Optogenetics: the age of light

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The optogenetic revolution is transforming neuroscience. The dramatic recent progress in using light to both control and read out neural activity has highlighted the need for better probes, improved light delivery and more careful interpretation of results, which will all be required for optogenetics to fully realize its remarkable potential.

Use light to read out and control neural activity! This idea, so easily expressed and understood, has fired the imagination of neuroscientists for decades. The advantages of using light as an effector are obvious[1]: it is noninvasive, can be precisely targeted with exquisite spatial and temporal precision, can be used simultaneously at multiple wavelengths and locations, and can report the presence or activity of specific molecules. However, despite early progress[2] and encouragement[3], it is only recently that widely usable approaches for optical readout and manipulation of specific neurons have become available. These new approaches rely on genetically encoded proteins that can be targeted to specific neuronal subtypes, giving birth to the term 'optogenetics' to signal the combination of genetic targeting and optical interrogation[4]. On the readout side, highly sensitive probes have been developed for imaging synaptic release, intracellular calcium (a proxy for neural activity) and membrane voltage. On the manipulation side, a palette of proteins for both activation and inactivation of neurons with millisecond precision using different wavelengths of light have been identified and optimized.

The extraordinary versatility and power of these new optogenetic tools are spurring a revolution in neuroscience research, and they have rapidly become part of the standard toolkit of thousands of research labs around the world. Although optogenetics may not yet be a household word (though try it on your mother; she may surprise you), there can be no better proof that optogenetics has become part of the scientific mainstream than the 2013 Brain Prize being awarded to the sextet that pioneered optogenetic manipulation (http://www.thebrainprize.org/flx/prize_winners/prize_winners_2013/) and the incorporation of optogenetics as a central plank in the US National Institutes of Health BRAIN Initiative[5]. Moreover, there is growing optimism about the prospect of using optogenetic probes not only to understand mechanisms of disease in animal models but also to treat disease in humans, particularly in more accessible parts of the brain such as the retina[6].

Despite all of this understandable exuberance, notes of caution are being sounded. The widespread use of optogenetic tools has led to a transition from proof-of-principle demonstrations to experiments that try to address difficult biological questions. This transition has been accompanied by a growing recognition of the limitations of current optogenetic approaches, which is in turn spurring further developments. This Commentary will briefly review recent successes in applying optogenetic strategies to understanding brain function, discuss some of the pitfalls that have been identified and point to future developments.

The good

A remarkable feature of the optogenetic approach is the ability to target probes to genetically defined cell types and subcellular compartments, which allows the probes to be used for investigating multiple levels of nervous system function (**Fig. 1**). As a consequence, optogenetics has penetrated all areas of neuroscience, from exploring the properties of individual synapses to investigating defined cell types within and across neural circuits to imaging entire brains and manipulating complex behaviors. These approaches are increasingly being used to study not only basic mechanisms of brain function but also mechanisms underlying animal models of disease[7]. The fact that optogenetic activators and inhibitors can be expressed in the same cells is crucial for testing both necessity and sufficiency, the twin elements required to establish causal relationships. The development of optogenetic inhibitors is particularly notable because scientists previously lacked the ability to conduct millisecond-precise loss-of-function experiments in neural circuits. Together, this combination of specificity, bimodality and breadth of implementation gives optogenetics the power to connect different levels of nervous system function, providing researchers with direct causal explanations for how the machinery of the brain drives high-level functions such as behavior and cognition.

Crucial to this new experimental landscape has been the development of novel optogenetic probes, which is continuing to progress at a stunning pace. Channelrhodopsin and its variants have recently been subjected to extensive molecular tinkering, producing such gems as C1V1, a variant with properties more favorable for two-photon excitation[8–10], and ReaChR, a red-shifted variant that enables activation deep in the brain or through the intact skull[11]. Most dramatically, the high-resolution crystal structure of a channelrhodopsin[12] has recently helped guide

