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Optogenetic pharmacology for control of native neuronal signaling proteins

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Abstract

The optical neuroscience revolution is transforming how we study neural circuits. By providing a precise way to manipulate endogenous neuronal signaling proteins, it also has the potential to transform our understanding of molecular neuroscience. Recent advances in chemical biology have produced light-sensitive compounds that photoregulate a wide variety of proteins underlying signaling between and within neurons. Chemical tools for optopharmacology include caged agonists and antagonists and reversibly photoswitchable ligands. These reagents act on voltage-gated ion channels and neurotransmitter receptors, enabling control of neuronal signaling with a high degree of spatial and temporal precision. By covalently attaching photoswitch molecules to genetically tagged proteins, the newly emerging methodology of optogenetic pharmacology allows biochemically precise control in targeted subsets of neurons. Now that the tools for manipulating endogenous neuronal signaling proteins are available, they can be implemented *in vivo* to enhance our understanding of the molecular bases of brain function and dysfunctions.

Microbes have given neuroscience a great gift in the form of genes encoding light-sensitive ion channels and transporters¹. These opsin-based proteins, including channelrhodopsin-2 and halorhodopsin, are being introduced to neurons so that endogenous electrical activity can be replaced with user-defined patterns of action potential firing, an approach known as optogenetics¹ (Figs. 1 and 2a). Although this is a boon to systems neuroscience, there remains the reductionist imperative to understand how neuronal circuits operate at a molecular level. If we want a high-resolution view of how particular ion channels or receptors drive a neural circuit, we need high-resolution tools for perturbing their functions.

Conventional pharmacology has given us a slew of drugs that affect many types of ion channels and neurotransmitter receptors, but this approach has some serious limitations. First of all, the delivery of conventional drugs to intact neural structures is slow and imprecise. Second, drugs are almost never perfectly specific for an individual subtype of a channel or receptor, and there are many of these. Third, conventional drugs cannot

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distinguish between the same receptor expressed in different classes of neurons, especially if they are intertwined in a neural circuit.

The solution to these thorny problems lies in combining optics and chemistry in various ways. Optopharmacology refers to photosensitive reagents that act on channels and receptors (Fig. 1b). As they can be precisely regulated with light, these tools offer a potential solution to the problem of delivery. The optopharmacological tools that have been most widely used in neuroscience are caged agonists of neurotransmitter receptors, particularly glutamate receptors. Two photon-sensitive caged glutamate compounds give users the opportunity to rapidly and locally release a puff of agonist with spatial and temporal precision that rivals that of a real synaptic contact². Recent developments are extending this approach to two photon-caged GABA reagents and caged neuropeptides³.

Chemical photoswitches (or photochromic ligands) are another type of optopharmacological tool⁴. These are compounds in which the ligand is attached to a photoisomerizable group that controls whether the ligand can fit properly into a binding site on a protein, either a channel or a receptor. Thus far, azobenzene is the photoisomerizable group of choice, and the ligand is either a pore blocker (for K⁺ channels) or a neurotransmitter agonist or antagonist (for ionotropic or metabotropic receptors). One important advantage over caged compounds is the reversibility of azobenzene photoswitches. The compounds can repeatedly transition between active versus inactive photoisomeric states, either in response to different wavelengths of light or as a consequence of thermal relaxation⁴. Azobenzene-containing photoswitch molecules have been used to confer light sensitivity on neurons *in vitro* and *in vivo*, but, similar to caged compounds or other drugs, they lack specificity for a given molecular target.

Optogenetic pharmacology combines optics and chemistry and adds genetics to the mix, simultaneously solving both the subtype-specificity and cell-targeting problems. The idea is to attach a synthetic photosensitive ligand onto a genetically engineered protein to allow activation or inhibition of only that specific protein with light (Fig. 1c). The power of this approach is that it combines the absolute specificity that only genetics can provide with the unique precision that only light can provide to regulate function of targeted neurons at the molecular level. These opportunities come at a cost: optogenetic pharmacology is technically demanding because it requires the introduction of two exogenous components, the chemical ligand and the gene encoding the target protein.

Here we discuss the current state of optopharmacology and optogenetic pharmacology, focusing on the opportunities, challenges and limitations in using these tools to understand the molecular basis of signaling in the intact nervous system. For a more comprehensive discussion of caged neurotransmitters and azobenzene-based photoswitches, the reader is referred to other recent reviews^{3,4}. Table 1 shows a listing of many of the photosensitive tools that are available for controlling neuronal function. This is a rapidly changing field and we run the risk of being instantly out of date about the tools that are available. Because of this, we have tried to put equal emphasis on why and how these tools might be used.

