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Kokaia, Merab; Toft Sörensen, Andreas

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The treatment of neurological diseases under a new light: the importance of optogenetics

by Merab Kokaia and Andreas Toft Sørensen

Running title: Optogenetic control of neurological diseases

Merab Kokaia*, PhD, Professor, and Andreas Toft Sørensen, PhD, Experimental Epilepsy Group, Division of Neurology, Lund University Hospital, Lund, Sweden.

*Correspondence: Merab Kokaia, Experimental Epilepsy Group, Division of Neurology, Wallenberg Neuroscience Center, BMC A-11, Sölvegatan 17, Lund University Hospital, 221 84 Lund, Sweden.

Tel.: +46 (0)46 2220547

Fax: +46 (0)46 2220560

E-mail: merab.kokaia@med.lu.se

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SUMMERY

Controlling activity of defined populations of neurons without affecting other neurons in the brain is now possible by a new gene- and neuroengineering technology termed optogenetics. Derived from microbial organisms, opsin genes encoding light-activated ion channels and pumps (channelrhodopsin - ChR2; halorhodopsin - NpHR, respectively), engineered for expression in the mammalian brain, can be genetically targeted into specific neural populations using viral vectors. When exposed to light with appropriate wavelength, action potentials can be triggered in ChR2-expressing neurons, whereas inhibition of action potentials can be obtained in NpHR-expressing neurons, thus allowing for powerful control of neural activity. Optogenetics is now intensively used in laboratory animals, both *in vitro* and *in vivo*, for exploring functions of complex neural circuits and information processing in the normal brain and during various neurological conditions. The clinical perspectives of adopting

optogenetics as a novel treatment strategy for human neurological disorders have generated considerable interest, largely because of the enormous potential demonstrated in recent rodent and non-human primate studies. Restoration of dopamine-related movement dysfunction in parkinsonian animals, amelioration of blindness, and recovery of breathing after spinal cord injury are a few examples of such perspective.

INTRODUCTION

Neurological diseases can be difficult to treat by traditional pharmacotherapy or surgical interventions, such as deep brain stimulation (DBS). Treatment, cure, and prevention can be obtained in many cases. However, beneficial therapeutic effects achieved by altering brain (mal)functions are often accompanied by harmful and undesired side effects. One major problem is lack of cellular specificity and spatiotemporal targeting exerted by current therapies. More effective approaches that regulate and control specific brain regions and/or specific neural populations *when* it is required are highly desirable, but are unmet needs of current treatment strategies. With a new bio-genetic technology termed optogenetics (1), highly precise spatiotemporal control of neural activity within defined neural population can be obtained by externally applied light. Acting like a switch to turn "on" and "off" action potential activity, genetically targeted neurons encoding and expressing microbial light-sensitive transmembrane ion conductive proteins can be controlled in intact neural circuitries (Figure 1). This review article will address recent advances of optogenetics in neuroscience, mainly exemplified by the microbial light-sensitive proteins *Chlamydomonas reinhardtii* Channelrhodopsin-2 (ChR2) and

Natronomonas pharaonis Halorhodopsin (NpHR), and discuss its potential clinical application for several human neurological disorders.

OPTOGENETIC TOOLS

The field of optogenetics has advanced rapidly since 2005 when the technology was first described by a group of scientists from Stanford University headed by Karl Deisseroth (1,2). They demonstrated for the first time that neurons expressing the light-sensitive ChR2 protein can generate action potentials in a timely and precise manner when illuminated by light (2).

Channelrhodopsin-2 (ChR2)

Derived from a unicellular alga, ChR2 is a light-sensitive cation channel that is engineered for stable membrane expression and can be introduced into mammalian cells using viral vectors without perturbing the cell integrity (2). When exposed to ~470 nm blue light, ChR2-expressing neurons are depolarized by a strong and ultrafast current sufficient to induce single or multiple action potentials (Figure 2A, B). This responsiveness is precise and controllable in a high temporal manner (2). Further molecular modifications of the gene encoding the ChR2 protein have generated variants with improved functionality, including faster deactivating kinetics and long-lasting activation (3,4).

Halorhodopsin (NpHR)

NpHR is a bacteria-derived light-sensitive chloride-pump, and when activated by ~570 nm yellow light, it generates fast chloride ion influx. NpHR has also been genetically engineered for mammalian application and display similar fast

temporal control as the ChR2 cation channel. In neurons, activation of NpHR strongly hyperpolarizes the membrane whereby it can effectively suppress action potentials (5,6) (Figure 2C, D). Improved NpHR variants that are more well-tolerated and efficient for neural silencing have been created (7,8).

