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Precise modulation of neuronal activity with synthetic photoswitchable ligands

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Introduction

To tackle some of the most fundamental questions in neurobiology at the molecular level, classical genetics and pharmacology have been effective tools to perturb the activity of neuronal receptors and ion channels. However, conventional pharmacology has several drawbacks that are especially problematic when studying neural circuits and synapses. First, standard diffusion and partitioning of the ligand mean poor spatial and temporal control of ligand activity. Second, even in cases of high specificity for ligand action, it is relatively common for the receptor or channel of interest to be expressed on multiple nearby cell types, or even on both sides of a synapse, making it difficult to isolate the effects of the target receptor within the circuit or synapse of interest. Light-based techniques that operate at the intersection of chemistry, biology, and neuroscience have been developed to overcome these challenges. We focus here on systems that contain a synthetic photoswitch, a small molecule that absorbs light to reversibly change its shape. The most commonly used photoswitch in biological applications is azobenzene due to its synthetic tractability, tunable photochemical properties, and biological compatibility (Fig. 1a). The lowest energy isomer, the straight *trans*-azobenzene, isomerizes to the bent *cis*-azobenzene configuration upon irradiation with near-UV light. Subsequent irradiation with longer wavelength visible light, or thermal relaxation, leads the metastable *cis*-azobenzene to revert to the *trans*-azobenzene isomer.

To make a light-sensitive probe, the photoswitch is chemically attached to a biologically active ligand. Freely diffusible photoswitch-ligand constructs, called photochromic ligands (PCLs) (Fig. 1b), interact with the native target protein such that one photoisomer is active and the other is not. PCLs have the capability to operate as blockers (e.g. of a pore or active

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site), agonists, antagonists, or allosteric modulators. This one-component system, called photopharmacology, is an improvement upon conventional pharmacology in that PCLs can be rapidly photoswitched to provide exceptional spatial and temporal control. Despite this, selectivity for a particular receptor among related subtypes can vary, and diffusion makes it impossible to confine PCL action to a particular cell or group of cells. To overcome these drawbacks, photoswitchable tethered ligands (PTLs) incorporate a covalent linkage to a modified version of the target protein, thereby enabling subunit-specific control and cell-specific genetic targeting in a two-component approach called chemical optogenetics (Fig. 1c). Until recently, photoswitch linkage mainly used a maleimide on the PTL and an introduced cysteine on the target protein. While cysteines are not unique in proteins, on external domains they tend to be disulfide bonded (and not subject to interaction with maleimide) or absent from “sensitive areas” near the mouth of a pore or ligand binding site. Nevertheless, there would be advantages for a new bio-orthogonal linkage. Such a strategy was achieved recently. It merges the properties of PCLs and PTLs by placing a PCL on a leash that is covalently attached via highly selective chemistry near the ligand binding site on the target protein. The leash restricts binding to the targeted protein and assures a sufficiently high local concentration for high occupancy in the switched-on state. These compounds are called photoswitchable orthogonal remotely tethered ligands (PORTLs) (Fig. 1d), and they provide a gateway to multiplexed optical control of distinct photoswitches and their targets.

In this work, we highlight recent developments, including the expansion of the available toolbox to include new receptors, channels, and binding sites, the incorporation of new bio-orthogonal attachment chemistry, the increased range of useful light wavelengths, and new *in vivo* applications. We will not address several related techniques and tools that have been well reviewed elsewhere, including optogenetics[1], chemogenetics[2], Photo-uncaging[3], and quasiparmacological interactions of cells with light[4].

Glutamate receptors (GluRs)

Glutamate is the primary excitatory neurotransmitter in the mammalian brain, with dozens of different receptor subunit types that mediate fast synaptic transmission and short- and long-term modulation of synaptic strength. Glutamate receptors (GluRs) comprise ionotropic receptors (iGluRs), which are tetrameric ion channels, and metabotropic receptors (mGluRs), which are class C dimeric G-protein coupled receptors (GPCRs). The iGluRs consist of three classes of receptors: NMDARs, AMPARs, and kainateRs.

