

Synapses in the spotlight with synthetic optogenetics

Shai Berlin^{1,*}  & Ehud Y Isacoff^{2,3,4}

Abstract

Membrane receptors and ion channels respond to various stimuli and relay that information across the plasma membrane by triggering specific and timed processes. These include activation of second messengers, allowing ion permeation, and changing cellular excitability, to name a few. Gaining control over equivalent processes is essential to understand neuronal physiology and pathophysiology. Recently, new optical techniques have emerged proffering new remote means to control various functions of defined neuronal populations by light, dubbed optogenetics. Still, optogenetic tools do not typically address the activity of receptors and channels native to neurons (or of neuronal origin), nor gain access to their signaling mechanisms. A related method—synthetic optogenetics—bridges this gap by endowing light sensitivity to endogenous neuronal receptors and channels by the appending of synthetic, light-receptive molecules, or photoswitches. This provides the means to photoregulate neuronal receptors and channels and tap into their native signaling mechanisms in select regions of the neurons, such as the synapse. This review discusses the development of synthetic optogenetics as a means to study neuronal receptors and channels remotely, in their natural environment, with unprecedented spatial and temporal precision, and provides an overview of tool design, mode of action, potential clinical applications and insights and achievements gained.

Keywords native receptors; neurons; optogenetics; photoswitches; synthetic optogenetics

DOI 10.15252/embr.201744010 | Received 30 January 2017 | Revised 2 March 2017 | Accepted 9 March 2017 | Published online 10 April 2017

EMBO Reports (2017) 18: 677–692

See the Glossary for abbreviations used in this article.

Introduction

The need to better understand the function and roles of neuronal membrane proteins, synaptic receptors and ion channels in particular, has become one of the more pressing goals in neuroscience for

the reason that their function and dysfunction underlay some of the most prevalent and devastating neurodegenerative diseases, neuropsychiatric disorders, and synaptopathies [1–6]. The better we understand their mechanisms of actions, in health and disease, the better we may design novel drugs and treatments.

The study of neuronal receptors and channels has been extensively addressed over the years by conventional pharmacology, electrophysiology, structural studies, molecular and genetic manipulations, etc. [7–13]. Despite the countless insights gained, these and other contemporary techniques display inherent drawbacks, such as specificity and off-targets, slow kinetics, poor spatial confinement, lack of or slow reversibility, etc. These appreciably limit our understanding of the processes under study [14–17]. Consequently, new methods are still vigorously pursued.

One novel approach developed for the remote study of neurons is optogenetics [18–20]. Optogenetics is a momentous and rapidly evolving field that leverages naturally occurring light-sensitive proteins (hereinafter referred to as photoreceptors, see [21]) and repurposes them into genetically encoded light-gated cellular actuators. Thereby, optogenetic tools allow the user to control cellular activity by light. The type of modulation depends on the photoreceptor's biological function. For instance, the widespread optogenetic tool ChR2 is a light-gated cation channel [22]. When expressed at the membrane of neurons, light illumination causes rapid channel opening resulting in strong membrane depolarization and AP firing [23–27]. This methodology has drastically transformed the way we approach and study biological systems, neuroscience in particular, owing to the method's non-invasive and reversible nature and, owing to the inherent properties of light, *fantastic* spatiotemporal resolution. Notably, with cell type-specific promoters [28], the expression of optogenetic tools can be confined to select cells and neuronal circuits to be exclusively interrogated by light. Optogenetic tools are extensively employed today to control neuronal circuits and behavior by regulating membrane excitability, though they can also control a wide array of additional cellular mechanisms ranging from protein trafficking, to inducing genomic engineering, to humbly name but a few of the achievements and applications described to date [19,22,23,29–38].

However, optogenetic tools are not without fault. These tools may suffer from slow kinetics of activation or deactivation,

1 The Ruth and Bruce Rappaport Faculty of Medicine, Technion- Israel Institute of Technology, Haifa, Israel

2 Helen Wills Neuroscience Institute, University of California, Berkeley, CA, USA

3 Department of Molecular and Cell Biology, University of California, Berkeley, CA, USA

4 Physical Bioscience Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA

*Corresponding author. Tel: +972 4 8295346; E-mail: shai.berlin@technion.ac.il

Glossary

2PE	two-photon excitation
AAQ	acrylamide–azobenzene–QA
ACH	acetylcholine
AP	action potential
BGAG	benzylguanine–azoglutamate
ChR2	channelrhodopsin-2
CNS	central nervous system
DPCC	diphenylcarbonyl-chloride
ER	endoplasmic reticulum
GABA	γ -aminobutyric acid
GIRK	G-protein activated inwardly rectifying potassium channel
GPCR	G-protein-coupled receptor
LBD	ligand-binding domain
LiGluN	light-gated NMDA glutamate receptors
LiGluR	light-gated kainate glutamate receptors
LimGluRs	light-gated metabotropic glutamate receptors
L-MAG	maleimide–azobenzene and 4'L stereochemistry glutamate
LTP	long-term potentiation
mAChR	muscarinic acetylcholine receptor
MAQ	maleimide–azobenzene–QA
MscL	mechanosensitive channel of large conductance
nAChR	nicotinic acetylcholine receptor
nAChR	nicotinic acetylcholine receptors
NMDAR	N-methyl-D-aspartate receptors
P2X	purinergic (ATP-gated) receptors
PALS	photoswitchable affinity labels
PAPC	<i>p</i> -azophenyldiphenylcarbonyl-chloride
PCL	photochromic ligand
PM	plasma membrane
PNS	peripheral nervous system
PTL	photoswitchable tethered ligands
PTX	pertussis toxin
PX	photoswitchable tweezers
QA	quaternary ammonium
RGC	retinal ganglion cell
SNAP	O6-alkylguanine-DNA alkyltransferase
SPARK	synthetic photoisomerizable azobenzene-regulated K ⁺ channel
TCP	targeted covalent photoswitch
TM	transmembrane
TREK	two-pore-domain potassium channel
V-LOGO	visible light operated GIRK opener
wt	wild type

small channel conductance, poor expression in mammalian cells, undesired secondary effects (e.g., acidifications), unwarranted basal activity prior to light illumination, unexpected inverse effects (i.e., depolarization instead of hyperpolarization), and more [19,39–44]. With regard to neuronal synapses, optogenetic tools are limited in their ability to control endogenous signaling mechanisms. For instance, though several tools have been specifically expressed at the synapse (e.g., [39,45–48]), very few were able to provide direct control over native components or endogenous signaling.

This gap can be bridged by an analogous method—synthetic optogenetics—that similarly employs light to regulate the function of proteins but, instead of the use of photoreceptors, relies on the appending of synthetic chromophores to “blind” proteins (i.e., proteins that do not naturally respond to light), denoted photoswitches.

There are many names by which this approach has been described (chemical-optogenetics, photopharmacology, or optogenetic

pharmacology [49–51]). However, as new tools are appearing in the literature, most names no longer cover all cases. For example, although the method initially employed pharmacophores in the design of photoswitches, several recent advances show the possibility to design pharmacophore-less photoswitches (e.g., photo-switchable tweezers, see below). Therefore, names embracing the term “pharmac” are less relevant today, warranting the re-naming of the approach. The name of synthetic optogenetics deems the most appropriate for several reasons: The method requires the usage of *synthetic* photoswitches, several key tools are denoted synthetic (e.g., SPARK, see below) and, importantly, as the field of optical control of cellular activity is widely recognized today by the name of optogenetics, the name “synthetic optogenetics” allows it to remain under this umbrella term, whilst emphasizing the distinction of the approach from others. In addition, we hypothesize that this name should also cover future developments in this area too (see discussion).