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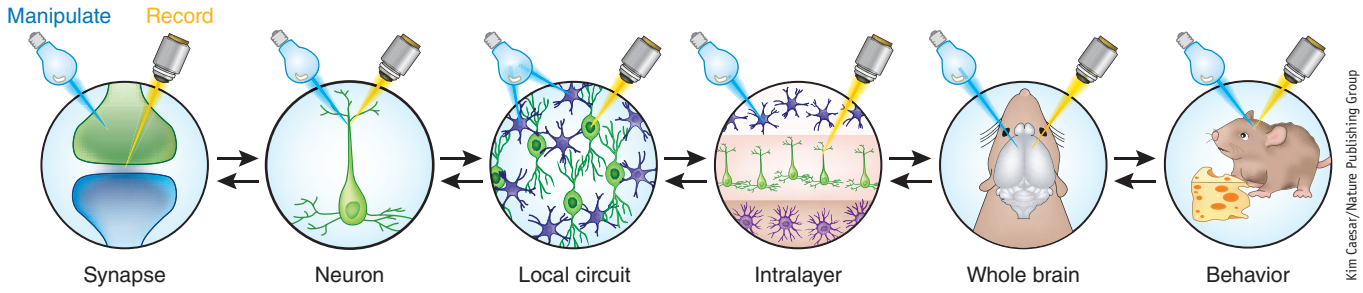

Figure 1 | Optogenetics can be applied at all levels of brain function. A variety of applications use optogenetic probes to both read out and manipulate activity. Specificity can be achieved either by targeting probe expression to relevant cellular compartments or network elements or by targeting light to these elements (see ref. 25 for discussion). The ability to implement optogenetics at different levels of nervous system function provides a powerful way to make causal links between these levels. Figure adapted from Hegemann, P. & Sigrist, S. (eds.), *Optogenetics*. De Gruyter, 2013, p. 109, fig. 10.1.

the molecular reengineering of the channel pore from cation to chloride conducting, converting it from an excitatory to an inhibitory channel[13,14]. New inhibitory opsins have also been identified from natural sources, including the now widely used proton pump Arch[15] and the recently discovered Jaws, which has been engineered to be red shifted to allow for more effective inhibition deeper in the brain[16]. On the readout side, structure-guided design of a new generation of genetically encoded calcium sensors such as GCaMP6 have finally brought the dream of *in vivo* single-spike sensitivity within reach[17]. Furthermore, the recent introduction of Twitch, a new family of ratiometric calcium sensors, may have advantages for long-term *in vivo* imaging of basal calcium levels[18]. There has also been significant recent progress in designing genetically encoded voltage sensors, most notably the newly developed QuasAr family, which offers unprecedented voltage sensitivity and rapid kinetics[19].

These remarkable advances have highlighted the emerging principles governing the development of optogenetic tools, which is rather different from the type of tool development that has pushed neuroscience forward in the past. In particular, progress in optogenetics depends on using molecular and biophysical tools to discover, identify and characterize promising molecules in the natural world; this is followed by structurally guided molecular engineering of these proteins (in combination with high-throughput screening), including changing their spectral properties, kinetics and ionic specificity. Molecular and cell biology tools are then used to target the engineered proteins to the elements of neural circuits over which the investigator wishes to exert control (**Fig. 1**). Thus, optogenetics relies on, and is helping to create, new and fascinating intersections between fields as disparate as botany, microbiology, structural biology, biophysics, cell biology and neuroscience.

The bad

Several criticisms have been leveled against the typical use of optogenetic activators, in which large numbers of genetically specified neurons in a circuit express an optogenetic probe that is then activated by bulk illumination of the tissue. First, the level of stimulation risks driving neuronal responses outside the physiological range, which is particularly hard to assess because it is not usual practice to record neural activity simultaneously in such experiments. This may in turn cause unnatural plasticity in the circuit, as well as engage (or disengage) downstream elements that may not normally be affected by the target population, leading to physiologically incorrect conclusions about circuit function. The problem is not restricted to the use of activators because silencers can drive the cells below their normal operating range, and the subsequent release of optogenetic silencing may cause rebound excitation[20]. Second, light stimulation and optogene expression are not uniform across the target neuron population, generating heterogeneity in the magnitude and spatial extent of optogenetic manipulation. Third, bulk stimulation precisely synchronizes the target neural population, potentially driving the circuit into unphysiological patterns of activity; this is particularly worrying given that millisecond-precise synchrony across large populations of neurons appears to be rare in mammalian neural circuits. Finally, conventional optogenetic stimulation usually activates neurons indiscriminately within a genetically defined population and cannot selectively activate subtypes within that population, for example, on the basis of stimulus selectivity or other functional signatures. Scientists are beginning to address this problem by using optogene expression driven by immediate early genes[21,22], but these experiments still lack any functional readout independent of immediate early gene activation.

