Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/19727
DOI URL: http://dx.doi.org/10.21474/IJAR01/19727
ISSN NO. 2320-5407

RESEARCH ARTICLE

ADVANCING NEUROSCIENCE THROUGH CHEMICAL TECHNIQUES IN OPTOGENETICS

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Manuscript Info

Manuscript History
Received: 2nd Nov 2024
Final Accepted: 6th Nov 2024
Published: 12th Nov 2024

Keywords:-
Optogenetics, Channelrhodopsin (Chr), Halorhodopsin (NpHR), Microbial opsins, Gussia Luciferase, Archaerhodopsin, Arches, Animal opsin, Luminopsin, Optochemical Genetics, Luciferase, Luciferin, Bioluminescence, Depression, Chlamydomonas reinhardtii, Anhedonia, Neurodegenerative diseases, Parkinsons disease chemical genetics, Gussia Luciferase, Renilla luciferase

Abstract

Optogenetics is a technique that allows the control of neuronal activities using light. This can be expressed by different engineered enzymes and light-sensitive ion channels such as **Channelrhodopsin (ChR)** and **Halorhodopsin (NpHR)**. Optogenetics has seen significant advancements from 2000 to 2024. This review article highlights the development of microbial opsins over the years, focusing on the most important ones, like Channelrhodopsin and Halorhodopsin, which led to the emergence of Optochemical genetics. Moreover, it analyzes the combination of optogenetics with chemical approaches rather than just relying solely on chemogenetic or optogenetic methods, which enhances the techniques capabilities and makes it more efficient. This combined approach, often called **optochemical genetics**, typically makes use of bioluminescence for its applications. This review also outlines the various types of luminopsins—combinations of light-sensing opsins and light-emitting proteins—and explores the applications of optochemical genetics.

Introduction

Optogenetics, a field of optics and genetics, allows scientists to authorize cellular activity using light-sensitive proteins called microbial opsins. The two foundational parameters of neuron excitability or neuron stimulation are ionic conductivity and membrane capacitance [7]. Until now, optogenetic techniques have been a powerful tool to temporarily manipulate a neuron's ionic conductivity, though it has been unable to influence the membrane capacitance.

Optogenetics saw many significant developments in the early 2000s when scientists tried to find specific biochemical photoreceptors. In 2002, Channelrhodopsin-1 (ChR1) was discovered followed by Channelrhodopsin-2 (ChR2) in 2005. Scientists also created chimeric versions of Channelrhodopsins [4][11]. Channelrhodopsins are typically excitatory channels, that when activated by light, allows the flow of cations through cell membranes [28]. In contrast to channelrhodopsin, halorhodopsins are the light-driven inhibitory channels that pump chloride ions into the cytoplasm when exposed to yellow light [29].

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The first ever halorhodopsin was discovered in 1977, decades before it was utilized as a research tool in 2007[38].

Channelrhodopsin and other microbial opsins saw significant development in the following years. In 2008, scientists identified **Volvox Channelrhodopsin**, which allowed red-shifted light activation [12]. The optogenetic toolkit nearly doubled with the development of a new technique called **topological inversion**, which facilitated the creation of additional luminopsins for optochemical genetics [3]. In 2013, Optochemical genetics emerged, and scientists combined **Gussia Luciferase (Gluc)**[1] with channelrhodopsin, leading to the creation of luminopsins [1]. This novel hybrid approach is still under development. Optochemical genetics, in essence, leverages bioluminescence through opsins (proteins) to produce light [14].

Microbial opsins: Types and Functions

A microbial opsin is a gene encoding light-responsive protein that generate ion flow across cell membranes. This unique structure in biology enables scientists to gain valuable insights into brain function [16]. The most commonly used light-driven microbial opsins in optogenetics are channelrhodopsins, halorhodopsins (used to inhibit specific neurons), and archaerhodopsins (arches).

Significance of Microbial opsins

Microbial Opsins play a significant role in neuronal manipulation using optics and chemical techniques. These proteins allow the ions to flow through the neurons and stimulate them. This is the reason Microbial opsins are capable of influencing ionic conductivity and temporarily controlling and directing neuronal excitability [17]. Most importantly, these microbial opsins contribute a major role in the development of Optogenetics into a novel technique known as **Optochemical genetics**, which combines luciferases (light-emitting proteins) and microbial opsins to form luminopsins, later discussed in this article. As a result, optochemical genetics provides a more efficient method to manipulate ionic conductivity [5].

