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CELL SCIENCE AT A GLANCE

# Rhodopsins at a glance

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## ABSTRACT

Rhodopsins are photoreceptive membrane proteins consisting of a common heptahelical transmembrane architecture that contains a retinal chromophore. Rhodopsin was first discovered in the animal retina in 1876, but a different type of rhodopsin, bacteriorhodopsin, was reported to be present in the cell membrane of an extreme halophilic archaeon, *Halobacterium salinarum*, 95 years later. Although these findings were made by physiological observation of pigmented tissue and cell bodies, recent progress in genomic and metagenomic analyses has revealed that there are more than 10,000 microbial rhodopsins and 9000 animal rhodopsins with large diversity and tremendous new functionality. In this Cell Science at a Glance article and accompanying poster, we provide an overview of the diversity of functions, structures, color discrimination mechanisms and optogenetic applications of these two rhodopsin families, and will also highlight the third distinctive rhodopsin family, heliorhodopsin.


**KEY WORDS: Animal rhodopsin, Microbial rhodopsin, Optogenetics, Photocycle, Retinal, Rhodopsin structure**

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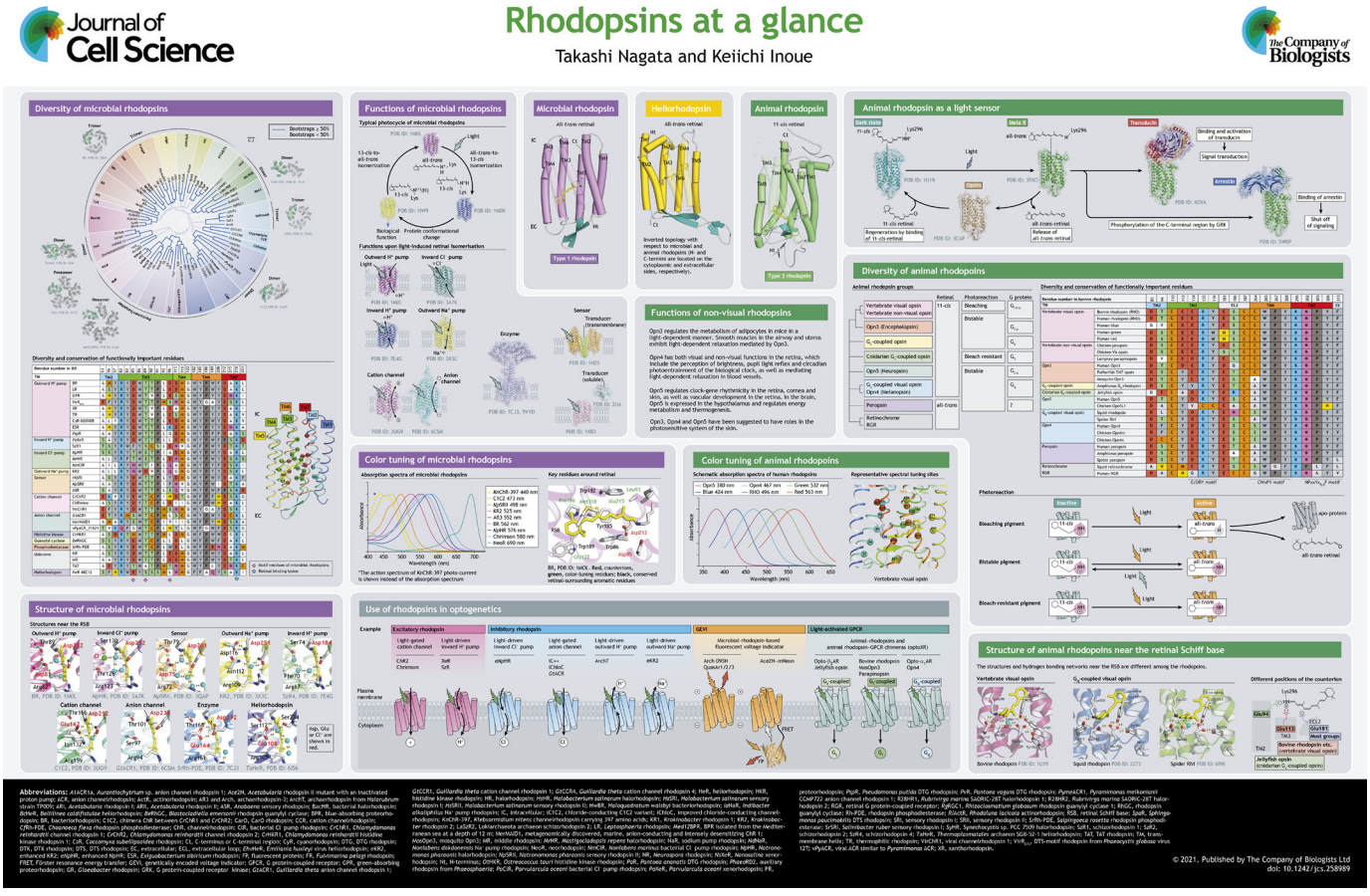
## Introduction

