Current Challenges in Optogenetics

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Introduction

Studying intact systems with simultaneous local precision and global scope is a fundamental challenge of biology, and part of the solution may be found in optogenetics. This field combines genetic and optical methods to achieve gain or loss of function of temporally defined events in specific cells embedded within intact living tissue or organisms. Such precise causal control within the functioning intact system can be achieved by introducing genes that confer to cells both light-detection capability and specific effector function. For example, microbial opsin genes can be expressed in neurons to mediate millisecond precision and reliable control of action potential firing in response to light pulses (Boyden et al., 2005; Ishizuka et al., 2006; Yizhar et al., 2011). Indeed, this approach has now been used to control neuronal activity in a wide range of animals and systems, yielding insights into fundamental aspects of physiology as well as into dysfunction and possible treatments for pathological states (Fenno and Deisseroth, 2013). Many other strategies for optical control (besides the microbial opsin gene approach) may be applied as well (Möglich and Moffat, 2007; Wu et al., 2009; Airan et al., 2009; Stierl et al., 2011). Yet despite the field's diversity of approaches, rapid growth, and wide scope of applications, fundamental challenges remain to be addressed in basic technology development. In this chapter, we review these challenges as well as the opportunities at hand, and aspects of the figures and text build on the findings of recent reviews (Yizhar et al., 2011; Zhang et al., 2011; Fenno and Deisseroth, 2013; Zalocusky and Deisseroth, 2013).

Background: Current Functionality of Tools

Diverse and elegant mechanisms have evolved to enable organisms to harvest light for survival functions (Fig. 1). For example, opsin genes encode 7-transmembrane (7-TM) proteins that, when bound to the small organic chromophore all-*trans* retinal, constitute light-sensitive rhodopsins, which are found across all kingdoms of life. Many prokaryotes employ these proteins to control proton gradients and to maintain membrane potential and ionic homeostasis, and many motile microorganisms have evolved opsin-based photoreceptors to modulate flagellar motors and thereby direct phototaxis toward environments with optimal light intensities for photosynthesis. Owing to their structural simplicity (both light-sensing and effector domains are encoded within a single gene) and fast kinetics, microbial rhodopsins can be treated as precise and modular photosensitization components for introduction into non-light-sensitive cells to enable rapid optical control of specific cellular processes (Yizhar et al., 2011). Alternatively, the light receptor can be a small organic molecule that is introduced into the biological system, with or without a designed binding protein as effector. Many other nonopsin classes of naturally occurring proteins have been explored as well. These include flavin chromophore–utilizing light-activated enzymes, such as adenylyl cyclases, as well as engineered systems in which light-sensation modules become physically linked to effector modules (Möglich and Moffat, 2007; Airan et al., 2009; Wu et al., 2009; Stierl et al., 2011).

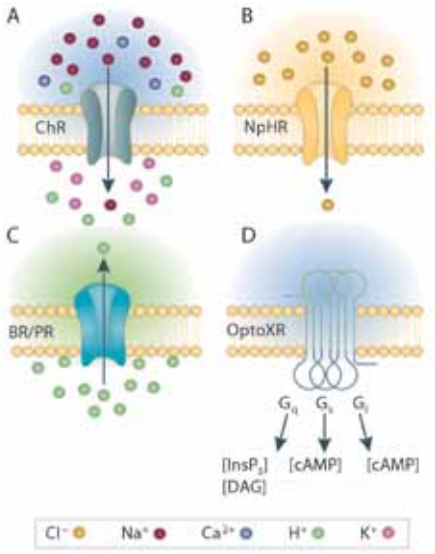

Figure 1. Single-component optogenetic tool categories. Four major classes of opsin commonly used in optogenetics experiments, each encompassing light sensation and effector function within a single gene, include: (1) ChRs, which are light-activated cation channels that give rise to inward (excitatory) currents under physiological conditions; (2) halorhodopsins (NpHR shown), which are inhibitory (outward-current) chloride pumps; (3) bacteriorhodopsins and proteorhodopsins (BR/PR), proton pumps that tend to be inhibitory and include archaerhodopsins; and (4) OptoXRs, which modulate secondary messenger-signaling pathways. Adapted from Zalocusky and Deisseroth (2013), their Figure 1.

