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Optogenetic tools for analyzing the neural circuits of behavior

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In order to understand how the brain generates behaviors, it is important to be able to determine how neural circuits work together to perform computations. Because neural circuits are made of a great diversity of cell types, it is critical to be able to analyze how these different kinds of cell work together. In recent years, a toolbox of fully genetically encoded molecules has emerged that, when expressed in specific neurons, enables the electrical activity of the targeted neurons to be controlled in a temporally precise fashion by pulses of light. We describe this optogenetic toolbox, how it can be used to analyze neural circuits in the brain and how optogenetics is impacting the study of cognition.

The neural circuits of behavior

In order to understand how the brain generates behaviors, it is important to be able to determine how the neural circuits that make up the brain integrate multiple kinds of information, including sensory experiences and internal information (such as long-term memories, short-term memories, goals and prior histories of rewards or aversive events) towards the generation of decisions, movements and other actions. One key problem in understanding such circuit information processing is that the brain is made up of a great diversity of cells, including neurons of varying morphology and molecular composition, as well as many non-neuronal cell types, such as glial cells and smooth muscle cells found in blood vessels. Furthermore, these neurons are wired up in complex patterns, involving both local and distant connections between cells. The ability to activate or silence a specific cell type or connection in a temporally precise fashion during a behavior could elucidate the role played by that cell type or connection in the behavior. For example, by activating or entering information into specific neurons within a region, it would be possible to assess the processes that those neurons initiate or sustain that contribute to the behavior. By silencing specific neurons (i.e. by deleting the information they carry) within a region, it would be possible to assess the processes that those neurons are necessary for that contribute to the behavior. In this review, we discuss one such toolset, optogenetic molecular tools, which helps solve these problems, as well as how it can be applied to the systematic analysis of how complex, three-dimensionally distributed neural circuits that generate behaviors. Finally, we explore examples of

how optogenetics can be applied towards the understanding of cognition.

Optogenetic molecular tools

Over the past few years, a toolbox of fully genetically encoded molecules has been developed that, when expressed in neurons, enables the electrical potentials of the neurons to be controlled in a temporally precise fashion by brief pulses of light. Some of the molecules enable the neurons to be electrically activated and others enable the neurons to be electrically silenced. Because the tools are genetically encoded and optically driven, they have come to be known as 'optogenetic'. These molecules are microbial (type I) opsins (seven-transmembrane proteins; see Glossary) found in organisms throughout the tree of life, where they mediate light-sensing or photosynthetic functions, capturing light

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Glossary

Channelrhodopsin: an opsin from algae, which, when illuminated, opens up an ion channel pore that is permeable to positively charged ions, such as sodium and protons. Algae use channelrhodopsins to sense light, to navigate so as to optimize photosynthesis. When expressed in neurons and illuminated, channelrhodopsins let positively charged ions into neurons, thus exciting the neurons.

Halorhodopsin: an opsin from archaea, which, when illuminated, pumps chloride ions into cells. Archaeal species use halorhodopsins to convert light into energy. When expressed in neurons and illuminated, halorhodopsins pump chloride into neurons, thus hyperpolarizing the neurons.

Light delivery methods: light must be delivered into the brain to activate or silence opsin-expressing neurons that are not at the very surface. Typically, this is done by inserting an optical fiber or other device into the brain, the other end of which is attached or coupled to a light source (e.g. laser or LED). For surface structures, a small light source can be directly mounted atop the head.

Light-driven proton pump: an opsin from archaea, bacteria, or fungi, which, when illuminated, pumps protons out of cells. Archaeal species use light-driven proton pumps to convert light into energy. When expressed in neurons and illuminated, light-driven proton pumps pump protons out of neurons, thus hyperpolarizing the neurons.

Microbial (type I) opsin: microbial opsins are proteins that are embedded in the internal or surface membranes of different organisms, such as plants, bacteria, fungi and archaea. When expressed in neurons, they can embed in the plasma membrane of the neurons. The protein amino acid chain crosses the membrane seven times. Inside the protein is a vitamin A derivative, all-*trans*-retinal, which acts as the light capturing chemical. When light hits one of these proteins, the all-*trans*-retinal undergoes a transformation, causing the protein to change conformation. As a result of this change in conformation, charged particles (ions) can translocate across the membrane via the opsin protein.

Opsin delivery methods: opsins are proteins and, thus, genetically encoded. Therefore, the gene must be delivered to neurons for it to be expressed at the protein level. Practically any method for delivering genes into cells can, and has, been used to deliver opsin genes into neurons for opsin expression. For example, viruses whose genomes encode opsins can be used to infect neurons, causing the neurons to express the opsin proteins. Transgenic methods, in which DNA is incorporated into the genomes of transgenic animals, such as mice, can also be used to express opsin genes in neurons. In many cases, inserting a DNA regulatory element called a promoter in front of the opsin gene will enable it to be selectively expressed in some neurons and not others.