Discovery and Development of Channelrhodopsin

In the quest to uncover the secrets of light detection in cells, the Hegemann research group embarked on an extensive journey to understand how light-sensitive microorganism's function and the cellular mechanisms they utilize. During the initial phases of their research, the scientists used traditional biochemical methods which failed to reveal the elusive photoreceptors. These light-sensing proteins proved to be scarce, unstable, and highly diverse, making detection difficult using these techniques. The biochemical approach faced challenges, as isolated proteins often lost functional properties without their native cellular environment, hindering the accurate understanding of channelrhodopsins roles in photoreception and signaling.

However, in 2001, a breakthrough occurred when Suneel Kateriya, a member of the Hegemann group, successfully discovered two previously unknown DNA sequences within the Chlamydomonas cDNA database. These sequences encoded a large microbial-type rhodopsin, sparking newfound hope in the scientific community. Collaborating with Georg Nagel, Kateriya conducted experiments on frog oocytes (eggs), revealing the remarkable ability of these DNA sequences to open cell channels upon exposure to light. This led to the discovery of **channelrhodopsin-1 (ChR1)** in 2002, followed by channelrhodopsin-2

[1] Gussia Luciferase is a bioluminescent enzyme derived from the Gussia species, used in bioluminescence assays to produce light in the presence of its substrate, luciferin.

(ChR2) in 2005. Expression of ChR-2 in various mammalian cells, including human kidney cells, demonstrated its remarkable ability to depolarize cell membranes upon light stimulation [11].

The year 2005 marked another milestone as scientists successfully engineered **channelrhodopsin chimeras**—hybrid proteins created by combining different channelrhodopsins. These chimeras allowed for the manipulation of specific properties, such as light sensitivity and ion selectivity. This breakthrough provided invaluable insights into their molecular structure through high-resolution crystallography [4]. It unveiled the intricate architecture of channelrhodopsin, wherein specific helical configurations created light-sensitive pores containing a trans-retinal chromophore, as shown in Figure 1.

There are two types of helical configurations in channelrhodopsins: **monomeric** and **dimeric** configurations of helices. In the monomeric form, the seven α-helical transmembrane domains (TMs) form the structure of the channel. When the protein absorbs light, the retinal chromophore undergoes a conformational change that opens the ion channel. In the dimeric form, two monomers come together, and the helices in each monomer align to form a functional ion channel. Dimerization stabilizes the channel's structure and ensures that the pore formed by the helices is large enough to allow the flow of ions. The alignment of helices between monomers may induce slight conformational changes that optimize the opening and closing of the channel in response to light. Dimer formation typically enhances the stability and activity of channelrhodopsin in its functional state [31][35]. Remarkably, this discovery eliminated the need for exogenous chromophore injection in vertebrates, although **Drosophila**[2] required trans-retinal supplementation in their diet. The crystal structure also facilitated the design of mutants with tailored functionalities and enabled the development of potassium-selective pores.

In 2008, Feng Zhang's identification of **Volvox channelrhodopsin-1 (VChR1)** introduced the groundbreaking concept of red-shifted light activation [12]. Derived from the multicellular algae Volvox, VChR-1 enabled optogenetic manipulation using longer wavelength light, broadening the scope of optogenetics research. Volvox, with its distinctive features like sunlight-sensitive eye spots and flagella, thrives in diverse aquatic habitats, showcasing nature's adaptability. Its discovery also underscored the evolutionary connection between multicellular organisms like Volvox and their unicellular counterparts such as Chlamydomonas.

Later, in 2013, it was also demonstrated that the integration of ChR2 into multiple tissues of mice could activate the neuronal circuits, control heart muscle contractions, and restore breathing after spinal cord injury. Furthermore, it has been shown that the artificial expression of channelrhodopsins in retinal ganglion cells can contribute to restoring visual sensing in mice with retinal degeneration [9]. Additionally, ChR2 has potential therapeutic applications, including the treatment of depression [15][18].

