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Chemical Genetics

Optochemical Genetics

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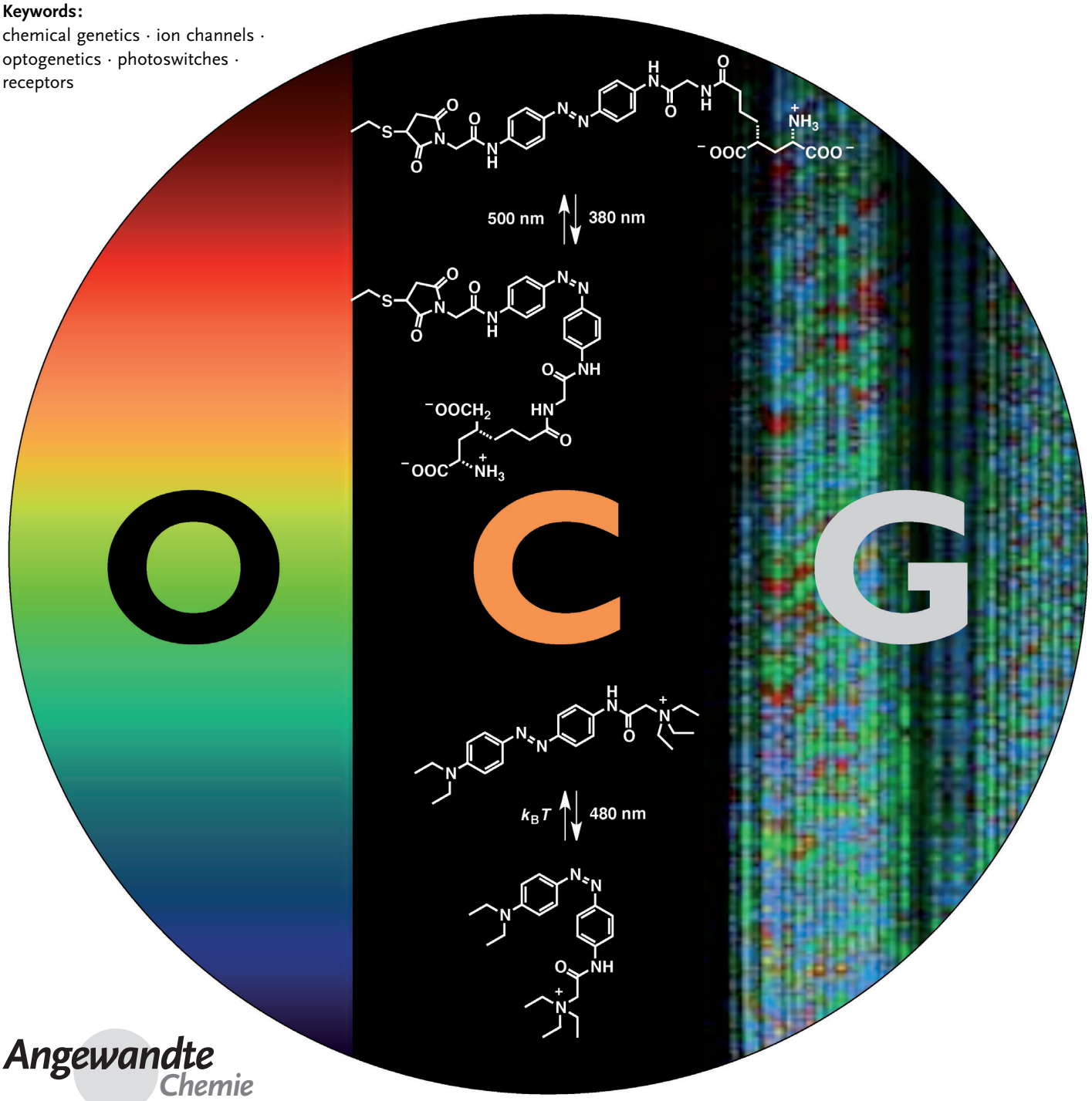

Transmembrane receptors allow a cell to communicate with its environment in response to a variety of input signals. These can be changes in the concentration of ligands (e.g. hormones or neurotransmitters), temperature, pressure (e.g. acoustic waves or touch), transmembrane potential, or light intensity. Many important receptors have now been characterized in atomic detail and our understanding of their functional properties has markedly increased in recent years. As a consequence, these sophisticated molecular machines can be reprogrammed to respond to unnatural input signals. In this Review, we show how voltage-gated and ligand-gated ion channels can be endowed with synthetic photoswitches, and how the resulting artificial photoreceptors can be used to optically control neurons with exceptional temporal and spatial precision. They work well in animals and might find applications in the restoration of vision and the optical control of other sensations. The combination of synthetic photoswitches and receptor proteins contributes to the field of optogenetics and adds a new functional dimension to chemical genetics. As such, we propose to call it "optochemical genetics".

> *"The trick then is not to use the clumsy and inefficient techniques of classical organic chemistry by themselves but to make use of Nature's tools."*
> *Sir Francis Crick, 1999*[1]

From the Contents

1. Introduction

Nature's molecular devices are unsurpassed in their beauty, efficiency, and ability to integrate into complex systems. This is not entirely surprising, given that they have evolved over billions of years. Organic chemistry, by contrast, is about two hundred years old. The synthesis of complex molecules is barely a third of that age, and far from being a mature and efficient technique. Nevertheless, synthetic chemistry has made remarkable progress over the last few decades and many molecules have been created that have no structural or functional counterpart in nature.

Still, what we can make today comes nowhere near to what nature can achieve. The drugs we synthesize are comparatively simple molecules and the switches, motors, and machines we forge are conceptually beautiful, but pale in comparison with nature's devices.[2] These have been studied in great detail by using various biophysical methods, and our understanding of how they work has dramatically increased in recent years. Some famous examples of molecular machines that have now been elucidated in atomic detail include ATP synthase,[3] the ribosome,[4] RNA polymerase,[5] various molecular motors,[6] and ion channels that control nervous activity.[7] Although they operate on a scale where the peculiarities of quantum mechanics apply, they can often be described in terms of relatively simple and intuitive mechanical models.

This much-improved understanding of nature's molecular machines opens the door for their functional manipulation using synthetic chemistry. Just as macroscopic engines can be taken apart, "souped up", and fitted with an ignition key, nanomachines can be manipulated and endowed with additional features and control elements. This can be done to a certain extent at a genetic level, that is, through classical protein engineering, but also by adding and attaching synthetic molecules. The trick is then not to use the traditional techniques of synthetic organic chemistry by themselves, but to make use of them in combination with nature's tools. This approach creates hybrid devices that can be controlled with unnatural input signals and can be easily integrated into highly complex biological systems. As such, they not only function in vitro or in single cells, but also in complex cellular networks—even in neural tissues and in living animals.

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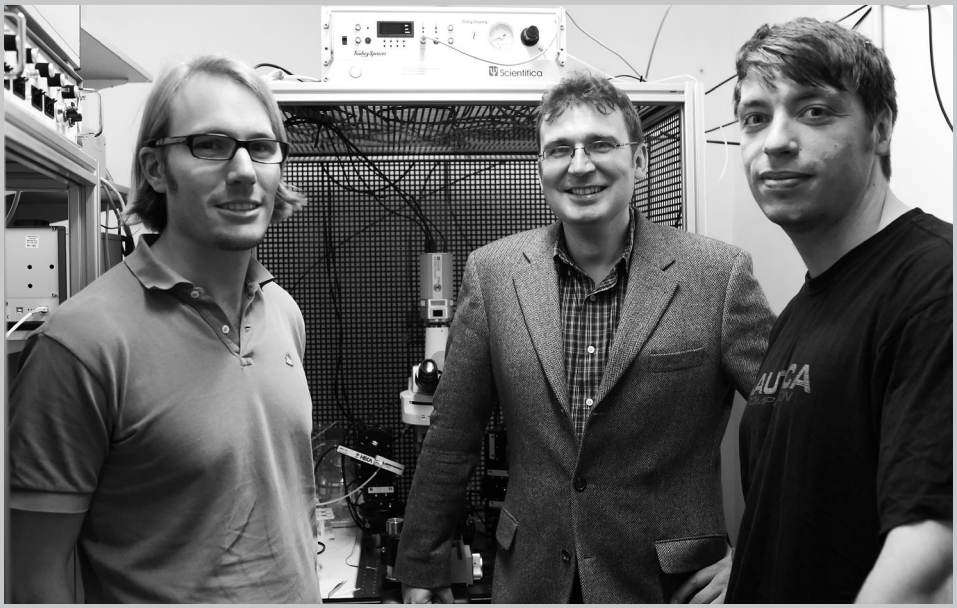
In this Review, we will show how the paradigm—that nature's molecular machines can be harnessed with synthetic chemistry—can be applied to a set of transmembrane proteins that play key roles in neurophysiology. To this end, we will first review some of the elementary machinery that is involved in sensory perception, synaptic transmission, and the generation of action potentials. We will then discuss in general terms how it can be reprogrammed to become sensitive to a very useful stimulus—light—and how the resulting artificial photoreceptors can be used to control and study neural networks. This is also the goal of "optogenetics", a new field of neuroscience that is currently undergoing rapid expansion.[8] We will show how the addition of a chemical component complements and extends optogenetics and how "optochemical genetics" contributes to the dissection and functional enhancement of nervous systems in new and exciting ways.[9]

2. Transmembrane Receptors and Their Role in Neurobiology

Transmembrane receptors underlie cellular communication, including the electrical and chemical communication within and in-between neurons. For the purposes of this Review, we define these receptors as transmembrane proteins that respond to changes in an input signal, be it light intensity, pressure, voltage, temperature, or the concentration of a small ligand. These include ion channels (ionotropic receptors), G-protein-coupled receptors (metabotropic receptors), and receptor-linked enzymes. For many years, these proteins were considered to be very difficult to study by structural methods, particularly by X-ray crystallography. This perception changed in 1998, when MacKinnon and co-workers disclosed the X-ray structure of KcsA, a potassium-selective ion channel sensitive to changes in pH value.[10] Since this groundbreaking study, representatives of several fundamental receptor classes have been characterized in atomic detail, including G-protein-coupled receptors (GPCRs),[11] ionotropic glutamate receptors (iGluRs),[12] trimeric ATP receptors (P2X),[13] voltage-gated ion channels,[14] and pentameric ligand-gated ion channels (pLGICs).[15] For convenience, these receptors are grouped in Figure 1 according to their symmetry. Note that these receptors can exist as homomultimers, but often assemble as heteromultimers composed of several similar, but not identical, subunits.

With the exception of rhodopsin,[11b] none of the receptors shown in Figure 1 are inherently light-sensitive. Three of them, GluA2,[12] $P2X_4$,[13] and the nicotinic acetylcholine receptor,[15a] are ligand-gated ion channels that respond to changes in the concentration of neurotransmitters or extracellular metabolites. Voltage-gated ion channels, such as



Dirk Trauner (middle) was born in Linz, Austria. After studying biology and then biochemistry at the University of Vienna, he joined the group of J. Mulzer at the Free University of Berlin to pursue natural product synthesis. In the late 1990s, he was a postdoctoral fellow with S. J. Danishefsky at the Memorial Sloan-Kettering Cancer Center in New York City. In 2000, he joined the University of California, Berkeley, where he rose through the ranks to become an Associate Professor of chemistry. In 2008, he moved to the University of Munich, where he is currently a Professor for Chemical Biology and Genetics.
Timm Fehrentz (left) studied chemistry in Konstanz, Uppsala, and Heidelberg and performed his Diploma research at the Max Planck Institute for Medical Research, Heidelberg, under the supervision of P. H. Seeburg and J. Spatz. In 2008, he joined the laboratory of D. Trauner at LMU Munich as a graduate student. Part of his graduate work has been carried out as a visiting researcher with R. H. Kramer at the University of California, Berkeley. His research focuses on new PCL strategies to target different voltage-gated ion channels.
Matthias Schönberger (right) studied biomedicinal chemistry at the University of Mainz and graduated in 2009. His Diploma research was carried out in the Brookhaven National Laboratory under the supervision of J. Fowler and J. Hooker and focused on positron emission tomography. Since 2010, he has been a graduate student with D. Trauner at the LMU Munich, working on optochemical genetics. He is a member of the International Max Planck Research School of Life Science and a recipient of a German National Foundation Graduate Fellowship.

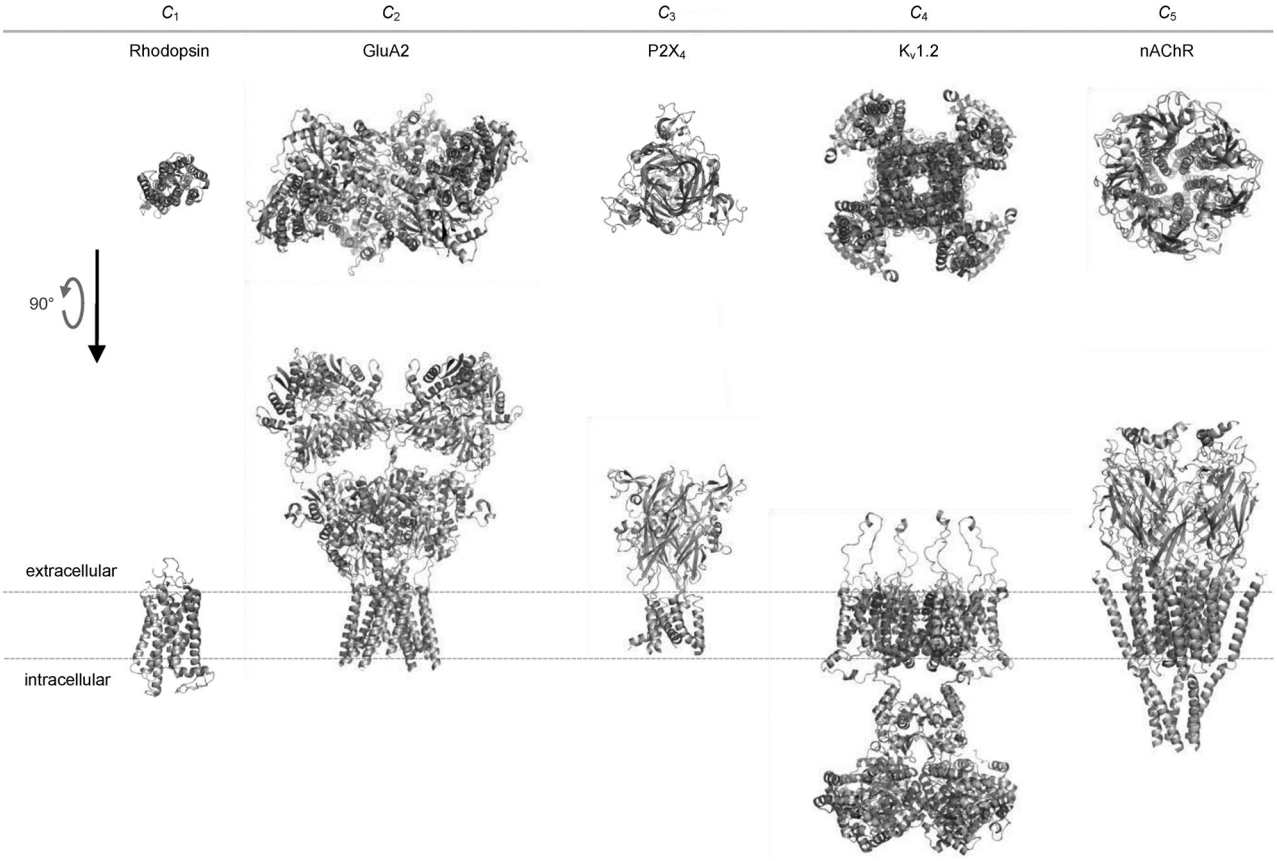


Figure 1. Selected transmembrane receptors of different symmetry characterized by X-ray crystallography and electron microscopy. Each receptor is shown in side and top views (from the extracellular side) and fitted to the same scale. Left to right: Rhodopsin, a G-protein-coupled receptor responsible for vision (pdb 1U19); GluA2, a glutamate-gated ion channel involved in excitatory neurotransmission (pdb 2KG2); $P2X_4$, an ATP-gated cation channel that mediates pain sensation (pdb 3H9V); $K_v1.2$, a voltage-gated potassium channel involved in controlling cellular excitability (pdb 2A79); nAChR, a nicotinic acetylcholine receptor that mediates the communication between nerves and muscles (pdb 2BG9). The dotted lines in this and other figures describe the boundaries between the cell interior, the membrane, and the space outside the cell.

$K_v1.2$,[16] are opened and closed by changes in transmembrane potential. However, they can also be influenced by ligands, such as channel blockers. As a consequence, all of the receptors shown in Figure 1 can be regulated in one form or another by a small molecule. Provided this small molecule can be persuaded to change its activity in response to light, the whole receptor–ligand assembly could be transformed into a photoreceptor.

