Light-induced regulation of ligand-gated channel activity

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Abstract

The control of ligand-gated receptors with light using photochromic compounds has evolved from the first handcrafted examples to truly engineered receptors whose development is supported on rational design, high-resolution protein structures, comparative pharmacology and molecular biology manipulations. Photoswitchable regulators have been designed and characterized for a large number of ligand-gated receptors in the mammalian nervous system, including nicotinic acetylcholine, glutamate and gamma amino butyric acid receptors. They conform a well-equipped toolbox to investigate synaptic and neuronal circuits in all-optical experiments. This focused review discusses the design and obtained properties of these photoswitches, their applications and shortcomings, and future perspectives of the field.

Abbreviations: **AMPAR** – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; **AP2** – azobenzene-propofol; **ATA** – azobenzene-tetrazolyl-AMPA; **azo-CarCh** - N-p-phenylazophenyl-N-phenylcarbamylcholine; **azo-PTA** – p-phenyl-azophenyl-trimethylammonium chloride; **GABAR** – gamma-aminobutyric acid receptor; **GluAzo** – glutamate-azobenzene; **IR** - infrared; **LiGABAR** – light-activated gamma-aminobutyric acid receptor; **LiGluR** – light-activated ionotropic glutamate receptor; **MAG** – maleimide-azobenzene-glutamate; **MAB** – maleimide-azobenzene-4-hydroxybenzylamine; **MAM** – maleimide-azobenzene-muscimol; **nAChR** - nicotinic acetylcholine receptor; **NMDAR** – N-methyl-D-aspartate receptor; **PCL** - photochromic ligand; **PTL**- photochromic tethered ligands; **TCPs** - targeted covalent photoswitches; **UV** – ultraviolet.

Introduction

Starting with Antoni van Leeuwenhoek and following highly important contributions of Camillo Golgi and Santiago Ramón y Cajal, optical methods are embedded at the heart of scientific research and are widely used in elucidating the morphology and functioning of different cell types, as well as determining principles of the organization of biological organisms. Rapid progress of molecular biology, fluorescent microscopy in combination with the use of genetically encoded sensors significantly expanded the possibilities of optical studies. The development of methods for the specific integration of proteins in certain cell types, as well as the detection of light-sensitive proteins, stimulated the explosion in the areas for remote control of cellular activity with high precision and specificity.

As a result, in recent years, new areas, namely optogenetics, optopharmacology/photopharmacology, optogenetic pharmacology have been developed (Fenno et al., 2011; Repina et al., 2017; Broichhagen et al., 2015).

The origin of optogenetics has been stimulated by cloning of the first light-gated cation-selective membrane channel, channelrhodopsin (Nagel et al., 2003) and observations that its

expression in cells results in the ability of these cells to be activated by light, i.e. change the membrane potential, generate ion currents and cause light-evoked firing (Nagel et al., 2003; Boyden et al., 2005). Moreover, it was found that integration of the photosensitive proteins in neurons of multicellular organisms allows changing their behaviour upon application of light (Lima et al., 2005). Thus, it become evident that bacterial light-sensitive proteins represent rather simple and easy to use tools for rapid control of cell excitability and neural networks function. New light-sensitive proteins and their derivatives were embedded in cells of different species of animals from worms and insects to monkeys (Diester et al., 2011; Fenno et al., 2011; Han et al., 2011; Gerits et al., 2012; Welberg, 2012) and in human pluripotent cells (Busskamp et al., 2010; Steinbeck et al., 2015).

Controlling the activity of cells with the help of light one can investigate their function (Tye and Deisseroth, 2012), measure the concentration of ions (Bregestovski et al. 2009), ATP (Imamura et al., 2009; Berg et al., 2009) and other cellular components (Marvin et al., 2013; Bilan et al., 2013), to control the behaviour of organisms (Covington et al., 2010; Haubensak et al., 2010; Miesenbock, 2011), as well as to seek for novel ways to treat certain diseases (Laprell et al., 2015; Rossi et al., 2015). Optogenetic approaches were used in many models with medical orientation, including the study of stress (Covington et al., 2010), schizophrenia, memory disorders, drug addiction, psychiatry and motor functions (Rossi et al., 2015); vision, pain, functional recovery after stroke and epilepsy (Tønnesen et al., 2009; Wagner et al, 2015; Gaub et al, 2015). However, one of the critical limitations of the optogenetics is the necessity to integrate foreign genes into organisms using viral gene therapy or development of transgenic animals.

