Optogenetics: Perspectives in Biomedical Research (Review)

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Optogenetic tools, photochromic switches and genetically encoded biosensors revolutionized contemporary neuroscience research. These approaches provided unprecedented opportunities for monitoring and modulating the function of specific neurons and have literally shed light on the mechanisms of neuronal networks function in the brain.

A number of light-sensitive biosensors for non-invasive monitoring of ions and enzymes have been developed. These molecular designs expand extremely rapidly and a number of new approaches for image analysis of various proteins in living cells have being proposed. In this review we discuss new tools for molecular imaging and remote activation of receptors, ionic channels and synaptic networks, as well as its potential for biomedical research.

Key words: optogenetics; genetically encoded sensors; Förster resonance energy transfer; FRET; photosensitive ligands; rhodopsins; optogenetic neuromodulation; optogenetic therapy.

Introduction. Rapid progress of molecular biology, fluorescent microscopy in combination with using of genetically encoding proteins significantly expanded the possibilities of experimental studies. The development of methods for the specific integration of proteins in certain cell types, as well as the detection of light-sensitive proteins, stimulated the explosion in the areas for a distant control of cellular activity with high precision and specificity.

As a result, in recent years, has been developed new areas: optogenetics, optopharmacology and optogenetic pharmacology, thanks to which with the help of light, we can investigate the function of cells, to control their activity, to measure the concentration of ions and other cellular components, to perform control of the behavior of organisms, as well as to seek for nowel ways to treat certain diseases.

Expression "optogenetics" appeared 10 years ago [1]. This has been preceded by cloning of the first photosensitive transmembrane proteins and observations that its expression in cells results in the ability of these cells to be activated by light, i.e. change the membrane potential, generate ion currents and cause light-evoked firing [2–4]. Moreover, it was found that integrating of the photosensitive proteins in neurons of multicellular organisms allows changing their behavior upon application of light [5].

Optogenetic tools and methodologies

These approaches can be divided on two main classes:

the genetically encoded sensors: tools which provide the functional analysis of cells and non-invasive estimation of intracellular ion concentrations;

genetically encoded activators and inhibitors of cells: light-sensitive molecules which provide a highly specific control of cellular activity.

A number of genetically encoded systems allowing modulation of protein activity using light have been developed in recent years. By using light-sensitive probes, it is possible to control the activity of entire populations of neurons.

The genetically encoded sensors are macromolecular protein complexes having fluorophore groups capable of selectively changing fluorescence intensity upon interaction with certain ions, specific proteins or molecular groups. These optogenetic probes combine power of molecular genetics and optical physiology. Genetically-encoded sensors of intracellular ion activity, specifically calcium (Ca^{2+}) and chloride (Cl^{-}) ions, become a powerful tool for functional analysis of neurons and neuronal circuits.

Majority of currently used optogenetic probes are almost exclusively based on the green fluorescent protein cloned from the jellyfish *Aequorea victoria* and its various

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mutants. The main approaches consist in fusing one or two fluorescent proteins (FPs) with various proteins or peptide linkers offering changing the sensitivity to different specific signals such as transmembrane potential, ions (calcium, pH, chloride, or zinc), cyclic nucleotides or neurotransmitters (glutamate, glycine or GABA).

Two main strategies are mainly used to design these probes. First, *single FP probes*: the fluorescent spectral properties of a single FP are modulated by conformational changes imposed by specific target molecule (Figure 1 (a)).

They are used to evaluate the membrane potential [6, 7], changes in the level of glutamate [8], H_2O_2 [9] or ratio of ATP/ADP in the cells [10, 11]. Unfortunately, most of these sensors do not allow ratiometric measurements, to avoid the artifacts related to the thickness of the test sample and variations fluorophore concentration. Despite this, the probes operate with remarkably high resolution [12].

Second, *FRET* (Förster resonance energy transfer) *sensors:* conformational changes are used to modify the distance or orientation of two FPs with spectral properties allowing FRET (Figure 1 (b)). On this basis biosensors for non-invasive monitoring concentration of intracellular calcium [13, 14], chloride [15–19], pH [20], ATP [21] and other components [22] has been developed.

