Channelrhodopsin engineering and exploration of new optogenetic tools

Peter Hegemann & Andreas Möglich

Rhodopsins from microalgae and eubacteria are powerful tools for manipulating the function of neurons and other cells, but these tools still have limitations. We discuss engineering approaches that can help advance optogenetics.

Channelrhodopsins are protein channels that transport cations across the plasma membrane in response to stimulation with light. To date, four channelrhodopsin variants have been discovered: ChR1 and ChR2 from *Chlamydomonas reinhardtii* and VChR1 and VChR2 from *Volvox carteri*.

All channelrhodopsins discovered so far function in nature as sensory photoreceptors that guide microalgae toward or away from light (phototaxis) and thus optimize the light conditions for photosynthetic growth. This cellular response must be graded over several orders of light intensity and must be attenuated under strong light to prevent complete depolarization of the cell or costly counterbalancing by outward ion pumping. Thus, the number of ions transported per absorbed photon by a single channelrhodopsin must be small, and the current must decrease under conditions of extended illumination to a relatively small stationary level[1].

Despite the great success that channelrhodopsins have had in recent years as tools to modulate the activity of neurons via optogenetics, their small current and strong inactivation properties, are less than ideal for certain biological applications. Moreover, the natural channelrhodopsin absorption spectrum does not normally extend beyond 520 nanometers (the exception being VChR1 with an absorption maximum at 535 nanometers), which limits their *in vivo* application in high light-scattering media such as the brain.

In recent years, strategies have been used to create channelrhodopsin variants with new and improved properties for optogenetic applications. Four directions have been pursued: site-directed mutagenesis; domain swapping between different channelrhodopsin species; modification of N and C termini; and genome mining in search of new channelrhodopsin sequences. All four approaches will need to be combined to strengthen the field of optogenetic tool development.

Here we will discuss some of the channelrhodopsin properties that have been optimized using engineering strategies and comment on how future developments will help to advance the function of channelrhodopsin as well as other optogenetic tools.

Improving photocurrents and inactivation properties

Photocurrents mediated by channelrhodopsin upon activation with light in the host cell can be improved by optimizing channelrhodopsin expression, by extending channelrhodopsin's open-state lifetime and by improving ion conductance. Strategies to optimize expression of exogenously expressed rhodopsins in the host-cell membrane are discussed by Karl Deisseroth in this issue[2].

To model the photocurrent kinetics of channelrhodopsin, including its fast inactivation kinetics and slow recovery of the full sensitivity in the dark, at least two closed dark states—one dark adapted (DA) state and one light adapted (LA) state—and two open conducting states, O1 and O2, are needed. Two separate light reactions mediate the transition between these states (**Fig. 1**). When photons activate channelrhodopsin in its DA state, the channel reaches the O1 state. In conditions of continuous light, channelrhodopsin reaches an equilibrium consisting of O1 and O2 states. As the O2 state is less permeant for Na^+ than the O1 state is, the transition from the O1 state to the O2 state results in reduced current amplitudes. This results in a decline of the photocurrent toward a stationary level with progressive inactivation of the channel and population of the LA state. This transition is strongly pH-dependent, and it is thought to be mediated by a protonatable residue that has yet to be identified. To engineer channelrhodopsin variants that do not exhibit this inactivation and have an extended open-state lifetime, we would have to mutate this still unknown residue, preventing the transitions between the two open states in the light.

In addition, the transition kinetics between the DA state and open states of channelrhodopsin can be altered by mutating the chromophore-binding pocket (**Fig. 2**). Examples of this are the step-function rhodopsins, which have open-state lifetimes extended from the millisecond range for wild-type channelrhodopsin to the minute range and can be closed by orange light. But these variants transition into an inactive state during long illumination—a problem that still needs to be resolved. Other variants with an accelerated photocycle and a concomitant reduction of the open-state lifetime are called ChETA mutants. The ChR2 E123T variant has the additional advantage that the channel kinetics are fast and that the rise as well as the decay of the O1 state is voltage-independent in contrast to the case for wild-type ChR2

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(A. Berndt, personal communication), rendering them particularly useful for inducing fast action-potential firing[3]. However, the short open-state lifetime of these variants results in a reduction of their photocurrents because it restricts even further the number of ions passing through the channel during a single opening event. This can create difficulties for certain applications, as in conditions in which light is limiting, and the only way to override this is by improving the ion conductance of these variants.

One approach to improve conductance of channelrhodopsins, is to modify their pore in such a way that cations pass through it more efficiently. For example, one could improve the conductance of Na^+ ions by widening the pore to admit water, creating new Na-binding sites. This type of modification results in decreased ion selectivity of the channel and improvement of the conductance of larger Na^+ ions. The H134R (ref. 4) and E90Q (ref. 5) variants, which are thought to resemble modifications of the water-filled pore, improve conductance twofold, but this might be insufficient. Several research groups have promising high-conductance variants under construction, which generates great expectations for the near future.

