HHS Public Access

Author manuscript

Epilepsy Res. Author manuscript; available in PMC 2016 October 01.

Published in final edited form as:

Epilepsy Res. 2015 October ; 116: 15–26. doi:10.1016/j.eplepsyres.2015.06.010.

Optogenetic tools for modulating and probing the epileptic network

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Abstract

Epilepsy affects roughly 1% of the population worldwide. Although effective treatments with antiepileptic drugs are available, more than 20% of patients have seizures that are refractory to medical therapy and many patients experience adverse effects. Hence, there is a continued need for novel therapies for those patients. A new technique called “optogenetics” may offer a new hope for these refractory patients. Optogenetics is a technology based on the combination of optics and genetics, which can control or record neural activity with light. After delivery of light-sensitive opsin genes such as channelrhodopsin-2 (ChR2), halorhodopsin (NpHR), and others into brain, excitation or inhibition of specific neurons in precise brain areas can be controlled by illuminations at different wavelengths with very high temporal and spatial resolution. Neuromodulations with the optogenetics toolbox have already been shown to be effective at treating seizures in animal models of epilepsy. This review will outline the most recent advances in epilepsy research with optogenetic techniques and discuss how this technology can contribute to our understanding and treatment of epilepsy in the future.

Keywords

optogenetic; epilepsy model; optical imaging; Photostimulation

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Introduction

Epilepsy affects more than 50 million people worldwide, or roughly 1% of the population. Although most patients can be adequately treated with antiepileptic drugs, more than 20% of patients continue to have seizures that are refractory to medical therapy and many additional patients experience adverse side effects(Hauser and Hesdorfer, 1990). Hence, there is a continued need for the development of novel anti-epileptic therapies. Recently, a new type of light-sensitive molecule, or “opsin”, has been developed which combines light-sensitivity with the modern genetic toolbox to control and monitor brain activity on a range of spatial resolutions from individual neurons to complex neural circuits (Boyden et al., 2005). “Optogenetics” is a novel field, which uses opsins for neuromodulation at extremely high spatial and temporal resolution, which can control the activity of specific types of neurons, or populations of neurons, in preparations ranging from cultured neurons to freely moving animals. The optogenetic toolbox has already demonstrated remarkable potential in epilepsy research and epilepsy therapy (Bentley et al., 2013; Kokaia et al., 2013; Krook-Magnuson and Soltesz, 2015; Ritter et al., 2014). A second generation of optogenetic tools, including indicators as well as actuators, permits the use of light to report on, as well as control, molecular processes in specific cell sub-populations within networks of heterogeneous cell types (Knöpfel et al., 2010). Since epilepsy involves complex neurochemical changes including synaptic and non-synaptic transmission, ion channels interactions, intracellular signaling pathways and glia–neuron signaling, these new optogenetic indicators can be used to probe those changes to understand the molecular and neurochemical basis of epilepsy to develop new targets for antiepileptic therapy.

Opsins

The possibility of using light for controlling precise neural activity was first proposed by Francis Crick in 1999 (Crick, 1999). However, a functional gene-based light-sensitive technique was not reported until 2002 by Gero Miesenböck's laboratory (Zemelman et al., 2002). They employed Drosophila rhodopsin photoreceptors to control neural activity in cultured mammalian neurons. Georg Nagel's lab first discovered the Channelrhodopsins including Channelrhodopsin-1 (ChR1) and Channelrhodopsin-2 (ChR2) from Chlamydomonas *reinhardtii*, which functioned as light-gated cation-selective membrane channels (Nagel et al., 2002; Nagel et al., 2003). These early genetic photostimulation techniques were only studied in a few laboratories due to technical limitations ((Banghart et al., 2004; Lima and Miesenböck, 2005; Volgraf et al., 2006). The revolutionary breakthrough in optogenetics occurred in Dr. Karl Deisseroth's laboratory in 2005, where a single-component ChR2 optogenetic system was used for millisecond control of neural firing in cultured neurons (Boyden et al., 2005). Intermittently illuminating and then extinguishing a light source caused a cell to fire, or stop firing, action potentials. Soon after, a series of studies using multiple opsins extended this new technique to *in vivo* preparations (Zhang et al., 2007). Further genetic manipulations have also made brain region- and cell type-specific modulation possible (Cardin et al., 2010). Optogenetics now includes both actuators and reporters. Optogenetic actuators are proteins with a light-controllable biological function and optogenetic reporters are proteins which provide readouts of biochemical processes that occur in the context of living tissue (Alford et al., 2013).

The key element of an optogenetic actuator is the opsin. Opsins are a group of light-sensitive proteins that underlie the molecular basis of various light-sensing systems including phototaxis, circadian rhythms, eyesight, and certain types of photosynthesis. The two major classes of opsins are defined and differentiated based either on the primary protein sequence, the chromophore chemistry or the signal transduction mechanisms. Type I opsins are present in bacteria, archaebacteria, and unicellular algae including bacteriorhodopsin, bacterial sensory rhodopsin, ChR, halorhodopsin (NpHR), and proteorhodopsin (Zhang et al., 2011). Type II opsins are present in eumetazoans (animals not including sponges) and have varied function, including phototransduction, vision, circadian rhythm entrainment, papillary reflexes and photoisomerization(Sakmar, 2002; Shichida and Yamashita, 2003). Commonly used optogenetic opsins are type I microbial opsins, including light-gated cations such as ChRs and light-driven pumps such as NpHR or archaerhodopsin (Fig 1).

ChRs are light-gated cation-selective ion channels that can be used to excite cells. ChR2, from *Chlamydomonas reinhardtii*, is the first fully genetically-encoded optogenetic tool used in neuroscience. It has been the major ChR prototype for optogenetic applications since it is expressed more highly in most host cells than ChR1(Nagel et al., 2003). The wild-type ChR2 absorbs blue light with and action spectrum maximum at 480nm. With blue light illumination, ChR2 induces a conformational change from all-trans to 13-cis-retinal, which opens a transmembrane protein pore to at least 6 Å depolarizing ChR2-expressed cell. Within milliseconds, the retinal relaxes back to the all-trans form, closing the pore and stopping the flow of ions.

Although wild-type ChR2 is a very useful tool to target light-induced neuronal activation, ChR2 mutants have been developed to increase light sensitivity to improve future clinical applications. Replacement of the active site residue E123 by Thr and Ala (ChETA variants) causes faster channel closing, eliminating the voltage sensitivity of the temporal kinetics, and inducing a 20 nm bathochromic shift (Gunaydin et al., 2010). Mutation of E90, E123, L132, or H134 does not change photocycle kinetics but alters ion selectivity in favor of H+, Na+ or Ca2+, and reduces inactivation after light step-up or multiple light flashes, respectively (Hegemann et al., 2005; Kleinlogel et al., 2011). For example, the L132C mutation (calcium translocating channelrhodopsin, CatCh) increases the permeability for calcium and generates very large currents (Kleinlogel et al., 2011). Red-sensitive ChR (ReaChR) can improve membrane trafficking and enhance steady-state response to light with wavelengths longer than 600 nm for deep transcranial optogenetic excitation (Lin et al., 2013).

