

Optogenetics in the nonhuman primate

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Abstract: The nonhuman primate brain, the model system closest to the human brain, plays a critical role in our understanding of neural computation, cognition, and behavior. The continued quest to crack the neural codes in the monkey brain would be greatly enhanced with new tools and technologies that can rapidly and reversibly control the activities of desired cells at precise times during specific behavioral states. Recent advances in adapting optogenetic technologies to monkeys have enabled precise control of specific cells or brain regions at the millisecond timescale, allowing for the investigation of the causal role of these neural circuits in this model system. Validation of optogenetic technologies in monkeys also represents a critical preclinical step on the translational path of new generation cell-type-specific neural modulation therapies. Here, I discuss the current state of the application of optogenetics in the nonhuman primate model system, highlighting the available genetic, optical and electrical technologies, and their limitations and potentials.

Keywords: monkey; genetic manipulation; optical; channelrhodopsin; archaerhodopsin; halorhodopsin; rat.

Introduction

Optogenetic technologies utilize light to control the activity patterns of neurons that are genetically modified to express light-activated opsin proteins. Recent advances in improving the functions of opsin proteins have made it possible to effectively activate or silence many types of brain cells with light at the millisecond timescale.

The efficiency of optogenetic control depends upon a number of factors, including the intrinsic physiological properties of the cell, the architecture of the neural network, the number of opsin proteins present on the cell membrane, the optical response kinetics of opsins, and the amount of light that reaches the cell. The major challenge in using this technology in genetically intractable animals, like primates, is the ability to target specific cells or anatomical connections. Here, I discuss details on genetic transduction methods, optical illumination strategies, and electrical monitoring techniques, with a main focus on the use of

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optogenetics in the genetically intractable nonhuman primate system.

Genetic transduction of brain cells

A major advantage of optogenetic control technology is the ability to control specific genetically modified cells that express light-activated opsin proteins. In genetically intractable animal models, such as the nonhuman primate, the technique to transduce specific cells has been largely limited to viral methods. The most commonly used viruses are lentivirus and adeno-associated virus (AAV). Other virus types, such as adenovirus and herpes simplex virus (HSV) that are effective in transducing brain cells in many model systems, have not yet been well adapted to monkeys, perhaps due to the concerns about the potential adverse immune responses and the limited duration of transgene expression. Although lentivirus and AAVs have been successfully used to transduce brain cells, there has been limited success in targeting specific cell types, and it remains to be a major challenge in realizing the full potential of optogenetics in this model system.

Lentivirus

Lentivirus is an enveloped retrovirus with a single-stranded RNA genome. Current recombinant lentivectors are derived from human immunodeficiency virus (HIV) and other nonhuman lentivirus, such as feline immunodeficiency virus (FIV) and equine infectious anemia virus (EIAV). The potential use of lentivirus in human gene therapy has led to major advances in improving the safety of these vectors. With over 95% of the parental viral genome removed, these recombinant lentiviral vectors induce minimal inflammatory responses. Lentivectors have a modest packaging capacity of ~8kb, large enough to deliver many genes of interest for gene therapy and basic research (Federico, 2003; Thomas et al., 2003).

Effective transduction of the target cells by lentivirus is influenced at each step from virus entry into

the cytosol to gene expression within the nucleus. The tropism of a lentivirus is determined by the interaction of the glycoproteins on its viral envelop and the cell surface receptors on the target cell. To facilitate the entry into target cells, lentivirus can be pseudotyped with different viral envelop glycoproteins that recognize membrane surface receptors on a broad range of cell types. However, pseudotyped lentivirus is often unstable and cannot be easily manufactured to produce high titer virus. Over the past two decades, a few lentiviruses have been successfully pseudotyped including HIV, FIV, and EIAV. For example, lentivirus pseudotyped with the glycoprotein (G) from *Vesicular stomatitis virus* (VSV-G) is stable and can be easily concentrated to a titer of 10^9 in a laboratory and can transduce a wide range of cell types. Upon entering a target cell, lentiviral genomic RNA is reverse transcribed into DNA in the cytoplasm, which is then actively transported into the nucleus and integrated into the host genome. Lentivirus tends to insert themselves to the genomic regions undergoing active transcription, which has raised the concerns for mutagenesis, especially in targeting proliferating cells, such as hematopoietic cells (Schroder et al., 2002). However, in the brain, where most cells are terminally differentiated, the chance of inducing brain cancer is extremely low (Thomas et al., 2003).

It has been well established that lentivirus can mediate widespread and long-term gene expression in the brain, capable of transducing neurons with high efficiency (Blomer et al., 1997). Recently, we have successfully used lentivirus pseudotyped with VSV-G to deliver channelrhodopsin (ChR2), Archaelhodopsin-3 (Arch), and Archaelhodopsin-TP009 (ArchT) into the monkey cortex (Han et al., 2009, 2011). For example, lentivirus is able to mediate ChR2-GFP expression in widespread and healthy neurons months after viral injections, and the expression of ChR2-GFP is well targeted to the plasma membrane (Fig. 1a and b). Detailed examination of the cell-type specificity revealed that lentivirus with a CaMKII promoter preferentially labels