The light-sensing moiety of rhodopsins is a covalently bound chromophore, retinal, which is a derivative of vitamin A, that is available in seemingly all vertebrate cells (**Fig. 2b**). The retinal chromophore isomerizes upon light absorption from a *cis* to a *trans* configuration or vice versa. The general consensus is that photochemical isomerization of the retinal is the trigger for channelrhodopsin activation and gates the channel. *In vitro* and probably also in cells, a channelrhodopsin population consists of a 2:1 mixture of molecules with their chromophores in an all-*trans* or a 13-*cis* retinal conformation (**Fig. 2b**). Notably, the *cis*:*trans* population ratio does not substantially change during light adaptation or even under exposure to strong continuous light[1,6]. Thus, both light reactions—*trans* to *cis* as well as *cis* to *trans*—occur in parallel, and it is unclear if one or both reactions trigger channel opening. To solve this question and fully understand the gating mechanism of channelrhodopsin, we would need to stabilize one isoform in the dark and study its gating efficiency and kinetics. Understanding the efficiency of both isomerization processes will help us select one or the other for future optogenetic applications.

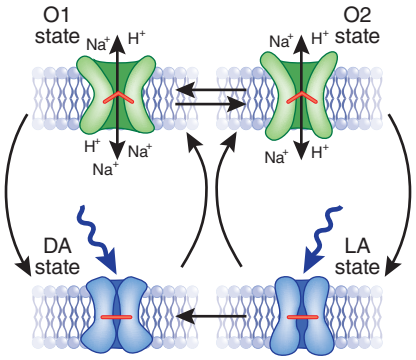

Figure 1 | A simplified kinetic model of channelrhodopsin function. The model includes two closed states; one prevails after dark adaptation (DA state), whereas the other (LA state) is only occupied after several hundred milliseconds in the light. Light absorption and subsequent isomerization of the retinal chromophore (red) converts both dark states into open conducting states, O1 and O2.

Changing the spectral properties of channelrhodopsin

The maximal extinction coefficient, ε, which is the probability that a molecule of channelrhodopsin will absorb a photon of light, is around 50,000 $M^{-1}cm^{-1}$, and the quantum efficiency, Φ, which is the probability that an excited chromophore will undergo a reaction, is 30–70%, values that are typical for rhodopsins. Together, both values define channelrhodopsin's light sensitivity. This parameter can hardly be improved by molecular engineering because it is an intrinsic property of the retinal chromophore. However, a rich and pleasant property of retinal-binding proteins is the enormous versatility of absorption bands and the fact that these can be shifted using engineering.

To expand channelrhodopsin's spectrum from the UV-light range (345 nanometers)[7] to beyond 600 nanometers[8], one can mine algal genomes in search of variants with different spectral properties (http://botany.si.edu/projects/algae/index.htm) or, as has been done with many rhodopsins in the past, mutate its retinal-binding pocket (**Fig. 2**). But color-shifting a certain rhodopsin species without losing function is a difficult task. Single mutations rarely shift the absorption by more than 20 nanometers, and thus multiple mutations often have to be introduced. Owing to the highly complex hydrogen bonding network needed for ion transport across membranes, multiple mutations generally affect the channel's ion transport efficiency, kinetics and protein stability.

An alternative approach is 'helix swapping', in which a defined number of transmembrane helices from the channel pore are exchanged with similar ones from a related species with a different absorption spectrum. The Yawo group pioneered this approach; they constructed a hybrid between ChR2 and ChR1, called ChRGR[9]. ChRGR is highly expressed in the membrane, as is typical for ChR2, but has a 505-nanometer absorption spectra and the low inactivation kinetics characteristic of ChR1 (ref. 9). Following this same strategy and in collaboration with the Deisseroth group, we recently developed C1V1, a hybrid between ChR1 and VChR1, which combines high membrane expression with red-shifted absorption at 535 nm (unpublished data). Given that we still lack sufficient knowledge to fully understand or predict the factors that determine channelrhodopsin's absorption spectra, these two hybrids might be the best starting points for the next round of color-tuning.

For application to cultured cells or tissues the absorption of channelrhodopsin might be shifted over a much wider range by using retinal analogs. Retinal derivatives with a reduced number of double bonds have an absorption spectrum in the UV-light range[10,11] and those containing an azulene ring might absorb beyond 750 nanometers[12]. But these chromophores have to be chemically synthesized, and to use them in cells or tissues one would need to exogenously add them in sufficient amounts (a strategy called 'chemo-optogenetics').