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energy and using the energy either to actively convey ions across cell membranes or to open up a channel that passively conducts ions across cell membranes. These molecules have been studied since the 1970s for the biophysical and biological insights they yield. More recently, these molecules were found to express well in neurons (perhaps surprisingly, given that they function natively in organisms such as fungi and algae) and to be able to mediate light-driven depolarizations and hyperpolarizations [1]. Furthermore, although these molecules require the vitamin A derivative all-*trans*-retinal to function (as the light-capturing chromophore component), enough all-*trans*-retinal is present in mammalian neurons in culture or *in vivo* to sustain the function of these molecules (and, for organisms such as *Caenorhabditis elegans, Drosophila* and other non-mammalian species, the all-*trans*-retinal is easily enough supplemented in the food supply). The illumination power required to activate these molecules is typically in the range of $0.1–10$ mW/mm^2. This illumination power is easily achieved *in vivo*, as will be described in the next section, and is far higher than observed in the ambient lab environment, thus reducing worries about background side effects.

The use of light to activate specific neurons preceded the use of microbial opsins in neuroscience, through several pioneering efforts. For example, in 2002, a three-protein *Drosophila* photoreceptor signalling cascade was genetically expressed in cultured mammalian neurons, thus enabling them to be activated by light, even though the temporal dynamics of the ionic currents that resulted was slow [2]. In 2003, the optical uncaging of chemicals [3–8], long used in neuroscience to drive neurons in a non-cell-type-specific fashion through the uncaging of chemically caged neurotransmitters, was also made genetically targetable [9,10], by expressing heterologous receptors that bind the uncaged chemicals only in defined cell types embedded within a neural circuit. In addition, in 2004, a paper reported a method in which engineered ion channels were expressed in neurons, with chemical channel-binding ligands linked to the channels via a light-drivable domain [11]. Upon illumination, the light-drivable domain would change conformation, and the ligand would alter its binding to the channel pore, thus activating the neurons in which the channel was expressed.

The microbial opsins offer high-speed neural activation and silencing, without requiring the use of chemicals in the mammalian brain. Three classes of such molecule are in widespread use in neuroscience (Figure 1). Channelrhodopsins (Figure 1a) are light-driven inward cation channels from green algae. When expressed in neurons, they localize to the cell membrane and, when illuminated, they open up a channel that lets in positive charge (chiefly sodium ions and protons, but also potassium and calcium), thus depolarizing the cell (Figure 1ai; [12]). The first one to be used in neurons was channelrhodopsin-2 (ChR2), from the green alga *Chlamydomonas reinhardtii* [13]; when expressed in neurons, it reacts rapidly to brief pulses of blue light, with large enough depolarizing photocurrents to mediate action potentials at rates of tens of hertz (Figure 1aii). Owing to the utility of ChR2 to mediate the driving of specific cells or pathways *in vivo*, many variants of channelrhodopsins have been found or

engineered, including the calcium permeability-enhanced channelrhodopsin CatCh [14], channelrhodopsins that exhibit higher amplitude currents or currents slower to run down than the original [e.g. several channelrhodopsin mutants and chimeras, including ChR2(H134R), ChR2(T159C), ChRGR and ChIEF [15–19]], channelrhodopsins that are faster or slower to turn off after illumination [e.g. several channelrhodopsin mutants, including ChETA, SFO and the ChR2(D156A) mutant [20–22]] and color-shifted channelrhodopsins (e.g. VChR1, MChR1 and C1V1 [23–26]), with new variants arising at a rapid pace [27].

Halorhodopsins (Figure 1b) are light-driven inward chloride pumps from archaeal species that live in very high-salinity environments. When expressed in neurons, and illuminated, they pump chloride ions into the cells (Figure 1bi), thus hyperpolarizing them. The first halorhodopsin to be used in neurons was the halorhodopsin from the archaeon *Natronomonas pharaonis* (Halo/NpHR) [28,29]. When expressed in neurons and illuminated with yellow light, halorhodopsins mediate hyperpolarizations, enabling the quieting of neural activity (Figure 1bii). This halorhodopsin is capable of supporting the perturbation of specific neurons to study their role in the brain functions of behaving mice [30]. Many other organisms bear halorhodopsins that express and can mediate hyperpolarization in neurons [23]. Halorhodopsins in general, however, have poorer expression in neurons than do channelrhodopsins, and require the addition of trafficking sequences (e.g. from potassium channels) to express well, at very high levels, in mammalian cells [31–34]. They also recover slowly after extended illumination, taking tens of minutes to recover full photocurrent amplitude after use [23,35,36].

A third class of light-activated protein, the light-driven outward proton pumps (Figure 1c), express well and also recover rapidly after use. One subclass of these light-driven proton pumps, the archaerhodopsins, as exemplified by the molecule archaerhodopsin-3 (Arch) from *Halobacterium sodomense*, are also found in archaeal species. When expressed in neurons and illuminated with yellow or green light, they pump positive charge out of the cells, hyperpolarizing them (Figure 1ci). Arch enables 100% neural silencing of neurons in the cortex of awake behaving mice (Figure 1cii) [23]. Recently, the molecule ArchT, which is 3.5 times more light-sensitive than Arch, has become available [37], enabling silencing of large brain regions. This, for example, might be of use in the brain of non-human primates. Light-driven proton pumps also exist in many different color variants: for example, the light-driven outward proton pump Mac (from the fungus *Leptosphaeria maculans*) supports blue-light driven neural silencing [23], thus enabling, alongside the earlier molecule Halo (which is drivable by yellow and, to some extent red, light), two-color neural silencing of two separate neural populations expressing Mac and Halo [23].

