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Review

The Impact of Optogenetics on Regenerative Medicine

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Abstract: Optogenetics is a novel strategic field that combines light (opto-) and genetics (genetic) into applications able to control the activity of excitable cells and neuronal circuits. Using genetic manipulation, optogenetics may induce the coding of photosensitive ion channels on specific neurons: this non-invasive technology combines several approaches that allow users to achieve improved optical control and higher resolution. This technology can be applied to optical systems already present in the clinical-diagnostic field, and it has also excellent effects on biological investigations and on therapeutic strategies. Recently, several biomedical applications of optogenetics have been investigated, such as applications in ophthalmology, in bone repairing, in heart failure recovery, in post-stroke recovery, in tissue engineering, and regenerative medicine (TERM). Nevertheless, the most promising and developed applications of optogenetics are related to dynamic signal coding in cell physiology and neurological diseases. In this review, we will describe the state of the art and future insights on the impact of optogenetics on regenerative medicine.

Keywords: optogenetics; regenerative medicine; biomedical applications

1. Introduction

The central nervous system (CNS) consists of many different types of neurons interconnected and able to manage the entire physiopathology of the human body. Neuroscience researchers constantly work to find optimal strategies. Optogenetics has revolutionized the study of the nervous system by providing adequate tools to analyze the functions of neurons within the brain without changing their interconnections [1]. Optogenetics is a novel approach that integrates optical (opto-) and genetic (genetic) methods to control the activity of excitable cells and neuronal circuits. The stimulation of a neuron can be followed by the depolarization of its membrane and the generation of an action potential that travels as an electrical signal along its axon. Using genetic manipulation, optogenetics may induce the coding of photosensitive ion channels on specific neurons: this non-invasive technology combines several approaches that allow users to achieve improved optical control, and higher resolution [2]. This technology works on genetic targets to control specific neurons for the study and the modulation of complex neuronal networks in humans. The first experiments were carried out on the opsins, a natural protein that can convert light into an electrochemical signal. More precisely, through specific

wavelength stimulation of specific proteins, it is possible to activate entire areas of the human brain [3]. The potential of this technique could affect many fields of science, including the medical field. In tissue engineering and regenerative medicine (TERM), optogenetics can be used in several applications, such as in signaling communications between cells, in the cell-to-materials interaction, and in the photo-modulation of biomaterials [4]. In this review, we have described the optogenetics method and its applications: specifically, we reported the main medical applications, such as retinal therapies, ventricular fibrillation, and pain therapy. Finally, we have described the main tools developed in the field of optogenetics, which may be applicable to the field of regenerative medicine (RM).

2. Molecular Pathways in Optogenetics

Optogenetics is a technique that uses that uses specific wavelengths as an activator of specific neural activities, and the proteins as a pathway for this activation. When light stimulates these proteins, such interaction rapidly influences specific ions across the cell membrane. Precisely, to fully modulate and control specific neural populations, light-sensitive genes are inserted and stimulated to act on specific transmembrane channels [2]. Optogenetic neuromodulation is useful to highlight several pathophysiological mechanisms and to explain the function of specific neural networks. Neuromodulation acts on opsins, a family of proteins susceptible to luminous stresses. Neuromodulation can be performed by using viral vectors that infect such neurons carrying specific proteins: these proteins will be activated and will be finally able to modulate specific areas located in the brain [5]. The light-sensitive proteins most commonly used for neural activation and inhibition are respectively Channelrhodopsin-2 and Halorhodopsin. Channelrhodopsin-2 is activated with blue light (~472 nm), and it excites the target neurons by the opening of cation channels. On the other hand, Halorodopsin is activated with yellow light (~590 nm), and it inhibits neural activity by the activation of the chloride pump (Figure 1). With the combined use of these proteins and specific light wavelengths, it is possible to study the behavior of a specific area of the human brain in different conditions [6]. The first trials involving optogenetic techniques were carried out in the field of ophthalmology [7]. These trials mainly involved the Opsins family. Opsins can be classified into two macro-classes: type I (microbial) and type II (animal) Opsins [8].