Channelrhodopsin emerged as a game-changing optogenetic tool, allowing scientists to dissect cellular processes with unprecedented temporal precision, akin to capturing fleeting moments in the blink of an eye. However, its limitations include challenges in specific cell targeting, such as the ability to target multiple cells simultaneously. In other words, its spatial precision is relatively low.

To overcome some of these limitations, **channelrhodopsin dimers**—complex structures formed by the pairing of two channelrhodopsin molecules—have been developed. These dimers work in tandem to

[2] Drosophila is a genus of fruit flies widely used as a model organism in genetics and neuroscience research due to its genetic similarity to humans and ease of study

provide more precise control over ion flow upon light activation. Dimerization enhances the stability and effectiveness of channelrhodopsin, improving its efficiency for optogenetic applications and enabling more controlled and reliable manipulation of cellular processes [31]

The Figure 1 given below illustrates the formation of the channelrhodopsin dimer and the steps involved in its assembly. It also depicts the crystal structure of the **channelrhodopsin dimer**

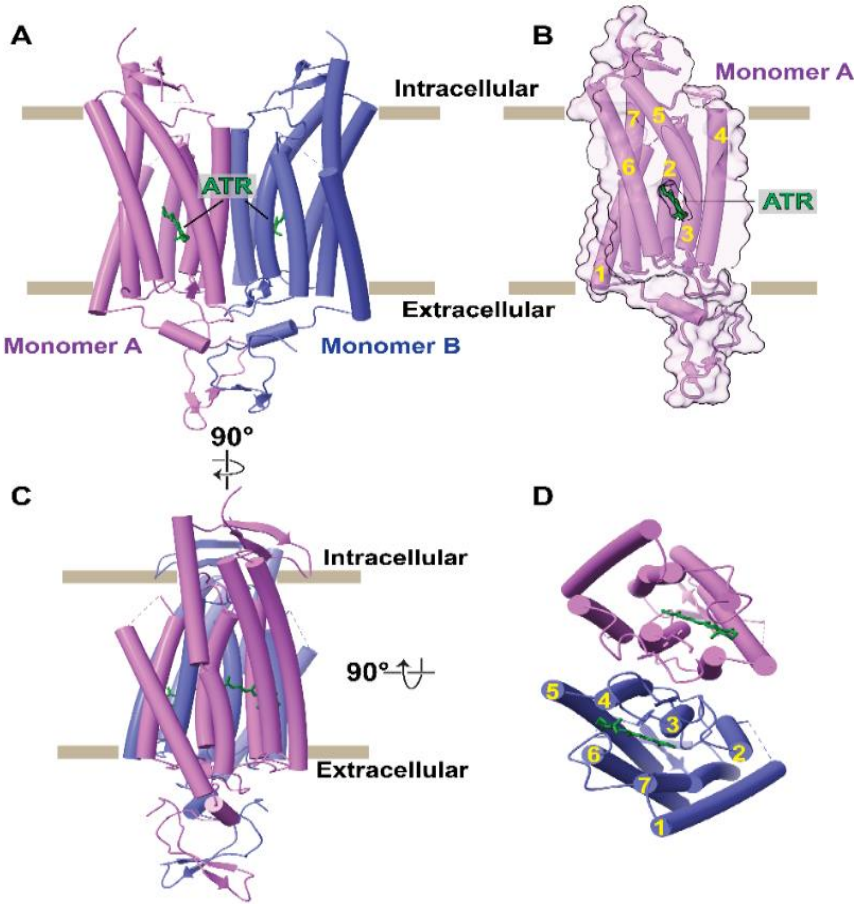

Figure 1: Channelrhodopsin dimer structure and formation of helical configurations

Functioning of Channelrhodopsin and Halorhodopsin

It's also proven that microbial opsins such as channelrhodopsins and halorhodopsins are ion-selective. Channelrhodopsin-2 (ChR-2) is a cation pump and mainly allows Na+, H+, and Ca++. Halorhodopsins (NpHR), which are inward chloride pumps, act as strong inhibitors, capable of inhibiting a neuron even when the intracellular region is more negative than the extracellular region. When yellow light is illuminated on the halorhodopsins expressed in the target cell, hyperpolarization occurs, gradually silencing the target cell [22][23].