Before we address this topic, however, it may be worthwhile to briefly review how some of these receptors are involved in neurotransmission and the all-important generation of action potentials (APs).[17] These can be studied by electrophysiology, a very powerful technique that allows for microsecond to millisecond resolution. The generalized shape of an AP and a schematic view of a typical neuron are shown in Figure 2. Ligand-gated cationic ion channels, such as ionotropic glutamate receptors (in the human central nervous system) or nicotinic acetylcholine receptors (mostly in the periphery) are responsible for the initial depolarization of the postsynaptic membrane. Binding of the neurotransmitter to its respective channel leads to pore opening, thereby allowing sodium and potassium ions to pass. Given the resting potential of the neuron and the tightly controlled relative concentrations of sodium and potassium on either side of the membrane, this will lead to a net influx of positive charge, which changes the membrane potential (Figure 2). Once a certain value has been reached (typically around -40 mV), voltage-gated sodium channels begin to open. As sodium rushes in, the membrane is further depolarized and the transmembrane potential inverts its sign (up to $+50$ mV). The neuron is quickly repolarized, however, as the voltage-gated sodium channels deactivate, and voltage-sensitive potassium channels begin to open after a brief delay. Once those are deactivated, the cell regains its resting potentials through the action of transporters and pumps, such as Na/K-ATPase.

Ion channels can have both excitatory and inhibitory effects on neurons, depending on whether they make the transmembrane potential less or more negative when opened. This is not only dependent on the charge of the ions they conduct but also on the relative concentration of ions on either side of the membrane. Consequently, at physiological conditions, nonselective cation channels and sodium channels will depolarize, whereas potassium channels hyper- or repolarize cells when opened. Chloride channels, such as $GABA_A$ or glycine receptors are generally inhibitory on AP firing.[17a]

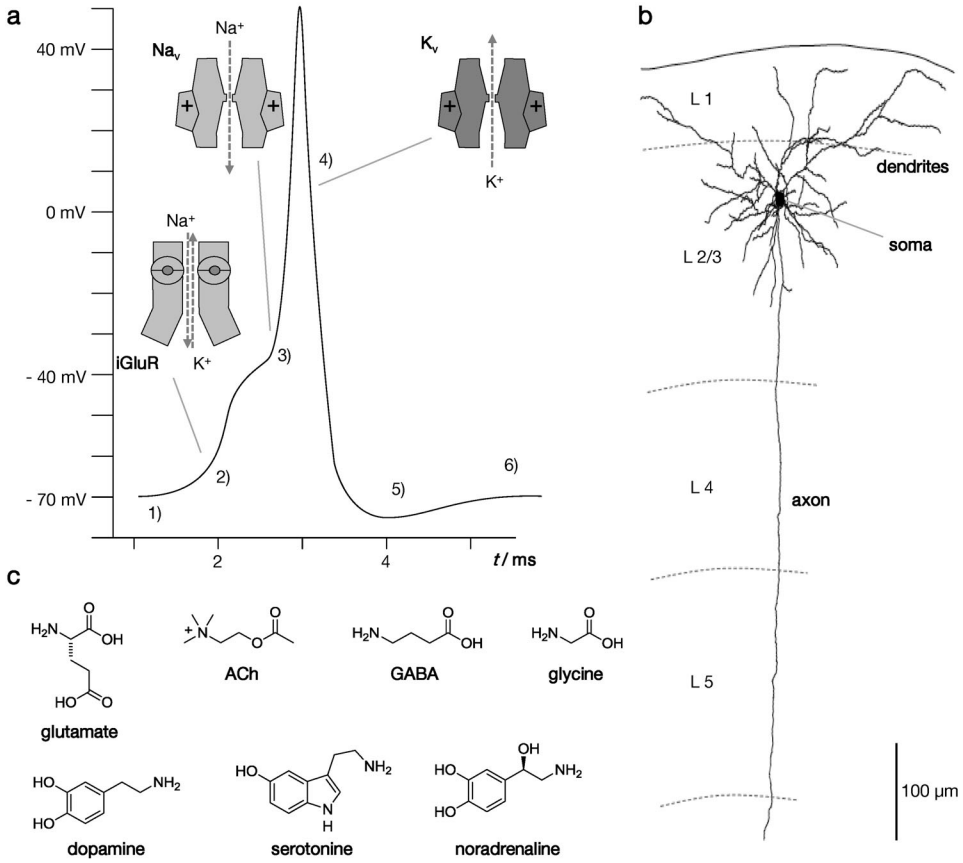


Figure 2. An overview of neurotransmission. a) The action potential: 1) the transmembrane potential of a neuron at rest; 2) a neurotransmitter binds to a ligand-gated ion channel, for example, a glutamate receptor (iGluR), and evokes an excitatory postsynaptic potential; 3) voltage-gated sodium channels (Na_V) respond and further depolarize the membrane, but quickly deactivate; 4) voltage-gated potassium channels (K_V) follow suit and repolarize the membrane, even beyond its resting potential (5); 6) the undershoot is removed through the concerted action of channels that are open at the resting potential and by ATP-driven ion pumps. b) A representative neuron from the visual cortex. The dendrites, the soma, and the axon are clearly visible. The soma resides in layer 2/3 (L2/3) and is much less extended than the axon, which spans several layers of the cortex. c) The most important neurotransmitters and neuromodulators.

In addition to these primary ion channels, there are receptors that perform a more modulatory role and influence the shape, duration, and frequency of the action potentials. These receptors can also be ion channels (such as KCNQ channels)[18] or, more often, G-protein-coupled receptors (GPCRs).[17a,19] In comparison to ion channels, GPCRs are "slow acting", since their effect is mediated by heterotrimeric G proteins, enzymes, second messengers, and transcription factors. Notably, the same neurotransmitter, for example, glutamate, acetylcholine, or GABA, can act on both the ionotropic receptors and the metabotropic receptors. Even neuromodulators that primarily target metabotropic receptors, such as serotonin, have an occasional ion channel target.[20]

3. Lighting up the Brain

Amongst different input signals that can actuate a receptor, light stands out for a variety of reasons: light can be modulated in its intensity within femtoseconds, can be focused onto very small areas (on the order of its wavelength), and can carry enough energy to trigger larger molecular motions (such as isomerizations). As such, it is unsurpassed in its temporal and spatial precision, as well as in its ability to remote-control molecular devices and systems, including neural systems.

Light has been used for eons to glean information from the environment. Visual systems have emerged at almost all levels in evolution and have been greatly enhanced by human technology, such as microscopy. This is still a highly active area of research, with new imaging technologies continuing to be developed. Recent examples include superresolution microscopy[21] and two-photon imaging.[22] As a consequence of these technical advances, neurons can now be described in incredible detail, action potentials can be visualized with fluorescent calcium sensors, and the activity of many neurons in a network can be monitored simultaneously in live animals.[23]

Light, however, can also be used to put information into systems—provided suitable photoreceptors are present. As in imaging, this can be done with exquisite temporal and spatial precision, and the optical setups needed are largely the same. If the light intensities are not too high and the wavelengths are not too short, it can be done with little damage to the tissues, especially when compared with the invasiveness of multiple electrodes. The usefulness of light as an input signal for neuroscience was realized by none other than Sir Francis Crick, who stated in 1999: "*The ideal signal [to study and control a brain] would be light, probably at an infrared wavelength to allow the light to penetrate far enough. This seems rather far-fetched but it is conceivable that molecular biologists could engineer a particular cell type to be sensitive to light in this way.*"[1]

It did not take long for molecular biologists to take up this challenge, which gave rise to a new field called "optogenetics" (Figure 3).[8a,b] In essence, optogenetics is an effort to control neurons, or other cells of interest, with genetically targeted photoreceptors. As a result of this genetic component, the sensitivity to a stimulus and not the stimulus itself can be precisely located. Therefore, the light beam itself does not need to be spatially controlled with very high resolution, and light scattering is less of an issue than it is in imaging.

The targeting of innately "blind" neurons with genetically encoded photoreceptors has been achieved in several ways. Historically, the first system used was "ChARGe", which

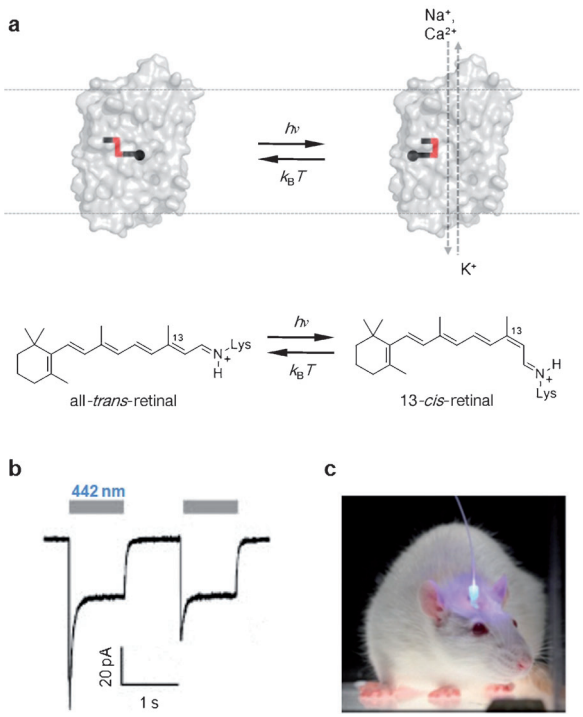


Figure 3. Optogenetics. a) Schematic representation of ChR2, a light-gated ion channel. The chromophore 13-*trans*-retinal (shown here conjugated as a Schiff base) undergoes isomerization with blue light and thermal relaxation, thereby gating the channel. b) Shining blue light on ChR2 triggers depolarization. c) Control of mouse behavior with blue light. Note the glass fiber cable that delivers light deep into the brain.[8b]

essentially consists of the primary components of the *Drosophila* visual cascade (rhodopsin, the associated heterotrimeric G protein, and arrestin).[24] This multicomponent system was shown to trigger APs when heterologously expressed in dissociated hippocampal neurons. This was quickly followed by early optochemical systems, which will be discussed in much more detail below. In 2004, a natural photoreceptor called channelrhodopsin-2 (ChR2) was introduced, which has since established itself as the most practical and popular tool.[25] ChR2 is an excitatory cation channel isolated from the alga *Chlamydomonas reinhardtii*.[25a] It can be activated with blue light and is rapidly deactivated once the light is gone, which allows for the control of AP firing with high precision.[25b] Similar to the rhodopsins, it uses retinal as a photoswitch, but unlike the mammalian visual pigments, the retinal is not excised after photoswitching. Therefore, ChR2 can be repeatedly used to trigger action potentials. As an added advantage, retinal is endogenously produced in many tissues and does not need to be added externally.[26]

Shortly after the excitatory photoreceptor channel ChR2 was identified, an inhibitory counterpart, namely a light-driven chloride pump called *Natromonas pharaonis* halorhodopsin (NpHR) was introduced.[27] NpHR can be activated with bright yellow light and is capable of silencing neurons, also with millisecond resolution. Importantly, as a consequence of little spectral overlap, ChR2 and NpHR can be expressed and used in the same neuron simultaneously.[8b,27] Variants of ChR2 and NpHR with modified spectral and kinetic features continue to be developed,[28] and other systems, such as light-driven proton pumps, have recently been discovered that can control neuronal activity with light.[29]

Since its inception at the beginning of the new millennium, optogenetics has found many applications in dissecting neuronal circuitries and has helped to answer fundamental questions in neuroscience. As a testament to its enthusiastic reception by the scientific community, it was deemed "Method of the Year" in 2010.[8a,c] In addition to its important role in basic neuroscience, optogenetics has, within the last few years, also found its first applications in clinical research. For example, ChR2 and NpHR have been used to investigate the mechanism of deep-brain stimulation, which ameliorates the symptoms of Parkinson disease.[30] A second study was aimed at Retinitis pigmentosa, a condition which involves the loss of photoreceptor cells. Here, the light sensitivity of the retina could be restored by expressing NpHR, which resulted in visually guided behavior in previously blind mice.[31] In another application of ChR2 and NpHR, cardiac pacemaker cells of zebrafish could be optically stimulated or inhibited, thus enabling the control of heart beat patterns.[32]

4. Optochemical Genetics

Given that the molecular tools used in optogenetics are mostly derived from bacteria and protozoa, they work amazingly well in the neurons of worms, flies, and furry animals. These neurons, however, express numerous receptors on their own that are easily accessible on their extracellular side but are not inherently photosensitive. The challenge then is to persuade endogenous receptors to become sensitive toward light.

Three general strategies have emerged to do just that by using small synthetic molecules (Figure 4). The simplest and oldest approach employs caged ligands (CLs).[33] Here, a ligand is endowed with a protecting group that renders it pharmacologically ineffective. This protecting group is rarely a true molecular cage (in the sense a chemist would use this term), but typically a photolabile moiety that masks a functional group crucial to the ligand–receptor interaction. Photochemical cleavage of the protecting group then sets the active ligand free and triggers the desired biological effect.

Caged ligands have indeed been applied to great effect in neuroscience. Caged glutamate, for example, has been very useful for unraveling neural systems, and two-photon cages have enabled the stimulation of single synapses through the spatial precision that can be achieved with two-photon techniques.[34] The photosensitization of $P2X_2$ receptors and TRPV1 channels with caged ATP and capsaicin, respectively, was the first approach shown to work in living animals (in this case decapitated fruit flies, to ensure that the visual system was inactivated).[35]

There are, however, certain functional disadvantages associated with caged ligands. Uncaging is an irreversible process and it is difficult, if not impossible, to "stuff the beast

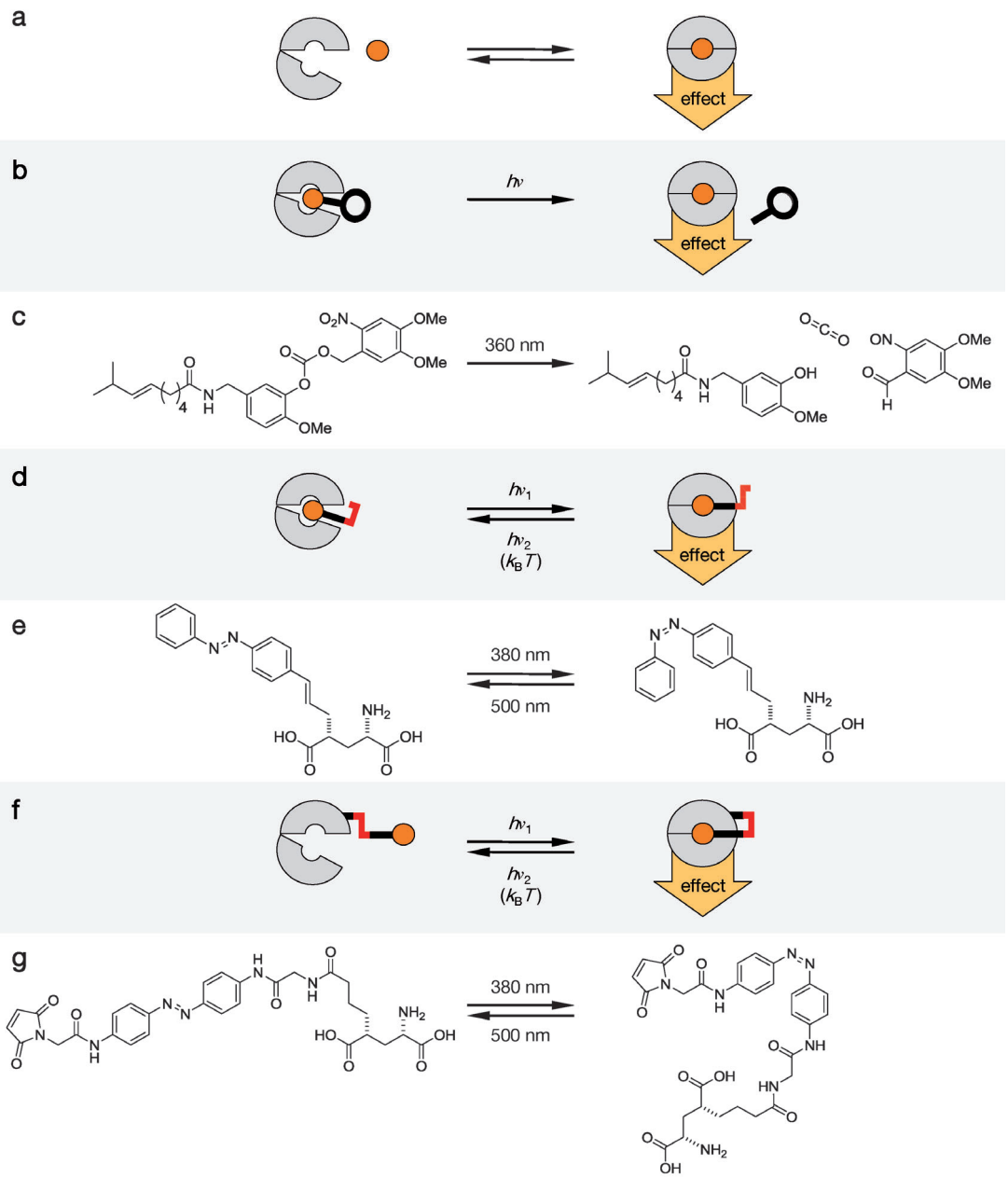


Figure 4. Three strategies used in optochemical genetics. a) A ligand binds to a generic receptor (not necessarily the clamshell-like receptor domain shown here), triggering a biological response. b) A caged ligand (CL) is broken apart with light, thus releasing its active form. c) Caged capsaicin, used to stimulate TRPV1 channels. d) A photochromic ligand (PCL) reversibly acts on a receptor. e) 4-GluAzo, a PCL that functions as "reversibly caged glutamate". f) A covalently attached photoswitchable tethered ligand (PTL) can optically regulate a receptor. g) MAG-1, a typical PTL in its unconjugated form.

back into the cage". Unless one is interested in tonic effects, such as a sustained receptor activation or inhibition, one can only hope that the ligand leaves the active zone as quickly as possible or is cleared by either a reuptake pump or a deactivating enzyme. The former exists for glutamate itself but not for more-selective synthetic agonists and antagonists. In addition, uncaging produces by-products, that is, the remnants of the protecting group, which can be toxic. The background release of the neurotransmitter, for example, through thermal hydrolysis, can also be a problem, as can be the fact that some caged compounds have off-target effects, for example as antagonists to other receptors.