In order to inhibit neuronal activity, a different set of light-driven pumps is used. A light-sensitive inwards chloride pump called halorhodopsin (NpHR), derived from the *halobacterium Natronomonas pharaoni*, is the major inhibitory opsin(Schobert and Lanyi, 1982). With yellow light illumination, NpHR generates an influx of chloride ions and generates hyperpolarization of cells (Han and Boyden, 2007; Zhang et al., 2007). A trafficking-enhanced NpHR known as eNpHR3.0 is the latest optimized version of NpHR (Gradinaru et al., 2010). Recently, a red-shifted cruxhalorhodopsin from *Haloarcula salinarum* (strain Shark), “Jaws”, was developed that causes robust neuronal inhibition and the red-shifted wavelength enables noninvasive transcranial photostimulation up to 3mm

deep (Chuong et al., 2014). An alternative inhibitory option is to use the light-activated outwards proton pump, archaerhodopsin. Three popular archeorhodopsins are Arch from *Halorubrum sodomense*, ArchT from Halorubrum genus, and Mac from fungus *Leptosphaeria maculans* (Chow et al., 2010; Han et al., 2011). Arch can be activated by orange-yellow light. Activation of Arch, ArchT, or Mac leads to an outwards flux of protons and thus generates a hyperpolarizing photocurrent that can be used to silence neuronal activity (Fig. 1). The mutation of E90 in the central gate of ChR converts the light-gated cation channel into a light-gated anion channel. This chloride-conducting ChR (ChloC) can be also used to inhibit neuronal activity (Wietek et al., 2014). Berndt et al produced iC1C2 variant and SwiChR variants (for Step-waveform inhibitory ChR) to demonstrate structure-guided conversion of a cation-selective ChR into a light-activated Cl– channel(Berndt et al., 2014).

Another type of optogenetic ligand-gated ion channel (LGIC) provides rapid, remote control over conductances for different ions (Magnus et al., 2011). Photoswitchable tethered ligands (PTLs) enable fast and reversible control of mammalian ion channels or proteins, allowing optical control of neuronal activity (Carroll et al., 2015; Srinivas et al., 2005). A group of optogenetic biochemical modulators was developed to manipulate G-protein and cAMP signaling, among others (Nagahama et al., 2007; O'Neill and Gautam, 2014). These photoactivated dimerizers are useful to investigate biochemical signaling pathways with high spatiotemporal resolution.

Optogenetic reporters are genetically encoded fluorescence proteins (FPs) that can probe the level of ions, metabolites, and enzyme activities as well as protein conformation and even membrane voltage (Alford et al., 2013; Tantama et al., 2012). Förster resonance energy transfer (FRET)-based genetically encoded reporters such as GCaMP3 and Clomeleon A provide a ratiometric readout of calcium and Cl^- (Berglund et al., 2008; Tian et al., 2009). Single FP-based reporters exhibit sensitivities to pH (Miesenbock et al., 1998). Bimolecular fluorescence complementation (BiFC)-based reporters and ddFP-based reporters can be used to detect protein-protein interactions (Alford et al., 2012).

Methods of genetic delivery

The delivery of opsins into cells requires genetic manipulation. For optogenetic actuators, opsins can be delivered into the brain through a variety of transfection techniques including viral transfection, electroporation, and gene gun. Transgenic mice with expression of optogenetic tools in a cell type-specific manner offer a powerful approach for examining the role of particular cells in discrete circuits (Zeng and Madisen, 2012). Ready-made transgenic mice that show stable opsin expression under the control of promoters such as ChAT (specific to cholinergic neurons) or VGAT (GABAergic interneurons) throughout the brain have become possible. If performed in embryonic mouse, in utero *in vivo* electroporation provides a powerful tool for quick gene delivery by expressing plasmids in the cortex and targeting specific neuronal layers using an electric field (Petreanu et al., 2007).

Acute viral vector injection into various brain regions may be the most utilized delivery method. Retrovirus/lentivirus and adeno-associated virus (AAV) are the two commonly used vectors. A major limitation of lentiviruses and AAV is the limited genetic payload length

that is often incompatible with the full size enhancer/promoter needed for strong and cell-type-specific expression. The optimal size of inserted transgene for AAV vectors is 4.1 and 4.9 kb, while the packaging limitation of lentiviruses is 10 kb. (Dissen et al., 2009; Dong et al., 1996). AAV has been the most popular because of its safety, the lack of immunogenic viral proteins and its efficient transgenic expression in a very broad range of hosts (Samulski et al., 1989). AAV are considered biosafety level (BSL) 1, while retrovirus/lentivirus belong to BSL2+. However, BSL2+ viruses are now easily obtained through core virus production facilities (University of Pennsylvania, and University of North Carolina), which make the performance of optogenetic experiments much easier than before. Using a simple surgical procedure, viral vectors carrying opsins can be injected into different brain regions at all stages of life with narrow spatiotemporal targeting (Yizhar et al., 2011a). (Fig. 2)

Cell-specificity can be easily achieved by using cell-type specific promoters(Betley and Sternson, 2011). Although stereotactic injection in the targeted brain region will infect many cell types, only those driven by the selected promoter will manufacture the opsin protein. Strong ubiquitous promoters, such as elongation factor 1α (ELF-1α), synapsin, cytomegalovirus (CMV) or CAG will give robust opsin expression in almost any cell type. Cell-type specific promoters such as α-calcium/calmodulin-dependent kinase II (αCamKII) will express opsins uniquely in forebrain pyramidal neurons. A popular alternative is to inject virus with the flip-excision (FLEX) switch system into the brain of Cre-expressing lines (Atasoy et al., 2008; Kuhlman and Huang, 2008). Cre recombinase is an enzyme that catalyzes site-specific recombination between two DNA recognition sequences known as loxP sites. Any DNA that is present between two loxP sites of the same orientation (‚floxed' DNA) will be excised(Kühn and M. Torres, 2002). However, the size limitations imposed by viral vectors mean that only relatively small stop cassettes can be used, and this can lead to ‚leakiness' with expression of the opsin gene in Cre-negative cells. To overcome this problem, a flip-excision (FLEX) switch system give selective Cre-mediated opsin expression without requiring the use of stop cassettes(Atasoy et al., 2008). This system is also named as double-floxed inverse open reading frame (DIO) (Sohal et al., 2009). In brief, an inverted version of the opsin gene is flanked by two incompatible loxP variants. Thus, the opsin with a DiO promotor can be expressed more precisely in different classes of neurons. This FLEX/DiO and Cre-Lox system makes optogenetics a powerful tool for genetic dissection of brain function, which is very useful in epilepsy research. Recently, Gradinaru et al used a dual-virus approach in which one virus expresses WGA-Cre, a fusion between the Cre and the transcellular tracer protein wheat germ agglutinin (WGA), while the other expresses an optogenetic opsin under the control of a FLEX/DIO cassette (Gradinaru et al., 2010). This system can activate the transcription of the tool of interest only in neurons of region B projecting to region A, which results in tissue-topologic control of optogenetic delivery.

Another example of selective expression based on neuronal activation couples activity-dependent opsin expression to the c-fos promoter, an immediate early gene often used as a marker of recent neuronal activity, to the tetracycline transactivator (tTA), a key component of the doxycycline (Dox) system, for inducible expression in a gene of interest (Liu et al., 2012). The presence of Dox inhibits c-fos-promoter-driven tTA from binding to its target tetracycline-responsive element (TRE) site, which in turn prevents it from driving opsin

expression. In the absence of Dox, training-induced neuronal activity selectively labels active c-fos-expressing neurons with opsin, which can then be reactivated by light stimulation during testing. Overall, it is critical to select opsins with specificity, efficacy, and selectivity for successful optogenetic experiments (Paz and Huguenard, 2015).