The experimental potential of optogenetics has triggered a surge of genome prospecting and molecular engineering to expand the repertoire of tools and generate new functionality. This expansion, in turn, has catalyzed further mechanistic studies of microbial proteins (Zhang et al., 2011). High-resolution crystal structures are now available for most of the major photoreceptor modules, including most recently, channelrhodopsin (ChR) (Kato et al., 2012). This information has been important not only for enhancing understanding of mechanism but also for guiding optogenetics in the generation of variants with novel function related to spectrum, selectivity, and kinetics. For example, ChR variants

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have been engineered with shorter or longer open-state lifetimes, shifted absorption spectra, reduced desensitization, increased expression, and increased photocurrent magnitude (Yizhar et al., 2011; Mattis et al., 2012). Likewise, high-resolution crystal–structural insights have been used to help guide the assembly of light-sensitive modules, together with effector modules, into artificial proteins. In this way, parallel information streams have been created that are capable of carrying optogenetic control signals for modulation purposes (Möglich and Moffat, 2007).

This diversity of optogenetic tool function will be important for making significant headway in our understanding both of normal brain function and of dysfunctional processes in neuropsychiatric disease. For example, many disease states arise in part from impaired interaction of multiple distinct cell or projection types. This etiology points to the experimental value of achieving multicolor excitation and multicolor inhibition optogenetically within the same living mammalian brain for neuropsychiatry research. It is encouraging that optogenetic interventions have now built a secure foothold for the study of both normal function and brain disease states. Nevertheless, major areas of optogenetic tool advancement are required in the future, as detailed next.

Unsolved Problems and Open Questions in Technology

Addressing the technological challenges that follow, all squarely in the domain of modern neuroscience, will help provide experimental leverage that may lead to key insights into neural circuit function and dysfunction. Such insights would be difficult or impossible to establish by other means.

Cell biology

One group of technological challenges to be addressed by optogenetics lies within the natural domain of metazoan biology. The development of guided subcellular trafficking will be an important step, and membrane trafficking strategies have already improved the expression of opsins at the membrane (Gradinaru et al., 2008). Further exploration in this area may produce targeting strategies that will allow selective optogenetic tool expression in subcellular compartments such as dendrites, somata, or axon terminals. Indeed, while efforts have been made in this regard, achieving truly robust (near 100%) exclusion of heterologously expressed optogenetic proteins from axons would prevent the undesired optical drive of axons of passage during illumination of a transduced brain region. The expression of optogenetic tools in axons is one of the most useful features of this approach in that it allows "projection targeting"–based recruitment of cells defined only by selective illumination and projection pattern (Yizhar et al., 2011; Fenno et al., 2013; Fenno and Deisseroth, 2013; Zalocusky and Deisseroth, 2013). However, this effect also confounds certain kinds of functional mapping procedures that employ optogenetics.

Optics

It also would be valuable to develop a robust and versatile optical (nonpharmacological) strategy. This would prevent (when desired) the propagation of optogenetically elicited action potentials in the antidromic direction or along axon collaterals during projection-targeting experiments. Although this antidromic drive is sometimes desired, in other cases, it is not, i.e., when the experimenter seeks to allow generalizable selective excitation only of spatially defined projections and does not wish to take advantage of the existing capability to recruit cells defined by projection (Yizhar et al., 2011).