Rhodopsins are photoreceptive membrane proteins to which a retinal chromophore is covalently bound; they have a common architecture, with N- and C-termini facing the extracellular and cytoplasmic sides, respectively, and seven transmembrane helices (TM1–TM7) (Ernst et al., 2014; Govorunova et al., 2017; Rozenberg et al., 2021). Rhodopsin was first reported as a pinkish photoreactive substance in the animal retina (Boll, 1876), and the word ‘rhodopsin’ was coined from the Greek words ‘rhódon’ and ‘opsis’ meaning rose and sight, respectively. Animal rhodopsins are members of the G protein-coupled receptor (GPCR) family (Dixon et al., 1986), and they absorb photons and convert the information



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into electrophysiological signals, a primary event in vision. A different type of heptahelical-transmembrane protein binding a retinal chromophore was found in an extreme halophilic archaeon, *Halobacterium salinarum* (formerly *Halobacterium halobium*) and was named bacteriorhodopsin (BR) (Oesterhelt and Stoeckenius, 1971). BR converts light into electrochemical energy by outwardly transporting $H^+$ in a light-dependent manner (Oesterhelt and Stoeckenius, 1973). Following the discovery of BR, homologous proteins have been identified in diverse microorganisms, and together this group is named microbial rhodopsins; microbial and animal rhodopsins are also referred to as Type 1 and Type 2 rhodopsins, respectively (Spudich and Jung, 2005) (see poster).

The extremely rapid progress of genomic and metagenomic analyses in recent decades has led to the identification of a large number of homologous proteins with different functions in diverse species. In addition, rhodopsins have received widespread attention as optogenetic tools to manipulate neural activity and other physiological processes. Here, we review our current understanding of both types of rhodopsins from a functional, molecular and optogenetic point of view.

## Molecular functions and diversity of microbial rhodopsins

In addition to BR, *H. salinarum* has three additional rhodopsins – halorhodopsin (HR), an inward $Cl^-$ pump (Matsuno-Yagi and Mukohata, 1977; Schobert and Lanyi, 1982), and sensory rhodopsins (SRI and SRII) (Bogomolni and Spudich, 1982; Spudich et al., 1986; Takahashi et al., 1985; Tomioka et al., 1986) (see poster). SRI mediates a positive phototactic response toward orange light and also a negative response toward near-UV light under orange background (Spudich and Bogomolni, 1984), whereas SRII is a negative phototactic sensor for blue light (Spudich et al., 1986; Tomioka et al., 1986). While similar microbial rhodopsins exist in various halophilic archaea (Ihara et al., 1999), a homologous opsin gene (*Neurospora* Opsin-1) was identified in the filamentous fungus *Neurospora crassa* (Bieszke et al., 1999). *Neurospora* Opsin-1 can bind retinal to form the photo-reactive pigment (*Neurospora* rhodopsin, NR); however, the molecular function of NR is still unknown, although it has been suggested to be involved in sexual reproduction (Wang et al., 2018). Beyond NR, there are dozens of fungal rhodopsin genes that have been identified to date (Brown, 2004; Wang et al., 2018). Among them, rhodopsins from *Leptosphaeria maculans* (LR) and *Fusarium fujikuroi* (CarO) work as outward $H^+$ pumps (García-Martínez et al., 2015; Idnurm and Howlett, 2001; Waschuk et al., 2005).

The rapid development of metagenomic analysis techniques has provided genetic information on microbial rhodopsins in uncultured microorganisms. One of the main findings by these approaches was the discovery of proteorhodopsin (PR) in marine bacteria that are abundant in oceans (Béjà et al., 2000). PR is an outward $H^+$ pump, like BR, but its cytoplasmic $H^+$ donor is a glutamic acid homologous to Asp96 of BR, one of the three residues, along with Asp85 and Thr89, in TM3 that have distinctive and important functions as part of three-residue motifs (the DTD and DTE motifs for BR and PR, respectively; see poster). Extensive genomic and metagenomic analyses have revealed that several thousands of bacteria have PR-like genes (de la Torre et al., 2003; Koh et al., 2010; Pushkarev and Béjà, 2016; Rusch et al., 2007; Sabehi et al., 2007; Venter et al., 2004; Yoshizawa et al., 2012). Interestingly, some of them do not have an acidic residue at the BR Asp96 position. For example, *Exiguobacterium* rhodopsin (ESR) is a $H^+$-pumping rhodopsin with a lysine residue at this position (Petrovskaya et al., 2010), which functions as a $H^+$ donor (Balashov et al., 2013). Furthermore, a new clade of $H^+$-pumping rhodopsins harbor a glycine or serine residue at the same position (Harris et al., 2015; Maliar et al., 2020; Sudo and Yoshizawa, 2016). In this case, $H^+$ binds directly to the retinal Schiff base (RSB) from the cytoplasmic bulk phase rather than from a specific $H^+$ donor residue (Harris et al., 2015; Maliar et al., 2020; Sudo and Yoshizawa, 2016). Xanthorhodopsin (XR) is an outward $H^+$-pumping rhodopsin from the extremely halophilic bacterium *Salinibacter ruber* that binds a carotenoid (salinixanthin) on the surface of transmembrane helices (Balashov et al., 2005; Luecke et al., 2008). Resonance energy transfer occurs from salinixanthin to retinal upon light absorption, leading to the isomerization of retinal, similar to when it absorbs light (Polívka et al., 2009). As such, XR can use the energy of light of a wider wavelength range for $H^+$ transport compared with other $H^+$ pumps that bind only to the retinal chromophore.