Methods of illumination

The final key component for successful optogenetic experiments is precise spatiotemporal wavelength-specific illumination. Common scientific light sources are arc lamp, laser, and light-emitting diode (LED). Arc lamp provides continuous bright light across visible wavelengths but has slow shutter control. Laser provides a high power collimated light but is more expensive and requires a complex shutter to control the timing of illumination. LEDs are cheaper, smaller, more reliable, and can be rapidly switched on and off. A high-power LED microarray can generate arbitrary optical excitation patterns on a neuronal sample with micrometer and millisecond resolution (Nir et al., 2010). For *in vivo* experiments, head-mounted LEDs offer a simple way of delivering light to the surface of the brain in unrestrained animals. One of disadvantages of LEDs is that the light is emitted in all directions, rather than in a coherent beam. Compared to the laser, the coupling efficiency from an LED is lower. Illuminating deep brain areas requires the use of light guides such as optical fibers. Fiberoptic light delivery can be implemented easily in freely behaving animals for photostimulation (Yizhar et al., 2011b). Fast excitation, inhibition and bistable optical control can be done both *in vitro* and *in vivo* using two-photon laser-scanning microscopy (Prakash et al., 2012; Rickgauer and Tank, 2009). Combined with functional imaging, optogenetic photoactivation provides full optical control of signal transmission in the brain. (Anselmi et al., 2011b). Moreover, spatial light modulation, DLP projection, digital holography, acousto-optic deflection and galvanometric scanning can be applied to generate specific light patterning stimulation (Anselmi et al., 2011a; Guo et al., 2009; Nikolenko et al., 2008; Wang et al., 2011).

Use in therapy for neurodegenerative disorders

The potential to use optogenetics as therapeutic tools for neurological disorders has been investigated since its early development. The expression of ChR2 in the basal ganglia or motor thalamus circuitry in a mouse model of Parkinson's disease (PD) was used to modulate the direct-pathway circuitry as an effective therapeutic strategy for ameliorating Parkinsonian motor deficits (Kravitz et al., 2010; Seeger-Armbruster et al., 2015). Alzheimer's Disease (AD), the most common form of memory loss in humans, has been approached with optogenetics using a photoactivatable Rac protein that might help prevent the loss of synapses and improve electrical impulse conduction in animal models (Zahedi et al., 2013).

Strategies for controlling epilepsy

Epilepsy is another chronic neurological disorder for which optogenetics may hold great promise. Based on their actions, current antiepileptic drugs are conveniently categorized into those that (1) modulate voltage-gated ion channels (including sodium, calcium and potassium channels); (2) enhance synaptic inhibition; and (3) inhibit of synaptic excitation

(Rogawski and Loscher, 2004). Current optogenetic approaches focus on either inhibition of synaptic excitation or enhancement of synaptic inhibition.

A. Inhibiting pyramidal cells—The formation of new recurrent excitatory circuits after brain injuries is a major contributor to epileptogenesis. Pyramidal neurons are the primary excitation unit in forebrain structures such as the cerebral cortex, hippocampus, and amygdala. Enhanced excitation of pyramidal neurons had been found in chronically injured epileptogenic neocortex (Jin et al., 2006). One strategy of optogenetic therapy is to use the CaMKII IIα promoter linked to an inhibitory optogenetic opsin such as NpHR3.0. After transfection, the CaMKII IIα promoter will restrict expression only to pyramidal cells. Photostimulation of those cells will induce inhibition of excitatory neurons, which can reduce epileptic activity. Using lentivirus containing CaMKII IIα promoter NpHR and both field and whole-cell patch clamp recording in hippocampal organotypic slice cultures, Tonnesen et al. found that light-induced inhibition of pyramidal neurons expressing NpHR was effective in attenuating stimulation train-induced bursting (Tonnesen et al., 2009) (Fig. 3A). *In vivo* results have shown some effect as well in both hippocampal and neocortical models. In an *in vivo* temporal lobe epilepsy (TLE) model induced by lithium-pilocarpine injection, Sukhotinsky et al. used eNpHR3.0 under the control of a CaMKIIα were able to delay the onset of seizures by 6 minutes compared with controls and reduced high gamma activity (a biomarker for excitatory activity). In another *in vivo* TLE model induced by kainic acid injection in which the investigators crossed a CamKIIα promoter Cre mouse with an inhibitory opsin NpHR Cre dependent mouse, 57% of seizures were stopped within 1 s of light delivery and seizure duration was decreased by 70% (Krook-Magnuson et al., 2013). In a tetanus toxin focal injection model of neocortical epilepsy Wykes et al. used an NpHR 2.0 lentivirus under a CaMKII IIα promoter (Wykes et al., 2012). Wireless recording and automated seizure detection were used for quantitative assessment of epileptogenesis and a 20-s on/20-s off duty cycle of 561 nm laser was used for photostimulation. The behavior of the animals was not visibly affected. The authors report a statistically significant decrease in high frequency activity and a decrease in automatically detected events, however, careful inspection of their data show that reductions were not dramatic. Animals still had large number of events detected as well as persistence of high frequency activity. These data indicate potential utility of optogenetics for neocortical epilepsy but indicate that much work is still needed to improve the strategy.

Although halorhodposins (light-driven inward Cl^- pump) and archeorhodopsins (a light-driven outward H+ pump) are both effective neuronal silencers, Raimondo et al found that they differed in their effect on GABA inhibitory transmission beyond the light-activation period (Raimondo et al., 2012). The eNpHR3.0 activation increased spiking probability in response to a volley of presynaptic action potentials, while Arch activation did not. The use of eNpHR3.0 can significantly affect GABAergic synaptic transmission during and after prolonged tissue illumination. This post-hyperpolarization effect most probably came from a collapse in the Cl^- transmembrane gradient which caused a depolarizing shift of the reversal potential of Cl^-. The rate of recovery of ECl- after eNpHR3.0 activation had a time constant of around 15 s. Although there were difference in cell types or variation in experimental

design with other studies (Ferenczi and Deisseroth, 2012), this potential drawback of halorhodposin parameter must be considered when using those silencing strategies.

B. Exciting inhibitory interneurons (or sub populations of interneurons)— Inhibitory interneurons play an important role in epilepsy in limiting propagation as well as initiation(de Lanerolle et al., 1989). Another optogenetic strategy for modulating epileptiform activity and preventing seizure initiation is to enhance inhibitory interneurons by targeting depolarizing actuators. Using excitatory opsins such as ChR2 or C1V1 with different promoters, this strategy can be implemented by targeting either all interneurons as a group γ-Aminobutyric acid (GABA), or selectively targeting interneuron subpopulations such as parvalbumin (PV), cholecystokinin (CCK), NPY (neuropeptide Y), and somatostatin (SST). Those subclasses of interneurons have different functional connectivity to the principal neurons. For example, SST-expressing cells, target dendritic domains, whereas others (e.g., PV-expressing), target perisomatic compartments, with different functional outcome for action potential generation in principal cells (Freund and Buzsáki, 1996) (Lovett-Barron et al., 2012). Transgenic mouse lines containing a GFP gene under the control of a glutamic acid decarboxylase (GAD) promoter show restricted expression in different subsets of inhibitory interneurons rather than ubiquitous expression in all GABAergic interneurons (Chattopadhyaya et al., 2004). On the other hand, the FLEX/DiO promotor can bring opsins to the Cre-expressing only cell and most of sub interneuron-Cre mice are available now. This combination makes it possible to selectively excite subclasses of interneurons. Selective photoactivation of either PV interneurons or SST interneurons can selectively enhance peri-somatic (basket cells) or peri-dendritic inhibition.

