



Emerging Diversity of Channelrhodopsins and Their Structure-Function Relationships

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Cation and anion channelrhodopsins (CCRs and ACRs, respectively) from phototactic algae have become widely used as genetically encoded molecular tools to control cell membrane potential with light. Recent advances in polynucleotide sequencing, especially in environmental samples, have led to identification of hundreds of channelrhodopsin homologs in many phylogenetic lineages, including non-photosynthetic protists. Only a few CCRs and ACRs have been characterized in detail, but there are indications that ion channel function has evolved within the rhodopsin superfamily by convergent routes. The diversity of channelrhodopsins provides an exceptional platform for the study of structure-function evolution in membrane proteins. Here we review the current state of channelrhodopsin research and outline perspectives for its further development.

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INTRODUCTION

Channelrhodopsins (ChRs) are microbial proteins that act as photoreceptors in photomotility of flagellate algae (Sineshchekov et al., 2002). ChRs are members of the rhodopsin superfamily, which comprises integral membrane proteins sharing common topology of seven transmembrane α -helices (TM1-TM7) and the retinal chromophore covalently bound *via* a Schiff base linkage to the ϵ -amino group of a specific lysine residue in TM7 (Spudich et al., 2000; Ernst et al., 2014; Kandori, 2020). According to their primary sequence homology and chromophore geometry, ChRs are type 1 rhodopsins (Govorunova et al., 2017a; Rozenberg et al., 2021), distinct from visual pigments (type 2 rhodopsins). So far type 1 rhodopsins are found only in microorganisms and their function is either photoenergy capture by light-driven ion pumping (Brown, 2014; Kandori, 2015) or photosensory signaling by three distinctly different mechanisms (Govorunova et al., 2017a): (i) participation as the photoactive subunit in sensory rhodopsin-transducer (SR-Htr) complexes in haloarchaea (Sasaki and Spudich, 2008); (ii) function as light-sensitive enzymes with guanylyl cyclase activity discovered in fungi (Avelar et al., 2014); and (iii) photocontrol of membrane electrical potential in algae (Sineshchekov et al., 2002). Each of these signaling processes controls flagellar motility, thereby mediating photomotility behavior in prokaryotes (i) or eukaryotic microorganisms (ii and iii).

Photoexcitation of ChRs results in passive ion transport across the membrane (Nagel et al., 2002, 2003). This unique light-gated channel activity, not found in any other natural proteins, enables stimulation or inhibition of specific cellular populations or even individual excitable cells with light on the submillisecond time scale, applying the transformative technique known as “optogenetics” (Boyden et al., 2005; Deisseroth, 2011). Recently, partial recovery of visual function in a human patient upon intraocular injection of an

adeno-associated viral vector encoding a ChR was reported (Sahel et al., 2021). Further development of ChR-based gene therapy will depend on the availability of molecules with desired biophysical properties. Many excellent reviews discuss perspectives of using ChRs in biomedical research and therapy (Deisseroth, 2011, 2015; Lin, 2012; Yawo et al., 2013; Wiegert et al., 2017; Schneider-Warme, 2018). Here we focus on the molecules themselves and report recent advances on our understanding of their evolution, and structure-function relationships that determine biophysical characteristics of ChRs relevant for optogenetics: ionic selectivity, rectification, desensitization and color tuning.

CONVERGENT EVOLUTION OF CHANNELRHODOPSINS

An important insight gained from recent studies on ChR diversity is that channel function cannot always be deduced from the primary sequence alone, i.e., several ChR families exist that show very low sequence homology to each other yet similar function (Govorunova et al., 2021). A striking example is the family of cryptophyte CCRs known as “bacteriorhodopsin-like CCRs” (BCCRs; Sineshchekov et al., 2017). These proteins show higher sequence homology to haloarchaeal proton pumps than to chlorophyte CCRs, and yet passively conduct protons and metal cations (Govorunova et al., 2016b; Yamauchi et al., 2017; Marshel et al., 2019; Sineshchekov et al., 2020). In particular, their sequences retain the two carboxylate residues that define the vectorial proton path in bacteriorhodopsin, in which Asp85 and Asp96 serve as acceptor and donor, respectively, of the photoactive site Schiff base proton (Lanyi, 2006). In contrast, nearly all chlorophyte CCRs contain Glu in the first position, and a non-carboxylate residue (most frequently, His) in the second position. Analysis of laser flash-induced photocurrents and transient absorption changes in CCR2 from the cryptophyte *Guillardia theta* (*GtCCR2*), a representative of BCCRs, has revealed a tight coupling between channel gating and intramolecular proton transfers involving the same residues that define vectorial proton transport in bacteriorhodopsin (Sineshchekov et al., 2017).

A high-resolution structure of a BCCR known as ChRmine, recently obtained by cryo-electron microscopy, revealed a trimeric organization (Kishi et al., 2021) typical of bacteriorhodopsin (Luecke et al., 1999). In contrast, X-ray structures of all other so far crystallized ChRs are homodimers (Kato et al., 2012; Volkov et al., 2017; Kim et al., 2018; Oda et al., 2018; Li et al., 2019; Zabelskii et al., 2020), confirmed by biophysical and biochemical analyses. Unlike canonical oligomeric channels, in which the channel pore is formed by several subunits, each ChR protomer is presumed to be conductive, and there is no evidence that dimerization plays any functional role. However, based on a combination of structural, functional and computational studies it has been suggested that ChRmine (and possibly other BCCRs) possess a cation conduction pathway along the trimer interface, in addition to that within individual monomers, as in other ChRs (Kishi et al., 2021).

TAXONOMIC DISTRIBUTION

Exploration of natural ChR diversity may yield variants better suited for optogenetic applications than currently known molecules. But where should we look for new ChR sequences? The best-characterized *Chlamydomonas reinhardtii* ChRs serve as photoreceptors in phototaxis (Sineshchekov et al., 2002), so phototactic flagellates seem to be a promising hunting ground. Indeed, high-throughput polynucleotide sequencing projects have yielded >150 ChR homologs in the species of the class Chlorophyceae (Klapoetke et al., 2014; Rozenberg et al., 2020), to which *C. reinhardtii* belongs (Figure 1). ChR sequences were also found in the other three classes of the “core” chlorophytes, i.e., Chlorodendrophyceae, Trebouxiophyceae and Ulvophyceae, in *Pedinomonas minor* (class Pedinophyceae), and in the three classes of the polyphyletic group known as “prasinophytes” (Mamiellophyceae, Nephroselmidiophyceae and Pyramimonadophyceae; Klapoetke et al., 2014; Rozenberg et al., 2020). ChR homologs have also been found in the several representatives of the phylum Streptophyta: the flagellate alga *Mesostigma viride* (Govorunova et al., 2011), included in Streptophyta as an early diverging lineage, the filamentous alga *Klebsormidium nitens* (Klebsormidiophyceae; Awasthi et al., 2020; Tashiro et al., 2021) and thallus-forming alga *Coleochaete irregularis* (Coleochaetophyceae; Rozenberg et al., 2020), the latter of which is considered as the closest relative of land plants (Leliaert et al., 2012). However, the sequenced genomes of land plants do not seem to encode ChRs (nor rhodopsins of any kind). Rhodopsin fragments reported in *Oryza sativa* most likely originate from fungal contamination (see discussion in Ruiz-Gonzalez and Marin, 2004).

Cryptophytes and dinoflagellates are two other algal lineages, representatives of which are capable of genuine phototactic orientation (Forward, 1974; Watanabe and Furuya, 1982; Erata et al., 1995; Horiguchi et al., 1999). More than 40 type I rhodopsin genes are predicted by the fully sequenced genome of the marine cryptophyte *G. theta* (Govorunova et al., 2015), at least 29 of which are expressed in native cells (Konno et al., 2020). Remarkably, in this alga and many other cryptophyte species analyzed by transcriptome sequencing projects there are two structurally and functionally distinct ChR families: ACRs and BCCRs (Klapoetke et al., 2014; Govorunova et al., 2015, 2016a,b, 2017b, 2018; Wietek et al., 2016; Yamauchi et al., 2017; Sineshchekov et al., 2020). Besides ChRs, cryptophyte genomes also encode type I rhodopsins of other families, such as the proton pump *GtR3* (Gradinaru et al., 2010). The absorption spectrum of *GtR1*, the first rhodopsin identified in *G. theta* and purified upon expression of its gene in *E. coli*, overlapped with the action spectrum of *G. theta* photobehavior, so this protein was suggested as a candidate photoreceptor for this response (Sineshchekov et al., 2005). However, neither *GtR1* nor homologous *GtR2* generate channel currents in heterologous systems, so *G. theta* phototaxis is most likely guided by BCCRs and ACRs subsequently found in this organism.

As those of cryptophytes, the genomes of dinoflagellates encode multiple rhodopsins of different families. In particular, many rhodopsin transcripts were identified in the predatory

dinoflagellate *Oxyrrhis marina* (Zhang et al., 2007; Slamovits et al., 2011). However, most *Oxyrrhis* rhodopsins are proteorhodopsin homologs (Rhiel et al., 2020). H⁺ pumping in one of them (OR1) was experimentally verified by heterologous expression followed by patch clamp recording (Janke et al., 2013). A group of rhodopsin sequences from dinoflagellates of the genera *Ansanella*, *Pelagodinum*, *Breviolum*, and *Symbiodinium* contain the Thr-Cys-Pro motif in the middle of TM3 that is conserved in most so far known ChRs. Two of such rhodopsins from the coral endosymbiont *Symbiodinium microadriaticum* exhibited channel activity upon heterologous expression (Govorunova et al., 2021). Their spectral sensitivity roughly matched that of phototactic accumulation observed in the Symbiodiniaceae and unclassified coral symbiotic dinoflagellates (Hollingsworth et al., 2005; Aihara et al., 2019), which suggests their photoreceptor role for this response. ChR-like sequences are also encoded by the genomes of the flagellate algae *Chromera velia* and *Vitrella brassicaformis* (Woo et al., 2015), representatives of the group Chromerida that belongs to the same clade Alveolata as dinoflagellates.