Some of these shortcomings can be overcome with a second approach called the photochromic ligand (PCL) approach. Here, the ligand carries a photoswitchable side chain that can be switched between two configurations. As the photoswitch toggles between these states, the efficacy of the ligand changes, thereby triggering the desired biological effect in a reversible fashion. The ligand can change its efficacy upon photoswitching and could even be an agonist in one form and an antagonist in the other.

PCLs have all the advantages of small-molecule drugs, including their ease of application and fast distribution in tissues. As with drugs, selectivity between receptor subtypes can be a challenge, but this can often be overcome through systematic variation of the molecule. In addition, one could be concerned that their photoisomers show relatively small differences in efficacy. However, in our experience PCLs work remarkably well in complex systems that have nonlinear responses, particularly in neural networks. Here, it is often the case that small changes in the activity of a modulator have dramatic effects on the output. Since the AP is an "all or nothing" response that is triggered only when a certain threshold is reached after a complex cellular integration, compounds that subtly influence this integration can have pronounced effects.

Of course, there are situations where receptor-subtype selectivity and cellular targeting is highly desirable. In this case, a third approach, which we call the photoswitched tethered ligand approach (PTL approach), can be employed. Here, the ligand is covalently attached to its receptor through a tether that contains a photoswitch. As the photoswitch toggles between long and short forms, the local concentration and/or efficacy of the ligand changes, thereby triggering a biological response in a reversible fashion. Importantly, PTLs can be genetically encoded, since the point of attachment is an engineered cysteine residue or any other encodable chemical motif that allows for specific bioconjugation. Since the PTL is covalently tethered, its local concentration at the site of attachment is very high in the active form of the photoswitch, which means that the affinity of the ligand is not a major concern. In fact, low-affinity ligands are usually preferred to ensure that photoswitching removes the ligand from the binding site.

The PTL approach is essentially a variant of optogenetics, since it combines a genetically encodable receptor with light to precisely control neural activity. In contrast to "classical" optogenetics, a synthetic component, that is, a reactive chemical, is needed, which is not endogenously produced, but needs to be synthesized and supplied by a chemist. The PCL and the CL strategies, on the other hand, are more akin to "chemical genetics".[36] Chemical genetics aims to address every protein target with a selective small-molecule ligand. Although such pharmacological control has a rapid onset, it is still not fast enough for many applications in neuroscience, where millisecond precision is required. This limitation can be overcome by optically controlling the small molecules that function as ligands.

Taken together, the CL, PCL, and PTL approaches provide the basis for what we call "optochemical genetics". It is an effort to control neural activity (or any network activity) with light and light-responsive synthetic molecules, with or without a genetically determined component. It shares with chemical genetics a certain desire to overcome the limitations of conventional genetic manipulation, but it also acknowledges that the targeted expression of proteins can be very powerful.

Which variant is used depends on the exact application. For therapeutic applications, where a certain lack of selectivity can be tolerated or is even desirable,[37] the PCL approach may be more suitable. On the other hand, in the analysis of functional pathways, for example, in neural circuitry mapping, the genetically targeted transfection of a specific cell is clearly advantageous. In addition, PTLs could be extremely useful in the functional dissection of closely related receptor subtypes, since selectivity can be achieved through covalent attachment to genetically engineered isoforms (Figure 5). Following conjugation, tonic activation or inhibition of the receptor can be prevented through action of the photoswitch. Overall, this PTL approach to selective pharmacology bears a certain resemblance to the "bump-hole" technique, which has been used so successfully in dissecting the human kinome, that is, the total of all human kinases.[38] In both cases, engineered proteins and unnatural ligands are needed. The PTL approach, however, not only provides a precise answer to the question "who", but also to "when" and "where".

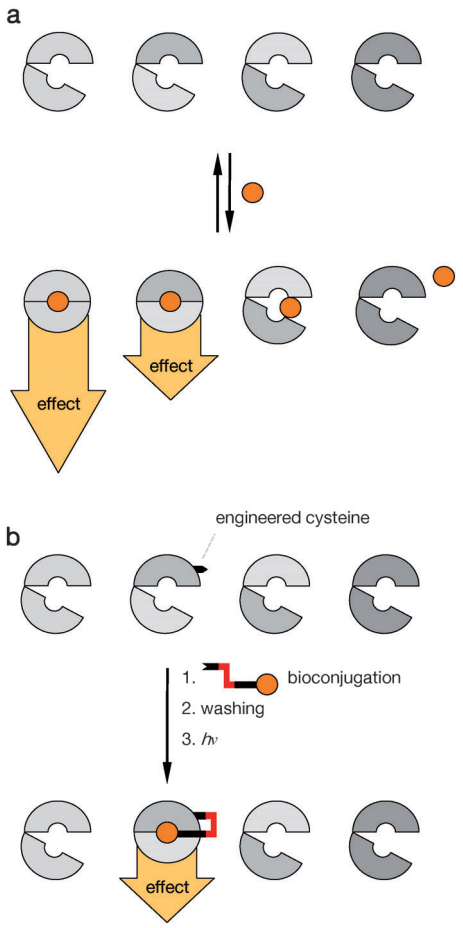


Figure 5. The PTL approach to selective pharmacology. a) Chemical genetics and pharmacology generally aim at individually targeting receptors and receptor isoforms. This requires ligands that bind with high affinity and selectivity. b) In optochemical genetics, selectivity can be achieved through genetic engineering of a bioconjugation site. In addition, the photoactivatable ligand can have low affinity to the receptor subtype.

Caged ligands have been mostly developed by other research groups and have been extensively reviewed elsewhere,[33] which is why our discussion here will focus on

synthetic photoswitches, that is, on PTLs and PCLs. Of the different photoswitch architectures investigated, azobenzenes have so far proven to be the most versatile and reliable ones.[39] This is due to several distinguishing functional features (Figure 6). For example, their geometries in their

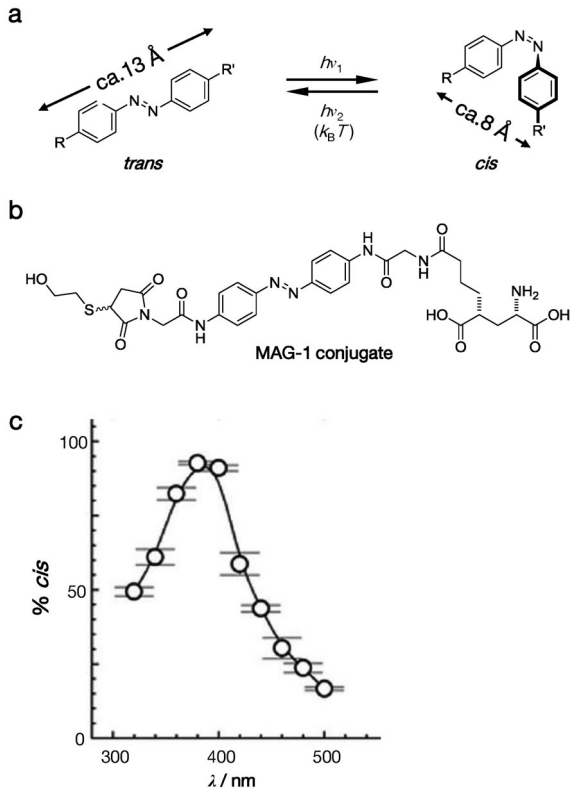


Figure 6. The logic of azobenzene photoswitches. a) Azobenzenes change their configuration and length upon irradiation with light of different wavelengths. They revert thermally or photochemically to their thermodynamically more stable state, which is usually the *trans* state. b) The mercaptoethanol conjugate of MAG-1, a model for the tethered PTL. c) The photostationary *cis*/*trans* ratio of the MAG-1 conjugate as a function of wavelength.

cis and *trans* states are well defined and the conformational space that can be mapped by both isomers has relatively little overlap. Substituents in the 4- and 4′-positions are substantially closer in their *cis* form than in their *trans* form, an effect that can be amplified further by appropriate substitution. Although azobenzenes are helically chiral in their *cis* form, which is not planar, they are typically not optically active because of facile racemization, and they do not generate stereocenters upon switching between a long and short form.

Azobenzenes often display high extinction coefficients and quantum yields, which mean that light of relatively low intensity can be used for photoisomerization. In addition, azobenzenes undergo photoswitching at very fast rates, which prevents intersystem crossing and the formation of triplet diradicals. These would react with triplet oxygen to generate singlet oxygen, a highly reactive and cytotoxic species, which is also damaging to the chromophore itself. Consequently, azobenzenes are relatively photostable and can be switched over many cycles. Their spectral tuning through substitution is straightforward and follows well-established rules. While most azobenzenes used to date are isomerized to their *cis* form with UV-A or deeply violet light (315–380 nm), red-shifted versions that are less harmful upon prolonged application to tissue are also known (see Section 6). Finally, azobenzenes are relatively easy to synthesize and modify, as several synthetic strategies are available, including diazonium coupling, Mills reactions, and transition-metal-catalyzed cross-coupling strategies.[40] One potential disadvantage is their comparatively low solubility, but this can usually be overcome with appropriate functional groups, in particular with charged substituents.

Notably, azobenzene photoswitches are marked by photostationary states that are a function of the wavelength. While they can exist 100% in the thermodynamically more stable *trans* form (also the dark-adapted form) and their photostationary *cis*/*trans* ratios can exceed 9:1 at short wavelengths (Figure 6),[41] it is practically impossible to push them fully into the *cis* state through irradiation. Therefore, the background activity of the remaining *trans* isomer is a concern, but for the reasons stated above this is often not a problem in neural networks. In fact, the dependence of the photostationary ratio on the wavelength also offers an opportunity to tune the response by gradually tuning the color. The thermal bistability of photoswitches can be further influenced through appropriate substitution. PTLs wherein one end of the *cis*-azobenzene interacts with the protein covalently and the other noncovalently show slow thermal relaxation, at least on a neurobiological time scale. On the other hand, certain red-shifted azobenzene PCLs can revert to their dark-adapted state within milliseconds.[42]

Once a photoswitch has been chosen, the design of PTLs and PCLs is straightforward, provided both the structural coordinates for the receptor and extensive structure–activity relationship data for the ligands are available. The latter is often the case, since neuropharmacology is a well-developed field. Today, one cannot complain about a lack of the former either, since relevant structures appear in the literature almost every week.

The design of PCLs and PTLs is closely intertwined. It typically starts out with a structure of a ligand, gleaned from the crystal structure or from the pharmacology literature. From this, it is often immediately clear where and how to attach the photoswitch to the ligand and which stereochemistry to choose at the point of attachment. In an intermediate stage, a so-called "tether model" is sometimes synthesized, which retains one phenyl ring of an azobenzene. This is then extended to a full-blown PCL that contains the entire chromophore and, finally, after addition of an electrophilic functional group, to the corresponding PTL. In general, extensive structure–activity data are sufficient to design a PCL with a reasonable chance of success, whereas X-ray structures are normally needed for a PTL. The latter requires cysteine sites for covalent attachment, and choosing those can be difficult without structural coordinates. Increasingly, sophisticated computational tools are used for that purpose.

Since most PTLs that have been used to date are cysteine-reactive compounds, unspecific labeling of the cell-surface might be a concern. However, one has to bear in mind that reduced, accessible cysteine residues are rare on the surface

of cells and the bioconjugation typically proceeds through affinity labeling. This means that the noncovalent interaction of the PTL with its ligand-binding site precedes formation of the covalent bond, thereby enhancing the rate and selectivity of the labeling, which will also depend on the state of the photoswitch. In addition, cells seem to be remarkably tolerant toward molecules attached to their surface, as long as they do not interfere with vital cell–cell interactions.[43]

The design of suitable PTLs and PCLs also goes hand in hand with their syntheses. These can present considerable challenges since the chemistry of photoswitches needs to interface with the chemistry of polar and charged ligands, which requires intricate protecting-group operations. In the case of PTLs, this is acerbated by the presence of an electrophile, which should be introduced into the molecule as late as possible. The syntheses have to be practical and efficient enough to support a sustained biology program that includes in vivo investigations and, eventually, behavioral studies in animals.

In the following sections we will show how optochemical genetics can be applied to some of the most basic molecular machines involved in synaptic transmission: voltage-gated potassium channels and ionotropic glutamate receptors. To this end, we will first discuss their innate functions and systemic roles in some detail and then show how they can be converted into hybrid photoreceptors through covalent or noncovalent attachment of azobenzene photoswitches. Afterwards, we will address other targets, such as the nicotinic acetylcholine receptor (nAChR) or the $P2X_4$ receptor, where this has been previously studied to a limited extent. Finally, we will address a few receptors that have not yet been converted into photoreceptors, but which are "sitting ducks" for the optochemical approach.

5. Voltage-Gated Potassium Channels

Together with voltage-gated sodium channels (Na_v), voltage-gated potassium channels (K_v) modulate cellular excitability and play a key role in the generation of action potentials.[17c] K_v channels are transmembrane proteins that assemble as tetramers from four single polypeptide chains that are known as α subunits.[4] In humans, 40 genes encode 12 α-subunit families (K_v1–K_v12).[44] Within each subfamily, different genes exist, as indicated by an additional number, for example, $K_v1.2$. This diversity is further increased by heterotetrameric assembly of the α subunits, which results in a very large number of potential combinations. K_v1 channels additionally interact with intracellular tetrameric β subunits that alter the gating behavior.[45]

To date, several potassium channels have been elucidated in atomic detail by X-ray crystallography. The first structure to be reported was that of KcsA, a simple bacterial channel from *Streptomyces lividans*. Its disclosure in 1998 stands as a milestone in biophysics.[10] Subsequently, the structures of a calcium-gated potassium channel (MthK),[46] several inward-rectifier potassium channels (e.g. $K_{ir}3.1$),[47] a sodium- and potassium-conducting channel (NaK),[48] and several prokaryotic and eukaryotic voltage-gated potassium channels have been reported.[14b,16] The structure of $K_v1.2$, a mammalian voltage-gated ion channel that modulates the electric excitability of neurons, in its open form is shown in Figure 7.[14b] In this representative structure, the voltage sensors, the pore, and both the inner cavity and outer vestibule of the channel are clearly visible (the β subunit is removed here, but present in Figure 1). Each single subunit contains an intracellular

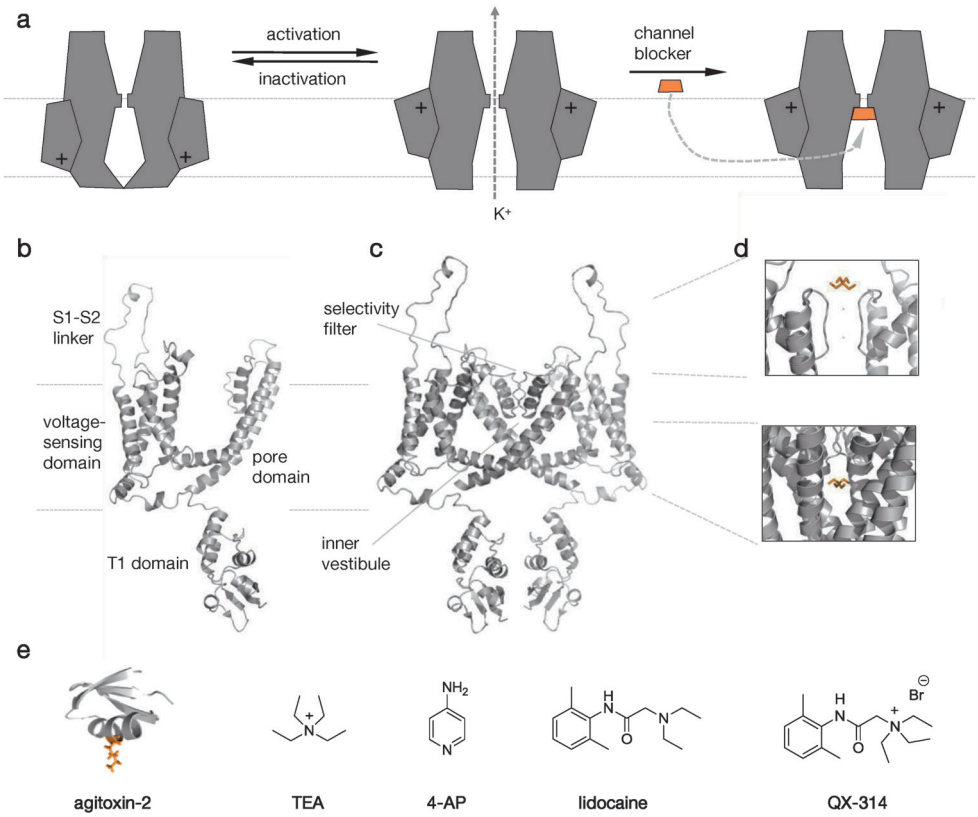


Figure 7. Structure and function of voltage-gated potassium channels. a) A highly schematic representation of K_v channels illustrating their functional cycle and channel blocking. b) A single $K_v1.2$ subunit showing the extracellular S1-S2 linker, the voltage-sensing domain, the pore domain embedded in the membrane, and the intracellular T1 domain (pdb 3LUT). The β subunit has been removed and the ball peptide is not resolved in this structure. c) Two of the four subunits, indicating the architecture of the selectivity filter and the inner vestibule. d) Expanded view of the TEA binding sites in the inner and outer vestibules (pdb 2BOB and 2BOC). e) Chemical structures of extracellular and intracellular potassium channel blockers.

tetramerization domain, a voltage sensor domain (with helices S1–S4), and a pore domain (containing helix S5, a short pore helix, a pore loop, and the C-terminal helix S6).[44] Both the N and the C termini are located in the cytosol, but the N-terminal domain is not resolved in the structure.