NMDARs are cation channels with both ligand and voltage dependence. They function as synaptic regulators that play an important role in learning and memory. Recently both PCL and PTL approaches were developed for NMDARs. The PCL agonist ATG is unreactive in its relaxed *trans*-isomer, but when irradiated with UV light, the *cis*-isomer is an agonist for multiple NMDAR subunits (GluN2A,B,C,D)[5]. Experiments in slices of mouse cortex show that ATG can be used to induce currents and action potentials, even in place of synaptic stimulation for signal coincidence detection. A two-component system was also developed for three NMDAR subunits to create “LiGluNs”[6]. GluN1, GluN2A, and GluN2B were cysteine-substituted to enable PTLs called MAGs to generate photo-agonism

(GluN2A, GluN2B) or photo-antagonism (GluN1a and GluN2A). In mouse hippocampal slices, photo-agonism can stimulate—and photo-antagonism inhibit—the dendritic spine growth associated with long-term potentiation (LTP) with single-spine resolution. Photo-antagonism also blocks the induction of LTP by synaptically-released glutamate. The relaxation of the active *cis* to inactive *trans* state of the photo-antagonist is slow, allowing for sustained light-block with brief pulses of light at long intervals. This was used over days of development to block NMDAR-dependent pruning of retinal ganglion cell axonal arbors innervating the brain of developing zebrafish[6].

AMPA receptors mediate fast excitatory synaptic transmission, and their modulation has strong effects on synaptic strength. Initial PCLs were agonists of GluA2, with the best compound being ATA[7]. The active *trans*-isomer of ATA allows photo-control of action potential firing in layer 2/3 neurons from mouse cortical slices. A complimentary PCL antagonist, ShuBQX-3, is quite selective over NMDARs, partially selective over kainate receptors, and provides photo-antagonism of hippocampal CA1 neurons[8].

Kainate receptors also mediate fast excitatory synaptic transmission. In part due to limited selectivity of conventional pharmacological probes, the kainate receptors were the first GluRs targeted for photocontrol with both one- and two-component systems[9]. Recent experiments showed that viruses containing LiGluR (GluK2 L439C) stereotactically injected into the V1 region of a mouse's visual cortex led to targeted production of LiGluR. Subsequent labeling with a PTL made these LiGluRs photoactivatable *in vivo*: light pulses in the cortex led to neuronal firing with a frequency of up to 5 Hz[10]. This study exemplifies the type of experiments possible when using cell-type specific promoters to express the target protein with high specificity. A study on the effects of multiple ligands binding to multiple subunits of tetrameric kainate receptors was only possible with LiGluR, and it suggested that multiple subunits must be occupied by ligand before the channel desensitizes[11].

mGluRs regulate signaling pathways within neurons to control synaptic strength and plasticity. An initial two-component system was developed for light-gated mGluRs, or "LimGluRs"[12]. They have been used to examine dimerization effects, receptor conformational dynamics, and receptor glutamate sensitivity[13], as well as to photoactivate the G_i GPCR pathway to show this pathway plays a role in astrocyte glutamate uptake[14]. A PCL negative allosteric modulator (NAM) targeting mGluR5, called alloswitch-1, was recently developed[15]. Alloswitch-1 photomodulates the activity of mGluR5 *in vitro* and tadpole swimming behavior *in vivo*. Medicinal chemistry refinements to find structure activity relationships for alloswitch-1 led to a series of phenylazopyridine-containing PCLs that also function as NAMs[16]. Impressively, some of these PCL NAMs produce photoswitchable analgesia in mice. Whereas mGluR5 has a potentiation effect on neural activity, mGluR4 works to inhibit glutamate release at the synapse. Accordingly, the PCL NAM for mGluR4, OptoGluNAM4.1, inhibits the activity of mGluR4 *in vivo* and has an opposite regulatory effect on the swimming behavior of zebrafish[17].

With these newest additions to the glutamate receptor family of tools, it is now possible to target at least one subunit from all three types of iGluR as well as to target or modulate at least one member of all three groups of mGluR.