Genetically encoded inhibitors and activators include two categories of photoactive molecules:

photosensitive ligands — for controlling of receptors or other cellular components;

photosensitive bacterial proteins — for the excitation or inhibition of cells.

To control the activity of ion channels with light, several strategies have been devised, including “caged” ligands [23], photoisomerizable ligands [24–26], and tethered photoisomerizable ligands [27–30].

“Caged” ligands. These compounds are chemically engineered molecules in which the ligands to specific receptors (i.e. acetylcholine, GABA or glutamate), is connected with an additional molecule, which prevents the interaction with the receptor.

This allows equilibration of the “caged” neurotransmitters with cell-surface receptors before light-induced releasing the neurotransmitter. Once equilibrated, the compound can be rapidly cleaved in the microsecond time scale by a pulse of UV light, thus releasing free neurotransmitter and performing extremely rapid activation of receptors (Figure 2 (a)). “Caged” compounds can also provide spatial resolution, depending on the illuminated area and the duration of illumination (Figure 2 (b)), thus allowing modeling of synaptic-like conditions [31].

With the help of these “caged” molecules can be produced also quick release of intracellular calcium ions, cAMP and other regulators or ions within the cells [32, 33]. This approach allows to test the receptor density distribution along neurons, to perform rapid activation or inhibition of postsynaptic receptors using a narrow beam of an ultraviolet laser or by using two-photon microscope [34].

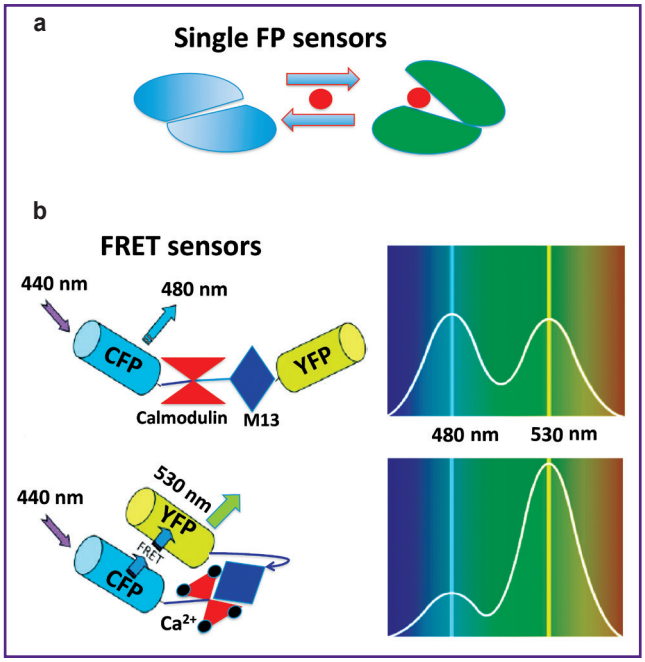

Figure 1. Basic principles of biosensors organization. (a) Single FP-based sensors. They consist of a protein changes its conformation and luminosity for specific binding to the ligand. (b) FRET sensors. It consists of two fluorescent proteins, connected by a linker containing in some cases a ligand receptor. In the absence of ligand fluorescent proteins are far from each other and light releasing by the first protein do not excite the second one. Upon binding of the ligand linker changes its conformation and proteins come together, as a result, the radiation from the first protein excites the second one and its luminosity increases. Organization of cameleon, biosensor of Ca^{2+} [13] is shown in (b). It consists of a cyan fluorescent protein (CFP), calcium-binding protein calmodulin, a calmodulin-binding domain of myosin light chain kinase (M13) and yellow fluorescent protein (YFP). In the absence of Ca^{2+} distance between fluorescent proteins large and the energy transfer is absent. As a result, the amplitudes of emission spectra of proteins are approximately the same (*right, top*). In case of Ca^{2+} elevation, calmodulin is activated and reacts with M13. Due to conformations distance between CFP and YFP decreases, creating conditions for energy transfer (FRET). As a result, the amplitude of the emission spectrum of CFP become reduced and YFP-increased (*right, bottom*)

On this base are designed activators of glutamate and GABA receptors (See Figure 2), as well as other ligand-gated ion channels [35]. Especially fruitful this approach proved to study the functional properties of synaptic and dendritic spine microdomains. For example, it has been shown that some dendritic spines are diffusely isolated and diffusion across the neck of dendritic spines is regulated by neuronal activity [36].