Behavior

Improved high-speed volumetric (three-dimensional) light delivery strategies with single-cell resolution would be of great value, in that populations of cells (even within intact mammalian brain tissue) could be recruited optogenetically with any required extent of synchrony or asynchrony. For example, optogenetics applications to questions of mammalian circuit dynamics and behavior *in vivo* have typically involved synchronous optogenetic control of entire genetically targeted cell populations over millimeter-scale spatial domains. Examples include studies of sleep–wake transitions, parkinsonian circuitry, gamma rhythms, feeding behavior, olfaction, aggression, and memory consolidation. Yet methods for guiding spatial delivery of multiple wavelengths of light excitation in three-dimensional volumes could yield much improved precision and complexity in optogenetic modulation. These methods would take the next step beyond the single-photon, guided-light strategies that have already been used, even in mammalian tissue, for applications such as highly refined optogenetic circuit mapping and dissection of anxiety circuitry. Optogenetic two-photon illumination could provide a distinct means of manipulating single or multiple genetically and spatially targeted cells with high temporal resolution over sustained intervals and within intact tissue volumes. This technique would then be able to delineate and define components that work in concert to generate circuit dynamics or behavior.

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One pioneering two-photon study was able to overcome the low, single-channel conductance of ChR2 and to produce action potentials in cultured neurons using complex scan patterns in order to open sufficient channels on individual neurons. Two-photon optogenetic manipulation of spatially and genetically defined cells within intact tissue volumes with simpler (standard) raster scanning would further broaden the reach of this approach to many laboratories worldwide. Two other reports of neuron activation in slice preparations with optogenetics relied on elegant hardware innovations and larger focal spots of laser illumination to overcome the modest conductance of individual channels. Other nonscanning methods, such as spatial light modulators (SLMs) and light-field microscopy, could allow myriad opportunities to probe the temporal mechanisms by which population codes are set up and employed in neural circuit function. Two recent reports have made headway in developing two-photon raster scanning and SLM-based methods for versatile optogenetic control in intact tissue or *in vivo* in mammals (Packer et al., 2012; Prakash et al., 2012).

Extension to other species and cell types

Robust extension of optogenetic tool-targeting strategies to non–genetically tractable species or cell types will be enormously helpful. The generation of Cre-driver rats has been important, and projection targeting provides an independent step forward.