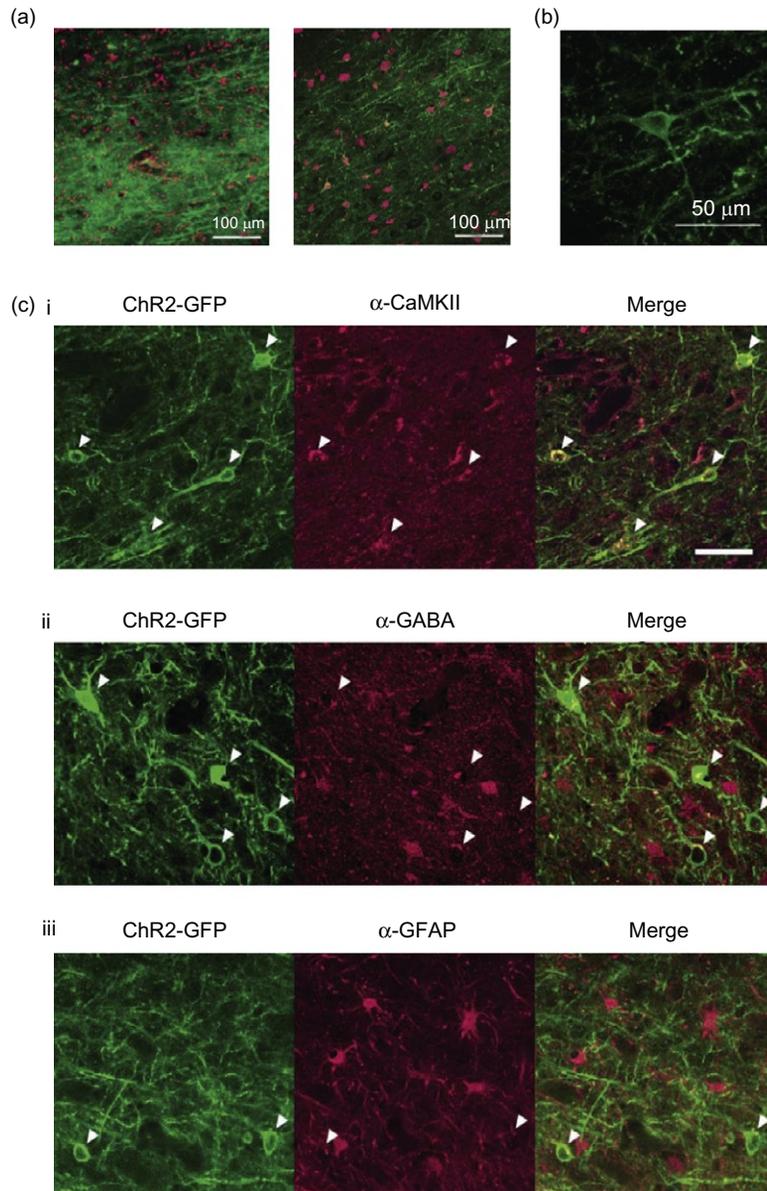


Fig. 1. Cell-type-specific transduction of monkey cortical neurons with light-activated opsin proteins. (a) Widespread and healthy neurons expressing ChR2-GFP (green) months after viral transduction (a-i, red, To-Pro3 nuclear DNA staining; a-ii, red, anti NeuN neuronal staining). (b) ChR2 expression is well targeted to the neuronal plasma membrane and processes. (c) Expression of ChR2-GFP by lentivirus with a CaMKII promoter is restricted to the excitatory neurons. Images of anti-GFP fluorescence (left) and immunofluorescence of cell-type makers, CaMKII (i), GABA (ii), and GFAP (iii; middle), and their overlay with anti-GFP fluorescence (right). Arrowheads indicate ChR2-GFP positive cell bodies. (Adapted from [Han et al., 2009](#).)

monkey cortical excitatory neurons (Fig. 1c), consistent with that observed in the mouse cortex (Nathanson et al., 2009b). Various tests examining immune responses, tissue pathology revealed no detectable damages on neural tissues expressing high levels of opsin molecules (Han et al., 2009).

High titer lentivirus is easy to produce in a laboratory. However, the lifetime of lentiviral particles is rather short, perhaps due to the presence of its fragile envelop. We have observed significant reduction in viral titer after storing them at -80°C for only half a year. In addition, the infectivity is easily destroyed by repeated freeze–thaw processes or simply sitting at room temperature for an extended period of time. Thus, precautions have to be made when using lentivirus. We found it necessary to use fresh stocks of lentivirus, to avoid repeated freeze–thaw process, and to limit the exposure to room temperature.

Another promising application of lentivirus is the capability of retrogradely transducing neurons projecting to the area of viral injections. Most promisingly, EIAV pseudotyped with rabies glycoprotein can be transported through axons, labeling large number of cells retrogradely (Mazarakis et al., 2001). However, it remains to be determined whether this retrograde strategy is capable of introducing sufficient amount of opsin molecules for optical modulation. Since significant number of opsins within a single cell is required for efficient optical modulation, amplification strategies may be helpful in amplifying opsin expression in retrogradely labeled projecting cells. For example, one could retrogradely express Cre enzymes by injecting rabies glycoprotein-pseudotyped EIAV and then transduce the Cre-expressing projecting neurons in the upstream brain regions with VSV-G-pseudotyped lentivirus through a second local viral injection to express opsins under the control of Cre enzymes. This way, the retrograde expression of a small number of Cre enzymes can be used to initiate the production of a large number of opsins in these projecting cells. However, as it is already difficult to perform genetic modification with a single virus in monkeys, the feasibility of performing such two-step viral transduction method remains to be tested.

Adeno-associated virus

AAV is becoming a common vector of choice for human gene therapy or basic research because of its low pathogenicity, low immunogenicity, high efficiency, and long-lasting transgene expression (Muzyczka, 1992; Peel and Klein, 2000). AAV, a nonpathogenic human parvovirus, $\sim 20\text{nm}$ in size, is a nonenveloped, single-stranded DNA virus with a 4.7-kb genome surrounded by coat proteins. AAVs are present in $\sim 80\%$ of human adults but cause no known pathology. AAV has been developed as a human gene therapy vector since 1984 (Hermonat and Muzyczka, 1984). Current recombinant AAV virus has $\sim 96\%$ of its viral genome removed, resulting in a greater reduction of possible immune responses for gene therapy. In non-active, nonamplifying conditions, AAV integrates into a specific site on human chromosome 19. However, when introduced as a gene therapy vector, only $\sim 10\%$ of AAV particles integrate into the genome and in a random nonspecific fashion (Thomas et al., 2003).

It has been of great interest to engineer different serotypes for effective and cell-type-specific transduction with AAVs (Muzyczka and Warrington, 2005). Infection efficiency of AAVs to target cells can be influenced by a variety of factors involved in the viral mediated gene expression process, that is, binding efficiency of the viral particles to specific cell surface receptors, rate of virus endocytosis, intracellular trafficking to cell nucleus, removing of the viral coat proteins, synthesis of the second strand of the viral genome, and transcription and translation of the gene of interest in the nucleus. Engineering novel AAV serotypes has been mostly through modifying AAV capsids, the protein shell, because such approaches are expected to have minimal influence on vector assembly, packaging, and particle stability. So far, over 100 unique AAV capsid sequences have been identified, among which AAV1–9 and Rh10 have been characterized in greater detail.

To produce AAVs, the capsid sequence for each serotype is often engineered into a separate vector,

in addition to the genomic vector that contains the gene of interest. Thus, the same genomic vector can be packaged with different capsids, enabling direct comparison of the efficiency of each capsid serotype in transducing specific cells. Note, since in most protocols, the genomic vectors used contain the inverted terminal repeats (ITRs) from AAV2, the pseudotyped AAV virus is often called AAV2/*, such as AAV2/5, often simplified as AAV5 in the literature, as well as in the following text, meaning the virus is made from the genomic vector containing AAV2 ITRs but pseudotyped with AAV5 capsid.

Tropism of different AAVs varies between developmental stages, cell types, and species. So far, AAV1, 2, 5, 7, 8, 9, and Rh10 seem to transduce adult brain cells with various efficiencies. In the adult rodent brain, direct comparison of AAV2, 4, 5 revealed that AAV5 has the highest efficiency in transducing striatum, both neurons and glia (Davidson et al., 2000). (Note, in neonatal mice brain, AAV1 seems to be better than AAV2 in transducing brain cells, whereas AAV5 failed to achieve a significant amount of transduction (Passini et al., 2003).) Direct comparison of AAV1, 2, 5, 7, and 8 suggested that AAV5, 7, 8 are able to transduce comparable brain volume at high titers, but at lower titers AAV5 and 7 transduced larger brain volume than AAV8 (Taymans et al., 2007). Direct comparison of AAV7, 8, 9, and Rh10 revealed that they are all quite efficient in transducing neurons, but not glia, in the cortex, hippocampus, striatum, and thalamus. Their transduction efficiencies slightly vary in different brain structures, with AAV9 being most effective in the hippocampus and cortex, and Rh10 being most effective in the thalamus (Cearley and Wolfe, 2006). Cearley further screened and identified new serotypes hu.32, hu.37, pi.2, hu.11, rh.8, and hu.48R3 that are all more efficient than AAV9 (Cearley et al., 2008). In addition, Lawlor et al. demonstrated that cy5, rh20, and rh39 are more efficient than AAV8 (Lawlor et al., 2009). Retrograde transport of AAVs has also been observed for various

serotypes in different brain structures. Most prominently, Cearley et al. observed that AAV9 and Rh10 are effectively transported retrogradely in many brain structures. AAV9 seems to result in more efficient retrograde transduction in the hippocampus and septal nuclei, whereas Rh10 is more effective in the thalamus (Cearley and Wolfe, 2006). With the identification of over 100 new serotypes, detailed characterization of these different serotypes should identify more efficient and more specific serotypes for different types of cells in different brain structures (Gao et al., 2002).