Other optogenetic tools have also been developed. For example, naturally occurring light-driven G protein-coupled receptors (GPCRs) from mammals, such as rhodopsin and melanopsin, have been adapted from nature for use in neurons or other cell types to drive G protein-coupled signaling, including Gi and Gq-mediated effects, such as
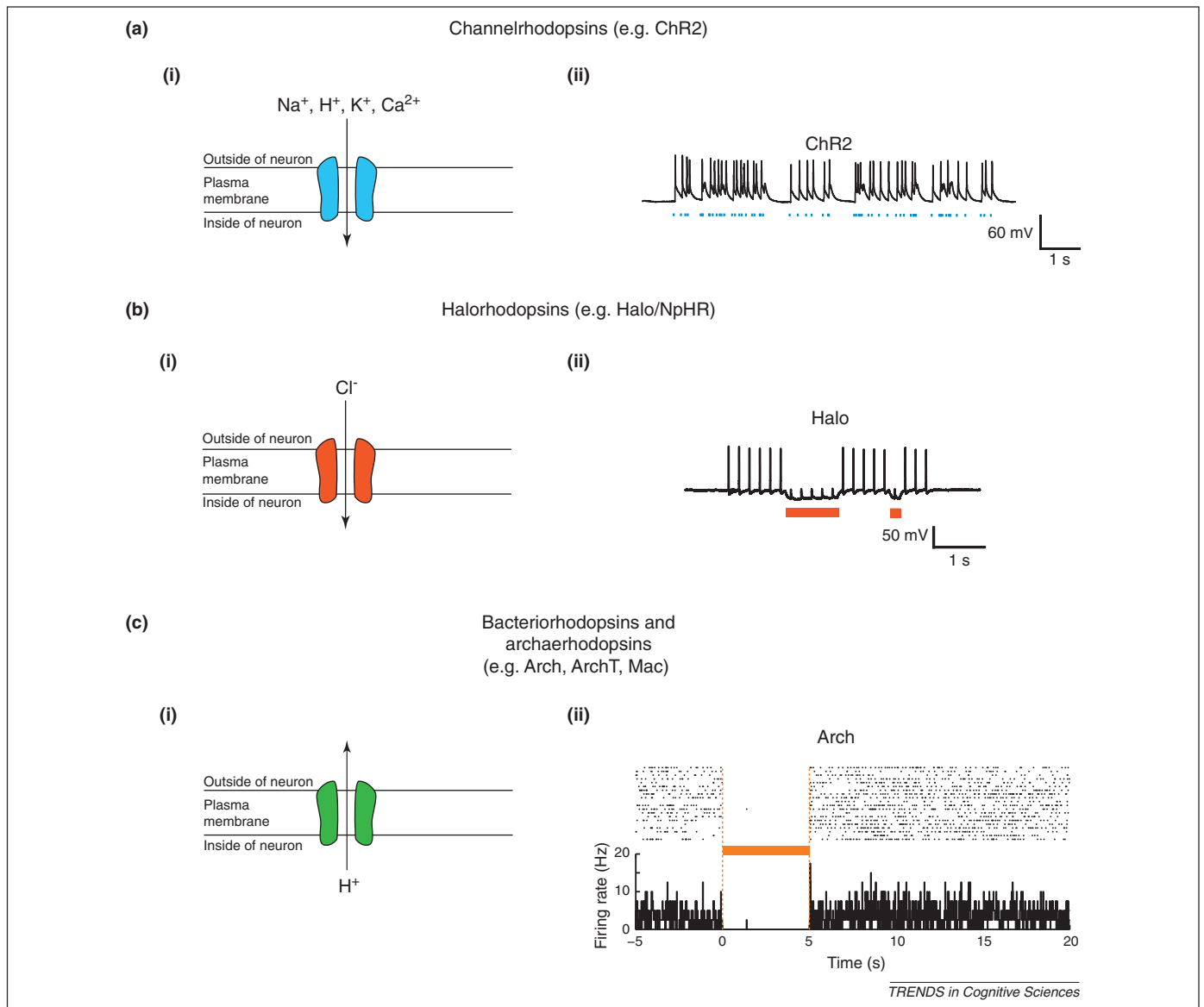
Figure 1. Molecular tools enabling activation and silencing of neurons with light. **(a) (i)** Diagram showing the physiological effect of expressing the gene for the light-driven inward cation channel channelrhodopsin-2 (ChR2) from the green alga *Chlamydomonas reinhardtii* in a neuron and illuminating the cell. Positively charged ions (chiefly sodium and protons, but also to a lesser extent potassium and calcium) flow into the intracellular space, from the extracellular space. **(ii)** Raw voltage trace (black trace) recorded, using whole-cell current clamp, from a cultured hippocampal neuron expressing ChR2 and illuminated with brief pulses of blue light (blue dashes under trace) from a mercury arc lamp through a GFP excitation filter, showing light-driven action potentials. **(b) (i)** Diagram showing the physiological effect of expressing the gene for the light-driven inward chloride pump halorhodopsin (Halo/NpHR) from the archaeal species *Natronomonas pharaonis* in a neuron and illuminating the cell. Negatively charged chloride ions flow into the cell. **(ii)** Raw voltage trace (black trace) recorded, using whole-cell current clamp, from a cultured hippocampal neuron expressing Halo and illuminated with orange light (orange dashes under trace) from a xenon lamp through a Texas Red excitation filter, showing light-driven action potential silencing. Action potentials were elicited by injecting brief pulses of current into the cell body (approximately 300 pA, 4 ms-duration pulses) at a rate of 5 Hz. **(c) (i)** Diagram showing the physiological effect of expressing the gene for the light-driven outward proton pump archaerhodopsin-3 (Arch) from the archaeal species *Halobacterium sodomense* in a neuron and illuminating the cell. Positively charged protons flow out of the cell. **(ii)** Neural activity in a representative neuron recorded using extracellular electrode recording in an awake mouse before, during and after 5 sec of yellow light illumination, shown as a spike raster plot (top), and as a histogram of instantaneous firing rate averaged across trials (bottom, bin size, 20 ms). Adapted from [13] (aii) and [28] (bii).