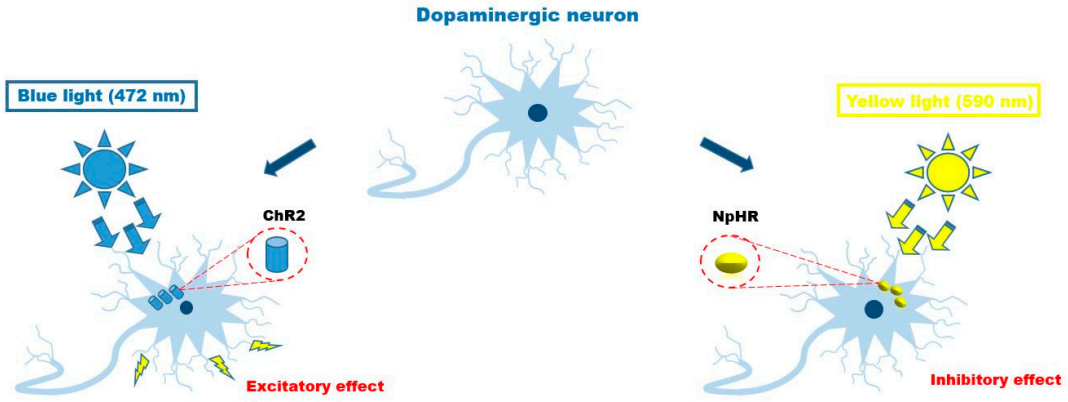

Figure 1. Neuromodulation with Optogenetic technique: blue light activates ChR2 with an excitatory effect, while yellow light activates NpHR with an inhibitory effect.

Type I Opsins can be found in algae and prokaryotes. Type II Opsins are receptor proteins, able to be coupled with other different types of proteins. The difference between the two types of Opsins is related to how the water molecules are positioned in the protein chains. The discovery of natural forms of protein chains has allowed researchers to study the optogenetics method in depth [9].

A well-known example of type II Opsins is the rhodopsin, also known as GPCR (G-Protein-Coupled receptor). Rhodopsin, under luminous stresses, activates hundreds of G downstream of proteins and provides different physiological responses (pupillary constriction, circadian rhythm, etc.). There are

different G protein subfamilies: Gt-coupled opsins, Gi/o-coupled opsins, encephalopsin, and TMT (Teleost Multiple Tissue) opsins (Gq-coupled opsin, Gi-coupled neuropsin, cnidarian Gs-coupled opsin) [10]. These G-proteins have a very important feature, used for optogenetic applications: the bistability. Indeed, G-proteins are able to convert the chromophores associated with them into a function of the absorbed light wavelength [11]. Among the numerous proteins used in the manipulation of the neuronal membrane potential, the Channelrhodopsin-2 (ChR2) has the peculiarity to work as light-gated ion channels [12]. Its structure can be easily changed with specific aminoacids that allow different cellular responses, obtained in response to the time of exposure to a specific light. Kleinlogel et al. [13] observed that proteins exposed to the pulsed blue light generated a potential action by muscle cells. This experimental finding could suggest a large-scale control of muscle contractions, through the optogenetics technique. Another protein successfully used in optogenetics is the Halorhodopsins (NpHR). In vitro and in vivo experiments reported that the NpHR, stimulated with yellow light (~590 nm), modifies the cell membrane potential in the opposite direction, thus increasing its absolute value. NpHR is also activated by the blue light, and it can inhibit or activate neurons based on the time of exposure to the blue light [14].

The optogenetics strategies can be used to selectively activate a specific group of neurons through the activation of membrane channels or pumps, via second messengers. Recently, this technique has been used to better understand the functional properties of nociceptive cutaneous activation of the Nav 1.8 tetrodotoxin-resistant voltage-gated channel. The sensitivity to blue light (470 nm) of the ChR2 is decisive for the activation of Nav 1.8 channels and the stimulation of several nociceptors. The stimulation by specific lights can lead to the depolarization of the neuronal membrane, inducing the opening of Nav 1.8 [15]. The neurons activated by Nav 1.8 are typically sensitive to thermal and mechanical stimuli. Thus, with optogenetics, light provides a non-invasive stimulation that activates specific biological structures that are difficult to stimulate with standard techniques [16,17].