Caged ligands for controlling neurotransmitter receptors

Caged molecules have a photolabile protecting group that is removed following illumination, enabling the uncaged ligand to bind to a receptor. Prior to the invention of caged neurotransmitters, direct application by superfusion or iontophoresis was the only means of investigating neurotransmitter activations on neurons in a circuit. However, the temporal and spatial resolution of this approach is very limited, restricting the interpretation of the resulting effects. In contrast, the timing and spatial precision of neurotransmitter uncaging is limited only by the speed of photolysis and the spatial resolution of the illuminating system. With efficient caged neurotransmitter analogs and two-photon light sources, uncaging can be directed to a single dendritic spine (Fig. 2b) and the response can be identical in both amplitude and time course to that of a single quantum of synaptically released neurotransmitter². Moreover, transmitter can be released in many different regions of a neural preparation, either sequentially, with scanning lasers⁵, or simultaneously, with holographic illumination⁶. This enables comprehensive two- or three-dimensional mapping of the biological sensitivity to a neurotransmitter, which was not a realistic option before the introduction of caged neurotransmitters.

There are at least four critical features of a caged neurotransmitter that are essential for its utility: efficiency of uncaging, the time constant of photolysis, stability in the absence of light and the inertness of the caged molecule. This last parameter has proven to be the biggest challenge, as caged molecules must often be applied at high (millimolar) concentrations that generate off-target effects, with many caged analogs acting as antagonists at the same receptors they are meant to probe⁷. Until recently, the number of caged molecules that fit these criteria was limited, with caged glutamate representing the most successful example by far.

Given that glutamate mediates the majority of fast excitatory transmission in the brain and is expressed on nearly all central neurons, a caged form of glutamate (DMNB-Glu) immediately found experimental utility. Pioneering studies used its photolysis to map synaptic connections in brain slices⁸. However, light absorbance and scattering limited the precision and effectiveness of glutamate uncaging, especially deeper than ~100 μm into tissue. These problems were alleviated by the development of MNI-caged glutamate, which has a more advantageous two-photon cross section^{2,7}. Armed with this tool, laser-scanning photostimulation to map neural circuits gained widespread use and has contributed to our understanding of circuits from the cerebral cortex⁵ to the basal ganglia⁹.

Yet even as caged glutamate was finding its permanent place in the toolbox of the neurophysiologist, identifying useful analogs of GABA, glycine and other transmitters seemed elusive, even when relying on the same caging groups. Several candidate molecules intended to act as agonists of receptors after photolysis actually acted as antagonists before photolysis^{7,10}. Recent advances in inorganic ruthenium chemistry and the development of biaryl derivatives have produced caged neurotransmitters that are sensitive to visible light and to two-photon excitation^{11,12}. However, it is not clear yet whether RuBi-GABA will be inert at high concentrations required for two-photon excitation, given that RuBi-Glu itself blocks GABA receptors at submillimolar concentrations¹².

This technology has matured to the point where there is now a wide palette of caged ligands for nearly all of the small-molecule neurotransmitter receptors, including neuropeptide receptors¹³, as well as caged agonists that are receptor-subtype specific (for example, MNI-NMDA and MNI-AMPA)¹⁴ (Table 1). Photolysis of these molecules may be helpful for dissecting the functions of different receptor subtypes. However, because they are not effectively removed by neurotransmitter reuptake mechanisms, these ligands will tend to persist in the extracellular space longer than the natural neurotransmitter, which could complicate the interpretation of uncaging results.

Controlling native ion channels with photochromic ligands

Azobenzene-containing photochromic ligands are another type of optopharmacological tool that confer light sensitivity onto endogenous neuronal ion channels⁴. In contrast with the uncaging of ligands, which is irreversible, photochromic ligands undergo a reversible photoisomerization and can therefore be switched on and off with light. Given an adequate light intensity, azobenzene will photoisomerize from *trans* to *cis* in milliseconds and will remain there for minutes. This ensures that the effects on ion channels are quick and persistent and can be triggered with only a brief flash of light. Longer wavelength light (for example, 500 nm) accelerates the return from the *cis* to the *trans* configuration. Under ordinary photostimulation conditions, azobenzene does not photobleach and can be used repeatedly with no decrement in activity. The first generation of photoswitch compounds required near-ultraviolet light (360–395 nm) for *trans* to *cis* conversion, but chemical modification of the azobenzene moiety has produced red-shifted compounds that operate at longer wavelengths (420–520 nm)¹⁵.

