focusing on chromophore'), such the molecule complex can be used in the place of the UCNP.

ii) Targeting a single neuron deep inside brain

As we described before, optogenetic researchers generally aim to manipulate the activities of neural populations by using 'diffused light' (Fig. 3A). Meanwhile, manipulating a single neuron with high-spatial resolution 'focused light', termed targeted optogenetics, has also drawn attention recently[59, 60] since it may address important neuroscientific questions such as the precise definition of neural identity and the number of neurons required to influence animal behaviors.[61] In such targeted optogenetics, the applicable depth in the brain is limited by the degradation of the quality of focused light due to the light scattering. The current strategy employed for the targeted

optogenetics is a multi-photon excitation using NIR focused light since it can simply reduce the number of light scattering due to the use of the longer wavelength.[62] However, this method still relies on non-scattered light (ballistic light) and the manipulation of a single neuron at more than a 1 mm depth in the brain is intrinsically difficult (Fig. 3C).

Complex wavefront shaping is one promising technique to greatly extend this limited depth by effectively exploiting scattered light (non-ballistic light). To determine an optimal wavefront that can gather scattered light into a spot within the disordered medium, a 'focused ultrasound spot' as a guidestar needs to be placed at the target position of interest. This ultrasound spot modulates scattered light in frequency, in other words the scattered light passing through the ultrasound spot is frequency-labelled. By retrieving the wavefront of such labelled light with a camera and reconstructing that wavefront with the SLM, the shaped light will be refocused deep within the disordered medium (Fig. 3D). The ability to focus scattered light has been validated at a depth of 2.5 mm within *ex-vivo* chicken tissue.[55] More importantly, this guidestar-assisted wavefront shaping has already taken the first step in the optogenetics field, in which a single neuron has been successfully stimulated deep within the brain of a mouse.[56] Our question here is whether we can adapt the wavefront shaping into an *in-vivo* mouse brain. Fortunately, unlike measuring the whole transmission matrix, the calibration process for acquiring the optimal wavefront only requires a single snapshot with a camera. The fastest calibration time reported so far was 3 ms,[63] which is slightly slower than the scattering response (~1 ms) of the mouse brain. Therefore, within the next several years we should be able to see the implementation of guidestar-assisted wavefront shaping for manipulating a single neuron in the deep mouse brain. Such deep-brain optogenetics using wavefront shaping could be extended to rats and monkeys with larger brain volumes.

Optical control of non-neural activities

Ion concentrations are involved not only in neural activities but also in non-neural activities. As mentioned above, microbial rhodopsins are representative molecules that can actively transport various kinds of ions and interact with various signal transduction molecules in a light-dependent manner.[22] So far, rhodopsins have been utilized mainly to control "neural" activity. To overcome that situation and to further exploit the potential of optogenetics, we have been developing "non-neural" optogenetics on the basis of the characteristics of novel rhodopsins.

In 2006, we succeeded in converting the function of an archaeal outward proton pumping rhodopsin into a photosensor by strategically introducing mutations.[64] The light illumination could indeed control the motility of a previously non light-sensitive null mutant of the archaeon *H. salinarum* by expressing the photosensor variant (Fig. 4A). Although this study has been carried out for experimentally verifying the functional conversion, it may be categorized as a first example of our "non-neural" optogenetics-like work in the context of successfully manipulating the biological activity of 'non light-sensitive' cells by the heterologous expression of photoactive proteins. Moreover, as a second example, we introduced the optical regulation of gene expression in bacteria. Anabaena sensory rhodopsin (ASR) was isolated in 2003 from the cyanobacterium *Anabaena* PCC7120.[65] Although the biological function of ASR was unclear, some researchers hypothesized that ASR regulates gene expression in response to light.[66-68] To experimentally validate that hypothesis, we introduced the ASR gene and the promoter sequence of the pigment protein phycocyanin with a reporter gene into *E. coli* cells.[69] The results revealed that the light-activated ASR inhibited the reporter gene expression. In other words, we succeeded in the optical control of the arbitrary gene/protein expression in bacteria expressing ASR (Fig. 4B).