The locus of stimulation also has important implications for the correct interpretation of optogenetic experiments. Stimulation of axons expressing optogenetic probes is a commonly used strategy for localizing the site of action for a particular projection. However, direct optical stimulation of axonal boutons can produce unphysiological transmitter release that can lead to the overestimation of the impact of a synaptic connection[23,24]. In addition, direct optogenetic stimulation of axons can cause antidromic activation, (i.e., reversed conduction of action potentials), which can in turn engage the cell bodies of origin and any collateral branches projecting to other brain areas. This problem is difficult to control for given that even chemical silencing of the cell bodies of origin may not prevent antidromic activation of collaterals emerging from the same parent axonal branch.

The ugly

Beyond the difficulties of interpreting the results of optogenetic manipulations, the implementation of optogenetic probes can itself perturb the system being investigated. One reason is that the available tools for driving expression of optogenetic probes in specific cell types remain relatively limited[25]. This difficulty is particularly relevant when considering the prospects for long-term therapeutic intervention in humans, for which transgenic approaches are not feasible. Although transgenic animals expressing the latest sensors are beginning

to become available[26], viruses have become the mainstay for expression of optogenetic tools in mammalian brains. However, major challenges remain regarding the specificity of viral targeting (especially in the absence of site-specific recombinases), the long-term toxicity of viruses and the stability of expression over long time periods. These are serious problems that have not been adequately addressed in the literature, with a few notable exceptions. For example, high-level, long-term expression of ChR2, either virally driven or via *in utero* electroporation, has recently been shown to cause abnormal axonal morphology[27], depending on the promoter used. Long-term expression of genetically encoded calcium indicators has also been associated with changes in the physiology of some cells[28]. These findings may well represent the tip of the iceberg, and they raise the concern that optogene expression may perturb the function of the circuits subject to investigation, highlighting the need for careful anatomical and physiological controls. More rational virus engineering, combined with the careful assessment of the long-term safety and efficacy of viral delivery systems, must be ensured long before optogenetic probes can be considered for treatment of human disease.

The future

Despite its power, optogenetics is not a panacea—as with any new method, its limitations are gradually being revealed and must be taken into account when designing, executing and interpreting optogenetic experiments. Many of the problems described here could be mitigated by an 'all-optical' approach combining expression of optogenetic actuators and sensors to allow readout from and control of the same neurons. This has been a difficult challenge for several reasons, not the least of which are hardware limitations for high-speed photostimulation and recording, insufficient sensitivity and temporal resolution of the available probes, and spectral overlap between actuators and sensors. The latter two problems have recently been addressed with the advent of new sensors for both calcium[17,18] and voltage[19,29]. By pairing a fast, new voltage sensor with a spectrally non-overlapping activator, researchers have demonstrated an all-optical system for electrophysiology in principle[19]. This is an important step forward as it allows the consequences of optogenetic manipulation to be directly read out and calibrated during the experiment in a more targeted way than is possible using optrodes.

However, such a system is still not sufficient to mimic natural patterns of activity in neural populations *in vivo*. An additional component is required: targeting multiple individual neurons *in vivo* for photoactivation or inactivation[25]. This can potentially be achieved using fast acousto-optical deflectors or a spatial light modulator[25,30], which can bridge the current 'scale gap' between optogenetic manipulation of single cells[9,31] and thousands of cells, and may eventually permit precise spatiotemporal patterns to be replayed or manipulated *in vivo*. This will not be easy: targeting individual neurons *in vivo* requires careful titration of light levels and probe expression to ensure reliable generation of individual spikes in a given neuron (and not in nearby neurons), necessitating more standardized expression of the optogenetic activator and sensor molecules coupled with careful control experiments (which should be an essential part of the standard repertoire of optogenetics). Such experiments will be particularly challenging in deep brain areas, which are less accessible to optical methods. But with further development of new probes and light-targeting techniques, it should hopefully soon be possible to achieve the magical trifecta of readout, activation and silencing—at physiological tempos in the same ensemble of neurons—that is required to unlock the secrets of neural codes in the mammalian brain.

The marriage between optogenetics and neuroscience, first consummated over a decade ago, has thus emerged from its honeymoon phase. The technical and interpretive challenges highlighted by the first generation of optogenetic experiments have spurred the development of novel probes and optical strategies that are leading to a new wave of more powerful and readily interpretable experiments. We can confidently predict an even brighter future for optogenetics in the coming decade.

COMPETING FINANCIAL INTERESTS
The author declares no competing financial interests.

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