GABA receptors (GABARs)

GABA is the primary inhibitory neurotransmitter in the mammalian brain. GABA_ARs are pentameric ion channels, while GABA_BRs are GPCRs. PCL versions of propofol[18,19] and gabazine[20] create photo-potiation and photo-antagonism of GABA_ARs, respectively, both *in vitro* and *in vivo*. Two-component systems using PTLs that act as either an agonist (MAB-0), a potentiator (MPC100), or an antagonist (PAG-1C) have been developed to generate “LiGABARs” for specific GABAR subunits (α 1-6, and γ). This has made it possible to map the subcellular location of different GABA_AR subunits in living neurons[18,21,22]. An exciting series of experiments shows the great potential of knock-in animal models, which possibly provide the best assurance that no compensatory changes, from the molecular to the systems level, have taken place due to overexpression[22]. When observing γ oscillations in mice, the oscillations decreased when a nonselective GABA_AR blockade was induced. However, in the α 1-GABA_AR photoswitch ready mouse (PhoRM, a knock-in mouse with all α 1-LiGABAR), infusion or brain surface application of PTL allows subtype specific photo-antagonism of only GABA_ARs with α 1, leading to enhanced γ oscillations. This opposite effect is evidence for inhibitory-inhibitory interactions for α 1 subunit-containing GABA_ARs[22]. In a notable shift from ion channels and GPCRs, a PCL based on nipecotic acid photo-inhibits GABA transporter mGAT1 *in vitro*, which led to light-dependent tonic currents in dentate gyrus granule cells in mouse slices[23].

Acetylcholine receptors/acetylcholine esterase (AChRs/AChE)

Acetylcholine (ACh), the first neurotransmitter discovered, is found throughout the brain and peripheral nervous system. It plays a well-known role as the main neurotransmitter of the neuromuscular junction, but in the brain operates as a neuromodulator through both ionotropic and metabotropic receptors. Nicotinic acetylcholine receptors (nAChRs) are pentameric ion channels, and muscarinic acetylcholine receptors (mAChRs) are GPCRs. The many historic firsts of ACh include the first-ever examples of a PCL and a PTL[24]. Recent work has focused on nAChRs with a two-component system developed for heteropentameric forms (α 3 β 4 and α 4 β 2) that have PTLs functioning as a photoagonist (MAACh) and a photoantagonist (MAHoCh) in *Xenopus* oocytes[25]. A reevaluation of a classic PCL, BisQ, showed it to be a photoagonist of muscle-type nAChR, while a new PCL, AzoCholine, is a photoagonist of α 7 nAChRs[26]. AzoCholine was further demonstrated to give photocontrol of bursting activity in mouse hippocampal brain slices. Additional regulation of ACh action at synapses is achieved by its degradation, an action performed by AChE. Photocontrol of AChE has recently been demonstrated with two different PCLs based on the AChE inhibitor tacrine[27,28].

Other Ion channels and promising targets

Several more of the channels and receptors involved in synaptic and neural function are targets for photocontrol. Recent work has targeted GIRK[29], TRPV1[30,31], TRPA1[32], P2X[33,34] and ASIC[33,35] channels. Of special note, two papers[33,34] on P2X channels employ a variant of the chemical optogenetic concept to create “molecular tweezers”: two subunits of the channel are tethered together with azobenzene via covalent cysteine-maleimide bonds at key positions in the channel, allowing the photoswitching azobenzene to open and close the pore.

The most light-accessible neurons in the central nervous system are those of the retina, and accordingly, much work has been done to make the non-photoreceptor cells of the retina light sensitive. Work using both one- and two-component systems on degenerated retinas lacking rods and cones (but possessing much of the rest of the retinal neural circuitry) shows great progress in restoring some degree of light sensitivity in both mouse and dog models of blindness[36–39].

Other developments outside of ion channels include a PCL derivative of the analgesic fentanyl that functions as a photo-agonist of the μ -opioid receptor, a family A GPCR[40]. Another paper describes a family of PCLs based on diacylglycerols, lipid signaling molecules, which provides photocontrol of a host of cellular targets (including protein kinase C) that, through phosphorylation of effector proteins, can modulate synaptic strength and the exocytosis of neurotransmitters[41]. Finally, two papers revealed PCL inhibitors of histone deacetylase, an enzyme that plays a role in gene expression and physiological processes like neuroplasticity[42,43].

