

# Channelrhodopsin-2 and optical control of excitable cells

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Electrically excitable cells are important in the normal functioning and in the pathophysiology of many biological processes. These cells are typically embedded in dense, heterogeneous tissues, rendering them difficult to target selectively with conventional electrical stimulation methods. The algal protein Channelrhodopsin-2 offers a new and promising solution by permitting minimally invasive, genetically targeted and temporally precise photostimulation. Here we explore technological issues relevant to the temporal precision, spatial targeting and physiological implementation of ChR2, in the context of other photostimulation approaches to optical control of excitable cells.

Electrically excitable cells include skeletal, cardiac and smooth muscle cells, pancreatic beta cells, and neurons. Malfunction of these cells can lead to heart failure, muscular dystrophies, diabetes, pain syndromes, cerebral palsy, paralysis, depression and schizophrenia, among many other diseases. Notably, unlike cells such as those in the immune and gastrointestinal systems that respond well to slow chemical modulation, electrically excitable cells signal and react on timescales as short as milliseconds. To date, stimulating electrodes have been a valuable tool for the study of excitable cells because they can accurately simulate natural cellular signals by controlling electrical activity on the millisecond time scale. Two experimental challenges, however, render investigation of excitable cells with electrodes difficult. First, it is generally infeasible or impractical to target multiple cells of a specific class simultaneously. Extracellular electrodes have limited spatial resolution for heterogeneous tissue, and although intracellular electrodes do target specific neurons, they are impractical for simultaneous targeting of many cells of a particular class. Second, electrode methodologies typically rely on mechanical stability because of the need to register electrodes with cells, and therefore can be cumbersome to use in awake, behaving animals. Solutions to these problems, which have challenged scientists and physicians alike, have begun to emerge from the neuroengineering and neuroscience fields.

Photostimulation provides a versatile alternative to electrode stimulation. Light beams can be easily and quickly manipulated to target one or many neurons, and could help relax the requirement for mechanical stability. Richard Fork at Bell Laboratories introduced the seminal idea in 1971 when he used an intense beam of blue light to evoke action potentials in *Aplysia* ganglion cells<sup>1</sup>. Although Fork's method was never widely adopted (presumably because it disrupted membranes, as later demonstrated for two-photon laser-based excitation<sup>2</sup>), several groups have since developed improved photostimulation techniques to address different aspects of what has been considered a 'holy grail' of neuroscience: minimally invasive, genetically targeted and temporally precise control of neural activity (for review see refs. 3,4). Here we will focus on physiological issues governing one such new approach—application of an algal light-gated ion channel Channelrhodopsin-2 for photostimulation<sup>5–8</sup>—and compare the technological capabilities of this approach with other optical methods.

## Photostimulation techniques and physiological considerations

Photostimulation techniques can be divided into three approaches: light-mediated 'uncaging' of chemically modified signaling molecules<sup>9–11</sup>, chemical modification of ion channels and receptors to render them

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light-responsive<sup>12,13</sup> and introduction of light-sensitive proteins into otherwise light-insensitive cells<sup>5–8,11,14–16</sup>. These techniques can modulate membrane potential and activate downstream signaling cascades with different levels of temporal and spatial control (summarized in **Table 1**).

**Light-mediated uncaging of signaling molecules.** In this approach, membrane potential or cellular signaling can be modulated when light releases a blocking moiety covalently attached to a biochemically active compound (for example, glutamate, GABA or second messengers such as Ca<sup>2+</sup>). Although glutamate is the most commonly used molecule, other types of caged compounds have been synthesized for controlling cellular activity by

activating G protein–coupled receptors and downstream signaling pathways<sup>17</sup>.

Uncaging can be used to stimulate individual parts of a neuron, single neurons or larger networks of neurons. In particular, glutamate uncaging has become an important and useful tool for studying dendritic integration<sup>18,19</sup>, and mapping neural connectivity in intact tissues<sup>20,21</sup> (for a detailed review of the pros and cons of glutamate uncaging, see ref. 22). Recently, researchers used glutamate uncaging to probe the cellular connectivity of cortical networks by exciting neurons at several hundred sites<sup>20</sup> and recording the resulting synaptic transmission in postsynaptic cells. Populations of neurons could be stimulated with a temporal precision on the order of several tens of milliseconds and spatial resolution down to fifty

**Table 1** | Temporal, spatial, and technical properties of different photostimulation techniques.

Optical method	Mechanism	Typical membrane voltage actuation	Typical single-photon excitation	Temporal spike jitter <sup>a</sup>	Sustained precisely timed spike trains <sup>b</sup>	Need for exogenous chemicals	Spatial resolution	References
Glutamate uncaging	Glutamate molecules are released from their caged form upon illumination by uncaging light. Free glutamate can activate both ionotropic and G protein–coupled metabotropic receptors.	Depolarizing	355 nm ultraviolet light	1–3 ms	NM	Caged glutamate (for example, nitrobenzyl ester form <sup>9</sup> )	5 μm <sup>c</sup>	18,20,41
ChARGe	Three-part <i>Drosophila</i> sp. G-protein phototransduction cascade is introduced into neurons, driving downstream endogenous ion channels.	Depolarizing	400–600 nm light	Seconds to minutes	NM	Requires retinal <sup>16</sup>	Genetically targetable	16
ChR2	Naturally occurring light-activated cation channel is opened upon exposure to blue light.	Depolarizing	480 nm blue light	1–3 ms	10 Hz routine, ≥30 Hz readily achievable in fast-spiking cells	Not needed for mammalian cells; non-mammalian systems may require added all- <i>trans</i> -retinal <sup>4</sup>	Genetically targetable	5–8,15,38
Modified glutamate receptor	Engineered iGluR receptor binds to a synthetic ‘switch’ molecule.	Depolarizing	380 nm for activation and 580 nm for inactivation	100 ms	NM	Azobenzene-tethered glutamate receptor agonist <sup>13</sup>	Genetically targetable	13
Ion channel ligand uncaging	ATP and capsaicin are uncaged to activate cells expressing exogenous ionotropic purinoceptors and capsaicin receptors.	Depolarizing	355 nm ultraviolet light	≤1 s	NM	Caged ATP, capsaicin, etc. (e.g. nitrobenzyl ester form <sup>10</sup> )	Genetically targetable	10,23
Modified potassium channel	Engineered potassium channel binds to a synthetic “switch” molecule.	Hyperpolarizing	380 nm for activation and 580 nm for inactivation	1–3 s	NA	Azobenzene-tethered potassium channel blocker <sup>12</sup>	Genetically targetable	12
Vertebrate rhodopsin	Vertebrate rat rhodopsin 4 is introduced into light-insensitive neurons, driving downstream endogenous ion channels.	Hyperpolarizing	475 nm blue light	1–3 s	NA	Requires retinal <sup>2</sup>	Genetically targetable	6