the opening of potassium channels or the release of calcium [38,39]. Additionally, chimeric GPCRs have been developed that contain the transmembrane domains of rhodopsin attached to the intracellular domains of GPCRs, additionally enabling Gs signaling [40–43]. Some of these tools have been utilized to control behavior in living animals, for example restoring photosensitivity to the blind retina or enabling silencing of neural activity in the spinal cord [38,39], or driving neurons involved with reinforcing behavior [43]. Finally, several groups have developed light-driven gene expression reagents that do not require chemicals, which could be of use in seeing how behaviors

change before versus after the induction of the expression of a given gene in specific cells [44,45].

Strategies for parsing neural circuits using light
The usage of these opsins spread rapidly throughout neuroscience in the months and years following the publication of the initial tool papers, with the molecules being expressed in many different kinds of targeted neuron, enabling them to be activated and silenced with light, to assess their contribution to behavior. These tools were used to investigate the role of specific neurons in behaviors such as breathing [46], awakening [47], sensing [15,48–53],

movement [38,54–56] and learning [57]; all of this within the first three years of the first paper on channelrhodopsin-2 in neurons being published. Currently, new papers using opsins in defined cell types to probe their role in behavior appear regularly, and the tools have been distributed to several hundreds of groups. These molecules are encoded by small genes (<1000 DNA bases long), and thus can be delivered into organisms for expression in targeted neurons via practically any commonly used method of gene delivery, including, for the case of the mammalian brain, viral delivery via lentiviruses, adeno-associated viruses (AAV) and other viruses [13,53,58], *in utero* electroporation [59] and transgenic mouse generation [60–62]. Viral delivery of opsin genes has been utilized to enable optical control of neurons in a wide variety of species, including non-human primates [63]. By choosing the promoter inside the virus so that the genetic payload expresses more highly