Here, we first review the historical development of the technique and proceed to provide an overview of modern developments of light-gated channels and receptors, through examples of their usage in the field of neuroscience. Lastly, we discuss several limitations, how these could be overcome and end with future prospects.

Photosensitizing proteins by synthetic optogenetics

The approach of synthetic optogenetics combines chemical and genetic methods to render potentially any protein sensitive to light. In contrast to optogenetic tools incorporating naturally occurring chromophores, this method employs *synthetic*, light-sensitive photoswitches [52]. Akin to chromophores found in nature (e.g., retinal [53,54]), photoswitches too undergo chemical reactions following the absorption of specific wavelengths. One highly relevant reaction to the design of biological tools is the *trans*-to-*cis* isomerization of azobenzenes, resulting in a large geometrical change and significant reduction in the length of the molecule (Fig 1A) [55]. Azobenzenes are mostly (> 95%) found in *trans* in the dark or following green-light illumination (e.g., ~500 nm), but isomerize to the shorter bent *cis* form (~80%) following near-UV light absorption (e.g., 350 nm; Fig 1B) [56]. Therefore, isomerization can be utilized to alter/modulate a protein's function. For instance, if the azobenzene-core is decorated with a specific receptor's ligand, isomerization from *trans*-to-*cis* could be used to expose the ligand making it accessible to the receptor, indirectly photoregulating it (Fig 1C). Other chemical headgroups can be added onto the azobenzene photoswitch, such as blockers, antagonists, allosteric modulators, conjugating moieties, lipophilic chains (see Fig 1, toolbox). These will typically determine the mechanism of action of the photoswitch. Today, the variety of the modes of action by which photoswitches can regulate protein function is extensive, involving varying the distance between a drug and its binding site, changing local polarity, inducing lateral pressure in the membrane, applying forceps-like motion to gate channels, piecing a fragmented drug, etc. (Fig 1C–J). Notably, the number of mechanisms employed by photoswitches exceeds the list enumerated here and, in fact, is limited only by the creativity of the designers.

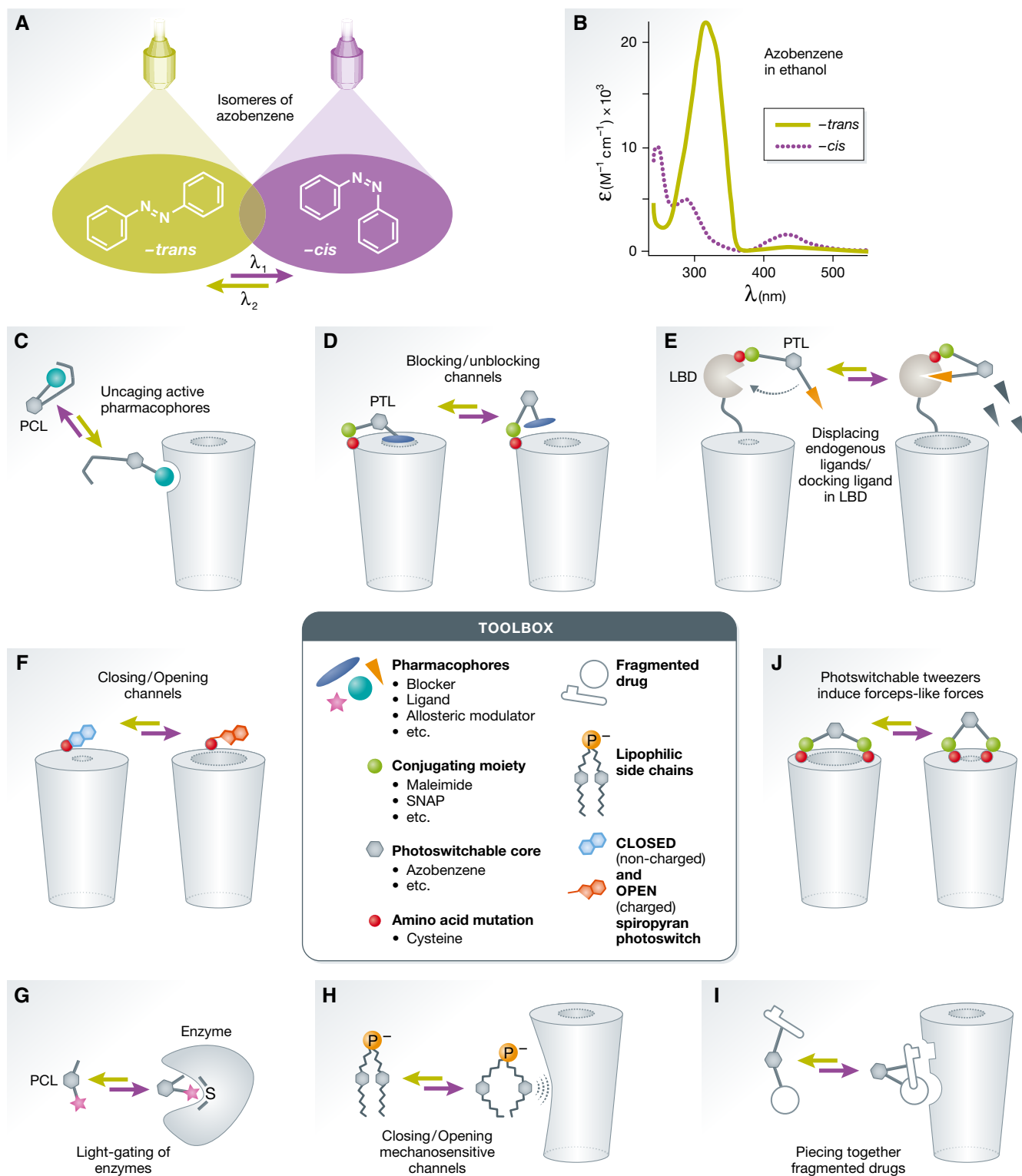


Figure 1.

The historical development of synthetic optogenetics

Synthetic optogenetics was pioneered by the Erlanger group, which initially demonstrated the photocontrol over the enzyme chymotrypsin [57]. Here, the authors synthesized a soluble photochromic

ligand (PCL) composed of a light-sensitive azobenzene-core linked to diphenylcarbonyl-chloride (DPCC—a specific inactivator of the enzyme) they termed PAPC (*p*-azophenyldiphenylcarbonyl-chloride; Fig 1G, purple). PCLs are advantageous as they do not require any genetic alterations of their target protein, enabling the

Figure 1. Isomerization of azobenzene to modulate protein function.

(A) Molecular structures of *–trans* and *–cis* isomers of azobenzene, depending on the wavelengths used. Near-UV light (violet) induces isomerization to *–cis*, whereas green light reverts the molecule back to *–trans*. (B) Absorption spectra of *–trans* and *–cis* isomers of azobenzene in ethanol. (C–J) Mechanisms of actions of azobenzene-based (gray hexagon) photoswitches. (middle) Toolbox: building blocks for the synthesis of photoswitches. Isomerization of PCLs can uncage an active pharmacophore (C). A PTL bearing a channel blocker (blue ellipse) is tethered to a modified channel (cysteine, red circle) via a conjugating moiety (maleimide, green circle). In *–trans*, the blocker reaches the pore of the channel (D). A receptor with a PTL bound to its LBD undergoing isomerization enables the ligand (orange triangle) to enter the LBD to activate or displace the endogenous ligand (black triangles) (E). Spiropyran undergoing isomerization from the closed (non-charged, cyan) to the open (charged, red) state induces channel opening (F). Light-gating of an enzyme by a PCL (G). Membrane incorporated photoswitches induce lateral mechanical pressure in the membrane during isomerization to open mechanosensitive channels (H). A fragmented drug is pieced together by the isomerization process (I). Photoswitchable tweezers bound to two cysteines (red circles) via maleimides (green circles) induce forceps-like forces (J).

regulation of *wt* proteins, *wt*-chymotrypsin in this case. The authors envisioned that the light-dependent isomerization of the azobenzene in PAPC could be used to gate (i.e., enable or disable) the ability of the pharmacophore (DPCC) to act on its site of action, a serine residue located deep within the enzyme. Indeed, they then showed that the *–cis* isomer of PAPC is five times more potent in inactivating chymotrypsin than the *–trans* isomer, suggesting that the DPCC has different pharmacological properties when the attached azobenzene is in the *–trans* or *–cis* conformation [57].