Moreover, a variety of ion-pumping rhodopsins other than $H^+$ pumps have been described. A new type of inward $Cl^-$ pump with an NTQ motif has been identified in marine bacteria (Inoue et al., 2014; Yoshizawa et al., 2014). In addition, cyanobacteria and marine bacteria belonging to the family *Rhodothermaceae* include another distinct group of $Cl^-$ pumps with TSD, TTD or TSA motifs (Hasemi et al., 2016; Nakajima et al., 2018; Niho et al, 2017). The first two residues of the motifs of $Cl^-$ pumps are involved in the $Cl^-$ binding in the dark state (Besaw et al., 2020; Hosaka et al., 2016; Kim et al., 2016; Kolbe et al., 2000; Kouyama et al., 2010; Yun et al., 2020).

The first $Na^+$-pumping rhodopsin (KR2) was discovered in the marine flavobacterium *Krokinobacter eikastus* (Inoue et al., 2013), and since then, many closely related $Na^+$-pumping rhodopsins have been identified in various bacteria (Balashov et al., 2014; Mamedov et al., 2016; Tsunoda et al., 2017; Yoshizawa et al., 2014). They constitute a subgroup, NaR, containing an NDQ motif.

*Anabaena* (Nostoc) sp. PCC7120 harbors a unique rhodopsin, *Anabaena* sensory rhodopsin (ASR), which is coupled to a cognate soluble transducer ($ASR_T$) to regulate the expression of light-harvesting and circadian clock proteins in a light-dependent manner (Irieda et al., 2012; Jung, 2007; Jung et al., 2003). Xenorhodopsins (XeRs) are a subgroup of microbial rhodopsins closely related to ASR (Ugalde et al., 2011), and most of them function as inward $H^+$ pumps (Inoue et al., 2016, 2018; Shevchenko et al., 2017). In addition, different types of inward $H^+$ pumps, including schizorhodopsin (SzR) and AntR, have been identified in the genome of Asgard archaea, a superphylum containing the closest living relatives to eukaryotes, reconstructed by assembling metagenomic sequences (metagenome-assembled genomes, MAGs), as well as in metagenomic contigs sampled from Antarctic freshwater lakes (Bulzu et al., 2019; Harris et al., 2020; Inoue et al., 2020; Kojima et al., 2020b).

New rhodopsins that passively transport various cations according to their electrochemical potential, the light-gated cation channelrhodopsins (ChRs), have been identified in the genome of the green alga *Chlamydomonas reinhardtii* (Nagel et al., 2002, 2003); the two ChRs from *C. reinhardtii* were demonstrated to mediate phototaxis by generating depolarizing photocurrents in algal plasma membranes (Sineshchekov et al., 2002). Apart from the ChRs in *C. reinhardtii*, many homologous ChRs from diverse algae have been characterized (Kianianmomeni et al., 2009; Klapoetke et al., 2014; Schneider et al., 2015; Zhang et al., 2011) (see poster and below). In addition, anion-selective ChRs have been artificially constructed (Berndt et al., 2014; Wietek et al., 2014) by designed mutagenesis of ChRs based on the X-ray crystallographic structure (Kato et al., 2012) in order to inhibit neural activity by light-gated $Cl^-$ influx. Natural anion-channelrhodopsins (ACRs)

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have also recently been discovered in the genome of the cryptophyte *Guillardia theta* (Govorunova et al., 2015), and since then, new subfamilies of anion-selective channelrhodopsins distinct from cryptophyta ACR have been identified in the genomes of diverse algae and giant viruses, as well as in metagenome sequences (Govorunova et al., 2020; Oppermann et al., 2019; Rozenberg et al., 2020).