in certain cell types than others, it is possible to attain a certain degree of cell-type specificity with such viruses. One particularly popular strategy is to take advantage of the large and increasing number of mice that express the molecule Cre recombinase in specific cell types, by injecting an AAV that encodes an opsin gene inserted in reverse orientation and flanked by strategically placed sites that undergo genetic recombination upon binding by Cre recombinase; when this AAV virus is injected into the Cre mouse, it undergoes recombination, thus enabling opsin expression in only the cell type expressing Cre [64–66]. Anterograde labeling or retrograde labeling of defined projections that enter a target region, or that innervate a target cell type, is also a possibility, given specific viruses with this capability [32,67–69]. Viruses can be delivered into many sites in the brain at once, using multiple-injector arrays [70].
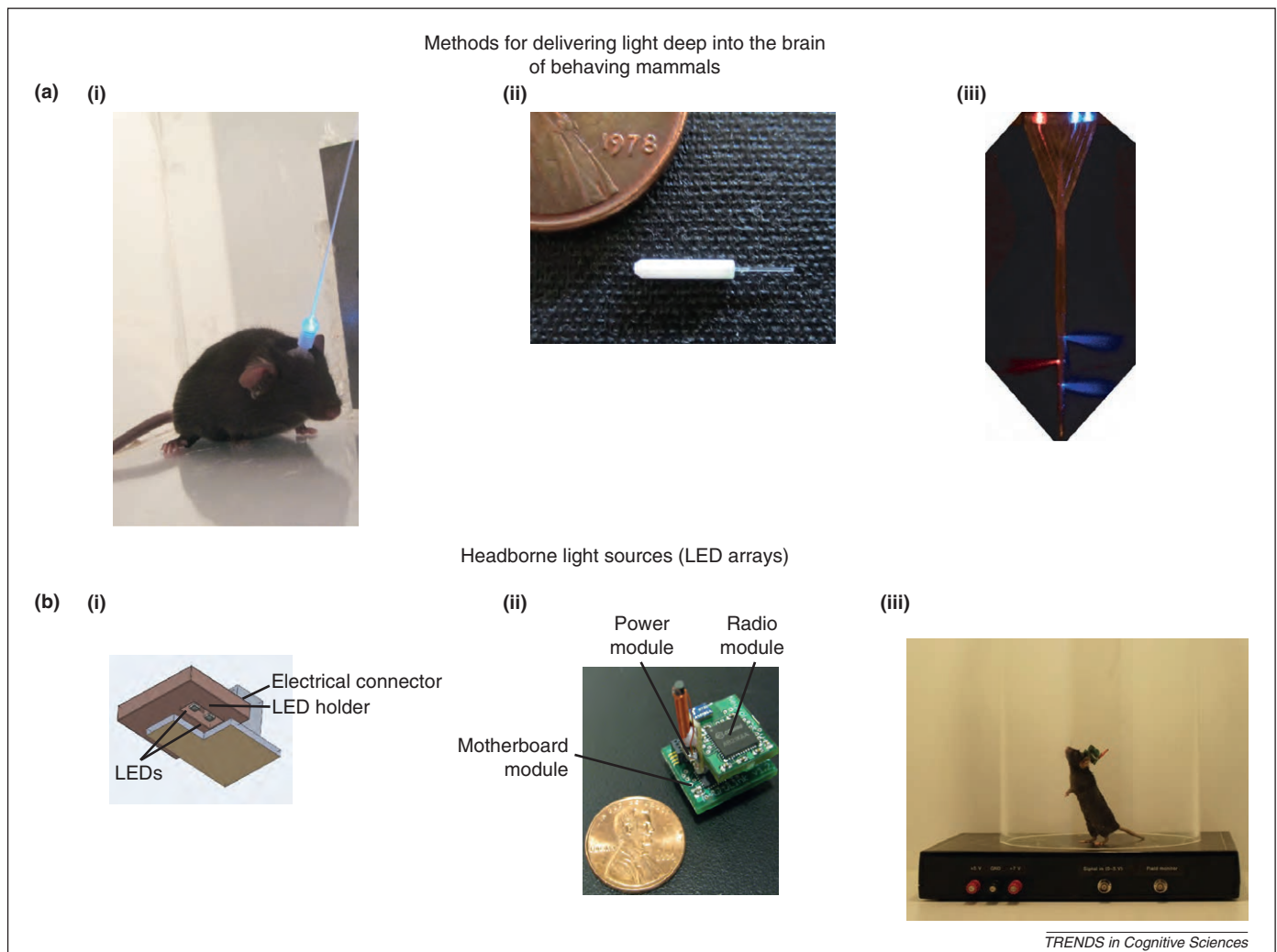

Figure 2. Methods for delivering light to the brain of behaving mammals, and miniature light sources for generation of light. **(a) (i)** Mouse with implanted cannula, into which is inserted an optical fiber (200 μm in diameter), for light delivery to deep brain regions. **(ii)** Chronically implantable optical fiber (200 μm in diameter), mounted inside a ferrule. The fiber is inserted into the brain, with the ferrule projecting outwards for later connection to a corresponding ferrule-mounted fiber coupled to a laser. **(iii)** Microfabricated multi-waveguide arrays, for delivery of light to multiple points along the insertion axis of the probe. The probe shown here is similar in thickness to a typical optical fiber inserted into the brain, but can deliver light to a dozen different sites along the axis of the probe. **(b) (i)** Schematic depicting a head-borne device comprising an array of light-emitting diodes (LEDs) (500–1000 μm in dimension; e.g. raw die LEDs from Cree or other vendors) mounted on a LED holder (which serves as heat sink as well as an electrical connection), for illumination of multiple (e.g. a dozen or more) structures on the surface of the brain. Such a device weighs a gram or less and can contain many independently controlled illuminators; the electrical connector conveys currents from LED driver chips. **(ii)** Circuitry that mounts on top of the LED array shown in (bi), enabling fully wireless power delivery and wireless control of the LEDs. The power module receives broadcast energy and the motherboard module enables control of the LEDs below; the radio module enables programs to be uploaded to the module. **(iii)** A mouse with implanted optics module (bi) and bearing the wireless power module (bii). Adapted from [77] (aiii) and [78] b(i,ii).

Multiple opsins can be expressed in the same cell; for example, a cell expressing both a channelrhodopsin and a halorhodopsin [28,29], perhaps linked by a ribosomal skip peptide [71,72], can be depolarized by blue light and hyperpolarized by yellow light. The ability to depolarize and hyperpolarize the same cell enables sophisticated manipulations of neural activity: for example, the ability to insert electrical spikes as well as to delete them can be used to change the timing of spikes in a spike train, while leaving their average rate unchanged [28], enabling the testing of hypotheses about the importance of spike timing to specific neural computations or behaviors.

Light delivery into the brain has been supported by the wide availability of fiber-coupled lasers that can be inserted into the brain via cannulae [54] (Figure 2ai) or coupled to fibers that are chronically implanted into the brain (Figure 2aii). As optics is a rapidly changing field, the Synthetic Neurobiology Group (Massachusetts Institute of Technology) maintains a web page with current part numbers and best-practice methods for assembling, calibrating and utilizing these fiber-coupled lasers and accessory parts (http://syntheticneurobiology.org/protocols). For example, light-emitting diode (LED)-coupled fibers are becoming increasingly commercially available, and these have improved stability and are cheaper than lasers, suggesting that at some time in the near future, LED-coupled fibers will be of increasing use in the application of optogenetics to the investigation of behavior [48,73] (see also below).