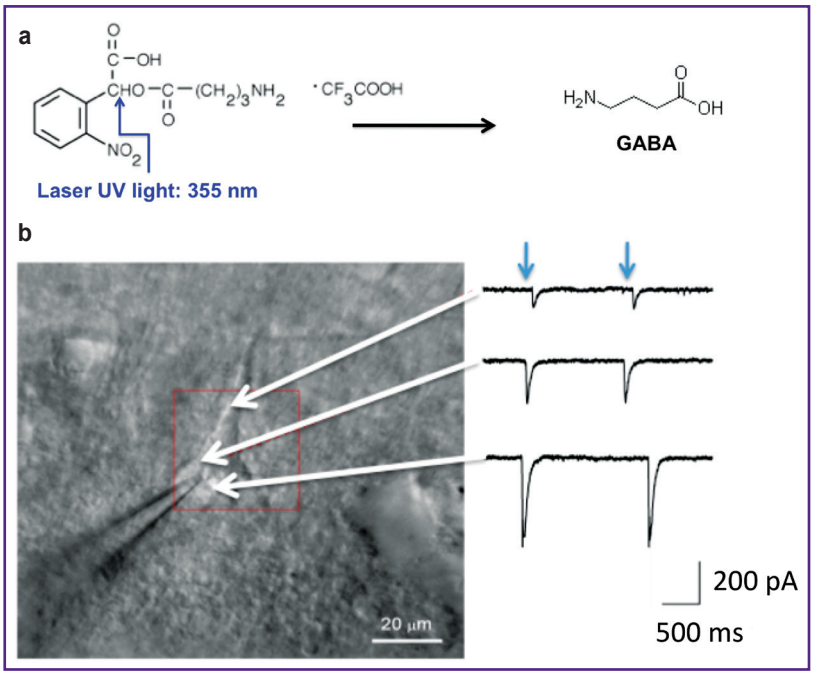

Figure 2. Light-induced activation of receptors using "caged" compounds. (a) Structure of "caged" GABA — inhibitory neurotransmitter. The arrow indicates a photosensitive chemical bond that could be broken by ultraviolet light. (b) Example of GABA receptor activation by "caged" GABA. *Left*: photo of hippocampal pyramidal neurons, to one of them is approached the recording pipette. The arrows indicate points on the neuron that are irradiated with a very narrow light beam (about 2 μm in diameter) from an ultraviolet laser. Computer program allows very accurately set the coordinates of the points for illumination and rapidly change the position between them. *Right*: ionic currents induced by flashes of UV light. The arrows indicate the moments of illumination. Patch-clamp whole-cell recording. External solution contains the α-carboxy-2-nitrobenzyl ester ("caged" GABA). Mukhtarov and Bregestovski, unpublished results

Moreover, these approaches allow to investigate structure-function relationships in the glutamatergic postsynaptic structures of the cerebral cortex *in vivo* in rats [37]; it has been found that the dendritic branches, and not the individual synapses are, apparently, the main functional units in the formation of memory [38].

Photoisomerizable ligands — light-controlled chemical switches for biological research. In this method, the ligand is covalently joined to a photochrome, a molecule that is capable to photoisomerization. In biological implications azobenzene (Figure 3 (a)) is widely used as it is capable to *cis–trans*-isomerization around the central nitrogen-nitrogen double bond [39]. Upon light stimulation, thousand photocycles can be performed on remarkably short time scales and with high quantum yield. In the dark or at visible light, the dominant is *trans*-configuration. Photoisomerization to *cis*-azobenzene can be initiated using UV light (~360 nm). The relaxation to *trans*-isoform can either occur thermally or be catalysed by visible light (~500 nm).