The recent discovery that AAVs can pass the blood–brain barrier has created tremendous enthusiasm in the use of these vectors. Duque et al. found that AAV9 with double-stranded genome can pass the blood–brain barrier when delivered intravenously in neonatal mice and resulted in the transduction of motor neurons in the spinal cord and all brain structures being tested including cortex, striatum, thalamus, hippocampus, cerebellum, and brain stem (Duque et al., 2009; Zhang et al., 2011). However, intravenous delivery of AAV9 in adult mice almost exclusively transduces astrocytes throughout the entire CNS, with little neuronal transduction (Foust et al., 2009). Another study showed that Rh10 is comparable if not more potent than AAV9 in transducing brain cells when delivered through systematic intravenous delivery (Zhang et al., 2011). Although it remains to be determined whether such delivery route will be effective in monkeys, it is highly plausible that some or many of the new AAV serotypes will be able to transduce brain cells through systemic injections.

In primates, direct comparison of AAV1–6 showed that AAV5 is the most efficient vector in substantia nigra and striatum, which transduces both neurons and glia (Markakis et al., 2010). AAV5 seems to be more efficient than AAV8 (Dodiya et al., 2010). AAV1 can effectively transduce both neurons and glia in monkey brains but unfortunately seemed to induce strong humoral and cell-mediated immune responses (Hadaczek et al., 2009). AAV1 has been successfully used to

transduce striate cortex in monkeys for two photon imaging (Heider et al., 2010; Stettler et al., 2006). Recently, Diester et al. used AAV5 with a synapsin promoter or a Thy-1 promoter to express ChR2, ChR2-C128S mutant, and eNpHR2.0 in the monkey cortex (Diester et al., 2011). These viruses induced strong expression in cortical neurons, and the transduced neurons responded well to light modulation. Unexpectedly, Diester et al. found no superficial layers of the cortex expressed any of these opsins regardless of promoters. It remains unclear whether this lack of labeling is due the specific serotype used, the promoters used, or the cortical regions transduced.

In summary, AAV1, 5, 8, 9, and Rh10 are all effective in transducing neurons. At lower titers, AAV5 seems to be more efficient than AAV1 and 8, but AAV5 seems to transduce both neurons and glia. In contrast, AAV8, 9, and Rh10 seem to be more specific to neurons. Perhaps due to the lack of lipid envelop, AAV can be easily concentrated to a high titer of 10^{12} or higher. AAV is also much more stable during storage, and the titer of AAV does not seem to decline noticeable even after a couple years of storage at -80°C . It is difficult to manufacture AAVs in a research laboratory due to the complicated procedures in purifying and concentrating AAVs, which may even influence the tropism of the virus (Klein et al., 2008). However, many viral core facilities are providing standard service for packaging AAVs with different serotypes with typical titers of 10^{12} and above, such as University of North Carolina gene therapy center (<http://genetherapy.unc.edu/>), University of Florida Powell gene therapy center (<http://www.gtc.ufl.edu/>), and University of Pennsylvania gene therapy program (<http://www.med.upenn.edu/gtp/>).

Cell-type specificity achieved with viral methods

Genetic targeting of specific cell types has been successful in transgenic mice, facilitated by the bacteria Cre–Lox system (Yizhar et al., 2011). Most recently, several new lines of transgenic

mice have been made to facilitate the targeting of specific cell types using the Cre–Lox system, in which opsin expression is regulated by Cre enzymes (Katzel et al., 2011). These advances and the continued effort in improving the expression levels of opsins in particular cell types in transgenic mice will certainly revolutionize the analysis of the causal role of specific cells in neural circuit functions in rodent models.

Transgenic monkeys, on the other hand, are costly and time consuming to generate and maintain. So far, there are two lines of transgenic rhesus monkeys reported, one expressing GFP alone (Chan et al., 2001), and the other expressing human huntingtin gene (Yang et al., 2008). In addition, transgenic marmosets have also been successfully generated (Sasaki et al., 2009). In monkeys, and other genetically intractable models, viral methods will remain the main methods of expressing opsins. However, limited success has been made in targeting specific cell types with viral technologies. This could be due to the limited packaging capacity of lentivirus or AAV, the lack of understanding of the interaction between target cells and viral particles, and the difficulty of predicting cellular regulation mechanisms of viral mediated gene expression. The glycoproteins on the envelops of the lentivirus or the capsid proteins of the AAV will determine the entry of viral particles to specific cell types through interactions with the surface receptors presented on target cells. So far, the commonly used lentivirus pseudotyped with VSV-G or AAV pseudotyped with the capsids from AAV1, 5, 8, 9, and Rh10 are able to effectively transduce neurons in rodents and monkeys. AAV1 and 5 are able to transduce both neurons and glia, whereas AAV 8, 9, and Rh10 seem to be more specific toward neurons. Lentivirus can infect both neurons and glia but prefers neurons at lower titers.

Detailed analysis of AAV1 tropism revealed that the transduction efficiency of excitatory neurons, inhibitory neurons, and nonneuronal glia cells depends upon promoters, viral titers, and cortical layers. For example, AAV1 with a CAG promoter can transduce both neurons and

glia cells effectively, but AAV1 with hSyn promoter preferentially transduce layers 2/3 inhibitory neurons at lower titer of $\sim 3 \times 10^{11}$ and preferentially transduce excitatory neurons at a higher titer of $\sim 8 \times 10^{12}$ (Nathanson et al., 2009b). In monkeys, AAV1 selectively labeled neurons (Heider et al., 2010), whereas AAV5 showed no specificity toward different cell types, transducing both excitatory, inhibitory neurons and glia (Diester et al., 2011).

Lentivirus, with its ability to package larger transgenes, has the potential to carry slightly larger promoter sequences for cell-specific targeting. Lentivirus with a CaMKII promoter has been demonstrated to selectively label excitatory neurons in mouse (Dittgen et al., 2004) and monkey cortex (Fig. 1c; Han et al., 2009). Such specificity may be partly attributed to the tropism of lentivirus particles themselves, as it has been suggested that lentivirus with hSyn promoter can selectively label excitatory neurons in the rodent cortex (Nathanson et al., 2009b). However, we have observed that high titer lentivirus with a CAG promoter readily labeled GFAP positive glia cells in the primate frontal cortex. In addition, the purification procedure during lentivirus production may influence the tropism of lentivirus.

There are a few successes in identifying promoters specific for particular cell types. A synthetic promoter or enhanced promoter based on the promoter sequence of human dopamine β -hydroxylase promoter, when used in adenovirus, was able to mediate efficient and specific transduction of noradrenergic neurons in rat locus coeruleus (Hwang et al., 2001, 2005). Lentivirus with a 3-kb promoter region of neuroactive peptide cholecystokinin (CCK) was found to be specific in transducing CCK positive cells (Chhatwal et al., 2007). Recently, a detailed systematic analysis was performed by the Callaway group in examining over 20 short promoters derived from fugu, mouse, human, and synthetic ones, packaged in AAV1 or lentivirus (Nathanson et al., 2009a). They identified a number of short promoters that showed selectivity toward inhibitory neurons versus excitatory neurons in rodents. However,

none of these promoters targeted a specific set of inhibitory neurons that can be coordinated with our current classification of inhibitory neurons based on protein markers, that is, parvalbumin, somatostatin, CCK, calretinin, etc. It remains to be determined whether these short promoters will be able to achieve higher specificity in monkeys.