Acrylamine-azobenzene-quaternary ammonium (AAQ) is a photochromic ligand that blocks many types of K⁺ channels, but has no apparent effects on voltage-gated Na⁺ or Ca²⁺ channels or on neurotransmitter receptors^{16,17}. When AAQ is in the *trans* configuration (in darkness or in long-wavelength light), K⁺ channel blockade promotes neuronal firing. In the *cis* configuration (in short wavelength light), the relief of K⁺ channel block inhibits firing. The equilibrium between *cis* and *trans* determines exactly how many K⁺ channels are blocked in a neuron, thereby determining its level of electrical excitability.

Because it affects so many types of K⁺ channels, AAQ affects many types of neurons. The lack of specificity may make it difficult to sort out the various light-sensitive effects in intact neural tissues. For example, AAQ can simultaneously affect both pre- and postsynaptic neurons in a neural circuit. However, the widespread photosensitization has proven to be beneficial in the retina, a neural circuit that involves a high degree of parallel processing. AAQ can bestow light sensitivity on retinas taken from genetically blind mice whose rods and cones have been lost to degenerative disease¹⁸. The restoration of light sensitivity can be observed electrophysiologically, with multi-electrode array recordings, and behaviorally, by analyzing light-elicited locomotor behavior and pupil constriction in these blind mice. Indeed AAQ, and now a new generation of red-shifted derivatives, are potential therapeutic tools for restoring a degree of visual sensitivity in human blinding diseases, such as retinitis pigmentosa.

Another photochromic ligand, QAQ, is a symmetrical molecule containing a central azobenzene flanked by two quaternary ammoniums, both carrying a permanent positive charge¹⁹. Ordinarily, QAQ is membrane impermeant and only affects ion channels when added to the cytoplasmic side of the membrane. QAQ can be dialyzed into cells through a whole-cell patch pipette and, subsequently, light can be used to influence excitability. A nice feature of QAQ is that focal illumination enables local control, a unique way to manipulate excitability in selected parts of a dendritic tree. However, there is a method for getting QAQ into the cytoplasm without using a pipette. Certain ion channels, including the capsaicin receptor (TRPV1) and a receptor for ATP (the P2X receptor), possess pores that dilate wide when opened for a long time, allowing large molecules to pass. If either of these channels is present and activated, QAQ will permeate and accumulate in the cytoplasm. In the dorsal root ganglion, TRPV1 channels and P2X receptors are selectively expressed in nociceptive neurons, and activation of these channels enables QAQ to enter the cell. The result is selective photosensitive silencing of these neurons. *In vivo* tests in mice have shown that QAQ can serve as a light-regulated analgesic, which could have important scientific and clinical implications.

In addition to voltage-gated channels, photochromic ligands have also been developed for GABA_A²⁰ and glutamate receptors, including a photo-reversible agonist²¹, and a photosensitive irreversible antagonist, ANQX²², which has been used to track membrane turnover of AMPA receptors during long-term plasticity in hippocampal neurons. Very recently, a small molecule known as optovin was shown to serve as a photochromic activator of TRPA1 channels, enabling optical control of behavior in zebrafish²³ (Table 1).

Optogenetic pharmacology

Optogenetic pharmacology combines the advantages of photochemical control with the specificity of genetics to enable precise manipulation of particular signaling proteins in particular cells (Fig. 2c). Optogenetic pharmacology has emerged in the form of designer ion channels and receptors that are made sensitive to light by conjugating a synthetic, small, light-sensitive molecule (that is, a photoswitch) onto a genetically modified protein subunit⁴. A common design principle is employed in these light-regulated proteins, whether they are voltage-gated ion channels, ligand-gated ion channels or metabotropic receptors. A point mutation is introduced into the channel or receptor protein, resulting in a single cysteine substitution on its extracellular side, near the binding site for a ligand (for example, the neurotransmitter binding site or the pore-lining domain). The chemical photoswitch is designed to be complementary to the engineered protein. It has a cysteine-reactive group (a maleimide), which irreversibly tethers it onto the cysteine-substituted protein, and a regulatory ligand, which is an agonist or antagonist for the neurotransmitter receptors or a pore blocker for the voltage-gated channels. In between the tethering group and the ligand is a photoisomerizable azobenzene, which interconverts between the short *cis* form and the long *trans* form in a light-dependent manner. This allows the ligand to be advanced or withdrawn from its binding site with different wavelengths of light, activating or inhibiting the channel or receptor. The tethering length can be adjusted by altering either the position of the cysteine in the protein or the length of the photoswitch molecule, to generate *cis*-binding or *trans*-binding systems.