In some algae and fungi, microbial rhodopsins fused with various types of enzymes on the C-terminal side have been identified and named enzymerhodopsins (Mukherjee et al., 2019). Histidine-kinase rhodopsins (HKRs), the enzymerhodopsins first identified in the genome of *C. reinhardtii*, contain a rhodopsin domain fused with a histidine kinase domain and a response regulator domain on the C-terminal side (Awasthi et al., 2020; Kateriya et al., 2004). Some HKRs contain additional adenylyl or guanylyl cyclase domains whose activities are downregulated by light sensed by the rhodopsin domains (Awasthi et al., 2020; Kateriya et al., 2004; Luck et al., 2012; Tian et al., 2018). Rhodopsins fused with a guanylyl-cyclase domain (RGCs, also called RhGC and CyclOp) and a phosphodiesterase (Rh-PDE) have been identified in fungi and choanoflagellates, respectively (Scheib et al., 2015; Yoshida et al., 2017). Rh-GC and Rh-PDE are relevant in the phototactic behavior of zoospores (Avelar et al., 2014) and light-dependent inversion of cap-shaped colony of cells (Brunet et al., 2019), respectively. Rh-GC increases cGMP levels under illumination, whereas Rh-PDE exhibits enhanced hydrolysis of cAMP or cGMP under illumination, with substantial basal activity in the dark and different specificities for each protein (Scheib et al., 2018; Sugiura et al., 2020; Yoshida et al., 2017).

## Structure of microbial rhodopsins

Most microbial rhodopsins are composed of seven transmembrane helices (TM1–TM7) and an all-*trans*-retinal chromophore covalently bound to a conserved lysine residue in TM7 (Lys216 in BR) via a Schiff-base linkage (Ernst et al., 2014) (see poster). Enzymerhodopsins have an additional TM (TM0) on the N-terminal side (Ikuta et al., 2020). The Schiff base is generally protonated to shift the absorption of the retinal chromophore into the visible region (Fujimoto et al., 2010), and the positive charge is stabilized by two negatively charged aspartate counterions in outward $H^+$-pumping rhodopsins (Asp85 and Asp212 in the case of BR) (Luecke et al., 1999). These counterions are altered in other microbial rhodopsins depending on their functions (see poster). In $Cl^-$-pumping rhodopsins, $Cl^-$ works as a counterion instead of the Asp85 in BR (Besaw et al., 2020; Hosaka et al., 2016; Kim et al., 2016; Kolbe et al., 2000; Kouyama et al., 2010; Yun et al., 2020). In $Na^+$-pumping rhodopsins, the position of the counterion in TM3 is displaced to the cytoplasmic side by one α-helical turn (Inoue et al., 2013; Kato et al., 2015a). In the inward $H^+$-pumping rhodopsins (XeR and SzR), ACR and HeR, only one counterion exists (Higuchi et al., 2021; Kim et al., 2018; Shihoya et al., 2019; Vogeley et al., 2004). In ChR and Rh-PDE, a glutamate residue substitutes for Asp85 of BR and is directly hydrogen-bonded with the RSB (Ikuta et al., 2020; Kato et al., 2012; Volkov et al., 2017). These examples highlight that the RSB region plays a critical role in determining the function of microbial rhodopsins.

Microbial rhodopsins exhibit different oligomeric states (see poster). While the oligomeric states do not correlate with molecular functions (microbial rhodopsins with the same function may exhibit different oligomeric states and vice versa), phylogenetically close rhodopsins exhibit the same oligomeric structure (Shibata et al., 2018). Microbial rhodopsins from halophilic archaea and phylogenetically close XeRs from bacteria are known to exist as trimers [SRs without halobacterial transducers (Htrs) also form trimers] (Kolbe et al., 2000; Kouyama et al., 2010; Luecke et al., 1999; Shevchenko et al., 2017; Shibata et al., 2018). Most bacterial ion-pumping rhodopsins, including PR, ESR, XR, ClR and NaR, mainly form pentamers, but a mixture of pentamers and hexamers has been observed for PR (Klyszejko et al., 2008; Shibata et al., 2018). ChR, ACR and enzymerhodopsins that are from eukaryotes form dimers (Ikuta et al., 2020; Kato et al., 2012; Kim et al., 2018). SzR, which is distinct from other microbial rhodopsin subfamilies, exhibits a trimeric structure (Higuchi et al., 2021). Finally, dimeric structures bridged by long loops of each protomer have been observed for HeRs (Kovalev et al., 2020; Shihoya et al., 2019).