Like most ion channels, K_v channels have three elementary functions that are physically represented by their protein domains and their movements: 1) an "activation gate" that interacts with the input signal (i.e. voltage) and opens the channel, 2) a "selectivity filter", which determines which ions can pass through the channel, and 3) an "inactivation gate", which is responsible for their desensitization. Activation is initiated by the voltage sensor once the membrane potential reaches −40 mV. The S4 helix of the voltage sensor domain in each subunit contains several positively charged residues, usually arginines, that move across the membrane as the cell depolarizes. This movement is mechanically coupled with a hinged motion of the inner helix bundle (S6) of the pore domain, which opens a gate through which the ions can pass.[7,44,45] Driven by the electrochemical gradient, the ions then permeate the selectivity filter, thereby generating a current that is specific for K_v channels. The selectivity filter is essentially a stack of backbone carbonyl groups that compensate for the hydration sphere of the cation as it passes through. This compensation is energetically more favorable for potassium ions than sodium ions.[7]

There are two native mechanisms of inactivation of K_v channels: N-type and C-type inactivation. N-type inactivation is mediated by the positively charged intracellular N terminus, which functions like a "ball" (or plug) on a chain. Once the voltage gate is opened, the N terminus follows the outward K^+ current. This pushes the "ball" into the inner vestibule of the channel and blocks further ion conduction.[49] In comparison, slow or C-type inactivation depends on permeant ions and blockers, and is believed to occur through a conformational change near the extracellular pore region.[48a,49] In a physiological context, fast inactivation of the sodium channels and currents through the K_v channel repolarize the neurons after an AP and determine the duration and frequency of the APs.[51]

K_v channels can be blocked by Cs^+ ions, small organic cations, and venom peptides. Venom peptides, such as agitoxin-2, are produced by some of the most dangerous animals known, for example, the death-stalker scorpion, certain sea anemones, and the green mamba.[45,52] These peptides bind to the outer vestibule of the channel and literally plug its pore from the extracellular side, usually with a protonated lysine side chain. Typical small organic K_v channel blockers are quaternary ammonium ions, such as tetraethylammonium (TEA) and 4-aminopyridine (4-AP; Figure 7).[17c,53]

Interestingly, K_v channels feature both an internal and external binding site for quaternary ammonium ions.[54] External TEA mimics the protonated lysine side chain that functions as the plug in agitoxin-2, whereas internally applied TEA acts similar to the positively charged ball peptide involved in N-type inactivation. This blockade requires opening of the activation gate. Therefore, charged blockers that act from the internal side are called "open-channel blockers" or "use-dependent blockers."[17c] The KcsA structure was also solved in the presence of analogues of TEA, which helped to exactly locate its intracellular and extracellular binding sites.[54]

6. Photosensitizing Voltage-Gated Potassium Channels

With their structures elucidated and their functions reasonably well understood, voltage-gated potassium channels have become prime targets for optochemical genetics. The first system to emerge was the "synthetic photoisomerizable azobenzene-regulated K^+ channel" (SPARK), which is an example of the PTL approach (Figure 8).[55] As a consequence of its hyperpolarizing effect, it was later renamed H-SPARK. H-SPARK consists of the channel blocker MAQ (maleimide/azobenzene/quaternary/ammonium) covalently attached to a genetically introduced cysteine residue on an extracellular loop of a K_v1-type channel. The location of this cysteine residue (E422C) could be determined on the basis of existing X-ray structures and previous experiments with "molecular tape measures."[56] Once attached, MAQ essentially functions as TEA on a leash that can be lengthened or shortened with light. MAQ was designed to block the channel in its elongated *trans* state, that is, at 500 nm or in the dark, whereas the blockage would be lifted in the *cis* state of the photoswitch, that is, at 380 nm. Slow and fast inactivation of the channel had to be prevented by using genetic engineering to make H-SPARK an effective modulator of membrane potential. The activation voltage was shifted from −35 to −70 mV through another point mutation (L366A), which led to a constantly open channel before conjugation of *trans* MAQ. As a result, the potassium channel is blocked in the dark or under 500 nm light. Illumination with light of wavelength 380 nm unblocks the pore and the resulting outward current leads to re- or hyperpolarization of the membrane. To our amazement, SPARK not only worked in excised patches from Xenopus oocytes but also in excitable cells, such as dissociated hippocampal neurons.[55] Indeed, when SPARK was introduced in 2004, it was the first system that allowed for optical silencing of neuronal activity.

As a complement to the silencing H-SPARK, a depolarizing version, termed D-SPARK, was engineered.[57] A single additional mutation (V443Q) was sufficient to convert the potassium selective H-SPARK into a nonselective cation channel. With this mutation in the selectivity filter, the permeability ratio of the K^+/Na^+ ions changes to 0.7:1, and Na^+ influx into the cell dominates the net effect on the membrane potential. After covalent attachment of MAQ, the channel depolarizes the membrane upon UV illumination instead of hyperpolarizing it, thus allowing for AP firing rather than silencing. This is a nice case of "sign inversion," which is often encountered in neuroscience.

The diversity of K^+ channels, the lack of selective pharmacology, and the success of H/D-SPARK provided the motivation to extend the PTL strategy to other members of the family.[58] $K_v1.3$, which is involved in membrane repolarization after AP firing of neurons and has immunosuppres-

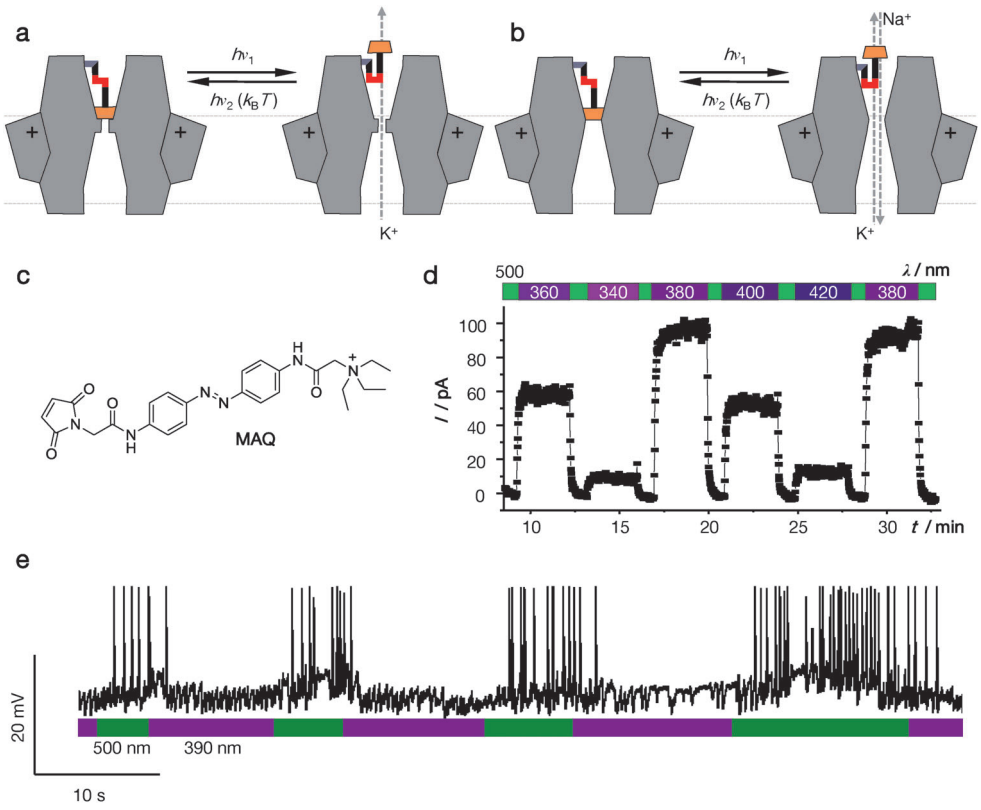


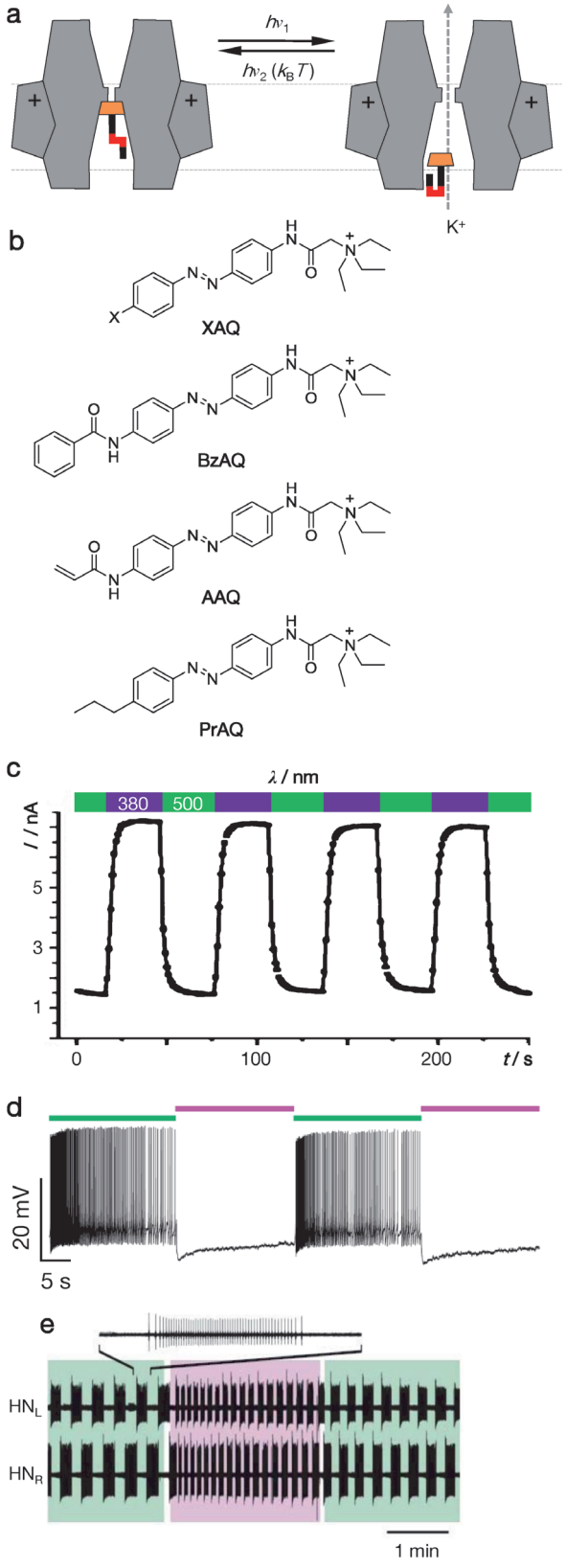
Figure 8. SPARK, the light-sensitive potassium channel. a) A highly schematic representation of H-SPARK, the hyperpolarizing channel. b) A schematic representation of D-SPARK, the depolarizing variant. c) The chemical structure of MAQ, the PTL used for SPARK. d) Reversible light control of potassium currents by H-SPARK. Irradiation in the UV range unblocks channels, while 500 nm light induces blocking. By convention, outward currents are plotted upwards. e) Light-dependent induction of AP firing by H-SPARK expressed in neurons. This is the original recording of the first experiment where neuronal activity was controlled reversibly by a PTL.

sive effects when blocked, became the next channel to be rendered light-sensitive by attachment of MAQ. Its very low affinity for TEA could be overcome by introducing a point mutation (H401Y). Both $K_v3.1$ and $K_v7.1$ could be converted into photoreceptors in a similar fashion. As a consequence of their fast deactivation, $K_v3.1$ channels play an important role in generating high-frequency APs, which occur in neurons of the auditory brainstem and the cerebellum. Finally, the PTL concept could be applied to the small conductance Ca^{2+}-activated K^+ channel 2 (SK2), a channel that is naturally sensitive to voltage and an intracellular ligand.[58] Taken together, these studies provide a first example for the PTL-driven selective pharmacology discussed in Figure 5.

As an alternative to PTLs, PCLs offer a way to control native K_v channels and neuronal activity with light. For this purpose, the cysteine-reactive maleimide group of MAQ was replaced with various moieties. This yielded a series of photochromic blockers, termed XAQs, which display different pharmacological and photophysical properties.[59] These compounds include the benzoate BzAQ, the propyl derivative PrAQ, and the acrylamide AAQ (Figure 9). Detailed investigations showed that the XAQs act as use-dependent, photochromic open-channel blockers.[59a] This also applies to AAQ, which does not react with the extracellular surface of native channels, as initially hypothesized. To reach the inner vestibule, the amphiphilic XAQs have to cross the membrane or they can be added to the cytosol through a patch pipette. External application of AAQ and BzAQ to cells or tissues blocks K_v channels in the *trans* state of the photoswitch, that is, in the dark or under 500 nm irradiation, while 380 nm light relieves the block. Interestingly, PrAQ preferentially blocks in its *cis* state (i.e. at 380 nm), which is another example of sign inversion.[59a]

Structurally, as well as functionally, XAQs resemble the well-known analgesic lidocaine or its permanently charged derivative QX-314, with the important difference that their efficacy is light-dependent. Similar to lidocaine, XAQs can be simply added to nervous tissues, which make them very attractive for therapeutic applications. After a brief waiting period, they reliably control action potential firing (Figure 9). When added to cerebellar slices, AAQ controls the activity of Purkinje neurons, mostly through its effect on inhibitory interneurons known as basket neurons.[59b] AAQ also has effects on the heartbeat of the medicinal leech *Hirudo medicinalis*.[59b] In this animal, central pattern generator interneurons (co-called HN cells) control the frequency of heart contractions. Earlier studies indicated that K^+ channels play a crucial role in burst firing of these HN cell. After application of AAQ, the activity of HN neurons could be modulated with light. Interestingly, burst periods decreased under 380 nm light illumination, whereas 500 nm light extended the period.