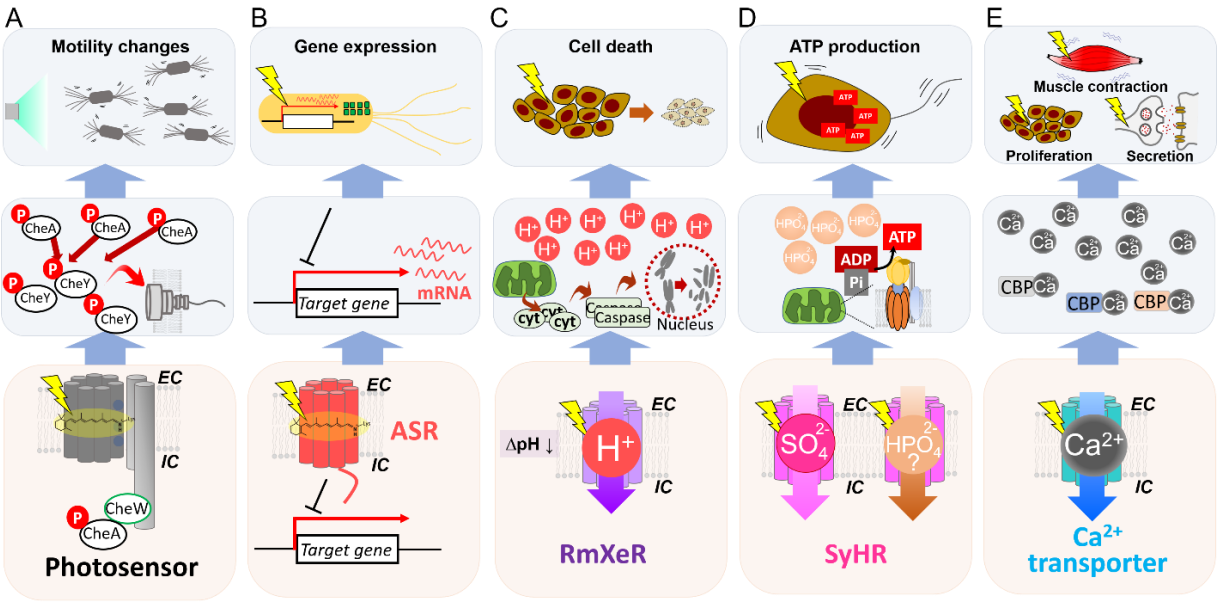

Figure 4. Optical control of non-neural activities.

(A) Optical control of the motility of archaeal cells. The engineered-photosensor variant triggers a signal transduction cascade and regulates the rotational direction of the flagellar motor. (B) Optogenetic regulation of gene/protein expression. The light-activated ASR inhibits gene expression in bacterial cells. (C) Optogenetic regulation of cell death. By intentionally decreasing the intracellular pH using the inward proton pump RmXeR, it may be possible to induce cell death. (D) Optogenetic ATP production. SyHR could be an optogenetic ATP production tool in living organisms. (E) Optogenetic regulation of Ca^{2+}-dependent biological activities. The light-driven Ca^{2+} transporter may enable us to control numerous biological activities such as proliferation, secretion and muscle contraction. Upper, middle and lower panels represent the schematic images of the functions of individual rhodopsins, those of biological and chemical reactions induced by the rhodopsins in the cells, and those of the activities of the cells and organisms, respectively. EC and IC indicate extracellular and intracellular sides in all panels, respectively.

Furthermore, the ability of rhodopsins to transport various ions can also be exploited to regulate non-neural activities. Recently, we identified two novel ion-transporting rhodopsins. The first such rhodopsin is RmXeR from a marine bacterium, *Rubricoccus marinus*, which we characterized as a light-driven inward proton pump (Fig. 4C).[70] It has been reported that intracellular acidification induces an apoptosis signal (e.g. release of cytochrome C from mitochondria, caspase activation and nuclear fragmentation) in mammalian cells.[71] Hence, by intentionally decreasing the intracellular pH inside cells using the inward proton pump RmXeR, it may be possible to induce cell death. Therefore, RmXeR could be an optogenetic regulator of cell death in living organisms (Fig. 4C). The second such rhodopsin is SyHR from a cyanobacterium *Synechocystis* sp. PCC 7509.[72] In the situation where transportable substrate ions have been believed to be limited to monovalent ions, we demonstrated that SyHR transports a divalent polyatomic sulfate ion (SO_4^{2-}) (Fig. 4D), indicating the possibility that SyHR controls intracellular SO_4^{2-} concentration. SO_4^{2-} is known to be involved in a variety of activation and detoxification processes of many compounds (e.g. steroids, catecholamines and isoproterenol) for biotransformation and excretion in mammalian cells.[73] Therefore, the optical control of intracellular sulfate ion concentration using SyHR should be useful to regulate those biological functions. In addition, SyHR may transport other divalent polyatomic ions such as HPO_4^{2-} (Fig. 4D). In living cells, the rate of ATP synthesis depends on the intracellular HPO_4^{2-} concentration because the phosphoric acid of HPO_4^{2-} is utilized for ATP synthesis.[74] ATP provides energy to drive almost all processes in living cells. Thus, SyHR could be an optogenetic energy regulation tool in living organisms (Fig. 4D). On the other hand, rhodopsin-based Ca^{2+} transporters should be significantly valuable since changes in Ca^{2+} concentration would trigger numerous intracellular signaling cascades such as muscle contraction, cell proliferation, cell death (apoptosis), cell