Functional ChRs have also been found in the haptophyte algae *Phaeocystis antarctica* and *P. globosa* (clade Prymnesiophyceae; Govorunova et al., 2020), although a non-homologous rhodopsin sequence from the haptophyte *Pavlova (Diacronema) lutheri* (order Pavlovaceae) was non-electrogenic upon heterologous expression (Klapoetke et al., 2014). Transcriptome sequencing within the frame of the 1,000 Plants (1KP) project (Matasci et al., 2014) has not identified rhodopsins in *Euglena gracilis*.

An important paradigm-shifting discovery of recent years has been that of ChRs in non-photosynthetic protists from the clade Stramenopiles. Labyrinthulea (also known as Labyrinthulomycetes) are a group of aquatic heterotrophic organisms that have independently evolved a fungus-like lifestyle (Pan et al., 2017). *Cafeteria roenbergensis* is a globally distributed marine bacterivorous flagellate that belongs to the same stramenopile class Bygira as Labyrinthulea (Hackl et al., 2020). ChRs from several Labyrinthulea species of the order Thraustochytrida, *C. roenbergensis* and the unclassified stramenopile strain TOSAG 23-3 have been functionally characterized by heterologous expression and patch clamp electrophysiology (Govorunova et al., 2020, 2021). ChRs from the latter source are highly homologous to metagenomically identified ChRs known as MerMAIDs (Metagenomically discovered, Marine, Anion-conducting and Intensely Desensitizing channelrhodopsins; Oppermann et al., 2019), which suggests that MerMAIDs also originate from stramenopiles. Most thraustochytrids produce flagellate zoospores, so the function of their ChRs in native cells is likely to be phototaxis, as that of algal ChRs. Indeed, phototaxis towards the light emitted by bioluminescent marine bacterium *Vibrio fischeri* has been documented in flagellate zoospores of the labyrinthulomycete *Ulkenia* sp. that prey on these bacteria (Amon and French, 2004). The labyrinthulomycete *Aplanochytrium stochinoi* does not produce flagellate zoospores (Moro et al., 2003), and yet its genome encodes ChR homologs (Rozenberg et al., 2020), the function of which is unknown. Very recently, functional ChRs have been identified in

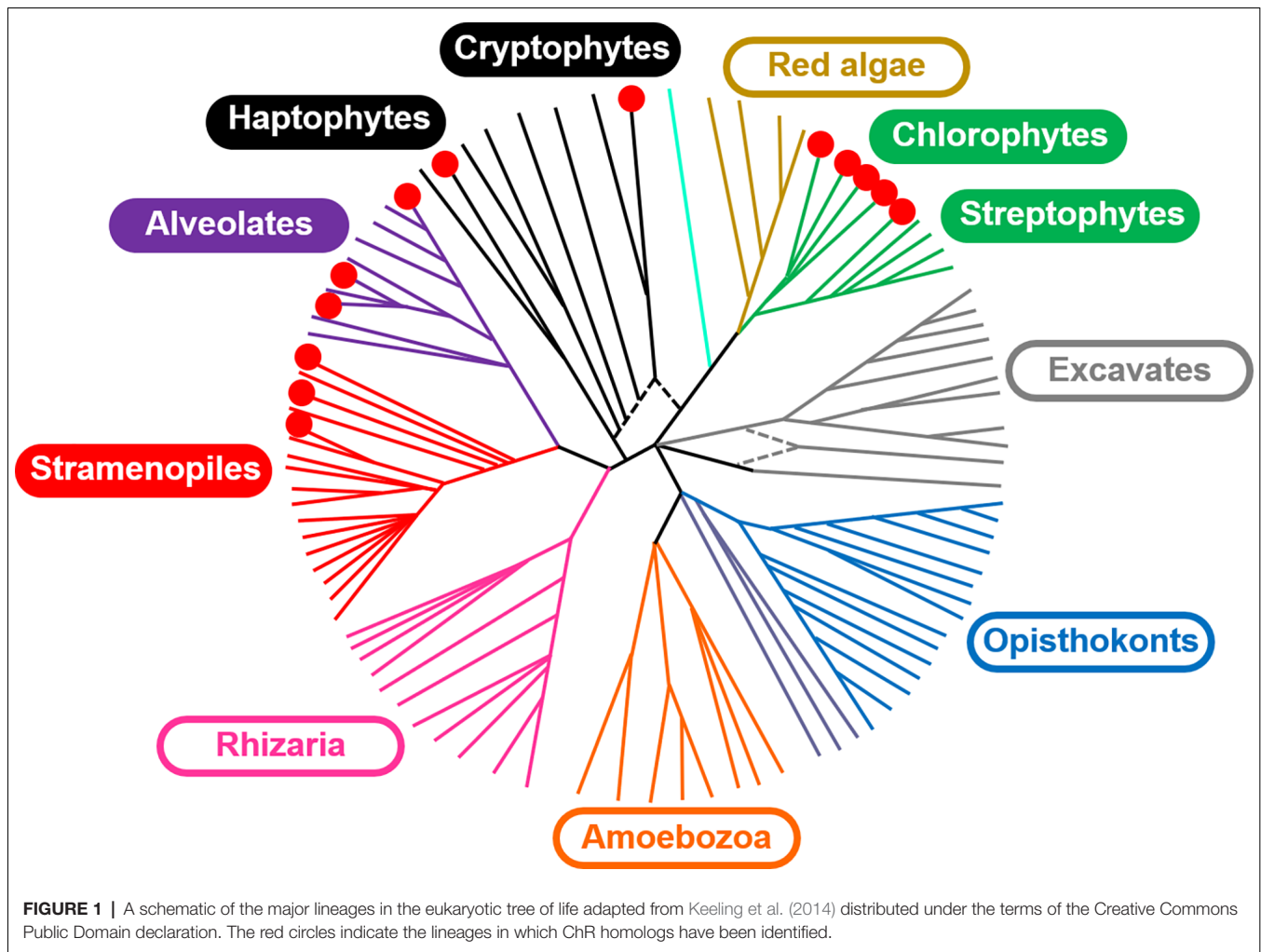
the *Hyphochytrium catenoides* from the stramenopile class Hyphochytriomycetes (e.g., Govorunova, O. A. Sineshchekov and J. L. Spudich, unpublished observations).

The high-throughput sequencing projects are a rich source of new ChR homologs, but the attribution of so identified sequences to particular organisms has to be treated with caution, even when axenic biomaterial is used. For example, a ChR sequence named ChRmine (Marshel et al., 2019) was found in the transcriptome of the ciliate *Tiarina fusus* within the frame of the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP; Keeling et al., 2014). However, this sequence is identical at the nucleotide level with a BCCR sequence derived from the cryptophyte *Rhodomonas lens* (Sineshchekov et al., 2020), which was used to feed *Tiarina* prior to RNA extraction. Similarly, other ChR homologs, attributed by MMETSP to the ciliates *Myrionecta rubra* and *Strombidinopsis* sp. (Rozenberg et al., 2020) are identical or very closely homologous to cryptophyte ChRs and almost certainly belong to the food organisms. The presence of rhodopsins in ciliates, based on retinal extraction and hydroxylamine inhibition experiments, has been suggested (Tokioka et al., 1991), but, to the best of our knowledge, no genetic evidence for this has so far been obtained.

ChR sequences are conspicuously absent in prokaryotes, most of which rely on enzymatic cascades for photosensory transduction (Armitage, 1997; Spudich, 2006). However, some eukaryotic ChR sequences are highly divergent, so it is plausible that prokaryotic ChRs simply could not be recognized by bioinformatic means alone. Patchy distribution of ChRs among eukaryotic taxa (Figure 1) suggests their acquisition by horizontal gene transfer and/or endosymbiosis. ChR genes found in giant viruses that infect marine protists (Rozenberg et al., 2020; Zabelskii et al., 2020) may represent one method of ChR transfer between different taxa, and endosymbiosis may represent another method. The origin of cryptophytes, dinoflagellates and haptophytes by secondary endosymbiosis (Hackett et al., 2004; Gentil et al., 2017) may explain a particularly large number of genes of different rhodopsin families in their genomes, transferred from the symbionts to the host nucleus.

PERMEATION PATHWAY AND ION SELECTIVITY

In contrast to oligomeric ion channels, the selectivity of which is determined by a defined structural feature (called the “selectivity filter”), ion selectivity in ChRs appears to be controlled by several residues that form the ion conduction pathway within each protomer. No high-resolution structures of the open state are available yet for any ChR, but those of the closed state together with the results of functional studies strongly suggest that the channel pore is formed by TM1-3 and 7. In particular, *GtACR1* structures (Kim et al., 2018; Li et al., 2019) reveal a narrow intramolecular tunnel connecting the cytoplasmic and extracellular aqueous phases (Li et al., 2019) that is expected to expand upon illumination (Figure 2). A bromide ion resolved near the cytoplasmic entry into this tunnel in the Br-soaked *GtACR1* structure strongly confirms this hypothesis



(Li et al., 2021). Crystal structures of chlorophyte CCRs reveal a series of unconnected intramolecular cavities in the dark state (Kato et al., 2012; Volkov et al., 2017; Oda et al., 2018). A time-resolved serial femtosecond crystallographic study using an X-ray free electron laser has shown an outward shift of TM3 and a local deformation of TM7 upon photoactivation of a hybrid chlorophyte CCR known as C1C2 (Oda et al., 2021).

Most chlorophyte CCRs contain five conserved Glu residues in TM2 and the TM2-TM3 loop along the predicted channel pore (Glu82, Glu83, Glu90, Glu97, and Glu101 in *CrChR2*; **Figure 2**). Mutagenetic studies have shown that Glu90 is required for cation conductance in *CrChR2*, as its replacement with Lys or Arg made the channel permeable for anions (Wietek et al., 2014), and its residual permeability for protons was eliminated by mutagenetic neutralization of two other glutamates (the E83Q and E101S mutations; Wietek et al., 2015). Alternatively, permeability for anions could be engineered in chlorophyte CCRs by multiple mutations in the channel pore reducing its overall negative charge (Berndt et al., 2014, 2016; Wietek et al., 2017). These results led to the notion that cation or anion selectivity of ChRs is determined by the electrostatic potential of the ion conducting pathway (Rappleye and Berndt, 2019).