Single-photon excitation wavelengths are indicated; two-photon excitation methods are typically suitable as well. The modified potassium channel has been recently modified to give depolarizing currents (R. Kramer, personal communication). GABA uncaging is also possible, which could give rise to either depolarizing or hyperpolarizing responses, depending on membrane voltage. <sup>a</sup>Temporal spike jitter is determined by the timing precision with which single spikes may be reliably evoked. <sup>b</sup>Sustainability of precise firing refers to the ability of the technique to control repeated precisely timed spike trains (NM, not measured; NA, not applicable). <sup>c</sup>The 5 μm spatial resolution for glutamate uncaging is based on reference 18.

micrometers. In very recent work, acousto-optical deflectors were used to stimulate several different locations on the dendritic tree of a single cerebellar Purkinje cell in succession<sup>18</sup>; with this custom optical setup, rapid elicitation of uncaging responses may be more readily achieved than with what is commercially available (for a similar study of dendritic integration, see ref. 19). The authors also presented data demonstrating precise timing of individual evoked action potentials, with single-spike jitters as low as 1–2 ms, although uncaging has not been shown to support sustained trains of precisely timed spikes.

Glutamate uncaging is unique among the photostimulation techniques described here in that it releases a ligand that activates endogenous receptor pathways in target neurons. For example, uncaged glutamate, if targeted to a synaptic site and with a physiological spatial profile, could in principle engage the same panel of endogenous glutamate receptors (for example, AMPA, NMDA, kainate and metabotropic receptors) that would have been activated by native release of glutamate. While enabling certain kinds of experiment that depend on the direct activation of endogenous cellular signaling, this capacity for physiological mimicking comes at a price. First, as glutamate receptors are not functionally important in many excitable cells such as cardiomyocytes, glutamate uncaging is chiefly limited to neural tissue. Second, as glutamate receptors are expressed in nearly every cell of the central nervous system, glutamate uncaging in a volume of tissue cannot selectively activate one cell type to the exclusion of others.

These limitations were recently addressed by genetically introducing the heterologous capsaicin receptor TRPV1 and the purinergic receptor P2X<sub>2</sub> into neurons<sup>10</sup>. When caged capsaicin or ATP is released by ultraviolet light, cells respectively expressing either TRPV1 or P2X<sub>2</sub> fired action potentials. This technique was successfully used to specifically trigger locomotor activity in freely behaving flies<sup>23</sup>. Notably, this powerful system addresses the cell type–specificity issue, (as long as only the target cells express receptors to the uncaged compounds), and in principle can be applied in non-neural tissue as well. But perhaps owing to the transduction kinetics of these receptors, or to the temporal specifications of the optical equipment used, this technique has not been demonstrated to provide millisecond control over spike firing<sup>10</sup> and, like uncaging, has not been shown to support sustained trains of precisely timed spikes.

**Chemically modified ion channels and receptors.** It is also possible to generate light-activated forms of the Shaker potassium channel and the iGluR6 receptor by attaching a photoswitch close to the active site of each receptor<sup>12,13</sup> (the photoswitch consists of a photoisomerizable azobenzene group, covalently attached to either a potassium channel antagonist or an iGluR6 agonist, respectively). Switching between long (580 nm) and short (380 nm) wavelength illumination can alternate the azobenzene arm between *cis* and *trans* isomers, toggling the ligand in and out of the protein's target site (that is, the pore or ligand-binding domain). This approach is appealing, as the same azobenzene linker can work with multiple receptors and channels. The specific case of the potassium channel, however, involves closing an engineered leak conductance, and the existing system takes seconds for the cell to respond to light stimulation. Newer versions of the channel with altered pore selectivity will permit depolarizing currents, and combined versions of these approaches may allow fast bidirectional control of cellular membrane potential. But posing challenges for *in vivo* work (as with

uncaging methods), the azobenzene compound must be supplemented during intact-tissue studies.

**Naturally occurring photosensitive proteins.** A potentially more direct approach for bestowing light-sensitivity is to use light-sensing rhodopsins. The first attempt at this strategy exploited the multiple-component *Drosophila* sp. visual system rhodopsin cascade (ChARGe)<sup>16</sup>. This cascade required, at minimum, three separate components: the G protein–coupled rhodopsin, an arrestin, and a G protein. Because the invertebrate and vertebrate rhodopsins function through different signaling cascades with opposite net membrane voltage changes, it is possible to either depolarize (via ChARGe<sup>16</sup>) or hyperpolarize (via the rat rhodopsin RO4, which couples to inhibitory ion channels<sup>6</sup>) cells that are normally light-insensitive. Perhaps because non-photoreceptor cells are not structurally designed for this cascade, cells heterologously expressing this cascade begin to respond only after seconds of exposure to light. Analogous to ChARGe, the metabotropic photoprotein melanopsin has also permitted depolarization of cell membranes within seconds of illumination<sup>11,24</sup>. In addition to allowing targeting of genetically defined classes of excitable cells, use of genetic manipulations to introduce natural light-sensitive proteins may obviate the need to provide exogenous chemicals.