differentiation, secretion of neurotransmitters and gene expression.[75, 76] CrChR2 is permeable to Ca^{2+} and improved mutants with higher Ca^{2+} conductance has been reported,[3, 37, 77] however, Ca^{2+} permeability of the mutants was still lower than those of other cations such as Na^{+} and K^{+}. We are now aiming to characterize a native light-driven highly Ca^{2+}-selective transporter and to produce an artificial one by introducing mutations in cation transport rhodopsins such as sodium pumping rhodopsin and cation channelrhodopsin (Fig. 4E).

Focusing on the chromophore

As described above, the classic strategy to alter the functions and properties of rhodopsins is to genetically engineer the protein moiety, opsin.[41] In this section, we describe our efforts focusing on the chromophore as an alternative strategy. The spectral sensitivity of rhodopsins significantly depends on the structure of the chromophore in the opsin.[78] Indeed, it is known that retinal analogs cause spectral shifts in many rhodopsins.[79-81] We also succeeded in functional conversion by the replacement of the retinal chromophore. Specifically, we produced a light-gated proton channeling rhodopsin with a leaky outward proton pump activity from an outward proton pumping rhodopsin by replacing the natural chromophore (A1- and A2-retinal) of wild-type AR3 with a retinal analogue, C(14)-vinylene A2-retinal (Fig. 5A, lower panel).[81] The proton channel induces hyperpolarization or depolarization depending on the electrochemical gradients across the cell membrane, whereas the leaky proton pump activity induces hyperpolarization. Therefore, the produced proton channel with the leaky outward proton pump could be utilized as hyperpolarization and depolarization tool in neurons (Fig. 5A, upper panel). However, the passive and active photocurrents of the proton channel were as small as the current of typical proton pumps

Thus, the increase of photocurrents is required for its application as optogenetics tools. Thus, using a retinal analog as the chromophore would provide the ability to modulate the absorption maximum and functions of rhodopsins as optogenetics tools (Fig. 5A).

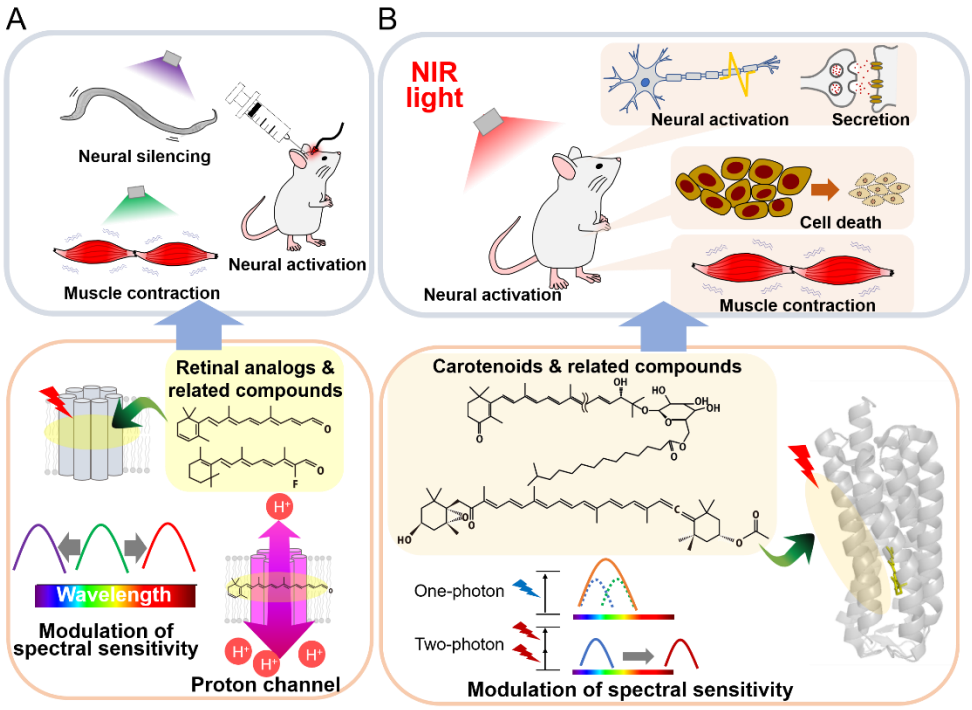

Figure 5. Modulating the spectral sensitivity and functions of microbial rhodopsins by retinal analogs and secondary chromophore attachment.

