CellPress

Review

Optogenetic Approaches to Drug Discovery in Neuroscience and Beyond

Hongkang Zhang[1] and Adam E. Cohen[1,*]

Recent advances in optogenetics have opened new routes to drug discovery, particularly in neuroscience. Physiological cellular assays probe functional phenotypes that connect genomic data to patient health. Optogenetic tools, in particular tools for all-optical electrophysiology, now provide a means to probe cellular disease models with unprecedented throughput and information content. These techniques promise to identify functional phenotypes associated with disease states and to identify compounds that improve cellular function regardless of whether the compound acts directly on a target or through a bypass mechanism. This review discusses opportunities and unresolved challenges in applying optogenetic techniques throughout the discovery pipeline – from target identification and validation, to target-based and phenotypic screens, to clinical trials.

Trends

Optogenetic approaches have the potential to contribute throughout the drug discovery pipeline from target identification to clinical trials.

Optogenetic assays can probe specific targets and cell types within a rich physiological context.

Optogenetic measurements in patient-derived neurons can elucidate genotype–phenotype relationships and probe disease mechanisms.

The integration of optogenetics with high-throughput screening requires advances in molecular tools, instrumentation, and data analytics.

Optogenetic Tools Contribute Throughout the Development Pipeline

The essence of drug discovery is to find compounds that modulate a specific physiological process in a particular subset of cells. The challenge is that minimalist high-throughput assays often have little physiological relevance, while richer phenotypic assays are slow and expensive. Typically, hundreds of thousands, or sometimes millions, of compounds fed into a screening campaign are whittled down to a few leads. A discovery team is fortunate if one lead molecule survives clinical trials. The high attrition arises because each step of the development pipeline challenges compounds against new aspects of biology which upstream assays did not address. Recently developed and emerging optogenetic tools provide a means to improve this tradeoff between throughput and realism at each step.

Optogenetics – the optical perturbation and optical measurement of physiological processes – has several attributes that make it a useful tool for drug discovery. Optical assays have the potential for high throughput and low cost. This feature has been appreciated at least since the introduction of the **fluorescent imaging plate reader** (FLIPR) (see Glossary) system more than 20 years ago [1]. Where optogenetics goes beyond traditional cell-based assays is in (i) the ability to deliver temporally and spatially precise stimuli to elicit defined patterns of molecular and cellular activity, (ii) the rapid proliferation of fluorescent protein reporters for a huge diversity of molecular and physiological signals, and (iii) the ability to use genetic techniques to target stimulus and readout to specified cell types within a possibly complex multicellular milieu. These capabilities give optogenetic assays throughput and information content that transcend the limits of more traditional approaches.

An optogenetic disease signature can aid discovery throughout the development process, from target identification to clinical trials (Figure 1, Key Figure). For instance, optogenetic measures

[1]Department of Chemistry and Chemical Biology, Howard Hughes Medical Institute, Department of Physics, Harvard University, Cambridge, MA 02138, USA

*Correspondence: cohen@chemistry.harvard.edu (A.E. Cohen).

of neuronal excitability can be used first to screen for targets via CRISPR-based knockout or knockdown, then to validate the efficacy and specificity of a screening hit via comparison of its effect on knockout and wild-type cells, and finally in a clinical trial with patient stem cell-derived neurons to segregate likely responders from likely non-responders.

Optogenetics in Neuroscience Drug Discovery

The impact of optogenetics will likely be greatest in drug discovery for neuroscience. On the one hand, genetic studies are implicating an ever-widening array of mutations, ranging from rare large effect-size mutations in the epilepsies [2,3] to common individually weak mutations in psychiatric disease [4,5]. On the other hand, the core functions of neurons are electrical spiking and synaptic transmission. Owing to the complex nonlinear interactions of multiple ion channels within cells, and of multiple cell types within circuits, the neurophysiological consequences of newly identified mutations can almost never be predicted *a priori*, nor can one reliably predict how pharmacological modulation of a particular target will affect brain function overall. Even as more targets are identified, neuroscience drug discoverers are turning toward a cell- and circuit-centric phenotypic approach [6,7] rather than focusing on specific targets.

Cell-based models provide a means to instantiate newly identified mutations in a physiological but pharmacologically addressable format, and thus are an attractive substrate for phenotypic assays. Human induced pluripotent stem cell (hiPSC) technology provides a direct link between *in vitro* models and real patients. Genome-editing technologies provide the ability to introduce and correct targeted mutations in human and rodent neuronal models. These rapidly improving cellular substrates hold the promise of connecting human genetics to the underlying physiology.

The weak link in this chain is the measurement: how to determine the effects of mutations and candidate therapeutics on neural function. The challenge of functional assays in neurons is severe. Electrical spiking and synaptic transmission cannot be purified or measured *post hoc*; they must be measured in live cells *in situ*. Neural firing occurs on the millisecond timescale, beyond the reach of most screening tools. State-dependent drugs bind to ion channels with affinities that vary by orders of magnitude depending upon the patterns of neural activity [8]. Feed-forward and feedback mechanisms can produce circuit-level effects that are opposite to the effects of a compound on isolated cells [9].

Optogenetic approaches are starting to address these challenges. Recent reviews have discussed optogenetic actuators [10,11] and reporters [12–14] in detail. In this review we discuss how optogenetics can facilitate target discovery and validation, high-throughput screening, functional phenotypic assays, safety pharmacology and toxicity, and patient stratification for clinical trials.

Optogenetics for High-Throughput Screening

Drugs and ion channels perform an intricate dance wherein the affinity of the drug depends on the state of the channel, and the dynamics of the channel depend on the binding of the drug. For instance, activity-dependent block of **Na_V channels** is crucial for targeting analgesic action to hyperactive neurons, while sparing less active neighbors. Ideally, a targeted screen for ion channel modulators would allow one to define state-dependent selection criteria, but conventional fluorescence screens are insensitive to the details of this dance (Box 1).

Optogenetic Control of Membrane Potential

Light-gated ion channels and pumps provide a robust means to modulate membrane potential rapidly and reversibly. The light-gated cation **channelrhodopsins** are the exemplars of this class [25,26]. Upon illumination, they mediate modestly inwardly rectifying currents, with a

Glossary

Bouton: a swelling in a neuronal axon that contains synaptic vesicles. A synapse comprises a junction of a presynaptic bouton and a postsynaptic dendritic spine.
Channelrhodopsin: a class of transmembrane proteins that transport cations across the plasma membrane in response to light stimulation.
CheRiff: a type of channelrhodopsin that shows good sensitivity to blue light illumination and rapid opening and closing kinetics.
Depolarization: in the resting state of most cells, the inside of the cell is electrically negative relative to the outside ('polarized'). Depolarization refers to a shift to a less negative voltage, often caused by a flow of positive ions into the cell.
Fluorescent imaging plate reader (FLIPR): a commercially available instrument that monitors the simultaneous fluorescence dynamics of each well in a multiwell plate. This instrument is widely used for high-throughput screening to identify modulators of GPCR and ion channel targets.
GCaMP: a class of protein-based calcium indicators that emit green fluorescence. The brightness of these reporters is highly sensitive to local Ca^{2+} concentration.
K_{ir} channel: inwardly rectifying potassium channel. These ion channels have greater conductance for potassium at negative potentials than at positive potentials. K_{ir} channels do not show voltage-gated behavior, in other words the current is only a function of the instantaneous voltage as opposed to also depending on past voltages. In neurons and cardiomyocytes K_{ir} channels are crucial for stabilizing a negative resting membrane potential close to the potassium reversal potential.
Na_V channels: voltage-gated sodium channels. When activated by a depolarizing shift in membrane voltage, these ion channels open and conduct sodium ions into the cell. This inward current leads to further depolarization, creating a positive feedback that produces a spike in the membrane voltage. Most Na_V channels spontaneously close after a short time, and thus Na_V activation only produces a brief spike in membrane voltage. Na_V channels

reversal potential of around 0 mV. Many channelrhodopsins have been discovered and engineered, with differing action spectra [27–31], kinetics [32,33], and ionic selectivities [34–36].

To modulate membrane potential optically, one can pair an optogenetic actuator with an inwardly rectifying potassium channel, such as $K_{ir}2.1$ or $K_{ir}2.3$. In the dark, the **K_{ir} channel** sets the cell resting potential to near the K^+ reversal potential, typically about −80 mV. Illumination **depolarizes** the cell toward 0 mV. Channels that activate within this window can be modulated in temporal patterns governed by the timecourse of illumination.