Neural activity readout methodologies, such as electrode recording, calcium imaging and other methods, are highly compatible with optogenetics. One nice property of optical stimulation, in contrast to electrical stimulation, is that electrical neural recording is possible without the artifacts that commonly result from electrical stimulation on recording electrodes. Optical artifacts do exist, especially in the lower frequency ranges, such as those utilized in local field potential recordings or electroencephalographic (EEG) recordings [63,74]. Moreover, recently, functional magnetic resonance imaging (fMRI), one of the most commonly applied methods for brain activity monitoring in behaving humans, was also adapted for use with optogenetics in awake behaving mice [75], opening up the possibility of conducting parallel experiments in behaving humans and behaving mice, and thus enabling the linking of the behavioral events and subjective experiences undergone by humans to causally mapped neural circuits analyzed in mice. Such opto-fMRI experiments have also been performed in anesthetized rodents [76].

Towards a systematic analysis of how neural circuits contribute to behavior

Although causal neural circuit analysis is powerful because it enables the determination of the functional roles played by specific cell types in complex behaviors, it is also low throughput, in the sense that typically only one region is perturbed at a time. Whereas observation of neural anatomy and physiology can be scaled up through strategies that enable many measurements to be performed and many analyses to be conducted simultaneously (such as robotic image processing and multi-electrode physiology, respectively), in optogenetics, relatively few sets of neurons can be manipulated at any one time, using the majority of commonly used technologies. Furthermore, only one neuromodulation pattern can be applied to that set of neurons at a time, via conventional approaches (Figure 2ai,ii). Given that there is an enormous diversity of cell types in the brain, understanding how they work together in intact, widely distributed, three-dimensional neural circuits would greatly benefit from new technologies for optically entering information into large numbers of sites in the brain, and to do these kinds of manipulation in a high-throughput fashion, ideally across many animals at once, to enable parallel experimentation in a scalable fashion.

Technologies capable of controlling large sets of neurons at a resolution of individual subcircuits within the brain, or even individual cells within subcircuits, would enable playback of more naturalistic activity patterns into the nervous system, thus enabling the testing of sophisticated hypotheses of how populations of neurons encode information or work together to implement neural computations. They could also be used to perform causal mapping of neural circuits; for example, enabling the silencing of many regions for brief periods of time in rapid succession, so that a scientist can hone in on the set of regions that is important for the performance of a given behavior. Such a 'high-throughput circuit screen' may be of use for resolving when and how specific regions play roles in behaviors. These screens could be conducted in many different algorithmic styles. For example, a large region could be silenced, and then if a behavioral effect is observed owing to the silencing, smaller and smaller subdivisions could be silenced within that region in turn, enabling the investigator to hone in iteratively on the specific subcircuits that most powerfully contribute to the behavior. As another example, the ability to enter information into a population of neurons could enable the testing of hypotheses about how population coding or cell assembly coding serves in the generation of behavior.

As one example, multi-waveguide probes that are as wide as an optical fiber, but can deliver light independently to many points along the axis of the probe, have been developed [77], thus minimizing brain damage while greatly increasing the number of points in the brain that can be controlled (Figure 2 (aiii). The probe shown in Figure 2aiii can deliver light to a dozen points along the probe axis. As another example, arrays of independently controlled LEDs (which are available in powerful blue, green and amber forms) can enable the stimulation or silencing of many surface structures, either alone or in combination (Figure 2bi), thus enabling the combinatorial driving or silencing of neurons in the cortex of the brain. These LEDs are used in raw die form (available from manufacturers, such as Cree; http://www.cree.com), and are fabricated in arrays of 12 to 24 LEDs, via self-assembly of LED chips on solder films on top of blocks of copper that serve as both a heat sink and electrical conductor [78]. These LED arrays weigh just a gram or two, are implanted atop the skull and can illuminate surface targets with appropriate light powers, even through the thinned skull. All parts of the arrays besides the commercially available LEDs and optical fibers can be fabricated in-house on low-cost, tabletop computer-numerically controlled (CNC) machines, allowing rapid customization

of fiber array geometries to targeted brain regions through computer-aided design tools. The LED arrays are easily controlled using circuits that contain LED-driver chips, and can illuminate the brain for many seconds at a time before resulting in appreciable heating, enabling optical modulation of behaviors, such as movement [78].

For high-throughput assessment of many mice at once, wireless power receiver modules (Figure 2bi, green circuit boards) that capture energy from oscillating magnetic fields emitted by under-cage or under-arena transmitters (Figure 2biii; black box under mouse) can be attached atop the implanted LED array before or at the time of experiment, enabling fully remote control of the LEDs for control of many mice (hundreds, using these technologies), without the need for tethering the mice via optical fibers or electrical cables [78], which can compromise repeated experimentation as well as complex behaviors.