(A) Modification of spectral sensitivities by replacement with synthetic retinal analogs. The retinal analogs modulate the absorption maximum of rhodopsins according to their structural characteristics. The retinal analog, C(14)-vinylene A2-retinal, converts a proton pump into a proton channel with a leaky proton pump activity. (B) Modification of spectral sensitivities by attaching a carotenoid as a second chromophore. The attachment of an appropriate carotenoid may allow us to produce NIR light-sensitive rhodopsins. The NIR light-sensitive rhodopsin-carotenoid complex with the wavefront shaping technique can be utilized for optogenetics deep inside biological tissues. Lower and upper panels represent the schematic images of modification of

rhodopsins by retinal analogs and secondary chromophore attachment and their application in the cells and organisms, respectively.

In addition to replacing the chromophore retinal, we are trying to modify the spectral sensitivity by attaching a carotenoid as a secondary chromophore. Recently, many enzymes which are related with the production of carotenoids have been identified and the heterologous production of target carotenoids from its precursors (e.g. β-carotene and lycopene) has been achieved in *E. coli* cells and yeasts.[82, 83] For the case of mammals, β-carotene and lycopene exist in blood serum,[84] which could ubiquitously supply them to the cells in the whole body. Using the same strategy, we speculate that the specific carotenoids can be heterologously produced from its precursors without their direct addition in mammalian cells by genetically expressing the enzymes. Carotenoids generally absorb light at wavelengths ranging from 400 to 500 nm (violet to green light),[85] after which the light energy absorbed by the carotenoid as the second chromophore is transferred to the first chromophore, retinal. Since rhodopsins mainly absorb light at wavelengths of 500 to 600 nm, the attachment of the antenna carotenoid is expected to extend the absorbed wavelength range and increase the absorption cross-section for rhodopsin activation (Fig. 5B, lower panel). In fact, an outward proton pump rhodopsin named xanthorhodopsin from the bacterium *Salinibacter ruber* forms a complex with the carotenoid salinixanthin (SX) in nature.[86] In a collaboration with Dr. Sheves, we demonstrated that SX also binds TR and the light energy absorbed by SX is transferred to the retinal in the complex.[87] Thus, the attachment of an antenna carotenoid can be an effective approach to expand the wavelength range at which rhodopsins are activatable. Interestingly, some carotenoids have been proven to have an attractive ability, two-photon absorption (excitation) of NIR light.[88] Therefore, the attachment of such carotenoids as the second chromophore may allow

us to produce NIR light-sensitive rhodopsins. Since NIR light is highly transparent in biological tissues, using a NIR light-sensitive rhodopsin-carotenoid complex is a promising molecular tool for optogenetics deep inside biological tissues (Fig. 5B, upper panel). The wavefront shaping technique (Fig. 3) can be combined with the NIR light-sensitive complex, which may enable optogenetics in the whole body.

Expansion of optogenetics

Our research group and others have demonstrated the great potential of microbial rhodopsins as optogenetics tools in bacteria, archaea and animals. On the other hand, there has been no report that microbial rhodopsins are useful in plants. Ion concentrations are well-known to be related to various biological activities of plants as well as animals and microbes.[89, 90] As a representative example, acidification of the inside of thylakoid membranes is generated by the photosynthetic activity of a photosynthetic electron transport chain consisting of photosystem I (PSI), photosystem II (PSII) and the cytochrome b_6f complex.[91] The proton gradient across thylakoid membranes drives the synthesis of ATP by coupling with ATP synthase. We expect that RmXeR as an inward proton-transporting rhodopsin can be used to produce proton gradients across thylakoid membranes that promote the synthesis of ATP (Fig. 6A, lower panel). Actually, BR expressed in the inner mitochondrial membrane could produce proton gradients across the membrane as produced by the respiratory chain for ATP synthesis in *Schizosaccharomyces pombe*.[92] The chromophore chlorophyll and carotenoids contained in PSI and PSII mainly absorb blue and red light (400 – 450 nm and 600 - 700 nm for chlorophyll, and 400 – 500 nm for carotenoids) for photosynthesis, while green light ranging from 500 - 600 nm is less utilized as an