This report paved the way for other groups to render their favored enzyme photolabile. To this end, other groups have replaced DPCC with other specific compounds from the toolbox of available pharmacophores. This mix and match quickly yielded photoregulated acetylcholinesterases [58,59], trypsin [60], aldolases [61,62], urease, amylase, and more (see list in [63]). Interestingly, the engineering of photoregulated enzymes subsided in the following years. However, the approach was not neglected, rather shifted from targeting soluble proteins to membrane proteins. The first demonstration of the photoswitching of membrane receptors was shown to involve nicotinic acetylcholine receptors (nAChR) [64]. Here too, the authors exploited the changes in geometry of the azobenzene so that the PCL, here containing a carbachol headgroup, would differently interact and affect nAChR. When in *–trans*, the photoswitch inhibited the receptor more potently than when in *–cis*. This report established that the approach was not limited to soluble proteins, namely enzymes, and further showed its advantages over conventional pharmacological reagents. Indeed, though several specific drugs acting on nAChRs were available at the time, this approach enabled the study of nAChRs in their native environment with unprecedented temporal resolution, resulting in a flurry of publications [65–69].

During these years, another development laid the groundwork for what will become one of the most employed strategies in synthetic optogenetics: conjugating PCLs to their target. This involved the design of tethered photoswitches or photoswitchable tethered ligands (PTLs). PTLs are designed to irreversibly bind to their protein target, creating a high local concentration of the drug. To the best of our knowledge, the first tethered photoswitch was the cysteine-reactive QBr photoswitch (3-(alpha-bromomethyl)-3'-[alpha-(trimethylammonium)methyl] azobenzene bromide) [70]. This azobenzene-based photoswitch included an additional chemical moiety at the other end of the azobenzene that would covalently conjugate the photoswitch onto existing outer cysteine residues in nAChRs [70]. Once bound, QBr selectively activated the channels when in its extended *–trans* form, and much less so when in *–cis* (e.g., Fig 1D). The use of PTLs is preferable over PCLs in certain instances as it keeps the photoswitch near the receptor at all times,

granting a high local concentration of the chemical headgroup. This can enable efficient competition of the photoswitch with the soluble ligand (e.g., Fig 1E, black filled triangles). It also enables persistent perfusion of the preparation without necessitating constant supplementation of the photoswitch. Owing to these advantages, the photoregulation of enzymes was revisited in the following years with the use of PTLs (e.g., [61,71,72]). Collectively, these pioneering experiments demonstrated the potential of the approach, laid the framework (i.e., toolbox) for the design of soluble (PCLs) or tethered (PTLs) photoswitches, and, importantly, hinted at the potential of the approach to render any blind protein light-sensitive [73–75].

Synthetic optogenetics revisited

Despite the method's first appearance almost 50 years ago, the approach was gradually ignored and almost completely neglected for many years. However, in 2004, the spark was re-ignited by a group of scientists from the University of California at Berkeley (amusingly befitting as UCB carries the motto “let there be light” on its seal). The Isacoff–Trauner–Kramer alliance (see perspective by [76]) designed a Synthetic Photoisomerizable Azobenzene-Regulated K⁺ channel based on the *Shaker* channel—dubbed SPARK—to control neuronal excitability [77]. This was the first report using a PTL and a genetically modified channel to control mammalian neuron excitability, only two years after the first optogenetic demonstration using rhodopsin to “chARGE” (depolarize) neurons ([33], but also see perspective by [78]).

To confer light sensitivity to the *Shaker* channel, Banghart and colleagues synthesized a PTL consisting of a maleimide, azobenzene and a quaternary amine (QA—a K⁺ channel blocker) denoted MAQ [77]. When in *–trans*, the end-to-end distance of MAQ spans ~17 Å. Knowledge of this distance was then used to locate an outer residue in the *Shaker* channel that was situated at a similar distance from the pore. This residue was mutated to a cysteine to conjugate the photoswitch (via its maleimide) so that when in *–trans*, the quaternary amine of MAQ would reach the pore and block the channel. In turn, this would inhibit membrane hyperpolarization resulting in AP firing by the neuron (Fig 2A and B). This process could be rapidly reversed by near-UV light, as isomerization of MAQ to *–cis* shortened the molecule (to ~10 Å), physically removing the blocker from the pore, which opened the channel and electrically silenced the neuron. By toggling between green and near-UV illumination, the authors were able to prevent or enable AP firing repeatedly and remotely with a high spatiotemporal resolution (Fig 2B). Importantly, the authors note that the molecular design of the channel

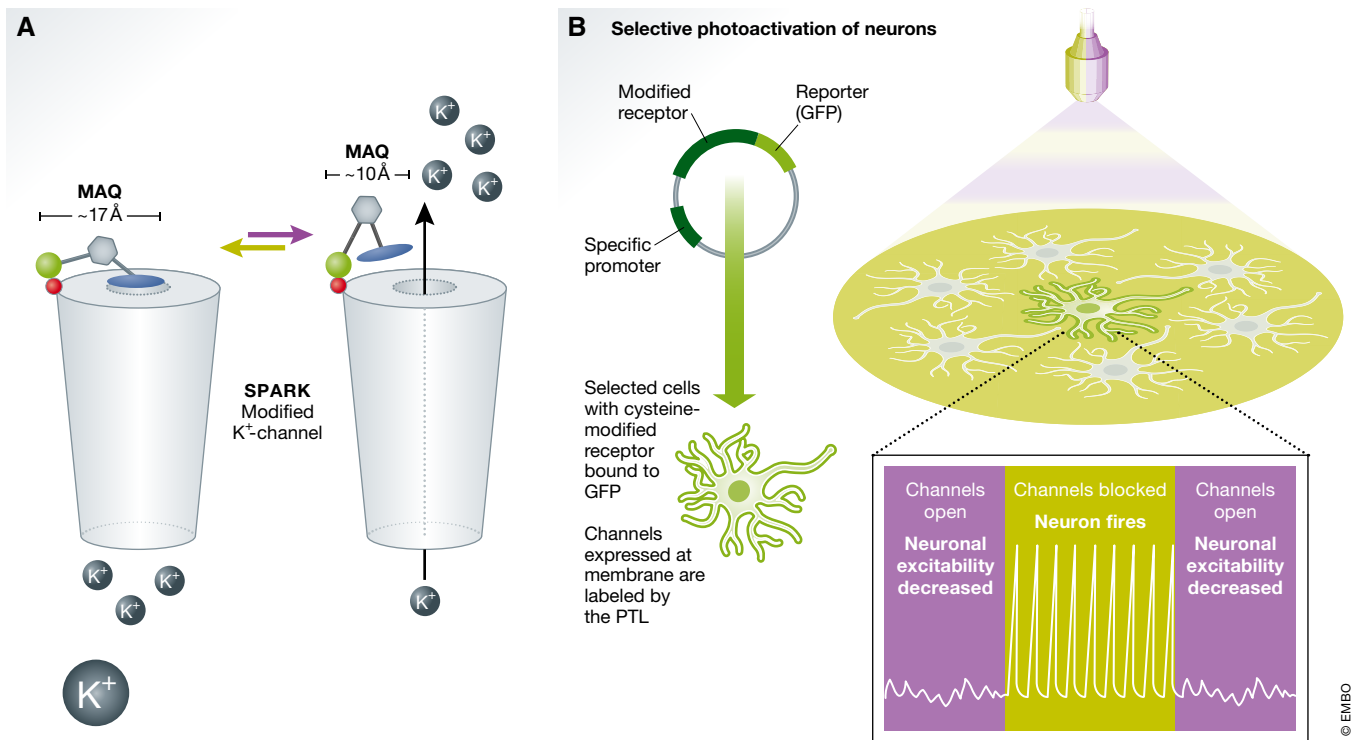


Figure 2. SPARK regulates neuronal excitability.