In parallel to optogenetics, in recent years, photopharmacology or optopharmacology - a direction based on the creation of chemical compounds capable of controlling the functions of biological molecules with the help of chemical photosensitive switches - has greatly developed (Kramer et al., 2013). Several classes of photochromic pharmacological compounds have already been successfully used to selectively modulate the activity of various proteins, including enzymes (Harvey and Abell, 2001), receptor-driven (Szobota et al., 2007; Tochitsky et al, 2012) and potential-dependent ion channels (Banghart et al., 2004; 2009; Fortin et al., 2008; 2011).

While the use of some photoswitchable compounds requires mutation of target proteins, a number of improved soluble compounds with a high specificity of action has been developed that does not require genetic manipulations. It turned out that synthetic light-controlled compounds capable of enhancing or inhibiting the activity of key cellular proteins, are powerful tools for non-invasive control of cellular activity, organs functioning and the behavior of living organisms.

In this mini-review we will focus on the control of ligand-gated ion channels by using light-sensitive molecules or photoswitches. In particular, we will introduce the early developments in

the photocontrol of cholinergic receptors of the neuromuscular junction, and more recent progress with the main excitatory and inhibitory receptor-channels in the vertebrate nervous system (glutamate and GABA, respectively). Wider scope reviews including voltage-gated, transient receptor potential and trimeric receptor ion channels have been recently published elsewhere (Bautista-Barrufet et al., 2014; McKenzie et al., 2015).

In general, photoswitches can be divided on two main classes: (i) those acting as soluble photochromic ligands (PCL); (ii) those being covalently tethered to the target protein (PTL- photochromic tethered ligands). Each class has its own advantages and limitations.

PCLs are convenient and simple to use in endogenous receptors and they do not require molecular modification (e.g. mutagenesis). However, it is difficult to obtain highly specific PCLs, as many receptor proteins show high similarity in ligand binding sites, which are conserved in most cases.

The PTL strategy allows controlling the activity of voltage-gated or receptor-operated ion channels due to irreversible tethering of PTLs to the proteins, often targeted at cysteine residues, either naturally occurring or genetically introduced (Lester et al., 1980; Gorostiza and Isacoff, 2007; 2008; Kramer et al., 2013). In general, this strategy requires both (i) chemical synthesis of PTL compounds and (ii) mutagenesis of the target protein to identify a suitable tethering site for optimal photoswitching. It offers the advantage of activating or inhibiting only this specific receptor or ion channel mutant. On the other side, because of the need in some cases of mutating target proteins, it poses limitations and difficulties to be used in experimental models *in vivo*, for instance, testing on humans.

Photoswitches (both PCLs and PTLs) are chemically synthesized molecules containing at least two components: a ligand molecule (agonist, antagonist or ion channel blocker) and a photoisomerisable group. PTLs, in addition, have a reactive group for irreversible tethering to the target protein (Fig.1).

The synthetic photoswitch that is most extensively used for channel applications is azobenzene (Fig.1,A), a molecule, which undergoes *cis-trans*-isomerization around the central double N=N bond (see rev Gorostiza and Isacoff, 2007; McKenzie et al., 2015). In the dark or under visible light azobenzene is in an extended *trans* configuration. Irradiation with near-ultraviolet (UV) light (360-380 nm) induces a change from *trans* to *cis* configuration, which shortens the molecule by about 0.6 nm. Visible light switches the azobenzene back to the *trans* form (Fig.1, A). Isomerisation of azobenzene occurs in picoseconds upon absorption of an UV photon (Bortolus and Monti, 1979) and this permits high-speed switching of many azobenzene-based molecules using bright light. Thermal back-relaxation lifetimes range between milliseconds

and days and can be adjusted by synthetic design according to application requirements (Velema et al., 2014).

Control of nicotinic acetylcholine receptor with light

Nicotinic acetylcholine receptor (nAChR) was the first light-modulated receptor-operated channel. Almost 50 years ago Erlanger and co-authors (Deal et al., 1969) introduced azobenzene-based photochromic ligands for regulating the activity of nAChR. Firstly the team demonstrated the capability of azobenzene-based compounds as photochromic regulators of enzymatic activity of chymotrypsin (Kaufman et al., 1968) and acetylcholinesterase (Bieth et al., 1969), and then extended the idea to the light-induced regulation of the excitability of the electroplax preparation of *Electrophorus electricus*. They used N-p-phenylazophenyl-N-phenylcarbamylcholine chloride (azo-CarCh) and p-phenyl-azophenyl-trimethylammonium chloride (azo-PTA) as light sensitive antagonists of acetylcholine receptor (Deal et al. 1969). This pioneer study was the first demonstration of the use of photochromic compounds to regulate the activity of cys-loop receptor channels.