Recently, work from our group and others has demonstrated that the first 315 amino-acid residues of the algal Channelrhodopsin-2 (abbreviated as ChR2 when coupled with retinal, or *Chop-2* for the gene) from *Chlamydomonas reinhardtii* can be used to impart fast photosensitivity<sup>5–7,15</sup>. ChR2 is a seven-transmembrane protein with a molecule of all-*trans* retinal (ATR) bound at the core as a photosensor<sup>15</sup> (the kinetic mechanism of a closely related molecule, channelrhodopsin-1, is reviewed in ref. 25). Upon illumination with ~470 nm blue light, ATR isomerizes and triggers a conformational change to open the channel pore. As ChR2 is itself a light-sensitive ion channel, it allows an inward current to be evoked within 50 μs of illumination<sup>15</sup>. Combining ChR2 with ultrafast light switching made it possible for the first time to activate neurons at the temporal precision of single action potentials, reliably over sustained multiple action potential trains<sup>5</sup>.

**Choosing a suitable photostimulation technique.** As summarized in **Table 1**, for each scientific question it is important to consider the underlying physical properties of the native signal when choosing the most suitable of these described photostimulation methods. Excitable cells distinguish inputs in part based on their temporal properties, channel recruitment patterns and amplitude or polarity characteristics. Regarding temporal properties, glutamate uncaging and ChR2 achieve responses on the millisecond time scale, optimal for photostimulating pathways triggered by fast synaptic events and action potentials, while the other methods based on current data provide slower rates of photostimulation most suitable for probing circuit connectivity, and hormone or neuromodulator responses (**Table 1**). Regarding channel recruitment patterns, glutamate uncaging directly activates native glutamate receptors and so may achieve physiological spatial patterns of subcellular excitation; however, it should be noted that the other photostimulation methods, via depolarization, will recruit native voltage-activated channels such as voltage-dependent calcium, sodium and potassium channels, and thereby activate native, spatially sensitive signaling pathways. With any method, channels could be activated experimentally that would

not normally be activated by physiological mechanisms (for example, nonphysiological levels of extrasynaptic glutamate receptor activation by uncaging, or abnormal calcium-entry patterns triggered by engineered ion channels). It is still unknown how well subcellular targeting of proteins like ChR2 to postsynaptic or presynaptic sites can mimic physiological activation of signaling pathways. Finally, the direction and magnitude of excitation is crucial in determining cellular responses. For reduction of cellular excitability, hyperpolarization methods can be achieved with a light-gated potassium pore or by introduction of the rat rhodopsin RO4 (Table 1). The

other methods increase excitability, and most have capacity for excitation intensity-tuning; for example, ChR2 stimulation can be readily toggled between subthreshold synaptic-like depolarizations and suprathreshold spike-generating events simply by modulating light-pulse duration or intensity<sup>5</sup>.

In the following sections, we delve into the detailed technological considerations that govern use of ChR2 technology, with quantitative comparisons to the other photostimulation methods. We focus on application to the rodent brain, but it is worth noting that ChR2 has been functionally expressed in non-mammalian vertebrate<sup>6</sup> and invertebrate organisms<sup>8</sup> via electroporation and microinjection of ChR2 cDNA, respectively.

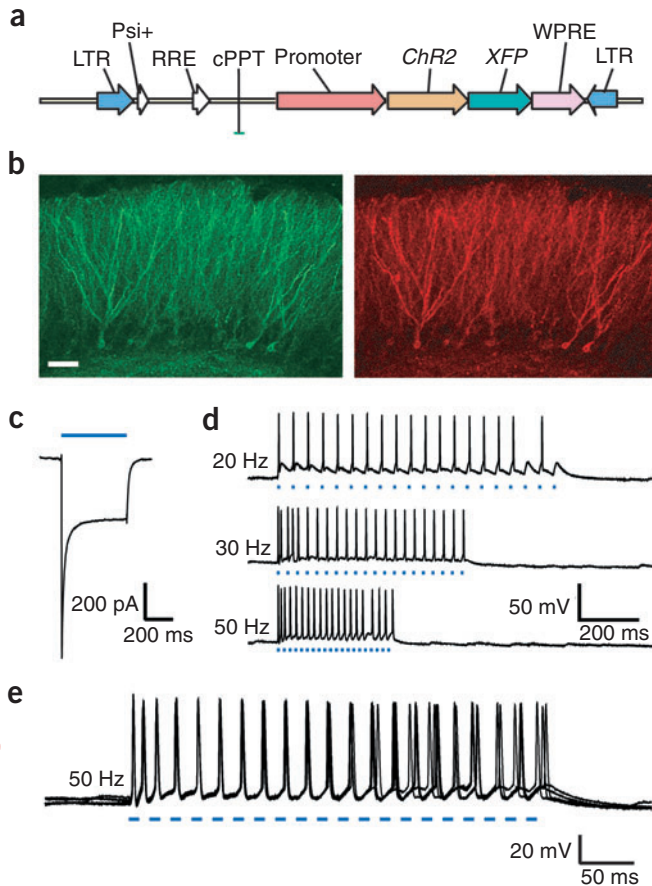
### Technological considerations

**Cellular targeting.** Genetic targeting techniques allow morphological and electrophysiological characterization of genetically defined cell populations<sup>26,27</sup>. Because the critical part of ChR2 is encoded by a single gene less than 1 kb long, it can be targeted readily to specific cell populations via genetic techniques<sup>3,28,29</sup>. For ease of identification of targeted cells, ChR2 can be tagged by a fluorescent marker such as YFP without affecting photosensitivity (we also have found that red variants of the coral fluorescent proteins, such as mCherry, can be used without affecting ChR2 function).