For the control of ligand-gated and voltage-activated ion channels, several complementary photoisomerizable types of ligands, agonists, antagonists and modulators, have been developed. The first photochromic ligands for modulation function of nicotinic acetylcholine receptor (nAChR) channels have been proposed more than 40 years ago. Bartels et al. [40, 41] studied the properties of *trans*-3-(α-bromomethyl)-3'-[α-(trimethylammonium) methyl]azobenzene (*trans*-QBr). This drug is a reversible partial agonist of nAChR. By comparing the agonist-induced conductance with the *cis*/*trans* ratio, Lester et al. [42] provided compelling evidences of the rapid conformational transitions of nAChR channels during activation. More recent designs are represented by photochromic ligands of ionotropic receptors for glutamate [25] or GABA [43, 44]. In the first design, the natural ligand, glutamate, is coupled to azobenzene (Figure 3 (b)), while for photochromic ligand of GABA, the aromatic group of the ligand propofol is added.

To ensure high ligand specificity of action, to the other end of azobenzene is joined a group capable of forming covalent bonds with certain molecules, ion channel or receptor to be activated. As a rule, azobenzene joins maleimide capable of forming covalent bonds with cysteines (See Figure 3 (b)). This technique used successfully to control nicotinic receptors [40, 42], ionotropic glutamate receptors [27, 45] (Figure 3 (c), (d)) and potassium channels [46–48]. Such compounds are called "tethered" agonists [42].

One major drawback of photochemical approaches with "*tethered*" *agonists* is the necessity of genetic manipulations (specific mutation in the target protein, its incorporation in the target etc.), which limits their use. In spite of this limitation these agonists were successfully used for specific activation of cultured neurons, brain slices, or small organisms such as fruit flies [5] or zebrafish larvae [49]. These observations have been reviewed earlier [50, 51].

Bacterial rhodopsins — main tools of cellular activity modulation by light

Channelrhodopsins are key excitatory tools of optogenetics. An extremely powerful way for remote, user defined control of neuronal activity opened microbial light-gated proteins (opsins) functionally expressed in mammalian tissue.

The first light-sensitive protein, proton ion channel, called channelrhodopsin-1 (ChR1) has been cloned from

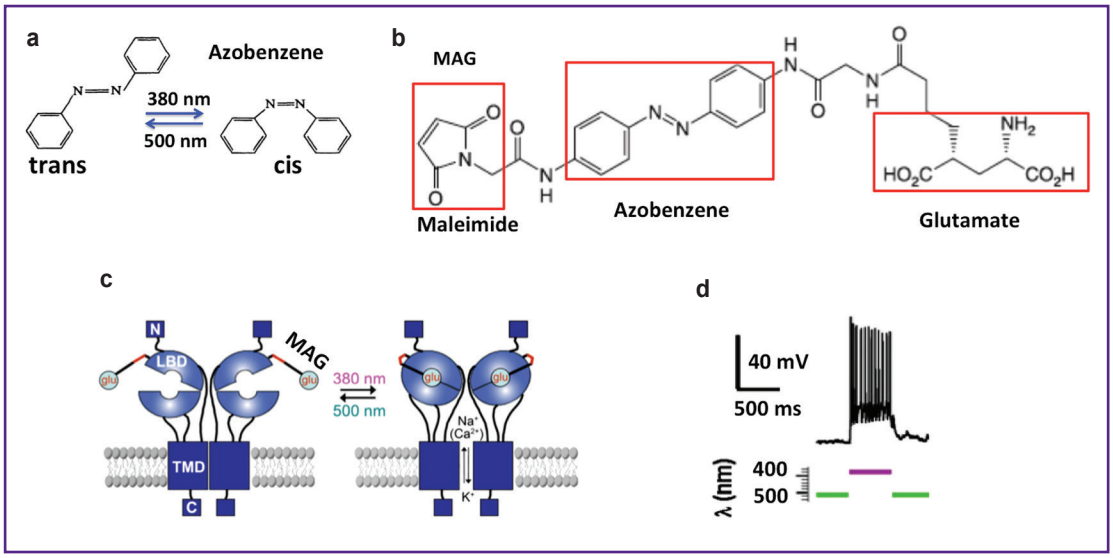

Figure 3. Light-controlled molecular switches. (a) Azobenzene. At visible light molecule is in a "stretched" *trans* configuration and on illumination with UV light conforms to *cis* configuration, thus becoming shorter on about 0.7 nm. (b) Light-controlled agonist of glutamate receptor (MAG). It consists of azobenzene (*center*) attached to the glutamate (*right*) and the maleimide (*left*). (c) Scheme of glutamate receptor activation by MAG. At illumination with wavelength 500 nm, MAG is not able to activate the glutamate receptor. When exposed to 380 nm, the MAG is capable of activating the ligand site, causing the protein conformation. (d) Light-induced generation of action potentials in hippocampal pyramidal neuron cultures expressing kainate subtype of glutamate receptor, containing the cysteine in the region of the ligand-binding domain. MAG is added to the solution and attached to the cysteine. When switching the lighting from 500 to 380 nm kainate receptor is activated and depolarizes the neuron, inducing the generation of action potentials. (c) and (d) from [45] with modifications