(A) SPARK, a modified K⁺ channel tethered to MAQ. In *trans* (green arrow), MAQ extends to ~17 Å, enabling block of the pore and no ion conductance (open circles denoted with K⁺). During isomerization (violet arrow), the shorter *cis* isomer (~10 Å) relieves the block and the channel opens, generating an efflux of K⁺ ions. (B) Selective photoactivation of neurons. The cysteine-modified receptor (bound to GFP) is genetically targeted to a defined neuronal population (via specific promoters). Channels expressed at the membrane (green highlight) of selected cells are labeled by the PTL. During green-light illumination (green bar, bottom), the channels are blocked, enabling firing by the neuron (bottom trace, APs). Neuronal excitability is decreased by isomerization back to *cis* (by near-UV light, violet bar) and opening of the channels. Cysteine, red circle; azobenzene, gray hexagon; maleimide, green circle; quaternary amine/QA, blue ellipse.

was possible mainly because of the rise in the availability of detailed structural and molecular information of ion channels, and K⁺ channels in particular (e.g., [79,80]). In point of fact, SPARK emerged at the time when the Nobel prize in Chemistry was attributed to Roderick MacKinnon for solving the structures of K⁺ channels [81]. Notably, the genetic manipulation of the channel enabled the exclusive expression of the channel in neurons, thereby limiting PTL conjugation to this defined population, unlike PCLs that cannot be typically confined to selected cells (but see below).

In parallel, two other groups were also toiling over the design of light-gated channels. Interestingly, both groups made use of the bacterial mechanosensitive channel of large conductance (MscL). The group of Bert Poolman synthesized a new type of an azobenzene-based photoswitch containing lipophilic phosphate tails, named 4-Azo-5P [82]. This photoswitch would incorporate within the leaflets of the membrane so that during *trans*-to-*cis* isomerization, the photoswitch would exert lateral pressure in the membrane. This would mechanically gate the closely situated MscL channel (Fig 1H). In 2005, the Feringa group also designed a photoregulated MscL, using another clever approach [83]. MscL, though mechanosensitive, also exhibits spontaneous channel opening when polar or charged amino acids are introduced next to the pore [84]. Koçer exploited this phenomenon and synthesized a spiroopyran-based PTL. To note, in contrast to azobenzenes, the isomerization

of spiroopyrans involves the interconversion between closed- and open-ring structures, which is accompanied by a large polarity change of the molecule [55]. Thus, when this photoswitch was accurately tethered next to the pore (via an iodoacetate tether), its isomerization by light absorption resulted in a local polarity change that induced channel opening (Fig 1F) [83].

Contemporary light-gated receptors and channels

The early developers of the method have provided the blueprint and building blocks for the development of the approach, which researchers are still using to date to devise more sophisticated photoswitches and light-gating mechanisms. Of the different light-sensitive cores (for detailed list see [55]) and protein tethering methods available [85], azobenzenes and maleimides are used most in the synthesis of PCL and PTL photoswitches, for good reasons. Azobenzenes are easily synthesized, exhibit high photostationary states, undergo fast photoisomerization, and photobleach slowly. Analogously, maleimides are commercially available, thiol conjugation is covalent, and the reaction is moderately specific under physiological conditions (pH and temperature). From a protein-engineering point of view, maleimide conjugation is highly favorable as it requires very minimal protein modification—a single

amino acid substitution to cysteine. Even though maleimides can potentially interact with other existing cysteines, free cysteines (i.e., unconjugated and water accessible) are naturally of low abundance in proteins (< 2% [86]). Nevertheless, it should be noted that maleimides hydrolyze rapidly (tens of minutes) in aqueous conditions, offering a narrow time-window for labeling proteins expressed on the membranes of live cells. Thus, as cysteine conjugation will mostly occur in the first hour after addition of the maleimide-PTL, this process will label only the receptors located at the membrane during that time. This could be both a curse and a blessing (see Box 1). Lastly, the use of maleimide-PTLs requires ectopic expression of the modified, cysteine-substituted protein. Despite the benefit of genetic-targeting (e.g., Fig 2B, a single highlighted cell), expression of ectopic genes can result in unwarranted overexpression of the receptors and their promiscuous cellular localizations. With these advantages and limitations in mind, below we describe several families of neuronal receptors and channels rendered photosensitive by either azobenzene-based PCLs or PTLs. We provide several examples where PCLs and PTLs target the same receptor, with the intent to showcase the alternatives the approaches offer, such as remaining under physiological conditions or enabling genetic-targeting of the tool. We regret that owing to space constraints, we cannot list all available examples.

GABA receptors

GABA is the major inhibitory neurotransmitter of the brain. It binds to ionotropic GABA_A or metabotropic GABA_B receptors found at the membrane of neurons. When activated, GABA_A receptors open and negatively charged chloride ions flow into the cell and inhibit neuronal firing [87]. The importance and variety of these receptors, and lack of specific pharmacology, has motivated several groups to tackle activation or antagonism of these receptors by light.

A couple of examples have shown that GABA_A receptors can be photoregulated by azobenzene-based PCLs employing propofols, compounds known to potentiate GABA_A currents, denoted azo-propofols [88,89]. For example, in the report by Stein *et al*, azo-propofol in *-trans* potentiates the GABA-induced currents by almost twofold, and this effect is fully reversed by near-UV light. Notably, azo-propofols interact with unmodified GABA_A receptors; thus, with restricted light illumination, these enable to map the localization of the receptors within neurons.

Examples of tethered photoswitches used with modified GABA_A receptors include a large toolbox of azobenzene-based PTLs containing various ligands as headgroups, namely muscimol, GABA, or its guanidinium analogs [90,91]. Their mechanism of action is postulated to resemble that of MAQ and SPARK (see above and Fig 1E), wherein the PTL is attached to an introduced cysteine on the receptor and the azobenzene modulates the pharmacophore's ability to physically reach its site of action on the receptor when in one conformation, but not when in the other. Notably, these photoswitches, and their cognate-modified GABA_A subunits, were the first to be used in mammalian brains *in vivo* [91], demonstrating the viability of this approach for manipulating receptors in living brains of behaving animals.