ChR2 can be stably introduced into tissues through techniques such as viral delivery, creation of transgenic lines or electroporation. Lentiviral technology provides a convenient combination of stable long-term expression, ease of high-titer vector production and low immunogenicity (for a detailed review of viral delivery techniques for the nervous system see ref. 30). Unlike the generation of transgenic animals, lentiviruses can be produced in days, and stable gene expression can be observed as early as 8 days after infection<sup>31</sup>. To mediate stable expression in genetically defined cell populations, ChR2 along with a cell-specific promoter can be incorporated into the lentiviral vector. The current generation of lentiviral vectors permits delivery of DNA sequences of up to 8 kb in length. Therefore, in addition to the ChR2 gene and an in-frame fused fluorescent protein tag at the carboxyl terminus, a promoter of up to 6 kb can be accommodated in the final viral vector (Fig. 1a). To increase viral titer and gene-delivery efficacy, the central polypurine tract (cPPT<sup>32</sup>) from wild-type human immunodeficiency virus-1 (HIV-1) as well as the Woodchuck hepatitis B virus post-transcriptional regulatory element (WPRE) should also be included in the vector (Fig. 1a). These are common elements in state-of-the-art lentiviral vectors<sup>33</sup>.

Using a lentiviral vector containing the ubiquitously functioning promoter for elongation factor 1- $\alpha$  (EF-1 $\alpha$ ), we have been able to achieve high levels of ChR2-EYFP expression in the mouse hippocampus. Concentrated virus (for a detailed protocol, see ref. 34) is stereotactically injected into the mouse hippocampus, and acute brain slices are prepared 8–21 days after injection. In agreement with the previously reported membrane-localized expression and nontoxicity of ChR2 in cultured mammalian neurons<sup>5</sup>, confocal images (Fig. 1b) of fixed brain slices show numerous intact neuronal somata and dendrites.

It is also possible to target cells in the absence of cell-specific promoters. For example, Moloney murine leukemia virus (MMLV)-based retroviruses can be used to selectively infect dividing cells, such as the stem and progenitor cells present in the adult brain, and thereby probe functional roles of cells born only at a particular time during development or adult life<sup>35</sup>. Additionally, specific cell



**Figure 1** | Functional expression of ChR2 in intact rodent hippocampus. (a) Lentiviral vector used for delivery of *ChR2* into the brain. *ChR2*, fused to the gene for a fluorescent protein (here *YFP* was used), is driven here by the EF-1 $\alpha$  promoter. The HIV-1 central polypurine tract (cPPT) and WPRE are also included for strong long-term expression. (b) Scanning confocal image of dentate gyrus granule cells expressing ChR2-EYFP in the adult mouse hippocampus (left, EYFP fluorescence; right, rhodamine-conjugated anti-GFP fluorescence; scale bar, 50  $\mu$ m). (c) Inward current in a voltage-clamped neuron in an acute slice evoked by 500 ms of 470 nm blue light (indicated by blue bar). (d) Voltage traces showing spikes in a current-clamped hippocampal dentate gyrus hilar interneuron in an acute adult mouse slice evoked by 20, 30 or 50 Hz trains of light pulses (each blue dash represent one 10-ms light flash). (e) The five traces of 50 Hz spike trains (95 spikes resulting from 100 light flashes) superimposed to demonstrate the low temporal jitter, reliability and sustainability of ChR2-based photostimulation. Traces were collected using a Sutter Lambda DG-4 rapid-wavelength switcher (Chroma HQ470/40X excitation filter, 40 $\times$  water immersion objective and 300 W Xenon lamp, giving rise to  $\sim$ 10 mW/mm<sup>2</sup> at the focus) and without the addition of exogenous ATR.

populations may be labeled via stereotactic injection of viruses that effect retrograde axonal transport, by taking advantage of region-specific axonal projections<sup>36,37</sup>. Just as with ChR2, other genetically based photostimulation methods can use these targeting strategies, although multicomponent systems such as ChARGed may be difficult to implement without the use of transgenic technologies.

For a genetically based photostimulation method to be viable, sufficient gene expression must be achieved to elicit physiologically relevant levels of current. To date, detailed quantification has yet to be conducted of the number of membrane-localized channels

required to drive spiking using these methods. Likewise, detailed studies of the promoter strengths or gene copy number needed to achieve sufficient expression have not been carried out. Although single-channel studies have not been performed, ChR2 has been estimated to possess a single-channel conductance as low as 50 femtoSiemens<sup>15</sup>. This would imply that between 100,000 and 1,000,000 ChR2 molecules would have to be generated and localized to the neuronal membrane to achieve the observed currents in the range of 1 nA (starting from a resting potential of  $-70$  mV and neglecting space-clamp issues and changes in driving force due to ion entry).

## BOX 1 EXPERIMENTAL CONSIDERATIONS FOR ChR2-BASED PHOTOSTIMULATION AND COMPARISONS WITH OTHER TECHNIQUES

The following criteria are among the important considerations for planning a photostimulation experiment.