Since NpHR and ChR2 proteins are activated by separate wavelengths of light, illumination with proper wavelength allows independent control of ChR2 and NpHR expressing cells for either initiation or inhibition of action potential activity. Ultimately, full bi-directional control of the membrane potential within a single neurons can be obtained, either by co-expression of ChR2 and NpHR (6) or by expression of both proteins together using a single viral vector encoding both opsin genes (termed eNPAC) (7).

Light sources

Light activation of ChR2 and NpHR proteins in neural circuits can be obtained by several means. For *in vitro* settings, such as brain slices preparation and cultured cells, light sources including xenon arg lamps, LEDs and lasers can provide the proper wavelengths, whereas for *in vivo* light delivery in living animals, LEDs can be applied for illuminating superficial brain areas (9,10). For deeper brain structures, implantable laser-coupled optical fibers are reliable and efficient sources for delivering light into the parenchyma (11,12).

Expression systems for controlling neural activity

One major aspect of optogenetics is the delivery of opsin genes into the brain and transduction of neurons. Up to now, direct *in vivo* gene transfer into the brain by stereotactic injection of viral vectors is the most widely used technique. This approach

allows for introducing genes into defined cell types, such as neurons, by using cell population-specific promoters that drive transgene expression. Stable and long-term expression of opsin proteins is also achieved by this approach, and has been proven to be successful in a variety of animal species, including non-human primates (2,11,13-16). Lentiviral and adeno-associated viral (AAV) vectors are both suitable mediators for such viral gene transfer (12). Notably, the AAV viral vector is considered innocuous and non-pathogenic for normal brain physiology, as all viral genes encoding wild type viral proteins are removed to avoid viral replication, toxicity, and reduce immunogenicity (17). For these reasons, AAV is considered a safe vector for gene transfer in the central nervous system (CNS) and has been used in various clinical trials (18,19). For driving gene expression, use of vectors with neuron-preferring promoters, like calcium/calmodulin-dependent protein kinase IIalpha (CaMKIIα) (11) and human synapsin-1 (20), have so far been the primary choice for obtaining neuron-specific expression of opsin proteins, but promoters for more distinct expression in neural subtypes, such as PRSx8 (targeting non-catecholaminergic glutamatergic neurons), Hcrt (targeting hypocretin peptide producing neurons) and VGlut2 (targeting glutamatergic neurons) has also been successfully employed (15,21-23). Genetic targeting of opsins is not limited to neural cell populations in the brain. For example, ChR2 expression in astroglia for triggering Ca^{2+} influx into these cells has been shown by using a lentiviral vector carrying the glial fibrillary acidic protein (GFAP) promoter (24), thus demonstrating that expression of opsin proteins can also be successfully obtained in non-neural populations.

Other expression systems are available, but these have generally limited potential in clinical application, and are mostly useful for basic and pre-clinical research. Such approaches include, for example, generation of transgenic mice expressing ChR2 in subset of neurons (15,25-27). Alternatively, using Cre-

recombinase knock-in mice in combination with injection of Cre-activated AAV vectors encoding ChR2 or NpHR allows more highly defined gene expression in anatomically and topographically distinct population of neurons (28). With this latter approach, expression of ChR2 or NpHR has been obtained in midbrain dopaminergic neurons in ventral tegmental area (VTA) (29-31), in striato-pallidal medium spiny neurons (32), or parvalbumin (PV) interneurons in neocortex (33,34), and their neural activity has successfully been controlled by illumination with respective spectra of light.

Besides controlling neuronal action potential generation within defined cell populations, downstream manipulation of intracellular messengers such as cGMP, cAMP and IP_3 has also been demonstrated with the use of light (35). This has been possible by molecular fusion of the intracellular domain of specific G-protein coupled receptors to opsins, generating synthetic opsin-receptor chimeras (termed OptoXR) (35). With this continuous expansion of optogenetic tools in various domains of cellular functions, more efficient and extended control of a broad range of cell types and functions in complex neural systems can be envisaged in the future.

LINKING NEURAL ACTIVITY TO SPECIFIC FUNCTION

To date, optogenetic approaches have proven superior to any other technologies for selective stimulation of defined cell populations. By contrast, the traditional metal electrode has limitations for such selectivity, primarily due to its low spatial resolution and inability to selectively activate different neuronal subtypes within the electrical field generated by current stimulation. Uncaging of drugs by light stimulation (36) can overcome some of the limitations of electrical

stimulation, but lacks ultrafast dynamics of optogenetics, which operates on a millisecond timescale.