The need for channelrhodopsin crystal structures

Most of the channelrhodopsin mutations generated so far have been chosen on the basis of the homology of channelrhodopsin to bacteriorhodopsin, which has been extensively characterized and is currently the best-understood membrane protein. Unfortunately the two proteins are homologous only in helices 3–7, and there is little sequence homology for helices 1 and 2 of the two proteins. To reach the goals defined above, high-resolution three-dimensional structures for the various dark and open states of channelrhodopsin are the most urgent requirements. Recombinant ChR2 has been purified from green monkey COS cells and *Pichia pastoris*, but the amount of pure protein is still limited. In addition, inhomogeneous dark states with unclear

retinal isomer composition and extent of glycosylation are still severe obstacles for obtaining crystals for high-resolution structure determination.

A few words on light-driven pumps

The light-driven ion pumps bacteriorhodopsin and halorhodopsin, which transport H^+ and Cl^- ions, respectively, fundamentally differ from light-gated channels: 'light driving' is more demanding than 'light gating'. In pumps, the transport is always uphill, that is, against the electrochemical gradient, and the stoichiometry between absorbed photons and transported ions is fixed at less than one. Pumps are fast, with a turnover in the 1–10-millisecond range, at small gradients and are slowed down 10–100-fold if the gradient is large. Engineering pumps is far more difficult than engineering channels. In pumps, both the photocycle kinetics and the driving force must be maintained to keep the transport going at the relevant membrane voltages. To date, most modifications have either slowed down the cycle or made the pump leaky, similar to what happens in naturally occurring proteorhodopsins. Unfortunately, most of the existing bacteriorhodopsin and halorhodopsin variants have been studied only at zero voltage in solution and would need to be reanalysed under voltage clamp before optogenetic applications may be considered. Acceleration of the photocycle at high voltage has, to our knowledge, never been realized by molecular engineering, but natural isolates with fast photocycle and good neuronal expression were recently found in archea and fungi[13]. Pumps with better properties than these found in nature are unlikely to be achieved by engineering. However, accelerated kinetics can only be capitalized on if the light intensity is so high that the pump is permanently active. For hyperpolarization of neuronal cells at low light intensities without flooding the host cell with light-driven pumps, we have only two options, either to find light-gated K^+ or Cl^- channels with high conductance in microbial genomes or to make existing K^+ or Cl^- channels light-sensitive.

Future directions

We also have to think in completely new directions for engineering light-activated channels in the future. Changing the ion selectivity and conductance of channelrhodopsins has fundamental limitations and will likely not turn channelrhodopsin into a light-switchable ion channel with both high conductance and high ion selectivity. Instead, a very different approach could be to confer light-sensitivity to existing channels, which are already highly ion-selective and have large conductance values. This has been approached in the past by chemically modifying specific channels using light-sensitive compounds such as azobenzenes (chemo-optogenetics). An alternative approach could be to fuse these channels with soluble flavin-based photoreceptor modules from plants such as the light, oxygen, voltage (LOV) domain and sensor of blue light using flavin adenine dinucleotide (BLUF domain). The integration of these light-sensitive domains into terminal extensions, loop regions or hormone-binding extracellular modules of the channels could be used to generate chimeric photosensitive proteins without the need for chemical modification.