**Light requirement.** Light sources must be carefully matched to the photostimulation technique (**Table 1**); for example, uncaging methods require UV illumination or two-photon uncaging. In contrast, ChR2 is optimally activated with blue light (excitation maximum  $\sim 470$  nm). Successful photostimulation of ChR2-expressing cells requires at least 5 mW/mm<sup>2</sup> of blue light at the sample. Sufficient blue light power can be generated by focusing light emitted by a bright blue LED, a blue laser (e.g., argon or diode laser) or by a mercury or xenon lamp filtered using a blue bandpass filter (e.g., HQ470/40X, Chroma Technology Corp). For application of ChR2 *in vivo*, the FiberTec488 from Blue Sky Research provides a convenient package for delivering 20 mW of blue light via a single fiber without elaborate focusing optics. For combining photostimulation with optical sensors (e.g., Ca<sup>2+</sup>-chelating dyes or voltage-sensitive dyes), consideration must be given to overlap of the action spectra to avoid photostimulation while intending to image, and vice versa. Dyes, filters and dichroics must be carefully chosen, particularly with photostimulation methods displaying broad excitation spectra (e.g. ChARGe and vertebrate rhodopsin); see **Table 1** and **Figure 2** for details.

**Exogenous chemical cofactors.** Adding required exogenous chemical cofactors poses particular challenges for *in vivo* and intact-tissue work. For *in vitro* work, however, this presents little problem. ATR must be present to allow ChR2 function, but native ATR is already present in sufficient quantity in some experimental systems (e.g. mammalian CNS tissue<sup>7</sup>). Other model organisms such as *D. melanogaster* or *C. elegans* can be supplemented with ATR to allow ChR2 photosensitivity. ATR is a natural molecule, and small amounts of ATR have been shown to be sufficient to allow channel function without toxicity to *C. elegans*<sup>8</sup>. Long-term toxicity of the exogenous chemical cofactors required for other photostimulation experiments is less clear.

**Protein expression.** The efficiency of photostimulation using ChR2 depends on the level of expression. Therefore, when targeting all cell types in the tissue, it is best to select a general promoter that drives strong expression (e.g., CMV, CAG, EF-1 $\alpha$ ). Cell type-specific promoters tend to be weaker, so when targeting specific cells, it may be important to devise a targeting strategy with a cell type-specific promoter that is still robustly expressed, such as CaMKII $\alpha$ , and to avoid weaker promoters. In the case where a strong promoter does not exist for the desired genetically specific cell population, a general floxed-stop transgenic line can be generated in which a series of stop signals separating the ChR2 gene and a strong promoter can be selectively removed by breeding with another transgenic line expressing Cre recombinase in the desired cell population<sup>42</sup>. In certain cases, spatially segregated cell populations can be selectively infected by virtue of precise stereotaxic injection. Also, newborn cell populations may be selectively targeted by using Moloney-type retroviruses that selectively infect dividing cells.

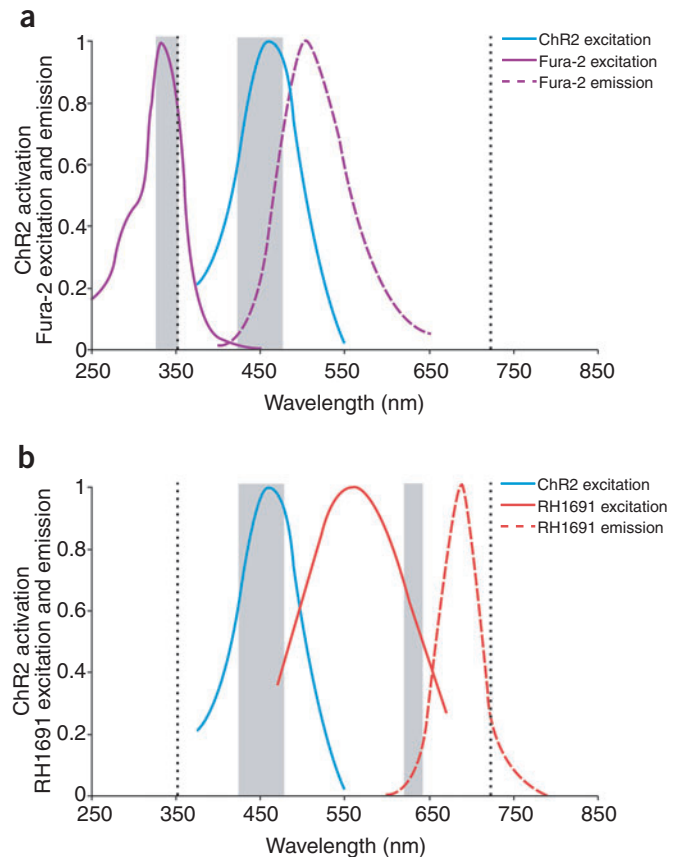
ChR2 can be introduced into cells or live animals via a number of techniques including electroporation, DNA microinjection, viral delivery, liposomal transfection and calcium-phosphate precipitation. We have had most success using lentiviral vectors for delivering ChR2 into cultured neurons as well as in mouse *in vivo*. For *in vivo* applications, high titers ( $>10^9$  p.f.u./ml) of viral vectors can be routinely obtained via calcium-phosphate cotransfection of 293FT cells (Invitrogen). Twenty-four hours after transfection, 293FT cells are switched to serum-free medium (ULTRACulture, Cambrex) containing 5 mM sodium butyrate; the supernatant is collected 16 hours later and concentrated in a ultracentrifuge at 50,000g with 20% sucrose cushion. The resulting viral pellet is resuspended in phosphate buffered saline at 1/1,000<sup>th</sup> of the original volume.

**Temporal and spatial control.** Precise temporal control of electrical activity using ChR2 also depends on the ability to manipulate light on the millisecond timescale. This can be accomplished in several ways, including via an ultrafast shutter (e.g., Lambda DG-4, Sutter Instruments) or by high-speed flashing of an LED. Upon illumination, all of the ChR2-expressing cells in the light path will be activated in concert, but pulsed lasers in principle may be used to achieve both high temporal resolution and selection of a subset of ChR2-expressing cells from a larger population, as with the uncaging methods; important constraints regarding temporal properties and channel recruitment patterns are discussed in the main text. Slower illumination strategies can, of course, be used with ChR2, as with the other methods.