To address deep structures distributed in three dimensions throughout the brain, arrays of precisely truncated optical fibers, trimmed to target sets of deep regions within the brain, and held in an aligner plate (made through standard electrical circuit board manufacturing means), can be rapidly docked to the planar arrays of LEDs described above (Figure 3a), thus enabling independent light delivery to dozens of sites distributed in a three-dimensional fashion throughout neural circuits (Figure 3b). When optical fibers are directly coupled to raw LED chips, an optimal configuration in many instances [79], irradiances of 200 mW/mm^2 are easily achievable at the ends of the fibers, similar to the peak irradiances used in laser-coupled-fiber experiments. These devices are also inexpensive to make, thanks to the low cost of LEDs and electronic circuit board fabrication. Shown in Figure 3 is an example of such a device, which tiles the bilateral mouse hippocampus, enabling independent control of dozens of sites within a circuit important for processes, such as memory formation and disorders, such as epilepsy. Electrodes for neural recording can be threaded through holes created in such devices, or attached aside these structures. These devices

are serving not only as tools for systematic neural circuit analysis, but may also in the future support new kinds of biological control prosthetics for the treatment of intractable disorders [63,80].

Using optogenetics to study the neural circuits of cognition and behavior

Over the past few years, many studies have appeared that utilize optogenetic tools to drive or silence specific populations of cells or neural pathways within the brains of behaving rodents, studying how the perturbation of the activity of specific cells alters behavior or neural dynamics in these animals. Many other studies have appeared that utilize optogenetic tools in behaving flies, worms and other species, as well as in rodent brain slices or in other preparations for neurophysiological analysis. Rather than attempting to review the long list of studies, we instead illustrate how optogenetics can be used in behavioral neuroscience by selecting a few examples of areas where optogenetics has played a helpful role, and then describe some of the results obtained over the past year or so within each area. In this way, we hope to describe realistic arcs of the use of optogenetics in the context of current neuroscience studies. For cohesiveness, we focus on rodent studies.

The first area that we examine is how specific cell types and pathways contribute to reward-seeking behaviors. One recent study attempted to determine what role the specific neural pathway connecting two regions involved in emotion-related learning [the basolateral amygdala (BLA) and the nucleus accumbens (NAc)] plays in the generation of motivated behavior [81]. In this study, the authors injected AAV encoding for channelrhodopsin-2 or *N. pharaonis* halorhodopsin under the CaMKII promoter (which preferentially drives expression in excitatory neurons, in many brain regions), into the BLA. This resulted in opsin expression along excitatory neuron axons that left the BLA and entered other targets, such as the NAc, where they formed glutamatergic synapses upon target neurons in the NAc. The mice were implanted with an optical fiber aimed at the
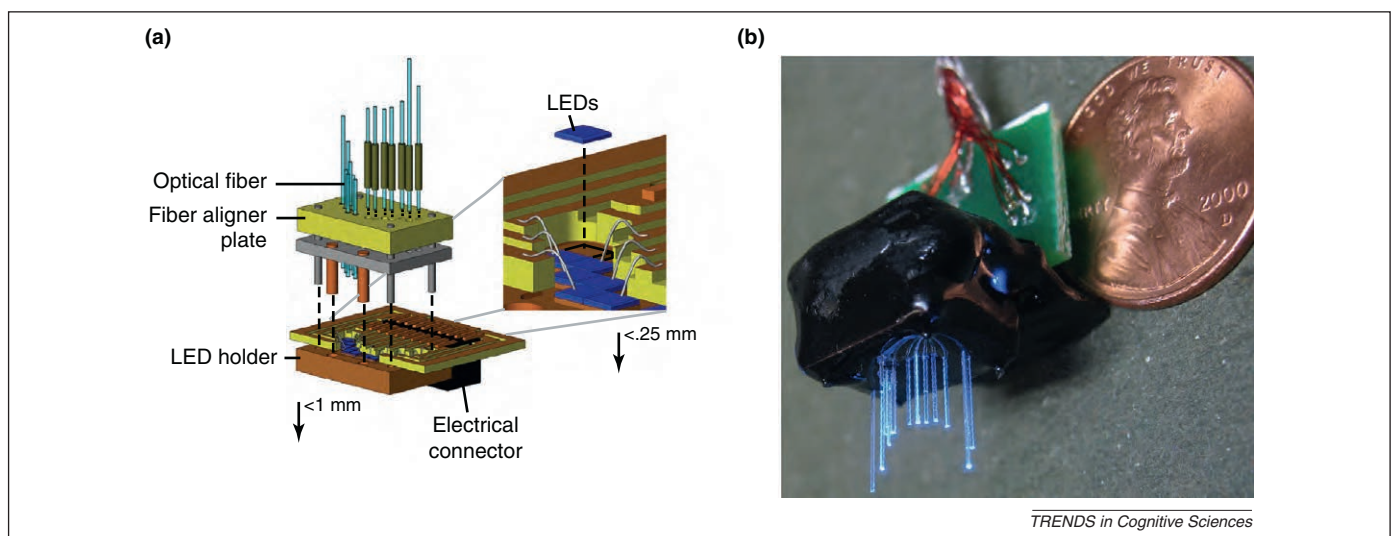

Figure 3. Strategies for targeting light to three-dimensionally distributed structures in the brain. **(a)** Example strategy for efficiently docking an array of optical fibers or waveguides (Figure 2a) to an array of light sources (Figure 2bi), for independent control of neural activity in a large number (e.g. a dozen or more) structures distributed throughout the brain. An aligner plate that holds the optical fibers can easily be docked to the array of light-emitting diodes. Shown is an example of a 14-fiber array for bilateral targeting of 14 independently accessible points within the mouse hippocampus. **(b)** Photograph of a device made according to the schematic shown in **(a)**.