energy source in plants, which is called the “green-gap” and is a crucial characteristic when expressing RmXeR that absorbs green light maximally. Hence, transgenic plants whose thylakoid membranes functionally express RmXeR are expected to utilize not only blue and red light but also green light for their photosynthesis (Fig. 6A, upper panel). In regions where sunlight is only available within a short period, many plants cannot produce sufficient amounts of ATP. This problem can be solved by using transgenic plants that can effectively exploit the energy of green light. We believe that creating vegetables that can produce ATP with green light would greatly expand farmland areas to improve food production.

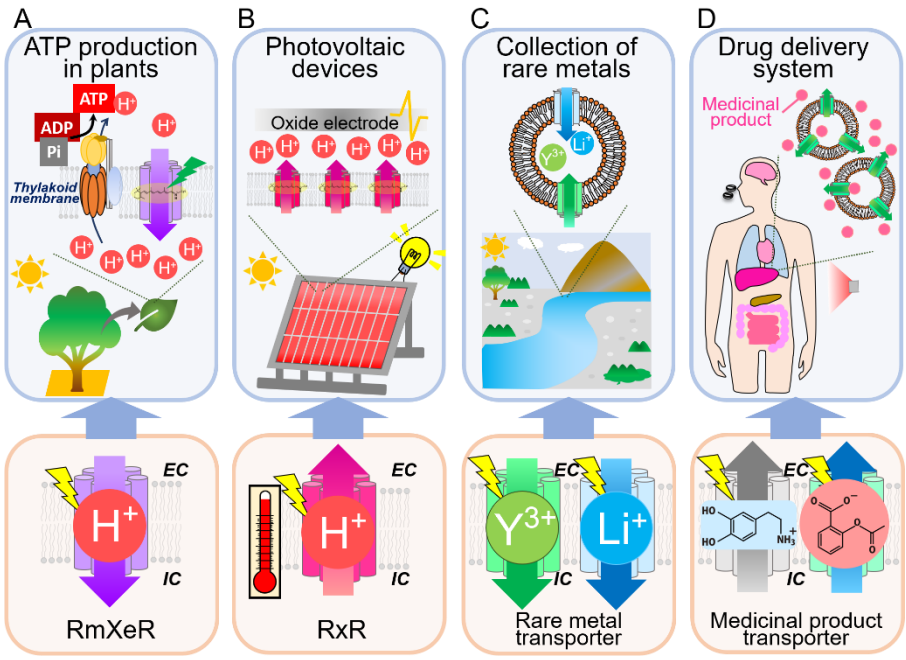

Figure 6. Unlimited possibilities of microbial rhodopsins.

(A) Green light induces ATP production in the chloroplasts of plants. Transgenic plants expressing RmXeR on their thylakoid membranes are expected to utilize green light for photosynthesis. (B) Rhodopsin-based photovoltaic devices. A photovoltaic device consisting of RxR and a conductive oxide electrode induces transient photocurrents. (C) Rhodopsin-based method to efficiently collect rare metals such as Li^+ and Y^{3+}. Long-term light illumination keeps activating rare metals-

transportable rhodopsin and rare metals are stored and enriched inside rhodopsin-expressing membranes. (D) Rhodopsin-based drug delivery system (DDS). Medicinal products and supplements are released only upon illumination. As representative medicinal products, dopamine and aspirin are shown. Lower and upper panels represent the schematic images of the functions of individual rhodopsins and those of their application, respectively. EC and IC indicate extracellular and intracellular sides, in all panels, respectively.

From the viewpoint of physical chemistry, we introduce here the potential of rhodopsins (Figs. 6B and 6C). Some researchers have produced photovoltaic devices consisting of BR-expressing purple membranes from *H. salinarum* and conductive oxide electrodes such as SnO_2 and indium-tin oxide.[93-95] The pH response of the surface oxide electrode due to proton release/uptake by BR can induce transient photocurrents in these photovoltaic devices.[95, 96] In 2017, we identified an extremely thermally-stable rhodopsin (RxR) from the bacterium *Rubrobacter xylanophilus* (Fig. 6C, lower panel).[97] RxR works as an outward proton pumping rhodopsin as well as BR. As far as we know, RxR is the most stable rhodopsin in nature and maintains its activity even at 85°C. Such an extremely high stability allows RxR to survive even in the abiotic environments (e.g. high temperature, salt-free and anhydrous environments) of photovoltaic devices. Therefore, we expect that RxR can be an alternative to BR for application to new photovoltaic devices (Fig. 6B, upper panel).