Comparative analysis of wild-type CCRs and ACRs from different taxa supports this notion. In general, ACRs sequences contain a smaller number of conserved glutamates than do chlorophyte and streptophyte CCRs (**Figure 3**). However, the same residue pattern (Glu82 and Glu90 are conserved, and the other positions are occupied with non-carboxylate residues) is found in some chlorophyte CCRs and most ACRs from cryptophytes, haptophytes and stramenopiles. This observation suggests that the role of the glutamate residues in ion selectivity depends on a wider protein context.

Another residue that appears to be involved in discrimination between ACRs and CCRs is that in the position of Asp85, the primary acceptor of the Schiff base proton in bacteriorhodopsin. In all so far known ACRs this Asp is replaced with a non-carboxylate residue, whereas in nearly all chlorophyte CCRs this carboxylate is conserved as Glu (and as Asp in BCCRs). However, this residue alone cannot be regarded as the sole indicator of anion selectivity, because some chlorophyte CCRs also contain a non-carboxylate residue in this position [e.g., *DsChR1* from *Dunaliella salina* (Zhang et al., 2011) and Chronos from *Stigeoclonium helveticum* (Klapoetke et al., 2014)]. Mutagenetic neutralization of this residue (Glu123) in

CrChR2 accelerated channel opening, but did not convert the cation channel into an anion channel (Gunaydin et al., 2010).

CCRs of both chlorophyte and cryptophyte families differ in their Na^+/H^+ permeability ratios. Among chlorophyte CCRs, ChR1 from *M. viride* (MvChR1) and ChR2 from *Platymonas* (*Tetraselmis*) *subcordiformis* (PsChR2) exhibit a higher relative permeability to Na^+ than CrChR2 (although they still are primarily H^+ channels; Govorunova et al., 2011, 2013; Duan et al., 2019). Most so far tested cryptophyte BCCRs also have an increased Na^+/H^+ permeability ratio (e.g., $\sim 5 \times 10^{-5}$ in GtCCR4), as compared to CrChR2 ($\sim 10^{-6}$) (Govorunova et al., 2016b; Shigemura et al., 2019). A number of chlorophyte and cryptophyte CCRs are almost purely H^+ channels (Berthold et al., 2008; Tsunoda and Hegemann, 2009; Zhang et al., 2011; Govorunova et al., 2021). Some CCRs are also permeable for Ca^{2+} , although to a lesser extent than for monovalent metal cations, e.g., the $\text{Ca}^{2+}/\text{Na}^+$ permeability ratio of CrChR2 is ~ 0.15 (Kleinlogel et al., 2011).

RECTIFICATION

For a simple aqueous pore in a lipid bilayer, the relationship between the membrane voltage and transmembrane current (the current-voltage relationship, or the IE curve) is linear as defined by Ohm's law. However, many ion channels show deviation from this linearity known as "rectification" (Hille, 2001). A likely reason for such behavior would be the difference in the concentrations of the permeated ions on the two sides of the membrane, as modeled by the Goldman-Hodgkin-Katz current equation (Hille, 2001), but many ion channels, including ChRs, show rectification even when probed under symmetrical ionic conditions.

Peak photocurrent generated by GtACR1 showed a linear dependence on voltage (Govorunova et al., 2015). A protein-wide Glu substitution screen has revealed that introducing a negative charge in the cytoplasmic half of the presumed ion pathway caused outward rectification, whereas that in the extracellular half, inward rectification (Sineshchekov et al., 2019). Wild-type GtACR1 photocurrents decay biphasically even under single-turnover conditions (Sineshchekov et al., 2015). The amplitude of the fast decay component shows outward, and that of the slow decay component, inward rectification. Ala61 (corresponding to Glu83 of CrChR2) is located on the cytoplasmic surface near the entry into the channel pore, and Ala75 (corresponding to Glu97 of CrChR2), near the extracellular entry into the channel pore (Figure 2). The A61E mutation enhanced the rate and contribution of the fast channel closing, whereas the A75E mutation enhanced the slow component. A similar correlation between rectification and photocurrent kinetics was observed in the outwardly rectifying Q46E and G242E mutants and the inwardly rectifying G80E and S227E mutants. In wild-type GtACR1 the fast channel closing component temporally correlated with the formation of the M intermediate of the photochemical cycle (i.e., deprotonation of the Schiff base), and the slow closing component, with M decay (Sineshchekov et al., 2016). This correlation also held for the A61E and A75E mutants, which suggests that an acidic group placed

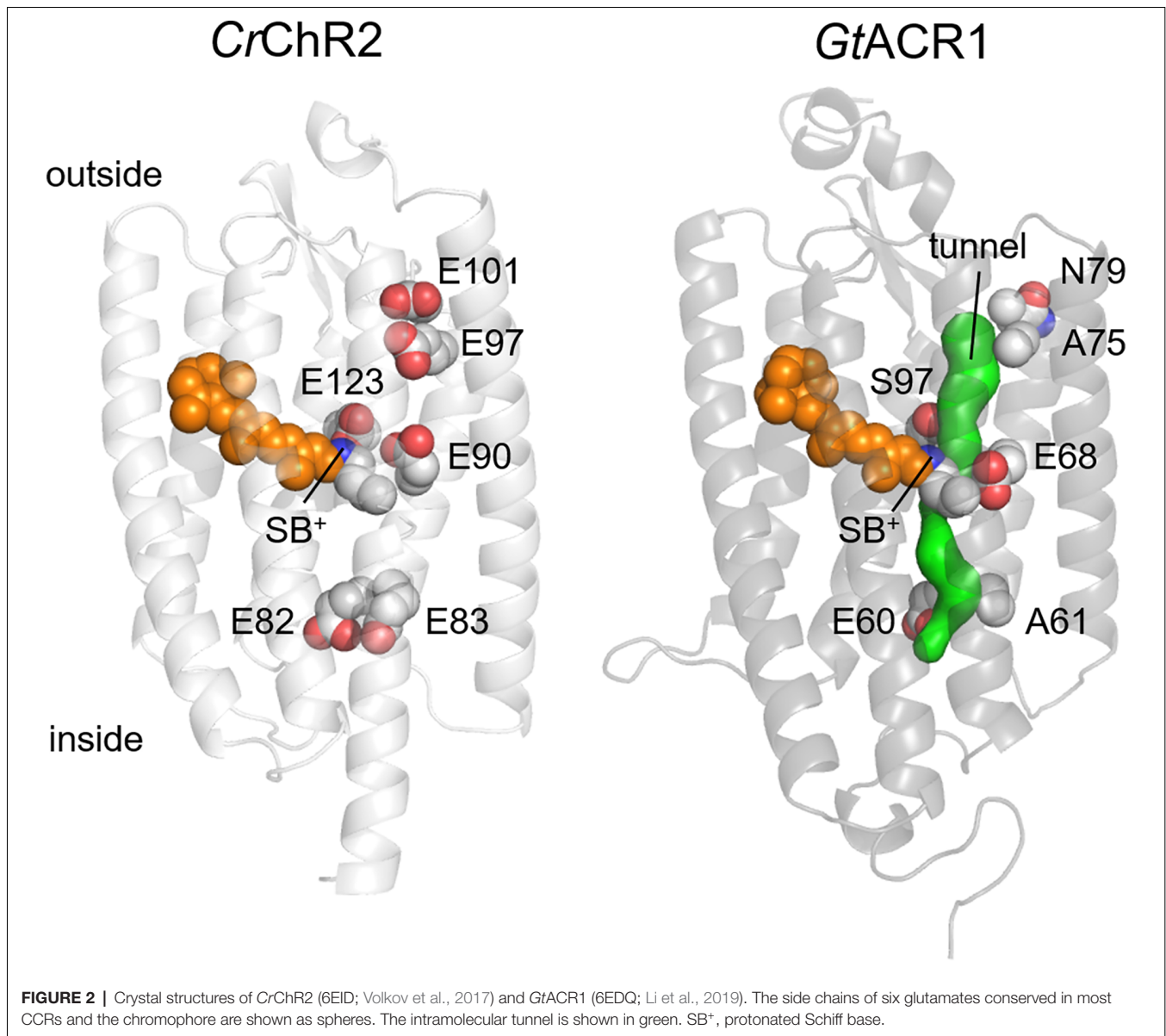
near the cytoplasmic or extracellular entry into the channel electrostatically influences the photoactive site.

The IE curves of most chlorophyte CCRs and cryptophyte BCCRs show inward rectification (Nagel et al., 2002, 2003; Zhang et al., 2008; Govorunova et al., 2011, 2016b; Hou et al., 2012; Yamauchi et al., 2017), which is not caused by voltage-dependent block by cytoplasmic Mg^{2+} (Gradmann et al., 2011), as in some oligomeric K^+ channels (Nichols et al., 1994). Quantitative modeling of the IE curves for CrChR2 using the formalism of enzyme kinetics led to the conclusion that inward rectification results from a combination of a nonlinear transport function and asymmetric competition between several cation species (Gradmann et al., 2011). The addition of Gd^{3+} or La^{3+} to the bath decreased inward rectification in CCRs, which was explained by a more selective block of cationic influx rather than efflux (Tanimoto et al., 2013). The residue in the Glu97 position of CrChR2 near the extracellular end of TM2 contributed to the Gd^{3+} effect (Tanimoto et al., 2013; Watanabe et al., 2016).