In vitro studies of this strategy have shown some efficacy. Ledri et al. used Mg-free and 4-aminopyridine (4-AP) perfusion to elicit epileptiform bursts in slice (Ledri et al., 2014). The Cre dependent ChR2 AAVDIO viral vector was injected into mouse hippocampus. They first targeted multiple populations of interneurons by using a Gad2-Cre mouse and then subpopulations with either a PV-Cre or SST-Cre mouse. Whereas optogenetic excitation of large “mixed” populations of interneurons was effective at reducing epileptiform events by 70-82%, the effects of selectively activating only PV or SST interneurons alone was less effective. Moreover, by altering the stimulation frequency and duration they concluded simply that the magnitude of the effect depends on the amount of GABA released by the light. However, the fact that the model was an acute pharmacologic model and was performed in slice limits the impact of the conclusion.

In vivo data support the slice findings. Using the kainic acid, hippocampal model *in vivo*, Krook-Magnuson et al. crossed Cre-dependent excitatory opsin ChR2 mice with PV-Cre mice to generate PV-ChR2 mice. After blue light delivery to the hippocampus by a closed-loop system they found that excitation of inhibitory PV-containing GABAergic cells stopped 59% of seizures within 5 s and decreased seizure duration by 43% (Krook-Magnuson et al., 2013) (Fig. 3B). Interestingly, illumination of the hippocampus contralateral to the seizure focus stopped 58% of seizures within 5 s and reduced duration by 37%. However, in this model a large percentage of seizures stop spontaneously within this time frame rendering the results slightly less impressive.

Due to the promoter complexities and the size limitations in AAV and Lentiviruses, current available short promoters in viral vector system drive selective expression in inhibitory neurons without a subtype specific manner (Nathanson et al., 2009). The exciting inhibitory interneurons has to combine a Cre-dependent viral construct like AAV-FLEX or AAV-DIO with the use of transgenic Cre recombinase driver mice. This limits the use of ChR2 for further clinical applications.

C. Controlling both types of cells—Another optogenetic strategy is to try and influence both excitatory and inhibitory cells simultaneously. This approach can take any one of a number of forms. However, experimentally, what has been attempted was to use a ubiquitous promoter such as Synapsin linked to an inhibitory opsin that can hyperpolarize all cell types. Recently, Berglind et al. used the eNpHR3.0 vector and the human Synapsin (hSyn) promotor. The pan-neuronal hSyn promoter drove eNpHR3.0 expression in both excitatory and inhibitor neurons. Firstly, *in vitro* studies showed that kainite injected animals whose hippocampal slices were bathed in Mg-free ACSF with picrotoxin (PTX) and 4-AP and found an 80% reduction in epileptic bursting. *In vivo* studies, however, were less impressive showing a reduction in bicuculline (BMI) induced spikes by only 20% (Berglind et al., 2014). One explanation may be that NpHR3.0 is also expressed in interneurons and induces interneuron hyperpolarization and thus reduces GABA release with yellow light exposure. This may not be desirable in clinical applications. Another strategy which has not yet been pursued would be to simultaneously excite interneurons and inhibit pyramidal cells. Further experiments are clearly needed.

D. Targeting other components of the epileptic network—Rather than directly modulating the seizure focus itself, which may comprise a large and diffuse network, optogenetics has been utilized to modulate seizures by targeting other areas of the brain that may interact with the seizure network. One such target is the thalamus. In a cortical stroke seizure model induced by photothrombosis of Rose Bengal dye, Paz at al inject the eNpHR3.0 virus under the CamKIIα promotor into thalamus. Both *in vitro* thalamic slice and freely behaving chronic recording were performed. A programmable real-time digital signal processor was used for real-time seizure detection and disruption. Using this close-loop system (see below), the 594-nm yellow light illumination through a multisite optrode interrupted ongoing electrographic epileptic activities in thalamus and cortex, as well as the behavioral seizure (Paz et al., 2013) (Fig 3C). Furthermore, they also found that the laser light at the intensity used to silence the seizures did not affect normal physiological rhythms in control non-injured animals or the interictal EEG. However, while the authors show examples of complete termination of some seizures it is unclear if this occurred in all seizures or only some seizures. Moreover, the power of the EEG was only slightly diminished during ictal illumination.

Another potential target is the cerebellum. Krook-Magnuson et al. demonstrated modest reduction in seizure duration of 33% and no effect on inter-seizure interval with illumination of the cerebellar hemisphere in a mouse with ChR2 in parvalbumin-expressing cells using the intrahippocampal kainite injection model (Krook-Magnuson et al., 2014). Illumination of the midline cerebellum was more effective at delaying seizures, with a 175% increase in

time to next seizure. Opsin expression restricted to Purkinje neurons, the output of the cerebellum, was generated by crossing the Pcp2 (Purkinje cell protein 2) gene mice with ChR2 opsin mouse lines. With this technique and midline illumination moderate decreases in seizure duration and increases in time to next seizure were achieved.

In addition to the key role played by pyramidal cells and interneurons in epileptogenesis, glial cells especially astrocytes and microglia, also play an important role in the pathophysiology of epilepsy (Devinsky et al., 2013), which may comprise an important target for photostimulation (Ji and Wang, 2015). AAV serotype 2/5 has glial tropism. A promoter of the GFAP gene has been used for many years to target astrocytes but has a low transcriptional activity. A new viral vector based on transcriptional amplification strategy to enhanced the activity of compact glial fibrillary acidic protein (GfaABC1D) promoter, which has the same level of specificity as the longer versions of GFAP promoter, has been developed along with an optogenetic opsin (Gourine et al., 2010). Studies in glia have not been attempted but will hopefully be forthcoming.

Closed-loop optogenetic system

Targeted, time-sensitive abortive therapy requires a method of automated seizure detection and prediction to trigger the therapeutic intervention. Although constant optogenetic illumination is feasible, such therapy would likely interrupt normal cortical processing that may occur in the pathological network. Cortical stimulation is a similar time-sensitive method, which has been currently implemented into clinical use. In 2013, the U.S. Food and Drug Administration (FDA) first approved such a RNS® deep brain stimulation system from NeuroPace to treat epilepsy.

A detailed modular protocol for the establishment of a closed-loop optogenetic experiment was recently described and has been used in several *in vivo* animal experiments (Fig. 4) (Armstrong et al., 2013). In support of this theory, a model-based analysis of open loop optogenetic control in a meso scale model of the human cortex has suggested that incorporating a feedback loop would make the optogenetic method more effective at inhibiting epileptiform activity because control could be applied only when seizure waves were detected (Selvaraj et al., 2014).