To hyperpolarize a membrane one can use light-powered inward chloride pumps (e.g., halorhodopsin [37]), outward proton pumps (e.g., archaerhodopsin 3 [38]) or outward sodium pumps (e.g., KR2 [35]). Light-gated chloride channels (ChloC [39,40] and recently discovered gtACR [36]) also provide hyperpolarizing drive if the chloride reversal potential is negative. In HEK cells, the chloride reversal potential is typically about −50 mV.

Fluorescent Reporters Compatible with Optogenetic Stimulation

An optical readout is necessary to monitor the activity of a target protein. The key challenge is to avoid optical crosstalk between the measurement and the optogenetic actuation. Near-IR **QuasAr** proteins provide a fast and sensitive, but dim, readout of membrane voltage and avoid spectral crosstalk with blue-excited channelrhodopsins [41]. A recently developed red-shifted voltage-sensitive dye, BeRST1, is also spectrally compatible with channelrhodopsin activation and shows excellent speed, sensitivity, and brightness [42]. For Ca^{2+}-based readouts, red-shifted Ca^{2+}-reporter dyes (e.g., CaRuby-Nano [43]) or proteins (e.g., **RCaMP2** [44], jRGE-CO1a, and jRCaMP1a,b [45]) have recently been introduced. Unfortunately, most protein-based fluorescent reporters are based on GFP or spectrally similar fluorophores, and thus suffer optical crosstalk with most optogenetic actuators, in other words the light used to excite the reporter spuriously activates the actuator, or vice versa. Development of far-red fluorescent reporters remains a pressing need.

Examples of Optogenetically Enabled Screens

Voltage-gated sodium (Na_V) channels are a natural target for screening via all-optical electrophysiology (Figure 2A), and are of interest as targets in pain and epilepsies [46]. In HEK293 cells heterologously expressing a K_{ir} channel and a Na_V channel, a depolarizing influence can activate the Na_V channel, causing a sharp voltage spike to the Na^+ reversal potential, followed by Na_V channel inactivation and return to baseline driven by the K_{ir} channel. Electrically spiking HEK cells have been demonstrated with Na_V 1.3 [47], Na_V 1.5 [48], Na_V 1.7 [49], and Na_V 1.9. A

produce the upstroke of the action potential in neurons and cardiomyocytes.

QuasAr: a near-IR fluorescent protein reporter of membrane voltage. This protein responds to changes in membrane voltage quickly and sensitively, but is very dim.

RCaMP: a class of protein-based calcium indicators that emit red fluorescence. The red-shifted spectrum of these reporters facilitates pairing with blue-activated channelrhodopsins.

Reversal potential: the value of the membrane potential at which an ion will neither flow into or out of a cell. The reversal potential for each ion depends on its concentrations inside and outside the cell, via the Nernst equation.

Box 1. Ion Channel Screening Tools

Several fluorescence assays have been developed for screening ion channel modulators. Typically, a static membrane voltage is set via the ionic content of the extracellular medium. Sometimes a tool compound is applied to force the channels into a particular state (e.g., veratridine for Na_V channels). The response to a test compound is then monitored either via fluorescence of a voltage-sensitive dye or via modulation of the flux of a readily detected ion (e.g., Ca^{2+} for Ca_V channels [15], Tl^+ for Na_V [16] and K_V [17] channels, and I^- for chloride channels [18]). Screens of this type cannot select for particular binding modes or kinetics.

Automated patch-clamp electrophysiology [19] provides accurate programming of membrane potential and readout of membrane current, and is becoming widely used in ion channel screening [20]. However, this approach becomes expensive for large-scale screens and still suffers from technical limitations, including a ~17% drop-out rate of individual wells [21] and difficulty in probing channels with small overall currents. For instance, the voltage-gated sodium channels Na_V 1.8 and Na_V 1.9, which play an important role in mediating pain transmission, are difficult to express at sufficiently high levels for automated patch-clamp measurements [22,23]. Automated patch-clamp measurements are typically restricted to stable cell lines, although applications in cardiomyocytes have been demonstrated [24].

Key Figure

Optogenetics Throughout the Discovery Pipeline

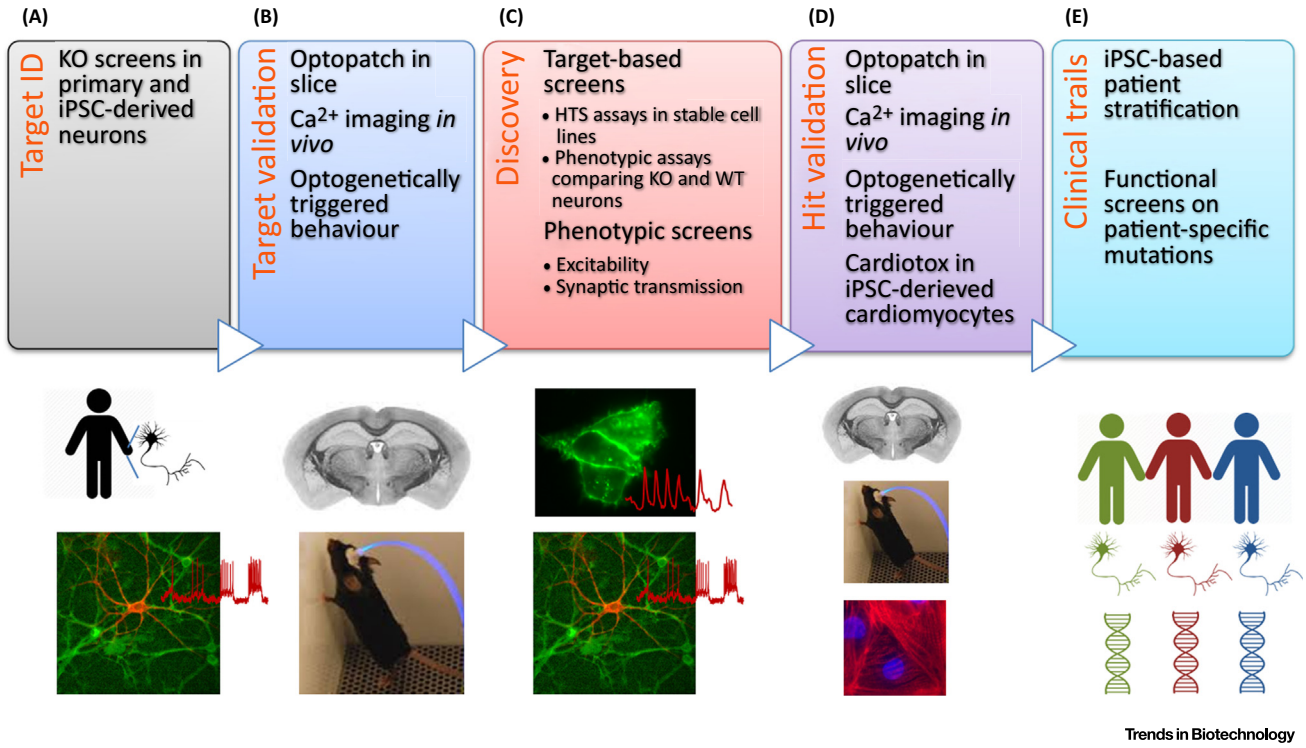

Figure 1. Optogenetic assays can contribute at each stage of drug discovery. (A) Comparative measures on rodent or human neurons +/− a disease-causing mutation can establish a screenable phenotype. Alternatively, optogenetic screens of genetic knockouts can identify novel targets which can then be the subject of optogenetic or conventional drug screens. (B) Targets identified in culture-based assays should be validated via knockout and functional measurements in acute slice, and ideally *in vivo*. Optogenetically triggered behaviors can provide a low-variance phenotype for testing the effects of mutations. (C) Target-based optogenetic screens can be performed in a wide variety of cell-based assays. Alternatively, phenotypic optogenetic screens can identify compounds that modulate a disease-relevant functional phenotype regardless of mechanism. (D) Screening hits should be validated in the same assays used for target validation. In addition, optogenetic assays in human iPSC-derived cardiomyocytes can help with cardiotoxicity assessment. (E) Functional optogenetic measurements in human iPSC-derived neurons can seek to distinguish likely responders from non-responders before a clinical trial, or to match genetically heterogeneous patients with existing medications. Abbreviations: iPSC, induced pluripotential stem cell; KO, knockout; WT, wild type.

recent all-optical screen of 320 FDA-approved compounds for activity-dependent block of $Na_V1.7$ yielded results in close concordance with manual patch clamp (Figure 3) [49]. By varying the intensity and duration of the optical stimulus pulses, one could probe subtle features of state-dependent pharmacology in a high-throughput format.