A major goal of our research program is to shift the absorption and action spectra of PTLs and PCLs toward the red, which would allow for deeper tissue penetration and would diminish the phototoxicity. This can easily be done through chemical derivatization of the azobenzene chromophore. For example, the introduction of an electron-donating substituent yielded XAQs that can be isomerized to their *cis* isomers with blue light. As an added advantage, these compounds revert to their *trans* form in the dark within milliseconds, effectively turning themselves off (Figure 10). Therefore, it is not necessary to toggle between two different wavelengths. An example of this type of compound is DENAQ, whose action spectrum reaches a maximum at



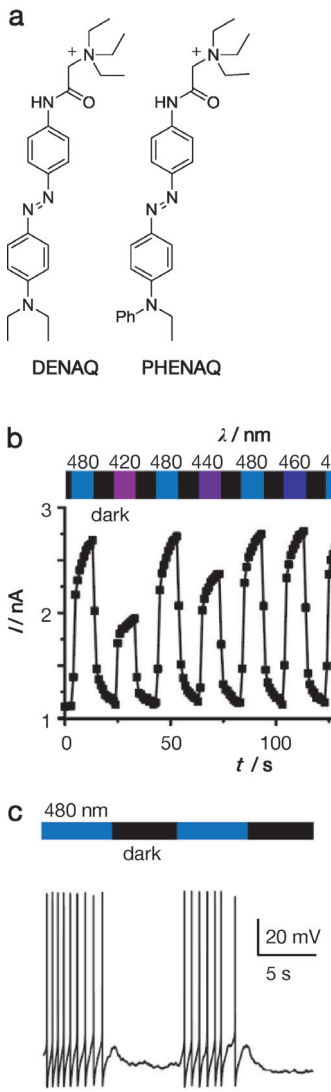


Figure 9. PCLs for voltage-gated ion channels. a) A schematic depiction of a XAQ that functions as a photochromic open-channel blocker of K_v channels. b) The chemical structures of BzAQ, AAQ, and PrAQ, three typical XAQ PCLs for potassium channels. c) AAQ reversibly blocks potassium currents of a Kv1 family channel. d) AP firing of hippocampal neurons controlled by AAQ. e) Controlling the heart beat of a leech with AAQ.

Figure 10. Red-shifted photochromic blockers. a) The chemical structures of DENAQ and PhENAQ, two red-shifted XAQs. b) Action spectrum of DENAQ. c) Controlling neuronal firing with PhENAQ.

480 nm. Interestingly, its analogue PhENAQ does not act as a blocker in the *trans* state but rather in the *cis* state (another case of sign inversion). It can also be switched to the blocking state with blue light and reverts to the inactive state automatically. Therefore, neuronal firing could be triggered with irradiation and quickly silenced by turning the light off.[42]

7. Ionotropic Glutamate Receptors

Ionotropic glutamate receptors (iGluRs) play a central role in synaptic transmission.[17a,50,60] Located primarily in postsynaptic membranes, these ligand-gated ion channels respond to the neurotransmitter glutamate released from vesicles on the presynaptic side. Upon glutamate binding, iGluRs generate a depolarizing current, which results in an excitatory postsynaptic potential (EPSP). With their fundamental involvement in neuronal communication, iGluRs are

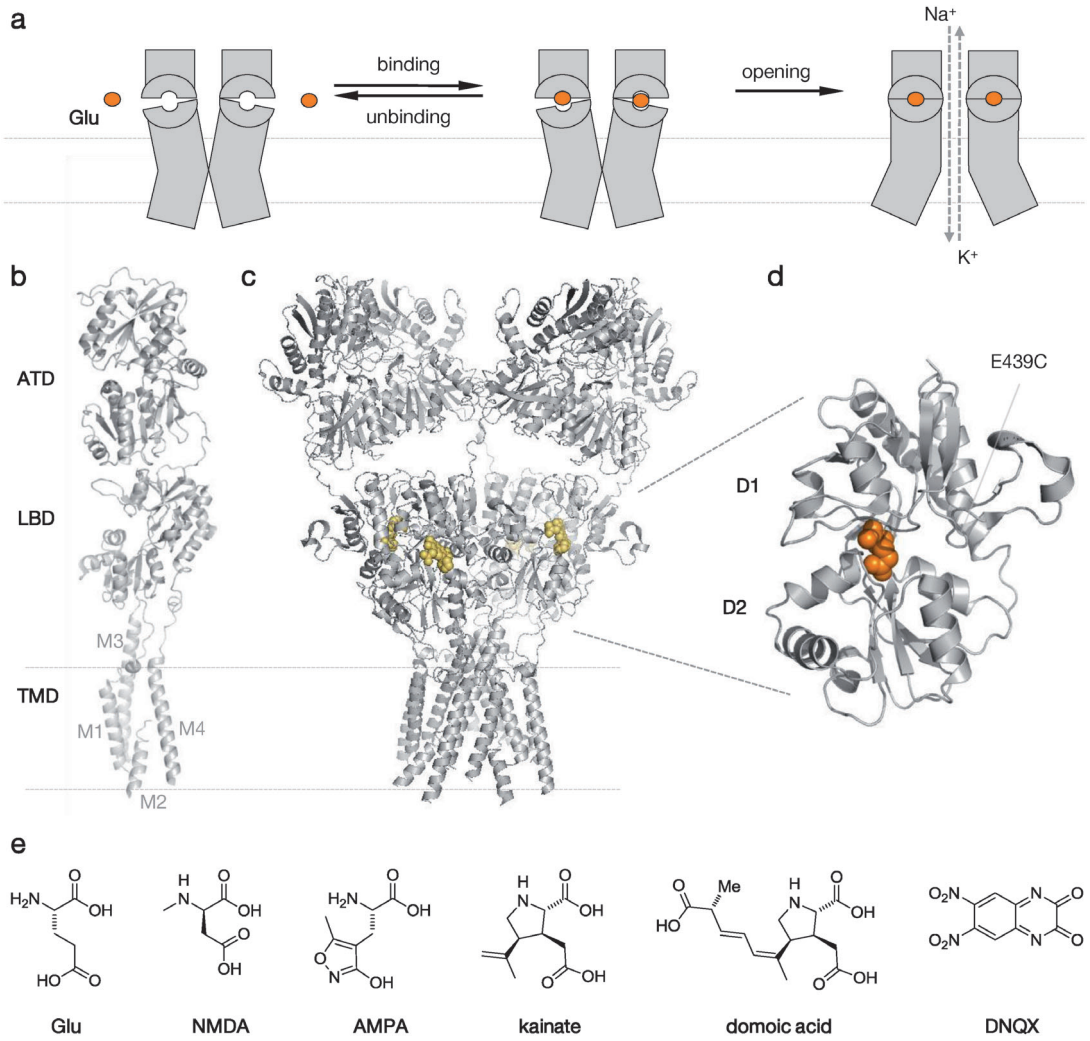


Figure 11. Ionotropic glutamate receptors. a) A highly schematic illustration of glutamate receptor gating. b) X-ray structure of a single GluA2 subunit, indicating the TMD (including M1–4), the extracellular LBD, and TMD. c) The fully functional tetrameric GluA2 channel cocrystallized with an antagonist (yellow; pdb 3KG2). d) Expanded view of a GluA2 LBD cocrystallized with glutamate, emphasizing the clamshell (pdb 1FTJ). e) The chemical structures of various agonists and antagonists for iGluRs.

associated with a wide variety of neurological diseases, including Alzheimer's disease, epilepsy, and neuronal damage from stroke, and have therefore been very important neuropharmacological targets.[61]

All ionotropic glutamate receptors in higher animals assemble as tetramers and allow the permeation of monovalent cations with little selectivity (Figure 11). Some are also permeable to calcium. On the basis of functional differences and their response to synthetic ligands, they can be classified into *N*-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors. The latter can be divided into three subgroups, namely so-called AMPA, kainite, and δ receptors.[51,62]

AMPA receptors, named after a selective agonist, form homo- and heteromeric channels composed of GluA1–GluA4 subunits.[51,62] They mediate postsynaptic depolarization after release of glutamate from the presynaptic side and exhibit fast activation and deactivation kinetics (within a few milliseconds). This feature makes them the principle molecular component of fast excitatory synaptic transmission.[51,62]

By contrast, kainate receptors play a role in the modulation of neuronal excitability rather than in fast excitatory transmission.[62,63] Their mode of action, however, is not as well understood as in the case of AMPA receptors. Homo- and heteromeric channels assemble from so-called GluK1–GluK5 subunits. δ Receptors assemble as homomeric receptors of GluD1 or GluD2 subunits. The function of these receptors, however, remains largely unknown.[62]

NMDA receptors assemble as obligate heterotetramers from a pool of seven subunits. Activation requires not only the simultaneous binding of glutamate and glycine (or D-serine), but also an elevated membrane potential of greater than -30 mV. This renders NMDA receptors coincidence detectors, since they are sensitive to both the release of ligands by the presynaptic neuron (and from glial cells)[51,62] and changes in voltage on the postsynaptic side. In addition, NMDA receptors are not only permeable for Na^+ and K^+, but also for Ca^{2+} ions. These activate various intracellular targets, such as the Ca^{2+}/calmoduline-dependent protein kinase II (CamKII). As such, NMDA receptors are involved in the

strengthening of synapses, which is believed to underlie learning and memory.[51,64]

A single iGluR subunit contains an extracellular amino-terminal domain (NTD), a ligand-binding domain (LBD), a transmembrane domain (TMD), and an intracellular carboxy-terminal domain (CTD). The NTD is involved in subtype-specific assembly and modulation of the receptor, while the LBD provides a binding site for agonists (and antagonists). Two subdomains called D1 and D2 form the LBD in a clamshell-like arrangement. The TMD is composed of three transmembrane helices (M1, M3, M4) and a pore helix (M2). The CTD plays a role in channel localization, stabilization, and targeting for degradation.[51,62]

X-ray crystallographic studies on GluRs began with soluble versions of LBD clamshells carved out of the full-length receptors through clever protein engineering.[63] These structures were obtained in conjunction with various ligands and proved to be remarkably insightful, since they could explain the activity of agonists, antagonists, and modifiers that affect desensitization.[63] For example, they showed that the binding mode of glutamate to the LBD clamshell is highly conserved in glutamate receptors and that the degree of closure of the clamshell is roughly correlated with the activity of a partial or full agonist.[62,65] In line with this logic, antagonists, such as DNQX, bind to the clamshell but do not allow it to close tight enough to trigger the biological effect.[61] This "foot-in-the-door" mechanism of antagonism also explains why relatively small changes in the molecular structure can turn an agonist into an antagonist.

In a recent breakthrough in structural biology, the first crystal structure of a full-length homomeric GluA2 receptor was determined at 3.6 Å resolution (Figure 11b).[12] This AMPA receptor was cocrystallized with a strong competitive antagonist, thus resulting in a closed, nonconducting state of the receptor. The structure confirmed that the subunits arrange as a dimer of dimers with an overall C_2 symmetry. The TMD architecture resembles an "inverted" K^+ channel, which is linked to a ligand sensor (the LBD) instead of a voltage sensor.

The activation cycle of glutamate receptors can be divided into an inactive resting state, an active nondesensitized state, and an inactive desensitized state. The LBDs of the four subunits are arranged in such a way that two clamshells on either side of the C_2 axis are sitting back to back. The binding of glutamate to the LBD of the inactive receptor initiates closure of the clamshell cleft. The movement of the lower lobe of the clamshell (D2) during this event rearranges the connector between the LBD and the TMD, which exerts a mechanical force that opens the channel. However, this active non-desensitized state creates tension at the LBD interface. This tension can be lifted by dissociation of the agonist or by rearrangement at the LBD dimer interface to give a closed, desensitized state.[62]

8. Photosensitizing Ionotropic Glutamate Receptors

Just as the outer vestibule of K_v channels had provided a suitable platform for the attachment of MAQ, the structurally well-characterized extracellular clamshell of glutamate receptors presented an opportunity to explore tethered ligands (i.e. PTLs). In this case, we opted for an agonist, instead of a blocker or antagonist. This approach yielded LiGluR, the "light-gated ionotropic glutamate receptor," which combines a genetically engineered kainate receptor (GluK2) with a PTL called MAG-1 (Figure 12).[66] MAG-1 consists of a maleimide for covalent attachment, an azobenzene photoswitch, and a substituted glutamate as the ligand. Once MAG-1 is attached to a cysteine residue at the LBD of GluK2 (L439C), light-induced *cis* and *trans* isomerization of the azobenzene moiety results in reversible binding of the tethered agonist, which translates into opening and closing of the channel.

The design of LiGluR was based on the crystal structure of a closed GluK2-LBD containing the agonist (2*S*,4*R*)-4-methylglutamate (pdb 1SD3).[63] This structure suggested the existence of a small "exit tunnel" from the bound ligand to the surface of the closed clamshell. It was, therefore, hypothesized that a tethered glutamate with this stereochemistry could bind to the LBD and still activate the receptor as an agonist or at least a partial agonist. A so-called tether model (T-Mod) was synthesized to support this hypothesis. It could be shown by calcium imaging in HEK293 cells that the tether model, and later MAG-1, indeed act as agonists.[66] Other crystal structures, such as the one of the partial agonist domoic acid bound to GluK2 (pdb 1YAE) also facilitated the design of suitable PTLs.[67]

Once the tether model was found to work, the molecule was extended to MAG-1 by installing the azobenzene photoswitch and adding a maleimide as an electrophile for cysteine conjugation. Several cysteine mutants of GluK2 were generated by site-directed mutagenesis to find a suitable attachment site for this maleimide. After screening about a dozen mutants, L439C turned out to be the best position for MAG-1 attachment and light-dependent activation of the channel.[66]

Since it was not clear whether and how the attachment of the PTL to the protein would affect the photophysics of its azobenzene moiety, the optimal wavelength for activation and inactivation was determined experimentally (Figure 12).[66] It was found that 380 nm light gave the maximum current, whereas 500 nm led to the fastest channel closure. Interestingly, this observation corresponds very well to the photostationary states of MAG-1 in solution (Figure 12). In addition, the thermal relaxation of *cis*-MAG-1 bound to the channel was found to be much slower than in solution, probably because of the noncovalent interaction of the glutamate ligand with the clamshell, which stabilizes the *cis* configuration.[41]

When expressed in neurons, which contain the full machinery to generate APs, LiGluR allows for the optical control of neuronal firing with millisecond resolution.[68] Brief light pulses (1–5 ms) alternating between 380 nm and 500 nm are sufficient to generate reproducible patterns of APs. APs can be induced in dissociated hippocampal neurons with a frequency of up to 50 Hz without missing a beat (Figure 12).[68] The *cis* stability of MAG means that it is possible to induce constant AP firing for seconds following a single flash of 380 nm light that lasts only a few milliseconds.

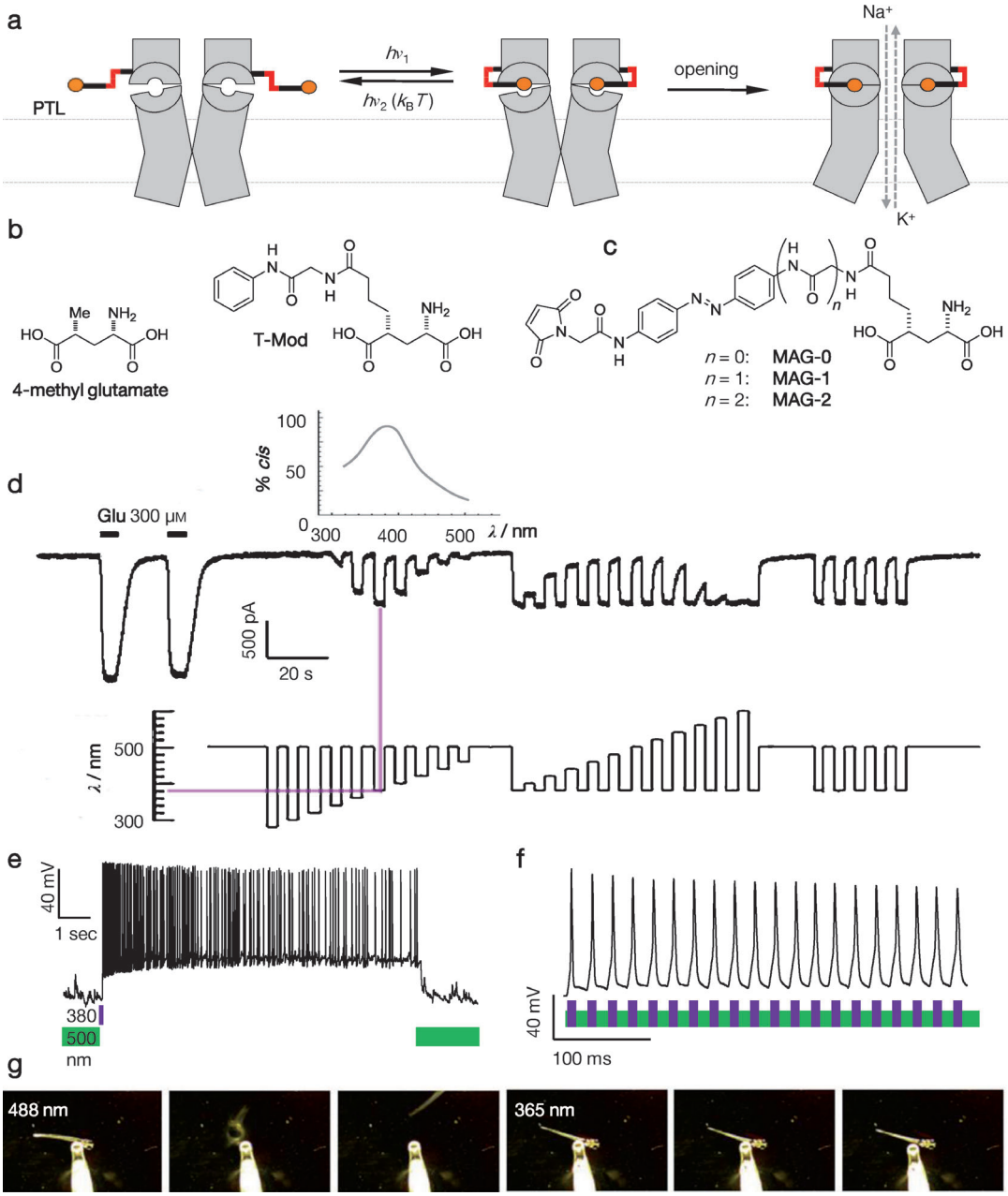


Figure 12. The light-gated glutamate receptor LiGluR. a) A highly schematic illustration of LiGluR. b) The chemical structure of 4-methylglutamate and the "tether model" (T-Mod), two important milestones on the way to LiGuR. c) The chemical structure of MAG-0, MAG-1, and MAG-2. d) The action spectrum of LiGluR. By convention, inward currents are plotted downwards. The phototunability of the system is apparent. e) LiGluR controls AP firing with millisecond precision. A very brief UV flash induces a train of APs, thus demonstrating the *cis* stability of the system. f) LiGluR enables AP firing of up to 50 Hz. g) Controlling the escape reflex in zebrafish.