Initiating and probing the epileptic network optogenetically

Animal models of epilepsy are very important not only for understanding the fundamental mechanism of epilepsy but also for testing the efficacy of new antiepileptic drugs or other therapeutic interventions (Löscher, 2011). Electrical stimulation and chemicals have been used to elicit epilepsy in animal models for many years. These techniques have drawbacks. Electrical stimulation generates large artifacts which interferes with electrical recording and it is impossible to stimulate specific subclasses of neurons (Tye and Deisseroth, 2012). Chemical injections into the brain lack spatial-temporal precision. Optogenetic methods overcome the above disadvantages. However, there are only a few studies using optogenetics to trigger seizures. In 2013, Osawa et al. induced focal seizure-like afterdischarges in rat hippocampus dentate granule cells using ChR2 opsin (Osawa et al., 2013). Repetitive pulse or continuous photostimuli were delivered to CA3-DG expressing

the excitatory opsin by either transgenic ChR2 rat under Thy promoter or via injection of ChR2 virus under a nonspecific hybrid cytomegalovirus enhancer/chicken beta-actin ‚CAG' promoter. Afterdischarge induction was confirmed in both acute electrophysiology and behavioral seizures. Stimulation frequency of 10-20 Hz appeared to be an important factor at increasing efficacy.

Wagner et al. used the an AAV virus encording C1V1(T/T) under the CaMKIIα promoter to infect WAG/Rij rat neocortex excitatory neurons and studied the spatiotemporal dynamics of optogenetically-induced and spontaneous seizure transitions by microelectrode array (Wagner et al., 2014). The WAG/Rij rat is a model of absence epilepsy with a cortical focus in the somatosensory cortex. They found that local neocortical rhythmic bursting at particular frequencies around 10Hz, under susceptible ongoing brain states, was sufficient to trigger primary generalized seizures. The probability of inducing seizures consisting of self-sustained spike-wave discharges (SWDs) was frequency-dependent, reaching a maximum at 10 Hz.

A critical role of interneurons at triggering both ictal and inter-ictal events has been suggested. The entorhinal cortex contains both PV- and SST- expressing interneuron. In an adult mouse medial entorhinal cortical slice model, it was shown that optogenetic triggering of action potential firing either in PV or SST interneurons caused a robust $GABA_A$-receptor mediated inhibition in pyramidal cells. During perfusion with 4-AP, brief photostimulation (300 ms) activating either PV or SST interneurons in ChR2 transgenic mice induced patterns of epileptiform activity. Interneuron photostimulation, however, even with prolonged flashes lasting several seconds, was ineffective in blocking ongoing seizure-like events. These results suggest that entorhinal PV and SST interneurons are nearly equally effective in triggering interictal and ictal discharges (Yekhlef et al., 2014).

Medial ganglionic eminence (MGE) cells are the major progenitors for GABAergic interneurons. Embryonic MGE cell transplants have been shown to have anti-epileptogenesis properties (Baraban et al., 2009; De la Cruz et al., 2011). Henderson et al. used optogenetic activation of transplanted MGE cells expressing ChR2 to demonstrate robust hyperpolarizations in granule cells (Henderson et al., 2014). This enhanced synaptic inhibition in local neural circuits may be one mechanism for the anti-epileptogenesis effect MGE transplants. Ellender et al. also used optogenetic techniques to selectively control PV interneuron activity during ongoing seizures activity with ChR2-mediated activation and Arch-mediated silencing. ChR2 or Arch AAV virus were injected into the hippocampus of PV-Cre mice. Epileptiform activity was induced in organotypic hippocampal slices using four different models: (1) 0 Mg^{2+}; (2) 0 Cl^{-}; (3) 4-AP; and (4) spontaneous (slice culture maintained for >2 weeks). Short, 1 ms blue laser light pulses delivered to the stratum pyramidale of the slice generated single action potentials in fast-spiking PV interneurons. ChR-2-mediated activation of PV interneurons during the clonic phase generated excitatory GABAergic responses in pyramidal neurons. Inhibiting PV interneurons by Arch-mediated photostimulation during epileptiform activity reduced afterdischarges. This study suggested that activity-dependent Cl^{-} accumulation subverts the actions of PV interneurons to perpetuate rather than terminate pathological network hyperexcitability during the clonic phase of seizures (Ellender et al., 2014).

Another novel use of optogenetics is to explore the role of interictal spikes in cognitive processing. Using a cultured neuronal network and random dot blue laser photostimulation delivered to the multielectrode array dish through a reflective spatial light modulator microdisplay, Dranias et al. modeled the effect of interictal spikes on cortical processing (Dranias et al., 2015). Neurons were transfected with plasmid DNA encoding ChR2 using electroporation. IISs were triggered during encoding, delay and readout phases and the found that regardless of which phase the synchronized network burst occurs, stimulus-specific information was impaired.

The impact of optogenetics on epilepsy has exploded in recent years and the pace of evolution is rapid. While early optogenetic approaches in epilepsy were done *in vitro* with a lentiviral vector opsin (Tonnesen et al., 2009), more recent studies have been performed in *in vivo* including freely moving rodent epilepsy models (Paz et al., 2013; Wykes et al., 2012). The use of AAV vectors has increased. Most studies have concentrated on hippocampal epilepsy rather than neocortical epilepsy. A summary of the more impactful publications in the field are presented in Table 1.

Challenges in epilepsy research

Several outstanding questions loom large over the field of optogenetics with respect to the impact it will have on epilepsy research. Unanswered questions abound, many of which are fundamental questions that are critical to frame and address in the next few years to allow optogenetics to evolve into clinically relevant treatments.

A. Photostimulation protocol—Optimizing illumination parameters are the key for optogenetic control. The light intensity and light transmission through brain tissue have been well studied in both experimental measurements and theoretical calculations (Adamantidis et al., 2007; Aravanis et al., 2007; Huber et al., 2008). However, the spatiotemporal stimulation parameters are still not fully understood in optogenetic epilepsy research. For how long should photostimulation occur? Is continuous illumination better than an intermittent stimulation? What is the ideal frequency of illumination? As with electrical brain stimulation, the duration and frequency of stimulation are important parameters in order to deliver efficient modulation. Alternatively, frequency and duration of illumination may have little impact on the ultimate result. For example, in cultured ChR2 hippocampal neurons, low frequency photo-stimulation protocols are sufficient to induce potentiation of network bursting, modify bursting dynamics, and increase interneuron synchronization (El Hady et al., 2013). However, in both *in vivo* and *in vitro* experiments, Chiang et al. compared the difference between optical stimulation with pulse trains at 20 and 50 Hz in Thy1-ChR2 transgenic mice and found little difference. They found that high frequency stimulation was able to suppress 82.4% of 4-AP induced seizures at 50 Hz with light power of 6.1 mW compared with a similar 80.2% seizure suppression at 20 Hz with light power of 2.0 mW (Chiang et al., 2014).

As another example, Sukhotinsky et al. showed inhibition of hippocampal eNpHR-expressing excitatory pyramidal neurons in behaving rats in a lithium-pilocarpine acute status epilepticus model with intermittent illumination for 1–2 minutes at either 1 or 3 min

intervals. Again little difference was identified. They found that both modes of illumination delayed electrographic and behavioral initiation of status epilepticus similarly and equally altered the dynamics of ictal activity development (Sukhotinsky et al., 2013).