DESENSITIZATION

When ChRs are activated with pulses of continuous light, their photocurrents first rise and then decline to a lower steady-state level even if the light is still on (the phenomenon known as desensitization). Desensitization reflects formation of an equilibrium mixture of photocycle intermediates and in different ChRs varies from nearly zero to practically 100% in MerMAIDs (Oppermann et al., 2019) and some *Rhodomonas* BCCRs (Sineshchekov et al., 2020). In a particular ChR, desensitization depends on the wavelength and intensity of the light (Ishizuka et al., 2006), membrane voltage (Ernst et al., 2008; Oppermann et al., 2019) and pH (Hegemann et al., 2005; Tsunoda and Hegemann, 2009), as all these factors are known to influence the photocycle.

Strong desensitization is a problem for most optogenetic applications, so elucidation of its molecular mechanisms is an important direction of ChR research. Theoretically, accumulation of any long-lived non- or less-conductive intermediate of the photocycle would result in desensitization. Experimental evidence shows that these intermediates are different in different ChR families. In CrChR2 desensitization is correlated with accumulation of the P480 state that is considered either as a late intermediate in a single branched photocycle (Lorenz-Fonfria and Heberle, 2014; Saita et al., 2018) or the initial state of a parallel photocycle that is formed in the primary photoreaction of the initial dark state (Kuhne et al., 2019). A recent study using DNP (dynamic nuclear polarization) enhanced solid-state MAS (magic-angle spinning) NMR spectroscopy has shown that P480 contains 13-*cis*, 15-*syn* retinal Schiff base and occurs late in the photocycle (Becker-Baldus et al., 2021). To return to the dark state, this chromophore configuration has to change, which could explain the long lifetime of the desensitized state. In MerMAID1 desensitization results from accumulation of the M intermediate (Oppermann et al., 2019), and in similarly rapidly desensitizing *Rhodomonas* BCCRs, from formation of an additional extremely blue-shifted intermediate P330 (Sineshchekov et al., 2020).



The E44Q and C84T mutations reduced desensitization in MerMAID1 (Oppermann et al., 2019). However, the mutated residues (corresponding, respectively, to Glu90 and Cys128 of CrChR2) are conserved in many ChRs that do not show strong desensitization, and therefore cannot be the sole cause of strong desensitization in MerMAIDs. As in the case of ion selectivity, the phenotypic effect of a particular residue substitution depends on a wider protein context.

COLOR TUNING

Most optogenetic applications benefit from red-shifted ChRs, because light of longer wavelengths is less scattered by biological tissues resulting in a longer penetration depth. Combinatorial (multiplex) optogenetics, such as simultaneous activation (or inhibition) of two different neuronal populations in the same

preparation, or combination of neuronal activation/inhibition with imaging of neuronal activity, requires both blue- and red-shifted ChRs with narrow absorption bands to avoid spectral overlap. Many individual residues (primarily in the retinal-binding pocket) have been shown to control the absorption wavelength in ChRs. However, their mutations intended to produce a spectral shift are usually associated with reduction of the photocurrent amplitude and/or deceleration of the photocurrent kinetics. Therefore, identification of natural spectrally shifted ChR variants, optimized by evolution, is particularly important.

A strongly red-shifted CCR variant from *Chlamydomonas noctigama* named Chrimson has already been known for several years (Klapoetke et al., 2014; Oda et al., 2018), but its counterparts among ACRs have been identified only recently (Govorunova et al., 2020). Four ACRs, found in

| Name | Taxonomy | 82 | 83 | 90 | 97 | 101 | 123 | Selectivity |
|------------|--------------|-----|----|----|----|--------------|-----|-------------|
| | | TM2 | | | | TM2-TM3 loop | TM3 | |
| CrChR2 | chlorophyte | E | E | E | E | E | E | cation |
| DsChR1 | chlorophyte | E | E | E | V | E | A | cation |
| MvChR1 | streptophyte | E | V | E | A | E | E | cation |
| CsCCR | chlorophyte | E | V | E | H | P | G | cation |
| MsCCR | chlorophyte | E | V | E | H | P | G | cation |
| GtACR1 | cryptophyte | E | A | E | A | N | S | anion |
| RapACR | cryptophyte | E | A | E | L | S | A | anion |
| PgACR1 | haptophyte | E | A | E | S | P | G | anion |
| CarACR1 | stramenopile | E | V | E | G | P | G | anion |
| sTACR1 | stramenopile | E | I | E | L | P | S | anion |
| PymeACR1 | chlorophyte | E | V | G | K | A | G | anion |
| Py2087ACR1 | chlorophyte | E | V | G | K | T | Q | anion |
| HfACR1 | stramenopile | H | P | T | S | T | F | anion |
| CarACR2 | stramenopile | T | K | M | S | S | G | anion |

FIGURE 3 | Residue motifs and ionic selectivity of representative ChRs. The residue numbers are according to the CrChR2 sequence. CrChR2, *Chlamydomonas reinhardtii* channelrhodopsin 2; DsChR1, *Dunaliella salina* channelrhodopsin 1; MvChR1, *Mesostigma viride* channelrhodopsin 1; CsCCR, *Crustomastix stigmatica* cation channelrhodopsin; MsCCR, *Mantoniella squamata* cation channelrhodopsin; GtACR1, *Guillardia theta* anion channelrhodopsin 1; RapACR, rapid anion channelrhodopsin from *Rhodomonas salina*; PgACR1, *Phaeocystis globosa* anion channelrhodopsin 1; CarACR1, *Cafeteria roenbergensis* anion channelrhodopsin 1; sTACR1, Stramenopiles sp. TOSAG23–3 anion channelrhodopsin 1; PymeACR1, *Pyramimonas melkonianii* anion channelrhodopsin 1; Py2087ACR1, *Pyramimonas* sp. CCMP2087 anion channelrhodopsin 1; HfACR1, *Hondaea fermentalgiana* anion channelrhodopsin 1; CarACR2, *Cafeteria roenbergensis* anion channelrhodopsin 2.

Labyrinthulea and collectively designated as RubyACRs, generate large photocurrents with the most red-shifted action spectra of any microbial rhodopsins known so far (up to 610 nm in *Hondaea fermentalgiana* anion channelrhodopsin 1 (HfACR1), the spectrum of which is shown in **Figure 4**, red). Their retinal-binding pockets show a distinctly different residue pattern as compared to that of Chrimson, indicating that the red spectral shift is achieved in RubyACRs by a different biophysical mechanism. Yet another residue pattern was found in two prasinophyte CCRs with the spectra red-shifted to 585 nm (Govorunova et al., 2021).

The isomer composition and stability of the chromophore also contribute to color tuning in ChRs. For example, according to the crystal structure, the polar S atom of the Cys102 sidechain in GtACR1 is oriented towards the Schiff base, which destabilizes the singlet excited state S_1 (Tsujiyama et al., 2021). Replacement of this Cys with a non-polar Ala is expected to red-shift the spectrum, but a 10-nm blue shift has been observed experimentally (Govorunova et al., 2018). Chromatographic analysis revealed an increased fraction of the blue-shifted 13-*cis* isomer in the C102A mutant as compared to the wild type, which suggests a decreased stability of the all-*trans* retinal configuration in the unphotolyzed state of this mutant (Tsujiyama et al., 2021).

The ring/chain coplanarity of the chromophore is an important steric factor in color tuning. Mutations of the

binding pocket residues that stabilized the twisted chromophore conformation led to the blue shift of the peak absorption from 476 to 455 nm in C1C2 (Kato et al., 2015). Two closely related CCRs, PsChR2 (Govorunova et al., 2013) and TsChR from *Tetraselmis striata* (Klapoetke et al., 2014), are the most blue-shifted ChRs known to date (**Figure 4**). In these proteins, the positions of Thr198 and Gly202 of C1C2 are occupied by Gly and Ala, respectively. Such a residue pattern strongly suggests that their chromophore is fixed in the twisted 6-*s-cis* configuration, which contributes to their blue-shifted absorption.

Microbial rhodopsins with absorption in the UV spectral region have been found among histidine kinase rhodopsins (HKRs), which do not generate transmembrane ionic currents, but exhibit an intrinsic enzymatic function (Mukherjee et al., 2019). The best studied representative, *C. reinhardtii* HKR1, binds 13-*cis*, 15-*anti* retinal via an unprotonated Schiff base (Luck et al., 2012; Penzkofer et al., 2014) and undergoes a unique dual isomerization upon photoexcitation (Hontani et al., 2020). At present it is unclear whether such photochemistry can support channel function.

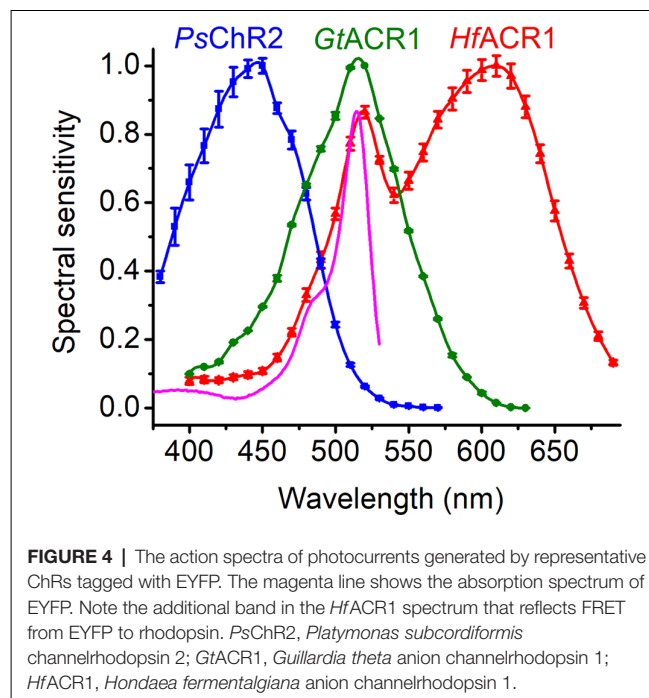
Finally, the absorption spectra of ChRs can be shifted by replacement of the natural chromophore with natural or synthetic analogs. 3,4-dehydroretinal, also known as A2 retinal, naturally occurs in the eyes of some animals and has an additional double bond in the ring, which extends its π -

conjugated system and red-shifts the absorption spectrum. Incorporation of all-*trans* A2 retinal shifted the action spectra of photocurrents generated by chlorophyte CCRs (Sineshchekov et al., 2012) and RubyACRs (Govorunova et al., 2020) 10–40 nm to longer wavelengths without significant effects on photocurrent kinetics. In contrast, a red-shifting effect of a synthetic analog with a modified ring (dimethylamino-retinal, or DMAR) was accompanied by deceleration of the channel kinetics in CrChR2 mutants (Azimi Hashemi et al., 2014). Extension of the chromophore π -system can alternatively be achieved by elongation of the polyene chain upon the insertion of a vinylene group. A series of such analogs has been tested in purified CCRs mutated to improve their affinity for such compounds (Shen et al., 2018; Okitsu et al., 2020). Channel activity of these molecules has not yet been tested, but measurements of flash-induced absorbance changes have revealed that the kinetics of the photocycle was altered, as compared to that of all-*trans* retinal-bound proteins.