Thus, long illumination periods leading to toxic side effects can be avoided.[68,69]

LiGluR shows some fundamental functional differences compared with SPARK. Both irradiation and depolarization are needed to open the SPARK channel (corresponding to a logic AND gate). By contrast, in LiGluR, either soluble glutamate or light is sufficient to allow ions to flow along their electrochemical gradient (corresponding to a logic OR gate). LiGluR is a light-powered molecular machine, since the energy of the photoisomerization translates into the free energy released upon ligand binding, triggering clamshell-closure and channel opening. It can be reset through slow thermal isomerization or active photoswitching with a longer wavelength. SPARK, on the other hand, is a more passive system, since the tethered blocker functions as an extracellular gatekeeper but does not trigger large molecular motions itself. These need to be generated by membrane depolarization, which moves the voltage sensor and opens the voltage gate.

One of the hallmarks of our systems in general, and LiGluR in particular, is their remarkable flexibility and tunability. This is evident at the level of the photoswitches and

also at the level of the protein. In addition to MAG-1, further derivatives called MAG-0 and MAG-2 with different lengths and stiffnesses were synthesized, and additional attachment sites were generated by site-directed mutagenesis. This "mix and match" approach led to some interesting results. For example, MAG-0 anchored at L439C provided the biggest change in the effective concentration in competition with the antagonist DNQX. In comparison, MAG-1, our original PTL, and MAG-2 are less effective.[69]

In another case of "sign inversion," we found that the point of attachment can determine whether a PTL activates the channel in the *cis* or in the *trans* form of the photoswitch (Figure 13).[69] MAG-0 bound to L439C, our original attachment site, acts as a *cis* agonist but becomes a *trans* agonist when attached to L486C. This is somewhat surprising given that these two sites are only a few Ångstroms apart in the X-ray structures of GluK2. As a consequence of this inverted wavelength sensitivity, the transfection of neurons with L486C followed by MAG-0 attachment triggers AP firing at 500 nm and not at 380 nm, as previously observed.

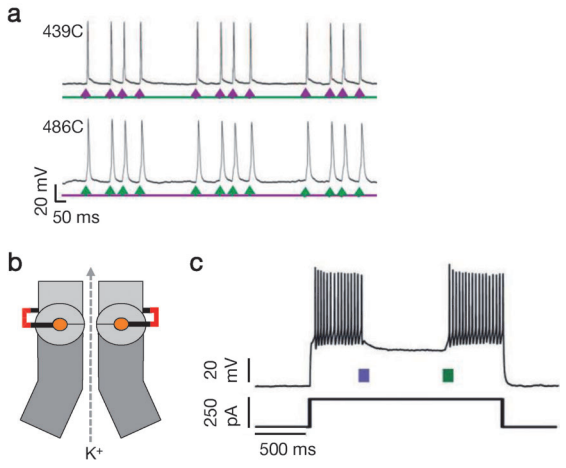


Figure 13. Sign inversion through variation of LiGluR. a) MAG-0 attached to mutant 439C activates at 380 nm, whereas MAG-0 attached to mutant 486C induces neuronal firing at 500 nm. b) Schematic representation of HyLighter, a hyperpolarizing light-gated glutamate receptor. c) HyLighter can be used to silence neurons. Evoked AP firing can be stopped and re-induced by light pulses at 380 nm and 500 nm, respectively, thus demonstrating the bistability of the system.

Just as H-SPARK could be converted into D-SPARK, LiGluR, a light-gated depolarizing ion channel, could be converted into a hyperpolarizing ion channel, called HyLighter.[70] As before, this could be achieved by changing the ion selectivity of the transmembrane domain through genetic manipulation. However, in the case of HyLighter, not a single point mutation, but the exchange of a whole protein domain was necessary. HyLighter combines the GluK2-derived photosensitive LBD of LiGluR with the potassium-selective TMD of the prokaryotic glutamate receptor SGluR0, which contains the signature GYG motif of K^+ channels.[71] Expression of HyLighter in hippocampal neurons showed that inhibition of neuronal firing can be reversibly turned on and off by irradiation with 380 nm and 500 nm light, respectively (Figure 13). The main advantage of HyLighter in comparison to fully genetically encoded hyperpolarizing systems is the thermal stability of *cis*-MAG-1 bound to the LBD. Therefore, in analogy to LiGluR, a single flash of 380 nm light of a few milliseconds is sufficient to prevent neurons from firing for seconds to minutes.

One of the big surprises in the development of optochemical systems was how quickly they could be transferred to animals and could be used to dissect behavior.[68,70] The first in vivo experiments with LiGluR were performed in zebrafish larvae.[68] Zebrafish at this stage of development are fully transparent, which is a necessary requirement for reaching neuronal circuits with light. To implement LiGluR, iGluK2-L439C was genetically encoded in small subsets of neurons which are involved in touch sensation and located at the head and trunk of the animals. For the delivery of the photoswitch to the tissue of interest, it was enough to bath the larvae in a solution containing MAG-1. Following this treatment, an escape reflex initiated by touching the trunk with a pipette tip could be controlled by optical activation of LiGluR. This reflex could be suppressed by brief illumination with 365 nm light and restored with 488 nm light.[68] HyLighter could be applied to zebrafish larvae in a similar way.[70]

In more sophisticated subsequent studies, LiGluR was genetically targeted to a single type of cell and used to elucidate the functional role of so-called Kolmer–Agduhr neurons—a mystery unsolved for 75 years. They turned out to be the central pattern generators of locomotion in the early development of vertebrates.[72]

In addition to PTLs, PCLs also turned out to be applicable to glutamate receptors (Figure 14). Their design was based on

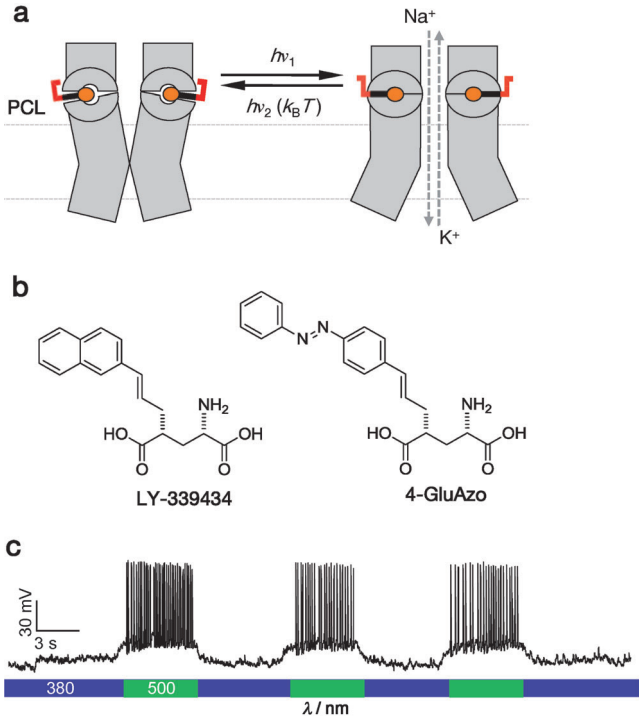


Figure 14. The PCL logic applied to ionotropic glutamate receptors. a) Schematic view of a PCL operating on LiGluR. b) The structures of LY-339434 and its derivative 4-GluAzo, a PCL that targets kainate receptors. c) Controlling the AP firing of hippocampal neurons with 4-GluAzo.

the extensive pharmacology of iGluR agonists, antagonists, and blockers, but was also supported by the LBD X-ray structures. Replacement of the naphthyl moiety of LY339434, a know agonist of GluK1 and GluK2, with an azobenzene moiety gave 4-GluAzo. This compound turned out to be a highly effective photochromic agonist of certain kainate receptors.[73] Its application to GluK1 and GluK2 channels expressed in HEK293 cells demonstrated the reversible control of inward currents in a light-dependent manner. In this case, the *trans* state of the azobenzene preferentially activates the receptors, and 4-GluAzo showed a slightly higher selectivity for GluK1 than for GluK2. When applied to dissociated rat hippocampal neurons, 4-GluAzo functioned as "reversibly caged" glutamate.[73] Action potential firing could be triggered by changing from 380 to 500 nm light and stopped by switching back to the lower wavelength. In subsequent studies, the burst firing of cerebellar Purkinje neurons could be effectively controlled with 4-GluAzo.[74]

Detailed biophysical studies will be needed to understand how 4-GluAzo and other PCLs work. In particular, it would be interesting to know whether the photoisomerization can take place while they are attached to their binding sites or whether have to dissociate before switching can occur. X-ray crystallographic studies could also provide important insights into the binding modes of PCLs (and PTLs) and guide their further development.

9. Pentameric Ligand-Gated Ion Channels

Pentameric ligand-gated ion channels (pLGICs) also play important roles in synaptic transmission.[51] In contrast to mammalian glutamate receptors, they can have both excitatory and inhibitory effects on the postsynaptic cell, which can be a neuron or a muscle cell. The superfamily of pLGICs includes nicotinic acetylcholine receptors (nAChR), γ-aminobutyric acid receptors ($GABA_A$ and $GABA_C$), 5-hydroxytryptamine receptors ($5\text{-}HT_3$), and glycine receptors (GlyRs).[75] They are sometimes also called Cys-loop receptors because of the fact that all pLGICs subunits feature an extracellular disulfide bond in a conserved region. $GABA_A$ and $GABA_C$ are chloride-selective ion channels that mediate fast inhibition of neuronal activity in the brain and the retina.[76] The serotonin receptor $5\text{-}HT_3$ is an excitatory cation channel that modulates neurotransmitter release. Similar to $GABA_A$ receptors, GlyRs have an inhibitory effect on neuronal communication. They are ligand-gated chloride channels that are mostly expressed in the spinal cord and the brain stem (Figure 15).[77]

Nicotinic acetylcholine receptors are excitatory cation channels that are expressed throughout the mammalian nervous system and at the neuromuscular junction.[78] Five classes of subunits exist for the muscle-type receptor nAChR: α_1, β_1, γ, ε, and δ.[51,78b] Together, they form a cation-selective pentameric ion channel with $(\alpha_1)_2\beta_1\gamma\delta$ stoichiometry. In contrast, the neuronal types are composed of α_2–α_{10} and β_2–β_4 subunits and can form both heteromeric (e.g. $(\alpha_4)_2(\beta_2)_3$) and homomeric (e.g $(\alpha_7)_5$) pentamers. Notably, only α_7, α_8, and α_9 form homomers when expressed heterologously.[78b,79]

Each subunit of the nAChR is divided into an extracellular, N-terminal ligand-binding domain, a transmembrane region with four transmembrane spanning helices, and an intracellular region (Figure 15). Both the N and C terminus are located in the extracellular space. The transmembrane helices of five single subunits line up to form the pore of the ion channel. The $(\alpha_1)_2\beta_1\gamma\delta$ receptor at the neuromuscular end plate has two ligand-binding sites located between the α–γ and α–δ subunits, which have distinct affinities for the neurotransmitter acetylcholine (Ach). Both binding sites must be occupied to open the channel pore.[51,78b]

The structural biology of nAChR has a long history, since it was accessible from the electric organ of the ray *Torpedo californica* well before the advent of molecular cloning and heterologous expression.[17c,51] By using electron microscopy, a structural model of the *Torpedo* nAChR could be gradually built and ultimately refined to 4 Å resolution.[15a,80] More recently, X-ray structures of the two bacterial homologues ELIC and GLIC were solved in their closed and open states, respectively, which provided a detailed view of the pentameric assembly and shed some light on channel gating.[15b,c] Unfortunately, none of the abovementioned receptors were crystallized and elucidated in conjunction with agonists or antagonists.

The X-ray structure of two acetylcholine-binding proteins, isolated from the sea snails *Limnia stagnalis* (L-AChBP) and *Aplysia californica* (A-AChBP) provided more insight into the interaction of pLGICs with their ligands.[81] These soluble proteins are homologous to the extracellular ligand-binding domain of pLGICs, but lack their transmembrane domain. Sequence alignment of L-AChBP with various pLGICs gave a 20–24% match with the nAChR subunits and a 15–18% match to the $5\text{-}HT_3$, $GABA_A$, $GABA_C$, and glycine receptor subunits.[81b] The X-ray structures of L-AChBP cocrystallized with the agonists nicotine and carbachol showed that the cationic ligands bind at the interface between two subunits in an "aromatic box" made up mostly of tyrosine and tryptophan side chains. This confirmed the importance of cation–π interactions that had been previously proposed on the basis of intricate biophysical studies with labeled amino acids.[81c] One side of the binding site is covered by the so-called C loop, which closes like a flap upon binding of the agonist. This flexible C loop also carries a cysteine disulfide at its tip but is not to be confused with the Cys loop, which is located closer to the membrane.

Subsequently, other ligands for pLGICs, such as epibatidine (an agonist for nAChR),[81d] lobeline (a mixed agonist/antagonist for nAChR),[81d] methyllycaconitine (an antagonist for nAChR),[81d] as well as strychnine (an antagonist for GlyR),[82] were cocrystallized with A-AChBP. These structures showed that the C loop indeed acts as an induced-fit sensor for ligands, and that the degree of C-loop closure correlates with the agonist versus antagonist activity. How this movement is mechanically linked with the opening of the channel is still a matter of debate.

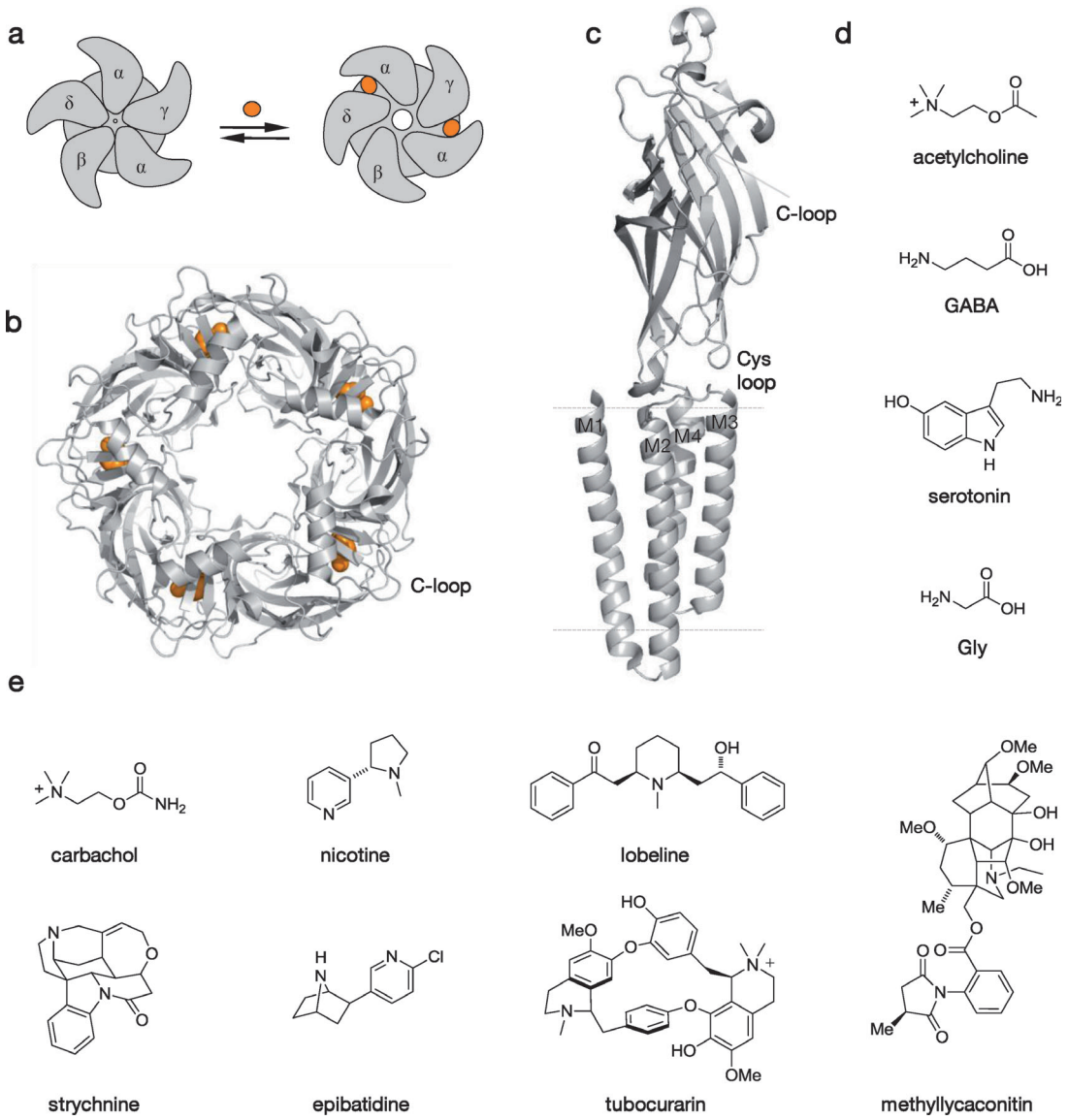


Figure 15. Pentameric ligand-gated ion channels (pLGICs). a) A highly schematic view of nAChR gating. b) Crystal structure of the acetylcholine-binding protein (AChBP) cocrystallized with nicotine (pdb 1UW6). c) A single subunit of a pLGIC, showing the transmembrane domain, the extracellular ligand-binding domain, and the Cys loop (pdb 3P4W). d) Endogenous ligands that gate pLGICs. e) Other well-known ligands of pentameric ligand-gated ion channels that have been cocrystallized with the AChBP.