***In vivo* optical control of transduced cells**

Success in the adaptation of optogenetic techniques to control the activities of specific cells in monkeys has raised significant excitement in the field (Diester et al., 2011; Han et al., 2009, 2011; Fig. 2). The effectiveness of optically controlling neural activities is determined by the absolute amount of light reaching the neuron, the number of opsin molecules presented on the neuronal plasma membrane, and the sensitivity of the opsins to light. The final output of the control precision, the time course and the magnitude of light modulation, is also influenced by the intrinsic physiological properties of the neuron and its surrounding neural network environment, which cannot be controlled by experimenters. In monkeys, a few unique challenges remain to be overcome to achieve effective optical control of larger brain volume and to perform long-term recordings that is often required for monkey experiments. The tissue volume that can be controlled optically is determined by the optical properties of the brain, the biophysical properties of the opsins, and the efficiency of genetic transduction of cells. Long-term repeated optogenetic experiments within the same brain region require minimal tissue damage during each recording and optical control session.

Optical properties of the brain

The absolute power of light reaching a neuron depends upon the input of light power at the optical fiber tip and the pattern of light propagation in the tissue. Light propagation is determined by

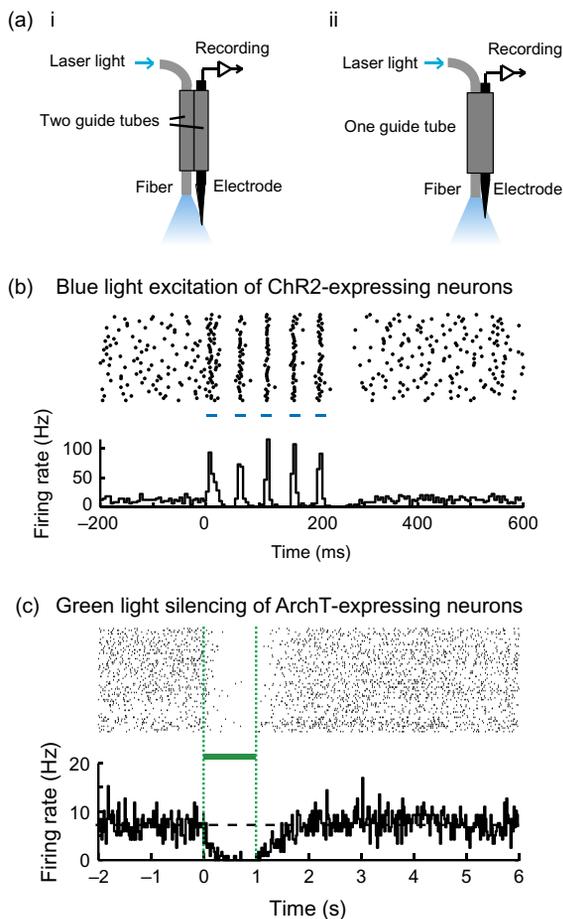


Fig. 2. Temporal precise optical activation or silencing of cortical neurons. (a) Two configurations of coupling an optical fiber to a recording electrode. (a-i) The optical fiber and the electrode are guided separately in two individual guide tubes. The relative distance between the optical fiber and the electrode can be easily adjusted during experiments. (a-ii) The optical fiber is directly glued to the electrode and is guided together within the same guide tube. (b) Temporal precise optical activation of ChR2-expressing cortical excitatory neurons. Top, spike raster plot displaying each spike as a black dot and each trial as a horizontal row; bottom, the histogram of instantaneous firing rate, averaged across all trials. Periods of light illumination are indicated by horizontal blue dashes. (c) Temporal precise optical silencing of ArchT-expressing cortical excitatory neurons. Top, spike raster plot; bottom, histogram of instantaneous firing rate. Periods of light illumination are indicated by horizontal green dashes. (Adapted from Han et al., 2009, 2011.)

the optical properties of brain tissue, with major considerations being tissue absorption and tissue scattering (Mobley and Vo-Dinh, 2003). Brain tissues are heterogeneous, with spatial variations in their optical properties. This spatial variation and the density of this variation make brain tissues strong scatterers. Tissue scattering and absorption will reduce the intensity of light as it propagates within the tissue, away from the light source. At locations within the close proximity of the light source, where light intensity is at the saturation level for opsin functions, the efficiency of optically controlling neurons will not vary with locations. But at locations further away from the light source, where the light intensity falls below the saturation level, the power to control neural activities will reduce with distance, and eventually at locations where the light power falls below the threshold of opsin activation, light will be unable to modulate neural activities. In addition, the presence of electrodes and optical fibers will influence the pattern of light propagation.

Brain tissues have been successfully modeled as a two-component system, a homogeneous continuum with randomly positioned scattering and absorbing particles (Bevilacqua et al., 1999; Yaroslavsky et al., 2002). Tissue absorption is determined by its molecular compositions. In the brain, hemoglobins, both oxygenated (HbO_2) and deoxygenated (Hb), are the major light absorbers at visible wavelength relevant for optical control (Fig. 3, summarized by Scott Prahl, <http://omlc.ogi.edu/spectra>). Current available classes of opsins, channelrhodopsins, halorhodopsins, and archaerhodopsins, are mainly excited by visible light of 450–600nm. Hb/HbO₂ absorbs highly in this wavelength range. To increase light propagation, it is useful to develop novel opsin molecules that can be sensitized with red light, that is, >650nm, where both Hb and HbO₂ absorption coefficients are drastically reduced. Development of these novel optogenetic molecules would be particularly useful in experiments in monkeys where controlling larger brain volumes may be necessary to perturb enough neurons to influence information processing.

Monte Carlo simulation of light propagation

To model light propagation in brain tissue, a radiation transport model has been established to simulate photon energy transport that explicitly ignores the complex multiple scattering effects. To simulate light transport and to visualize the distribution of light in tissue, the widely accepted and most commonly used method is Monte Carlo simulation (Mobley and Vo-Dinh, 2003). Monte Carlo methods include a broad class of computational algorithms that employ random numbers in simulating complex systems. In predicting light propagation in tissues, the Monte Carlo method tracks the trajectory of each photon and calculates the light intensity at each position within the tissue based on the distributions of photons. Photon trajectory is estimated based on the random walk that each photon performs in a specific tissue, and the specific parameters for each step, that is, length and direction, are calculated using random numbers. The accuracy of the simulation increases with the number of photons launched. Typically more than 100,000 photons are required for three-dimensional simulations of light propagation in the brain.

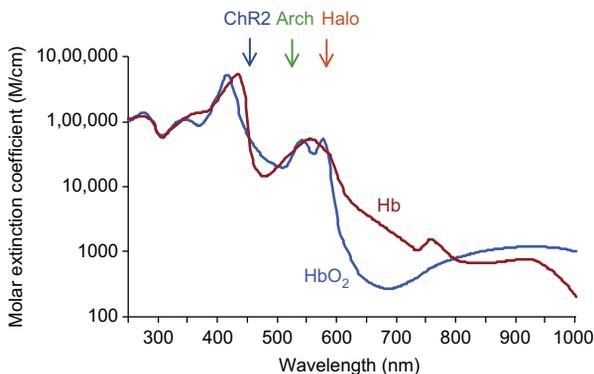


Fig. 3. Hemoglobin, oxygenated (HbO_2) and deoxygenated (Hb), absorption spectrum. Values are based on that summarized by Scott Prahl, <http://omlc.ogi.edu/spectra>. The peak excitation wavelengths for ChR2, Arch, and Halo are indicated.