Improving synthetic photoswitch utility: wavelength-shifting, two photon (2P) activation and orthogonal attachment chemistry

For synthetic photoswitches to reach their full potential, two goals must be met. First, in order to use these compounds simultaneously, their absorbance spectra shouldn't overlap. To achieve this goal, a collection of compounds activated by various wavelengths of light is necessary. Particularly desirable is to red-shift the absorbance to red and infrared light, as these wavelengths better penetrate tissue. Much of the appeal of using azobenzene photoswitches is the ability to change the spectral properties of both isomers by changing chemical substitution patterns on the benzene rings[44,45]. Because chemical optogenetic methods are modular (individual tuning of both PTL and receptor), it is possible to use previously validated mutant receptors with a new PTL that has different photochemical properties. We have demonstrated this permissive labeling by attaching different red-shifted PTLs to LiGluR[46,47].

In addition to red-shifting photoswitches, it is also possible to activate these compounds with two photon (2P) excitation. When two photons hit a photoswitch nearly coincidentally, the photoswitch will isomerize as if it were hit by single photon of their combined energy. While this technique requires a high intensity laser, it allows activation of a photoswitch with significantly lower energy/longer wavelength light. The feasibility of 2P activation has been

demonstrated for both PCLs and PTLs. Neurons[48,49] and astrocytes[48] expressing LiGluR and labeled with a PTL give a photocurrent with 2P excitation, and even neuron spiking. Further, 2P activation of LimGluRs[49,50], as well as the PCL ATG, which targets NMDARs[5] were reported.

The second goal is to implement PTLs with new bio-orthogonal attachment chemistry. To that end, targeted covalent photoswitches (TCPs) are a type of PTL that use a reactive group (e.g. NHS ester) to covalently bind to an endogenous protein and make it light sensitive. Light-dependent affinity labeling is possible, so patterns of light can shape where the TCP binds in the sample. When endogenous GluK1 channels are labeled with a TCP in DRG neurons and degenerate retina, the neurons become light sensitive[51].

We recently reported a two-component bio-orthogonal labeling technique taking advantage of genetically-encoded self-labeling proteins (e.g. SNAP-tag) fused to a target receptor[52]. The complimentary switches, called PORTLs (photoswitchable orthogonal remotely tethered ligands), covalently bind to the self-labeling protein via selective chemistry (e.g. a benzylguanine group in the case of SNAP). The technique was applied to a SNAP-mGluR2 fusion labeled with a glutamate-containing PORTL, making it possible to optically hyperpolarize and silence hippocampal neurons[52]. Perhaps the most exciting advantage with the addition of a new orthogonal chemical labeling strategy is the ability to individually express, label, and control more than one protein with light. When HEK293 cells expressing both SNAP-mGluR2 and LiGluR were labeled with different PTLs, each channel could be activated independently[52]. We recently expanded this technique to another orthogonal labeling system (the CLIP-tag and its benzylcytosine-selective PORTLs), as well as making the complete set of group II/III SNAP-mGluRs[50].

Conclusions/future directions

Chemical optogenetics and photopharmacology continue to mature. The major differences between these techniques and conventional optogenetics as tools to study neurons and synapses are significant and bear elaboration. Traditional optogenetics relies on the expression of an exogenous light sensitive channel or receptor, frequently an opsin, to generate or inhibit action potential firing. However, this top-down drive of function can obscure the behavior of endogenous receptors and channels. Chemical optogenetics shines in that by requiring minimal structural changes to endogenous proteins, minimal perturbations to the native modulation of that protein can be expected. This is especially important for GPCRs as they can affect downstream signaling in multiple pathways (e.g. G-Protein vs β -arrestin)[53]. Many of the known neurotransmitter small molecules and peptides have not yet been brought into the photopharmacology realm, and expansion to other neurotransmitters and neuropeptides is ongoing. Targets of great interest to our labs include the neuromodulatory class A GPCRs, especially the dopamine and serotonin receptors, due to their essential but unresolved role in many brain behaviors including mood and cognition.

Expanded bio-orthogonal labeling chemistries and photoswitch color palettes will give researchers the ability to construct elaborate experiments with multiplexed photoswitches and fluorophores with a high degree of spatial, temporal, and genetic targeting. Indeed, the

proof of concept combining PTLs and PORTLs for both orthogonal labeling and light activation is described above, and we are excited to see this concept expanded to other attachment strategies in addition to SNAP- and CLIP-tags (e.g. HALO-tags). In fact, this and other recent chemical developments [54] encourages us to envision an ideal scenario where orthogonality of labeling a photochemistry allows precise targeting of a synapse (Fig. 2).