As mentioned above, we would like to modulate ion selectivity by introducing mutations in cation and anion transporters. As part of such a challenge, we would like to produce light-driven inward pumps of rare metal ions such as Li^+, Y^{3+} and Nd^{3+} (Fig. 6C, lower panel). Because rare metals are utilized for the production of electric devices and cars, developing a way to efficiently

collect rare metals from nature are highly desired.[98] If they exist or if we can produce a rhodopsin that inwardly pumps rare metal ions, we could collect rare metals from nature using proteoliposomes containing an inward rare metal pumping rhodopsin, which would result in the enrichment of rare metals (Fig. 6C, upper panel).

Finally, we introduce the possible applications of rhodopsins in humans from the viewpoint of medical and pharmaceutical sciences. As well as ions, medicinal products and supplements (e.g. nucleotides, hormones and related small compounds) often possess positively and negatively charged groups such as dopamine and aspirin (Fig. 6D, lower panel). We would like to produce a rhodopsin-based transporter by introducing mutations in SyHR having a large ion transport pathway. As a rhodopsin-based drug delivery system (DDS),[99, 100] we propose the application of proteoliposomes containing substrates (medicinal products and supplements) with the engineered-transporter. The substrates within proteoliposomes would be released only upon irradiation, which would allow us to control the timing of substrate release and the amounts of released substrates in the body by light (Fig. 6D, upper panel). Therefore, such rhodopsin-based drug delivery systems would enable us to precisely control the release and administration of medicinal products and supplements in specific regions of the body without undesired side effects.

Conclusion

In this perspective, we discussed challenges in designing and producing novel rhodopsin-based optogenetics tools to optically control a wide variety of biological functions in animals, bacteria, archaea and plants. We also considered unlimited future applications of rhodopsins in agriculture, physicochemical, medical and pharmaceutical sciences. Thus, a colorful future is ongoing with rhodopsins in science.

Supporting Information

Table S1: The lists of GenBank accession numbers of amino acid sequences used to construct the phylogenetic tree of Figure 1B with their source organism names.

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Notes

All authors declare that they have no conflict of interest.

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ABBREVIATIONS

AR3, Archaerhodopsin-3; ASR, Anabaena sensory rhodopsin; BR, bacteriorodopsin; CrChRs, *Chlamydomonas reinhardtii* channelrhodopsins; DDS, drug delivery system; GtACR1, *Guillardia theta* anion channelrhodopsin-1; GtACR2, *Guillardia theta* anion channelrhodopsin-2; RmXeR, *Rubricoccus marinus* xenorhodopsin; RxR, *Rubrobacter xylanophilus* rhodopsin; SLM, spatial light modulator ; SyHR, *Synechocystis* halorhodopsin; SX, salinixanthin; TR, Thermophilic rhodopsin.

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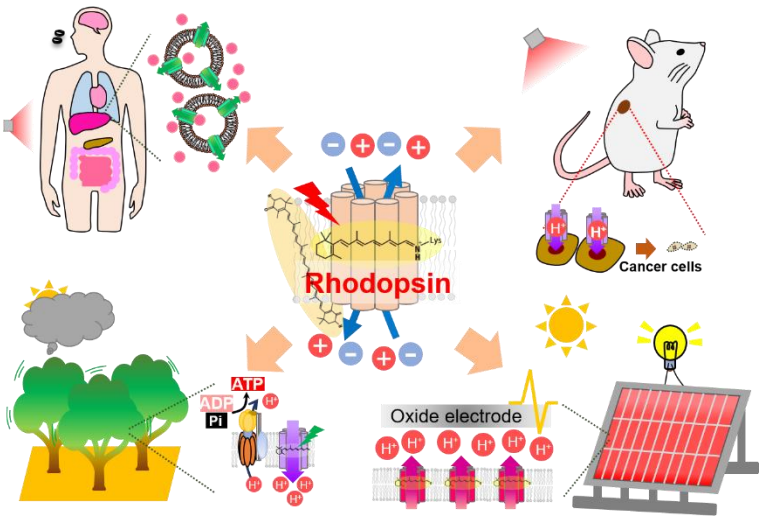

Rhodopsins will open new avenues not only as tools for controlling neural activity, but also as materials in agriculture, physicochemical, medical and pharmaceutical sciences.