10. Photosensitizing Pentameric Ligand-Gated Ion Channels

The nicotinic acetylcholine receptor has been studied for many decades, since it was the first ligand-gated ion channel to be isolated and characterized as a protein.[17c] It occurs in high concentrations in the electric organs of certain fish, such as the ray *Torpedo electroplax* and the eel *Electrophorus electricus*, which is why it could be investigated with biophysical methods that were not applicable to other types of receptors at the time. This may be the reason why the nAChR was also the first receptor to be photosensitized, both with a PCL and a PTL.[83] Amazingly, this was done in the early 1970s, well before the advent of molecular cloning, heterologous expression, modern structural biology, and patch-clamp electrophysiology. It took more than three decades to repeat this achievement with other types of receptors!

The PCL used in this case was an azobenzene unit called Bis-Q, which bears a quaternary ammonium ion (Q) on both sides of the chromophore. This simple, symmetric molecule functions as a photoswitchable version of the natural agonist acetylcholine.[83] Whereas *trans*-Bis-Q activated nAChRs in *Electrophorus electricus*, the corresponding *cis* isomer was found to be less active. The activity of the two isomers was assayed by measuring the transmembrane potential of *Electrophorus* electroplaques, which are specialized cells from its electric organ. The system was later used to determine the opening and closing rates of nAChRs.[84]

The corresponding PTL system was based on careful reasoning and a bit of luck, since the native receptor could be used without genetic manipulation. It turns out that nAChRs

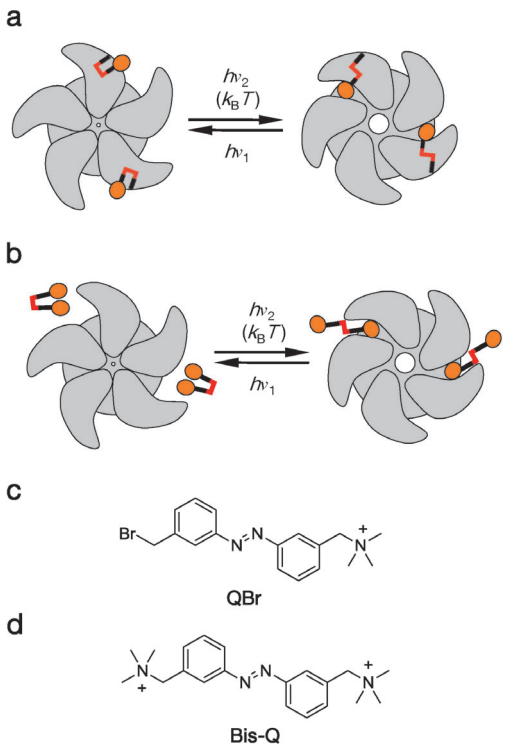


Figure 16. Photosensitizing the nicotinic acetylcholine receptor. A highly schematic depiction of a) a PTL reversibly acting on a nAChR, and b) a PCL controlling the nAChRs. c) The structure of QBr, a PTL, and d) BisQ, a PCL.

possess a disulfide bond on the tip of their C loops in the α subunits, in proximity to the ligand-binding site (Figure 16). This disulfide could be reduced without greatly affecting the function of the receptor, thereby providing a reactive thiol for the covalent attachment of a PTL.[83] The PTL was a benzylic bromide, called QBr, which also carried the requisite quaternary ammonium ion for activation of the receptor. Conveniently, QBr could be obtained through a slight modification of the synthetic protocol used to make Bis-Q. Once attached to nAChRs, QBr could be used to reversibly stimulate *Electrophorus* electroplaques, frog muscles, and rat myoballs with light.[85]

Of course, in the 1970s and 1980s the light-activated nAChR could not be heterologously expressed and genetically targeted, and it was, therefore, never used to control AP firing. Similarly, Bis-Q was mostly seen as a tool to study the innate function of nAChRs and not as a practical way to optically control nervous systems or animal behavior. However, with detailed structures and the modern tools of molecular biology now available, it is highly likely that light-gated nAChRs will soon resurface and be used in optochemical genetics. In addition, PCLs and PTLs for other pLGICs are bound to be developed.

11. An Abundance of Targets

From the preceding paragraphs it is clear that a variety of receptors from different structural classes can be rendered photosensitive by using synthetic PTLs and PCLs. However, the vast majority of potential candidates with sufficient structural and pharmacological data have not yet been investigated, and opportunities abound to explore the optochemical genetics of these targets. For example, given the many structural and functional similarities between voltage-gated potassium (K_v), sodium (Na_v), and calcium channels (Ca_v), the photochromic open-channel blockers discussed above could be applied to the latter as well. In addition, Na_v and Ca_v channels have extensive pharmacology of their own, which could be exploited to design more selective PCLs. The long-awaited X-ray structure of a Na_v channel has just been reported and should greatly facilitate the design of the corresponding PTLs.[86]

Even if one were confined to clamshell-bearing receptors, there would be a large number of interesting targets to work on. Clamshell-like ligand-binding domains appeared early in evolution and are a prime example of how a successful structural motif has been used again and again in different functional contexts.[87]

Originally evolved as bacterial periplasmic binding proteins (such as the well-known maltose-binding protein or amino acid binding proteins), clamshells have subsequently been integrated as functional and structural domains into prokaryotic and eukaryotic transmembrane proteins. Apparently, they were first captured by bacterial ABC transporters that initially interacted with soluble periplasmic binding proteins in a noncovalent fashion. At some point, they made their way into ion channels, such as ionotropic glutamate receptors. These include several prokaryotic glutamate-gated channels,[71] a plant receptor,[88] and one that was recently found in a comparatively simple eukaryotic rotifer.[89] As discussed in Section 7, glutamate-gated ion channels have assumed a major role in the fast synaptic transmission of higher animals. However, glutamate-binding clamshells can also be found in metabotropic glutamate receptors (mGluRs), which are family C GPCRs.[90] This large and important class also includes $GABA_B$ receptors[91] and T1R taste receptors,[92] and is found in many places in neurophysiology. The clamshell of several of these mGluRs has been characterized by X-ray crystallography in atomic detail and their pharmacology is very well developed (Figure 17).[93] Therefore, they are logical next targets for manipulation with PTLs or PCLs. Finally, clamshell-like ligand binding domains have been found in certain receptor-linked enzymes, which represent the third major class of receptors involved in signal transduction, in addition to ion channels and GPCRs. An example is the ANP receptor, the clamshell-dimer of which has been crystallized bound to the atrial natriuretic peptide (Figure 17).[94] Clamshell-containing receptor tyrosine kinases have also been recently described.[95]

The X-ray structure of a full-length GPCR of family C that includes the transmembrane domain has not yet been reported. In contrast, several GPCRs belonging to family A have been recently elucidated by X-ray crystallography (Figure 18). These receptors comprise the largest class of GPCRs and have a ligand-binding site that is embedded more- or less-deeply within the membrane. They include such

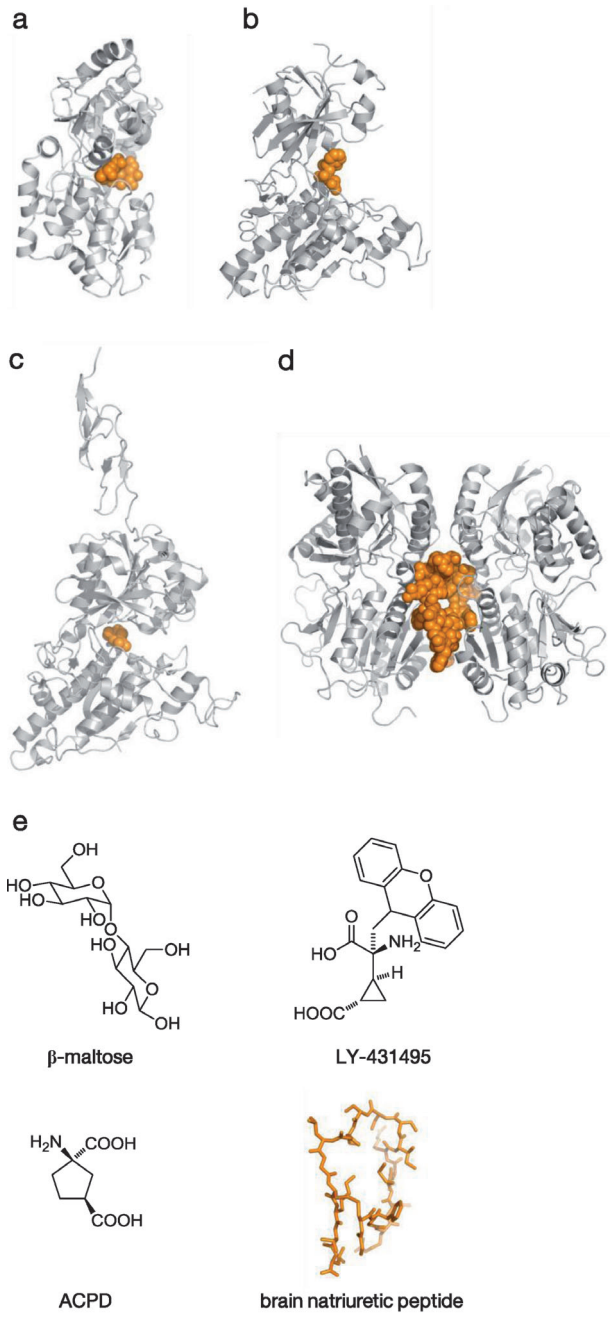


Figure 17. Clamshell-like LBDs as potential targets for optochemical genetics. a) The maltose-binding protein, a typical periplasmic binding protein (pdb 1ANF). b) The extracellular clamshell of mGluR3 with an agonist (pdb 2E4X). c) The extracellular clamshell of mGluR1 with an antagonist (pdb 3KS9). d) The clamshell dimer of the ANP receptor, a receptor-linked enzyme (pdb 1YK0). e) The chemical structures of ligands for clamshell-containing receptors.

important pharmacological targets as adrenergic receptors (e.g. β2A),[96] adenosine receptors (e.g. A_{2A}, which is a target of the antagonist caffeine),[11c] and dopamine receptors (e.g. D3).[11d] Rhodopsin, the photoreceptor used in animal vision, also belongs to this category.[11b] In this case, however, the photoswitch retinal is covalently bound in an inactive form (11-*cis*) and undergoes photoisomerization to the active form (all-*trans*), followed by hydrolysis and recycling, instead of reversible binding. As such, it resembles a PTL to a certain extent. Given this analogy, it is entirely conceivable that well-characterized ligands for other class A GPCRs could be replaced with PTLs and PCLs, thus turning them into photoreceptors.

Trimeric ion channels are also interesting targets, since they have recently been structurally characterized in atomic detail and have good pharmacology (Figure 19). They include purinergic receptors (P2X receptors), acid-sensing ion channels (ASICs), and epithelial sodium channels (ENaCs). The ionotropic P2X receptors are nonselective cation channels that are activated by extracellular adenosine 5′-triphosphate (ATP). They are widely expressed in the nervous and immune system, and are involved in numerous neurological functions, such as pain sensation.[97]

The first X-ray crystal structure of a P2X receptor, the zebrafish $P2X_4$ receptor, was solved in its closed state at 3.1 Å resolution.[13] The structure confirmed the trimeric channel architecture and provided important insights into the ion-conducting pore. The position of the ATP binding site was proposed to be located between each subunit at the outer extracellular surface of the receptor. However, the $P2X_4$ structure was solved without ATP or an antagonist bound (e.g. the azobenzene PPADS), thus leaving the exact binding mode of ligands undefined.

One particular P2X receptor, $P2X_2$, holds a special place in the development of optochemical genetics. After heterologous expression, this cationic channel could be optically stimulated with caged ATP. This system was reported in 2003, and was one of the first systems to work in neurons. Since ATP is hydrolyzed rapidly, even in the extracellular space, photostimulation could be carried out repeatedly and with relatively good temporal resolution. Incidentally, caged ATP is also one of the first caged ligands, if not the first.[35]

ASIC channels belong to the degenerin/epithelial sodium channel (DEG/ENaC) family. They are ligand-gated trimeric cation channels that are activated by extracellular protons and favor Na^+ over K^+ ions by a factor of ten. Isoforms of ASICs are distributed throughout the mammalian central and peripheral nervous system. They play important physiological roles, for example, in the detection of tissue acidosis during ischemia.[98] Two crystal structures of homotrimeric ASIC channels have been reported recently.[99]

Unfortunately, this is not yet the case for ENaC, a heterotrimeric channel that is constitutively open and is extremely selective for sodium. It plays a key role in sodium reabsorption and the perception of salt taste, and is the target of widely used diuretics, such as Amiloride.[100]

Transient-receptor potential channels (TRP channels) have been identified as major molecular players in sensory perception.[101] They are tetrameric cation channels that are polymodal and sensitive to a wide variety of input signals, including temperature, small molecules, and ligands. One famous member of this large superfamily is TRPV1, which is activated upon heating, but also responds to capsaicin, the active component of chili peppers.[102] Therefore, on a molecular level, "hot" as in "hotplate" and "red-hot chili peppers" are really the same thing. In one of the first applications of optochemical genetics, TRPV1 was hetero-