During these years, both classes of photoswitches (PCL and PTL) for modulation function of nAChR have been developed. They represent Bis-Q, compound whose two ligand moieties can bind reversibly to the receptor, and tethered QBr, which can be covalently linked to the native sulfhydryl groups in the vicinity of acetylcholine binding site (Fig. 1, B). Irreversible targeting could be achieved after the treatment of receptors with dithiothreitol, a reducing agent that causes reduction of disulfide (S-S) bonds and exposes free thiols to reaction (Karlin, 1969; Bregestovski et al., 1977). These compounds, called "tethered" agonists were successfully used to induce light-flash relaxations and to analyse rate-limiting steps governing the opening and closing of channels (Bartels et al., 1971; Bartels-Bernal et al., 1976; Lester et al., 1979; 1980, Chabala et al. 1986).

These developments were to a large extent empirical and cumulative, but determination of the amino acid sequences and atomic structures of the channel proteins brought about the possibility of rationally designing photochromic compounds to regulate the activity of ligand- and voltage-gated channels, and a rebirth of the field in the XXI century (Banghart et al. 2004; Volgraf et al., 2006; Szoboda et al., 2007; Gorostiza et al., 2007; Stein et al., 2012; Yue et al., 2012; Damijonaitis et al., 2015a,b).

Recently, new generations of PCLs and PTLs for effective light-dependent modulation of nAChRs were proposed. Based on the X-ray structure of an acetylcholine binding protein in complex with carbamylcholine (Celie et al., 2004) and distance measurements in the protein structures, in the beta-subunit of nAChR, several positions have been identified that face the ligand-binding site as a potential region for the attachment of agonists and antagonists. This

allowed performing site directed insertions of cysteines and synthesis of appropriate PTLs.

After expressing in *Xenopus* oocytes of mutated nAChRs and labelling it with PTL agonist (MAACh) or antagonist (MAHoCh), illumination with 380 nm light produced either an inward current that could be reversed with 500 nm light (labelling with MAACh), or inhibition of acetylcholine-induced currents (labelling with MAHoCh (Fig.1, C). These PTL compounds enabled heteromeric neuronal nAChRs to be activated or inhibited with UV light, but respond normally to acetylcholine in the dark, which is important for more profound analysis of their physiological and pathological cholinergic functions (Tochitsky et al., 2012).

Recently the team of D. Trauner reported a photoswitchable agonist for neuronal α7 nAChRs, AzoCholine (Damijonaitis et al., 2015a). In heterologously expressed α7 nAChR/glycine receptor chimera in HEK293T cells, this compound was not effective upon illuminating with UV light, however it caused a large current at illumination with visible light, i.e. at transition to *trans* configuration (Fig. 1,D). AzoCholine thus activates α7 receptors in the dark, but on the other hand it displays subtype selectivity versus the muscular nAChR. Importantly, AzoCholine is a PCL compound, i.e. its application does not need molecular modification of the α7 nAChRs. AzoCholine effectively modulated neuronal activity of rat sensory neurons from dorsal root ganglia, in mouse hippocampal brain slices and it was able to perturb in a light-dependent manner swimming behaviour of *C. elegans* (Damijonaitis et al., 2015a). This demonstrates the main advantage of AzoCholine, as the other PCLs, is their ease of use for light-dependent control of cellular processes *in vitro* and *in vivo*.

This area of research now develops extremely rapidly and in various directions. Below we will discuss just some of the studies, concentrating on two main functional classes of ionotropic receptors determining synaptic excitation and inhibition of nervous system in vertebrates.

Photochromic modulators of glutamate receptors

Glutamate receptors provide the main excitatory drive in the mammalian nervous system and are involved in a large variety of physiological processes, including brain development, synaptic plasticity, memory formation, pain, excitotoxicity and neurodegenerative diseases (Gonzalez et al., 2015; Zhuo, 2017). Disorders of glutamatergic transmission lead to imbalances of inhibition-excitation and have dramatic consequences for both cellular and network functions. These receptors are among the primary targets for development of photopharmacological regulators.