Monte Carlo simulation for both blue and yellow light propagation in the brain was performed by Ed Boyden's lab, for both light emitted out of LEDs and optical fibers (Bernstein et al., 2008; Chow et al., 2010). For example, they simulated the trajectory of yellow light emitted from the end of an optical fiber in a cube of gray matter of $200 \times 200 \times 200$ grid of voxels, corresponding to $10\text{mm} \times 10\text{mm} \times 10\text{mm}$ in dimension, using previously published parameters and algorithms (Binzoni et al., 2006; Wang et al., 1995; Yaroslavsky et al., 2002). In this simulation, a scattering coefficient of 13mm^{-1} and an absorption coefficient of 0.028mm^{-1} were used from the interpolated data in Yaroslavsky et al. (2002). 5×10^6 photons were launched in a pattern through a model fiber with a numerical aperture of 0.48 (Optran 0.48 HPCS, Thorlabs; Wang et al., 1995). The anisotropic scattering model based on the Henyey–Greenstein phase function with an anisotropy parameter of 0.89 was first used before randomizing the photon trajectories (Binzoni et al., 2006; Yaroslavsky et al., 2002). In the simulation, the photon was absorbed according to the distance it traveled for each step. When a photon enters a voxel, the stimulation probabilistically calculates the forecasted traveling distance for the next step, and the direction of the photon packet propagation is randomly chosen according to the Henyey–Greenstein function. Using this model, the estimated light propagation in brain tissue can be plotted (Fig. 4a). The simulated light distribution generally agrees with that measured in brain slices (Wang et al., 1995).

Experimental examination of light propagation on optical control efficiency

To estimate the influence of light propagation on the efficiency of optical modulation *in vivo*, we have performed detailed analysis of light modulation of neural activities recorded with the same electrode at the same location in the brain of awake monkeys (Han et al., 2009). First, we systematically reduced the light power out of the fiber tip while

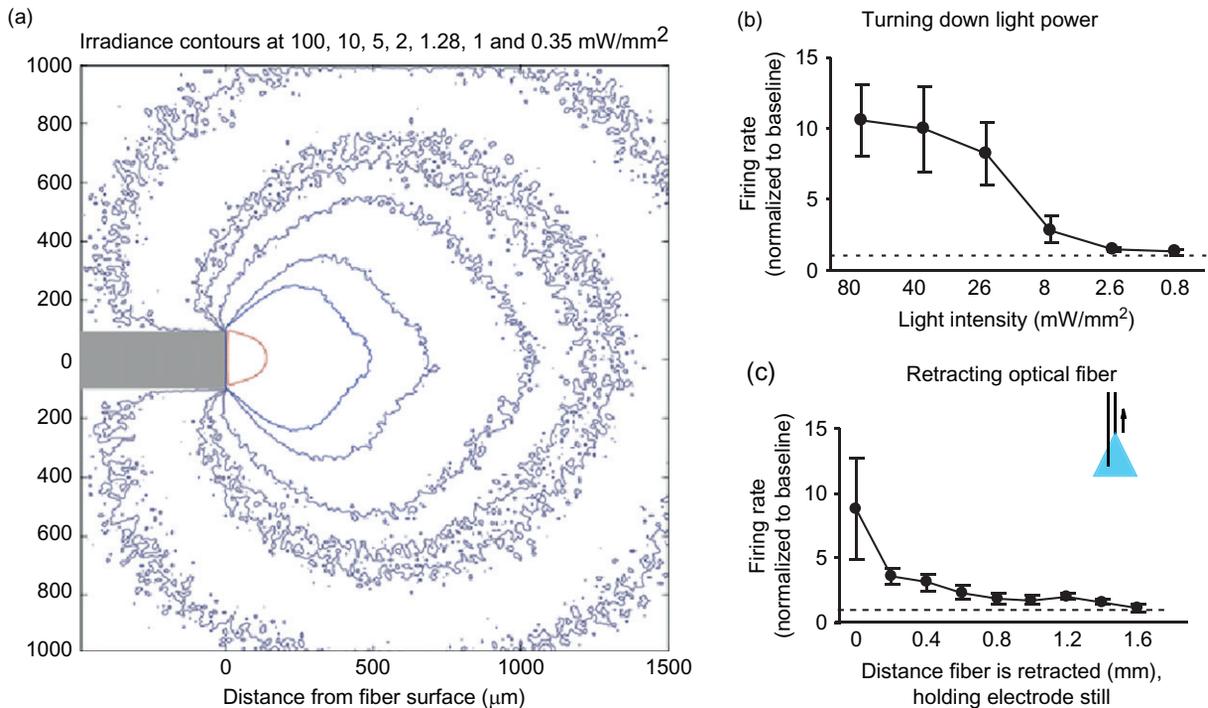


Fig. 4. Influence of light propagation on optogenetic control. (a) Monte Carlo simulation of light propagation in the brain. 593nm yellow light irradiance in the brain gray matter is plotted as a function of location with respect to the tip of an optical fiber (200 μ m in diameter, represented by the gray rectangle shown at left; the irradiance at the surface of the fiber tip was set at 200mW/mm²). The contours display 100, 10, 5, 2, 1.28, 1, and 0.35mW/mm² irradiances. (b) Light intensity dependence of optical control. Optical modulation of neural activity driven by ChR2-mediated activation of excitatory neuron is determined upon illumination with different light power intensities. (c) Distance dependence of optical control. Modulation of neural activities is determined when optical fibers were retracted in 200 μ m steps, while holding the electrode still. (Adapted from Han et al., 2009.)

keeping the distance between the optical fiber and the recording electrode constant (Fig. 4b). Reduction of light intensity reduced the efficiency of light modulation. However, the effect of light power on modulation efficiency is not linear. Efficiency of light modulation drops sharply from 26 to 8mW/mm², and the effect of optical modulation is nearly abolished at \sim 2.6mW/mm². In a second experiment, we kept the light power constant but systematically retracted the optical fiber away from the recording electrode in 200 μ m steps (Fig. 4c). The change in optical modulation drops sharply when the optical fiber is retracted from the recording

electrode, with most of the reduction happening in the first 200 μ m step. Together, our experimental observations agree with the Monte Carlo simulation of light propagation, in which light falls off nonlinearly and falls to \sim 1% at a location \sim 1mm away from the fiber tip.

Tissue damages from device insertion and heat

Since many experiments conducted in awake behaving monkeys are chronic, often extending to several years, a major consideration for optogenetic

experiments is tissue damage introduced by device insertion, that is, viral injection cannula and optical fibers, and the tissue damage from heat produced by light. Viral injection-induced tissue damage is a somewhat minor concern because viral mediated gene expression, either with lentivirus or with AAV, is long lasting. One successful injection of a few microliter of virus is often sufficient to label a spherical volume of a few millimeters in diameter. A critical consideration is the success of injecting virus into targeted areas in the monkey brain. MRI or electrophysiology mapping would be helpful in targeting the desired locations. To perform an accurate injection, it is important to eliminate as much as possible the dead space between the syringe that holds the virus and the needle tip in the brain. It would be optimal to position the syringe directly over the injection site and connect the needle directly to the syringe without using tubing in between. Many commercially available syringe pumps are compact and can be easily mounted onto a manipulator, for example, UltraMicroPump from World Precision Instruments. However, if tubing is necessary, it is important to use thin and nonelastic tubing to efficiently transduce the force from syringe pump to the tip of the needle. Before and after each injection, it is useful to check possible leaks.