The constructs used for heterologous expression of ChRs in animal cells usually encode fluorescent proteins as tags. Provided there is sufficient spectral overlap between the tag and ChR, Förster resonance energy transfer (FRET) occurs, which is manifested by an additional band or shoulder in the action spectrum of photocurrents. Such bands have been reported for C-terminal EYFP fusions of RubyACRs (Figure 4) and red-shifted chlorophyte CCRs (Govorunova et al., 2021), and can be expected in other cases as well. A contribution of FRET explains some discrepancies in the reported action spectra of photocurrents by the same ChR. For example, an EYFP (absorption peak at 513 nm) fusion of *Psu*ACR1 from *Proteomonas sulcata* exhibited peak sensitivity at ~520 nm (Govorunova et al., 2016a), as compared to that of a GFP (absorption peak at 485 nm) fusion with the peak at 540 nm (Wietek et al., 2016). The addition of fluorescent tags serving as FRET donors might be used as an engineering strategy to design tools for optogenetic applications in which a broad-band efficiency is desired, as has previously been achieved by fusing two ChRs with different spectra (Batabyal et al., 2015).

PERSPECTIVES

Rapid progress in ChR research in recent years suggests that neither their natural diversity, nor molecular mechanisms have been fully explored. Better understanding of these mechanisms has not only fundamental, but also practical importance for improving their utility as optogenetic tools, e.g., obtaining more selective light-gated channels. In particular, a purely Na⁺-selective CCR would eliminate photoinduced acidification of the cytoplasm (Lin et al., 2009) that may activate various endogenous ion channels and receptors (Beppu et al., 2014), and an elevation of the cytosolic Ca²⁺ (Lin et al., 2009) may cause release of Ca²⁺ from intracellular stores and influence the activity of cellular kinases and transcription factors. On the other hand, a purely Ca²⁺-selective CCR would be invaluable for photocontrol of many Ca²⁺-regulated physiological and biochemical processes such as synaptic release, and a K⁺-selective CCR would be a powerful optogenetic tool for neuronal silencing.



So far, electrophysiological characterization has lagged behind bioinformatic identification of new ChR sequences. The advent of automated patch clamping is likely to change that (Suk et al., 2019; Obergrussberger et al., 2021). Recently, a high-throughput, automated planar patch clamp system has been used to determine ion permeability of novel cryptophyte ACR homologs (Govorunova et al., 2021). In this approach, a suspension of dissociated cells is loaded into wells of a 384-well plate with a glass bottom, in which microscopic orifices are bored. Suction is applied to capture a cell in each well and form a gigaohm seal. This technique allows simultaneous recording from 384 or even 768 cells (in two-module systems), which greatly reduces the time of experimentation. Alternatively, fluorescent voltage, proton and/or Ca²⁺ imaging can be used for rapid screening of multiple CCR variants and mutants (Cho et al., 2019).

Rational protein engineering can improve biophysical properties of ChRs to address specific needs of optogenetic applications. However, mutations that bring about desired changes such as faster current kinetics or red-shifted absorption are usually accompanied by undesired effects, such as reduction of expression and/or conductance. Machine learning algorithms might be a means to solve this problem. They have already been used successfully to generate chimeric ChRs (Bedbrook et al., 2017), and to improve expression, plasma membrane localization, and light sensitivity of ChRs (Bedbrook et al., 2019). Machine learning has also been employed to predict and tune spectral properties of ion-pumping microbial rhodopsins (Inoue et al., 2021), and the same procedures can be applied to ChRs. A systematic analysis of already identified ChR variants and mutants is needed to produce a reliable training set for machine learning.

AUTHOR CONTRIBUTIONS

EG, OS, and JS conceived the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

- Aihara, Y., Maruyama, S., Baird, A. H., Iguchi, A., Takahashi, S., and Minagawa, J. (2019). Green fluorescence from cnidarian hosts attracts symbiotic algae. *Proc. Natl. Acad. Sci. U S A* 116, 2118–2123. doi: 10.1073/pnas.1812257116
- Amon, J. P., and French, K. H. (2004). Photoresponses of the marine protist *Ulkenia* sp. zoospores to ambient, artificial and bioluminescent light. *Mycologia* 96, 463–469. doi: 10.1080/15572536.2005.11832945
- Armitage, J. P. (1997). Behavioural responses of bacteria to light and oxygen. *Arch. Microbiol.* 168, 249–261. doi: 10.1007/s002030050496
- Avelar, G. M., Schumacher, R. I., Zaini, P. A., Leonard, G., Richards, T. A., and Gomes, S. L. (2014). A rhodopsin-guanylyl cyclase gene fusion functions in visual perception in a fungus. *Curr. Biol.* 24, 1234–1240. doi: 10.1016/j.cub.2014.04.009
- Awasthi, M., Sushmita, K., Kaushik, M. S., Ranjan, P., and Kateriya, S. (2020). Novel modular rhodopsins from green algae hold great potential for cellular optogenetic modulation across the biological model systems. *Life (Basel)* 10:259. doi: 10.3390/life10110259
- Azimi Hashemi, N., Erbguth, K., Vogt, A., Riemensperger, T., Rauch, E., Woodmansee, D., et al. (2014). Synthetic retinal analogues modify the spectral and kinetic characteristics of microbial rhodopsin optogenetic tools. *Nat. Commun.* 5:5810. doi: 10.1038/ncomms6810
- Batabyal, S., Cervenka, G., Birch, D., Kim, Y. T., and Mohanty, S. (2015). Broadband activation by white-opsin lowers intensity threshold for cellular stimulation. *Sci. Rep.* 5:17857. doi: 10.1038/srep17857
- Becker-Baldus, J., Leeder, A., Brown, L. J., Brown, R. C. D., Bamann, C., and Glaubitz, C. (2021). The desensitized channelrhodopsin-2 photointermediate contains 13-*cis*,15-*syn* retinal Schiff base. *Angew. Chem. Int. Ed. Engl.* 60, 16442–16447. doi: 10.1002/anie.202015797
- Bedbrook, C. N., Rice, A. J., Yang, K. K., Ding, X., Chen, S., LeProust, E. M., et al. (2017). Structure-guided SCHEMA recombination generates diverse chimeric channelrhodopsins. *Proc. Natl. Acad. Sci. U S A* 114, E2624–E2633. doi: 10.1073/pnas.1700269114
- Bedbrook, C. N., Yang, K. K., Robinson, J. E., Mackey, E. D., Gradinaru, V., and Arnold, F. H. (2019). Machine learning-guided channelrhodopsin engineering enables minimally invasive optogenetics. *Nat. Methods* 16, 1176–1184. doi: 10.1038/s41592-019-0583-8
- Beppu, K., Sasaki, T., Tanaka, K. F., Yamanaka, A., Fukazawa, Y., Shigemoto, R., et al. (2014). Optogenetic countering of glial acidosis suppresses glial glutamate release and ischemic brain damage. *Neuron* 81, 314–320. doi: 10.1016/j.neuron.2013.11.011
- Berndt, A., Lee, S. Y., Ramakrishnan, C., and Deisseroth, K. (2014). Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. *Science* 344, 420–424. doi: 10.1126/science.1252367
- Berndt, A., Lee, S. Y., Wietek, J., Ramakrishnan, C., Steinberg, E. E., Rashid, A. J., et al. (2016). Structural foundations of optogenetics: determinants of channelrhodopsin ion selectivity. *Proc. Natl. Acad. Sci. U S A* 113, 822–829. doi: 10.1073/pnas.1523341113
- Berthold, P., Tsunoda, S. P., Ernst, O. P., Mages, W., Gradmann, D., and Hegemann, P. (2008). Channelrhodopsin-1 initiates phototaxis and photophobic responses in *Chlamydomonas* by immediate light-induced depolarization. *Plant Cell* 20, 1665–1677. doi: 10.1105/tpc.108.057919
- Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G., and Deisseroth, K. (2005). Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci.* 8, 1263–1268. doi: 10.1038/nn1525
- Brown, L. S. (2014). Eubacterial rhodopsins - unique photosensors and diverse ion pumps. *Biochim. Biophys. Acta* 1837, 553–561. doi: 10.1016/j.bbabi.2013.05.006