Incorporation of photoswitch unnatural amino acids is also an area of active research seeing rapid development; replacing photoswitchable ligands with photoswitch amino acids incorporated into proteins of interest at key positions near pores or fulcrums of movement is becoming feasible as our understanding of structure-function relationships grow[55]. We are also optimistic that methods to achieve both cell specificity and native expression levels will soon be practical, e.g. with cell-targeted CRISPR knock-ins[56]. Finally, some of the photopharmacology tools mentioned above show promise for applications in pain regulation via photoswitchable analgesia, a field in its infancy, and recently reviewed elsewhere[57].

We hope that we have enticed the reader into considering use of some of the many photoswitchable tools discussed in this review to address their research questions, as they now modulate many key synaptic proteins. At the same time, we hope that by showing so many different functioning systems, we can reinforce the general applicability of these approaches to molecular neuroscience. Should an interested reader find no tool currently in existence to deal with their research questions, joining forces with a local collaborator with complimentary skills is a realistic possibility. Indeed, while most of the effort in chemical optogenetic and photopharmacology has focused on ion channels and neurotransmitter receptors, the approaches are applicable to a host of proteins whose function can be controlled by an orthosteric or allosteric ligand or an active site blocker, a design process that was recently explained in detail[58,59].

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Glossary

Optogenetics

A technique now common in neuroscience that uses genetically encoded light-activated proteins to control neuronal activity.

Chemogenetics

A technique that relies on an orthogonally active chemical (usually synthetic) to selectively activate a genetically encoded engineered receptor.

Photoswitch A

structure that reversibly changes its shape and/or function on absorption of light.

Photochromism

A reversible light dependent chemical change in a molecule that results in the formation of spectrally distinct compounds sometimes visible to the eye as a color change.

Photoisomerization

A light dependent structural change in a molecule generally, but not exclusively, applied to reversible processes.

PCL - Photochromic ligand

A freely diffusible photoswitchable ligand.

Photopharmacology

A technique using a freely diffusible biologically active small molecule synthetically modified to include a photoswitchable element. Photoisomerization toggles between a more and less active form of the molecule.

Optopharmacology

See photopharmacology.

One-component system

Used here to mean a receptor or channel rendered photo-controllable with a just one synthetic non-endogenous component. For example, photopharmacology.

chemical optogenetics

A technique for photocontrol of protein activity that relies on the combination of two synthetic elements—a genetically encoded engineered protein and a covalently bound photoswitchable ligand.

optochemical genetics

See chemical optogenetics.

optogenetic pharmacology

See chemical optogenetics.

Two-component system

Used here to mean a receptor or channel rendered photo-controllable using both a one synthetic photoswitch and an engineered receptor. For example chemical optogenetics.

PTL - photoswitchable tethered ligand

A photoswitchable ligand that is covalently attached to its target protein such that one photoisomer binds significantly better than the other.

PORTL - photoswitchable orthogonal remotely tethered ligand

Conceptually a cross between a PCL and PTL it covalently binds to the target protein, but with a long tether, so photoisomerization affects binding efficacy and not access to the binding site.

LiGluRs

A collection of engineered kainate receptors channels designed to become light-sensitive when a PTL covalently binds.

LimGluRs

A collection of engineered group 2/3 glutamate receptor GPCRs designed to become light-sensitive when a PTL covalently binds.

LiGABARs

A collection of engineered GABA_AR channels designed to become light-sensitive when a PTL covalently binds.