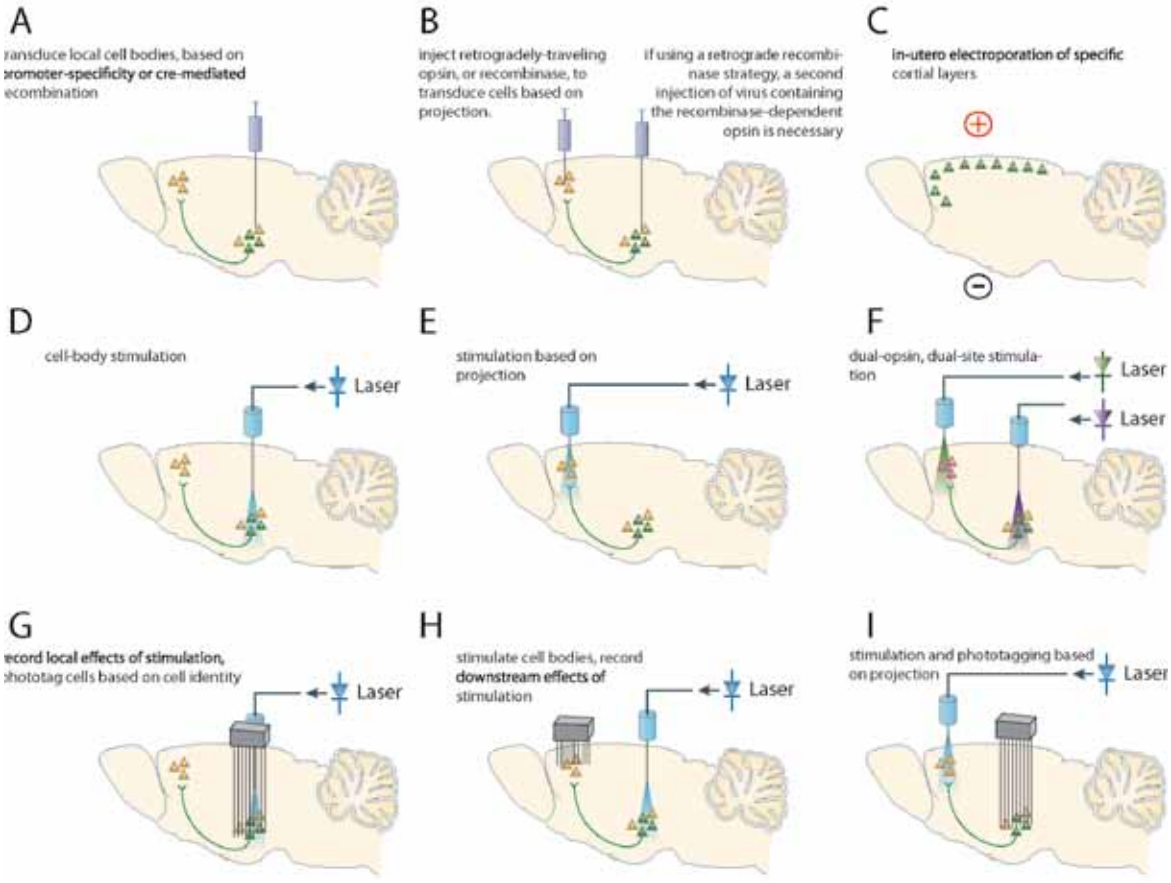

Figure 2. Optogenetic targeting and experimental design suitable for any vertebrate species, including mouse, rat, and primate. Panels *A–C* illustrate strategies for transducing the cell population of interest with an opsin. These include *A,* transduction of cell bodies via viral injection, *B,* single- or dual-virus retrograde strategies for projection-specific opsin expression, and *C, in utero* electroporation for cortical-layer-specific expression. Panels *D–F* illustrate possible configurations for optical stimulation, including *D,* illumination at the site of transduced cell bodies, *E*, illumination of downstream projections, and *F,* illuminating multiple distinct populations of cells at the same or different locations, which can express opsins sensitive to different wavelengths of light. Panels *G–I* illustrate combinations of electrical recording with optical stimulation. Possible configurations include *G,* recording at the site of optical stimulation, *H,* recording downstream of optical stimulation, and *I,* recording at transduced cell bodies while stimulating downstream projections. Adapted from Fenno and Deisseroth (2013) and Zalocusky and Deisseroth (2013), their Figure 2.

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But improved intersectional targeting strategies will also be crucial since few relevant cell types can be specified by only a single descriptor, e.g., cell body location, projection target, or activity of one promoter/enhancer region. Thus, designing and validating optogenetic tool-carrying viruses or other vectors that depend on multiple recombinases (for example, with Boolean and or other logical gates) will be essential. Also, developing improved methods to selectively exclude optogenetic tool expression in cells with a given genetic identity will be useful.

Wiring-based (connectomic) strategies

Finally, true retrograde and anterograde wiring-based strategies (i.e., targeting cells that project to a particular region, or cells that receive projections from a particular region) would greatly enhance the flexibility of optogenetic control, both in mice and in other species. Although such strategies exist, they are not always robust or well tolerated (Fenno and Deisseroth, 2013; Zalocusky and Deisseroth, 2013) (Fig. 2).

It would be immensely valuable to develop methods to rapidly and efficiently extract brainwide wiring (connectomic) patterns, or at least projection patterns, from optogenetically driven cells that had been shown to have a known and quantifiable impact on behavior in the very same animal. Further, it would be of great value to rapidly and efficiently extract the brainwide elicited-activity patterns arising from optogenetic control of a targeted population. This can be achieved to some extent with optogenetic functional magnetic resonance imaging (ofMRI), an optogenetic method that enables unbiased global assessment of the neural circuits upstream and downstream of focal stimulation. However, fMRI methods in general suffer from poor spatial and temporal resolution. Overall, improving the integration of optogenetic control with readouts—whether behavioral, electrophysiological, or imaging—will be important. Moreover, closing the loop so that neural activity or behavioral readouts can feed back and control the inputs played in via optogenetics will be of great interest, as will the development of computational methods to begin "reverse engineering" the studied circuitry by identifying the underlying transformations of information carried out in the tissue.