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- Cho, Y. K., Park, D., Yang, A., Chen, F., Chuong, A. S., Klapoetke, N. C., et al. (2019). Multidimensional screening yields channelrhodopsin variants having improved photocurrent and order-of-magnitude reductions in calcium and proton currents. *J. Biol. Chem.* 294, 3806–3821. doi: 10.1074/jbc.RA118.006996
- Deisseroth, K. (2011). Optogenetics. *Nat. Methods* 8, 26–29. doi: 10.1038/nmeth.1324
- Deisseroth, K. (2015). Optogenetics: 10 years of microbial opsins in neuroscience. *Nat. Neurosci.* 18, 1213–1225. doi: 10.1038/nn.4091
- Duan, X., Nagel, G., and Gao, S. (2019). Mutated channelrhodopsins with increased sodium and calcium permeability. *Appl. Sci.* 9:664. doi: 10.3390/app9040664
- Erata, M., Kubota, M., Takahashi, T., Inouye, I., and Watanabe, M. (1995). Ultrastructure and phototactic action spectra of two genera of cryptophyte flagellate algae, *Cryptomonas* and *Chroomonas*. *Protoplasma* 188, 258–266. doi: 10.1007/BF01280378
- Ernst, O. P., Lodowski, D. T., Elstner, M., Hegemann, P., Brown, L. S., and Kandori, H. (2014). Microbial and animal rhodopsins: structures, functions and molecular mechanisms. *Chem. Rev.* 114, 126–163. doi: 10.1021/cr4003769
- Ernst, O. P., Sanchez Murcia, P. A., Daldrop, P., Tsunoda, S. P., Kateriya, S., and Hegemann, P. (2008). Photoactivation of channelrhodopsin. *J. Biol. Chem.* 283, 1637–1643. doi: 10.1074/jbc.M708039200
- Forward, R. B., Jr. (1974). Phototaxis by the dinoflagellate *Gymnodinium splendens* Lebour. *J. Protozool.* 21, 312–315. doi: 10.1111/j.1550-7408.1974.tb03659.x
- Gentil, J., Hempel, F., Moog, D., Zauner, S., and Maier, U. G. (2017). Review: origin of complex algae by secondary endosymbiosis: a journey through time. *Protoplasma* 254, 1835–1843. doi: 10.1007/s00709-017-1098-8
- Govorunova, E. G., Sineshchekov, O. A., Hemmati, R., Janz, R., Morelle, O., Melkonian, M., et al. (2018). Extending the time domain of neuronal silencing with cryptophyte anion channelrhodopsins. *eNeuro* 5:ENEURO.0174-0118.2018. doi: 10.1523/ENEURO.0174-18.2018
- Govorunova, E. G., Sineshchekov, O. A., Li, H., Janz, R., and Spudich, J. L. (2013). Characterization of a highly efficient blue-shifted channelrhodopsin from the marine alga *Platymonas subcordiformis*. *J. Biol. Chem.* 288, 29911–29922. doi: 10.1074/jbc.M113.505495
- Govorunova, E. G., Sineshchekov, O. A., Li, H., Wang, Y., Brown, L. S., Palmateer, A., et al. (2021). Cation and anion channelrhodopsins: sequence motifs and taxonomic distribution. *mBio* 12:e0165621. doi: 10.1128/mBio.01656-21
- Govorunova, E. G., Sineshchekov, O. A., Li, H., Wang, Y., Brown, L. S., and Spudich, J. L. (2020). RubyACRs, non-algal anion channelrhodopsins with highly red-shifted absorption. *Proc. Natl. Acad. Sci. U S A* 117, 22833–22840. doi: 10.1073/pnas.2005981117
- Govorunova, E. G., Sineshchekov, O. A., Liu, X., Janz, R., and Spudich, J. L. (2015). Natural light-gated anion channels: a family of microbial rhodopsins for advanced optogenetics. *Science* 349, 647–650. doi: 10.1126/science.aaa7484
- Govorunova, E. G., Sineshchekov, O. A., Li, H., and Spudich, J. L. (2017a). Microbial rhodopsins: diversity, mechanisms and optogenetic applications. *Annu. Rev. Biochem.* 86, 845–872. doi: 10.1146/annurev-biochem-101910-144233
- Govorunova, E. G., Sineshchekov, O. A., Rodarte, E. M., Janz, R., Morelle, O., Melkonian, M., et al. (2017b). The expanding family of natural anion channelrhodopsins reveals large variations in kinetics, conductance and spectral sensitivity. *Sci. Rep.* 7:43358. doi: 10.1038/srep43358
- Govorunova, E. G., Sineshchekov, O. A., and Spudich, J. L. (2016a). *Proteomonas sulcata* ACR1: a fast anion channelrhodopsin. *Photochem. Photobiol.* 92, 257–263. doi: 10.1111/php.12558
- Govorunova, E. G., Sineshchekov, O. A., and Spudich, J. L. (2016b). Structurally distinct cation channelrhodopsins from cryptophyte algae. *Biophys. J.* 110, 2302–2304. doi: 10.1016/j.bpj.2016.05.001

- Govorunova, E. G., Spudich, E. N., Lane, C. E., Sineshchekov, O. A., and Spudich, J. L. (2011). New channelrhodopsin with a red-shifted spectrum and rapid kinetics from *Mesostigma viride*. *mBio* 2, e00115–e00111. doi: 10.1128/mBio.00115-11
- Gradinaru, V., Zhang, F., Ramakrishnan, C., Mattis, J., Prakash, R., Diester, I., et al. (2010). Molecular and cellular approaches for diversifying and extending optogenetics. *Cell* 141, 154–165. doi: 10.1016/j.cell.2010.02.037
- Gradmann, D., Berndt, A., Schneider, F., and Hegemann, P. (2011). Rectification of the channelrhodopsin early conductance. *Biophys. J.* 101, 1057–1068. doi: 10.1016/j.bpj.2011.07.040
- Gunaydin, L. A., Yizhar, O., Berndt, A., Sohal, V. S., Deisseroth, K., and Hegemann, P. (2010). Ultrafast optogenetic control. *Nat. Neurosci.* 13, 387–392. doi: 10.1038/nn.2495
- Hackett, J. D., Anderson, D. M., Erdner, D. L., and Bhattacharya, D. (2004). Dinoflagellates: a remarkable evolutionary experiment. *Am. J. Bot.* 91, 1523–1534. doi: 10.3732/ajb.91.10.1523
- Hackl, T., Martin, R., Barenhoff, K., Duponchel, S., Heider, D., and Fischer, M. G. (2020). Four high-quality draft genome assemblies of the marine heterotrophic nanoflagellate *Cafeteria roenbergensis*. *Sci. Data* 7:29. doi: 10.1038/s41597-020-0363-4
- Hegemann, P., Ehlenbeck, S., and Gradmann, D. (2005). Multiple photocycles of channelrhodopsin. *Biophys. J.* 89, 3911–3918. doi: 10.1529/biophysj.105.069716
- Hille, B. (2001). *Ion Channels of Excitable Membranes*. Sunderland, MA: Sinauer Associates.
- Hollingsworth, L. L., Kinzie, R. A., Lewis, T. D., Krupp, D. A., and Leong, J. A. C. (2005). Phototaxis of motile zooxanthellae to green light may facilitate symbiont capture by coral larvae. *Coral Reefs* 24:523. doi: 10.1007/s00338-005-0063-8
- Hontani, Y., Broser, M., Luck, M., Weissenborn, J., Kloz, M., Hegemann, P., et al. (2020). Dual photoisomerization on distinct potential energy surfaces in a UV absorbing rhodopsin. *J. Am. Chem. Soc.* 142, 11464–11473. doi: 10.1021/jacs.0c03229
- Horiguchi, T., Kawai, H., Kubota, M., Takahashi, T., and Watanabe, M. (1999). Phototactic responses of four marine dinoflagellates with different types of eyespot and chloroplast. *Phycol. Res.* 47, 101–107. doi: 10.1046/j.1440-1835.1999.47220158.x
- Hou, S. Y., Govorunova, E. G., Ntefidou, M., Lane, C. E., Spudich, E. N., Sineshchekov, O. A., et al. (2012). Diversity of *Chlamydomonas* channelrhodopsins. *Photochem. Photobiol.* 88, 119–128. doi: 10.1111/j.1751-1097.2011.01027.x
- Inoue, K., Karasuyama, M., Nakamura, R., Konno, M., Yamada, D., Mannen, K., et al. (2021). Exploration of natural red-shifted rhodopsins using a machine learning-based Bayesian experimental design. *Commun. Biol.* 4:362. doi: 10.1038/s42003-021-01878-9
- Ishizuka, T., Kakuda, M., Araki, R., and Yawo, H. (2006). Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels. *Neurosci. Res.* 54, 85–94. doi: 10.1016/j.neures.2005.10.009
- Janke, C., Scholz, F., Becker-Baldus, J., Glaubit, C., Wood, P. G., Bamberg, E., et al. (2013). Photocycle and vectorial proton transfer in a rhodopsin from the eukaryote *Oxyrrhis marina*. *Biochemistry* 52, 2750–2763. doi: 10.1021/bi301412n
- Kandori, H. (2015). Ion-pumping microbial rhodopsins. *Front. Mol. Biosci.* 2:52. doi: 10.3389/fmolb.2015.00052
- Kandori, H. (2020). Biophysics of rhodopsins and optogenetics. *Biophys. Rev.* 12, 355–361. doi: 10.1007/s12551-020-00645-0
- Kato, H. E., Kamiya, M., Sugo, S., Ito, J., Taniguchi, R., Orito, A., et al. (2015). Atomistic design of microbial opsin-based blue-shifted optogenetics tools. *Nat. Commun.* 6:7177. doi: 10.1038/ncomms8177
- Kato, H. E., Zhang, F., Yizhar, O., Ramakrishnan, C., Nishizawa, T., Hirata, K., et al. (2012). Crystal structure of the channelrhodopsin light-gated cation channel. *Nature* 482, 369–374. doi: 10.1038/nature10870
- Keeling, P. J., Burki, F., Wilcox, H. M., Allam, B., Allen, E. E., Amaral-Zettler, L. A., et al. (2014). The marine microbial eukaryote transcriptome sequencing project (MMETSP): illuminating the functional diversity of eukaryotic life in the oceans through transcriptome sequencing. *PLoS Biol.* 12:e1001889. doi: 10.1371/journal.pbio.1001889
- Kim, Y. S., Kato, H. E., Yamashita, K., Ito, S., Inoue, K., Ramakrishnan, C., et al. (2018). Crystal structure of the natural anion-conducting channelrhodopsin GtACR1. *Nature* 561, 343–348. doi: 10.1038/s41586-018-0511-6
- Kishi, K. E., Kim, Y. S., Fukuda, M., Kusakizako, T., Thadhani, E., Byrne, E. F. X., et al. (2021). Structural basis for channel conduction in the pump-like channelrhodopsin ChRmine. *bioRxiv* [Preprint]. doi: 10.1101/2021.08.15.456392
- Klapoetke, N. C., Murata, Y., Kim, S. S., Pulver, S. R., Birdsey-Benson, A., Cho, Y. K., et al. (2014). Independent optical excitation of distinct neural populations. *Nat. Methods* 11, 338–346. doi: 10.1038/nmeth.2836
- Kleinlogel, S., Feldbauer, K., Dempski, R. E., Fotis, H., Wood, P. G., Bamann, C., et al. (2011). Ultra light-sensitive and fast neuronal activation with the Ca²⁺-permeable channelrhodopsin CatCh. *Nat. Neurosci.* 14, 513–518. doi: 10.1038/nn.2776
- Konno, M., Yamauchi, Y., Inoue, K., and Kandori, H. (2020). Expression analysis of microbial rhodopsin-like genes in *Guillardia theta*. *PLoS One* 15:e0243387. doi: 10.1371/journal.pone.0243387
- Kuhne, J., Vierock, J., Tennigkeit, S. A., Dreier, M. A., Wietek, J., Petersen, D., et al. (2019). Unifying photocycle model for light adaptation and temporal evolution of cation conductance in channelrhodopsin-2. *Proc. Natl. Acad. Sci. U S A* 116, 9380–9389. doi: 10.1073/pnas.1818707116
- Lanyi, J. K. (2006). Proton transfers in the bacteriorhodopsin photocycle. *Biochim. Biophys. Acta* 1757, 1012–1018. doi: 10.1016/j.bbabi.2005.11.003
- Leliaert, F., Smith, D. R., Moreau, H., Herron, M. D., Verbruggen, H., Delwiche, C. F., et al. (2012). Phylogeny and molecular evolution of the green algae. *Crit. Rev. Plant Sci.* 31, 1–46. doi: 10.1080/07352689.2011.615705
- Li, H., Huang, C. Y., Govorunova, E. G., Schafer, C. T., Sineshchekov, O. A., Wang, M., et al. (2019). Crystal structure of a natural light-gated anion channelrhodopsin. *eLife* 8:e41741. doi: 10.7554/eLife.41741
- Li, H., Huang, C.-Y., Govorunova, E. G., Sineshchekov, O. A., Yi, A., Rothschild, K. J., et al. (2021). The crystal structure of bromide-bound GtACR1 reveals a pre-activated state in the transmembrane anion tunnel. *eLife* 10:e65903. doi: 10.7554/eLife.65903
- Lin, J. Y. (2012). Optogenetic excitation of neurons with channelrhodopsins: light instrumentation, expression systems and channelrhodopsin variants. *Prog. Brain Res.* 196, 29–47. doi: 10.1016/B978-0-444-59426-6.00002-1
- 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. doi: 10.1016/j.bpj.2008.11.034
- Lorenz-Fonfria, V. A., and Heberle, J. (2014). Channelrhodopsin unchained: structure and mechanism of a light-gated cation channel. *Biochim. Biophys. Acta* 1837, 626–642. doi: 10.1016/j.bbabi.2013.10.014
- Luck, M., Mathes, T., Bruun, S., Fudim, R., Hagedorn, R., Tran Nguyen, T. M., et al. (2012). A photochromic histidine kinase rhodopsin (HKR1) that is bimodally switched by ultraviolet and blue light. *J. Biol. Chem.* 287, 40083–40090. doi: 10.1074/jbc.M112.401604
- Luecke, H., Schobert, B., Richter, H. T., Cartailier, J. P., and Lanyi, J. K. (1999). Structure of bacteriorhodopsin at 1.55 Å resolution. *J. Mol. Biol.* 291, 899–911. doi: 10.1006/jmbi.1999.3027
- Marshall, J. H., Kim, Y. S., Machado, T. A., Quirin, S., Benson, B., Kadmon, J., et al. (2019). Cortical layer-specific critical dynamics triggering perception. *Science* 365:eaaw5202. doi: 10.1126/science.aaw5202
- Matasci, N., Hung, L. H., Yan, Z., Carpenter, E. J., Wickett, N. J., Mirarab, S., et al. (2014). Data access for the 1,000 Plants (1KP) project. *Gigascience* 3:17. doi: 10.1186/2047-217X-3-17
- Moro, I., Negrison, E., Callegaro, A., and Andreoli, C. (2003). *Aplanochytrium stochinoi*: a new Labyrinthulomycota from the southern ocean (Ross Sea, Antarctica). *Protist* 154, 331–340. doi: 10.1078/143446103322454103
- Mukherjee, S., Hegemann, P., and Broser, M. (2019). Enzymerhodopsins: novel photoregulated catalysts for optogenetics. *Curr. Opin. Struct. Biol.* 57, 118–126. doi: 10.1016/j.sbi.2019.02.003
- Nagel, G., Ollig, D., Fuhrmann, M., Kateriya, S., Musti, A. M., Bamberg, E., et al. (2002). Channelrhodopsin-1: a light-gated proton channel in green algae. *Science* 296, 2395–2398. doi: 10.1126/science.1072068
- Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., et al. (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc. Natl. Acad. Sci. U S A* 100, 13940–13945. doi: 10.1073/pnas.1936192100