Chlamydomonas reinhardtii and expressed in *Xenopus* oocytes [52]. The following year, from the same species of bacteria was expressed another channelrhodopsin (ChR2), structurally similar to vertebrate rhodopsin ion channel that opens in response to blue light. It proved cation-selective ion channel permeable to sodium, potassium and even to calcium [2]. This ChR2 become particularly attractive to researchers, since many receptor-operated channels in the synapses of multicellular organisms, including humans are permeable to these cations.

Several factors indicated that ChR2 is extremely powerful and useful tool for neurobiologists. Indeed, it demonstrated reliable photostimulation within milliseconds time scale. Using lentivirus, ChR2 has been expressed in cultured hippocampal neurons, and induced responses to a flash of blue light (450–500 nm) with generation of action potentials [3, 4]. Activation can be carried out with very high frequency (20 Hz), while weaker light intensities could cause the generation of excitatory potentials, similar to postsynaptic potentials observed at the release of neurotransmitters from the presynaptic terminals (Figure 4 (a), insert). Moreover, it becomes clear that at expression in neurons of vertebrates activation of ChR2 does not need cofactor, i.e. all-*trans* retinal, as it is naturally expressed in these cells. Also for expression in specific populations of cells ChR2 can be combined with specific promoter [1, 53, 54].

Subsequent studies have shown that with moderate expression ChR2 perfectly integrates in cells of multicellular organisms, including mammals without causing damage or severe toxicity. It turned out that with the help of specialized promoters, these light-sensitive proteins can be embedded in certain subpopulations of cells and tissues to study their physiological functions at activation in the millisecond time range, typical for mammals [55].

The family of channelrhodopsins expanded with new light-sensitive proteins derived from *Volvox carteri* [56] and from *Mesostigma viride* [57], as well with new variants obtained through the creation of chimeras and mutagenesis [58]. Currently are described more than 12 channelrhodopsins which exhibit unique properties [59].

Also channelrhodopsins with excitation in the red range of wavelengths, suitable for using two-photon microscopy, has been obtained. For example, the opsin S1V1 has been used for analysis of synaptic networks in the neocortex of rats and for activation of individual spines [60], while its analog, ReaChR, provided the way to activate neurons in the deeper layers of the brain without the need for surgery thin skull [61].

Thus, the introduction of a variety of proteins capable of performing excitation cells by light laid the foundation