Tracing cell activity in neural circuits

Exploring functional connectivity of complex neural circuits can be done by expressing ChR2 in defined presynaptic neurons and axons in combination with whole-cell recordings of postsynaptic target neurons in slice preparations. Such *in vitro* approach has unveiled detailed mapping of long-range callosal projections (37), classified individual afferent excitatory synapses on pyramidal cells in the barrel cortex (37), explored the functional properties of the reciprocally connected thalamocortical and corticothalamic pathways (20) and determined newly established efferent connections made by adult born neurons in the dentate gyrus (38). In addition, mediators of intracellular signaling cascades involved in the regulation of synaptic plasticity in the hippocampus (39,40) and the contribution of striatal dopamine receptor D2 subtype for regulating synaptic plasticity in glutamatergic synapses in striatum (32), previously inaccessible by traditional electrophysiology (41), have been uncovered via optical stimulation of ChR2 expressing neurons.

Linking cell activity to animal behavior

Another powerful asset of optogentics is that it can establish a causal link between activity within a specific cell type to behavior in living animals. This is possible by targeting specific neurons for ChR2 expression in combination with light illumination of target areas in freely moving animals. This *in vivo* approach has, for example, identified that perceptual decisions and learning can be controlled by a subset of excitatory (ChR2-expressing) neurons in the barrel cortex of mice (10), and fast-spiking (ChR2-expressing)

parvalbumin interneurons in barrel cortex are important mediators for the generation of cortical gamma oscillations (33,34) and are central for processing of efferent sensory signaling (34). Furthermore, fear behavioral responses induced by aversive stimuli can be replicated (without an aversive stimulus) by light-activation of ChR2-expressing pyramidal cells in the lateral amygdala, thus linking associative fear learning to activity within these cells (42). Thus, by combining genetic targeting for high spatial resolution of defined cells with *in vivo* light illumination for their activation enables definition of neuronal subtypes for their participation in behavioral events.

As optogenetics also offers high temporal resolution, patterns of action potentials within single neuron subtypes driving behavioral conditions can also be explored. This is important for expanding our current understanding of causal relationships between frequency-dependent activity within defined cell types and a specific behavior. For example, alteration of dopamine signaling within the VTA is known to be centrally involved in reward behavior (including drug addiction), but how dopaminergic activity contributes to this behavior is unknown. By selectively stimulating ChR2-expressing dopaminergic neurons in VTA of living animals in a conditional place preference paradigm, only high frequency phasic firing (50 Hz), but not low frequency tonic firing (1 Hz), released sufficient dopamine levels to drive behavioral conditioning (30). Likewise, the role of hypocretin (Hcrt)-expressing neurons in the lateral hypothalamus for participating in the transition from sleep to wakefulness has been elusive. However, by genetic targeting of ChR2 and probing of Hcrt neurons in behaving animals, it was demonstrated that elevation of the action potential

frequency in Hcrt neurons led to increased probability of the transition to wakefulness (14,23).

AMELIORATION OF NEUROLOGICAL CONDITIONS

It is clear from the studies described above that optogenetics has an enormous potential for exploring the CNS and its functions in previously unprecedented ways. This technique is therefore instrumental for extending our current understanding of normal brain processing, but clearly also opens totally new opportunities for more systematic delineation of disease mechanisms. This may in some cases prove beneficial for better treatments, as described below.

Activation and recovery of breathing

Within the rostral ventrolateral medullary reticular formation, the retrotrapezoid nucleus (RTN) contains propriobulbar neurons, which are suspected to express central respiratory chemoreceptors working as generators for breathing. To selectively address these neurons and delineate their role in respiration, a cluster of non-catecholaminergic glutamatergic neurons expressing paired mesoderm homeobox protein 2B (Phox2b) were targeted in the RTN of rats using a lentiviral vector containing ChR2 (22,43). In two independent studies, light stimulation of the ChR2-transduced neurons via implanted optic fibers vigorously and repeatedly increased both phrenic nerve activation and respiratory activity (22,43), thus providing compelling evidence that these cells play a role in central respiratory chemoreception. Since no other behavioral effects were detected during light stimulation, apart from a small rise in blood pressure (22,43), these studies also demonstrate that an optogenetic

approach can be used as a strategy to enhance functional respiration. In particular, activation of the phrenic motor nucleus, either directly or via Phox2b expressing neurons in RTN, appears to be an interesting target for enhancing breathing. In line with this notion, repeated optogenetic stimulation of the phrenic motor nucleus (expressing ChR2) significantly improved the respiratory insufficiency observed after partial disruption of descending axons to respiratory motor neurons in the injured spinal cord (44). Together, these studies demonstrate that novel optogenetic strategies aimed at selectively activating neural populations within the CNS can be a viable approach to enhance breathing and improve respiratory recovery after spinal cord injuries, which lack any other viable therapy at the moment.