Considering its peculiarities, Optogenetics may be considered as a reliable tool for the study of the dopaminergic system. Recently, Lippert et al. [18] conducted a study on the effects of dopamine obtained by the stimulation with optogenetic techniques. As known, dopamine is a neurotransmitter with several functions; its control depends on the mesolimbic system in which dopamine regulates the synaptic weights. The study performed by Lippert et al. [18] consisted of in vivo experiments aimed to evaluate the efficacy of optogenetic techniques, in comparison with the electronic stimulation. In this context, the optogenetic-induced intracranial self-stimulation is a helpful tool to study the effects of dopamine release in the healthy and pathological human brain. Under light stimulation, the ChR2 located in the VTA (ventral tegmental area) cells can induce dopaminergic self-stimulation, capable of evading sensory limitations. For example, a sense of appetite or satiety can be evoked, and thus investigated in depth [19].

Applications for Cellular Communication

During the embryonic development, a dynamic coding can play an important role in cell-to-cell communication. The duration of the stimulus is an important factor for the development of proper cell responses: in this context, an optogenetics approach may offer an interesting opportunity to modulate signaling paths with high precision and in real time. In several studies on somitogenesis, the somites have been studied during their generation by the presomitic mesenchymal mesoderm (PSM). These somites will develop bones, skeletal muscles, and subcutaneous tissues. The optogenetics approach has been used to investigate the processes of the spatial evolution and the temporal genetic coding, analyzing the frequencies of such signals that stimulate and inhibit embryonic development [20,21]. Several optogenetic-based techniques have been developed in recent years: some of them are currently applicable to TERM. Functional optogenetic tools are safely usable in human cells, by modulating specific parameters, such as wavelength, duration, or energy emitted by laser devices [22]. Ultraviolet (UV) receptor systems are also used in optogenetic applications: they are easily controllable, and they work with both normal light or lasers [23].

A novel technique is the light-oxygen-voltage (LOV): LOV tools are used to control such signaling characterized by a rapid kinetic, such as ion channels and protein kinases [24].

Cryptochrome systems are characterized by Cryptochrome 2 photosensors (CRY2) and cryptochrome-interacting basic-helix-loop-helix (CIB1), sensitive to blue light. Such systems are also suitable for the detection of signal cascades showing a rapid kinetic [25].

In 2017, Zhou et al. [26] reported two-way photocontrol systems without cofactors, using a specific protein, the Dropna145N, able to ensure more flexible manipulation of the target proteins, revealing all the possible states of activation.

Recently, cobalamin-based systems (CBD) have been involved in optogenetic tools, in the green spectrum with a specific activation with vitamin B12 and AdoB12. Vitamin B12 is naturally present in mammals, playing an important role in hematopoiesis, neuroprotection, and protein synthesis. AdoB12 is a ubiquitous coenzyme derived from vitamin B12, synthesized in the mitochondria of eukaryotic cells. CBD-based optogenetic systems require the integration of AdoB12 for proper light sensitivity; however, compared to other optogenetic systems, CBD-based optogenetic systems have a slow activation and a long deactivation, probably due to the natural degradation of AdoB12 [27].

3. Biomedical Applications of Optogenetics

The optogenetics strategy can be an excellent support for clinical applications. Optogenetics showed good results in ophthalmology, neurodegenerative diseases (Parkinson's and Alzheimer's), cardiac stimulation, and other systemic therapies. Furthermore, recent studies have shown that optogenetics may control the cellular contractility and the production of several pigments [28].