Parameters of illumination may also be less important than the chemistry of the opsin. For example, constant activation of ChR2 requires lengthy illumination of tissue with millisecond pulses, which may lead to damage due to overheating. A class of step-function opsins (SFOs) has been developed in which a single pulse of blue light can turn on and maintain their activities for up to a minute even after the light has been switched off. By contrast, other variants of ChR2 such as the ChETA family and ChIEF allow neurons to be triggered to fire at higher frequencies than is possible with ChR2.

B. Human implementation—Another question is how to deliver opsins as well as light in humans. Optogenetics has been used in different species including mouse, rat, zebrafish, and fly. Recently, optogenetic-induced changes in behavior in nonhuman primates were reported (Han et al., 2011; Ruiz et al., 2013). As a therapeutic tool in human epilepsy, there are a number of problems that must be overcome before optogenetic therapeutic techniques become a reality (Chow and Boyden, 2013). This includes the safe and effective opsin gene delivery, implantable or external light-delivery device, and reliable seizure detection algorithms. Optogenetic therapeutics will require the delivery of genetic constructs into the body by means of a viral vector, but this technology is far from fully established in humans. Although there no FDA approved viral gene therapies, several recent AAV phase II and path II clinical trials have successfully transduced cells in the central nervous system. AAV-based gene delivery is a promising gene therapy for human disease. There are three general approaches of AAV delivery (Samulski and Muzyczka, 2014). The first approach is to use a depot organ such as liver or muscle to effect the production and secretion of a protein. In a second approach, systemic (intravenous) injection is used to treat diseases that affect all cells, such as lysosome storage diseases. A major challenge for systemic delivery in epilepsy has been identifying vectors that are capable of crossing the blood-brain barrier. The third approach is surgical injection into a specific, diseased brain region including convection-enhance delivery or intra-venous with mannitol to break down the blood brain barrier (Sanftner et al., 2005). Intravenous (IV) administration of a viral vector delivery is comparably less invasive. Microbubble-facilitated focused ultrasound (FUS) has been used to locally and temporally disrupt the BBB (Hynynen et al., 2005). Contrast-enhanced magnetic resonance imaging (CE-MRI) can be used to observe, monitor and guide the distribution of BBB-opened regions. A recent study showed successful AAV gene delivery and stable production in mice using AAV systemic injection coupled with MRI-guided transcranial focused ultrasound(Hsu et al., 2013; Thévenot et al., 2012).

For the potential of human therapeutics, red-shifted light-sensitive opsins would be useful for noninvasive optogenetic inhibition because red and near infrared light penetrate further in tissue(Tromberg et al., 2000). . A new red-shifted cruxhalorhodopsin, Jaws, derived from *Haloarcula (Halobacterium) salinarum* (strain Shark) has displayed sensitivity in the red wavelength where light penetrates further, even through the skull. Using Jaws, illumination could be done transcranially (Chuong et al., 2014).

C. Real-time detection of epileptic activity—Seizures rarely occur with any sort of reliable periodicity. Hence, successful optogenetic therapy will likely require a close-loop device with reliable seizure prediction algorithms. Although it may be possible to abort a seizures once it begins, ideal therapy will likely require the ability to prevent the seizure from occurring in the first place (Berényi et al., 2012). As already demonstrated, the mechanics of a closed-loop device have already been described (Krook-Magnuson et al., 2013; Paz et al., 2013). However, the success of such a device is only as good as the seizure prediction algorithm. The current system mostly uses parameters of the EEG or LFP signal such as amplitude, frequency, or power as markers to predict seizures. Complex non-linear mathematic algorithms have been used to predict onset with increasing reliability (Feldwisch-Drentrup et al., 2011; Litt and Echauz, 2002). Recently, a new class of ‚microseizures' has been identified in human epileptic tissue (Schevon et al., 2008; Stead et al., 2010). Those microseizures that occur within extremely small neuronal networks play an important role in the initiation of epileptic seizures(Kramer et al., 2010). Focal hemodynamic changes or blood vessel constriction have been found before electrophysiological seizure onset in human lesional neocortical case and in animal models (Zhao et al., 2011; Zhao et al., 2007). The latter provides an optical method for a non-invasive measurement, which can avoid the brain damage caused by implanted electrodes (Cox et al., 2010). An implanted optical grid with a combination of intrinsic imaging and LED stimulation would be possible to apply in human patients as an implant not unlike the NeuroPace. A flexible organic light-emitting diode (OLED) has already been tested to provide blue light photostimulation (Smith et al., 2014).

Future Perspectives

It has only been 10 years since optogenetics were introduced into the field of neuroscience. Despite enormous progress in the genetic toolbox and optical hardware, the application of optogenetics as a therapy for epilepsy has been limited. Although preliminary results are promising, seizure control has only been moderate.

Besides therapeutic avenues, optogenetics has potential for applications to understand basic mechanisms of epileptogenesis and optical mapping of seizures onsets and propagation. Voltage and calcium dyes have been recently used in epilepsy mapping (Ma et al., 2014; Ma et al., 2009). Genetically encoded voltage, calcium, chloride and pH indicators will further improve our ability to map seizure events. Glutamatergic mechanisms play an important role in the transition from inter-ictal to ictal state (Huberfeld et al., 2011). An intensity-based glutamate-sensing fluorescent reporter (iGluSnFR) has recently been developed to visualize glutamate neurotransmission *in vivo* (Marvin et al., 2013). This optogenetic reporter will be a useful tool not only for mapping seizure activity but also for possibly finding novel pre-ictal changes that can be used for seizure prediction.

Advances in genetic manipulation will be an important aspect of optogenetic development. One new genetic technology, called clustered regularly interspaced short palindromic repeats (CRISPR)/Cas 9system, is heralding a new revolution in gene-editing, targeting and regulation(Cong et al., 2013; Mali et al., 2013). The CRISPR-Cas9 method can manipulate the genome of neurons in adult mouse brain (Swiech et al., 2015). Current optogenetic

techniques are limited by manipulating a single gene one time. CRISPRs have been used to cut as many as five genes at once with reversible knock-outs and activations. Combining this genome editing technique with multiple opsins, will allow multicolor optogenetic controlling and probing of the epileptic network. Future developments will usher in a revolution in epilepsy treatment possibilities.

Acknowledgments

This work was supported by the NINDS RO1 NS49482 (T.H.S), the AES seed grant (T.H.S), the Clinical and Translational Science Center (CTSC) Grant UL1 RR 024996 Pilot Grant (M.Z), and the Cornell University Ithaca-WCMC seed grant (M.Z.). We thank Drs. MG Kaplitt, C Liston, and Y Liou for helpful input.

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Highlights

- Optogenetics toolbox have already been shown to be somewhat effective at treating seizures in animal models of epilepsy.
- Outline the most recent advances in epilepsy research with optogenetic techniques
- Discuss how this technology can contribute to our understanding and treatment of epilepsy in the future.