The first photoswitch of glutamate receptors was engineered ten years ago (Volgraf et al., 2006; Gorostiza et al., 2007) and termed MAG to highlight its components: a cysteine-reactive maleimide group, an azobenzene photoswitch and a glutamate ligand (Fig. 2,A,a). Maleimide

allowed tethering the compound to the GluK2 kainate receptor after introducing a cysteine substitution (L439C), close to the glutamate-binding site (Fig. 2,B,a). This type of receptors was called LiGluRs – light-activated ionotropic glutamate receptors. Illumination with 380 nm light induced transition of MAG to the *cis* state, in which the glutamate head was bound to the agonist-binding site with subsequent activation (opening) of the ion channel. Back isomerisation of MAG and receptor deactivation were triggered with 500 nm light (Volgraf et al., 2006). Light pulses reliably induced depolarization and firing of neurons due to activation of GluK2 channels by MAG (Fig.1, B,b; Szobota et al., 2007).

Later the same group proposed the first nontethered photochromic agonist of iGluRs that could modulate function of wild-type receptors, GluAzo (Fig.2,A,d). This compound, representing PCL series, was based on using a potent and selective agonist of GluK1 kainate receptor chemically conjugated to an azobenzene (Volgraf et al., 2007). The photoswitchable ligand was controlled with the same wavelengths as MAG and its activity was competitively blocked by the non-NMDA receptor antagonist DNQX. In contrast to MAG, this compound activated kainate receptors in the *trans*-state and lost its activity in the *cis*-configuration induced by UV light. This PCL successfully caused light-induced modulation of depolarization in cultured hippocampal neurons from wild type rats demonstrating that the cysteine substitution in the target receptor is not required for its action (Volgraf et al., 2007).

The results of these pioneering works were further elaborated in a number of subsequent studies, demonstrating that photoregulation of glutamate receptors represent an efficient tool to control glutamatergic neurotransmission. One of the aspects to be optimised was the photoswitch action spectrum. The requirement of UV light for azobenzene isomerisation is not ideal for biological systems because (i) prolonged UV exposure can be damaging and (ii) UV light poorly penetrates mammalian tissue. To overcome this problem, synthesis of the light-sensitive azobenzene-based GluR ligand with about 100 nm redshift of the absorption has been performed (Kienzler et al., 2013). The compound, called MAG_{460} (Fig.1,A,b), can be switched into *cis*-configuration by visible light (460 nm) and rapidly return to the *trans*-state by thermal relaxation in the darkness (Fig.2). Whole-cell patch-clamp recording from HEK 293 cells expressing GluK2(439C) and incubated with the red-shifted L-$MAG0_{460}$ showed that illumination with blue light induced a large inward currents (Fig. 2, C, a). In the dark the recovery of currents was observed due to closing of the channels after transition of MAG_{460} to the *trans* configuration. Other MAG variants allowed, with slow kinetics, activation with red light (625 nm) (Rullo et al., 2014).

Recently LiGluRs have been expressed in the visual cortex of mice using an adeno-associated virus under the control of the specific promoter. In conjunction with fiber-based optogenetic technologies it has been shown that $MAG0_{460}$ can activate LiGluRs in cultured

hippocampal neurons (Fig. 2,C,b) and *in vivo* conditions increasing neuronal cell firing in mouse cortex upon blue light illumination (Levitz et al., 2016). These experiments have proven that LiGluRs-MAG technique is compatible with existing fiber-based *in vivo* light control technologies and can be used to manipulate the activity of neuronal circuitry.

Similar blue-shifted MAG derivatives were developed for the purpose of enhancing two-proton activation of the azobenzene switch using pulsed infrared (IR) light (Izquierdo-Serra et al., 2014; Gascón-Moya et al., 2015), which enables deeper penetration in tissue and focal activation in neurons and astrocytes. Two photon activation and digital holography were further used to shape stimulation patterns in three dimensions for the purpose of studying neural circuits (Carroll et al., 2015).

The development of MAG derivatives isomerised by visible light also expanded the application of LiGluRs in vision restoration research (Kienzler et al., 2013). The first attempt at using light-sensitive glutamate ligands for vision restoration has been performed with UV-modulated MAG. The gene encoding for cysteine substituted GluK2 subunit of glutamate receptor (LiGluR) was delivered to retinal ganglionic cells by intravitreal injection of adeno-associated virus (AAV), and the photoswitchable tethered ligand maleimide-azobenzene-glutamate (MAG) was delivered in a subsequent intravitreal injection. This resulted in restoration of light responses of blind retina degeneration mice (Caporale et al., 2011).