## Color tuning of microbial rhodopsins

Microbial rhodopsins exhibit a wide variety of absorption wavelengths (436–610 nm) (Govorunova et al., 2020; Inoue et al., 2021) (see poster). Controlling the absorption wavelength of rhodopsins allows the use of the most physiologically advantageous light for each species, and this feature is also exploited for multi-wavelength optogenetic manipulation or manipulation with long wavelength light, which is less affected by scattering in tissues. Recently neorhodopsin (NeoR), a member of the RGCs, was identified to have a surprisingly red-shifted absorption maximum wavelength ($\lambda_{max}$) at 690 nm and a narrow spectral peak width (Broser et al., 2020). The $\lambda_{max}$ of rhodopsin is determined by the energy gap between the electronic ground state ($S_0$) and the excited state ($S_1$) of the π-electron of the retinal chromophore. While $S_0$ has a positive charge on the Schiff-base side, it is partially transferred to the β-ionone ring side in $S_1$ (Marín et al., 2019). Hence, a negative charge on the Schiff-base and β-ionone ring side induces a blue- and red-shift of $\lambda_{max}$, respectively, and a positive charge has the opposite effect (Melaccio et al., 2012; Shtyrov et al., 2021). In addition to this electrostatic effect, the planarity and distortion of the polyene chain of the retinal chromophore and polarizability of the retinal binding pocket affect the $\lambda_{max}$ (Inoue et al., 2019; Kato et al., 2015b; Melaccio et al., 2016; Wanko et al., 2008).

Three positions are known as natural color-tuning 'switches' that regulate $\lambda_{max}$ without impeding molecular functions (Inoue et al., 2019; Man et al., 2003). The difference between an alanine (BR Ala215) and a threonine (Thr204 in SRII from *Natronomonas pharaonis*) in TM7 has been shown to contribute to the difference in absorption wavelength (9 nm out of a total difference of 70 nm) between BR and SRII (Shimono et al., 2001). In addition, mutations of this residue in other ion-transporting rhodopsins from alanine to threonine, serine or cysteine induce a blue-shift (Engqvist et al., 2015; Sudo et al., 2013), and mutations in the opposite direction (serine or cysteine to alanine) result in a longer $\lambda_{max}$ (Inoue et al., 2019; Kojima et al., 2020a). Green-light-absorbing proteorhodopsins (GPRs; $\lambda_{max}$=~520 nm), which are abundant in surface-water bacterial species, have leucine or methionine at the position of Leu93 in TM3 of BR (Gómez-Consarnau et al., 2007; Man et al., 2003). The replacement of this residue with glutamine shifts the $\lambda_{max}$ to ~500 nm, as, for instance, in blue-absorbing proteorhodopsins (BPRs) (Man et al., 2003; Ozaki et al., 2014; Pushkarev et al., 2018a). Pro186 in BR is a highly conserved residue in >98% microbial rhodopsins (Inoue et al., 2021); if this proline is mutated to threonine, in many microbial rhodopsins, $\lambda_{max}$ shifts to a 5–20 nm longer wavelength (Inoue et al., 2019; Kojima et al., 2020a).

## Animal rhodopsins are light sensors

Animal rhodopsins are light-sensitive proteins that typically function as GPCRs (Hofmann et al., 2009). Many animals use

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**Box 1. Functions of non-visual rhodopsins in mammals**

Animals of different lineages have different sets of rhodopsins (Davies et al., 2015; Ramirez et al., 2016) that have various physiological functions. Most mammals, including humans, possess five non-visual rhodopsins: Opn3, Opn4, Opn5, peropsin and RGR (Upton et al., 2021), which have photoreceptive functions. Opn3 is expressed in adipocytes and has been suggested to regulate the metabolism of adipocytes and enhance thermogenesis in mice in a light-dependent manner (Nayak et al., 2020; Sato et al., 2020). Furthermore, smooth muscles in the airway and uterus exhibit light-dependent relaxation mediated by Opn3 (Wu et al., 2021; Yim et al., 2020). Opn4 has both visual and non-visual functions in the retina, which include the perception of brightness, the pupil light reflex and circadian photoentrainment of the biological clock (Lucas et al., 2020; Provencio and Warthen, 2012), as well as mediating light-dependent relaxation in blood vessels (Sikka et al., 2014). Opn5 forms a UV-sensitive rhodopsin and has non-visual functions, such as regulation of clock-gene rhythmicity in the retina, cornea and skin, as well as vascular development in the retina (Buhr et al., 2019, 2015; Nguyen et al., 2019). In the brain, Opn5 is expressed in the hypothalamus and regulates energy metabolism and thermogenesis (Zhang et al., 2020). Multiple rhodopsins, including Opn3, Opn4 and Opn5, have been suggested to have roles in the photosensitive system of the skin (de Assis et al., 2021).

animal rhodopsins as light sensors for various physiological functions, such as vision and regulation of biological rhythms.