To reduce mechanical damage from optical fibers, ideally one would want to use optical fibers as thin as possible. Typical electrodes, even with a shank size of 200 μm , have fine tips within 10 μm . A 200- μm optical fiber with a blunt tip is thus orders of magnitude larger than an electrode tip. We have tried to taper the optical fiber to address this issue. But, it is difficult to polish a tapered fiber tip, and the distribution of light out of the fiber tip is different from that modeled out of a blunt end fiber tip. With these potential concerns, it may still be advantageous to use tapered fibers, since the variability in viral injections and the uncertainty of targeting the injection sites in monkeys are often more variable than the variability in light emission out of a tapered fiber tip. A potential strategy to avoid repeated penetration of brain tissue with optical fiber is to leave the optical fiber in the brain for as

long as possible. Another strategy of reducing the mechanical tissue damage is to use arrays of small optical fibers. For example, a single optical fiber of 200 μm in diameter is equivalent in volume to four optical fibers of 100 μm in diameter. But four optical fibers of 100 μm in diameter are capable of illuminating a much larger volume than a single 200 μm fiber. Adaption of high-density fiber arrays, such as those developed in Ed Boyden's lab, will be helpful to reduce mechanical tissue damage (<http://syntheticneurobiology.org/>).

Heat produced by shining light into the brain is another consideration for tissue damage. Measuring heat dissipation within the brain is rather difficult, since the introduction of the measuring device itself will influence heat dissipation. It has been observed that heating of the metal recording electrode with strong laser light will in turn activate wild-type neurons expressing no opsins, though this heat-induced neural activation has a much slower time constant (personal communication with Michael Fee). Thus, whenever possible, the power of laser light used should be limited to what is sufficient to drive neurons. Typically, a few hundred mW/mm^2 of irradiance or a few mW of total light power does not seem to produce detrimental damaging effects.

Neuronal and behavioral modulation with optogenetic control

Electrical recording of neural activities in monkeys has been a major driving force in our understanding of the neural basis of many brain functions, for example, sensation, action, decision making, attention, emotion, etc. Electrophysiological methods can establish a precise correlative relationship between neural activity patterns and brain state or behavioral phenomena with a high temporal resolution. However, optogenetic control provides a unique approach in examining the causal role of specific neural activity patterns in neural information processing and behavior. Often during optogenetic experiments, simultaneous optical

control and electrophysiology or optical imaging methods are performed to directly link the changes in neural activity patterns to neural network dynamics or behavior. Here, I focus on challenges in simultaneous electrophysiological recording during light illumination and the possibilities for current failures in modulating behavior with optogenetic control in monkeys.

Optical artifact on metal electrodes

Coupling an optical fiber to a metal electrode is a simple and reliable way to measure light modulation of brain activities. However, we and others have observed strong voltage deflection artifact when laser light was directed onto metal electrode tips, in the brain or in saline (Fig. 5; Ayling et al., 2009; Han et al., 2009, 2011). This effect was clearly observed when the electrode tip was positioned in the blue laser beam in saline. This artifact was also evident in the brain with a radiant flux of 80mW/mm^2 , a light intensity that is often needed for *in vivo* optogenetic experiments, when the tip of the optical fiber is 0.5–1mm away from the electrode tip. It is possible that part of the voltage deflection recorded in the brain reflects physiological changes in local field potential (LFP) upon optical stimulation of transduced neurons. However, it is not yet possible to isolate light evoked physiological responses from the optical artifact.

The light-induced artifact is slow evolving. Upon illumination with a long light pulse of 200 ms, the voltage deflection slowly reaches its peak after tens of milliseconds, which can be easily eliminated with a high-pass filter that electrophysiologists typically use for isolating spikes during extracellular recordings. For example, this artifact is completely removed by the band pass filter of 170–8000Hz used in Plexon data acquisition system. However, precautions are needed when laser light is pulsed at higher frequencies, since high-frequency artifact produced by brief light pulses cannot be removed by simply filtering

the signal with high-pass filters. But if the artifact produced by brief high-frequency light pulse trains is significantly different from the spikes recorded, it is possible to isolate the light artifact waveforms through spike sorting. In contrast, LFP that measures slow voltage fluctuations at lower frequencies in the range of Hz to tens of Hz cannot be isolated from slow evolving light-induced artifact. Thus, while this artifact typically does not influence the ability to record spikes at the site of illumination, it does prevent accurate measure of LFP at the site of illumination.

The magnitude of the artifact depends upon the precise power of light illumination, the relative position of the light source and the electrode, the properties of the electrode tip, and the optical properties of the brain tissue between the light source and the electrode. We have observed that the magnitude of the artifact is proportional to the power of light illumination but varies with the wavelength of the light. For example, we observed stronger voltage deflection artifact with 472nm blue light than with 532nm green light or 589nm yellow light.

The observed light-induced artifact is consistent with the Becquerel effect. The Becquerel effect describes a classical photoelectrochemical phenomenon first demonstrated by French scientist Becquerel in 1839 (Gratzel, 2001; Honda, 2004). Becquerel demonstrated that exposing metal electrodes, such as platinum, gold, and silver to sunlight produced very small electric current when these metals were positioned in electrolyte. This phenomenon has inspired major research interests in improving this photoelectrochemical effect in converting sun light to electrical powers. However, for the neuroscientists applying optogenetic techniques, it remains a critical challenge to minimize such photoelectrochemical effects.

Consistent with the generality of the Becquerel effect, we observed such artifact with metal electrode wires made of stainless steel, platinum–iridium, silver/silver chloride, gold, nichrome, copper, or silicon. However, we have never observed such

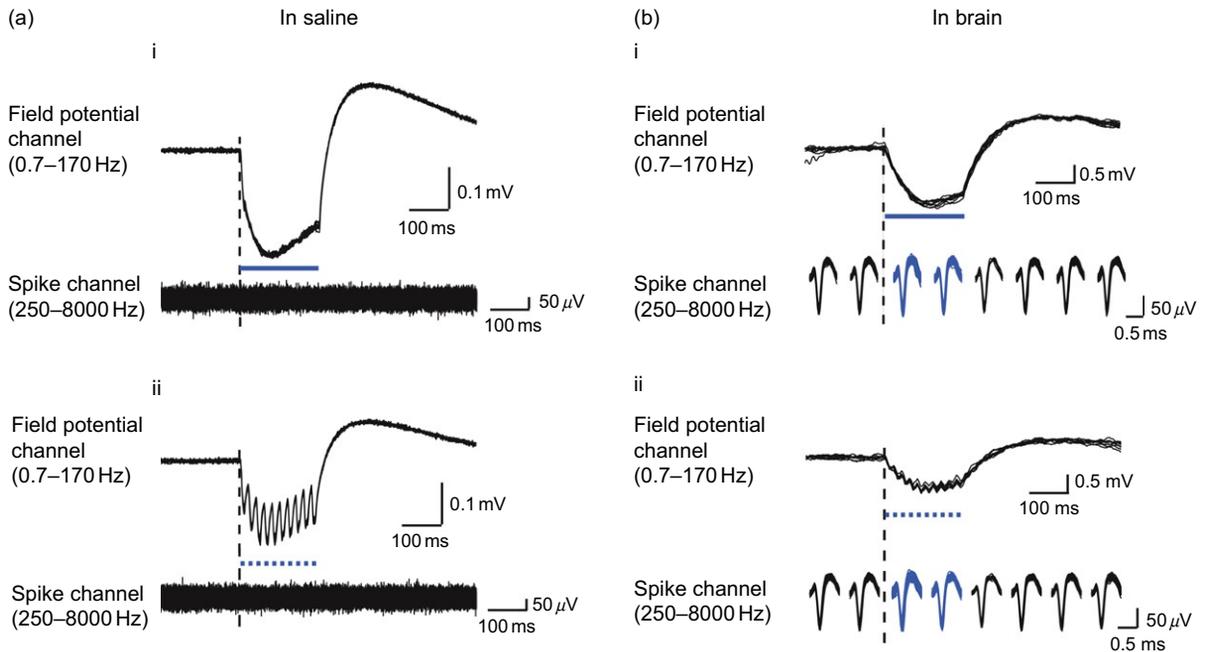


Fig. 5. Optical artifact observed on tungsten electrodes immersed in saline (a) or brain (b) upon tip exposure to 200ms blue light pulses (i) or trains of 10ms blue light pulses delivered at 50Hz (ii). Light pulses are indicated by blue dashes. Electrode data were hardware filtered using two data acquisition channels operating in parallel, yielding a low-frequency component (field potential channel) and a high-frequency component (spike channel). For the “spike channel” traces taken in brain (b), spikes were grouped into 100ms bins, and then the binned spikes were displayed beneath corresponding parts of the simultaneously acquired “field potential channel” signal. (Shown are the spikes in eight such bins—the two bins before light onset, the two bins during the light delivery period, and the four bins after light cessation.) (Adapted from [Han et al., 2009](#).)