A similar approach has been used to probe the $Ca_V1.3$ voltage-gated calcium channel (Figure 2B) [50], a potential target in Parkinson's disease [51]. Here the channel was paired with $K_{ir}2.3$ to set the resting potential, and a 'step-function' channelrhodopsin variant, ChR2 (D156A) [33], was used to induce a slowly varying membrane potential. Channel activity was probed via a small-molecule Ca^{2+} indicator, Fluo8. Although the Ca^{2+} reporter was excited at a blue wavelength that also drove the channelrhodopsin, crosstalk was minimized by performing the fluorescence measurement on a timescale much shorter than that of Ca^{2+} influx. The activity of Ca_V channels could likely also be probed with red-shifted Ca^{2+} reporters to avoid optical crosstalk.

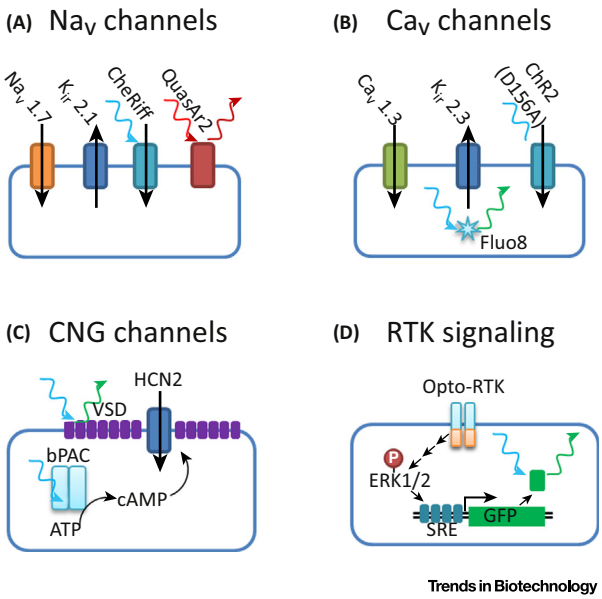

Figure 2. Optogenetic High-Throughput Target-Based Screens. (A) Screens for state-dependent modulators of Na_V channels can be carried out in Optopatch spiking HEK cells [49]. (B) Screens for state-dependent modulators of Ca_V channels use the gradual recovery of a step function channelrhodopsin, ChR2(D156A) [33], to induce a slowly varying membrane voltage [50]. (D) Screens for modulators of cyclic nucleotide gated (CNG) channels use a photo-activated adenylyl cyclase, bPAC [53], to induce a step in cAMP concentration. (E) Screens for modulators of receptor tyrosine kinase (RTK) signaling use a light-activated RTK and a transcriptional readout [54].

Cyclic nucleotide-gated (CNG) cation channels are a possible target for neuropathic pain [52]. Figure 2C illustrates an optogenetic screen for the HCN2 channel [50]. Upon blue illumination, a light-activated adenylyl cyclase, bPAC [53], produces cAMP which then activates the HCN channel. Channel activation was probed via a slow voltage-sensitive dye. Although the wavelength used to excite the dye also activated the bPAC, the dye measurement took place sufficiently rapidly that measurement-triggered bPAC activation did not affect the measurement. The HCN channels are also activated by membrane hyperpolarization, and thus optogenetic approaches could be used, in principle, to probe the voltage-dependent pharmacology of these channels.

Receptor tyrosine kinases (RTKs) are interesting targets in oncology, metabolic disorders, and neurodegeneration. However, a lack of known ligands presents a problem for screening against some RTKs. Figure 2D shows an optogenetic screen for modulators of RTK signaling [54]. Light-sensitive 'Opto-RTKs' were formed by incorporation of a light-oxygen-voltage-sensing (LOV) domain which caused dimerization and thereby activation of the RTK in the light. Downstream activation of the MAPK/ERK pathway drove the expression of a gene for a fluorescent reporter protein, and the pharmacological modulation of this response was readily detected in a high-throughput format. Remarkably, this approach worked even for RTKs with no known ligand. As in the screens of the Ca_V and HCN channels, crosstalk between stimulation and readout was avoided by using a slow reporter mechanism, in this case GFP expression.

In these examples, optogenetics offers several key advantages over ligand-based activation of the target pathways. First, for rapid processes, one can achieve precise temporal synchronization across a well, and correspondingly high time-resolution in whole-well fluorescence measurements. Second, it is possible to mix cell lines with different actuators paired with spectrally distinct reporters to provide in-well controls. For instance, one could screen for

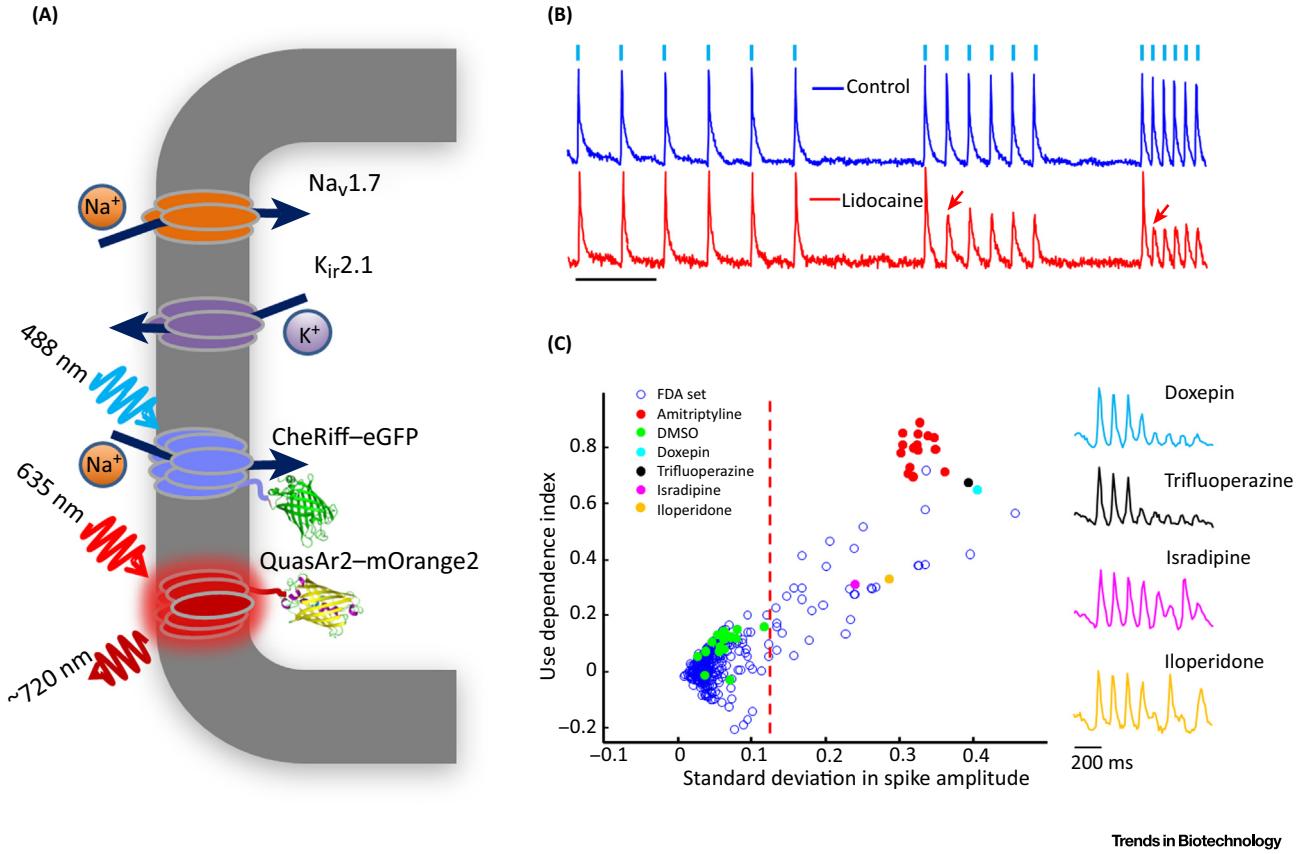

Figure 3. Optical High-Throughput Screen of Sodium Channel Modulators. (A) HEK293 cell engineered for optogenetic studies on $Na_V1.7$. The cell stably expressed the test channel, $Na_V1.7$, and $K_{ir}2.1$ to lower the resting potential to near the K^+ reversal potential. The blue light-activated ion channel CheRiff induced action potentials upon blue illumination. Fluorescence of the voltage indicator QuasAr2 reported the dynamics. (B) Optically induced and optically recorded spikes followed the stimulus pattern in the absence of drug, but in the presence of a state-dependent blocker, lidocaine, the spikes failed at high stimulus frequency (arrows). Scale bar, 1 s. (C) All-optical screen of 320 FDA-approved compounds for use-dependent block. Each response was characterized by the overall decay in the spike amplitude (use-dependence index) and the standard deviation of the spike amplitude. State-dependent blockers showed clear functional clustering. Image adapted from [49].

compounds that have differential activation of structurally similar targets. Third, one can vary the illumination intensity across a well to perform in-well dose–response measurements. While sophisticated instrumentation is necessary to produce precise optical gradients, a simple shadow mask can divide a well between optogenetically activated and unactivated cells for an in-well reference. These benefits could be applied to many novel optogenetic screens that have not yet been implemented (Box 2).