Light-regulated K⁺ channels

The first channel engineered in this manner was a light-regulated K⁺, named SPARK, which was generated by tethering a photoswitch compound onto a Shaker K⁺ channel protein²⁴. The channel had a genetically engineered cysteine on an extracellular loop, close to the pore. The photoswitch (MAQ) contained a cysteine-reactive maleimide, a photoisomerizable azobenzene and a pore-blocking quaternary ammonium. The resulting photoswitch-protein conjugate is blocked when the azobenzene is in the extended *trans* configuration, but not in the bent *cis* configuration.

Since the development of SPARK, the optogenetic pharmacology strategy has been used to engineer light sensitivity onto many other types of K⁺ channels that have different functions in the nervous system^{25,26}. These include Kv1.3, which controls axonal action potential repolarization, Kv3.1, which contributes to rapid-firing properties of certain brain neurons, Kv7.2, which underlies M current, SK2, which is a Ca²⁺-activated K⁺ channel that regulates the magnitude of synaptic responses in hippocampal neurons, and TREK, which is a polymodal, non-voltage-gated potassium channel that has been implicated in controlling resting membrane potential. The current through each of these channels could be selectively and reversibly downregulated in response to light. The selective control by light enabled one subtype of a channel to be differentiated from a closely related subtype, even if there were no pre-existing pharmacological distinctions. For example, there are no drugs known to distinguish between Kv3.1 and Kv3.4, which are thought to have different functions in the same fast-spiking brain neurons²⁷. Genetic insertion of a cysteine attachment site into one or the other generates photoswitch-ready channels with built-in pharmacological specificity, which is actualized by the covalent attachment of MAQ. This enables optical control of one specific type of K⁺ channel at a time, which will enable an investigator to better define what each channel does for a living.

Light-regulated ionotropic glutamate receptors

The optogenetic pharmacology approach was then extended to the ionotropic glutamate receptor family. But this time, instead of sterically blocking the ion channel pore, the photoswitchable ligand was designed to allosterically induce the opening of the channel gate²⁸. Cysteine mutations were engineered into the ligand binding domain of the kainate GluK2 receptor, in close proximity to the glutamate-binding site, to produce a light-gated glutamate receptor (LiGluR). After covalent attachment of the photoswitchable agonist MAG (maleimide-azobenzene-glutamate) onto the engineered cysteine, light was used to rapidly switch MAG between its active *cis* and its inactive *trans* forms, leading to rapid activation and deactivation of LiGluR.

Because glutamate receptors are nonselective cation channels, activation of LiGluR can be used to induce action potential firing in neurons both *in vitro*²⁹ and *in vivo*, in zebrafish larvae and mice retinas^{30,31}. As with channelrhodopsin-2, LiGluR can be used in neural circuit analysis as a tool for driving action potential firing in genetically targeted neurons. LiGluR has a much larger single-channel conductance than channelrhodopsin-2 (~250 fS versus ~40 fS)^{32,33}, so it provides more bang for the buck. This advantage is balanced by the

requirement for adding two exogenous components, a gene and a chemical photoswitch, which presumably makes implementation more difficult, especially in the mammalian brain.

Of course, LiGluR has something else that channelrhodopsin-2 does not: it can tell us about the physiological roles of GluK2. Kainate receptors are present throughout the CNS, yet their function remains a mystery, as the selectivity of the pharmacological tools available for the discrimination of kainate receptor subtypes is limited. The structural similarity between the clamshell-shaped ligand binding domains of kainate, AMPA and NMDA receptors should allow this strategy to be extended to other members of the glutamate receptor family.