3.1. Applications in Ophthalmology

Degenerative retinal disorders affect 1/3000 people worldwide. Early diagnosis and gene therapies have good chances to successfully treat most such pathologies [29]. Although the retinal disorders severely impact the sight, many neural retinal connections remain active and functional for a long time; this residual function is the main target of optogenetics. The therapeutic approach is based on intravitreal vectors carrying ChR2 to retinal ganglion cells. The main limitation of this technique is that high frequencies of light and prolonged exposures to it can saturate the response of the retinal cells [30]. In 2018, Gaub et al. [31] described in vitro and in vivo experiments performed on blind mice, providing rhodopsin GPCR vectors, and applying the optogenetic technique. It was possible to characterize how the visual recovery was different, based on the exposure to specific wavelength, and on the movement and the duration of exposition to pulsed light [32].

3.2. Applications in Bone Repairing

The development of optogenetic tools has improved research on the role of Ca^{2+} channels in cell regeneration. The opening of Ca^{2+} channels is typically allowed by proteins stimulated with blue light: this biological condition has been linked to cell proliferation in specific degenerative diseases [32]. Recent research has demonstrated that the application of electrical stimulation promoted the activation of calcium channels in osteoblasts: Ca^{2+} oscillations were monitored in mesenchymal stem cells during osteogenic differentiation. The role of Ca^{2+} in osteoblasts was investigated through the optogenetic technique, using the blue light stimulation (488 nm) to activate the Ca^{2+} channels (BACCS) BACCS is an optogenetic tool: MC3T3-E1 cells were used as a model for the differentiation towards osteoblasts, suggesting the effective expression of BACCS under luminous impulse [32] highlights how the Ca^{2+} channels play a fundamental role in osteoblast differentiation [33].

3.3. Applications in Heart Failure

Optogenetics in heart pathologies and therapy could achieve significant advantages [34]. In 2016, Karathanos et al. [35] performed a study to evaluate the induction of ventricular fibrillation (VF) through light-induced excitation of opsins present in the myocardium. Through electrophysiological

simulations in a human ventricular model, specific ChR2 was injected and irradiated by blue light 470 nm, 0.4 mW/mm^2. This experimental model allowed observing a VF induced by the optogenetic device, demonstrating that opsin is a determining factor to consider for future therapies of severe VF [36]. Several studies have been also carried out on the hearts of mice, especially in the field of atrial fibrillation (AF). The idea was to modulate the heart rhythm, using light radiation on genetically modified ChR2 proteins [37]. These proteins can activate or inhibit the potassium (K) channel, thus shortening the atrial refractoriness and improving the atrial conduction. The in vivo results obtained with the optogenetic technique will allow for creating new applications to treat AF [38].

3.4. Applications in Post-Stroke Recovery

Optogenetics allows us to selectively stimulate or inhibit specific populations of neurons and specific brain areas for targeted functional studies or possible rehabilitative therapeutic solutions. The main issues in cell manipulation are related to stimulating specific cells without impacting those surroundings [39]. Optogenetics can ensure high precision, as only those neurons that express ChR2 are selectively activated by blue light. In the post-stroke period, a stimulation on primary neurons of the motor cortex ipsilesional (iM1) can promote functional recovery. Experiments on mice with induced stroke condition, showed improvement in cerebral blood flow, after repeated light stimulation. Good results were also observed in the expression of neurotrophins in the contralesional cortex. Optogenetic stimulation also showed a significant increase in a protein associated with the plasticity growth marker. In conclusion, the future application of the technique to stimulate hemisphere neurons attacked by stroke may be a good solution to promote neurofunctional recovery [40].

3.5. Applications in Neurological Diseases

Useful applications of optogenetics also involve the study of the peripheral nervous system (PNS). Researchers have assessed that the expression of opsins can be induced through adequate viral concentration; on the other hand, the use of ATR (all-trans-retinal) acid has been used to increase the photosensitivity of tissues releasing ChR2 in the PNS. This strategy allowed an optogenetic stimulation, followed by an electrophysiological response of PNS [41]. The vestibular system is controlled by neurons within the vestibular nucleus (VN), stimulating the auditory, visual, and motor centers [42]. Generally, through fMRI (functional magnetic resonance), brain functions are mapped in response to different sensory or cognitive tasks; however, fMRI does not work well in the studies on VN. Optogenetics overcomes these limitations by stimulating VN neurons with light that activates neurons of the associated thalamic nuclei (auditory, visual, somatosensory, and motor). Combining optogenetics with fMRI, the researchers have answered the main questions related to the visual cortex, in the thalamus, for more precise medical diagnoses [43].