However, as mentioned above the use of UV illuminations raises some problems, particularly in the case of retina. The second generation of red-shifted LiGluR-$MAG0_{460}$ has been shown to be much more promising. Upon administration of $MAG0_{460}$ light-evoked responses in retinal ganglion cells as well as in ON-bipolar cells were recorded. Moreover, visual guided behaviour of animals was demonstrated in the functional tests in blind mouse and dog models (Gaub et al., 2014).

The structural data combined with mutagenesis and electrophysiological observations (Sobolevsky, 2015) greatly facilitated the design of efficient photoswitches for AMPA, kainate and NMDA receptors. Photoswitchable activators of AMPA receptors were developed on the basis of AMPA with azobenzene substitution and were called ATAs (azobenzene tetrazolyl AMPAs). They were proven to be potent AMPA-activators in *trans*-state, and could be used to control neuronal activity in acute cortical brain slices (Stawski et al., 2012; Reiner et al., 2015).

MAG-based ligands also enabled the photoregulation of NMDA-selective glutamate receptors. The previously described method of cysteine substitution yielded light-activated GluN2A, light-activated GluN2B, light-antagonized GluN2A and light-antagonized GluN1 subunits of NMDA receptor (Berlin et al., 2016). This model of light-controlled NMDA receptor subunits provides precise, fast and reversible remote control of specific receptor subtypes in

localized areas, modulation of excitatory synaptic currents, long-term plasticity and spine-specific regulation of intracellular calcium transients.

Variants in the reactive group of MAG derivatives have also been explored, with the aim of achieving covalent conjugation (PTL) without requiring the introduction of cysteine residues by mutagenesis, thus targeting endogenous receptors (Fig. 2,A,c; Izquierdo-Serra et al. 2016) A modular library of photoswitchable ligands and reactive groups was optimised for GluK1 and allowed identifying efficient photoswitches that covalently conjugated to a lysine residue in the receptor following an affinity labelling process. Thus they can be termed photoswitchable affinity labels (PALs) (Harvey and Trauner, 2008) or targeted covalent photoswitches (TCPs) in analogy with targeted covalent drugs, an important class of medicines including aspirin, penicillin and omeprazole. These compounds activate GluK1 under UV light and deactivate it under 500 nm illumination, providing photocontrol of untransfected neurons and restoration of the photosensitivity of degenerated retina.

Currently available photochromic modulators of glutamate receptors offer a wide choice of pharmacologic function (agonist, antagonist), selectivity (kainate, AMPA, NMDA) and optical properties (from violet to red to IR stimulation using multiphoton processes, and diverse relaxation lifetimes in the dark). In addition, it is possible to take advantage of genetic targeting using cysteine-conjugated MAG derivatives, or aim at endogenous receptors using either freely diffusible PCLs or lysine-targeted photoswitches that are conjugated by affinity. Overall, the photoswitch toolbox is well furnished to approach a systematic investigation of glutamatergic neurotransmission in the mammalian brain.

Light-induced modulation of GABA receptors

Since GABA provides the main inhibitory neurotransmission in the CNS of vertebrates, the search for specific photoswitchable regulators of GABA receptor function constitutes a very important task. Due to efforts of several teams, a rich pharmacology of optically switched ligands of $GABA_A$ receptors has been developed, including PTLs and PCLs, activators, allosteric potentiators and antagonists.

One of the first compounds that served as a basis for development of light-sensitive potentiators of $GABA_A$ receptor was propofol. This lipophilic anaesthetic has been shown to act as potentiator of GABA-induced currents (Sieghard, 1995). A propofol/azobenzene based photo-isomerisable soluble ligand MPC088 (Fig.3,Aa) was developed by the team of David Pepperberg (Yue et al., 2012). Using α1β2γ2 $GABA_A$Rs expressed in *Xenopus laevis* oocytes, authors showed that in *trans*-form this freely diffusible, i.e. PCL type, compound efficiently potentiated GABA-induced currents at concentration 1μM, while at higher concentrations it directly activates the

receptors (Fig. 3,B,a). In *cis*-form generated by UV-illumination (365 nm) the compound caused little effect on the amplitude of GABA-induced currents. Moreover, in cerebellar brain slices, MPC088 co-applied with GABA, caused bidirectional photomodulation of Purkinje cell membrane current (Fig.3,B,b) and changes in spike-firing rate (Yue et al., 2012). The results of this study suggest that MPC088 interact with GABA receptor in the same site of β subunit as propofol, but the efficiency of this interaction is higher than in the case of propofol.