Unsolved Problems and Open Questions in Genomics and Biophysics

Another group of technological challenges to be addressed by optogenetics falls more into the natural domain of microbial biologists and protein biophysicists. (Of course, many laboratories and investigators span the metazoan and the microbial realms.)

Engineering better tools

The ongoing identification of additional genomically identified tools (e.g., via databases searches, broad-based next-generation sequencing efforts, and ecological genome mining) is expected to profoundly improve our ability to perturb and understand biological systems (Zhang et al., 2011; Mattis et al., 2012). Many thousands of new light-sensitive modules will be accessible in this way. For example, even though known opsins already span most of the visual spectrum and a broad kinetic space, it is very likely that new kinds of light sensitivity, kinetic properties, and even ion selectivity will emerge. One important goal is to move toward the infrared,

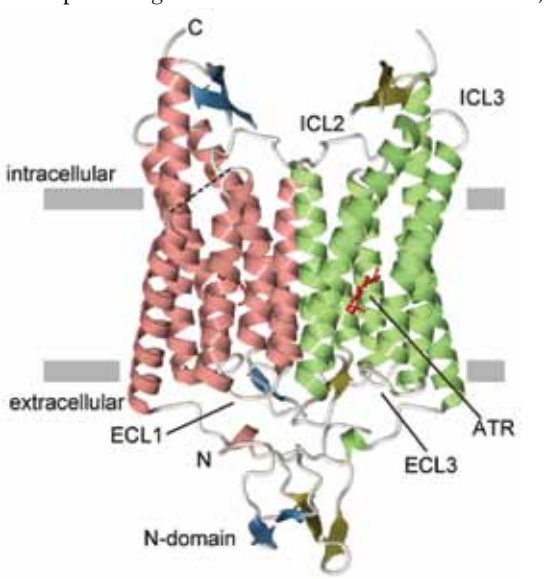

Figure 3. Channelrhodopsin crystal structure. This structure is of C1C2, a chimera between ChR1 and ChR2, consisting of the *N*-domain, with the 7-transmembrane helices connected by extracellular loops (ECLs) and intracellular loops (ICLs), and a truncated *C*-domain. All-*trans*-retinal (ATR) is red. This high-resolution structure includes description of the environment around the retinal-binding pocket, which will enable the optimized design of red- and blue-shifted ChR variants. In addition, the structure of the cation-conducting pathway may facilitate construction of ChR variants with improved photocurrents, photosensitivity, cation selectivity, and kinetics. Already, structure-guided mutagenesis has resulted in some degree of K^+ selectivity, which could be useful for suppressing neural activity. Further structural studies, including determination of crystal structures in intermediate states, are clearly needed that will help enable the principled design of ChR variants with new properties. These, in turn, will both accelerate the applications of optogenetics to intact-systems biology and further the basic mechanistic understanding of these remarkable photoreceptor proteins. Adapted with permission from Kato et al. (2012). © Nature Publishing Group.

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which will (1) achieve deeper light penetration at a given irradiance value; (2) reduce scattering (for improved resolution); and (3) provide an additional control channel. Infrared actuation has already been achieved for certain nonopsin-based optogenetic approaches, but it may encounter physics-based limitations for retinal-based photoreceptors.

Engineering these known or new tools for narrowed (as well as shifted) action spectra would enable cleaner separation of control channels. For example, engineering blue-shifted hyperpolarizing opsins with narrower activation wavelength spectra could ultimately allow researchers to enhance combinatorial neuronal inhibition experiments within scattering mammalian tissue volumes. Although action-spectrum peaks for existing tools span the visible spectrum and beyond, the broad shoulders of relevant action spectra might prevent the use of more than 2–3 channels of control at once, unless spectra can be narrowed. Such efforts might involve mutations that prevent access of the photocycle to specific states or intermediates that have shifted absorbance properties. This class of engineering will be facilitated by structure-based insights into the photocycle. For example, to understand the ChR photocycle in more detail, it will be necessary to undertake studies beyond the current closed-state structure ones (Kato et al., 2012; Fig. 3) and including open and intermediate photocycle states. These efforts may also lead to the generation of mutants with novel kinetic properties (Stehfest and Hegemann, 2010).