Noteworthy, the metabotropic GABA_B receptor family has not been addressed to date by the technique, though other PTL-gated

GPCRs have been described (see below). This is unfortunate, as light-gated GABA_B receptors could help unravel their unique molecular roles at the post-synapse, where they are suggested to modulate the function of ionotropic glutamate receptors, for example by reducing calcium permeability through NMDA receptors [92]. Other GABA-binding proteins have also been the target of light-aficionados. The most abundant GABA transporter in the brain, mGAT1, was recently made photoantagonizable via PCLs containing tiagabine, a potent mGAT1 inhibitor [93]. We note this, although beyond the scope of this review, as this is the first time that the synthetic optogenetic approach was applied on membrane transporters. Attractively, there are numerous other photochromic agents described in the literature that could, in principle, target GABA-binding proteins, but to date remain untested [94]. These are ready for the picking by laboratories interested in studying GABA-mediated synaptic processes by light.

Glutamate receptors

On the other side of the spectrum, there is glutamate. Glutamate is the major excitatory neurotransmitter that activates members of the glutamate-receptor superfamily, which includes 18 ionotropic receptors [95] and eight GPCRs [96]. These receptors are expressed at pre-, post-, and extra-synaptic regions, regulate essential processes in the brain, spinal cord, and peripheral nervous system, and are implicated in a myriad of diseases [97,98]. A large repertoire of pharmacological agents has been developed to probe the roles of glutamate receptors, but these agents attend to but a few receptor subtypes [95].

To address some of the limitations of pharmacological agents and of other methods used to study glutamate receptors, numerous synthetic tools have been developed, with the light-gated kainate receptor—LiGluR—as the prominent representative of this group (e.g., [99–108]). LiGluR tethers L-MAG (maleimide-azobenzene with a 4'L stereochemistry glutamate) by a cysteine introduced into the receptor's LBD (Fig 1E) [106]. As noted above, this residue was located by analyzing the structures of several LBDs of kainate receptors (e.g., Fig 3 and [109]). In particular, it is located at the surface of the protein and next to the opening of the LBD. To note, here the photoswitch does not interact directly with the pore of the receptor (as in SPARK, compare Fig 1D and E), but rather with allosteric elements of the receptor, explicitly the LBD. When bound at this site, the elongated *-trans* form of MAG does not allow the glutamate headgroup to reach into the glutamate-binding pocket located deep within the LBD. Isomerization to *-cis* by near-UV light propels the glutamate end into this groove, enabling LBD closure and, subsequently, channel opening (Fig 1E). Green-light illumination isomerizes L-MAG back to *-trans*, pulling the glutamate away from the receptor and triggering channel closure. LiGluR was initially expressed in neurons for the purpose of driving neuronal excitability *in vitro* [105] and extended to *in vivo* studies more recently [103], further validating the technique's applicability in the live mammalian brain. LiGluR was also used to study the desensitization process of the kainate receptor, showing the utility of the tool for biophysical studies of the receptor [110]. In the degenerating retina, LiGluR was expressed in RGCs where, following *in vivo* injections of L-MAG

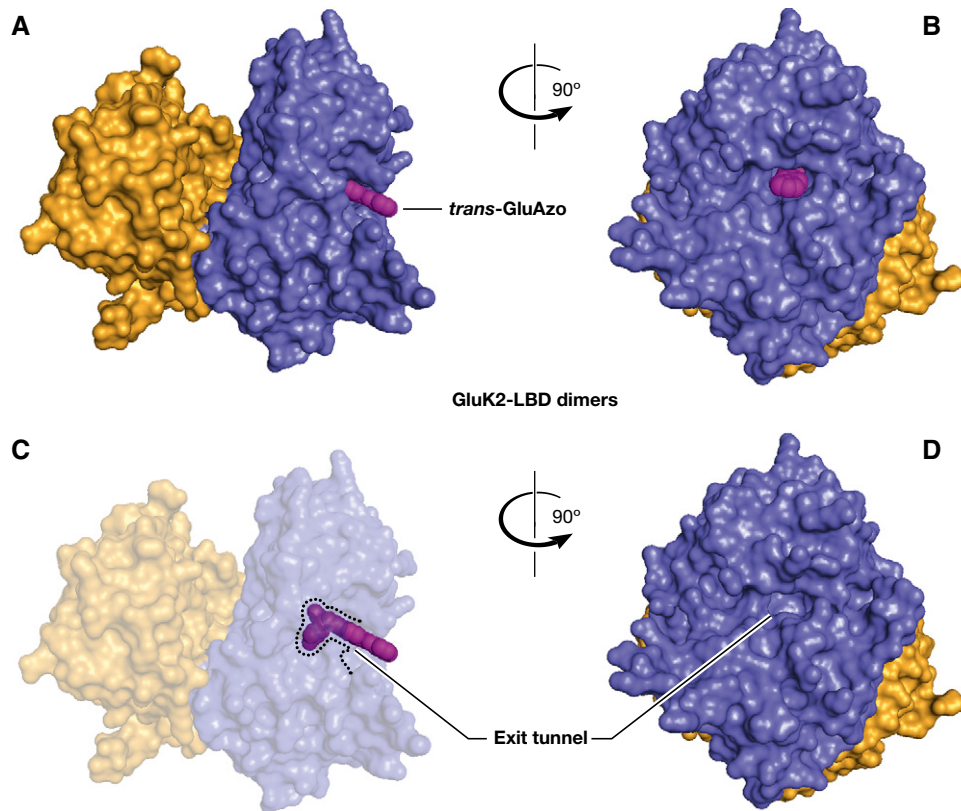


Figure 3. Crystal structure of the LBD of a kainate receptor bound to a photoswitch.

(A) Surface representation of GluK2-LBD dimers. One LBD (orange) is bound to domoate (not shown), whereas the second LBD (blue) is in complex with GluAzo (magenta), a specific iGluR5 and -6 PCL (structure is from PDB: 4H8I). The structure shows a closed LBD conformation. (B) Front view of the LBD with the tip of the azobenzene tail protruding from the LBD. (C) Transparent view of the dimer, depicting the partially embedded GluAzo photoswitch within the LBD (dashed line) and the exit tunnel through which the azobenzene protrudes. (D) Front view of the LBD (GluAzo removed).

into the retina, the now light-responsive LiGluR restored light sensitivity to the retina [100,102]. LiGluR has also been expressed in non-neuronal cells, namely astrocytes, to stimulate glutamate exocytosis owing to its relatively high Ca^{2+} -permeability [104].

We have recently described photoswitchable NMDA receptors (LiGluNs) [111]. We created a small library of GluN-subunits bearing cysteine mutations to tether L-MAGs, with a similar engineering logic as described for LiGluR, culminating in several photoswitchable NMDAR subunits. This process allowed us to photoagonize and photoantagonize the NMDA current [52,111]. The difference in activity was obtained by tethering L-MAG at different locations at the LBD of the subunit. At one position, when in *-cis*, the glutamate headgroup could properly dock within the glutamate-binding pocket resulting in proper clamshell closure and channel opening. However, when the photoswitch was tethered to another residue, even if only one amino acid away, the isomerization of L-MAG would incorrectly position the glutamate headgroup in the pocket, leading to the obstruction of clamshell closure and channel opening. This obstruction persisted in the presence of soluble agonists, acting as a non-competitive antagonist [111]. We have also used the glutamate-based L-MAG photoswitch to photoantagonize the glycine-binding GluN1 subunit. In this instance, the glutamate

moiety of L-MAG acts as an antagonist when positioned in the glycine-binding pocket, as it prevents the binding of the native ligand glycine. These examples display the versatility of the method and demonstrate that a single photoswitch can be used with multiple subunits of the glutamate-receptor family, even if those bind a different ligand than the one used in the photoswitch (e.g., glutamate and glycine).

To obtain modest expression levels of LiGluNs at the PM, we have taken advantage of the fact that GluN2 subunits are retained at the ER, unless bound to the GluN1 subunit. In this manner, the expression levels of LiGluN2 subunits are effectively controlled by the availability of the endogenous pool of GluN1 subunits in the neuron.