Suppression of seizure-like activity

Because NpHR is an effective chloride pump that can significantly hyperpolarize neurons and effectively suppress the generation of action potentials, it could theoretically be used to reduce aberrant hyperexcitation within defined neuronal networks. Gaining such powerful control of neuronal excitability would be of particular interest for controlling seizures in patients with drug resistant focal epilepsies. This concept has been explored in a study using organotypic hippocampal slice cultures, a model tissue system closely resembling pharmacoresistant epileptic brain tissue of both human and animal origin. NpHR was expressed in principal pyramidal and granule cells of the hippocampus, and upon electrical induction of epileptiform activity (i.e. stimulation train induced bursting, STIB), NpHR was simultaneously activated by yellow light illumination of the transduced slice cultures (45). During such conditions, epileptiform

activity could be repeatedly and significantly suppressed, and in some cases even completely abolished (45) (Figure 3A, B, C), thus proving a proof-of-concept for controlling seizure activity by optogenetic silencing of principal neurons.

Amelioration of parkinsonian symptoms

Parkinson's disease is a degenerative neurological disorder characterized by a progressive loss of midbrain domaminergic neurons leading to alteration of neural activity within the basal ganglia causing abnormal control and execution of movements. Besides dopamine agonist administration, DBS of the subthalamic nucleus (STN) in the basal ganglia has emerged as a highly effective method for ameliorating Parkinson's disease symptoms. Exactly how DBS exerts its therapeutic effects is unknown, since electrical stimulation indiscriminately influences a mixed cell population, in which both residing neurons and axons passing or terminating in the STN can be stimulated. However, by using the advantages of optogenetics, systematic delineation of DBS mechanisms have now been addressed in a study of freely moving hemiparkinsonian rodents, wherein amphetamine-induced rotational bias, among others, served as the behavioral test to evaluate the effect of optical stimulation of the STN (24). During light application, it was demonstrated that direct ChR2-mediated enhancement or NpHR-mediated silencing of STN neuron activity was insufficient to induce any symptomatic relief. However, during high frequency light activation of afferent axons projecting to the STN, pronounced therapeutic effects were observed. In this case, parkinsonian animals restored their normal motor behavior, a condition that could be fully reversed when terminating the light stimulation (24). This study is particularly interesting as it clearly demonstrates that by

gaining high spatiotemporal control over defined neuronal subtypes *in vivo*, while leaving other neurons unaltered, it is possible to dissect complicated disease-related mechanisms, gain new knowledge on how therapeutic action is achieved, and also reduce disease symptoms. This is an important step for translating optogenetic strategies into human clinical use.

Recovery of blindness using ChR2 and NpHR

Retinitis pigmentosa (RD) is a genetically inherited disease, characterized by a progressive degeneration of retinal photoreceptor cells, ultimately leading to blindness. At present, no effective treatment exists. However, since ChR2 and NpHR can convert light into electrical signaling, this approach has provided new strategies for restoring vision. The basic idea is to insert these light-sensitive proteins into surviving cells of the retina, those that are not totally degenerated but lack functionality, thereby making them intrinsically photosensitive capable of re-gaining neural signaling in the visual pathway. This has been attempted in several studies using blind RD rodents. Expression of ChR2 and/or NpHR in inner retinal cells (either in ganglion cells or ON bipolar cells) has been shown to produce visual-evoked potentials recorded in cortex and can facilitate visually guided behaviors (46-49). Another approach is to target cone cell bodies, which have lost their photoreceptive outer segment. Viral expression of NpHR in these light-insensitive cones has been shown to reactivate retinal circuits and visual function, and also restore visual behavior (50). Most importantly, after transduction of an AAV vector encoding NpHR in cone cells bodies of human *ex vivo* retinas, it was demonstrated that light-insensitivity of defective human photoreceptors could be restored (50). Together, these studies demonstrate a

breakthrough in strategies for restoring blindness in humans with RD, although further developments are needed to provide a more useful vision in man including generation of opsin variants that are more sensitive to normal light intensities (51) and recognize wavelengths other than blue (ChR2) and yellow (NpHR) light (52).