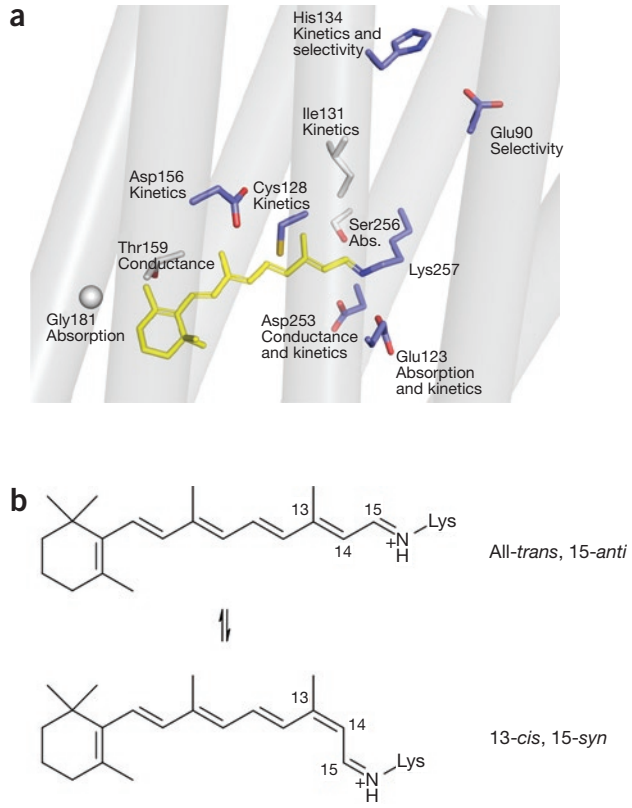

Figure 2 | Structural model of a channelrhodopsin. (**a**) Three-dimensional computer model of ChR2. Mutations of the amino acid residues shown in stick representation are known to substantially influence absorption, conductance (without selectivity change), kinetics and ion selectivity, as indicated for each residue. The retinal moiety is shown in yellow, residues conserved in all four known channelrhodopsins are colored blue, and residues that differ in various channelrhodopsins are colored gray. Oxygen, nitrogen and sulfur atoms are colored red, blue and dark yellow, respectively. Graphics are based on the coordinates of *H. salinarum* bacteriorhodopsin[28] and were drawn with Pymol (Schrödinger). (**b**) Structures of the two possible chromophore dark state isomers: the all-*trans*, 15-*anti* form and the 13-*cis*, 15-*syn* form.

Beyond natural photoreceptors

In addition to retinal-binding proteins, photoreceptors that use as chromophores flavin and tetrapyrrole (biliverdin) that are also widely available to cells should be more strongly considered for optogenetic experiments in the near future. Unlike rhodopsins, which have intrinsic activity, tetrapyrrole- and flavin-based photosensors occur as modular units in conjunction with effector domains. Notably, the photoactivated cyclases from the flagellate *Euglena gracilis*[14] or the soil bacterium *Beggiatoa* sp.[15], produce the general second messenger cyclic AMP in a light-controlled manner and guanylyl cyclase derivatives have been engineered but not yet tested for application[16]. Although only very few microbial photoreceptors have targets that have counterparts in mammals, customized synthetic light-activated proteins can be designed by recombining photosensor modules derived from natural photoreceptors such as LOV,

BLUF, cryptochromes or Phy with light-insensitive effector proteins or enzymes. A few promising light-activated proteins were recently produced and have paved the way for further applications.

Recent review articles cover in detail the design of synthetic photoreceptors[17,18], and their possible applications to cell biology are discussed in this issue by Lim and colleagues[19]. Although only a few synthetic light-activated proteins have been described, several initial conclusions can be drawn. First, where one might have assumed that bestowing light sensitivity on a desired protein effector would be exceedingly difficult, this is not necessarily the case. In all examples to date, small numbers of protein variants (usually 10–20 variants) sufficed to generate synthetic light-activated proteins. Second, design approaches have been successful even when the signaling mechanism of the underlying natural photoreceptors was nebulous. Third, quite disparate effectors such as the small GTPase Rac1 (ref. 20) or the DNA-binding protein TrpR[21] have been made light-sensitive, indicating that photoreceptor design by modular recombination is widely applicable. Fourth, despite having common photochemistry, certain photosensor modules use diverse signaling strategies[22,23]. Even a single photosensor domain can be used in vastly different ways to regulate effector modules[20,21]. Fifth, synthetic light-activated proteins can elicit meaningful physiological responses, even if the difference between their dark and light activities is small (for example, less than tenfold[20]).

Now that the basic proof of concept has been shown, engineering efforts should be concentrated on improved synthetic light-activated proteins suitable for optogenetic applications. Future light-activated proteins should display a high dynamic range of light regulation with only low residual activity in their 'off' state and sufficiently high activity in their 'on' state. Similarly to channelrhodopsin, synthetic light-activated proteins need to be fine-tuned for light sensitivity and response kinetics, which can be readily accomplished through mutations in the photosensor domains. In contrast, color-tuning of photosensors is difficult; a more viable strategy to change the action spectra of synthetic light-activated proteins is to change the entire photosensor, for example, substitute a blue-light sensor for a red-light sensor. The biological function of synthetic light-activated proteins needs to be chosen and optimized so that target systems are perturbed only to the required extent but not more. We expect that there will be demand both for synthetic light-activated proteins that affect fundamental cellular processes, for example, cytoskeletal dynamics[20,24], and for light-activated proteins with very specific effector modules, for example, certain mammalian kinases.

It remains to be seen whether any arbitrary effector function can be controlled by light. In marked contrast to fluorescent proteins, which serve as relatively inert monitoring units, photosensor modules need a much higher extent of interaction and communication with their associated effector modules to accomplish the regulatory function. Thus, generation of synthetic light-activated proteins generally requires more knowledge about photosensor and effector modules than necessary for labeling with a fluorescent protein. It is unlikely that a universal approach suffices to regulate arbitrary effector modules by light; rather, case-specific solutions will be required. Additional research into the properties and mechanisms of both natural and synthetic photoreceptors will benefit such customized approaches. However, certain mechanisms, such as the light-induced dimerization cassettes, are versatile and clearly transferable to other effector units[24–26]. The tremendous advances made in gene delivery discussed by Karl Deisseroth[2] and light application discussed by Simon Peron and Karel Svoboda[27] will facilitate *in vivo* deployment of synthetic photoreceptors. Creative learning from nature will empower optogenetics.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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