A unique example of a light-gated ionotropic glutamate receptor has been described by Janovjak *et al* [112]. The authors generated HyLighter (short for hyperpolarizing light-gated glutamate receptor), a chimeric receptor containing the LBD of the mammalian iGluR6 and the membrane-spanning domains (including the pore) of sGluR0, a prokaryotic homolog of mammalian iGluRs from cyanobacteria [113]. The interesting feature of sGluR0 is that, unlike all other glutamate receptors, it holds the signature of the selectivity filter of K^+ channels (GYG motif [114]) and, thereby, solely

conducts K^+ ions. Thus, when HyLighter was bound to L-MAG, near-UV illumination would open the channel and hyperpolarize the neuron due to K^+ -ion outflow.

Multiple light-gated metabotropic glutamate receptors (LimGluRs) have been engineered [115–119]. Levitz *et al* show that photocontrol over their LimGluR version requires 4'D stereochemistry of MAG, as opposed to 4'L (as for LiGluR and LiGluN). Broichhagen *et al* show that LimGluRs could support the fusion of a SNAP-tag [120] to tether a benzylguanine-based photoswitch, denoted BGAG [121]. Whereas the SNAP-tagging strategy overcomes several limitations of the maleimide chemistry, namely its short viable lifetime and potential off-target cysteines conjugation (see Box 1), it requires the incorporation of a large protein domain (SNAP, ~20 kDa) into the receptor, which might be more detrimental to the protein's function than a single amino acid substitution.

Photoswitchable tethered ligands, though extremely useful, require genetically modified channels that need to be ectopically expressed (Fig 2B, plasmid). Overexpression of ion channels may limit their usage in certain contexts and preparations (see Box 1). To bypass this drawback, and still make use of PTLs, Izquierdo-Serra *et al* devised a method to tether photoswitches to specific endogenous glutamate receptors without the necessity of cysteine mutagenesis. This was achieved by synthesizing a series of new photoswitches—targeted covalent photoswitches (TCPs)—containing a short-lived, highly reactive anchoring group, such as epoxide and N-hydroxysuccinimide esters [122]. These anchoring groups covalently react with lysine residues exposed on the protein's surface. The ligand headgroup of the photoswitch affords a kinetically controlled site-selective conjugation specifically in the vicinity of the LBD, ensuring target specificity (by affinity labeling [123]). Synonymously, there are photoswitchable affinity labels (PALs), which are conceptually identical to TCPs (some PALs even include epoxide as do TCPs) in the sense that they are also tethered to native channels by affinity labeling [124,125].

In sharp contrast, several soluble PCLs have been engineered to selectively target ionotropic glutamate receptors. For instance, GluAzo is a photoagonizing PCL specific to iGluR5 and -6 (Fig 3) [126]. Laprell *et al* [127] designed a similar caged photoswitch to non-specifically activate all types of NMDA receptors, termed ATG. Lastly, several potent PCLs that photoregulate AMPA receptors by a similar “uncaging” mechanism have also been synthesized, for example, ATA and ShuBQX-3 [128,129]. Importantly, while the ON photoswitching of PCLs can be compared to the photolysis of “conventional” caged compounds, PCLs can be turned OFF reversibly, contrasting the irreversible photolysis of caged drugs (see [130]).

Recently, the Trauner and Schiefner groups succeeded in crystallizing a kainate receptor's LBD bound to the GluAzo photoswitch (Fig 3) [131]. The structures show that the interactions of the glutamate moiety of GluAzo with its binding pocket are essentially the same as with soluble glutamate. In addition, the structure highlights how the LBD can assume a closed conformation with the photoswitch by allowing the azobenzene part of the molecule to protrude from the LBD via an exit tunnel only when in *-trans* (Fig 3A and B). Thus, because this cavity cannot accommodate the *-cis* isomer, it explains why only *trans*-GluAzo can induce channel opening. Whereas soluble glutamate is almost entirely buried within the LBD

after clamshell closure, GluAzo is not and results in a slightly more open LBD conformation (Fig 3C and D). This further elucidates why GluAzo acts as a partial agonist, as the degree of LBD closure correlates with the extent of channel activation [132,133]. Together, the structures validate many assumptions made in the design of light-gated glutamate receptors and should further assist in the design of better kainate receptor photoswitches, as well as of photoswitches for other glutamate receptors.

Potassium channels

The K^+ channel family is one of the largest ion channel families and the most widely distributed among organisms, consisting of more than 50 genes, found in both excitable and non-excitable cells [114]. Their roles are diverse (e.g., they control heart rate, insulin secretion, electrolyte transport, and cell volume regulation), but with respect to neuronal physiology, K^+ channels are fundamentally responsible for setting membrane potential, decreasing excitability, and shaping action potentials [134,135].

K^+ channels can be divided into several subfamilies, namely voltage-gated, calcium-gated, inward rectifying, and two-pore (2P) channels [136]. Many of these have been targeted by PCLs or subjected to genetic manipulation to tether PTLs. In general, most synthetic optogenetic tools based on K^+ channels have been developed for hyperpolarizing neuronal membranes, with a few exceptions. As briefly mentioned above, SPARK is a genetically modified channel based on the *Drosophila Shaker* potassium channel, belonging to the voltage-gated family of channels (K_v). SPARK, when bound to MAQ, was initially designed to hyperpolarize neurons (H-SPARK) [77]. However, SPARK was also further mutated and converted into a non-selective cation channel (as opposed to the exclusive K^+ conductance of *Shaker*) to depolarize neurons (D-SPARK) [137]. Similarly, several other potassium channels were designed to tether MAQ or MAQ-like PTLs to control channel gating and membrane excitability, such as $K_v1.3$, $K_v3.1$, $K_v7.2$, and the Ca^{2+} -activated SK2 channel (Fig 2) [125]. This long list of modified light-gated K^+ channels employing the same photoswitch, and likely photogating mechanism (i.e., channel block by the QA moiety), demonstrates once more the transferability of the photoswitch between members of the same family. Moreover, the MAQ photoswitch was even applied to photogate other ion channels (e.g., Ca^{2+} and Na^+ channels [138]). This is possible with photoswitches that are based on pharmacophores with non-specific and broad activity such as QX-314. Together, these demonstrations display the versatility of the technique.

The Kramer group (UC Berkeley) has extensively explored the photoregulation of endogenous K^+ channels by a large repertoire of PCLs and PALs ([124,139–141], for an extensive list see [138]). Of the many examples, we would like to highlight one interesting case involving a photoswitch denoted AAQ (acrylamide–azobenzene–QA). AAQ includes an acrylamide tethering group intended to react (to tether the photoswitch) with native extracellular residues located near the pore of the channels [124]. Indeed, Fortin *et al* found that AAQ photoregulated the activity of K^+ channels, presumably from the extracellular side of the membrane, meriting its classification as a PAL. Surprisingly, it was later discovered that the hydrophobic acrylamide group facilitated permeation of AAQ into the cell, before

it could react with external residues (Fig 4A) [139]. Thus, AAQ is a membrane-permeable PCL and its photoregulation mechanism involves its entry into the inner vestibule of the channel when in *-trans*, but not when in *-cis*. Once in the vestibule, the QA head-group exerts its block (Fig 4A). Though given lemons, the group was able to make lemonade from the AAQ misfortune by using these observations for the development of new hydrophobic photoswitches (e.g., BzAQ, PrAQ) that acted from the intracellular side of the membrane to block voltage-gated potassium channels, but also voltage-gated calcium and sodium channels [138,139].