OPTOGENETIC SYSTEMS IN CLINICAL PERSPECTIVES

Optical deep brain stimulation

Surgical treatment with DBS can induce remarkable symptomatic relief probably by changing aberrant activity within the brain. It can alleviate symptoms in several neurological disorders such as Parkinson's disease (53), tremor (53), chronic pain (54), epilepsy (55) and major depression (56). While helpful in some patients, its applicability is still restricted partly due to variability in treatment efficiency and potential side effects. This can be related to misplacement of the electrode and/or activation of heterogeneous populations of neurons as well as axons projecting through the field of stimulation, whereby also normal physiological activity is altered (see figure 1). These issues are resolved with optogenetics as only genetically targeted cells can be selectively activated (see figure 1). If better treatment efficacy can be demonstrated in animal models with improved therapeutic outcomes, and generation of fewer side effects as compared to conventional electrical DBS, the applicability of optogenetics is definitely pertinent for clinical testing as a refined substitute for electrical stimulation.

One could think that in patients with intractable focal onset epilepsy, it would be possible to genetically target the seizure focus or key propagation

areas with different opsins by intraparenchymal viral delivery. Excitatory projection neurons could be selectively targeted with NpHR for their inhibition and/or GABAergic inhibitory interneurons could be targeted with ChR2 for increasing the inhibitory signaling. Such strategies could dampen overall excitation and even interrupt hypersynchronization of neural activity, and thereby be an effective method for inhibiting seizures and perhaps even prevent them. This could be achieved by combining a prosthetic system that delivers preemptive light to the seizure focus triggered by a seizure detection device in a close-loop system (57). Technical advancements on these matters are needed, but a recent clinical study has shown that optimized patient-specific preonset seizure detection systems can be near 100% correct (58).

Clinical strategies using optogenetics is probably not limited for CNS diseases. Therapeutic approaches towards disorders affecting the peripheral nervous system are also plausible. Selective optogenetic recruitment of muscle fibers by sciatic nerve activation has been successfully implemented in experimental animals leading to refine physiological muscle contraction without fatigue (59). In the future, this type of treatment might enable patients with paralysis or motor neuron dysfunction to regain better physiological muscle control.

Concluding remarks

Optogenetics is still a relatively new technology, but has already proven to be a highly effective tool for dissecting normal brain functions and disease mechanisms. Although the therapeutic potentials for its human clinical application are still to be proven, several encouraging studies together with the

constant development of more advanced tools for optogenetics have outlined great hopes that appear reachable in the foreseeable future.

Certainly, less invasive procedures will be more likely to enter clinical trials first. Blind patients with RD eligible for AAV vector-mediated NpHR restoration of visual function have already been identified (50). Otherwise, optogenetic DBS seems to be one of the most suitable applications for human trials. However, similar to electrical DBS employed in for example Parkinson's disease patients, it carries the risk of major surgery. Nevertheless, prosthetic systems and devices for optogenetic activation or silencing of genetically targeted neurons within deep brain structures using LED arrays are being designed and constructed for practical human use (**60**). Such a clinical setup will in addition also require opsin gene transduction of target brain tissue. In recent clinical trials using AAV vectors, evidence of both efficacy and safety has been obtained (18,19), suggesting that safe transduction systems for both ChR2 and NpHR genes for human use are already available. The question remains, however, whether bacterial proteins are safe to be permanently expressed in the human brain. This aspect of optogenetic DBS may necessitate that the superiority of its therapeutic effects, as compared to traditional DBS, are well documented before it is considered for clinical application. This needs to be addressed in future studies.