Rhodopsin in rod photoreceptor cells in the retina of *Bos taurus* has been extensively studied, due to the large amount of protein that can be obtained from the bovine retina. The crystal structure of bovine rhodopsin in the dark state, which is an inactive state, revealed a seven TM helix structure with the chromophore retinal in its 11-*cis* state bound to Lys296 at the seventh helix via a Schiff base linkage (Palczewski et al., 2000). Upon light absorption, the chromophore is photoisomerized to all-*trans* form, which triggers conformational changes in rhodopsin, leading to the formation of the active state (Meta II) (see poster) (Choe et al., 2011; Hofmann et al., 2009). The Meta II state is thermally unstable and eventually releases an all-*trans*-retinal chromophore (Farrens and Khorana, 1995; Matthews et al., 1963; Wald, 1968). The opsin apoprotein then takes up 11-*cis*-retinal to form the original dark-state rhodopsin. The Meta II state binds to and activates the heterotrimeric G protein transducin ($G_t$) to drive an intracellular signal transduction cascade leading to hyperpolarization of photoreceptor cells (Yau and Hardie, 2009). Signaling by the Meta II state is inhibited by two events – phosphorylation of the C-terminal region of rhodopsin by a G protein-coupled receptor kinase (GRK) and subsequent binding of the regulator protein arrestin, which terminates G protein activation and downstream signaling (Yau and Hardie, 2009).

## Diversity of animal rhodopsins

Animal rhodopsins can be classified into eight groups, namely, vertebrate visual and non-visual opsin, Opn3 (encephalopsin), $G_q$-coupled visual opsin and Opn4 (melanopsin), Opn5 (neuropsin), $G_o$-coupled opsin, cnidarian $G_s$-coupled opsin, peropsin and retinochrome and retinal G-protein-coupled receptor (RGR) (Koyanagi et al., 2021; Terakita, 2005) (see poster). In addition, new phylogenetic groups of invertebrate opsins have been recently proposed, such as bathyopsin, xenopsin and chaopsin (Fleming et al., 2020). Apart from peropsin, retinochrome and RGR, all other rhodopsins have been shown to activate G proteins (Kühn, 1980; Kojima et al., 2011, 1997; Koyanagi et al., 2013, 2008; Koyanagi and Terakita, 2008; Lee et al., 1994; Terakita et al., 1993; Tsukamoto et al., 2005; Yamashita et al., 2010), suggesting that they act as light sensors for vision and/or other non-visual photoreception (see Box 1). Consistently, these rhodopsins contain E/DRY, CWxPY, and NPxxY$x_{5,6}$F motifs, which are highly conserved amino acid motifs among the GPCRs, located in TM3, TM6, and TM7, respectively. These rhodopsins mainly bind to 11-*cis*-retinal in the dark.

In contrast, peropsin, retinochrome and RGR bind all-*trans*-retinal in the dark, which photoisomerizes into the 11-*cis* form (Hao and Fong, 1999; Hara and Hara, 1968; Koyanagi et al., 2002; Nagata et al., 2010). Squid retinochrome and mammalian RGR act as retinal photoisomerases that enzymatically produce 11-*cis*-retinal by photoisomerization to restore visual rhodopsins (Chen et al., 2001; Morshedian et al., 2019; Terakita et al., 1989; Zhang et al., 2019). It has been reported that amphioxus and spider peropsins activate G proteins when their amino acids in the third intracellular loop are replaced with those of rhodopsins that activate G proteins (Nagata et al., 2018), although it is unclear which type of G protein is activated by the wild-type proteins. Since these mutant peropsins form an active all-*trans*-retinal-bearing state in the dark and convert to an inactive 11-*cis*-retinal-bearing state upon light absorption, they act as dark-active, light-inactivated GPCRs (Nagata et al., 2018). Similarly, chicken Opn5L1 is also activated in the dark and inactivated in the light (Sato et al., 2018).

The photoproducts of the various animal rhodopsin groups exhibit different photochemical properties. Upon light absorption, vertebrate visual rhodopsins, such as bovine rhodopsin, eventually release their chromophore and thus bleach (Imamoto and Shichida, 2014). In contrast, many of the other rhodopsins, including vertebrate non-visual opsin, Opn3, $G_q$-coupled visual opsin and Opn4, Opn5, $G_o$-coupled opsin, and peropsin form a stable photoproduct with a $\lambda_{max}$ in the visible region, which reverts to the original dark state upon absorption of another photon (Gartner and Towner, 1995; Koyanagi et al., 2013; Koyanagi and Terakita, 2008; Nagata et al., 2010; Tsukamoto and Terakita, 2010; Yamashita et al., 2010). This reversibility is called bistability (see poster). Jellyfish opsin (a cnidarian $G_s$-coupled opsin) does not bleach nor exhibit bistability (Koyanagi et al., 2008). Recently, chicken Opn5L1 was shown to exhibit thermal re-isomerization of the retinal chromophore from 11-*cis* to all-*trans,* followed by the formation of a covalent adduct between retinal and a cysteine residue (Sato et al., 2018). Thus, Opn5L1 is the first animal rhodopsin that has been shown to undergo cyclic photoreactions similar to microbial rhodopsins (Sato et al., 2018).