These rhodopsins significantly aid in membrane trafficking enhancements. Due to their different activation wavelengths, ChR2 and NpHR typically respond to 470 nm and 580 nm light, respectively. However, these microbial opsins can be sensitive to a range of wavelengths and can produce precise responses depending on experimental conditions. They offer millisecond precision and instantaneous

reversibility. That said, caution is needed when considering the effects of optogenetic interventions, as they influence not only the targeted neuron but also the activity in the surrounding extracellular fluid and neighbouring neurons [6].

From Figure 2, it is evident that Channelrhodopsin is a cation pump that responds to 470 nm blue light, while Halorhodopsin is an anion pump that is activated by 580 nm yellow light. The lower part of the figure illustrates the activation and deactivation of channelrhodopsin and halorhodopsin when blue and yellow light are turned on and off, respectively.

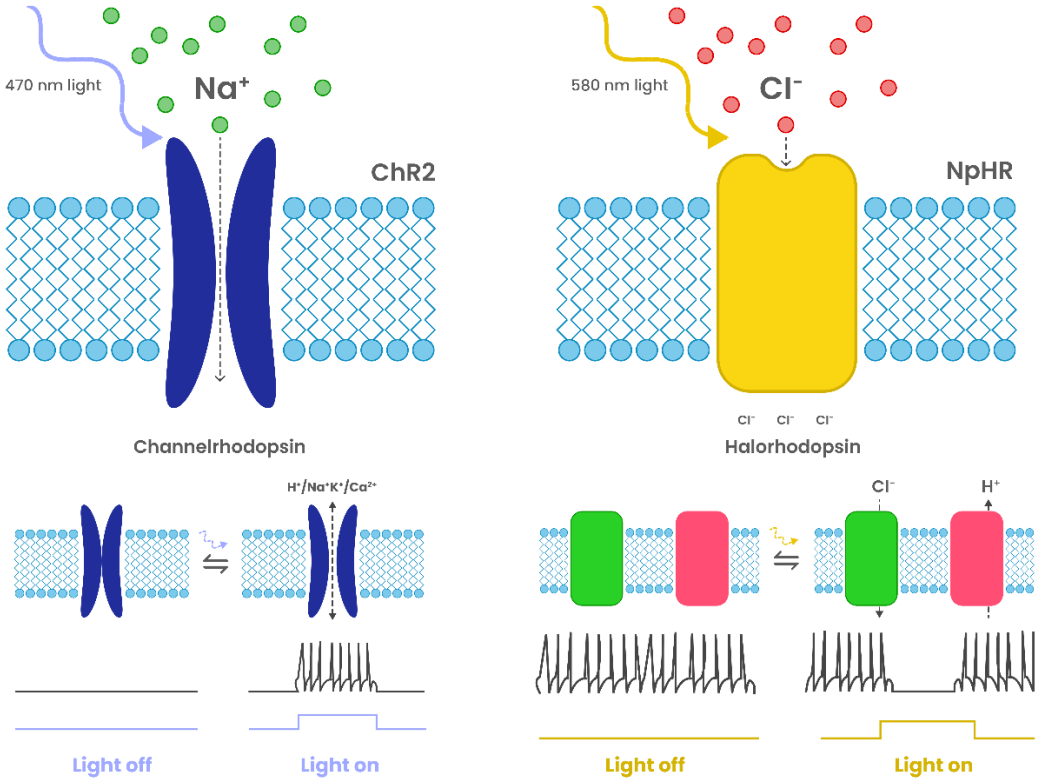

Figure 2: Photo responsive Channelrhodopsin and Halorhodopsin

Archaerhodopsins and Animal Opsins

Archaerhodopsins (Arches) such as Arch-3, are light-driven pumps that activate upon exposure to yellow or green light, leading to depolarization [6]. When neurons are manipulated using this technique, molecules having higher sensitivity are typically utilized to silence more significant and extensive areas of the brain. The excitation maxima for archaerhodopsin is approximately 566nm [21].

Animal opsins, such as rhodopsin and melanopsin, are a large group of naturally occurring light-driven G-Protein Coupled Receptors (GPCRs)[3] that are essential tools in optogenetics [23]. Compared to microbial opsins, animal opsins are much more light-sensitive, enabling more efficient research.