Another chemically synthesized azo-propofol compound, AP2, which contained azobenzene group at the *para*-position of phenol has been reported (Fig.3,A,b; Stein et al., 2012). In trans-configuration the AP2 potentiated GABA-induced currents with EC_{50} of micromolar, while irradiation with UV light, which transferred the compound in *cis*-configuration, prevented development of the potentiation. Activity of propofol-based AP2 was demonstrated on *Xenopus* oocytes, HEK cells and in animal model – *Xenopus laevis* tadpoles, where AP2 caused light-dependent anaesthesia. Future studies in other experimental models should demonstrate the usefulness of azo-propofols for acting as light-dependent anaesthetic and modulator of GABAergic activity in the brain. The broad possibilities remain that trans-MPC088 and AP2 could modulate the function of non-$GABA_A$Rs ion channels or other proteins of neural tissues.

More recently, two effective inhibitory PTLs, Li$GABA_A$R, were tethered to the mutant GABA receptor that contains cysteine-substituted α1 subunit (T125C) (Lin et al., 2014). One of the compounds (MAM-6) consists of muscimol (as element responsible for the specific interaction with GABA-binding site) combined with azobenzene photoswitch conjugated to maleimide. Effective allocation of the compound to the active site was achieved using a 6-carbon spacer between the muscimol pharmacophore and the azobenzene group (Fig. 3,A,c).

Although muscimol is an agonist for ionotropic GABA receptors (Johnston, 1996; Krogsgaard-Larsen et al., 1997), the MAM-6 acted as photoswitchable antagonist, capable to bind or to retract from the GABA-binding pocket with 500 and 380 nm illumination respectively (Fig. 3,C,a). This inhibitory effect of the agonist-based molecule was observed previously for the nicotinic acetylcholine receptor (Tochitsky et al., 2012) and could be caused by disrupting concerted reorganization of the agonist binding site during activation and consequent conformational changes required for ion channels opening (Miller and Smart, 2010).

Another compound, MAB-0, that contained neutral analogue of muscimol and did not contain any carbon spacer was even more effective in light-sensitive inhibition of GABA receptors. After treatment with MAB-0, cultured hippocampal neurons expressing α1(T125C) subunit were effectively modulated by light (Lin et al., 2014).

A series of PTLs were further developed for the efficient light-mediated control of all alpha subunits (alpha 1- alpha 6) of $GABA_A$ receptors. For each isoform the best PTL/mutant pair was

selected based on two criteria: (1) GABA-elicited currents are robustly photo-controlled (preferably >50% photo-antagonism at EC_{50}) and (2) receptor function is unaffected by cysteine mutation and PTL conjugation. Moreover, mutated GABA subunits were incorporated in living animals by generating a knockin mouse in which the ‘‘photoswitch-ready’’ version of a $GABA_A$ receptor subunit genetically replaces its wild-type counterpart, ensuring normal receptor expression (Lin et al., 2015). This elegant approach allowed to perform mapping of subcellular distribution of different alpha subunits in neurons and characterize the differential distribution pattern of $GABA_A$Rs in the brain of living animals (Fig. 3, D).

In general, the team of Kramer (Lin et al., 2014; 2015) proposed a "toolkit" for efficient optogenetic control of $GABA_A$ receptors. Similarly to proposed previously for nAChR and glutamate receptors (Volgraf et al., 2006; Tochitsky et al., 2012), it consists of (1) a photoswitchable tethered ligand composed of cysteine-reactive maleimide group for receptor conjugation, an azobenzene core for photoswitching and a GABA-site ligand for competitive antagonism like GABA or its guanidinium analogues and (2) the alpha subunits of GABA receptor with a genetically-engineered cysteine near the GABA-binding site. This optogenetic pharmacology toolkit allows scalable interrogation of endogenous $GABA_A$ receptor function with high spatial, temporal, and biochemical precision. However, antagonism of gabaergic transmission results in excitatory stimuli, which limits the applications of these switches to study neuronal circuits and complicates the interpretation of photomanipulation experiments. Future studies should be oriented on development of selective optopharmacological potentiators of GABAR function.