Optogenetics for Phenotypic Screening

Following the maxim 'you get what you screen for' [74], target-based screens identify modulators of specific targets. However, a modulator of a disease-relevant target might still make a poor drug for several reasons: (i) the hit compounds might also modulate other targets. Indeed, many neuroactive drugs developed around a nominal target turned out later to modulate other ion channels [75]. (ii) Selective modulation of a specific target can be a poor therapeutic strategy. Dysregulated neural circuits might not contain a unique target whose modulation will restore the defective function; selective modulation of a single broadly distributed target might still cause undesirable effects in other brain regions or organs. (iii) Many effective drugs have been developed without a clear target, or that modulate multiple targets. For instance, gabapentin, pregabalin [76], and levetiracetam [77], all widely prescribed anti-epileptic drugs,

Box 2. Optogenetics for Novel Target-Based Screens

The power of optogenetically enabled high-throughput screens is only beginning to be realized. We give several examples of prospective screening approaches.

Gap Junctions

Gap junctions, which are electrical conduits between neighboring cells, are an interesting target in epilepsy [55]. When HEK cells are grown to confluence, they form gap junction-mediated electrical contacts. Overexpression of gap junctions can further strengthen this electrotonic coupling [56]. In syncytial monolayers of spiking HEK cells, optogenetically induced depolarizations lead to propagating waves which are sensitive to gap junction modulators [48]. Mixed coculture of HEK cells expressing either an optogenetic actuator or a fluorescent voltage indicator would provide a robust means to probe gap junction-mediated electrical conduction.

Transporters and Pumps

Membrane voltage is important in the dynamics of many transmembrane proteins beyond ion channels [57]. Indeed, every transmembrane protein that binds a charged ligand, undergoes conformational shifts, or transports charged moieties across the membrane is, in principle, susceptible to changes in membrane voltage [58]. Electrogenic transporters are sensitive to membrane voltage, as are some GPCRs [59,60]. Voltage affects the activity of the multidrug resistance proteins MRP4 and MRP5 [61], although there has been controversy over whether voltage also affects the multidrug efflux transporter P-gp (also known as MDR1) [62,63]. Indeed, changes in membrane voltage have been implicated in regulating the cell cycle [64] and in the progression of cancer [65]. For these targets, optogenetic modulation of membrane potential could augment screening pipelines.

GPCRs and Other Signaling Pathways

Optogenetics can also augment drug discovery for targets where membrane voltage is not a factor. Optogenetic control has been exerted over the three major classes of GPCR-based signaling (G_s [66], $G_{i/o}$ [67], and $G_{q/11}$ [68]). Chimeras of bovine rhodopsins and endogenous receptors have been used to make light-activated mimics of the adrenergic receptor (optoXR) [68] and the serotonin receptor (Rh-$CT_{5\text{-}HT1A}$) [67]. Light-responsive soluble proteins have been coupled to the MAPK pathway, PI3K pathway, Rho GTPase signaling, and RTK signaling [69]. Broadly applicable strategies have been developed for controlling protein localization, activity, oligomerization, and secretion with light [69]. Recently, the optogenetic toolbox was further expanded with a light-controlled CRISPRa activator which can control endogenous gene expression with spatial and temporal precision [70]. These powerful capabilities are only beginning to enter drug discovery pipelines.

Target-Based Screening in Primary Cells

Target proteins can behave very differently in heterologous expression systems and in native cells because of alternative splicing, post-translational modifications, variations in trafficking, clustering, and degradation, and interactions with modulatory subunits. For instance, dynamic clustering of the soma-localized potassium channel $K_V2.1$ regulates its activity [71], while trafficking of the sodium channel $Na_V1.2$ along the axon initial segment regulates neuronal excitability [72]. Thus, even for target-based screens, there is merit in assessing target function in its native cellular context.

A few optogenetic tools have been developed to probe the function of specific targets in a complex cellular milieu. Fluorescently labeled toxins with state-dependent binding can be used to probe the activation of native $K_V2.1$ ion channels [73]. FRET-based reporters can probe the activation of specific kinases, GPCRs, and other targets [13,14]. With these tools one can perform target-based screens in native cells. There remains a strong need for improved fluorescent reporters for tracking the location and activity of specific drug targets within a complex cellular milieu.

were developed without a clear understanding of their mechanism of action. Screens that directly probe the effects of compounds on neuronal firing or synaptic transmission select for mechanisms relevant to disease biology, agnostic to the molecular target. Optogenetic assays now enable phenotypic measurements across a range of contexts relevant to neuroscience drug discovery (Figure 4).

Neuronal Excitability

Neuronal firing patterns arise from complex interactions of many ion channels and metabolic factors; neuronal firing converts these molecular interactions into sensations and behavior.

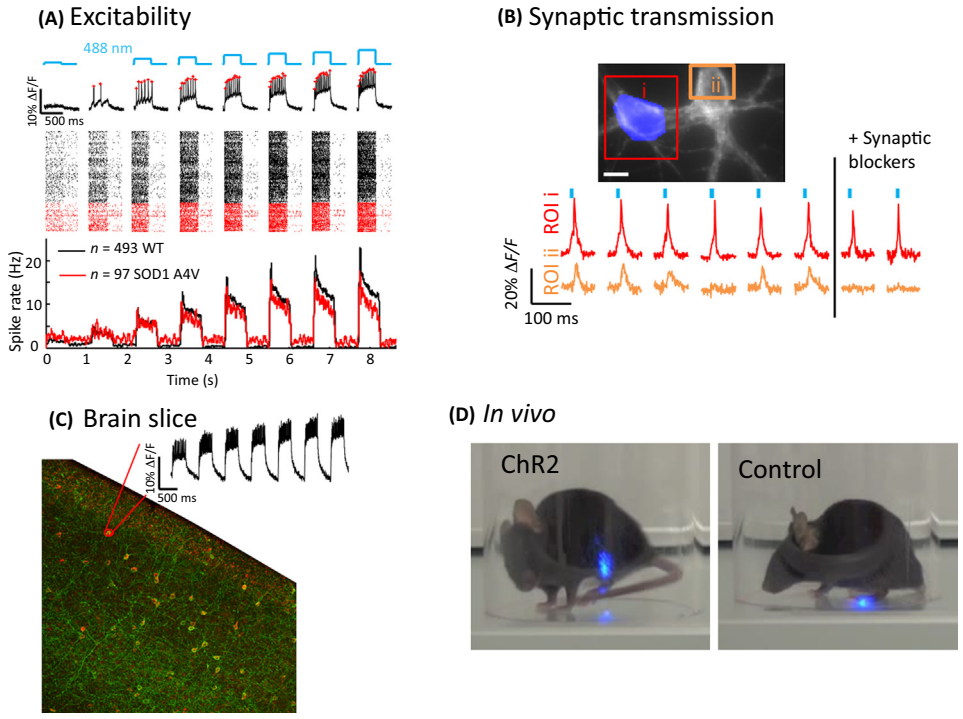

Figure 4. Optogenetics-Enabled Phenotypic Assays. (A) High-throughput measures of excitability in cultured neurons can characterize rodent and human iPSC-derived neurons. Here Optopatch recordings are shown from human iPSC-derived motor neurons comparing a wild-type genotype with cells containing an ALS-causing mutation, SOD1(A4V). The mutation causes differences in spontaneous and optogenetically induced firing patterns. Figure adapted from Kiskinis *et al.* (unpublished). (B) All-optical measures of synaptic transmission can identify mutations or compounds that modulate excitatory or inhibitory signaling in cultured neurons. Here a single rat hippocampal neuron is stimulated with patterned illumination, and excitatory postsynaptic potentials are recorded in a neighboring cell. Figure adapted from [41]. (C) All-optical measures of excitability in an acute brain slice can identify characteristic firing patterns in genetically defined neuronal subtypes in defined brain regions. Here an Optopatch recording from a somatostatin-positive interneuron is overlaid on an image of a brain slice expressing Optopatch under control of somatostatin-Cre. Figure adapted from [81]. (D) A mouse expressing channelrhodopsin 2 in its sensory neurons exhibits nocifensive behavior upon blue light illumination of its left hindpaw (left), while a control mouse does not (right). Figure adapted from [98]. Abbreviations: ALS, amyotrophic lateral sclerosis; iPSC, induced pluripotent stem cell; ROI, region of interest; WT, wild type.