Light-sensitive pumps and channels

Engineering optogenetic light sensors for higher quantum efficiency, greater light sensitivity, and/or increased biological effect (e.g., current) elicited per protein molecule would be of substantial value because it would enable the use of lower irradiances for targeting a given tissue volume or depth. Also, lower irradiance might be important for minimizing photo damage, heating, or power use/deposition constraints (Lin et al., 2009; Yizhar et al., 2011; Mattis et al., 2012). Although, for opsins, many orders of magnitude of increased light sensitivity can be achieved using the bistable or step-function opsin (SFO) approach, this comes at a kinetic cost: slowing down the deactivation after light-off (Stehfest and Hegemann, 2010).

Developing a potent electrically inhibitory optogenetic channel (rather than a pump) would be of immense value. Current hyperpolarizing tools are pumps rather than channels, and therefore do not provide shunting or input-resistance changes (also, they can move only one ion per photon). As a result, these optogenetic tools are not nearly as effective as the channels or native inhibitory receptors—especially in projection-targeting experiments whose goal is to intercept action potentials in axons. Developing a potent electrically inhibitory optogenetic channel would also rapidly enable the generation of a hyperpolarizing SFO or bistable optogenetic tool (Stehfest and Hegemann, 2010; Yizhar et al., 2011; Fenno et al., 2013). This tool would enable sustained inhibition of neurons without requiring constant illumination. New structural knowledge of the ChR cation-conducting pathway and pore vestibules may facilitate construction of ChR variants with potassium selectivity for this purpose, as well as improved photocurrents, light sensitivity, and kinetic properties (Kato et al., 2012).

Controlling for opsin effects

Elsewhere, we have cautioned that powerful and prolonged light delivery can cause heating effects that could, in principle, alter neural activity even in nonexpressing cells. We have provided quantitative estimates of the magnitude of this effect (Yizhar et al., 2011). This potential confound can be addressed by maintaining moderate-intensity or pulsed-light protocols and by including experimental cohorts in which no opsin is expressed but all other manipulations are performed in the target animals (e.g., surgery, viral transduction, hardware implantation, and light delivery) (Yizhar et al., 2011). Similar controls are useful for identifying and/or correcting for confounds linked to any perception of the light by the animal's sensory systems. Moreover, overexpression of any foreign protein could cause altered structure, function, or survival of host cells, and opsins are no exception to this rule.

Despite these caveats, optogenetic methods do intrinsically provide a powerful means for controlling for such effects by allowing light-on and light-off assessment of physiology or behavior in each experimental subject. This technique ensures normal baseline behavior in the same animal at virtually the same time. Further, overexpression of control proteins in parallel experimental animals allows the experimenter to ensure that light effects are not being observed only because the animal or tissue is in an unusual state imposed by opsin expression independent of optical activation. Fluorescent proteins (XFPs) are most often employed as this control protein, since opsins are often expressed as XFP fusions; ongoing work is focusing on developing