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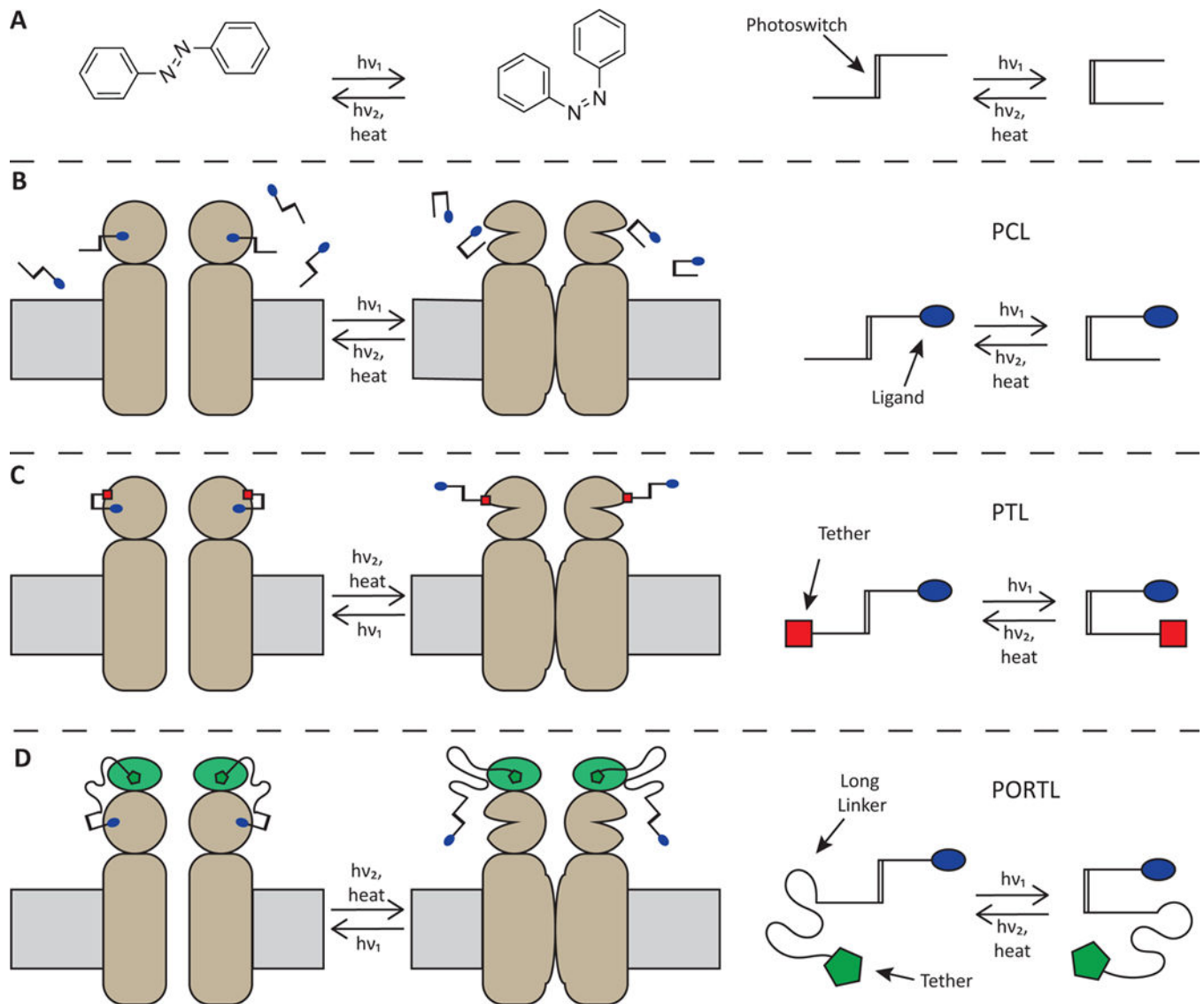
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Highlights

- Photoswitchable ligands allow high spatial and temporal control
- Combining with genetic targeting gives cell-type specificity
- Orthogonal attachment and photochemistry allows multiplexing

**Figure 1.**

Strategies for incorporating synthetic photoswitches into neuroscience tools. A) The *trans* and *cis* isomers of azobenzene can be interconverted with different wavelengths of light. Cartoons show how the core azobenzene structure can be elaborated into photoswitchable tools. B) Photochromic ligands (PCLs) switch between active and inactive compounds that freely diffuse and function with endogenous channels and receptors. C) Photoswitchable Tethered Ligands (PTLs) covalently attach to an engineered protein to provide photocontrol. D) Photoswitchable orthogonal remotely tethered ligands (PORTLs) are conceptually a cross between PCLs and PTLs, they use a self-labeling protein fused to the target protein and a photoswitch ligand that has long linker to accommodate attachment farther from the ligand binding site.