Finally, 2P K^+ channels and inward rectifiers (K_{ir}) have not escaped the attention of photoengineers. Briefly, to photocontrol endogenous 2P-TREK channels with the MAQ PTL, Sandoz *et al* devised a conditional expression scheme. To this end, the authors created a conditional TREK subunit bearing a cysteine and a truncated carboxy-terminus (Δ CT), required for ER export of the subunit when intact. Thus, only when the Δ CT-subunit dimerized with a *wt* subunit (endogenously present in the ER of the neuron) could the heteromeric channel complex exit the ER and reach the PM [142,143]. To open the GIRK channel, belonging to the family of inward rectifiers [144], the Trauner group designed a PCL denoted LOGO [145,146]. LOGO is an interesting PCL consisting of an azobenzene-based photoswitch containing VU0259369, a potent and selective GIRK1 channel activator [147]. Whereas GIRK channels are uniquely opened by the binding of $G\beta\gamma$ molecules derived from activated $G\alpha_i$ -coupled GPCRs [148], VU0259369, and hence LOGO, can open the GIRK1-containing channels even when the G-protein cycle is disrupted by PTX. This photoswitch can thereby bypass the need to activate GPCRs to elucidate the gating mechanisms of the GIRK channel in neurons.

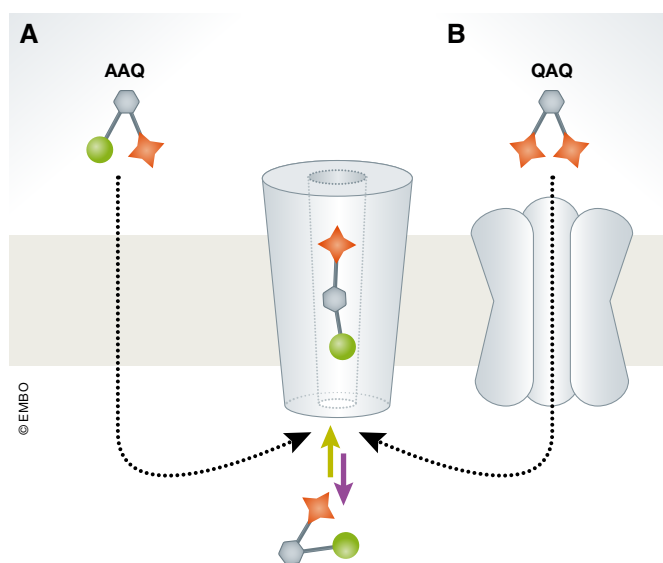


Figure 4. Intracellular photoswitchable blockers.

(A) AAQ (acrylamide-azobenzene-QA) crosses the membrane owing to its lipophilic acrylamide group, can enter the inner vestibule of the channel, and exert its block when in *-trans*. (B) The blocking mechanism of QAQ (QA-azobenzene-QA) resembles that of AAQ. However, QAQ cannot cross the membrane and needs to travel through pore-dilated channels (e.g., TRPV1) to enter the cell.

P2X receptors

P2X receptors are trimeric, ATP-binding cation channels [149]. With regard to the brain, P2X receptors are implicated in several diseases, such as neuropathic pain [150]. To photogate P2X receptors, the team of Grutter has employed a unique strategy consisting of using charge to induce channel opening, reminiscent of the strategy employed by Kocer *et al* [83]. To this end, the authors have synthesized a maleimide-azobenzene-based PTL comprising positively charged trimethyl ammonium derivatives at the end of the molecule [151]. When the PTL was tethered to cysteines at different TM domains, light-dependent isomerization induced changes in the local charge that was sensed by local residues of the TMs and induced channel opening or closing, depending on the tethering location. With the use of this approach, the authors describe a unique ATP-independent gating mechanism of P2X receptors. Accordingly, the authors suggest the name of opto-gating to describe this process, as the photoswitchable reagents do not include the ATP ligand, nor target any particular active or known allosteric sites of the receptors [151]. The same group next developed a new type of photoswitch they name photoswitchable tweezers (PXs). PXs are closely related to PTLs, but instead of tethering a single cysteine residue, they tether two cysteine residues resembling molecular rulers [152]. However, unlike the latter, PXs are capable of pulling or pushing on gating elements of the receptor during isomerization (Fig 1J) [153]. With these, the authors induce forceps-like motion to open the pore of the P2X channels, resembling the motion of a camera's iris.

Nicotinic acetylcholine receptors

Acetylcholine (ACh) is an important neurotransmitter in both central and peripheral nervous systems (CNS and PNS, respectively). Its actions are mediated by two types of receptors—the G-protein-coupled muscarinic receptor (mAChR) and the nicotinic AChRs (nAChRs) [154]. nAChRs function as non-selective, excitatory cation channels with roles in the sympathetic and parasympathetic nervous system, and in skeletal muscle, they mediate contraction. In the brain, however, their roles are much less understood. They are found throughout the neuron's membrane with no obvious concentration at synaptic junctions. In fact, a large proportion of receptors can be found at nerve terminals. These receptors are considered neuromodulatory agents for neurotransmitter release and are implicated in numerous diseases [154,155].

As noted above, the history of synthetic optogenetics intimately involves these receptors (e.g., PCL-Bis-Q [65,70] and PTL-Q-Br [70,156] and see above). However, Bis-Q was found to be a muscle-type photoagonist [157], and Q-Br could not be targeted to defined subunits, as it conjugated native cysteines located next to the pore of the channel, extant in several subunits [70]. Present studies have now elaborated on these earlier reports and produced several light-gated nAChRs via azobenzene-based PCLs and PTLs. Azocholine (azobenzene-choline) is a photoagonizing PCL that targets and activates $\alpha 7$ -type nAChRs [157]. Two genetically modified β subunits (2 and 4) have been engineered to covalently bind the photoactivating PTL, MAACH (maleimide-azobenzene-acylcholine), or the deactivating PTL, MAHoCh (maleimide-azobenzene-homocholine). The

latter enable to photocontrol unique $\beta 2/4$ -containing nAChRs receptors, denoted LinAChRs [158].

Next-generation photoswitches

Spatially confine photomanipulation of light-gated receptors located deep within scattering tissue is best achieved by 2PE [159]. Whereas 2PE is readily used for photolysis of caged compounds [160–162] or for the photoactivation of photoreceptor-based optogenetic tools (e.g., [163–168]), the activation of synthetic optogenetic tools by 2PE is far behind. This stems from the azobenzene's poor 2P absorption properties, owing to the molecule's symmetry [169]. Among the first to tackle this issue was the group of Gorostiza Pau. In order to increase the absorption cross section of the L-MAG photoswitch, the authors modified it to increase its push–pull characteristics or introduced light-sensitive antennae to sensitize the isomerization process by resonance. These modifications yielded MAG_{2p} and MAGA_{2p}, respectively [170]. The authors then used 2PE to photoactivate LiGluR in cultured neurons and astrocytes. Comparably, we have used a red-shifted MAG photoswitch, denoted L- and D-MAG₄₆₀ [171], to photoactivate LiGluR or LimGluRs, respectively, in neurons [101]. Additional 2P-compatible photoswitches have also been reported (e.g., [119,127]), showing that the field is steadily transitioning in the direction of 2PE, which is more suitable for *in vivo* applications. Notably, the transition from a 2PE-incompatible to 2PE-compatible photoswitch requires the design of an entirely new photoswitch by methods as described above. This may result in an altered or non-functional photoswitch. Therefore, as the number of 2PE-incompatible photoswitches exceeds, by far, that of compatible ones, it will take some time before we see photoswitches with revisited 2PE-compatible design.