Conclusion

Since the first reports almost 50 years ago, the engineering of light-gated receptors has greatly expanded. Highly efficient photoswitches of many neuronal receptor-channels have been reported, based on rational design, high-resolution protein structures, comparative pharmacology and molecular biology manipulations. Several studies demonstrated that photochromic compounds could be used for optical control of behaviour, and function of different organs. It has been shown that AzoCholine, which specifically activates neuronal nAChRs, modulates behaviour in the nematode *C.elegans.* The other photoswitches, AzoCarbachol, modulated in a light-dependent fashion the beat frequency of a whole heart preparation of the mouse (Damijonaitis et al., 2015b). Photoswitches can restore electrophysiological and behavioural light responses in mutant strains of blind mice (Polosukhina et al., 2012). Also ATA, a freely diffusible specific photochromic agonist for AMPA receptors, in a light-dependent manner modulated function of amacrine and retinal ganglion cells, although a minor effect on bipolar cells has been observed (Laprell et al.,

2015). These observations suggest the powerful potential of photochromic compounds for ophthalmology.

Still, several aspects can be improved considerably. Regarding the optical properties, red-shifted variants are not available for all photoswitches, and efficient two-photon switching is also desirable for localized activation at the micrometer (subcellular) scale. In addition, a disadvantage found for most PCLs and many PTLs is that ligand action (agonist or antagonist) is exerted in the dark (*trans* isomer) (nAChR: Tochitsky et al., 2012; $GABA_A$: Lin et al., 2014), and this results in the requirement to illuminate with UV light in order to maintain normal receptor activity. TCPs allow reversing the photoswitch action (Izquierdo-Serra et al., 2016) although this possibility depends on the actual localization of suitable reactive residues in each receptor protein.

In general, photopharmacological compounds represent efficient tools for reversible and reproducible activation or block of specific neurotransmitter-gated receptors and ion channels in specific cells. However, the subtype selectivity is a very desirable pharmacological property that is found in few cases. The covalent attachment of the PTL to the target protein provides high subtype specificity compared to soluble pharmacological agents. PTLs allow precise spatiotemporal control since the photoisomerization of azobenzene is a picosecond process and binding is not limited by diffusion (Levitz et al., 2016). Successfully engineered PTLs include light-gated glutamate receptors activated by MAG (Volgraf et al., 2006) or L-$MAG0_{460}$ (Kienzler et al., 2013), ATA (Laprell et al., 2015) and TCP (Izquierdo-Serra et al., 2016), and neuronal acetylcholine receptors activated or inhibited by MAACh or MAHoCh (Damijonaitis et al., 2015a). These approaches, however, need genetic manipulation of the target protein. Development of highly specific soluble pharmacological agents is still an urgent and important problem. Combination of expanding knowledge in crystal structure, pharmacological analysis and chemical synthesis will provide the basis for further precision of photochromic compounds.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16: Ligand-gated ion channels. Br J Pharmacol. 172: 5870-5903.

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Figure legends

Figure 1. Optical switches for modulation activity of nicotinic acetylcholine receptors.

A, Light-induced conformations of azobenzene.

B, Chemical structures of the first azobenzene-based photochromic ligands for regulating the function of nAChRs (modified from Lester et al., 1980).

C, PTLs for photochemical control of neuronal AChRs.

a, Modular organization of maleimide–azobenzene–acetylcholine (MAACh) in trans configuration.

b, Scheme of a tethered agonist action. At illumination, with visible light (500nm) or in darkness the compound is in trans-configuration and not capable of activating heteropentameric nAChRs (upper part). Under UV light (380 nm) the tethered agonist is converted into its cis-configuration and thus activates receptors causing channels opening (bottom part).

c, Photoactivation of the α3β4E61C mutant receptors by tethered MAACh in Xenopus oocyte. Illumination at 380 nm (violet line) triggers ionic current and at 500 nm (green line) shuts it off. For comparison, the right trace shows the response to acetylcholine 100 µM.

d, Photoinhibition of the current induced by 300 µM acetylcholine (green line) by tethered to the α3β4E61C mutant receptors antagonist MAHoCh at 380 nm illumination (violet line). (Modified from Tochitsky et al., 2012).