NAc, so that when light was delivered down the fiber, the BLA→NAc synapses would be excited or inhibited, depending on the identity of the opsin expressed. Mice were then placed into a box where they were given a train of light pulses if they poked their noses into a beam-break device. For mice expressing channelrhodopsin-2 in the BLA, their rate of nose pokes increased, indicating that the activation of BLA→NAc synapses served as a reinforcing or rewarding stimulus. By contrast, for mice expressing an increased-trafficking version of the *N. pharonis* halorhodopsin in the BLA, and placed in a box where tone and light stimuli indicated the availability of a sucrose solution reward, the silencing of BLA→NAc synapses during the period of stimulus presentation reduced pursuit of the sucrose. Thus, the BLA→NAc pathway seems to be not only sufficient to reinforce behavior, but also necessary during normal reward-seeking behavior.

As a second example, we discuss two recent results in the field of Pavlovian fear conditioning, a model of emotional learning in which animals learn to freeze in response to a tone, after the tone is paired with a brief co-terminating shock. In one study [82], rats were injected with AAV encoding channelrhodopsin-2 under the CaMKII promoter (the same kind of vector used in the previous study) into the lateral amygdala (LA), a region that has long been implicated in mediating the associative processing of aversive stimuli during fear memory acquisition, and implanted with an optical fiber aimed at the LA. In this case, the shock typically used in this behavioral paradigm was replaced by direct optical activation of LA. When rats were exposed to 20 sec of auditory stimulation, which co-terminated with 2 sec of light pulses (delivered at 20 Hz, with 10 ms pulse durations), mice learned to fear the tone, as evidenced by increased freezing to the tone after the training. Thus, direct activation of the LA can behaviorally recapitulate the associative effect of a shock presented at the end of the tone, in that both the LA activation and the shock cause the tone to become an aversive stimulus.

In another study [83], the role of the central nucleus of the amygdala (CE) in fear conditioning was investigated. The CE has been implicated in the expression of conditioned fear, and is subdivided into parts such as the medial and lateral CE (CEm and CEl, respectively). The CEl inhibits the CEm. To examine how the CEl and CEm directly regulate fear, the authors infused muscimol, a γ-aminobutyric acid (GABA)$_A$ agonist capable of neural silencing in many brain regions, into the CEl, and showed that this by itself was capable of inducing freezing. The authors also used an AAV to deliver channelrhodopsin-2 virally to the CEm, and implanted an optical fiber to illuminate the CEm; when blue light was delivered to the CEm, the animals would again freeze. Thus, through pharmacology and optogenetics, this study was able to piece together a picture of the CE in which fear responses can be directly mediated by neurons in this region.

Finally, we discuss a result in the field of learning and the representation of information in the brain. In one recent study [84], the authors investigated the ability of neurons in the piriform cortex, which receive axonal projections from glomeruli of the olfactory bulb in a diffuse fashion (thus suggesting a convergence of many kinds of odorant information on a given set of piriform cortex neurons), to come to represent behaviorally meaningful events. The authors used lentiviruses to label piriform cortex neurons with channelrhodopsin-2, using the synapsin promoter to drive expression in both excitatory and inhibitory neurons; they also used the Cre-dependent AAV vector described above, along with Emx1-Cre mice that express Cre recombinase in just the excitatory cells of the cortex, to target expression to excitatory pyramidal neurons; typically, 200–1000 neurons were labeled. The animals then received photostimulation just before either a footshock was delivered, or before a water reward was made available. In either case, mice learned to associate the activation of piriform cortex neurons with the outcome and act accordingly (e.g. fleeing the shock floor in the case of shock presentation; licking in the case of water presentation). Thus, despite the diffuse innervation of a given region of piriform cortex by olfactory glomerulus projections, which raises the question of what kind of information these neurons could be representing, mice could learn to act upon the information encoded by a given set of piriform cortex neurons.

Concluding remarks

Because optogenetics enables specific cell types and neural pathways to be driven or silenced by light, it enables causal, time-resolved tests of how specific neural circuit elements participate in the emergent functions of the neural circuit as a whole. The ability to control, for example, the electrical activity of a specific neural population and to assess the effects on behavior can resolve whether and how the neural population in question contributes to the computations that generate the behavior. Such experiments are increasingly widespread in neuroscience, thanks to the increasing commoditization of the molecular and optical technologies required for optogenetics experiments. Currently, most optogenetics studies activate or silence large populations of a given cell type or pathway, so tools for entering information into neural circuits in more realistic patterns will greatly increase the power of optogenetics to gauge the role of neural activity patterns in brain functions. At a molecular level, molecules with red-shifted spectra for activation will enable less-invasive, deeper-tissue optical neural control, and improvements in the kinetics and amplitude of the physiological responses of molecules will augment the precision of the perturbations that scientists incur. Finally, the ability to survey comprehensive neural dynamics downstream of an optogenetic perturbation event, will help reveal how a change in the activity of a given cell type alters the neural processing elsewhere in the circuit.

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