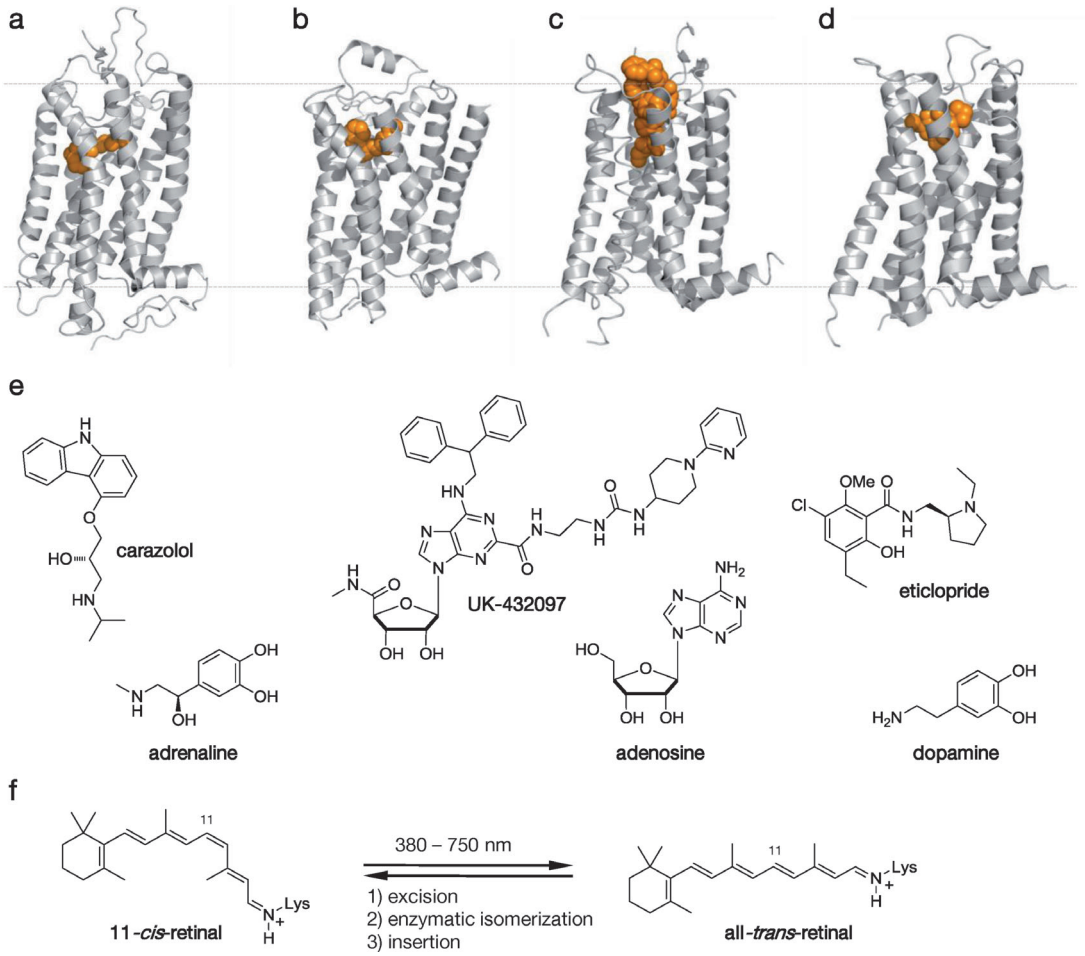


Figure 18. Family A GPCRs as potential targets of optochemical genetics. a) Rhodopsin, a natural photoreceptor with a covalently attached photoswitch (pdb 1U19). b) The β2 adrenergic receptor bound to the inverse agonist carazolol (pdb 2RH1). c) The A_{2A} adenosine receptor bound to the agonist UK-432097 (pdb 3QAK). d) The D3 dopamine receptor bound to the antagonist eticlopride (pdb 3PBL). Protein domains used to facilitate the crystallization have been cut off. e) Chemical structures of the cocrystallized and endogenous ligands for the receptors depicted above. f) Isomerization of 11-*cis*-retinal to all-*trans*-retinal in rhodopsin.

logously expressed in neurons and stimulated with caged capsaicin and light (Figure 4b,c).[35]

TRP channels are not only responsible for sensing heat and coldness but may also play a role in sensing pressure.[101] As such, they could have physiological roles in mechanoreception (touch), balance, pain, the regulation of blood flow, and hearing.[103] The regulation of pressure sensors with light would indeed be a very interesting exercise. This has not yet been done with a TRP channel, but proof of principle has been given with a comparatively simple bacterial mechanoreceptor isolated from *Mycobacterium tuberculosis* (Figure 20).[104] This homopentameric channel, called MscL, was crystallized in its closed form and its structure was elucidated by X-ray crystallography.[105] If the tension of the membrane in which it is embedded exceeds a certain value, it opens to form a very large pore, thus functioning like a valve that relieves osmotic pressure in the bacterium. The closed gate of the channel is formed by a ring of five hydrophobic residues that prevent ions and other solutes from passing through. Detailed biophysical investigations had shown that replacement of these hydrophobic residues with charged amino acids leads to a constitutively open channel, presumably as a result of electrostatic repulsion.[106]

In a first approach toward the photosensitization of MscL, a caged carboxylic acid was covalently attached to a cysteine residue to replace the hydrophobic residues at the gate. Upon irradiation with UV light, the cages were cleaved and the resulting negative charges triggered the opening of the channel. In a second step, the caged acids were replaced with a covalently attached spiropyran/merocyanine (SP/MC) photoswitch. Switching from the comparatively nonpolar SP state to the highly polar MC state resulted in the opening of the valve. This process was reversible over a few cycles.[104] Although this system was developed as a "nanovalve" for drug delivery and may never find its way into neurobiology, it is nevertheless a very nice example of the optical control of channel activity and it involves both cages and photoswitches. Interestingly, photoswitchable lipids have also been used to change the membrane tension with light and reversibly activate MscL.[107] Indeed, photoswitchable lipids may provide yet another general way to influence the function of transmembrane proteins and cells with light.

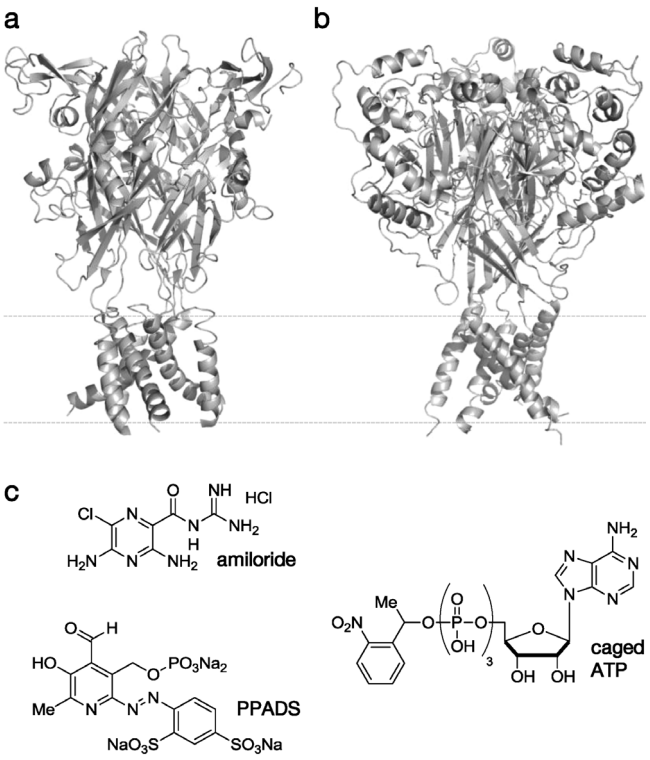


Figure 19. Trimeric ion channels as potential targets for optochemical genetics. a) The ASIC1 channel (pdb 3HGC). b) The $P2X_4$ receptor (pdb 3H9V). c) Chemicals acting on trimeric ion channels. Amiloride: a blocker for ASIC and ENaC channels. PPADS: an antagonist of purinergic receptors. Caged ATP: a CL for $P2X_2$ receptors.

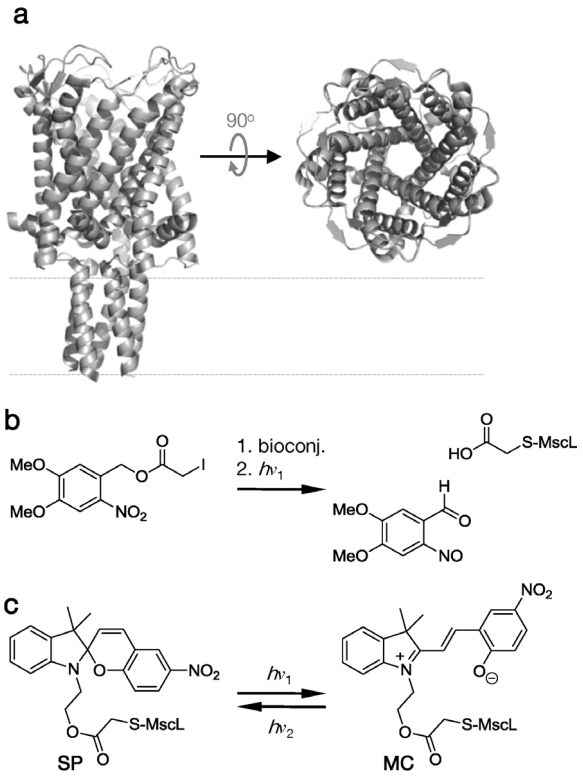


Figure 20. Photosensitization of a bacterial mechanosensitive ion channel. a) The structure of MsCl in its closed form: side and top view (pdb 2OAR). b) A caged acid used to trigger MscL opening. c) The SP and MC forms of a photoswitch used to reversibly gate MscL.

12. Summary and Outlook

As outlined above, there is certainly no shortage of protein targets that could be addressed with optochemical genetics. These include naturally occurring photoreceptors, such as the channelrhodopsins, which could be fitted with synthetic switches to tune their spectral properties. However, many variants of synthetic molecules attached to naturally blind receptors are also conceivable. For example, all of the PTLs and PCLs presented herein are either agonists or blockers, but it should be possible to turn them into antagonists or find photochromic molecules that function as channel openers. In addition, photoswitchable cross-linkers could be developed that could be hooked up to two cysteine residues.[108] These have been used with great success to control the helical content of peptides,[108b,109] and they have already found applications in governing the activity of proteins.[110] It is entirely possible that this approach could be extended to transmembrane receptors, thereby providing yet another way to control neural activity with light.

Other types of photoswitches, such as hemthioindigos or SPMCs, could be systematically explored and applied in regard to neurobiologically relevant receptors. However, much could be done even if one sticks with azobenzenes, since azobenzenes can be easily tuned toward specific photophysical and thermal requirements. For example, the red-shifted variants that operate far in the visible range of the spectrum and turn themselves off in the dark need to be further developed. Perhaps, one could even push the spectral sensitivity of azobenzenes into the near-infrared, in keeping with the grand challenge of Sir Francis Crick. Even if it is not possible to develop single-photon switches that respond to these wavelengths, one could explore two-photon photoswitches that would also allow for the precise activation of PCLs in very small volumes. These could be used, for example, to photochemically stimulate a single dendritic spine, which can be simultaneously monitored with two-photon fluorescence imaging.

Light, however, is not the only "unnatural" input signal that could be considered. For example, one could reprogram a ligand-gated ion channel to respond to an orthogonal ligand, that is, a molecule that does not interact with native receptors. This strategy has already been achieved with much success with kinases and GPCRs, and it should be possible to extend this approach to other types of receptors.[111] One could even speculate about sensitizing ion channels to signals that can penetrate deep into tissues, such as radiofrequency electromagnetic fields or static magnetic fields.

Yet, as tempting as these speculations are, light is, and will remain, the most interesting input signal for neuroscience. The technology needed to quickly modulate its wavelength and intensity is highly developed and new ways to deliver light with spatiotemporal control and deep within tissues continue to emerge. These include digital micromirror arrays[112] and fiber-optic microendoscopy.[113] At the same time, genetic targeting through viral transfection is becoming increasingly

precise and effective, and the application of synthetic photoswitches will certainly benefit from new drug-delivery techniques. Thus, we expect that many neurobiological questions will be tackled with optochemical genetics in the future.

Much could be learned, for example, about the innate function of receptors and their many isoforms, since the optical control of genetically defined receptor subtypes could clarify their individual contributions to various physiological processes (see Figure 5). So far, this has been carried out with a variety of K_v channels (see Section 6), but we anticipate that the optochemical genetic approach can be applied more broadly. Simple pharmacological approaches frequently fail because of a lack of selectivity, and genetic knock-out strategies are often inconclusive because of compensatory effects. Therefore, a knock-in animal, wherein the receptor of interest is substituted with a simple cysteine mutation for PTL attachment and can be conditionally activated and deactivated with light could be very useful.

In addition to basic neuroscience research, where the goal is to understand existing systems through systematic perturbation, one could apply optochemical genetics to what could be called "synthetic neurobiology". Here, the emphasis lies on rewiring nervous systems and altering their components to create new forms of neural processing or sensory perception. For example, the receptors that underlie our sense of temperature or mediate pain sensation could be reprogrammed to become photoreceptors. The mechanoreceptors responsible for our sense of hearing and balance could be engineered in a similar fashion. Perhaps, the day is not far away where taste receptors and odorant receptors can be stimulated with light!

This may sound a bit outlandish, but such a philosophy may actually lead to useful human therapies. A premier therapeutic target would be the retina, which can be easily reached with light, viruses, and small molecules. In many forms of blindness, such as retinitis pigmentosa, the natural photoreceptor cells of the retina, that is, the rods and cones, have been destroyed, but its remaining layers are largely intact. Their neurons express numerous receptors that could be persuaded to become light-sensitive by using PTLs or PCLs. Indeed, the first attempts to use optochemical genetics to restore vision are very promising (Figure 21). In a recent study, LiGluR was expressed in the retinal ganglion cells of blind mice after transfection with adeno-associated virus (AAV). Following injection of MAG-0 into the vitreous body, the retinal ganglion cells, which communicate directly with the brain trough the optical nerve, responded to stimulation with light. This information was relayed to the visual cortex and, as a consequence, the pupillary reflex as well as natural light-avoidance behavior of the animals was restored.[114] The first experiences with PCLs have also been promising and bode well for applications of synthetic photoswitches in human therapy.

For us, it is simply amazing that our attempts to teach old receptors new tricks have gone from a proof of principle to studies of vision restoration within seven years. This pace is likely to increase, as numerous new targets are on the horizon and a growing number of neurobiologists, biophysicists, and chemists are drawn into the field. We are convinced that these highly collaborative efforts will yield many useful techniques

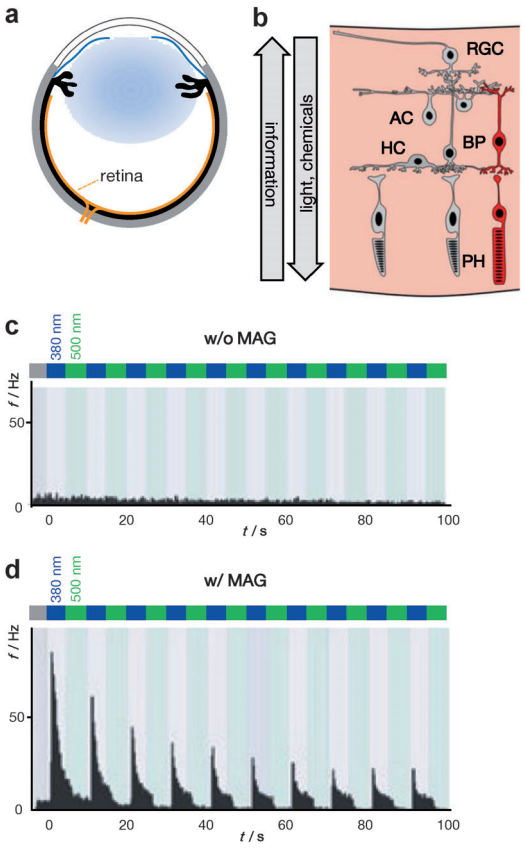


Figure 21. Restoring visual responses with synthetic photoswitches. a) Cross-section of a mouse eye with the retina shown in orange. b) A schematic diagram of the retina, showing its layered architecture. RGC: retinal ganglion cell, AC: amacrine cell, BP: bipolar cell, HC: horizontal cell, PH: photoreceptor cells (rods and cones). c), d) Electrical activity of RGCs expressing LiGluR before (c) and after addition of MAG (d).

with which to study neural networks or treat disorders of the nervous system. But usefulness aside, optochemical genetics will always remain an incredibly rewarding intellectual exercise and a nice demonstration that the complexities of

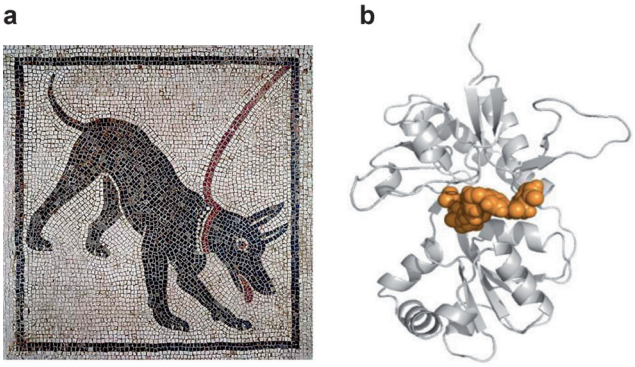


Figure 22. Harnessing the complexities of life. a) A dog (a natural creature slightly modified through breeding) with a leash (a crude device used to control its behavior, with mixed success). b) LiGluR (a complex receptor slightly modified through genetic engineering) on a comparatively simple synthetic leash.

life can be harnessed with comparatively simple synthetic chemistry (Figure 22).

13. Addendum (10. November 2011)

Orthogonal ligands, mentioned in Section 12, have recently been developed for pentameric ligand-gated ion channels.[115]

14. Abbreviations

AMPA	2-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AP	action potential
Ca_v	voltage-gated calcium channel
CL	caged ligand
GABA	γ-aminobutyric acid
GPCR	G-protein-coupled receptor
K_v	voltage-gated potassium channel
MscL	large-conductance mechanosensitive channel from *M. tuberculosis*
Na_v	voltage-gated sodium channel
NMDA	methyl-D-aspartate
pLGIC	pentameric ligand-gated ion channel
PTL	photoswitchable tethered ligand
PCL	photochromic ligand
SPMC	spiropyran/merocyanine photoswitch
TEA	tetraethylammonium ion
TRPV1	transient receptor potential channel, for example, subtype V1

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