D, Photoswitchable PCL agonist for neuronal α7 nAChRs.

a, Chemical structure of the AzoCholine.

b, Light-dependent effect of BisQ or AzoCholine on α7/GlyR chimera expressed in HEK293T cells. Note that illumination with light 440 nm triggered large inward current (bottom trace) while BisQ was not effective (top trace).

c, Effect of BisQ or AzoCholine on neuromuscular nAChR (α1/β1/δ/ε) expressed in HEK293T cells. Note that on this receptor AzoCholine is not active, in contrast to BisQ. (Modified from Damijonaitis et al., 2015a).

Figure 2. Optical switches for modulation activity of glutamate receptors.

A, Modular design of azobenzene−glutamate photoswitches.

a,b, PTLs in trans configuration for modulation of ionotropic glutamate receptor. They are composed of three parts: maleimide−azobenzene−glutamate (MAG). In section (**a**), for clarity different components of the synthetic photoswitcher are highlighted and labeled. For MAG_{380}

(**a**) the most efficient isomerization from *trans* to *cis* configuration is triggered by illumination at 380 nm (Volgraf et al., 2006), while for L- $MAG0_{460}$ (**b**) this transition occurs at visible light with optimal wavelength 460 nm (Kienzler et al., 2013).
c, PTL agonist for native affinity labeling via lysines. No need to introduce cysteine by mutagenesis (from Izquierdo-Serra et al 2016).
d, PCL version of azobenzene−glutamate photoswitcher, which reversibly interacts with glycine receptor (modified from Volgraf et al., 2007).
B, a, The ribbon structure of *apo*-iGluR2 together with the ball-and-stick structure of MAG attached to cysteine at L439C (yellow) in the extended (*trans*) and unbound conformation. (modified from Gorostiza et al., 2006).
b, A neuron transfected with iGluR6 (L439C) and labeled with MAG is illuminated at 380 nm for 500 ms, yielding reproducible depolarization that trigger trains of action potentials. Illumination at 500 nm turns the response off and permits repolarization. (Modified from Szopota et al., 2007).
C, The photo-induced activation of LiGluR with "red-shifted" covalently tethered MAG_{460}.
a, Patch-clamp recording from HEK 293 cells expressing GluK2 (439C). Illumination by 500 nm light causes generation of inward currents, while in the dark MAG_{460} relaxes back to *trans* configuration, resulting in closing of the channels (modified from Kienzler et al., 2013).
b, The effect of blue light illumination (blue bar) on activity of cultured hippocampal neuron expressing mutant of GluK2 with a cysteine substitution (L439C). Current-clamp recording. (Modified from Levitz et al., 2016).

Figure 3. Optical switches for modulation activity of ionotropic GABA receptors.
A, Examples of chemical structures of some PCL (**a,b**) and PTL (**c**) photochromic ligands of GABARs.
B, Ion current induced on *Xenopus* oocyte expressing $\alpha1\beta2\gamma2$ $GABA_AR$. **a,** *Left trace*; current induced by 3 μMGABA; *right trace:* co-application of 3 μMGABA and 1 μMMPC088 at visible light and during illumination with UV light.
b, Ion current induced by application of 15 μM MPC088 at visible light and during repetitive illumination of the oocyte with UV light. Note that UV illumination causes decrease of the responses, while at visible light the currents slowly recover.
c, Whole-cell recording from the mouse brain slice. Effect of MPC088 photoactivation on GABA-evoked currents in cerebellar Purkinje neuron. Cells were exposed to multiple UV/blue light flashes during application of GABA and MPC088 (indicated above the trace). (Modified from Yue et al., 2012).

C, a, Scheme of photoswitchable PTL antagonist MAM-6 action. Being conjugated to GABA receptor it reversibly isomerizes between *cis*- and *trans*- states. In *cis*-configuration (UV illumination) it is not active, while at illumination with visible light, it isomerizes to *trans*-configuration and prevents GABA binding and the subsequent opening of the channels.
b, Photoregulation of GABA-induced currents by the tethered MAM-6 on cells expressing the mutant S68C of α1 GABAR subunits. (Modified from Lin et al., 2014)
D, Differential photo-control of inhibitory postsynaptic currents (IPSCs) in cerebellar molecular layer interneuron (top traces) and a Golgi cell (bottom traces) of the mouse expressing the α1-$GABA_A$ with a single point mutation (T125C) and treated with PCL compound PAG-1C. Note that on a Golgi cell the currents are not modulated by light, suggesting absence of the α1-$GABA_A$ on these cells. (Modified from Lin et al., 2015).

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