[3] GPCRs (G-Protein Coupled Receptors) are a large family of cell surface receptors that transmit signals from external stimuli to the inside of the cell, playing a key role in many physiological processes [30]

The Emergence of Optochemical Genetics

Microbial opsins in optogenetics have vast potential across a broad spectrum of applications, such as their capacity to regulate cellular processes with exceptional precision and spatial accuracy. This technique introduces novel methods to investigate and activate cells, thereby creating opportunities to manage and modulate co-cultures, enhancing bioproduction efficiency, shaping biomaterials, and facilitating precise drug delivery for therapeutic purposes, among other applications. However, there are a few concerns regarding these opsins, which are expected to be solved in future research, such as Limited Tissue penetration[4] and decreased spatial precision in dense or thick tissue. In particular to optogenetics, the solution uses the fiber optics approach; however, fibre optic cables are fragile and can easily be damaged, and their flexibility is limited [19].

The Figure 3 portrays fibre optic method being used for sending the optogenetic signals inside the brain. It also shows the structure of the fibre optic system.

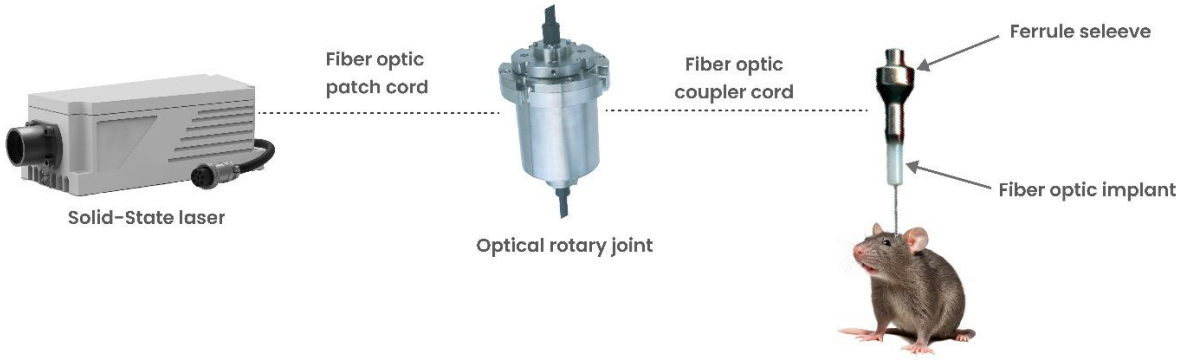

Figure 3: Fiber-optic Implantation for Chronic Optogenetic Stimulation of Brain Tissue of an animal

Due to the risks associated with fiber optics, scientists developed a novel technique called optochemical genetics. This technique combines optogenetic approaches with chemical methods, offering a high potential for enhanced spatiotemporal precision[5]. Optochemical genetics merges the advantages of both techniques while mitigating their drawbacks [2][13][8].

In 2013, scientists developed combined chemical genetic and optogenetic manipulation tools. The foundation of this technology was the integration of multiple light-emitting proteins of different types [1]. This system allows ions to flow by activating an enzyme called luciferase using its substrate, coelenterazine (CTZ), along with light. In response, the fusion of Gaussia Luciferase and Channelrhodopsin-2 (ChR2) occurs. By fusing Channelrhodopsin with Gaussia Luciferase, the scientists successfully created luminopsins (light-emitting channelrhodopsins that produce light when triggered by their own chemical substrate). Compared to ChR1, ChR2 is more effective, as it generates a higher photocurrent when combined with Gaussia Luciferase.

[4] Limited tissue penetration refers to a substance's inability to deeply reach or affect tissues beyond a certain point.

[5] Spatiotemporal precision in optogenetics refers to the ability to precisely control light-induced cellular activation in both space and time. This high level of accuracy is essential for studying neural circuits and complex biological processes, particularly in neuroscience [32].

Approaches	Advantages	Disadvantages
Chemical approaches	Firstly, chemical approaches allow the modulation of an entire cell or Neuron. Secondly, Chemical genetics is a non-invasive technique because scientists inject molecules into the target cell's body.	One of the significant disadvantages of chemical genetic approaches is slow diffusion - limited timescale.
Optical approaches	Optical approaches facilitate rapid neuronal control with a millisecond timescale and high spatial precision.	However, the need for an external light source limits the number of location(s) of neurons that can be photo-stimulated because a small dimension of optical fibers can be used for photo-stimulation

Table 1: Advantages and disadvantages of Optogenetics and Chemical genetics

Optochemical genetics also overcomes a significant limitation of optogenetics: the issue of optical penetration, which refers to the difficulty of using light to penetrate deeper tissues.