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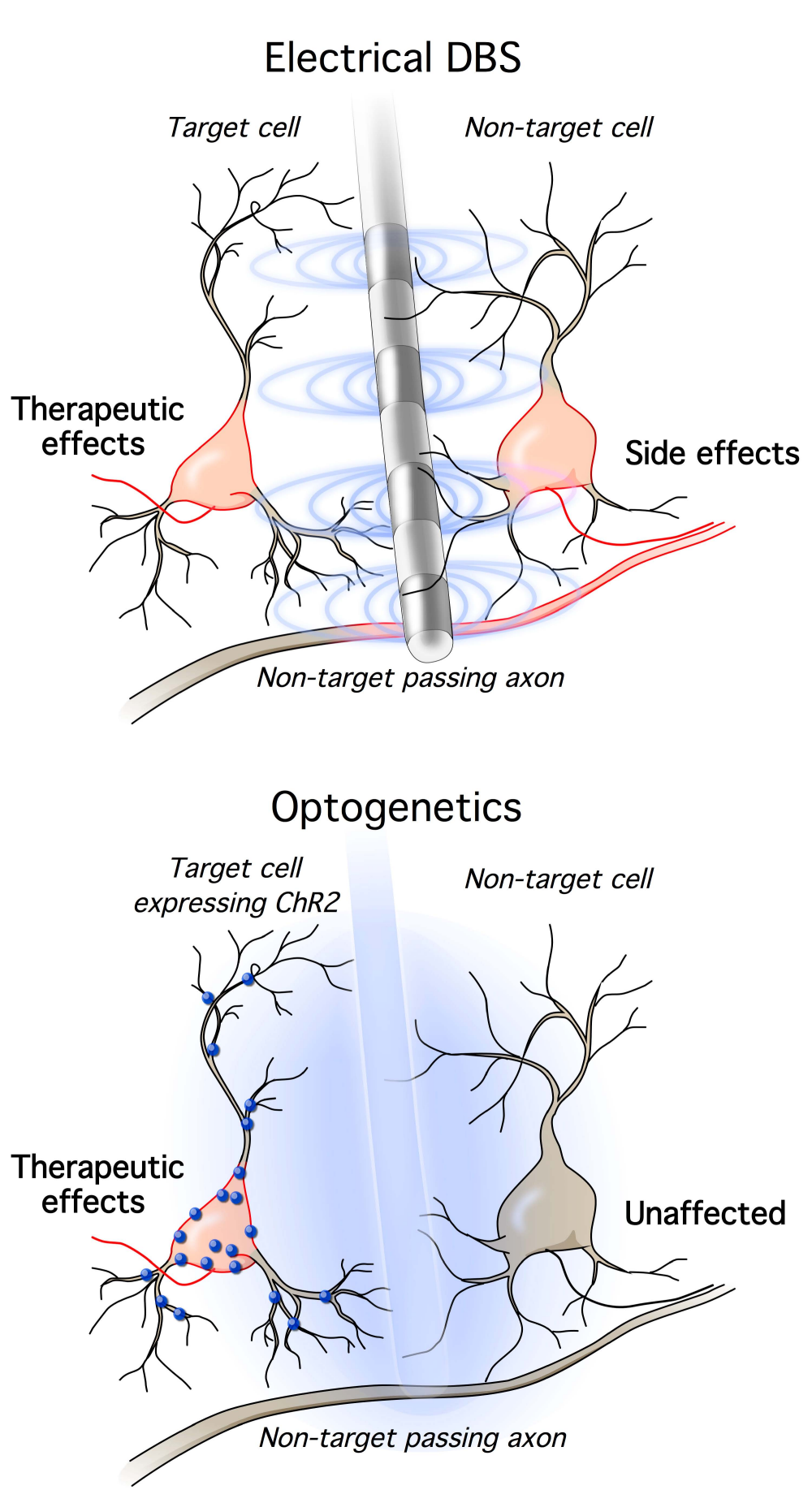
Figure Legends