Light-regulated acetylcholine and GABA_A receptors

GABA_A receptors and nicotinic acetylcholine receptors (nAChRs), which belong to the cyst-loop family of ligand-gated ion channels, can also be rendered photosensitive. Even before genetic manipulation of ion channels was feasible, a photoswitchable agonist was covalently tethered onto the nAChR³⁴. This tour de force was made possible by the presence in the acetylcholine-binding pocket of a reducible disulfide bond, which is found naturally in the nAChR. Controlling a specific nAChR subtype in neurons requires genetic engineering of the protein. Brain nAChR subtypes result from many different subunit combinations (for example, $\alpha 4\beta 2$, $\alpha 3\beta 4$, $\alpha 4\beta 2\alpha 5$, $\alpha 7$, etc.) and these combinations are differentially expressed in the CNS. nAChRs have been implicated in the pathophysiology of several psychiatric disorders, nicotine addiction and Alzheimer's disease. However, the role of specific subtypes in these disorders is unclear, largely because of limitations in subtype-selective nAChR pharmacology. We addressed this issue by engineering specific photosensitive nAChR subtypes³⁵. Structure-based design produced heteromeric $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs that could be activated or inhibited with light (LinAChRs). We designed two different photoswitchable ligands: a maleimide-azobenzene-acetylcholine (MAACh) agonist and a maleimide-azobenzene-homocholine (MAHoCh) antagonist. The same cysteine mutation, located on the β subunit in proximity to the acetylcholine binding site, was used for the conjugation of MAACh or MAHoCh, providing bidirectional control of nAChR function. The photo-inhibited receptor is useful for asking whether a given subtype is necessary for a physiological response and the photo-activated receptor is useful for addressing whether it is sufficient.

GABA_A receptors are also heteromultimers, and there are many subtypes containing different subunit combination. The subtypes are biophysically distinct and differentially distributed, not only between different neuronal types, but even within a single neuron. Thus, different GABA_A receptor subtypes are predominantly located synaptically versus extra-synaptically, exerting phasic versus tonic inhibition, respectively. The different subtypes also have different sensitivities to pharmacological agents, such as benzodiazepines and barbiturates. However, pharmacology alone gives ambiguous answers about which of the many subtypes mediates specific physiological effects. In principle, optogenetic pharmacology could provide answers to these questions. We have successfully conferred light sensitivity onto specific GABA_A receptor subtypes by genetically engineering a cysteine in the α subunit, close to the GABA binding site, and covalently attaching a photoswitchable competitive inhibitor (unpublished data).

Light-regulated metabotropic neurotransmitter receptors

Two strategies have been developed to optically control G protein–coupled receptor (GPCR) signaling with light. GPCRs are seven-transmembrane segment proteins that can be activated by various stimuli, such as neurotransmitters, odors, hormones and even light, as in the case of rhodopsin. Their activation triggers intracellular signal transduction pathways, the nature of which is dependent on which particular G protein(s) is coupled to the receptor. For example, rhodopsin, which has a covalently bound cofactor retinal, activates the G protein transducin G_t , which in turn activates cGMP phosphodiesterase. The similarity between class I GPCRs members has made it possible to engineer chimeras between rhodopsin and the β_2 (which couples to G_s) or the α_1 adrenergic receptor (which couples to G_q)³⁶. In these chimeras (OptoXRs), the binding site for catecholamines is swapped for the binding site for retinal and the intracellular loops that bind the G protein are conserved. This manipulation substitutes receptor sensitivity to catecholamines with receptor sensitivity to light, providing an optogenetic tool for controlling intracellular signaling cascades. However, given that retinal does not photoisomerize back to *cis* after activation, OptoXRs exhibit incomplete recovery following stimulation.

The second strategy for photosensitizing GPCRs is the optogenetic pharmacology approach, which leaves sensitivity to endogenous neurotransmitters intact. Recently, light-agonized and light-antagonized metabotropic glutamate receptors (mGluRs) of the mGluR2, mGluR3 and mGluR6 families have been generated, which all couple to the $G_{i/o}$ pathway to inhibit adenylyl cyclase³⁷.

Photocontrol of intracellular signaling proteins

On the horizon is the prospect of controlling other aspects of neuronal function with light. Investigators have again ‘borrowed’ light-sensitive proteins from a non-animal system for use in mammalian cells. For example, PAC is a microbial light-sensitive adenylate cyclase that, when expressed in mammalian cells, enables photocontrol of cAMP synthesis³⁸. Instead of using entire proteins, specific light-sensitive domains can be employed. For example, the flavin-binding LOV domain from the plant phototropin-1 protein was spliced onto Rac, a small GTPase protein involved in cell motility. The result was a light-sensitive regulator of cytoskeletal function, which enables photocontrol of cell shape³⁹. The LOV domain has also been spliced onto the membrane protein-localizing PDZ domain, yielding a fusion protein that rapidly and reversibly translocates to the membrane of mammalian cells when exposed to 500-nm light⁴⁰. Another example is the photochromic domain of the fluorescent protein Dronpa, which has been fused onto the N or C termini of recipient proteins, resulting in caged enzymes that can be relieved from blockade with visible light⁴¹. These and other light-sensitive constructs are just waiting to be applied to the nervous system and could enable *in vivo* manipulation. Perhaps the most exciting prospect is the development of a photosensitive transcription factor⁴², which could enable precise temporal and spatial control of the expression of particular genes in a light-dependent manner.