Feature and Method	Optogenetics	Chemogenetics	Optochemical genetics
Trigger	External Light	Exogenous chemical	External light or BRET
Orthogonal multiplexing	Wavelength	Substrate	Substrate
Mechanism	Ionotropic	Ionotropic or Metabotropic	Ionotropic
Hardware	Required	Independent	Optional
Region of Influence	Small	Variable	Variable
Kinetics	Fast	Slow	Fast or Slow
Off-target effects	Low	Possible	Low
Intrinsic activation monitoring	Inferred from light intensity and opacity of the tissue	None	Bioluminescence

Table 2: Comparison of Genetic Neuromodulatory Approaches: Optogenetics, Chemogenetics, and Optochemical genetics

Luminopsins and its Types

Luminopsins are combinations of light-sensing opsins and emitting luciferases. Their cognate substrates of the luciferase, "Luciferin," activate, which crosses the blood-brain barrier[6]. They generate light using the brain's enzymatic reaction, allowing scientists to form an internal light source. However, these light-emitting luciferases produce dim light compared to external light sources, and their internal operation can potentially damage nearby neurons. Scientists combined luciferases with higher luminescence emission and higher light-sensitive opsins in search of an improved version of Luminopsins [25]. As of 2024, approximately 12-15 Luminopsins have been created. Currently, all luminopsins use two different marine luciferases: Gussia luciferase and Renilla luciferase[7]. Both catalyze the marine luciferin called coelenterazine. The polarity of

[6] The blood-brain barrier is a semi-permeable protective layer that prevents harmful substances like toxins or pathogens from entering the brain while allowing nutrients [36].

[7] Renilla luciferase is an enzyme derived from the bioluminescent marine organism Renilla reniformis that catalyzes the oxidation of coelenterazine, producing light as a byproduct [33].

luminopsin action can be determined by the electrophysiological properties of the coupled opsin They can either be excitatory or inhibitory luminopsin. There are two major types of Luminopsins: **Excitatory Luminopsins** and **Inhibitory Luminopsins.**

Excitatory Luminopsins

Excitatory luminopsins are primarily designed to activate cells or neurons by merging luciferases with an excitatory opsin, such as channelrhodopsin. For example, in 2013, Ken Berglund created the first luminopsin by fusing Chlamydomonas reinhardtii Channelrhodopsin-2 (CrChR2) with Gussia Luciferase. The resulting molecule, known as Luminopsin-1, demonstrated the potential for bioluminescence, although with limited efficacy. To improve the efficacy, scientists experimented with a different opsin, Volvox Channelrhodopsin-1 (VChR1), leading to the creation of Luminopsin-2 (LMO2). They chose Volvox Channelrhodopsin-1 because its light sensitivity is superior to that of ChR1. Even in the blue spectrum, VChR1 showed a 1% increase in efficacy, and it is often referred to as a red-shifted version of CrChR2 due to its optimal excitation by green light [26].

In 2016, the same research group developed the novel luminopsin, LMO3, by replacing the wild-type Gussia luciferase in Luminopsin-2 with a triple-mutant variant. This new variant is long-lasting and nearly ten times more bioluminescent. Thereby, the results of LMO3 were ten times more efficacious than LMO2. LMO3 activation was able to induce a behavioural change when expressed in Substantia Nigra[8]. In 2020, scientists advanced this trend by combining the brightest blue-emitting luciferase known, the M23 variant of Gussia luciferase, with Volvox channelrhodopsin, resulting in the formation of Luminopsin-4. Compared with the previous LMOs, Luminopsin – 4 showed a better activation efficacy by bioluminescence, and using this new version of luminopsin, scientists also figured out that controlling neuronal activity was more efficient in the vitro cultured neurons as well as in vivo in awake behaving rats. To further improve the versatility and efficacy of **LMOs**, Ken Berglund and his team used a complementary approach as a solution for the naturally dim bioluminescence by replacing the opsin with CrChR2 variants. Due to long wavelengths, these step-function variants have enhanced light sensitivity and slower deactivation. These Luminopsins were named **Step-function Luminopsins (SFLMOs)**. Step-function Luminopsins were more efficiently activated in comparison to the previous **LMO1-LMO3**. As expected, the scientists demonstrated that the step-function luminopsins controlled the vitro and rational behavior (referring to the decision-making process based on choices that result in the optimal level of benefit or utility for an individual) in awake rats [25][24].