For different animals and methods of gene delivery, tuning of DNA dosage and promoter strength may be needed. One interesting possibility would be to use tunable promoters, such as the commercially available tet-on promoter, for which expression can be altered dynamically with doxycycline. Given that even different neurons in the same animal may have radically different sizes, basal conductances, voltage-gated channels and morphologies, it is possible that no single set of universal quantitative parameters may apply for genetically encoded transduction of light into electrical activity. Nevertheless, the published data on ChR2 in rat and mouse hippocampal neurons<sup>5–7</sup>, mouse retinal neurons<sup>38</sup>, chick spinal cord neurons<sup>6</sup> and worm neurons<sup>8</sup>, achieved using a variety of genetic targeting strategies, suggest that sufficient levels of expression can be acquired using several means.

Although genetic targeting allows simultaneous activation of a defined cell population, some experiments may necessitate selective activation of single cells or even different positions on the same cell. For those applications, glutamate uncaging has been shown to be an effective *in vitro* technique (for application notes, see ref. 18). Similar region-specific excitation methods should be adaptable to ChR2 experiments to activate subsets or even subregions of ChR2-expressing neurons in a single experiment, using a scanning laser directed at specific regions of interest. ChR2 expression may also be restricted to cellular substructures via specific localization sequences<sup>39,40</sup>, to facilitate triggering of specific subcellular signaling pathways. As noted above, ChR2 depolarization can activate native voltage-dependent channels by virtue of its depolarizing effect. Furthermore, this intrinsic depolarizing capability allows ChR2 to stimulate excitable cells that do not express glutamate receptors. In **Table 1** we compare important quantitative parameters governing the use of the different photostimulation techniques, and in **Box 1** we outline some practical considerations for designing photostimulation experiments.

**Photostimulation in intact tissue.** Within intact neural tissue, most neurons relay information through the circuit via millisecond–time scale action potentials and synaptic events. Owing to their underlying phototransduction mechanisms, most of the existing photostimulation techniques (apart from glutamate uncaging) control spiking on a much longer timescale (**Table 1**). Thus these techniques have been limited to studying neural connectivity and relatively coarse behavioral processes where temporally precise manipulation is less critical. The single-component nature of ChR2, however, allows light to be immediately transduced into an ionic current with a maximal rise rate of  $160 \pm 111$  pA/ms within  $2.3 \pm 1.1$  ms after light pulse onset<sup>5</sup>. This fast depolarizing photocurrent, in principle, can be used to achieve precise excitation of neurons within high-speed circuits at the millisecond time scale<sup>5–7</sup>, although the dependence of ChR2 on its cofactor ATR could limit the intact-tissue application of ChR2. Nevertheless, our recent work (**Fig. 1**) and two independent studies in the mouse hippocampus and chicken spinal cord<sup>6,7</sup> have shown that remarkably, exogenous retinal is not required for functional expression of ChR2 in vertebrate nervous systems, likely owing to the basal levels of retinoids present in the central nervous system (CNS). While no added cofactor is needed in the intact vertebrate CNS tissues tested, if some preparations are found to lack the necessary level of retinal (invertebrate organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* may not express sufficient levels of ATR), ChR2 function typically can be restored by supplementing ATR in the food supply<sup>8</sup>.



**Figure 2** | Spectral properties of photostimulation techniques and imaging dyes. (a) ChR2 excitation spectrum superimposed with Fura-2 excitation and emission spectra. (b) ChR2 excitation spectrum superimposed with RH1691 excitation and emission spectra. For coapplication with ChR2, suggested ultraviolet and two-photon uncaging laser lines (355 and 720 nm, respectively) are shown as black dashed lines. Shaded gray areas indicate excitation filter bands for Fura-2 imaging ( $340 \pm 13$  nm in a), RH1691 imaging ( $630 \pm 10$  nm in b), and ChR2 excitation ( $450 \pm 25$  nm in a and b).

ChR2 exhibits the same rapid kinetics in acute mouse hippocampal slices, with no added ATR, as was demonstrated in culture<sup>5</sup>. Illumination with  $\sim 470$  nm blue light evokes the same waveform of rapid inward current (**Fig. 1c**). With the same high-speed light switching strategy used previously<sup>5</sup>, ChR2 drives reliable action potential trains of up to 50 Hz (**Fig. 1d**), and spike timing precision can be preserved even in sustained high-frequency spike trains (**Fig. 1e**). The limiting factor in generating high-frequency spike trains using ChR2 will depend more on the membrane electrical properties of the ChR2-expressing cell (and the power and duration of the light pulses) than on the intrinsic kinetics of ChR2. We have found that, for example, dentate gyrus hilar interneurons can follow much higher frequencies of photostimulation compared to pyramidal neurons or dentate gyrus granule cells (50–100 Hz; data not shown). Moreover, by adjusting the duration of each light pulse, it is possible to toggle between action potentials and subthreshold depolarizations as in culture (data not shown).