Thus neuronal excitability is a powerful integrative phenotype for disease modeling and drug discovery. Optogenetic techniques now provide high-throughput access to this phenotype.

Optogenetic measures of neuronal excitability can be used in several contexts (Figure 5) (i) Target identification: genetic perturbations, for example via CRISPR/Cas9 technology, can identify prospective drug targets that modulate neuronal excitability, or validate targets suggested by other means, for example genetic studies. (ii) Target-based screens: comparisons of drug effects on the excitability of neurons +/− a specific target can distinguish on-target from off-target mechanisms of action. (iii) Purely phenotypic screens: comparisons of drug effects on different neuronal subtypes, for example excitatory versus inhibitory neurons, can identify compounds that have differential effects on excitability, regardless of mechanism of action. (iv) Patient stratification: comparisons of drug effects on patient iPSC-derived neurons and on wild-type controls can seek to match patients with therapeutics or stratify patients for clinical trials – even when the underlying genetic cause of the patient's illness is not known or is poorly understood.

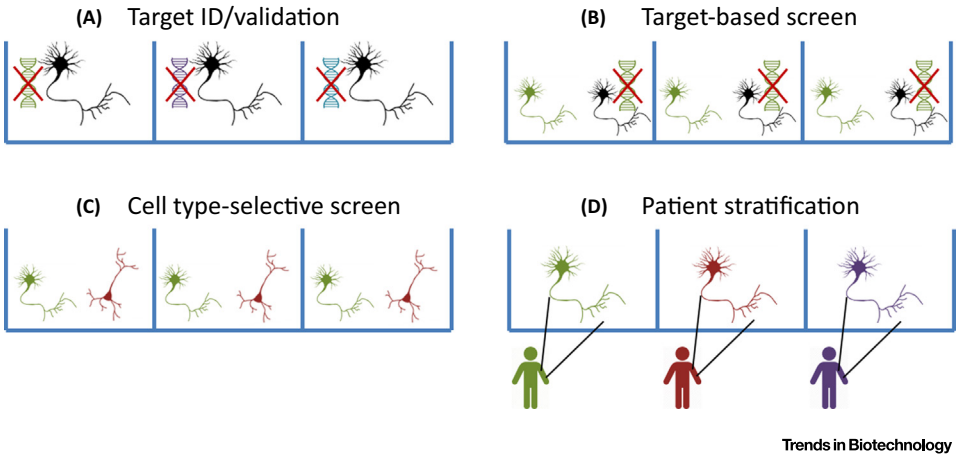

Figure 5. Optogenetic Excitability Assays Can Be Used Throughout the Discovery Pipeline. (A) Target identification. Each well contains neurons with a distinct genetic perturbation (e.g., gene knockout). Optogenetic measurements can identify genetic modulators of neuronal excitability. (B) Target-based screen. Each well contains a mixture of cells expressing and not expressing a specific drug target, as well as a fluorescent marker to distinguish the populations (green). Optogenetic excitability measurements are performed with single-cell resolution. Compounds that modulate the excitability only of the cells with the target are judged to be functionally selective for the target in the native cellular milieu. (C) Cell type-selective screen. Each well contains a mixture of two or more cell types with fluorescent markers identifying the cell type (green vs red). Optogenetic excitability measurements are performed with single-cell resolution. Compounds that modulate the excitability of only one cell type are judged to have cell type-selectivity regardless of the mechanism of action. (D) Patient stratification. Each well contains induced pluripotent stem cell (iPSC)-derived neurons from a different patient. Optogenetic excitability measurements in the presence of a test compound can determine how the cells of the patient will respond to the compound.

A paired stimulus and readout are needed to perform high-throughput measures of neuronal excitability. Electric field stimulation (EFS) is a convenient means of whole-well stimulation that leaves the visible spectrum free for a variety of optogenetic readouts. EFS has been paired with whole-well measures of Ca^{2+} dynamics (via **GCaMP** reporters) in screening format for central nervous system (CNS) [6] and dorsal root ganglion (DRG) [78] neurons, as well as with voltage-sensitive dyes [75]. By using genetically encoded reporters, one could also probe the effect of test compounds on neural activity-dependent changes in other parameters such as cAMP (via ICUE3 [79]), ATP (via Percival [80]), or other metabolic factors.

When EFS is applied in an imaging format, cell type-specific markers in one color channel, and broadly expressed activity markers in another channel, could be used to probe for cell type-specific effects of compounds on excitability. Such an approach could be useful, for instance, to detect compounds that differentially suppress firing in nociceptive versus non-nociceptive sensory neurons, or to detect compounds that differentially affect excitability in excitatory versus inhibitory CNS neurons.

While EFS leaves the entire optical spectrum available for functional measurements, this technique does not allow targeted stimulation of specific subsets of cells, or stimulation in precisely controlled temporal waveforms. Devoting the blue part of the spectrum to optogenetic actuation still leaves the yellow and red parts of the spectrum for imaging voltage, Ca^{2+}, or other functional reporters.

Simultaneous optogenetic stimulation and readout of membrane voltage has been achieved through pairing a blue-shifted channelrhodopsin variant, **CheRiff**, with near-IR QuasAr voltage indicators [41]. These paired 'Optopatch' constructs enabled wide-field all-optical stimulation and

readout of neural activity. Optopatch measurements have been used to probe the excitability of primary rodent and human iPSC-derived CNS [41] and sensory DRG [81] neurons. A recent application probed the excitability of human iPSC-derived motor neurons containing a mutation causal for amyotrophic lateral sclerosis (ALS) (Kiskinis *et al.*, unpublished).

Synaptic Transmission

Synaptic dysfunction is implicated in many neuropsychiatric and neurodegenerative diseases. The immense morphological and biochemical complexity of the synapse presents a forbidding obstacle to target-based drug development in this domain. Recently developed tools are beginning to enable optogenetic interrogation of synaptic function. Calcium indicators targeted to presynaptic **boutons** [82] report Ca^{2+} entry at the site of vesicle release. pH indicators targeted to the lumen of synaptic vesicles report the de-acidification that occurs during vesicle fusion [83]. A recently developed glutamate indicator, iGluSnfr, reports glutamate release [84]. On the postsynaptic side, spine-targeted Ca^{2+} indicators are in development [85]. By pairing optogenetic stimulation of a channelrhodopsin (CheRiff) in a presynaptic cell with fluorescence imaging of membrane voltage in a postsynaptic cell expressing QuasAr2, one can detect excitatory and inhibitory postsynaptic potentials. By adding selective blockers of AMPA, NMDA, or glutamate receptors one can isolate the contributions to the postsynaptic potential from specific classes of receptors. Furthermore, genetic targeting of presynaptic actuator and, when appropriate, postsynaptic reporter enables targeted measurements of synaptic transmission between genetically defined neuronal subtypes.

Several challenges must be addressed before synaptic assays can transition from the research domain to screening applications. Homeostatic feedbacks render synaptic strengths highly sensitive to the density of neurons and glia, as well as to the past history of activity [86]. It is technically challenging to achieve sufficiently tight control over these parameters for screening. Assays that measure the same cells pre- and post-compound are likely to be more useful in the near-term because one can null-out cell-to-cell variability. Microchannel devices that compartmentalize pre- and postsynaptic cells offer an intriguing possibility for assays in a standardized geometry [87], but scale-up engineering will be needed before these devices are useful for screening. Given the transformative promise of synaptic assays, one can anticipate rapid progress in this domain.

Measurements in Brain Slices and Tissue

Owing to circuit-level feedbacks, modulation of cell-autonomous excitability or monosynaptic transmission can have counterintuitive effects at the level of microcircuit function in intact tissue. Furthermore, one important goal is to identify compounds that selectively modulate the function of particular microcircuits. This modulation could arise from pleiotropic molecular mechanisms. Thus there is strong demand for optogenetic tools to map neuronal function in the context of intact tissue, for example in acute brain slices.