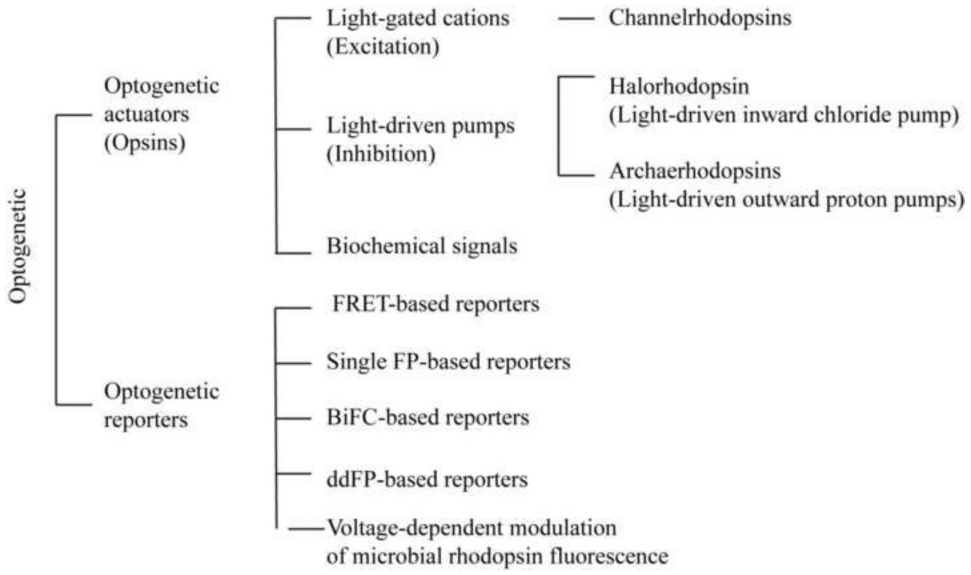

Figure 1.

Optogenetic tools. FRET: Förster (Fluorescence) resonance energy transfer. FPs: fluorescence proteins. BiFC: Bimolecular fluorescence complementation.

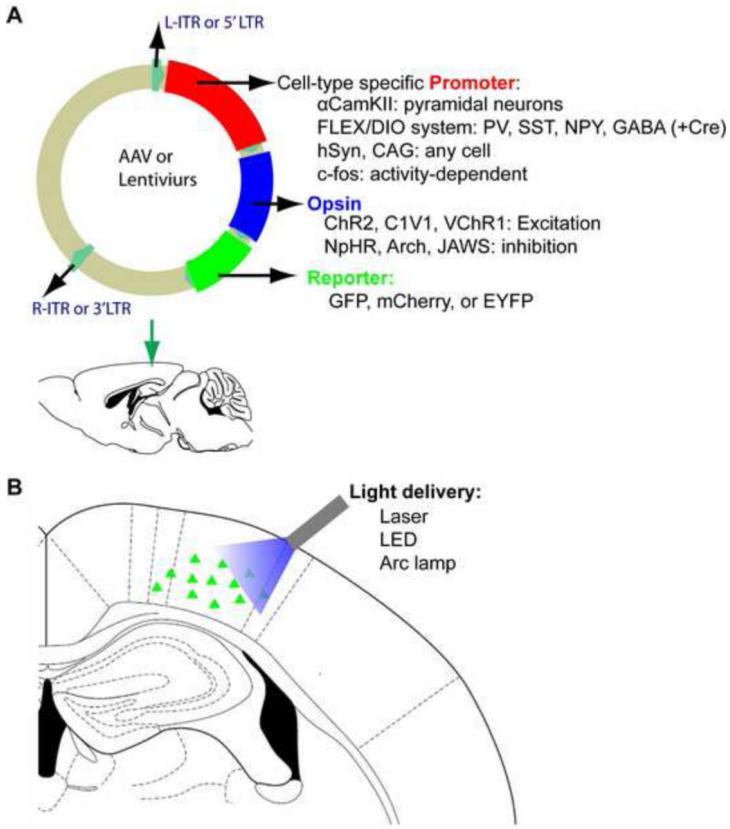

Figure 2.

Targeting optogenetic tools using viral injection. (A) AAVs or lentivirus can be directly injected into cortical and subcortical regions. A typical vector is constructed with a promoter, a opsin, and a reporter. Additional cell-type specificity is attained either by cell-type-specific promoters or via a recombinase-dependent virus, injected in a transgenic animal expressing a recombinase such as Cre in specific cells, leading to specific expression of the transgene only in defined cell types. The opsin gene is combined or fused to a reporter fluorescent protein such as GFP, mCherry, or EYFP. (B) After viral expression, photostimulation drives local excitation or inhibition using different color lights such as laser, LED, or Arc lamp. The detail strategies for spatial optogenetic targeting including local somata or/ and projection (axon) are discussed in previous review paper (Yizhar et al., 2011a).

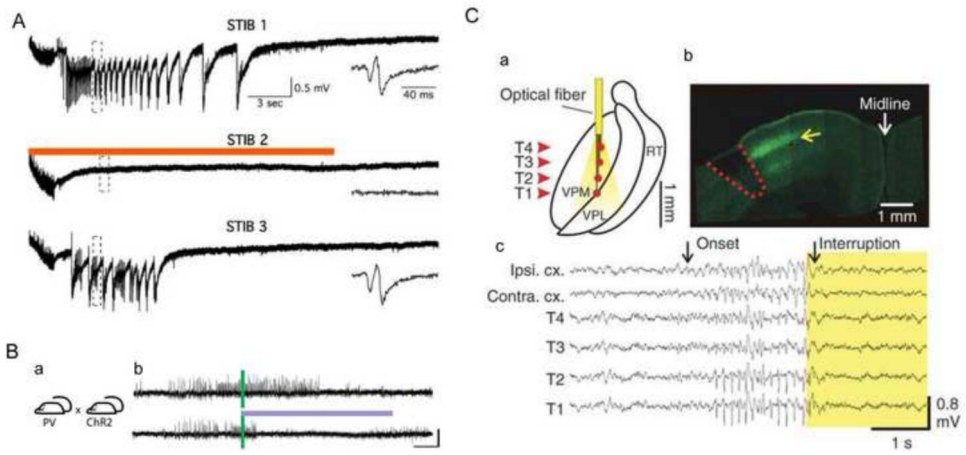

Figure 3.

Optogenetic strategies for controlling epilepsy. A. First optogenetic epilepsy experiment. "Stimulation Train Induced Bursting (STIB) in CA3 is strongly attenuated by orange-light activation of transgene NpHR in organotypic hippocampal cultures. Recordings of 3 consecutive STIB stimulations, with orange-light illumination on second stimulation, in NpHR-transduced slices. Insets: Magnification of traces showing epileptiform bursts after STIB stimulation. Scale bars apply for all traces" Adapted with permission from PNAS (Tonnesen et al., 2009). B. Ipsilateral and contralateral control of seizures in PV-ChR2 mice. (a) Crossing PV-Cre and Cre-dependent ChR2 mouse lines generated mice expressing the excitatory opsin ChR2 in PV-expressing GABAergic cells (PV-ChR2 mice). (b) Example electrographic seizures in a PV-ChR2 mouse (top, no-light) truncated by blue (473 nm) light delivery (bottom, blue line) to the hippocampus. Adapted with permission from Nat Comm (Krook-Magnuson et al., 2013). C. Selective optical inhibition of thalamocortical neurons interrupts ongoing epileptic seizures in awake, freely behaving animals. (a) Diagram of chronic multisite optrode (CMO) implanted into somatosensory thalamus for behaving recordings and optical stimulations. Arrowheads indicate thalamic recording sites (T1–4). (b) Confocal image of coronal brain section taken through the cortical lesion (red dashed line) showing eNPHR-expressing thalamocortical fibers terminating mainly in layer 4 (yellow arrow) from a rat killed after recordings. (c) Representative example of simultaneously recorded cortical EEGs and thalamic LFPs before and during 594-nm light delivery in the thalamus ipsilateral to stroke. Arrows indicate seizure onset and its interruption by light delivery in thalamus. Adapted with permission from Nature Neuroscience (Paz et al., 2013).