Experimental opportunities provided by optogenetic pharmacology

For many decades, conventional pharmacology has been providing us with a wealth of information about ion channels, neurotransmitters and receptors in the brain. The emergence of optogenetics is allowing us to answer questions at the level of neural circuits. However, in principle, optogenetic pharmacology can give us information that neither optogenetics nor conventional pharmacology alone can provide. Optogenetic pharmacology not only enables fine spatial and temporal control of neurons with cell-type specificity, but has the added value of differentiating between different postsynaptic receptors with biochemical precision, an elusive goal of conventional pharmacology. Thus far, optogenetic pharmacology has mainly been applied to synapses and circuits in reduced neural preparations *in vitro* (Fig. 3). *In vivo* use has been largely limited to studies in invertebrates and embryonic zebrafish^{30,37}, where photoswitch molecules can be introduced into the nervous system simply by including them in the water in which the fish swim. Only recently has optogenetic pharmacology been employed in the eye to restore light sensitivity to blind retinas of mutant mice³¹. Broader implementation of optogenetic pharmacology in the mammalian brain *in vivo* should soon be feasible and could open up major new avenues of research into the molecular basis of brain function.

Optogenetic pharmacology offers some unique opportunities for *in vivo* investigation, for example, linking particular neuromodulatory systems to brain state and behavior. The advent of microbial opsins has given investigators a way to optogenetically stimulate or suppress subsets of neuromodulatory afferents using different neurotransmitters, including acetylcholine, dopamine or serotonin^{43,44}. However, most neuromodulators act on multiple receptor classes (as many as 14 for serotonin)⁴⁵, with different subtypes able to exert diverse physiological effects through different intracellular signaling pathways. Unlike conventional optogenetics, which can only demonstrate that a given neuromodulatory pathway is involved in a physiological phenomenon, optogenetic pharmacology could unambiguously identify which receptor is involved and on which cell type. This could be accomplished by using *cre-loxP* technology to target the light-sensitive receptor to a particular cell class. After photoswitch conjugation, control by light is orthogonal to the normal regulatory mechanisms that activate the channels. Thus, voltage-gated channels retain their normal voltage sensitivity and neurotransmitter receptors retain their normal sensitivity to endogenous or exogenous agonists or antagonists. Although conventional optogenetics can be used to determine whether a specific neuromodulator is involved in a behavior, optogenetic pharmacology can take the further step of discerning which particular receptor(s) underlies the response. And with the advent of tools tuned to operate at different wavelengths, optogenetics and optogenetic pharmacology could be used together to transform our molecular to systems level analysis of synaptic information processing in the brain.

Although mapping of neural circuits with optogenetics is typically a presynaptic manipulation, we expect that optogenetic pharmacology will typically be a postsynaptic manipulation. Photosensitizing a particular receptor allows a user to distinguish the effect of a given neurotransmitter on different cell types. For instance, GABAergic inhibition between inhibitory interneurons is emerging as an important component of neural computation⁴⁶. With optogenetics, investigators typically activate or silence a given subtype of interneuron,

but this has the necessary consequence of suppressing or driving GABA release onto all of its targets, and the interpretation of the optogenetic effects are therefore limited^{47,48}. In contrast, optogenetic pharmacology allows control over the postsynaptic GABA_A receptors on a specific interneuron subtype. This will help to elucidate the role that inhibition onto specific cell types has in neural function. Of course, this also bears the limitation that GABAergic inhibition from any presynaptic source onto that cell type will be blocked. Thus, a combination of optogenetics and optogenetic pharmacology, with their complementary advantages, should prove to be powerful.

Challenges of implementing optogenetic pharmacology *in vivo*

What are the major obstacles to the successful application of optogenetic pharmacology *in vivo*? First, the gene encoding a photoswitch-ready (that is, cysteine substituted) channel or receptor must be introduced and expressed in the nervous system. Overexpression of the gene (for example, with a viral vector) could alter receptor density or localization in the cell. If the mutant gene is expressed in a cell still possessing the wild-type gene, light-sensitive and light-insensitive channels will co-exist (Fig. 4a), resulting in incomplete photocontrol. However, these issues are less problematic if the channel or receptor is an obligate heteromultimer²⁶. As long as the photoswitch-ready subunit cannot form functional channels by itself, the overall density and distribution of channels will be unadulterated (Fig. 4b). Of course, concerns about overexpression can be removed entirely by genetically substituting the wild-type channel with the photoswitch-ready version by making a knock-in mouse (Fig. 4c).