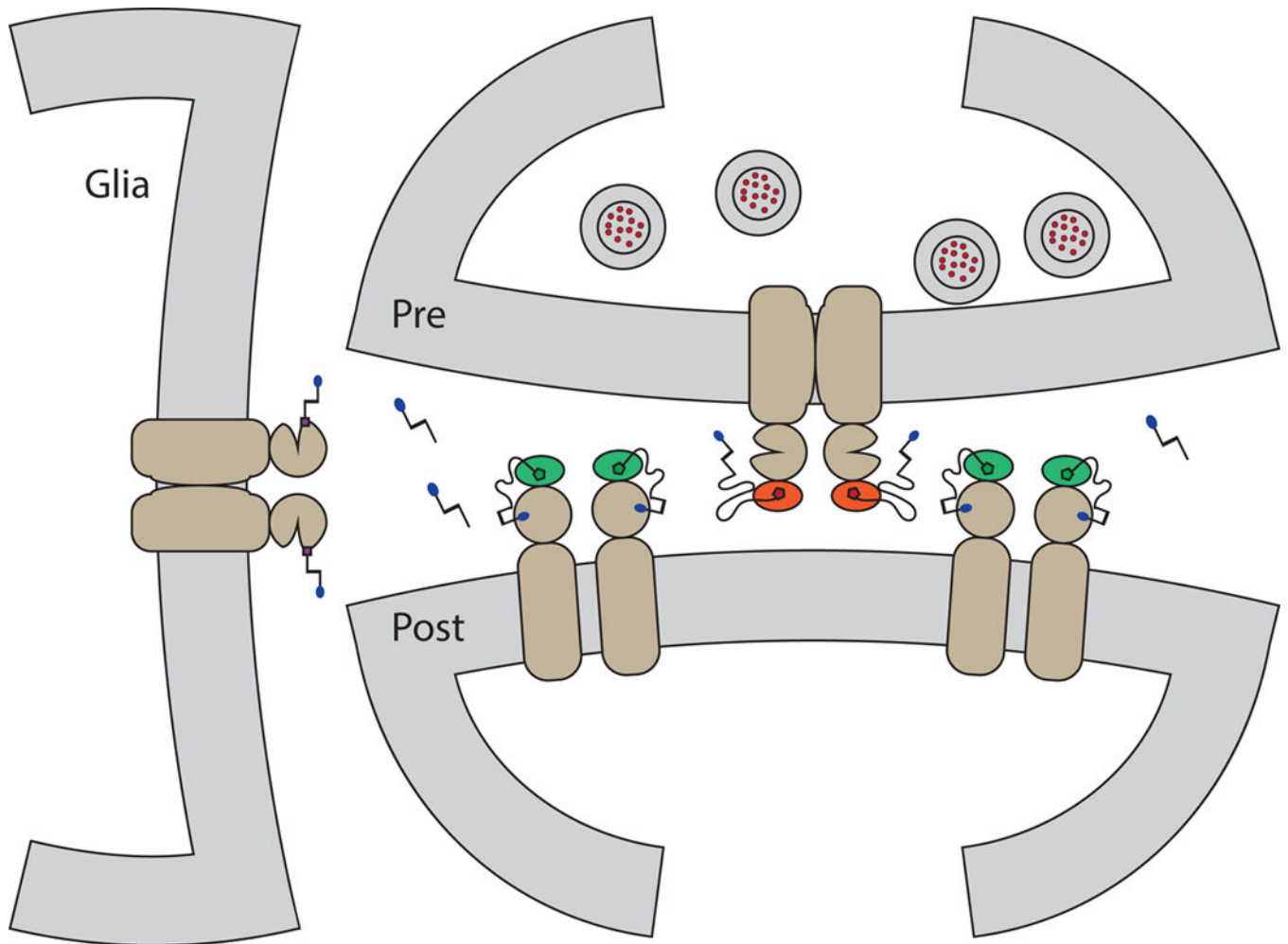


Figure 2.

A cartoon of a hypothetical tripartite synapse. Here each piece, glia, presynaptic cell, and postsynaptic cell are all expressing a different form of receptor, which is labeled with an orthogonal photoswitchable ligand (PORTLs and PTLs). The synaptic cleft also has a freely diffusing PCL. Distinctly different absorption spectra for each photoswitchable ligand would allow exact spatiotemporal control at the subcellular level.