## Structure near the Schiff base

The counterion of vertebrate visual opsins is Glu113, which directly interacts with the protonated Schiff base by forming a salt bridge. Glu113 is highly conserved in vertebrate visual and non-visual opsins, but not in other rhodopsin groups. Indeed, in most rhodopsin groups, including retinochrome, $G_q$-coupled visual opsin, $G_o$-coupled opsin and peropsin, site-directed mutagenesis studies have suggested that Glu181 is the conserved counterion (Kojima et al., 2017; Nagata et al., 2019; Terakita et al., 2004, 2000). Based on the crystal structures of squid rhodopsin and spider rhodopsin (spider Rh1), which both are $G_q$-coupled visual opsins, this conserved counterion indirectly interacts with the protonated Schiff base (Murakami and Kouyama, 2008; Varma et al., 2019). Jellyfish $G_s$-coupled opsin has Glu94 as its counterion (Gerrard et al., 2018). Thus, animal rhodopsins can accommodate a counterion at different positions while retaining the function as a light sensor.

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### Absorption spectra

The $\lambda_{max}$ of vertebrate visual opsin and the $G_q$-coupled visual opsin in arthropods ranges from the ultraviolet to the red region (Arikawa and Stavenga, 2014; Bowmaker, 2008; Henze and Oakley, 2015; Yokoyama, 2008). Vertebrate visual opsins are divided into five classes: RH1 (460–530 nm), RH2 (470–530 nm), SWS1 (355–450 nm), SWS2 (415–480 nm) and LWS (495–570 nm) (Bowmaker, 2008). Humans have four visual rhodopsins, RHO (rod opsin, also called OPN2; $\lambda_{max}$=496 nm), SWS1 (also called blue cone opsin or OPN1SW; $\lambda_{max}$=424 nm) and two LWSs (green cone opsin or OPN1MW; $\lambda_{max}$=532 nm, and red cone opsin or OPN1LW; $\lambda_{max}$=563 nm) (Imamoto and Shichida, 2014). Amino acid residues at positions 180, 277 and 285 are the major spectral tuning sites responsible for the difference in $\lambda_{max}$ between human green and red cone opsins (Asenjo et al., 1994; Neitz et al., 1991). To date, more than 20 spectral tuning sites in vertebrate visual opsins have been reported (Bowmaker, 2008; Lin et al., 2017; Yokoyama, 2008) (see poster), which underlie the visual diversity of vertebrates living in different environments.

### Heliorhodopsins

Recently, functional metagenomics analysis revealed a new large family of rhodopsins, heliorhodopsins (HeRs; 'helios' meaning sun in Greek), which is distinct from both microbial and animal rhodopsins (Pushkarev et al., 2018b). HeRs show no homology with animal rhodopsins, but a small but substantial homology (<15% identity in amino acid sequence) has been observed with Type 1 microbial rhodopsins (Pushkarev et al., 2018b). The most prominent molecular difference in HeR is an inverted topology compared with Type 1 and 2 rhodopsins, in that the N- and C- termini of HeR face the cytoplasmic and extracellular sides, respectively (see poster) (Pushkarev et al., 2018b; Shihoya et al., 2019). HeR-like genes are widely distributed in diverse microorganisms, such as bacteria, archaea, fungi and algae, as well as giant viruses (Flores-Uribe et al., 2019; Pushkarev et al., 2018b; Shihoya et al., 2019). The photoreactions of HeRs are similar to those of Type 1 microbial rhodopsins, which are photocyclic and induced by all-*trans*-to-13-*cis* photo-isomerization (Pushkarev et al., 2018b). The biological functions of HeRs are unknown, but they have been proposed to act as sensors with long photocycles, or to be involved in the transport of amphiphilic molecules or redox processes of nitrate or carbonate (Flores-Uribe et al., 2019; Kovalev et al., 2020; Pushkarev et al., 2018b).