In recent years, many studies have investigated the usefulness of optogenetics in pain therapy [44]. Chronic pain is often related to neuronal damage, immunological impairment, and inflammatory conditions: it is characterized by the production of molecules correlated with specific ion channels [45]. The connection between the modulation of ionic channels activating the nociceptors was observed. In the priming of immune cells, the receptors that couple to the G proteins (GPCR) are very important [45]. Light-mediated manipulation of G protein subunits of ChR2 and NpHR proteins can activate or inhibit the receptors of injured neurons. The sequencing of the RNA can promote novel research targeting such protein chains able to control the pain with optogenetic tools [46].

The optogenetic stimulation on ChR2 proteins expressed by spinal astrocytes leads to reversible and time-depending hypersensitivity to pain. Therefore, pain can be treated through optogenetics working on spinal astrocytes [47]. Cortico-trigeminal pathways can modulate nociceptive input from the spinal trigeminal caudal nucleus (SpVc) and regulate pain perception. Through optogenetic stimulation on ChR2, there is a suppression of the SpVc responses to noxious stimuli, leading to a pain modulation [48].

3.6. Clinical and Tissue Engineering and Regenerative Medicine (TERM) Applications

Optogenetic applications can be related to emerging biomaterials and photoelectric technologies. One of the strategies used to overcome the barriers to human tissue is to transform energy from near infra-red (NIR), X-rays, and ultrasounds into visible light. NIR light is highly promising since lipids, hemoglobin, and water have an absorption coefficient of 650–900 nm, which is lower than such tissues investigated by NIR [49]. Chen et al. [49] demonstrated that optogenetics could stimulate deep-brain neurons. Some neurological applications have taken advantage of the use of lanthanide-doped nanoparticles (UCNP): UCNPs are able to convert the low-energy photons into energy. The optogenetic activation with UCNPs only requires a host material (sodium fluoride ittria—NaYF4) able to ensure a proper photonic absorption (Yb^{3+}) [50].

Unfortunately, several tissues located in the deepest zone cannot be investigated for clinical purposes. For this reason, researchers are studying alternative solutions, such as X-optogenetics, which involves X-rays, and U-optogenetics, which exploits sonoluminescence [51,52].

It is well known that ultrasonographic waves and X-rays penetrate deeper into human tissues than NIRs. Although promising, these applications must be carefully evaluated for clinical applications and preclinical safety [53].

The inorganic light-emitting diodes (μ-ILED) are able to provide support during minimally-invasive in vivo treatments. The idea is to apply μ-ILEDs in multi-function systems, with mechanical, thermal, and electrophysiological sensors to obtain accurate feedback in complex clinical evaluations [54].

Mickle et al. [55] designed a modern example of closed-system technology in 2019, made from μ-ILED, combining optogenetic neuromodulation with optoelectronic technology, to control pathological conditions [55].

Wang et al. [56] have proposed a non-invasive technique based on ultrasounds and optogenetics. Through the application of ultrasounds, the blood–brain barrier (BBB) has been overcome, favoring the non-invasive introduction of channelrhodopsins and their related activation with a light-based activation. The approaches with photostimulation are preferred to those involving the emission of radiation or electrical impulses because of the low invasiveness and the lower possibility to induce side effects.

Advances in tissue engineering and regenerative medicine (TERM) have shed light on important scenarios in medicine, with a specific interest in tissue repairing. Optogenetics is pretty useful in the study of mechanisms supporting cell development and regeneration, in order to better understand the physiological processes of tissues and organs replacement [57].