artifact with hollow glass microelectrodes ([Boyden et al., 2005](#); [Han and Boyden, 2007](#)). A few cautions have to be made with glass electrodes in optogenetic experiments. For example, if laser light reaches the Ag/AgCl wire that is in direct contact with the solution inside the glass electrode, light will induce artifact. Since the Ag/AgCl wire is typically tens of millimeters away from the tip of the glass electrode under illumination, this can be easily controlled. Similarly, if light reaches the metal ground electrode, this optical artifact will also be picked up by glass electrodes. Even though glass electrodes offer a good way to circumvent the artifact problem with recording LFP at the site of illumination, it is difficult to record from multiple glass electrodes, and the use of glass electrodes in monkeys are

limited, in particular in chronic awake experiments where breaking of the electrode tip in the brain would lead to significant tissue damage at the recording sites.

It might be possible to develop computational methods to remove this artifact, since the amplitude and the time course of this artifact are stable with repeated light illumination when the electrode and the optical fiber remain at the same location and the light intensity remains the same. However, to isolate or average out the real physiological effects, some computational/experimental methods have to be used, which is yet to be developed. More promisingly, optimization of the electrode tip surface or electrode material may be proven useful in eliminating optical artifact.

Recently, Zorzos and Boyden eliminated this artifact by coating the surface of the electrodes with conducting material indium tin oxide (ITO; Zorzos et al., 2009). Continued advance in improving electrode coating strategy or developing novel electrodes is critical in enabling measurement of LFP at the site of illumination.

Homeostatic neural dynamics upon perturbing specific neurons

Altering the activities of a small set of neurons or even a single neuron can induce complex network changes, as demonstrated elegantly by recent experiments through intracellular current injection via whole cell patch clamp electrodes in anesthetized rats (Brecht et al., 2004; Li et al., 2009). For example, stimulating a single neuron in the superficial layers of the visual or somatosensory cortex can switch global cortical states from slow wave-like to rapid-eye-movement-sleep-like states (Li et al., 2009), whereas stimulating a single pyramidal cell in layer six of the motor cortex can evoke whisker movement (Brecht et al., 2004). Since light cannot be easily directed to only one cell as with a patch electrode, the major advantage of optogenetics is to control a set of genetically identified cell types. It might be possible to stimulate just a few cells when light is directed into areas with sparsely transduced cells.

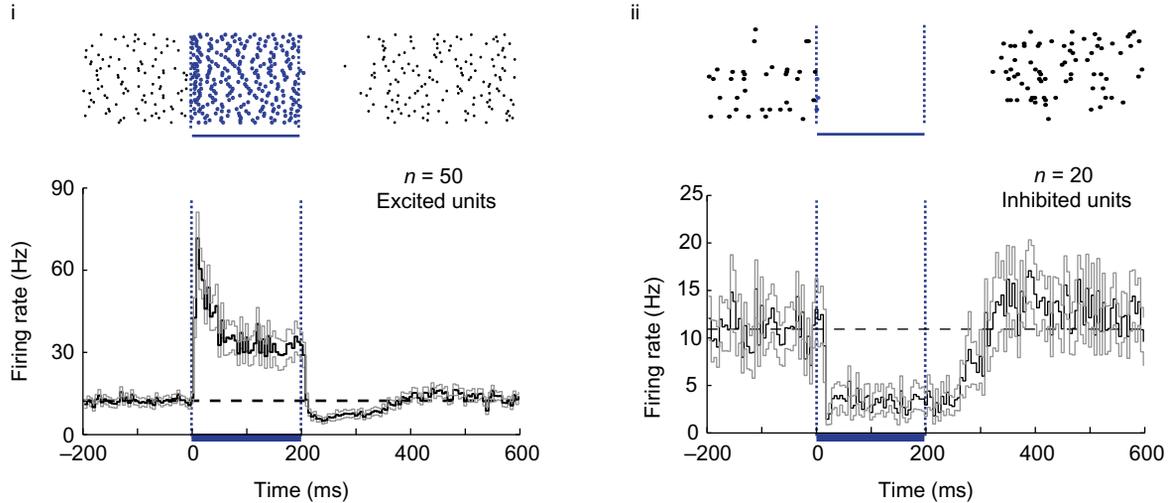
With the readily available genetic techniques in transgenic mice, rapid progress is being made in assessing the functions of specific cell types in transgenic mouse models, such as, parvalbumin-positive cells (Cardin et al., 2010; Sohal et al., 2009), hypothalamus POMC-positive neurons (Aponte et al., 2011), cholinergic neurons (Witten et al., 2010), specific dopamine receptor-expressing neurons (Kravitz et al., 2010; Lobo et al., 2010), and retina ganglion cells (Thyagarajan et al., 2010). However, the examination of the functional significance of specific cell types in genetically intractable animals is limited by the available genetic tools as described above. So far, we are only able to target cortical

excitatory neurons as recently demonstrated with a lentivirus with a CaMKII promoter in monkeys (Han et al., 2009).

When a population of pyramidal cells expressing ChR2 was excited with blue laser light in the monkey brain, we observed a major population of excited cells, and a significant minority of suppressed cells (Fig. 6a and b). In contrast, temporary silencing of a population of pyramidal cells expressing ArchT resulted in a major population of suppressed cells, and a significant minority of excited cells (Fig. 6c and d). Together, these results suggest that when a population of cells is directly controlled, either excited or silenced, a secondary component from a minority of cells reacted in the opposite fashion, which could in part homeostatically balance the network activity produced by direct optical perturbation. The secondary component responded with a longer latency than the primary response. Further, when a perturbation is induced repeatedly, the set of cells that undergo the secondary response does not change from trial to trial but instead retains its identity.