Recent advances in ultrawide-field imaging have opened the possibility of two-photon Ca^{2+} or glutamate imaging over areas almost covering a complete brain slice [88,89]. The challenge with passive imaging in acute slices is that, under typical conditions, there is little spontaneous activity. Recently, Optopatch measurements of excitability were demonstrated in acute slices using a transgenic Cre-dependent Optopatch mouse to target Optopatch expression to genetically specified subsets of neurons [81]. It has not yet been possible to perform Optopatch measurements of synaptic transmission in acute slices because postsynaptic potentials in tissue are ~20-fold smaller than in culture [90]. Owing to the low throughput of measurements slice-based measurements, these assays will likely find most use in target validation, for example via studies on genetically modified mice, and on hit validation or prioritization, where

a small number of molecules can be tested for effects on the target circuit and on other brain regions. Despite these limitations on throughput, optogenetic mapping of compound effects on circuit excitability is a promising frontier for drug discovery.

Measurements *In Vivo*

Most drugs go through tests of efficacy in rodent models before human clinical trials. Optogenetic activation of specific circuits can elicit a wide range of disease-relevant behaviors [91,92] including fear [93], aggression [94], feeding [95], pain [96,97], and sleep [98]. The temporal precision and repeatability with which these behaviors can be evoked via optogenetic stimulation provides a robust baseline against which to test the effect of perturbations in candidate target genes or to test application of candidate therapeutics, although one should keep in mind that patterns of neural activity induced by synchronous activation of large ensembles are likely to differ substantially from naturally occurring patterns.

Implantable microscopes [99] and ultra-wide-field imaging preparations [100] further enable optogenetic recording of Ca^{2+} dynamics in large populations of cells *in vivo*, which can be used to validate the effect of a test compound on circuit dynamics. Recently developed implants that combine optogenetic stimulation with drug delivery provide a means to test compound effects in a minimally invasive manner [101]. As with measurements in acute brain slices, the low throughput of optogenetic measurements *in vivo* will most likely restrict applications to target and hit validation.

Optogenetics in Safety and Toxicology

Safety remains a key hurdle in drug development, and cardiac safety is of paramount concern because of the potential severity of cardiac side-effects. Traditional safety assessments require testing against the cardiac hERG channel, a common cause of off-target cardiotoxicity, followed by tests in dogs and humans [102]. However, hERG testing may reject some potentially promising candidates. Indeed, several drugs known to be safe and approved before hERG testing became standard would not pass current safety tests. The cardiac *in vitro* proarrhythmia (CiPA) initiative aims to develop more accurate *in vitro* assays of cardiac risk [103]. Tests in human iPSC-derived cardiomyocytes (hiPSC-CM) provide one arm of this new approach. Optogenetic measurements of voltage [104] and calcium [105], combined with optogenetic pacing [106], provide a rich functional phenotype for exploring drug effects on human cardiac activity. Similarly, the all-optical electrophysiology method can also be used for neurotoxicity studies to evaluate compound effects on neuron intrinsic excitability and synaptic transmission.

Future Directions: Optogenetics in Patient Stratification and Precision Medicine

For many diseases of the nervous system, symptoms alone poorly predict the response of a patient to a candidate therapeutic. Despite some notable successes of genetic stratification [107], in many instances genetics provides little additional guidance. Optogenetic measurements on patient iPSC-derived neurons provide a means to instantiate the entire genome of a patient in a physiologically relevant and pharmacologically accessible format. A likely direction of future progress will be toward patient stratification based on functional cell-based measurements. Initially this work will occur in the presence of stratification for clinical trials, although ultimately the approach may be extended to individual patients, seeking to select among many possible therapeutic options. Patient iPSC-derived neurons are most likely to replicate disease pathology for monogenic channelopathies where one can reasonably expect a robust cell-autonomous phenotype. The extent to which these cells can recapitulate complex neurodevelopmental and neurodegenerative disease processes remains a topic of active research.

Challenges for Optogenetics-Enabled Drug Discovery

Optogenetic techniques offer great promise for accelerating drug discovery. However, technical challenges remain (see Outstanding Questions). The set of spectrally compatible optogenetic actuators and reporters is limited because of the very broad action spectra of microbial rhodopsin-based actuators and the reliance of most reporters on GFP or spectrally similar proteins. Development of far-red or near-IR genetically encoded reporters would greatly expand the scope of possible applications. One would also like to see reporters for more analytes than are currently available, particularly for neurotransmitters and neuromodulators, for example GABA, glycine, dopamine, serotonin, and acetylcholine.

A further challenge is quantification. In conventional patch-clamp measurements one knows the accurate value of the membrane current and voltage. For both optogenetic stimulation and measurement, the strength of the signal depends on the expression level and trafficking of the transducer, the membrane area, and the distribution of illumination intensity. These parameters are challenging to know precisely, although there are several promising remedies. Stable transgenic cell lines can have more homogeneous expression compared to transiently transfected cells, leading to less cell-to-cell variability. Coexpression of a stable fluorescent tag can further provide a means to estimate expression level ratiometrically. The electronic excited-state lifetime of some fluorescent reporters can also provide an expression level-independent measure of activation. These techniques have been applied for voltage [108,109] and calcium [110,111], but not yet in a high-throughput format. Ultimately, quantitative measurements come from careful experimental design, including for example mixed positive and negative control cells in the same well, pre- and post-drug measurements on the same cells, or tool pharmacology for post-measurement calibration of signal magnitudes.

We also need better instrumentation and software. Existing screening tools lack the capability for spatially and spectrally patterned illumination, and lack the temporal resolution, spatial resolution, and sensitivity needed for high-speed measurements of neuronal firing. While optical stimulation capabilities are starting to be added to existing screening platforms [112], these capabilities remain rudimentary. This challenge will likely be addressed through a series of instruments with differing tradeoffs in throughput and information content. A key divide is whether the instrument provides cellular resolution in a single-well format or averages over the whole well and probes multiple wells in parallel. For truly high-throughput screens (>105 compounds), the latter approach will be essential. Whole-well average measurements are most appropriate for screens on heterologously expressed proteins, for example in spiking HEK cells, or for measures of neuronal activity where the population-level response is expected to be homogeneous. Follow-up lower-throughput assays can use instruments with single-cell resolution and more complex stimulation, but with serial scanning over multiple wells. Both types of measurements have been demonstrated academically, but no company has yet undertaken the engineering to make these tools commercially available.

Finally, we need better software. Imaging a wide area at high speed and high resolution leads to prodigious data rates, typically ~1 GByte/s. Specialized software pipelines are needed to store, segment, and process these data. Each discovery project must address the challenge of converting terabytes of movies into actionable screening data.

Despite these challenges, optogenetic tools are already making headway in drug discovery. Further technical advances offer the promise to bring in a new age of functional screening for diseases of cellular excitability. Traditional screening approaches have led to high rates of expensive attrition during clinical trials. By testing drugs for functional modulation of neurons, circuits, and patient-derived cells, there is reason for optimism that the initial assays will more closely approximate the patient-relevant biology.

Disclaimer Statement

A.E.C. is a founder of Q-State Biosciences. H.Z. is an employee of Q-State Biosciences.

Acknowledgments

H.Z. and A.E.C. were supported by the Howard Hughes Medical Institute and the Gordon and Betty Moore Foundation. We thank the members of the Cohen Lab for their work on some of the technologies described in this manuscript.

Outstanding Questions

Can we develop red-shifted reporters for neurotransmitters and neuromodulators, which are compatible with optogenetic stimulation?

Can optogenetic measurements on human iPSC-derived neurons be used to predict patient response to drugs?

How best to parameterize and classify the complex stimulus-dependent firing patterns of neuronal disease models *in vitro*?

How can one attain optogenetic stimuli of precisely calibrated magnitudes, and fluorescence measurements of accurately calibrated concentrations or voltages?