- Nichols, C. G., Ho, K., and Hebert, S. (1994). Mg^{2+} -dependent inward rectification of ROMK1 potassium channels expressed in *Xenopus* oocytes. *J. Physiol.* 476, 399–409. doi: 10.1113/jphysiol.1994.sp020141
- Obergrussberger, A., Friis, S., Bruggemann, A., and Fertig, N. (2021). Automated patch clamp in drug discovery: major breakthroughs and innovation in the last decade. *Expert Opin. Drug Discov.* 16, 1–5. doi: 10.1080/17460441.2020.1791079
- Oda, K., Nomura, T., Nakane, T., Yamashita, K., Inoue, K., Ito, S., et al. (2021). Time-resolved serial femtosecond crystallography reveals early structural changes in channelrhodopsin. *eLife* 10:e62389. doi: 10.7554/eLife.62389
- Oda, K., Vierock, J., Oishi, S., Rodriguez-Rozada, S., Taniguchi, R., Yamashita, K., et al. (2018). Crystal structure of the red light-activated channelrhodopsin Chrimson. *Nat. Commun.* 9:3949. doi: 10.1038/s41467-018-06421-9
- Okitsu, T., Yamano, Y., Shen, Y. C., Sasaki, T., Kobayashi, Y., Morisawa, S., et al. (2020). Synthesis of one double bond-inserted retinal analogs and their binding experiments with opsins: preparation of novel red-shifted channelrhodopsin variants. *Chem. Pharm. Bull. (Tokyo)* 68, 265–272. doi: 10.1248/cpb.c19-01005
- Oppermann, J., Fischer, P., Silapetere, A., Liepe, B., Rodriguez-Rozada, S., Flores-Uribe, J., et al. (2019). MerMAIDs: a family of metagenomically discovered marine anion-conducting and intensely desensitizing channelrhodopsins. *Nat. Commun.* 10:3315. doi: 10.1038/s41467-019-11322-6
- Pan, J., Del Campo, J., and Keeling, P. J. (2017). Reference tree and environmental sequence diversity of Labyrinthulomycetes. *J. Eukaryot. Microbiol.* 64, 88–96. doi: 10.1111/jeu.12342
- Penzkofer, A., Luck, M., Mathes, T., and Hegemann, P. (2014). Bistable retinal Schiff base photo-dynamics of histidine kinase rhodopsin HKR1 from *Chlamydomonas reinhardtii*. *Photochem. Photobiol.* 90, 773–785. doi: 10.1111/php.12246
- Rappleye, M., and Berndt, A. (2019). Structural basis for ion selectivity and engineering in channelrhodopsins. *Curr. Opin. Struct. Biol.* 57, 176–184. doi: 10.1016/j.sbi.2019.04.008
- Rhiel, E., Westermann, M., Steiniger, F., and Hoischen, C. (2020). The proteorhodopsins of the dinoflagellate *Oxyrrhis marina*: ultrastructure and localization by immunofluorescence light microscopy and immunoelectron microscopy. *Protoplasm* 257, 1531–1541. doi: 10.1007/s00709-020-01530-z
- Rozenberg, A., Inoue, K., Kandori, H., and Beja, O. (2021). Microbial rhodopsins: the last two decades. *Annu. Rev. Microbiol.* 75, 427–447. doi: 10.1146/annurev-micro-031721-020452
- Rozenberg, A., Oppermann, J., Wietek, J., Fernandez Lahore, R. G., Sandaa, R. A., Bratbak, G., et al. (2020). Lateral gene transfer of anion-conducting channelrhodopsins between green algae and giant viruses. *Curr. Biol.* 30, 4910–4920.e5. doi: 10.1016/j.cub.2020.09.056
- Ruiz-Gonzalez, M. X., and Marin, I. (2004). New insights into the evolutionary history of type 1 rhodopsins. *J. Mol. Evol.* 58, 348–358. doi: 10.1007/s00239-003-2557-8
- Sahel, J. A., Boulanger-Scemama, E., Pagot, C., Arleo, A., Galluppi, F., Martel, J. N., et al. (2021). Partial recovery of visual function in a blind patient after optogenetic therapy. *Nat. Med.* 27, 1223–1229. doi: 10.1038/s41591-021-01351-4
- Saita, M., Pranga-Sellnau, F., Resler, T., Schlesinger, R., Heberle, J., and Lorenz-Fonfria, V. A. (2018). Photoexcitation of the P4(480) state induces a secondary photocycle that potentially desensitizes channelrhodopsin-2. *J. Am. Chem. Soc.* 140, 9899–9903. doi: 10.1021/jacs.8b03931
- Sasaki, J., and Spudich, J. L. (2008). Signal transfer in haloarchaeal sensory rhodopsin-transducer complexes. *Photochem. Photobiol.* 84, 863–868. doi: 10.1111/j.1751-1097.2008.00314.x
- Schneider-Warme, F. (2018). The power of optogenetics: potential in cardiac experimental and clinical electrophysiology. *Herzschrittmacherther. Elektrophysiol.* 29, 24–29. doi: 10.1007/s00399-017-0545-8
- Shen, Y. C., Sasaki, T., Matsuyama, T., Yamashita, T., Shichida, Y., Okitsu, T., et al. (2018). Red-tuning of channelrhodopsin spectrum using long-conjugated retinal analogues. *Biochemistry* 57, 5544–5556. doi: 10.1021/acs.biochem.8b00583
- Shigemura, S., Hososhima, S., Kandori, H., and Tsunoda, S. P. (2019). Ion channel properties of a cation channelrhodopsin, *Gt_CCR4*. *Appl. Sci.* 9:3440. doi: 10.3390/app9173440
- Sineshchekov, O. A., Govorunova, E. G., Jung, K.-H., Zauner, S., Maier, U.-G., and Spudich, J. L. (2005). Rhodopsin-mediated photoreception in cryptophyte flagellates. *Biophys. J.* 89, 4310–4319. doi: 10.1529/biophysj.105.070920
- Sineshchekov, O. A., Govorunova, E. G., Li, H., and Spudich, J. L. (2015). Gating mechanisms of a natural anion channelrhodopsin. *Proc. Natl. Acad. Sci. U S A* 112, 14236–14241. doi: 10.1073/pnas.1513602112
- Sineshchekov, O. A., Govorunova, E. G., Li, H., and Spudich, J. L. (2017). Bacteriorhodopsin-like channelrhodopsins: alternative mechanism for control of cation conductance. *Proc. Natl. Acad. Sci. U S A* 114, E9512–E9519. doi: 10.1073/pnas.1710702114
- Sineshchekov, O. A., Govorunova, E. G., Li, H., Wang, Y., Melkonian, M., Wong, G. K.-S., et al. (2020). Conductance mechanisms of rapidly desensitizing cation channelrhodopsins from cryptophyte algae. *mBio* 11, e00657–e00620. doi: 10.1128/mBio.00657-20
- Sineshchekov, O. A., Govorunova, E. G., Li, H., Wang, X., and Spudich, J. L. (2019). Opposite charge movements within the photoactive site modulate two-step channel closing in *GtACR1*. *Biophys. J.* 117, 2034–2040. doi: 10.1016/j.bpj.2019.10.009
- Sineshchekov, O. A., Govorunova, E. G., Wang, J., and Spudich, J. L. (2012). Enhancement of the long-wavelength sensitivity of optogenetic microbial rhodopsins by 3,4-dehydroretinal. *Biochemistry* 51, 4499–4506. doi: 10.1021/bi2018859
- 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. doi: 10.1073/pnas.122243399
- Sineshchekov, O. A., Li, H., Govorunova, E. G., and Spudich, J. L. (2016). Photochemical reaction cycle transitions during anion channelrhodopsin gating. *Proc. Natl. Acad. Sci. U S A* 113, E1993–E2000. doi: 10.1073/pnas.1525269113
- Slamovits, C. H., Okamoto, N., Burri, L., James, E. R., and Keeling, P. J. (2011). A bacterial proteorhodopsin proton pump in marine eukaryotes. *Nat. Commun.* 2:183. doi: 10.1038/ncomms1188
- Spudich, J. L. (2006). The multitasking microbial sensory rhodopsins. *Trends Microbiol.* 14, 480–487. doi: 10.1016/j.tim.2006.09.005
- 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. doi: 10.1146/annurev.cellbio.16.1.365
- Suk, H. J., Boyden, E. S., and van Welie, I. (2019). Advances in the automation of whole-cell patch clamp technology. *J. Neurosci. Methods* 326:108357. doi: 10.1016/j.jneumeth.2019.108357
- Tanimoto, S., Sugiyama, Y., Takahashi, T., Ishizuka, T., and Yawo, H. (2013). Involvement of glutamate 97 in ion influx through photo-activated channelrhodopsin-2. *Neurosci. Res.* 75, 13–22. doi: 10.1016/j.neures.2012.05.008
- Tashiro, R., Sushmita, K., Hososhima, S., Sharma, S., Kateriya, S., Kandori, H., et al. (2021). Specific residues in the cytoplasmic domain modulate photocurrent kinetics of channelrhodopsin from the alga *Klebsormidium nitens*. *Commun. Biol.* 4:235. doi: 10.1038/s42003-021-01755-5
- Tokioka, R., Matsuoka, K., Nakaoka, Y., and Kito, Y. (1991). Extraction of retinal from *Paramecium bursaria*. *Photochem. Photobiol.* 53, 149–151. doi: 10.1111/j.1751-1097.1991.tb08481.x
- Tsujimura, M., Noji, T., Saito, K., Kojima, K., Sudo, Y., and Ishikita, H. (2021). Mechanism of absorption wavelength shifts in anion channelrhodopsin-1 mutants. *Biochim. Biophys. Acta Bioenerg.* 1862:148349. doi: 10.1016/j.bbabi.2020.148349
- Tsunoda, S. P., and Hegemann, P. (2009). Glu 87 of channelrhodopsin-1 causes pH-dependent color tuning and fast photocurrent inactivation. *Photochem. Photobiol.* 85, 564–569. doi: 10.1111/j.1751-1097.2008.00519.x
- Volkov, O., Kovalev, K., Polovinkin, V., Borshchevskiy, V., Bamann, C., Astashkin, R., et al. (2017). Structural insights into ion conduction by channelrhodopsin 2. *Science* 358:eaan8862. doi: 10.1126/science.aan8862
- Watanabe, M., and Furuya, M. (1982). Phototactic behavior of individual cells of *Cryptomonas* sp. in response to continuous and intermittent light stimuli. *Photochem. Photobiol.* 35, 559–563. doi: 10.1111/j.1751-1097.1982.tb02609.x
- Watanabe, S., Ishizuka, T., Hososhima, S., Zamani, A., Hoque, M. R., and Yawo, H. (2016). The regulatory mechanism of ion permeation through a