Furthermore, Step-function Luminopsins also provide an additional layer of controllability through deactivation by longer wavelength light. It is worth to mention that all the opsins discussed in this section are non-selective cation channels The Figure 4 depicts the utilization of Chemogenetic Approach for activation. The light needed for the activation of Luminopsins is produced when the luciferase catalyses its substrate. The same molecule used in the figure to display the Optogenetic activation from the external light source.

[8] SN is a dopaminergic nucleus in the midbrain with important implications for motor movement and reward functions within basal ganglia circuitry.

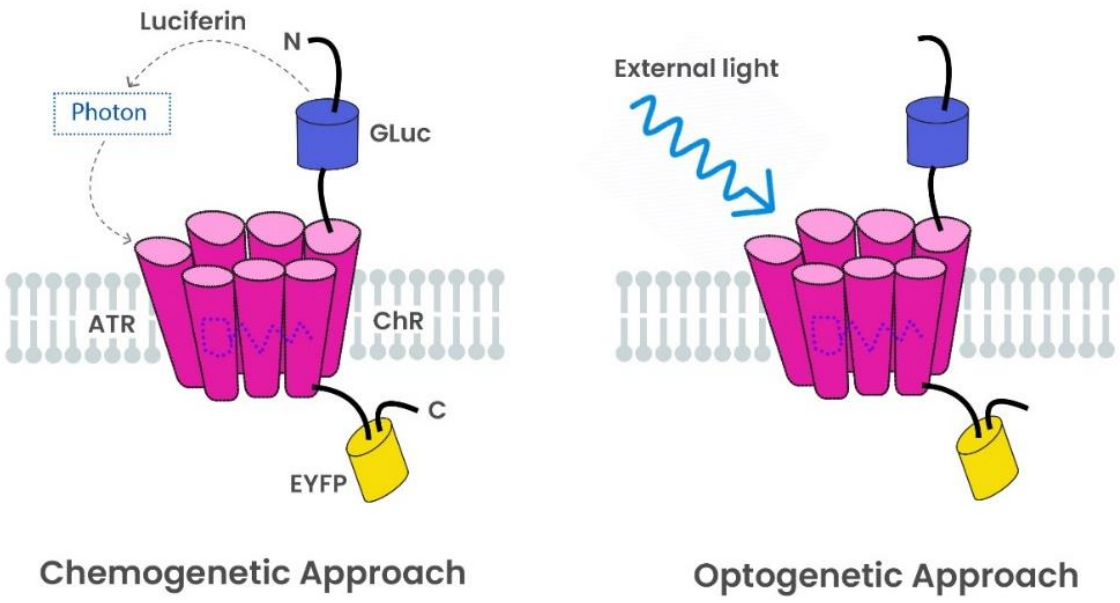

Figure 4: Chemogenetic and Optogenetic approach of Luminopsin Activation

Inhibitory Luminopsins

In contrast to the Excitatory Luminopsins, the Inhibitory luminopsins are designed to suppress cellular and neuronal activity. These luminopsins couple a luciferase with inhibitory luminopsins, such as halorhodopsin. Four inhibitory luminopsins have been discovered. Inhibitory Luminopsin-1 (iLMO1) and Inhibitory Luminopsin-2 (iLMO2) are created by fusing Np Halorhodopsin (NpHr) with engineered Renilla luciferase (RLuc) and Tag red fluorescent protein (RFP), respectively [24][27]. Inhibitory Luminopsin (iLMO) was formed by the fusion of a slow-burn variant of Gaussia Luciferase with a light-driven proton pump from Leptosphaeria maculans[9]. Inhibitory Luminopsin – 4 (iLMO4) consists of the M23 variant of Gaussia Luciferase with the improved chloride conducting channelrhodopsin. These inhibitory Luminopsin have been well demonstrated to bring out the neuronal inhibition in vitro and vivo in the presence of CTZ [25]. It is essential to mention that iLMO3 has not been officially published as an exception.