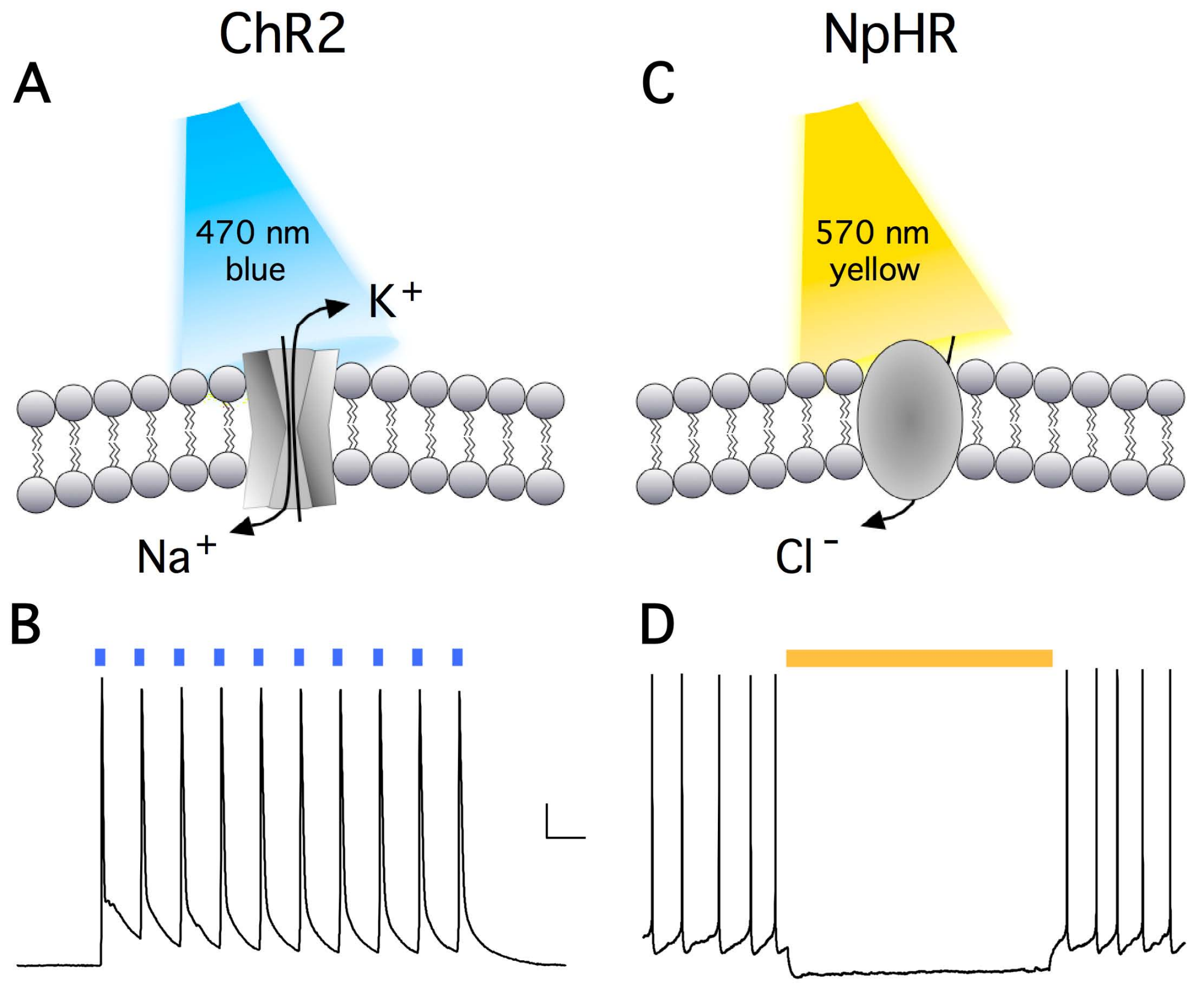
Figure 1. Optogenetic versus electrical deep brain stimulation. (*Left*) Electrical deep brain stimulation (DBS) can be used as a treatment strategy to reduce disease symptoms in various neurological disorders. Abnormal neural activity within neural circuitries can be altered to induce therapeutic effects. However, as the electrical field also influences other neurons and passing axonal fibers conveying normal physiological activity, side effects can be generated. (*Right*) With optogenetics, only neural activity of genetically targeted neurons is altered. Viral vectors carrying cell type specific promoters are employed to specifically target defined neural populations. Target cells, which for example express ChR2 (marked by blue dots), are selectively controlled externally by light for inducing therapeutic effects while other neurons and passing axonal fibers stay unaffected.