Bringing synthetic optogenetics to the clinic

Given that the use of photoswitches for clinical applications depends on the ability to deliver light into the tissue, it is not surprising that most of the translational progress made with synthetic optogenetics has mainly focused on the visual system [100,102,172–174]. Collectively, these reports show that a variety of photoswitches and modified receptors can endow surviving neurons of the degenerating retina with sensitivity to environmental light and partially restore visual function to blind mice (see review in [175]). However, there are still several hurdles to pass before this treatment transitions from bench to bedside, such as toxicity and solubility issues (see Box 1).

Another potentially relevant clinical application of photoswitches pertains to chronic pain. The Kramer group has developed a cell-impermeant PCL named QAQ, containing two QA groups spanning the azobenzene. QAQ enables photosilencing of neurons by non-specifically inhibiting several voltage-gated channels (Na_v, K_v, and Ca_v channels [176]). Interestingly, QAQ exerts its block from the intracellular side of the membrane. To reach the intracellular, QAQ must enter the cells via a membrane conduit, such as the pore-dilated TRPV1 channel—the major heat sensors in nociceptor neurons—that allows permeation of large and charged molecules

Box 1: In need of answers

- (i) Labeling efficiency—Whereas naturally occurring photoreceptors are found at the membrane at a 1:1 stoichiometry with their chromophores, synthetic optogenetics tools are not. Following expression of modified receptors and incubation with the relevant PTL, it is difficult to determine the number of modified receptors that have been labeled by the PTL. Labeling efficiency will determine the effectiveness of the tool. The extent of labeling can be approximated by equating the photocurrent with the maximal ligand-induced current. However, this method does not comply with photoswitches that exhibit partial agonism (e.g., GluAzo [131]). As a result, methods to increase labeling efficiency, means to measure it, and alternative labeling schemes are still under development.
- (ii) Narrow labeling time—Maleimides hydrolyze rapidly (tens of minutes). This presents the user with a narrow time-window, during which the photoswitch would need to be applied as close as possible to the site expressing the receptors. For prolonged *in vivo* experiments, this is limiting as the photoswitch may need to be reapplied during the course of the experiment to maintain a constant amount of labeled receptors. This is cumbersome and possibly damaging, as it entails multiple applications of the photoswitch. Photoswitches with better stability would broaden the labeling time-window and would also allow the user to apply them at a larger distance from the site of expression. This type of photoswitch could also be slowly and steadily administered to the brain via an implanted cannula, lessening the burden of multiple injections.
- (iii) Solubility—Azobenzene are lipophilic compounds, typically solubilized in DMSO prior their application onto the preparation [180]. Even more challenging are azobenzenes further decorated with lipophilic groups (such as fatty acids, e.g., [181]). Therefore, photoswitches with improved solubility are still under pursuit [182].
- (iv) Near-UV absorption—Most azobenzene-based photoswitches absorb near-UV (< 380 nm, see Fig 1B), to isomerize from *–trans* to *–cis*, wavelengths that may be harmful to cells. This justifies the synthesis of red-shifted photoswitches. Indeed, several red-shifted photoswitches have been synthesized (e.g., MAG₄₆₀ [171]), though this shift comes at the expense of bistability, resulting in the spontaneous relaxation of the photoswitch back to *–trans*. This may lower the photostationary population of the *–cis* isomers and would require longer or repetitive illumination to obtain continuous photoresponses. Therefore, better red-shifted photoswitches that maintain excellent switching behavior are necessary.
- (v) Broad absorption—Azobenzenes exhibit broad absorption, in particular the *–cis* isomer (Fig 1B [55]). This complicates the use of photoswitches with additional optical tools (such as fluorescent proteins and probes), because the photoswitch will be isomerized back to *–trans* during the imaging of the probes. Thus, narrower absorbing photoswitches are needed to achieve all-optical interrogation of cells (see [183]).
- (vi) Clinical use—Whereas several research groups have shown the utility of photoswitches *in vivo* (e.g., fish and rodents), there are still questions pertaining to the safety of the use of azobenzene-based photoswitches. This concern stems from their poor solubility and metabolic stability, both of which may lead to potential long-term toxicity.
- (vii) Availability—Most photoswitches are not commercially available, rather are typically obtained directly from the research groups. There are few exceptions, such as several MAG variants sold by Aspira Scientific (www.aspirasci.com). The lack of a chemical depository hinders the broad dissemination of the photoswitches. Unlike DNA depositories (e.g., Addgene), the synthesis process is much more expensive and laborious.

such as QAQ (Fig 4B). Because pathological pain is characterized by hyperactivity of nociceptor neurons (which may also exhibit increased activity of TRPV1), QAQ can thereby selectively enter hyperactive nociceptors to silence them. While the pore dilation mechanism is currently under debate [177], this mechanism has been established for a lidocaine-related anesthetic, QX-314 [178].

As a therapy for cancer, a new class of photoswitches—photostatins—has recently emerged. These PCLs bind to and interfere with microtubule dynamics, inhibit mitosis, and promote apoptosis [179]. These photoswitches are based on combretastatin A-4, a potential chemotherapeutic drug in clinical trials. The photoswitch consists of a fragmented drug, whose parts span the azobenzene-core. Following isomerization to *-cis*, the two fragments are combined, yielding the final active form of the drug (Fig 1I).

Conclusion

Synthetic optogenetics was once considered a highly specialized technique, benefitting but a handful of laboratories. Today, however, the technique has become more approachable, judging by the growing number of laboratories across the world employing and developing the technique. To date, the variety of tools makes it possible to explore the role and signaling mechanisms of numerous receptors subtypes, in their natural environment, *in vitro* as well as *in vivo*. Nevertheless, there are still several hurdles to cross before synthetic optogenetics becomes widely accepted and clinically used (see Box 1). Luckily, major steps have been taken in that direction, with better photoswitches and gating mechanisms increasingly appearing in the literature.

It is important to recognize that whereas photoreceptor-based optogenetic tools are unsurpassed for controlling mechanisms such as cellular excitability, synthetic optogenetic tools are more suited for controlling native receptor function or for modulating endogenous signaling mechanisms. In fact, the two techniques are highly complementary. For example, ChR2 and LiGluNs could be used concomitantly at the synapse to induce LTP. In such an instance, photoactivation of ChR2 would be required to depolarize the synapse and relieve the Mg^{2+} -block from LiGluNs. Then, precisely timed photoactivation of LiGluN would induce robust Ca^{2+} -entry, leading to LTP at the synapse.

Nevertheless, despite the numerous photoswitches produced, synthetic optogenetics is still not as broadly employed by the neuroscience community, especially when compared with opsin-based optogenetic tools. One of the main reasons for this situation is the lack of commercial availability or absence of a chemical depository (but see exceptions in Box 1). It is unlikely that chemical companies will start synthesizing photoswitches for several reasons: the arduous and expensive synthesis process, low demand, therefore narrow profit margin, and patent issues. Secondly, though a chemical depository would certainly help for research groups to gain access to the compounds, such a chemical bank would need to eventually synthesize the photoswitches in-house, which is expensive and thus highly unlikely. To date, the most common practice is to directly obtain photoswitches from the developing groups, despite potential conflicts of interests or competitions. In fact, as far as we are aware, most research groups are open to distribute their photoswitches and/or start collaborations.

Taken together, we expect the technique to be further perfected and optimized and hope to see many more exciting developments in the near future, such as novel light-gated receptors, multiphoton-compatible switches applied *in vivo*, and perhaps even acoustic and magneto-switches.

Conflict of interest

The authors declare that they have no conflict of interest.

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