### Optogenetics

Rhodopsins have been used as molecular tools in optogenetics to control neural activity and intracellular G-protein signaling. The light-gated cation channel ChR2 from *C. reinhardtii* can induce membrane depolarization in mammalian cells (Nagel et al., 2003) and action potentials in neurons upon light illumination (an excitatory optogenetics tool) (Boyden et al., 2005). Since these studied were performed, various types of ChRs with higher sensitivity, faster response, prolonged opening and blue- or red-shifted absorption have been obtained by either screening natural ChRs or through artificial mutagenesis (Govorunova et al., 2013, 2016; Kato et al., 2015b; Kianianmomeni et al., 2009; Klapoetke et al., 2014; Lin, 2011; Marshel et al., 2019; Prigge et al., 2012; Schneider et al., 2015; Tashiro et al., 2021; Wen et al., 2010; Yizhar et al., 2011) (see poster). Inward $H^+$ pumps are also able to excite neural activity (Shevchenko et al., 2017). In contrast, ACRs and hyperpolarizing outward $H^+$, inward $Cl^-$, and outward $Na^+$ pumps can inhibit neural firing by fixing the membrane potential at a negative value (an inhibitory optogenetics tool) (Berndt et al., 2014; Chow et al., 2010; Govorunova et al., 2015; Gradinaru et al., 2008; Grimm et al., 2018; Hoque et al., 2016; Wietek et al., 2014). Some of the outward $H^+$-pumping microbial rhodopsins generate fluorescence depending on the membrane potential and are used as genetically encoded voltage indicators (GEVIs) to visualize the dynamic membrane potential change in each neuron, even in tissue slices or *in vivo* (Box 2).

Taken together, optogenetic application of animal rhodopsins enables the optical control of G-protein signaling. Here, various types of G proteins, including $G_s$, $G_i$ and $G_q$, can be controlled either with natural animal rhodopsins, for example inhibition of neural activity via $G_i$ activation (Copits et al., 2021; Mahn et al., 2021; Rodgers et al., 2021) and regulation of gene expression via $G_q$ activation (Ye et al., 2011), or with chimeric proteins that contain intracellular loops of different types of animal rhodopsins or even non-rhodopsin GPCRs (Airan et al., 2009; Ballister et al., 2018; Copits et al., 2021; Hickey et al., 2021; Mahn et al., 2021; Nagata et al., 2018; Rodgers et al., 2021).

### Perspectives

With the development of genomic and metagenomic analysis techniques, the number of rhodopsin genes identified has rapidly increased in recent decades, and we anticipate that this will continue in the future. Although the number of rhodopsin structures in the dark state or photo-intermediate states, determined by the X-ray crystallography, has also rapidly increased, time-resolved serial millisecond crystallography (TR-SMX) with synchrotron radiation and time-resolved serial femtosecond crystallography (TR-SFX) with an X-ray free-electron laser facility have enabled direct observation of the dynamic structural change along with the functional photoreaction of rhodopsins at physiological temperature (Nango et al., 2016; Nogly et al., 2018; Skopintsev et al., 2020; Weinert et al., 2019). Furthermore, using single-particle cryo-electron microscopy (cryo-EM), the structure of animal rhodopsins has been determined at near-atomic resolution (Gao et al., 2019) and that of GPR at atomic resolution (Hirschi et al., 2021). Obtaining 3D

#### Box 2. Application of microbial rhodopsins for genetically encoded voltage indicators

Some microbial rhodopsins can be used as genetically encoded voltage indicators (GEVIs) to monitor a change in intracellular membrane potential and report it through a change in fluorescence intensity. The first microbial-rhodopsin-based GEVI is archaealrhodopsin-3 (called Arch or AR3) (Kralj et al., 2012). Wild-type Arch exhibits far-red fluorescence depending on membrane potential, but it is dim, and hyperpolarization due to $H^+$ transport by Arch occurs simultaneously. However, multiple directed and random mutagenesis has conferred bright fluorescence, fast kinetics and absence hyperpolarization to Arch, resulting in several types of practical GEVIs, including Arch D97N, Arch-EEN/EEQ, QuasArs and Archon1 (Adam et al., 2019; Gong et al., 2013; Hochbaum et al., 2014; Kralj et al., 2012; Piatkevich et al., 2018), which are useful for membrane potential imaging even in slice tissues or *in vivo*. Other types of microbial-rhodopsin-based GEVIs are fusion proteins between microbial rhodopsin and a fluorescent protein. In this case, electrochromic Förster resonance energy transfer (eFRET) between a fluorescent protein (FRET donor) and microbial rhodopsin (FRET acceptor) yields bright membrane-voltage-sensitive fluorescence. For the rhodopsin part, variants of LR (also called Mac) and *Acetabularia acetabulum* rhodopsins (Ace1 and Ace2, also called ARI and ARII) have been used (Gong et al., 2015, 2014) to achieve efficient FRET with fluorescent proteins, such as mNeon, mCitrine and mScarlet.

cryo-EM structures for other rhodopsins will not only increase our understanding of the molecular mechanisms underlying their function, but also pave the way for the rational design of next-generation optogenetic tools.

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**Competing interests**
The authors declare no competing or financial interests.

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**Cell science at a glance**
Individual poster panels and Table S1 containing the PDB codes shown on the poster are available for downloading at https://journals.biologists.com/jcs/article-lookup/doi/10.1242/jcs.258989#supplementary-data

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