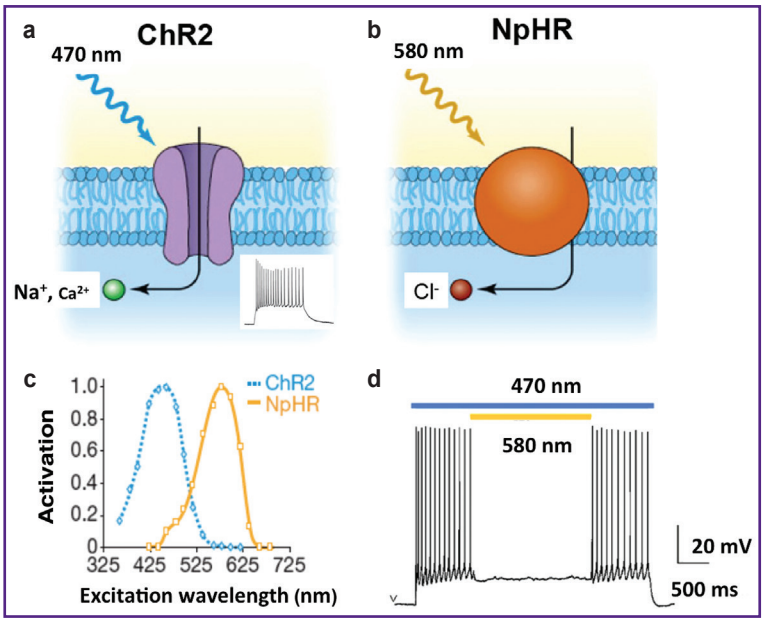

Figure 4. Optogenetic tools of excitation and inhibition. (a) Channelrhodopsin ChR2 — cation-selective ion channel permeable to sodium, potassium, calcium. When illuminated by blue light (470 nm), the channels is activated and, due to the concentration gradient, allows influx into the cell mainly of Na^+ ions, causing depolarization and generation of action potentials (example in the insert). (b) Halorhodopsin NpHR is the ion pump. At illumination by yellow light (580 nm), the molecule pumping into the cell chloride anions and causes hyperpolarization. (c) The excitation spectra channelrhodopsin (blue) and halorhodopsin (orange). Importantly, that the excitation maxima are separated by more than 100 nm. (d) Example of light-induced modulation of neuron, which expresses the ChR2 and NpHR. Illuminating of the neuron by blue light stimulates the generation of action potentials. This stimulation is inhibited when NpHR is activated by yellow light. (c) and (d) from [66] with modifications

of the technical revolution at almost all levels of neurobiological research.

Halorhodopsins — optogenetic tools of inhibition. However, to effectively control the operation of the cell is necessary not only to excite, but also to inhibit their activity. In search for such inhibitory tools, researchers paid attention on bacteria *Halobacterium halobium*, in which many years ago was discovered a proton pump, bacteriorhodopsin. More detailed studies have shown that the bacteria that live in super salty lakes express at least two retinal proteins: the previously described bacteriorhodopsin [62, 63], as well as halorhodopsin [64]. Both pigments absorb visible light in the yellow range of wavelengths (568 and 588 nm). However, unlike the bacteriorhodopsin, which is the proton pump, it has been shown that halorhodopsin is a chlorine pump. At illumination, it pumps this anion into the cells and thus increases the negative potential inside the bacteria [65].

This protein, halorhodopsin NpHR, has been cloned and expressed in neurons. It turned out that upon the illumination of these cells by yellow light (~580 nm), one could inhibit the spontaneous or induced neuronal activity in the millisecond time range [66] (Figure 4 (b), (d)).

Thus, it becomes evident that bacterial light-sensitive proteins represent rather simple and easy to use tools for rapid control of cell excitability and neural networks function. An additional important advantage was the fact that the excitation spectra of activating and inhibitory rhodopsins can be reliably separated (Figure 4 (c)).

Discovery and applying of these highly efficient molecular tools to control excitability of cells using light, produced an explosion in the creation of new experimental models and led to the birth of the novel branches of research, optogenetics and optophysiology [1, 67]. New light-sensitive proteins, like ChR2, NpHR and their derivatives are embedded in cells of different species of animals from worms and insects to monkeys [68–72] and even in human cells [73, 74].

Prospects of optogenetics for medicine

Optogenetic approaches are used in many models with medical orientation, including the study of stress, schizophrenia, memory disorders, drug addiction, psychiatry, motor function, vision, functional recovery after a stroke and many others. Results obtained on mammalian models indicate that optogenetics is potentially a powerful tool for the treatment of several diseases, especially a neuropathologies and visual impairment. Briefly introduce some approaches.

Optogenetics and neuromodulation. One of the promising applications of optogenetics in medicine could be the direction associated with high-frequency stimulation. Different types of neuromodulation via stimulation of the spinal cord or peripheral nerves represent an important part of the medical techniques for the prevention of painful symptoms associated with dysfunction of the central or peripheral nervous system.

Electrical stimulation devices are currently widespread in medical practice [75]. These methods are used to restore the normal functions in movement disorders [76], Parkinson's disease [77], epilepsy, psychiatry, recovery of motor functions, pain relief and others [75]. However, the use of electrical stimulation frequently causes undesirable effects. For example, high-frequency stimulation can cause neuropsychological disorders in patients with Parkinson's disease [78].