- channelrhodopsin derived from *Mesostigma viride* (MvChR1). *Photochem. Photobiol. Sci.* 15, 365–374. doi: 10.1039/c5pp00290g
- Wiegert, J. S., Mahn, M., Prigge, M., Printz, Y., and Yizhar, O. (2017). Silencing neurons: tools, applications and experimental constraints. *Neuron* 95, 504–529. doi: 10.1016/j.neuron.2017.06.050
- Wietek, J., Beltramo, R., Scanziani, M., Hegemann, P., Oertner, T. G., and Wiegert, J. S. (2015). An improved chloride-conducting channelrhodopsin for light-induced inhibition of neuronal activity *in vivo*. *Sci. Rep.* 5:14807. doi: 10.1038/srep14807
- Wietek, J., Broser, M., Krause, B. S., and Hegemann, P. (2016). Identification of a natural green light absorbing chloride conducting channelrhodopsin from *Proteomonas sulcata*. *J. Biol. Chem.* 291, 4121–4127. doi: 10.1074/jbc.M115.699637
- Wietek, J., Rodriguez-Rozada, S., Tutas, J., Tenedini, F., Grimm, C., Oertner, T. G., et al. (2017). Anion-conducting channelrhodopsins with tuned spectra and modified kinetics engineered for optogenetic manipulation of behavior. *Sci. Rep.* 7:14957. doi: 10.1038/s41598-017-14330-y
- Wietek, J., Wiegert, J. S., Adeishvili, N., Schneider, F., Watanabe, H., Tsunoda, S. P., et al. (2014). Conversion of channelrhodopsin into a light-gated chloride channel. *Science* 344, 409–412. doi: 10.1126/science.1249375
- Woo, Y. H., Ansari, H., Otto, T. D., Klinger, C. M., Kolisko, M., Michalek, J., et al. (2015). Chromerid genomes reveal the evolutionary path from photosynthetic algae to obligate intracellular parasites. *eLife* 4:e06974. doi: 10.7554/eLife.06974
- Yamauchi, Y., Konno, M., Ito, S., Tsunoda, S. P., Inoue, K., and Kandori, H. (2017). Molecular properties of a DTD channelrhodopsin from *Guillardia theta*. *Biophys. Physicobiol.* 14, 57–66. doi: 10.2142/biophysico.14.0_57
- Yawo, H., Asano, T., Sakai, S., and Ishizuka, T. (2013). Optogenetic manipulation of neural and non-neural functions. *Dev. Growth Differ.* 55, 474–490. doi: 10.1111/dgd.12053
- Zabelskii, D., Alekseev, A., Kovalev, K., Rankovic, V., Balandin, T., Soloviov, D., et al. (2020). Viral rhodopsins 1 are a unique family of light-gated cation channels. *Nat. Commun.* 11:5707. doi: 10.1038/s41467-020-19457-7
- Zhang, H., Hou, Y., Miranda, L., Campbell, D. A., Sturm, N. R., Gaasterland, T., et al. (2007). Spliced leader RNA trans-splicing in dinoflagellates. *Proc. Natl. Acad. Sci. U S A* 104, 4618–4623. doi: 10.1073/pnas.0700258104
- Zhang, F., Vierock, J., Yizhar, O., Fenno, L. E., Tsunoda, S., Kianianmomeni, A., et al. (2011). The microbial opsin family of optogenetic tools. *Cell* 147, 1446–1457. doi: 10.1016/j.cell.2011.12.004
- Zhang, F., Prigge, M., Beyriere, F., Tsunoda, S. P., Mattis, J., Yizhar, O., et al. (2008). Red-shifted optogenetic excitation: a tool for fast neural control derived from *Volvox carteri*. *Nat. Neurosci.* 11, 631–633. doi: 10.1038/nn.2120

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