Together, these results suggest a novel homeostatic principle for the cortex governing changes upon the control of the activity of specific cell populations. It is possible that specific neural circuit elements, such as inhibitory neurons, are important in balancing the overall network responses upon perturbation of pyramidal neurons. For example, the silencing of pyramidal neurons decreases their excitatory drive to inhibitory neurons, which in turn disinhibit downstream targeted cortical neurons; conversely, exciting pyramidal neurons increases the drive to inhibitory neurons, decreasing activity in targeted cortical neurons. It is important to point out that interactions between cortical areas may also contribute to the observed opponent responses. The complex neural network architecture also predicts that the secondary responses may also modulate the primary responses. For example, a neuron may increase its activity as a direct response to optical modulation. However, it also receives synaptic inputs from neurons responding in the opposite

(a) Blue light excitation of ChR2-expressing excitatory neurons



(b) Green light silencing of ArchT-expressing excitatory neurons

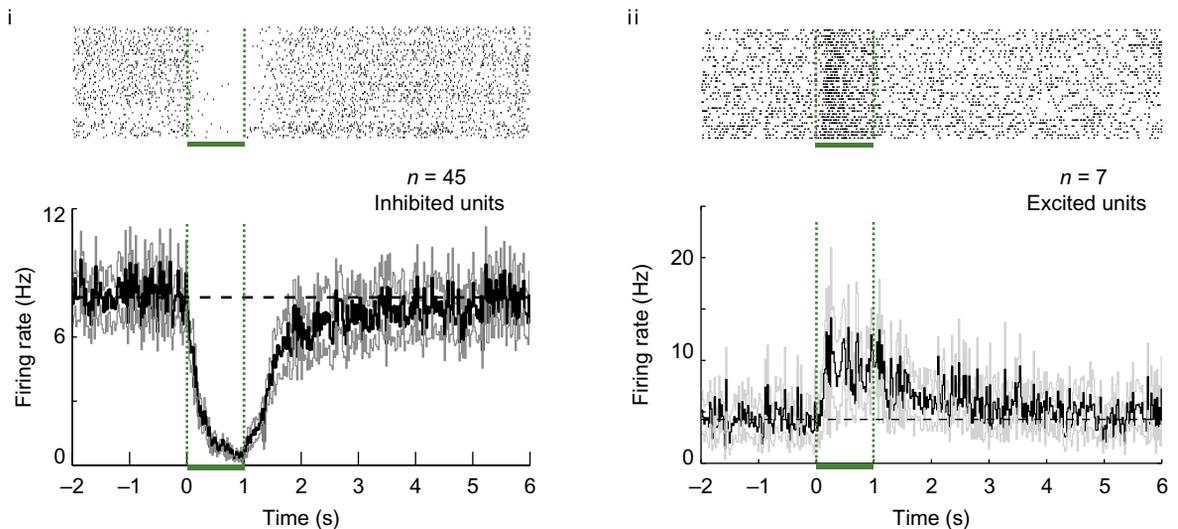


Fig. 6. Homeostatic neural network responses upon optical activation or silencing of cortical excitatory neurons. (a-i) Top, increase in spiking activity in one neuron during blue light illumination of ChR2-expressing neurons. Spike raster plot displays each spike as a black dot and each trial as a horizontal row; periods of blue light illumination are indicated by horizontal blue dashes. Bottom, instantaneous firing rate, averaged across all excited units recorded upon 200ms blue light exposure (black line, mean; gray lines, mean \pm standard error) ($n = 50$ units). (a-ii) Top, decreases in spiking activity in one neuron during blue light illumination of ChR2-expressing neurons. Bottom, instantaneous firing rate averaged across all suppressed units upon 200ms blue light exposure ($n = 20$ units). (b-i) Top, decreases in spiking activity in one neuron during green light illumination of ArchT-expressing neurons. Bottom, instantaneous firing rate averaged across all suppressed neurons upon 1s light exposure ($n = 45$ units). (b-ii) Top, increases in spiking activity in one neuron during green light illumination of ArchT-expressing neurons. Bottom, instantaneous firing rate averaged across all excited neurons upon 1s light exposure ($n = 7$ units). (Adapted from Han et al., 2009, 2011.)

fashion, which would lessen, slow, or reverse the primary responses. Indeed, we have observed complex time course upon optical stimulation, in particular in Chr2-expressing cortical neurons. Often a brief excitation is followed by a long-lasting inhibition, which sometimes last for hundreds of milliseconds after light illumination.

Given that brain homeostasis has previously been detected chiefly on the timescale of minutes to days, and at the level of proteins and synapses (Thiagarajan et al., 2005; Turrigiano et al., 1998), the ability to detect homeostasis at the network level at the millisecond timescale may open up new studies on how networks dynamically reconfigure during behavior. Principles that predict or govern how a neural circuit reacts to a particular kind of neural manipulation, activation or silencing of particular cells, will be increasingly important if such tools are to be used in an algorithmic fashion to control the state of a neural network.

Optical modulation of behaviors in monkeys

So far, with a single optical fiber illuminating a limited volume of brain tissue, no behavioral modification has been reported in monkeys, even though small electrical stimulation at the same site elicited clear behaviors (Diester et al., 2011; Han et al., 2009). This may be due to the limited volume of tissue that is effectively controlled with optogenetics, as compared to microelectrical stimulation. Although optogenetic modulation of behavior has been very successful in rodents, the volume of illumination that is required for modulating behaviors in monkeys may be much larger than that in rodents, or it could be due to the inherent difference in optical and electrical stimulation methods. For example, Chr2 fails to drive high-frequency firing that is often needed for evoking a movement behavior by microstimulating cortex, and the temporal precision with optogenetic stimulation is not as high as with electrical stimulation. In addition, optogenetic modulation relies mainly on the intrinsic physiological properties of the cell, in contrast to the artificial

electrical pulse activation through microstimulation. The lack of observable behavioral effect in the two studies employing optogenetic stimulation published to date remains unknown. With continued effort and tremendous enthusiasm in the field, I predict that scientists will be able to modulate behaviors in monkeys in the near future.

Conclusions and perspectives

The excitement in applying optogenetic techniques in various model systems has spread to the monkey model over the past couple years. With increasing available commercial resources, scientists can now easily obtain high-quality virus to genetically modify neurons and can purchase hardware at reasonably low cost to incorporate optical techniques into classical electrophysiology experiments. Although different viral methods vary in their transduction efficiency for different brain structures, reliable and highly efficient transduction of brain cells has been achieved with lentivirus pseudotyped with VSV-G coat protein, AAV pseudotyped with capsids from AAV1, 5, 8, 9, and Rh10. While the exact efficiency and transduction pattern for each virus type may differ significantly between monkey brain structures, it remains advisable to test different types of virus for the specific brain structure of interest. Fortunately, high-quality viruses can be easily obtained from commercial gene therapy programs, many available in small aliquots at low cost, for test injections. The simple strategy of coupling optical fibers to recording electrodes can be used to reliably monitor the effect of light modulation on neural activities in monkeys. The major advantages of optogenetic control in monkeys, at the current state, are the ability to directly stimulate neurons, instead of involving nonspecific antidromic stimulation of axon terminals as with electrical stimulations, and to silence neurons with unprecedented millisecond time resolution. It is also advantageous to activate or silence specific pathways in the monkey brain by expressing opsins retrogradely.

A few challenges remain in conducting long-term optogenetic control experiments. Specifically, development or implementation of semichronic or chronic optical fiber implants could eliminate mechanical tissue damage from repeated optical fiber insertions. Adaption of artifact free electrodes for *in vivo* recordings in monkeys will enable accurate measure of LFPs at the site of light illumination. Adaption of optical fiber arrays in monkeys or development of novel opsins with higher light sensitivity, larger photocurrents, and red shift action spectrum will increase the tissue volume effectively controlled by light, which may be necessary for perturbing enough brain tissues. Finally, and most challengingly, the development of new viral technologies is needed to target specific cell types in the monkey brain.

The success of using optogenetic molecules in monkeys has pointed to the serious translational potentials. Indeed, opsins have been shown to be functional when expressed in human *ex vivo* retinas last year (Busskamp et al., 2010). Several groups are hopeful in conducting clinical trials in the near future on the treatment of blindness (i.e., the groups in Switzerland (Busskamp et al., 2010) and Eos Neuroscience of California, USA (Doroudchi et al., 2011)) and spinal cord injury (Case Western Reserve University in Ohio, USA). It is exciting to see the rapid progress from identifying the first opsin in 2005 (Boyden et al., 2005), to the realization of its translational potential today. There is realistic potential that the continued rapid progress in the field will eventually lead to novel cell type specific neuromodulation therapies.

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