**Combining photostimulation and imaging.** Although photostimulation can simplify the study of excitable cells by reducing the need for mechanical electrodes, it is still most common to use electrodes

to record cellular responses. It would be a tremendous technical advance to eliminate the need for electrodes altogether by combining photostimulation with optical imaging techniques such as calcium or voltage-sensitive dye imaging to achieve a fully noninvasive experimental setup. Commercially available dyes already exist with excitation spectra that overlap little with ChR2, such as the calcium dye Fura-2 from Molecular Probes (used with 340 nm excitation; Fig. 2a) and the voltage-sensitive dye RH1691 from Optical Imaging (used with 630 nm excitation; Fig. 2b). Even with conventional epifluorescence microscopes, multiple excitation filters can be used in combination with rapid-wavelength switching devices such as the Sutter DG-4 first to activate neurons rapidly, and then to record the resulting activity in the stimulated and downstream neurons. ChR2 can be activated with a  $450 \pm 25$  nm excitation filter and the imaging dyes excited with nonoverlapping filters ( $340 \pm 13$  nm for Fura-2 or  $630 \pm 10$  nm for RH1691). Critical for the success of the combined optical stimulation and imaging strategy is the selection of dichroics that reflect both the ChR2-stimulating light and the dye-exciting light, while permitting dye-emitted light to reach the observation equipment. The 'extended-reflectance into the UV' (DCXRU) series of dichroic mirrors from Chroma Technology may be particularly useful for combined ChR2-excitation and dye readout. For example, in the Fura-2 case, a 480DCXRU dichroic would reflect both  $340 \pm 13$  nm light (for Fura-2 excitation) as well as  $450 \pm 25$  nm light (for ChR2 excitation) toward the sample, while letting emitted Fura-2 fluorescence (at  $510 \pm 20$  nm) pass to the eyepiece or charge-coupled device (CCD) camera, unfiltered. (Note that this setup could also suffice for simultaneous ultraviolet glutamate uncaging and ChR2 excitation.) For the red-excited voltage-sensitive dye RH1691, which is often used with longpass filters exceeding wavelengths of 670 nm, the 650DCXRU dichroic mirror from the same 'extended-reflectance into the UV' series may prove useful.

### Future directions

As chemically synthesized and genetically encoded optical sensor technologies mature, it will be a physiologist's dream-come-true to simply sit back and let light beams stimulate and assay the operation of a well-defined excitable tissue, such as a neural circuit. It may be possible to combine glutamate uncaging, or one of the other methods listed in Table 1, with photostimulation of ChR2 in a single experiment to allow selective excitation of two populations of cells in the same tissue. In addition to these basic science applications, ChR2 also shows promise for biomedical and bioengineering purposes. For example, insulin- or growth hormone-releasing cells may be amenable to driving with light-activated ion channels, allowing them to release their cargo in a temporally precise fashion while still embedded within tissue. The rapid transduction of light into ionic current also has clear biotechnological implications in high-throughput studies of activity-dependent signal transduction and gene expression, perhaps including guiding stem cell differentiation by precise depolarization patterns and screening for drugs that modulate cellular responses to depolarization. Indeed, the ability to depolarize cells with light, coupled with the ability to remotely read out the signals (using the dyes described above), may accelerate ion-channel drug discovery—presently done largely by patch-clamp methods in electrophysiology laboratories—by a factor of 1,000 or more.

The light power ( $5\text{--}12$  mW/mm<sup>2</sup>)<sup>5,7</sup> required to activate ChR2 makes it feasible to apply ChR2 *in vivo* to drive cellular activity and to assay physiological and behavioral responses<sup>8</sup>. In the spirit of apply-

ing photostimulation to address biomedical needs, a recent study demonstrated that expression of ChR2 in the retina of a mouse with photoreceptor degeneration restored the retina's ability to transduce light signals and relay information to the visual cortex<sup>38</sup>. Aside from being the most intact system application of ChR2 to date, the study demonstrated that safe, stable and functional expression of ChR2 can last for up to one year. These demonstrations of the ability of mammalian systems to tolerate and respond to ChR2 indicate that ChR2, alone or combined with the other photostimulation techniques described here, will serve as a valuable tool to allow minimally invasive, genetically targeted and temporally precise control of electrical activity, with applications ranging from neuroscience to biomedical engineering.

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### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Fork, R.L. Laser stimulation of nerve cells in *Aplysia*. *Science* **171**, 907–908 (1971).
2. Hirase, H., Nikolenko, V., Goldberg, J.H. & Yuste, R. Multiphoton stimulation of neurons. *J. Neurobiol.* **51**, 237–247 (2002).
3. Miesenbock, G. & Kevrekidis, I.G. Optical imaging and control of genetically designated neurons in functioning circuits. *Annu. Rev. Neurosci.* **28**, 533–563 (2005).
4. Kramer, R.H., Chambers, J.J. & Trauner, D. Photochemical tools for remote control of ion channels in excitable cells. *Nat. Chem. Biol.* **1**, 360–365 (2005).
5. Boyden, E.S., Zhang, F., Bamberg, E., Nagel, G. & Deisseroth, K. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci.* **8**, 1263–1268 (2005).
6. Li, X. *et al.* Fast noninvasive activation and inhibition of neural and network activity by vertebrate rhodopsin and green algae channelrhodopsin. *Proc. Natl. Acad. Sci. USA* **102**, 17816–17821 (2005).
7. Ishizuka, T., Kakuda, M., Araki, R. & Yawo, H. Kinetic evaluation of photosensitivity in genetically engineered neurons expressing green algae light-gated channels. *Neurosci. Res.* **54**, 85–94 (2006).
8. Nagel, G. *et al.* Light activation of channelrhodopsin-2 in excitable cells of *Caenorhabditis elegans* triggers rapid behavioral responses. *Curr. Biol.* **15**, 2279–2284 (2005).
9. Callaway, E.M. & Katz, L.C. Photostimulation using caged glutamate reveals functional circuitry in living brain slices. *Proc. Natl. Acad. Sci. USA* **90**, 7661–7665 (1993).
10. Zemelman, B.V., Nesnas, N., Lee, G.A. & Miesenbock, G. Photochemical gating of heterologous ion channels: remote control over genetically designated populations of neurons. *Proc. Natl. Acad. Sci. USA* **100**, 1352–1357 (2003).
11. Melyan, Z., Tarttelin, E.E., Bellingham, J., Lucas, R.J. & Hankins, M.W. Addition of human melanopsin renders mammalian cells photoresponsive. *Nature* **433**, 741–745 (2005).
12. Banghart, M., Borges, K., Isacoff, E., Trauner, D. & Kramer, R.H. Light-activated ion channels for remote control of neuronal firing. *Nat. Neurosci.* **7**, 1381–1386 (2004).
13. Volgraf, M. *et al.* Allosteric control of an ionotropic glutamate receptor with an optical switch. *Nat. Chem. Biol.* **2**, 47–52 (2006).
14. Nagel, G. *et al.* Channelrhodopsin-1: a light-gated proton channel in green algae. *Science* **296**, 2395–2398 (2002).