References

1. Schroeder, K.S. and Neagle, B.D. (1996) FLIPR: a new instrument for accurate, high throughput optical screening. *J. Biomol. Screen.* 1, 75–80
2. Epi4K Consortium (2013) De novo mutations in epileptic encephalopathies. *Nature* 501, 217–221
3. Epi4K consortium (2017) Ultra-rare genetic variation in common epilepsies: a case–control sequencing study. *Lancet Neurol.* 16, 135–143
4. Fromer, M. (2014) De novo mutations in schizophrenia implicate synaptic networks. *Nature* 506, 179–184
5. Purcell, S.M. (2014) A polygenic burden of rare disruptive mutations in schizophrenia. *Nature* 506, 185–190
6. Hempel, C.M. (2011) A system for performing high throughput assays of synaptic function. *PLoS One* 6, e25999
7. Zheng, W. (2013) Phenotypic screens as a renewed approach for drug discovery. *Drug Discov. Today* 18, 1067–1073
8. Eijkelkamp, N. (2012) Neurological perspectives on voltage-gated sodium channels. *Brain* 135, 2585–2612
9. Paz, J.T. and Huguenard, J.R. (2015) Microcircuits and their interactions in epilepsy: is the focus out of focus? *Nat. Neurosci.* 18, 351–359
10. Deisseroth, K. (2015) Optogenetics: 10 years of microbial opsins in neuroscience. 18, 1213–1225
11. Lin, J.Y. (2011) A user's guide to channelrhodopsin variants: features, limitations and future developments. *Exp. Physiol.* 96, 19–25
12. Lin, M.Z. and Schnitzer, M.J. (2016) Genetically encoded indicators of neuronal activity. *Nat. Neurosci.* 19, 1142–1153
13. Mehta, S. and Zhang, J. (2011) Reporting from the field: genetically encoded fluorescent reporters uncover signaling dynamics in living biological systems. *Annu. Rev. Biochem.* 80, 375–401
14. Bolbat, A. and Schultz, C. (2017) Recent developments of genetically encoded optical sensors for cell biology. *Biol. Cell* 109, 1–23
15. McManus, O.B. (2014) HTS assays for developing the molecular pharmacology of ion channels. *Curr. Opin. Pharmacol.* 15, 91–96
16. Du, Y. (2015) Development and validation of a thallium flux-based functional assay for the sodium channel NaV1. 7 and its utility for lead discovery and compound profiling. *ACS Chem. Neurosci.* 6, 871–878
17. Weaver, C.D. (2004) A thallium-sensitive, fluorescence-based assay for detecting and characterizing potassium channel modulators in mammalian cells. *J. Biomol. Screen.* 9, 671–677
18. Sui, J. (2010) Optimization of a yellow fluorescent protein-based iodide influx high-throughput screening assay for cystic fibrosis transmembrane conductance regulator (CFTR) modulators. *Assay Drug Dev. Technol.* 8, 656–668
19. Sawada, K. and Yoshinaga, T. (2012) Automated patch clamping. In *Patch Clamp Techniques: From Beginning to Advanced Protocols* (Okada, Y., ed.), pp. 323–332, Springer
20. Farre, C. (2008) Ion channel screening – automated patch clamp on the rise. *Drug Discov. Today Technol.* 5, e23–e28
21. Chambers, C. *et al.* (2016) High-throughput screening of Na(V) 1.7 modulators using a giga-seal automated patch clamp instrument. *Assay Drug Dev. Technol.* 14, 93–108
22. Zhang, Z.N. *et al.* (2008) The voltage-gated Na^+ channel Nav1.8 contains an ER-retention/retrieval signal antagonized by the beta3 subunit. *J. Cell Sci.* 121, 3243–3252
23. Vanoye, C.G. *et al.* (2013) Mechanism of sodium channel NaV1.9 potentiation by G-protein signaling. *J. Gen. Physiol.* 141, 193–202
24. Scheel, O. (2014) Action potential characterization of human induced pluripotent stem cell-derived cardiomyocytes using automated patch-clamp technology. *Assay Drug Dev. Technol.* 12, 457–469
25. Nagel, G. (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc. Natl. Acad. Sci. U. S. A.* 100, 13940–13945
26. Boyden, E.S. (2005) Millisecond-timescale, genetically targeted optical control of neural activity. *Nat. Neurosci.* 8, 1263–1268
27. Lin, J.Y. (2013) ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. *Nat. Neurosci.* 16, 1499–1508
28. Klapoetke, N.C. (2014) Independent optical excitation of distinct neural populations. *Nat. Methods* 11, 338–346
29. Erbguth, K. (2012) Bimodal activation of different neuron classes with the spectrally red-shifted channelrhodopsin chimera C1V1 in *Caenorhabditis elegans*. *PLoS One* 7, e46827
30. Govorunova, E.G. (2013) Characterization of a highly efficient blue-shifted channelrhodopsin from the marine alga *Platymonas subcordiformis*. *J. Biol. Chem.* 288, 29911–29922
31. Zhang, F. (2008) Red-shifted optogenetic excitation: a tool for fast neural control derived from *Volvox carteri*. *Nat. Neurosci.* 11, 631–633
32. Lin, J.Y. (2009) Characterization of engineered channelrhodopsin variants with improved properties and kinetics. *Biophys. J.* 96, 1803–1814
33. Berndt, A. *et al.* (2009) Bi-stable neural state switches. *Nat. Neurosci.* 12, 229–234
34. Kleinlogel, S. (2011) Ultra light-sensitive and fast neuronal activation with the Ca^{2+}-permeable channelrhodopsin CatCh. *Nat. Neurosci.* 14, 513–518
35. Inoue, K. *et al.* (2013) A light-driven sodium ion pump in marine bacteria. *Nat. Commun.* 4, 1678
36. Govorunova, E.G. *et al.* (2015) Natural light-gated anion channels: A family of microbial rhodopsins for advanced optogenetics. *Science* 349, 647–650
37. Gradinaru, V. (2008) eNpHR: a *Natronomonas* halorhodopsin enhanced for optogenetic applications. *Brain Cell Biol.* 36, 129–139
38. Chow, B.Y. (2010) High-performance genetically targetable optical neural silencing by light-driven proton pumps. *Nature* 463, 98–102
39. Berndt, A. (2014) Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. *Science* 344, 420–424
40. Wietek, J. (2014) Conversion of channelrhodopsin into a light-gated chloride channel. *Science* 344, 409–412
41. Hochbaum, D.R. (2014) All-optical electrophysiology in mammalian neurons using engineered microbial rhodopsins. *Nat. Methods* 11, 825–833
42. Huang, Y.L. (2015) A photostable silicon rhodamine platform for optical voltage sensing. *J. Am. Chem. Soc.* 137, 10767–10776
43. Collot, M. (2015) CaRuby-Nano: a novel high affinity calcium probe for dual color imaging. *Elife* 4, 05808
44. Inoue, M. (2015) Rational design of a high-affinity, fast, red calcium indicator R-CaMP2. *Nat. Methods* 12, 64–70
45. Dana, H. (2016) Sensitive red protein calcium indicators for imaging neural activity. *Elife* 5, e12727
46. Bagal, S.K. (2015) Voltage gated sodium channels as drug discovery targets. *Channels* 9, 360–366