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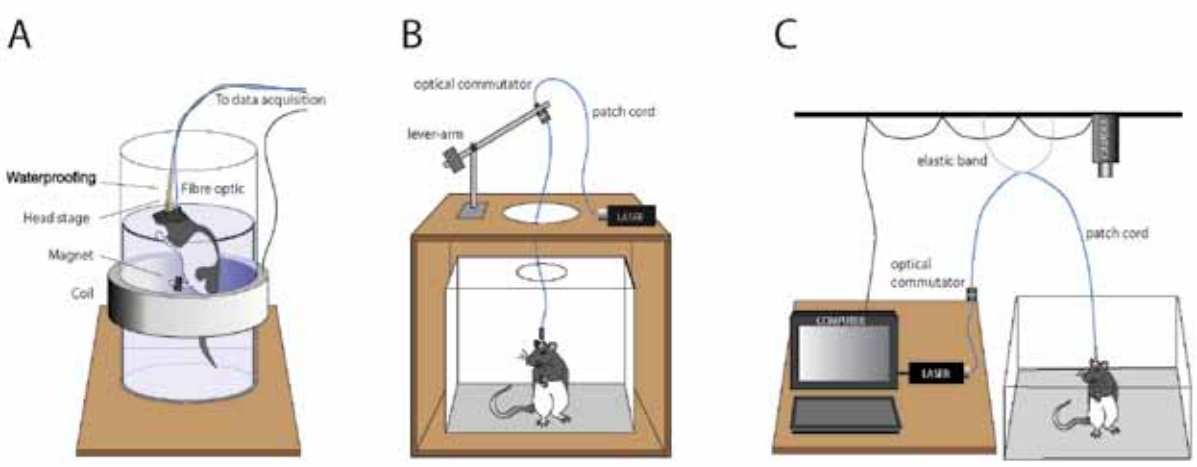

Figure 4. Integrating optogenetics with behavior. Diverse behavioral rigs can be outfitted for optogenetic experimentation. *A,* The forced swim test has been automated with magnetic induction–based detection of kicks combined with optogenetic stimulation and electrical recording. *B,* Operant behavior can also be combined with optogenetics. The chamber itself is modified to accommodate the entry of fiber optics and recording wires, and the stimulation/recording assembly is kept out of reach of the rodent with a counterweighted lever arm. *C,* Optogenetic manipulations can also be combined with behavior in open fields or large mazes. In these experiments, an elastic band, rather than a lever arm, is used to support stimulation/recording equipment. Video recording, combined with custom or commercially available software, can be used to synchronize optical stimulation with behavior. Adapted from Zalocusky and Deisseroth (2013), their Figure 4.

photocurrent-null opsins for improved experimental control purposes. Such truly "dead" optogenetic tool mutants, having expression and targeting properties comparable with active tools but with no light-induced effector function, would be useful as controls to ensure that effects seen are specifically the result of optical recruitment of opsins in targeted cells. However, it will be important to ensure that the photocurrents are truly zero even under high membrane expression levels *in vivo*. Knowledge of pore structure (and pump mechanisms) may facilitate the generation of such tools (Kato et al., 2012).

Optically recruited biochemical signaling

In addition to light-sensitive pumps and channels, continued expansion of optically recruited biochemical signaling will be a significant development. Increasing attention should be given to strategies for recruiting modular and easily programmable signaling pathways, improving specificity, expanding spectral responsivity bands, and adapting to additional classes of native chromophores (e.g., flavins and biliverdins) (Stierl et al., 2011). We expect to see the OptoXR family of light-activated 7-TM neurotransmitter/neuromodulator receptors adding novel tools based on chimeras between vertebrate rhodopsins and both well-known and orphan G-protein coupled receptors (GPCRs) (Airan et al., 2009). In addition, light-sensitive domains are being added to an increasing number of receptors and even intracellular signaling proteins. In this way, optogenetics promises to expand to occupy the full breadth of cell signaling, far beyond the study of neural activity (Wu et al., 2009).

Conclusion

The discovery and engineering of new and improved classes of optogenetic control will come from continued microbial and biophysical investigations into ecological diversity, high-resolution structures, photocycle properties, and functional phylogenetics of light-sensitive protein modules. Moreover, investigations from the neuroscience side will fundamentally advance the scope and precision of resulting insights into complex intact biological systems (Fig. 4). These investigations will explore targeting, trafficking, selective spatiotemporal properties of illumination, precise circuit-element recruitment, and diverse readout engineering and analysis. In optogenetics, existing methods represent only the tip of the iceberg in terms of what may be ultimately achieved in maximally enabling this technique's principled design and application.

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