15. Nagel, G. *et al.* Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc. Natl. Acad. Sci. USA* **100**, 13940–13945 (2003).
16. Zemelman, B.V., Lee, G.A., Ng, M. & Miesenbock, G. Selective photostimulation of genetically chARGed neurons. *Neuron* **33**, 15–22 (2002).
17. Adams, S.R. & Tsien, R.Y. Controlling cell chemistry with caged compounds. *Annu. Rev. Physiol.* **55**, 755–784 (1993).
18. Shoham, S., O'Connor, D.H., Sarkisov, D.V. & Wang, S.S. Rapid neurotransmitter uncaging in spatially defined patterns. *Nat. Methods* **2**, 837–843 (2005).
19. Losonczy, A. & Magee, J.C. Integrative properties of radial oblique dendrites in hippocampal CA1 pyramidal neurons. *Neuron* **50**, 291–307 (2006).
20. Yoshimura, Y., Dantzker, J.L. & Callaway, E.M. Excitatory cortical neurons form fine-scale functional networks. *Nature* **433**, 868–873 (2005).
21. Pettit, D.L., Helms, M.C., Lee, P., Augustine, G.J. & Hall, W.C. Local excitatory circuits in the intermediate gray layer of the superior colliculus. *J. Neurophysiol.* **81**, 1424–1427 (1999).
22. Callaway, E.M. & Yuste, R. Stimulating neurons with light. *Curr. Opin. Neurobiol.* **12**, 587–592 (2002).
23. Lima, S.Q. & Miesenbock, G. Remote control of behavior through genetically targeted photostimulation of neurons. *Cell* **121**, 141–152 (2005).
24. Qiu, X. *et al.* Induction of photosensitivity by heterologous expression of melanopsin. *Nature* **433**, 745–749 (2005).
25. Hegemann, P., Ehlenbeck, S. & Gradmann, D. Multiple photocycles of channelrhodopsin. *Biophys. J.* **89**, 3911–3918 (2005).
26. McBain, C.J. & Fisahn, A. Interneurons unbound. *Nat. Rev. Neurosci.* **2**, 11–23 (2001).
27. Gong, S. *et al.* A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* **425**, 917–925 (2003).
28. Miesenbock, G. Genetic methods for illuminating the function of neural circuits. *Curr. Opin. Neurobiol.* **14**, 395–402 (2004).
29. Callaway, E.M. A molecular and genetic arsenal for systems neuroscience. *Trends Neurosci.* **28**, 196–201 (2005).
30. Davidson, B.L. & Breakefield, X.O. Viral vectors for gene delivery to the nervous system. *Nat. Rev. Neurosci.* **4**, 353–364 (2003).
31. Ehrenguber, M.U. *et al.* Gene transfer into neurons from hippocampal slices: comparison of recombinant Semliki Forest Virus, adenovirus, adeno-associated virus, lentivirus, and measles virus. *Mol. Cell. Neurosci.* **17**, 855–871 (2001).
32. Zennou, V. *et al.* The HIV-1 DNA flap stimulates HIV vector-mediated cell transduction in the brain. *Nat. Biotechnol.* **19**, 446–450 (2001).
33. Dittgen, T. *et al.* Lentivirus-based genetic manipulations of cortical neurons and their optical and electrophysiological monitoring *in vivo*. *Proc. Natl. Acad. Sci. USA* **101**, 18206–18211 (2004).
34. Sena-Esteves, M., Tebbets, J.C., Steffens, S., Crombleholme, T. & Flake, A.W. Optimized large-scale production of high titer lentivirus vector pseudotypes. *J. Virol. Methods* **122**, 131–139 (2004).
35. Zhao, C., Teng, E.M., Summers, R.G., Jr, Ming, G.L. & Gage, F.H. Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. *J. Neurosci.* **26**, 3–11 (2006).
36. Mazarakis, N.D. *et al.* Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery. *Hum. Mol. Genet.* **10**, 2109–2121 (2001).
37. Wong, L.F. *et al.* Transduction patterns of pseudotyped lentiviral vectors in the nervous system. *Mol. Ther.* **9**, 101–111 (2004).
38. Bi, A. *et al.* Ectopic expression of a microbial-type rhodopsin restores visual responses in mice with photoreceptor degeneration. *Neuron* **50**, 23–33 (2006).
39. Weick, J.P., Groth, R.D., Isaksen, A.L. & Mermelstein, P.G. Interactions with PDZ proteins are required for L-type calcium channels to activate cAMP response element-binding protein-dependent gene expression. *J. Neurosci.* **23**, 3446–3456 (2003).
40. Garrido, J.J. *et al.* A targeting motif involved in sodium channel clustering at the axonal initial segment. *Science* **300**, 2091–2094 (2003).
41. Farber, I.C. & Grinvald, A. Identification of presynaptic neurons by laser photostimulation. *Science* **222**, 1025–1027 (1983).
42. Srinivas, S. *et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into the *ROSA26* locus. *BMC Dev. Biol.* **1**, 4 (2001).