47. Park, J. (2013) Screening fluorescent voltage indicators with spontaneously spiking HEK cells. *PLoS One* 8, e85221
48. McNamara, H.M. (2016) Optically controlled oscillators in an engineered bioelectric tissue. *Phys. Rev. X* 6, 031001
49. Zhang, H. (2016) Optical electrophysiology for probing function and pharmacology of voltage-gated ion channels. *ELife* 5, e15202
50. Agus, V. (2015) Bringing the light to high throughput screening: use of optogenetic tools for the development of recombinant cellular assays. *SPIE Proc.* 9305, 93052T
51. Kang, S. *et al.* (2012) CaV1.3-selective L-type calcium channel antagonists as potential new therapeutics for Parkinson's disease. *Nat. Commun.* 3, 1146
52. Maher, M.P. (2009) HCN channels as targets for drug discovery. *Comb. Chem. High Throughput Screen.* 12, 64–72
53. Stierl, M. (2011) Light modulation of cellular cAMP by a small bacterial photoactivated adenylyl cyclase, bPAC, of the soil bacterium *Beggiatoa*. *J. Biol. Chem.* 286, 1181–1188
54. Inglés-Prieto, Á. *et al.* (2015) Light-assisted small-molecule screening against protein kinases. *Nat. Chem. Biol.* 11, 952–954
55. Mylvaganam, S. (2014) Roles of gap junctions, connexins, and pannexins in epilepsy. *Front. Physiol.* 5, 172
56. Kirkton, R.D. and Bursac, N. (2011) Engineering biosynthetic excitable tissues from unexcitable cells for electrophysiological and cell therapy studies. *Nat. Commun.* 2, 300
57. Bezanilla, F. (2008) How membrane proteins sense voltage. *Nat. Rev. Mol. Cell Biol.* 9, 323–332
58. Cohen, A.E. and Venkatachalam, V. (2014) Bringing bioelectricity to light. *Annu. Rev. Biophys.* 43, 211–232
59. Mahaut-Smith, M.P. (2008) A role for membrane potential in regulating GPCRs? *Trends Pharmacol. Sci.* 29, 421–429
60. Vickery, O.N. (2016) Membrane potentials regulating GPCRs: insights from experiments and molecular dynamics simulations. *Curr. Opin. Pharmacol.* 30, 44–50
61. Kucka, M. (2010) Dependence of multidrug resistance protein-mediated cyclic nucleotide efflux on the background sodium conductance. *Mol. Pharmacol.* 77, 270–279
62. Luker, G.D. (2001) MDR1 P-glycoprotein reduces influx of substrates without affecting membrane potential. *J. Biol. Chem.* 276, 49053–49060
63. Howard, E.M. and Roepe, P.D. (2003) Purified human MDR 1 modulates membrane potential in reconstituted proteoliposomes. *Biochemistry* 42, 3544–3555
64. Zhou, Y. (2015) Membrane potential modulates plasma membrane phospholipid dynamics and K-Ras signaling. *Science* 349, 873–876
65. Yang, M. and Brackenbury, W.J. (2013) Membrane potential and cancer progression. *Front. Physiol.* 4, 185
66. Bailes, H.J. (2012) Reproducible and sustained regulation of Gαs signalling using a metazoan opsin as an optogenetic tool. *PLoS One* 7, e30774
67. Oh, E. (2010) Substitution of 5-HT1A receptor signaling by a light-activated G protein-coupled receptor. *J. Biol. Chem.* 285, 30825–30836
68. Airan, R.D. (2009) Temporally precise in vivo control of intracellular signalling. *Nature* 458, 1025–1029
69. Zhang, K. and Cui, B. (2015) Optogenetic control of intracellular signaling pathways. *Trends Biotechnol.* 33, 92–100
70. Polstein, L.R. and Gersbach, C.A. (2015) A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. *Nat. Chem. Biol.* 11, 198–200
71. Lim, S.T. (2000) A novel targeting signal for proximal clustering of the Kv2. 1K channel in hippocampal neurons. *Neuron* 25, 385–397
72. Grubb, M.S. and Burrone, J. (2010) Activity-dependent relocation of the axon initial segment fine-tunes neuronal excitability. *Nature* 465, 1070–1074
73. Tilley, D.C. (2014) Chemoselective tarantula toxins report voltage activation of wild-type ion channels in live cells. *Proc. Natl. Acad. Sci. U. S. A.* 111, E4789–E4796
74. Schmidt-Dannert, C. and Arnold, F.H. (1999) Directed evolution of industrial enzymes. *Trends Biotechnol.* 17, 135–136
75. Huang, C. (2006) Characterization of voltage-gated sodium-channel blockers by electrical stimulation and fluorescence detection of membrane potential. *Nat. Biotechnol.* 24, 439–446
76. Sills, G.J. (2006) The mechanisms of action of gabapentin and pregabalin. *Curr. Opin. Pharmacol.* 6, 108–113
77. Lynch, B.A. (2004) The synaptic vesicle protein SV2A is the binding site for the antiepileptic drug levetiracetam. *Proc. Natl. Acad. Sci. U. S. A.* 101, 9861–9866
78. Karila, P. and Blom, C. (2015) The development of cell-based assays for pain drug discovery. *Drug Discovery World* Summer Issue, 40–44
79. DiPilato, L.M. and Zhang, J. (2009) The role of membrane microdomains in shaping β2-adrenergic receptor-mediated cAMP dynamics. *Mol. Biosyst.* 5, 832–837
80. Tantama, M. (2013) Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio. *Nat. Commun.* 4, e2550
81. Lou, S. (2016) Genetically targeted all-optical electrophysiology with a transgenic Cre-dependent Optopatch mouse. *J. Neurosci.* 36, 11059–11073
82. Jackson, R.E. and Burrone, J. (2016) Visualizing presynaptic calcium dynamics and vesicle fusion with a single genetically encoded reporter at individual synapses. *Front. Synaptic Neurosci.* 8, 21
83. Miesenbock, G. (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394, 192–195
84. Marvin, J.S. (2013) An optimized fluorescent probe for visualizing glutamate neurotransmission. *Nat. Methods* 10, 162–170
85. Ohkura, M. *et al.* (2015) Optogenetic manipulation and probing. In *Optogenetics: Light-Sensing Proteins and Their Applications* (Yawo, H., ed.), pp. 133–147, Springer
86. Barral, J. and Reyes, A.D. (2016) Synaptic scaling rule preserves excitatory-inhibitory balance and salient neuronal network dynamics. *Nat. Neurosci.* 19, 1690–1696
87. Millet, L.J. (2007) Microfluidic devices for culturing primary mammalian neurons at low densities. *Lab Chip* 7, 987–994
88. Sofroniew, N.J. *et al.* (2016) A large field of view two-photon mesoscope with subcellular resolution for in vivo imaging. *eLife* 5, e14472
89. Tsai, P.S. (2015) Ultra-large field-of-view two-photon microscopy. *Optics Express* 23, 13833–13847
90. Magee, J.C. and Cook, E.P. (2000) Somatic EPSP amplitude is independent of synapse location in hippocampal pyramidal neurons. *Nat. Neurosci.* 3, 895–903
91. Bernstein, J.G. and Boyden, E.S. (2011) Optogenetic tools for analyzing the neural circuits of behavior. *Trends Cogn. Sci.* 15, 592–600
92. Tye, K.M. and Deisseroth, K. (2012) Optogenetic investigation of neural circuits underlying brain disease in animal models. *Nat. Rev. Neurosci.* 13, 251–266
93. Johansen, J.P. (2012) Controlling the elements: an optogenetic approach to understanding the neural circuits of fear. *Biol. Psychiatry* 71, 1053–1060
94. Lin, D. (2011) Functional identification of an aggression locus in the mouse hypothalamus. *Nature* 470, 221–226
95. Aponte, Y. (2011) AGRP neurons are sufficient to orchestrate feeding behavior rapidly and without training. *Nat. Neurosci.* 14, 351–355
96. Daou, I. (2013) Remote optogenetic activation and sensitization of pain pathways in freely moving mice. *J. Neurosci.* 33, 18631–18640
97. Iyer, S.M. (2014) Virally mediated optogenetic excitation and inhibition of pain in freely moving nontransgenic mice. *Nat. Biotechnol.* 32, 274–278
98. Tsunematsu, T. (2011) Acute optogenetic silencing of orexin/hypocretin neurons induces slow-wave sleep in mice. *J. Neurosci.* 31, 10529–10539

99. Hamel, E.J. (2015) Cellular level brain imaging in behaving mammals: an engineering approach. *Neuron* 86, 140–159

100. Kim, T.H. (2016) Long-term optical access to an estimated one million neurons in the live mouse cortex. *Cell Rep.* 17, 3385–3394

101. Jeong, J. (2015) Wireless optofluidic systems for programmable in vivo pharmacology and optogenetics. *Cell* 162, 662–674

102. Chi, K.R. (2013) Revolution dawning in cardiotoxicity testing. *Nat. Rev. Drug Discov.* 12, 565–567

103. Colatsky, T. (2016) The comprehensive in vitro proarrhythmia assay (CiPA) initiative – update on progress. *J. Pharmacol. Toxicol. Methods* 81, 15–20

104. Klimas, A. (2016) OptoDyCE as an automated system for high-throughput all-optical dynamic cardiac electrophysiology. *Nat. Commun.* 7, 11542

105. Cerignoli, F. (2012) High throughput measurement of Ca^{2+} dynamics for drug risk assessment in human stem cell-derived cardiomyocytes by kinetic image cytometry. *J. Pharmacol. Toxicol. Methods* 66, 246–256

106. Dempsey, G.T. (2016) Cardiotoxicity screening with simultaneous optogenetic pacing, voltage imaging and calcium imaging. *J. Pharmacol. Toxicol. Methods* 81, 240–250

107. Collins, F.S. and Varmus, H. (2015) A new initiative on precision medicine. *N. Engl. J. Med.* 372, 793–795

108. Hou, J.H. (2014) Temporal dynamics of microbial rhodopsin fluorescence reports absolute membrane voltage. *Biophys. J.* 106, 639–648

109. Brinks, D. (2015) Two-photon fluorescence lifetime imaging microscopy (2P-FLIM) of genetically encoded voltage indicators as a probe of absolute membrane voltage. *Biophys. J.* 109, 914–921

110. Thestrup, T. (2014) Optimized ratiometric calcium sensors for functional in vivo imaging of neurons and T lymphocytes. *Nat. Methods* 11, 175–182

111. Jensen, T.P. *et al.* (2017) Monitoring single-synapse glutamate release and presynaptic calcium concentration in organised brain tissue. *Cell Calcium* Published online March 30, 2017. http://dx.doi.org/10.1016/j.ceca.2017.03.007

112. Clements, I.P. *et al.* (2016) Optogenetic stimulation of multiwell MEA plates for neural and cardiac applications. *SPIE Proc.* 9690, 96902C