This is primarily due to the non-specific stimulation of the total volume of tissue surrounding the electrode. Optogenetic methods would allow circumventing these problems. In fact, studies conducted on animal models,

including primates, shows high specificity control of neuronal networks and brain functions [72]. It has been shown that the optogenetic stimulation decreases the depression in rodent models. In mice lines expressing strong depressive phenotype, optogenetic stimulation of areas of the medial prefrontal cortex has a powerful anti-depressant-like effect without affecting the general motor activity, social memory, or other forms of behavior [79].

These and other studies [80–82] suggest that optogenetic neuromodulation with its potential for high specificity can be a promising tool in the treatment of motor and psychomotor disorders [83].

Optogenetics and epilepsy. In epilepsy, the balance between excitation and inhibition in neural networks is disturbed. In many cases it is related to the disturbance of the basic functions of principal neurons and interneurons. Several studies used optogenetic methods to control the function of neurons in models of epilepsy.

In the pioneering work Tønnesen et al. [84], used a lentiviral vector, under the control of a specific promoter for expressing of halorhodopsin NpHR in the hippocampal pyramidal neurons. It was shown that upon applying of light, cells expressing NpHR were hyperpolarized and also inhibited the activity of target cells. An improved version of halorhodopsin (eNpHR3.0) was expressed in different models of epilepsy and it has been shown that illumination of neurons delivered via an optical fiber, significantly delayed electrographic and behavioral initiation of status epilepticus and altered the dynamics of ictal activity development [85].

The second strategy is based on the incorporation of depolarizing channelrhodopsins in inhibitory interneurons. On transgenic mouse line expressing channelrhodopsin interneurons, it has been shown that blue light stimulation significantly inhibits epileptic manifestations in these models [86, 87]. Despite the need to address many technological and physiological difficulties, these studies point to the possible prospects of optogenetics for the treatment of epilepsy.

Optogenetics and vision. Several experimental studies clearly indicate that the expression of light-sensitive proteins creates great prospects for the restoration of vision at degenerative diseases of the retina. Microbial opsins may be inserted into different subpopulations of retinal cells using viral vectors with the specialized promoters. It can convert the light-insensitive cells in artificial photoreceptors.

Different strategies are used in this direction. When photoreceptors are still alive but are not capable of operating properly, a chloride pump, halorhodopsin eNpHR, can be expressed in these cells creating a way to hyperpolarize them under illumination. This idea was first implemented for genetic reactivation of cone photoreceptors on the lines of blind mice with retinitis pigmentosa [73]. It has been shown that expression of archaebacterial halorhodopsin eNpHR in light-insensitive cones can substitute for the native phototransduction cascade and restore behavioral responses of animals. These findings suggest a potential for translating eNpHR-based rescue of visual function to humans [73].

Another approach is based on the expression of channelrhodopsin in bipolar cells, or in the ganglion cells, making them as artificial photoreceptors [88]. It has been shown that after a complete degeneration of photoreceptors in mice and rats, it is possible to restore the ability of the retina to perceive light and transmit signals to the visual cortex [89, 90]. Moreover, selective ChR2 expression using adenoviral vectors in bipolar cells allowed to recover components ON and OFF visual responses, as well as light-induced locomotor behavior of blind mice [91].

In a recent study, the vertebrate rhodopsin was expressed in retinal ON-bipolar cells of the blind mice allowing to recover the light-induced potentials in the visual cortex of animals *in vivo*, and also to form in mice visually-guided behavior [92].

These and other studies [93, 94] create a strategic framework for the development of therapies to restore function in degenerative disorders of the retina cells.

Conclusion. Optogenetics becomes an important tool not only for analysis of neurobiological problems, but also for highly specific functional control of different types of tissues and cells, from cardiomyocytes to embryonic stem cells. Moreover, steady progress in the development of new optical technologies and bioengineering of different opsins create important opportunities for therapeutic purposes, particularly treatment of human diseases as visual impairment, Parkinson's disease or epilepsy.

However, the optogenetic therapy is at the very early stages of development. To ensure that this new technology becomes applicable in medical practice, new research and developments are necessary, which would allow using a safe and efficient genetic methods suitable for human organisms and it should be combined with the development of reliable and convenient delivery of light.

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Conflicts of Interest. The authors declare no conflicts of interest.

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