The next issue is how to apply the photoswitch compound. Attachment of the photoswitch requires that the cysteine site on the channel be freely accessible, which may make it necessary to include a reducing agent, such as dithiothreitol or *tris*(2-carboxyethyl)phosphine. Fortunately, maleimide conjugation is irreversible, and the photoswitch can be applied hours (or perhaps days) before the actual experiment, depending on the turnover rate of the targeted receptor. Possible off-target effects of the photoswitch, stemming from attachment to reduced cysteines on other membrane proteins, will need to be evaluated. At least for *in vitro* systems, optogenetic pharmacology has proven to be remarkably specific for the targeted channel or receptor, with native versions devoid of the introduced cysteine remaining functionally unaltered after exposure to the photoswitch compound. There is the possibility of side effects that would not be apparent in patch-clamp experiments, but these may be irrelevant for the neural function in the time frame of short-term experiments.

Finally, there is the issue of light delivery. Most currently available photoswitches require ultraviolet (~380 nm) light to transition from the *trans*- to the *cis*- state. However, ultraviolet light poorly penetrates brain tissue and can result in cellular toxicity at high intensity. Fortunately, red-shifted photoswitches have recently been synthesized that can be activated with visible light¹⁵. Thus, although there are some technical challenges to making optogenetic pharmacology a practical and useful system for *in vivo* physiological and behavioral research, none of the obstacles are insurmountable.

Concluding remarks

The emergence of new optogenetic technologies for visualizing and manipulating neuronal firing has provoked efforts to construct maps that reveal brain activity at the cellular, circuit and systems level. Underlying all of the electrical firing and synaptic activity in the brain is a complex assortment of neuronal signaling proteins, including myriad ion channels and receptors. Changes in channel or receptor activity are at the root of many neurological disorders. By providing a new way to control these proteins with spatial, temporal and biochemical precision, optogenetic pharmacology could help to bring the ‘brain activity map’ down to the molecular level, giving us new insights into the mechanisms of brain function, both in health and in disease.

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Figure 1. Photochemical tools for controlling neural function. **(a)** Optogenetics. **(b)** Optopharmacology. **(c)** Optogenetic pharmacology.

Figure 2.

Three methods of photocontrol for mapping mechanisms of brain function. **(a)** Left, optogenetics with microbial opsins allows photocontrol of the activity of neurons expressing the foreign gene, such as with ChR2, without any exogenous cofactors. Right, the opsin can be targeted to a specific cell type using genetic approaches permitting one to selectively control the activity of a presynaptic population's activity (green cells) while monitoring the functional effect on postsynaptic cells downstream. **(b)** Optopharmacology, such as with glutamate uncaging, allows photocontrol of any neuron that expresses the appropriate

receptor (in this case, glutamate receptors). Two-photon uncaging can locally activate receptors in a small region, for example, on a dendritic spine (left). Laser-scanning photostimulation can activate receptors on a large scale, for example, in a population of presynaptic neurons (right). (c) Optogenetic pharmacology allows photocontrol of native receptor subtypes. This is receptor specific (left) and can be cell-type specific (right, green cells).

Figure 3.

Optogenetic pharmacology enables photocontrol of ion channels and neurotransmitter receptors in mouse hippocampal neurons. **(a)** In a neuron expressing photosensitive SK2 channels, depolarization leads to an afterhyperpolarizing tail current (I_{AHP}) that can be modulated by light. Depolarization causes Ca^{2+} influx and SK2 is a Ca^{2+} -activated K^+ channel. **(b)** Photocontrol of SK2 modulates the amplitude of NMDA receptor-mediated excitatory postsynaptic potentials (EPSPs). NMDA receptor activation causes Ca^{2+} influx, which activates nearby SK2 channels. Recordings are from a CA1 neuron in a hippocampal slice that virally expressed photoswitch-ready SK2. Modified from ref. 25. **(c)** In a neuron expressing photosensitive mGluR2 receptors, autaptic excitatory postsynaptic current

(EPSC) amplitude can be regulated by light. (d) Photosensitive mGluR2 enables light modulation of presynaptic release. Photocontrol of mGluR2 modulates presynaptic voltage-gated Ca^{2+} , leading to changes in paired-pulse facilitation, an indicator of release probability. Autaptic recordings are from a hippocampal neuron in primary culture. Modified from ref. 37. In all panels, green traces were obtained in 500-nm light (photoswitch in *trans*) and violet traces in 380-nm light (photoswitch in *cis*).