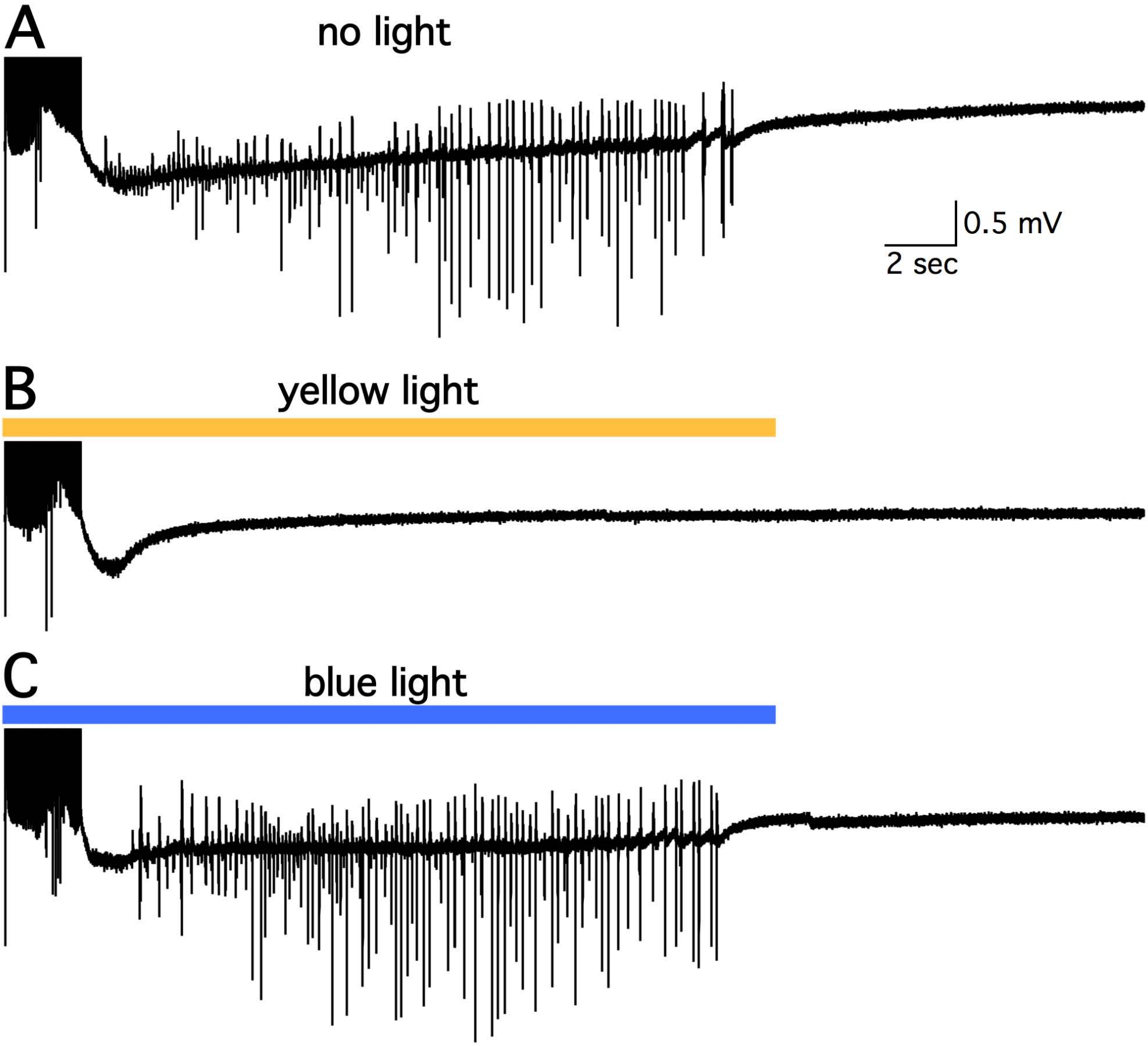
Figure 2. Optogenetic control of cellular activity. A) Channelrhodopsin-2 (ChR2) is a cation channel that can be expressed in the membrane of defined neurons. When activated by blue light (peak activation ~470 nm), cations diffuse instantly down their electrochemical gradient into the cell and the cell membrane is depolarized. B) Light-activation of ChR2 permits high temporal control of the action potentials. In this example, ten action potentials are induced by 10 pulses of 1 msec blue light (marked by blue color). The membrane potential was recorded by whole-cell patch-clamp technique from a cortical layer II/III pyramidal cell expressing ChR2 in a slice preparation. Scale bar 10 mV and 50 ms, respectively. C) Halorhodopsin (NpHR) is a light-activated chloride-pump, which hyperpolarizes the cell membrane by chloride ion influx, thus having

opposing effects of ChR2. It can be expressed in membranes of defined neural populations, and is activated by yellow light (peak activation ~570 nm). D) Activation of NpHR by yellow light can effectively inhibit the generation of action potentials. In this example, continuous action potentials are totally inhibited by yellow light illumination (marked by yellow color). The whole-cell patch-clamp recording is obtained from a hippocampal CA1 pyramidal neurons expressing NpHR. Action potentials were trigged by constant current depolarization of the membrane. Scale bar 10 mV and 300 ms, respectively.

Figure 3. Suppression of epileptiform activity by optogenetics. Organotypic hippocampal slice cultures, resembling pharmacoresistant epileptic tissue, were used as a model system to evaluate the effect of NpHR for suppressing epileptiform activity induced by electrical stimulation. NpHR was expressed in principal hippocampal neurons, i.e. granule and pyramidal cells, using a lentiviral vector with CaMKIIα promoter. A) Representative trace showing that during control conditions, i.e., without light illumination, electrical stimulation (initial dark field marks the stimulation artifacts) to CA1 stratum radiatum induces epileptiform activity as revealed by a field electrode placed in the same area. B) Illuminating the same slice with yellow light for activating NpHR (yellow bar), while applying an identical electrical stimulation as in *A,* totally inhibits the generation of epileptiform activity. C) Since NpHR is selectively activated by yellow light, no effect on epileptiform activity is observed during blue light illumination (blue bar). Scale bar applies to all traces. Modified with permission from *Proceedings of the National Academy of Sciences, PNAS* (45).


Electrical DBS
Target cell
Non-target cell
Therapeutic effects
Side effects
Non-target passing axon
Optogenetics
Target cell expressing ChR2
Non-target cell
Therapeutic effects
Unaffected
Non-target passing axon


ChR2
A
470 nm
blue
K+
Na+
NpHR
C
570 nm
yellow
Cl-
B
D


A
no light
0.5 mV
2 sec
B
yellow light
C
blue light