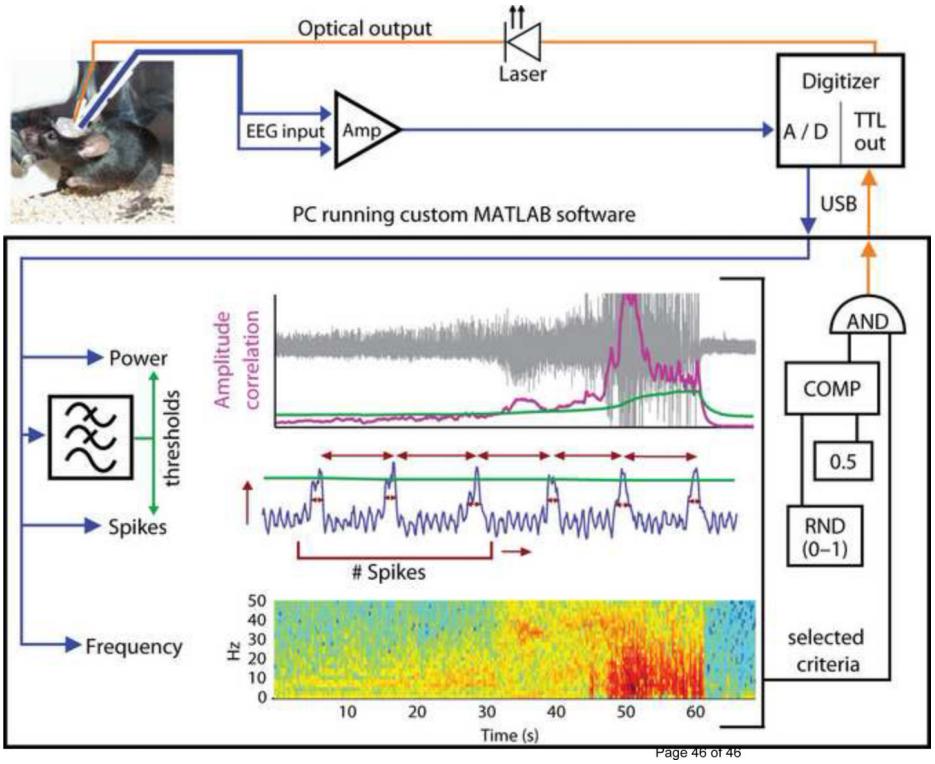

Figure 4.

Closed-loop system design. EEG input (blue) from the mouse hippocampus is amplified (Amp), digitized (A/D) and relayed to a PC running a custom-designed real-time seizure detection software. The signal is fed into a number of possible detection algorithms, which utilize features of signal power, spikes or frequency. Thresholds for power and spike properties (green) are determined using tunable leaky integrators acting as low-pass filters. Top: Amplitude Correlation (purple, during an example seizure, shown in grey); Middle: spike characteristics (for example, amplitude, rate, regularity and spike width, shown in red); Bottom: power of the signal in specific frequency bands during the same seizure, with warmer colors representing higher energy. Once a seizure has been detected using the selected criteria, for 50% of the events in a random fashion (RND), the software activates the optical output (orange) delivered to the hippocampus of the mouse, via a TTL signal from the digitizer to the laser. All trigger events, however, are flagged for later off-line analysis. COMP, digital comparator. USB, universal serial bus. Adapted with permission from ref Nature Commun (Krook-Magnuson et al., 2013).

Table 1

Selected optogenetic publications in epilepsy research by chronological order

Year	Opsin	Promotor	Virus	Light	In vitro/in vivo	Epilepsy model	Brain region	Refs
Therapeutic								
2009	NpHR	CaMKIIα	lentiviral	mercury lamp	organotypic slice cultures	STIB	hippocampus	(Tonnesen et al., 2009)
2012	NpHR 2.0	CaMKIIα	lentiviral	laser	*in vivo*	TTx injection	motor cortex	(Bernard, 2012)
2013	eNpHR3.0	CaMKIIα	AAV	laser	thalamic slice; awake, freely behaving animals	photothrombotic stroke[1]	somatosensory cortex/thalamocortical	(Paz et al., 2013)
2013	HR ChR2	CaMK-Cre PV-Cre	Cre-mice crossing	laser	Slice; *in vivo*	kainate injection	hippocampus	(Krook-Magnuson et al., 2013)
2013	eNpHR3.0	CaMKIIα	AAV	laser	*in vivo*	lithium-pilocarpine model; i.p.	hippocampus	(Sukhotinsky et al., 2013)
2014	ChR2	DIO	AAV	LED	slice	Mg2+ free / 4-AP	Hippocampus	(Ledri et al., 2014)
2014	ChR2				computational model	A meso scale model of the human cortex		(Selvaraj et al., 2014)
2014	ChR2	Thy-1	transgenic mice	laser	*in vitro* and *in vivo*	4-AP	hippocampus	(Chiang et al., 2014)
2014	ChR2/HR		transgenic	laser	*in vivo* and slice	focal hippocampal KA injection	cerebellum; hippocampus	(Krook-Magnuson et al., 2014)
Modeling & Mechanisms								
2013	ChR2	Thy-1 CAG	transgenic rat or AAV	laser	*in vivo*	seizure-like afterdischarges	hippocampus	(Osawa et al., 2013)
2014	eNpHR3.0	hSyn	AAV	mercury light	slice; *in vivo*	Mg2+-free / 4-AP/Picrotoxin; BMI	hippocampus	(Berglind et al., 2014)
2014	ChR2	VGAT	transgenic mice		slice	pilocarpine TLE/MEG graft	hippocampus	(Henderson et al., 2014)
2014	ChR2 or Arch	CAG	AAV	laser and LED	slice	1) 0 Mg^{2+}; 2) 0 Cl^{-} model; 3) 4-AP; and 4) spontaneous model.	hippocampus	(Ellender et al., 2014)
2015	ChR2		electroporation	laser/SLM	Cell culture	synchronized network bursts	cortical neuron	(Dranias et al., 2015)
2015	ChR2		Cre-mice crossing	laser	slice	Ictal; interictal/4-AP	medial entorhinal cortex	(Yekhlef et al., 2015)
2015	C1V1	CaMKIIα	AAV	laser	*in vivo*	SWD / WAG/Rij rats,	neocortex	(Wagner et al., 2014)

4- AP: 4-Aminopyridine; HR: halorhodopsin; SLM: spatial light modulator; SWD: spike-wave discharges; STIB: stimulation train-induced bursting (pharmacoresistant epilepsy); TLE: temporal lobe epilepsy; TTx: tetanus toxin;

[1] light-sensitive Rose Bengal dye