Moreover, optogenetics can monitor the dynamic signaling related to several cellular processes: this is very important to support the future 3D bioprinting techniques that will be aimed to exactly replace cells, growth factors and biomaterials in the conditions able to create tissue-like or organ-like parts of the human body [58]. Furthermore, the optogenetics approach, used with specific target genes, allows monitoring the cellular processes involved in the pathogenesis of some morphological anomalies or even some tumors [59].

The proper combination of photoreceptor and gene engineered effectors has widely improved the fields of application of optogenetics. Generally, this strategy requires guidance modules (GMs) [60], light-sensing modules (LMs), and effector modules (EMs). More in detail, GMs will find the proper target genes; LMs will determine the spectrum of light-based activation and kinetics of transcription [61]; EMs will be involved in different functions, such as gene activation and recombination, and epigenetic modification [62].

Optogenetic stimulation has been found to improve both bradykinesia and hypokinesia that characterize the clinical picture of patients with Parkinson's disease [63]. Optogenetics also has potentialities in the treatment of endocrine disorders; in fact, optogenetic tools may enhance blood–glucose homeostasis by regulating the expression of glucagon-like peptide 1, a therapeutic peptide for type-2 diabetes [64]. In oncological therapies, immune cells may be "optically-oriented"

directly into the tumor mass, in order to maximize the killing effect and, contemporarily, to minimize the local cytotoxicity [65] (Figure 2).

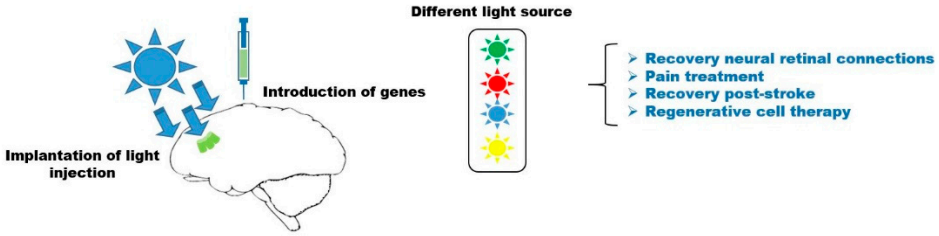

Figure 2. Strategic applications of Optogenetics strategy in different neurological diseases: different light sources, together with the introduction of specific genes, may have different outcomes that lead to different possible clinical applications.

4. Conclusions and Future Insights

Optogenetics has been used in several fields of medicine, and one of the most promising is undoubtedly regenerative medicine. Yu et al. [66] have performed experiments using the optogenetic technique to selectively excite cells. Precisely, induced pluripotent stem cell-derived neural progenitor cells (iPS-NPCs) of mice models were combined with specific proteins and activated by photo-stimulation: in vitro stimulation, increased post-synaptic density, and promoted neurite growth; in vivo stimulation, activated cells residing into the ischemic cortex of the mouse, creating novel pathways with a better functional recovery. In conclusion, non-invasive optogenetic treatments may provide a possible new regenerative cell therapy [67].

Optogenetics has been used to achieve insights into tissue regeneration and organ development: this novel technique also offers great opportunities for developing new tools to build artificial organs, via understanding and mimicking physiological processes. Combining optogenetics and existing technologies (e.g., 3D biological printing, functional materials, and gene-editing techniques) holds great potential for regenerative medicine and can provide substantial appealing for researchers across many fields. Future challenges will address the potential uses of this technique on stem cells [66–69] combined with innovative biomaterials or bioactive matrices [70,71]: the next breakthrough will be related to use of optogenetics in the treatment of the most severe clinical conditions, such as syndromic conditions and cancer [72–78]. Moreover, the use of optogenetics will improve "smart" diagnostic applications, based on the current knowledge on wearable devices, smart materials, and tissue engineering [79,80]: the next research will be used to develop new tools for forthcoming applications combining genetics and molecular biology. In conclusion, optogenetics is a promising technique that will provide several opportunities in several biomedical fields.

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