Figure 4.

Expression options for optogenetic pharmacology. **(a)** The photoswitch-ready protein (gray) can be overexpressed to form exogenous light-sensitive channels that co-exist with native light-insensitive channels (white). Overall channel number is increased. **(b)** The photoswitch-ready protein may form heteromers with endogenous subunits, thereby conferring light sensitivity onto the hybrid channels. Depending on the availability of the endogenous subunits, hybrid light-sensitive channels may partially or fully replace the native light-insensitive channels. Overall channel number may or may not be increased. **(c)** The photoswitch-ready protein can genetically replace the wild-type protein in a knock-in mouse. All of the channels are light-sensitive and overall channel number is unchanged.

Table 1

Optopharmacological and optogenetic tools for neuronal control

Optical tool	Light sensor (cofactor)	Signal	<i>In vivo</i> application	Spectral sensitivity	Kinetics	Refs.	Refs. <i>in vivo</i>
Optopharmacology							
DMNB-Glu	DMNB	Exogenous	Activation of iGluRs	Ultraviolet	ms	8	
MNI-Glu	MNI	Exogenous	Activation of iGluRs	Ultraviolet or two photon	ms	2,7,9	49
NI-Glu	NI	Exogenous	Activation of iGluRs	Ultraviolet	ms	5,7	
RuBi-Glu	RuBi	Exogenous	Activation of iGluRs	Blue or two photon	ms	12	
MNI-GABA	MNI	Exogenous	Activation of GABA _A Rs	Ultraviolet or two photon	ms	7	
RuBi-GABA	RuBi	Exogenous	Activation of GABA _A Rs	Blue	ms	11	
CANBP-GABA	ANBP	Exogenous	Activation of GABA _A Rs	Ultraviolet or two photon	ms	10	
CNB-L-enkephalin CNB-dynorphin	CNB	Exogenous	Activation of opioid receptors	Ultraviolet	ms	13	
AAQ	Azobenzene	Exogenous	Block of K ⁺ channels	Violet and green	ms to s	16,17	18
QAQ	Azobenzene	Exogenous	Block of K ⁺ , Na ⁺ and Ca ²⁺ channels	Violet and green	ms to s		19
DENAQ	Amino-azobenzene	Exogenous	Block of K ⁺ channels	Blue	ms to s	15	
GluAzo	Azobenzene	Exogenous	Activation of kainate receptors	Violet and green	ms to s	21	
Azo-propofol MPC088	Azobenzene	Exogenous	Potentiation of GABA _A Rs	Violet and green	ms to s	20,50	
ANQX	Azido	Exogenous	Irreversible block of AMPARs	Ultraviolet	s	22	
Optogenetics							
Channelrhodopsins (ChR2, H134R, ChIEF, ChETAs, VChR1...)	Retinal	Endogenous	Depolarization	Blue to red, or two photon	ms		1
Halorhodopsin	Retinal	Endogenous	Hyperpolarization	Yellow to red	ms		1,48
Archaeorhodopsin	Retinal	Endogenous	Hyperpolarization	Green	ms		1
Mac	Retinal	Endogenous	Hyperpolarization	Blue to green	ms	1	
OptoXRs	Retinal	Endogenous	Intracellular signaling	Blue to green	s		36
Optogenetic pharmacology							

Optical tool	Light sensor (cofactor)	Signal	<i>In vivo</i> application	Spectral sensitivity	Kinetics	Refs.	Refs. <i>in vivo</i>
SPARK (and other designer K ⁺ channels)	Azobenzene	Block of K ⁺ channels	N.R.	Violet and green	ms	24–26	
LiGluR	Azobenzene	Activation of iGluRs	Mice, zebrafish	Violet and green	ms	28	29–31
LinAChR	Azobenzene	Activation and inhibition of nAChRs	N.R.	Violet and green	ms	35	
LiGABAR	Azobenzene	Inhibition of GABA _A Rs	N.R.	Violet and green	ms		
LimGluR	Azobenzene	Activation and inhibition of mGluRs	Zebrafish	Violet and green	ms to s		37

N.R., not reported.