Applications of Optochemical genetics and Microbial opsins

Scientists have used channelrhodopsin to study the functioning of different parts of the brain by analyzing changes in animal behavior resulting from the depolarization of specific groups of neurons. One of the primary therapeutic applications of optochemical genetics is in treating depression. In 2014, scientists used Channelrhodopsin (ChR) to address a symptom of depression known as anhedonia, which is the inability to experience pleasure. By modulating Channelrhodopsin activity in the brain, they observed how specific brain activities influence behavior. One of the most remarkable aspects of ChR is that this light-sensitive protein has helped scientists analyze and understand various behavioural symptoms like anhedonia in depression. Furthermore, luminopsins could potentially be used as an alternative approach to delivering antidepressant effects. Microbial opsins could target neurotransmitters like serotonin or norepinephrine to positively impact mood.

[9] Leptosphaeria maculans is a fungal pathogen that causes blackleg disease in Brassica crops, particularly canola. It is also used in optogenetics for its light-driven proton pumps, like halorhodopsin, which enable neural inhibition upon light exposure. [34].

Secondly, Optochemical genetics is capable of targeting specific neuronal populations involved in motor control, such as the basal ganglia. By manipulating the activity of these neurons, researchers can potentially decrease the effect of the symptoms of Parkinson's disease or relieve the patient from them. Some symptoms consist of tremors and rigidity which could gradually be treated [20].

Conclusion

Optogenetics has made remarkable strides since the early 21st century, highlighted by the development of microbial opsins, including the discovery of channelrhodopsin, and the rise of hybrid technologies combining optogenetics and chemical genetics, known as optochemical genetics. This approach allows scientists to harness the benefits of both technologies while addressing key limitations in optogenetics, such as optical penetration and reduced spatial precision in deep or thick tissues. Luminopsins are combinations of light-sensing opsins that emit luciferases, enabling the generation of light internally. There are two types of luminopsins: excitatory and inhibitory. The excitatory luminopsins developed so far include luminopsin-1, luminopsin-2, luminopsin-3, luminopsin-4, step-function luminopsin-1, step-function luminopsin-2, and step-function luminopsin-3. The inhibitory luminopsins developed so far include inhibitory luminopsin-1, inhibitory luminopsin-2, and inhibitory luminopsin-4. Optochemical genetics offers a wide range of applications, including the treatment of depression, Parkinson's disease, and other neurodegenerative disorders. However, it still faces several challenges, with phototoxicity being one of the key concerns. High light intensity from luminopsins can potentially cause damage to brain tissue. Despite these limitations, researchers remain hopeful that advancements in the field will address these issues. The integration of light-sensitive proteins, such as microbial opsins, with chemical tools has the potential to greatly enhance neuroscience research.

Acknowledgements

I would like to extend my heartfelt gratitude to Greenwood High International School, Bangalore, for their unwavering support throughout the course of this research. My deepest appreciation goes to former Principal Mr. Aloysius D'Mello, whose initiative in establishing the mentorship program and assigning Dr. Sahana Rao as my mentor has been instrumental in the success of this work. Dr. Rao’s exceptional guidance, insightful feedback, and consistent encouragement played a crucial role in refining and developing this article. Her mentorship not only deepened my understanding of the subject but also significantly contributed to the intellectual rigor of this research. I am also thankful to the current Principal, Ms. Sheila Alexander, for her continued support and encouragement throughout this process.

About the Author

Nimit Akhawat is a Middle Years Programme (MYP) student at Greenwood High International School, Bangalore, under the IGCSE curriculum. He is deeply passionate about neuroscience and focuses on advanced studies in science, mathematics, and research methodologies. His research interests center around cognitive neuroscience, computational neuroscience, and molecular neuroscience, with the goal of exploring the depths of the human brain. Recognized for his academic achievements, Nimit holds records in **the India Book of Records, Asia Book of Records, Rising Youth Superstars of India** and actively shares his knowledge through YouTube channel **“Medical Learning with Nimit Akhawat”**. Currently, Nimit is particularly engaged in the fields of optogenetics and optochemical genetics, which leverage light and chemical signals to control cellular activity within living tissues. These cutting-edge areas are key to advancing neurological treatments and understanding brain circuit functions.

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