DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Expression and purification of Channelrhodopsin-2 in *Pichia pastoris*: generation of blue-shifted and red-shifted optogenetic variants

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Doutor João Peça (Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e supervisão académica do Doutor Professor Carlos Duarte (Departamento de Ciências da Vida, Faculdade de Ciências e Tecnologia, Universidade de Coimbra).

Fabio Mazza

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INDEX

ABBREVIATIONS

7-TM	seven transmembrane
Asp	aspartic acid
ATP	Adenosine-5'-triphosphate
ATR	*all-trans* retinal
BCA	bicinchoninic acid
BHK	baby hamster kidney
BMGY	Buffered Glycerol-complex Medium
BMMY	Buffered methanol-complex Medium
Bop	Bacterioopsin
BR	Bacteriorhodopsin
BSA	bovine serum albumin
Ca^{2+}	calcium
cDNA	complementary deoxyribonucleic acid
Chop1	Channelopsin-1
ChR1	Channelrhodopsin-1
ChR2	Channelrhodopsin-2
CIAP	calf-intestinal alkaline phosphatase
cRNA	complementary ribonucleic acid
Cys	cysteine
DCM	Direct Coupling Model
DDM	n-dodecyl-β-D-maltoside
DNA	deoxyribonucleic acid

dNTP	deoxyribonucleotide triphosphate
E	glutamic acid
ECL	extracellular loop
EDTA	ethylenediaminetetraacetic acid
Glu	glutamic acid
H^+	hydrogen
hChR2	humanized Channelrhodopsin-2
hCRBPII	human cellular retinol binding protein II
HEK293	human embryonic kidney 293
HR	Halorhodopsin
ICL	intracellular loop
K	lysine
LB	Luria-Broth
Lys	lysine
MeOH	methanol
$MgCl_2$	magnesium chloride
N	asparagine
NpHR	*Natromonas pharaonis* halorhodopsin
O.D.	optical density
PCR	polymerase chain reaction
PDB	Protein Data Bank
Phe	phenylalanine
PMSF	phenylmethylsulphonyl fluoride

PVDF	polyvinylidene difluoride
Q	glutamin
S	serine
Ser	serine
SFO	step-function opsin
SRI	Sensory Rhodopsin I
SRII	Sensory Rhodopsin II
T	threonine
TDDFT	time-dependent density functional theory
Thr	threonine
TM	transmembrane
Trp	tryptophan
UV	ultra-violet
VChR1	*Volvox* Channelrhodopsin-1
Y	tyrosine
YFP	yellow fluorescent protein
YNB	yeast nitrogen base
YPD	Yeast Peptone Dextrose
Y-PER	Yeast protein extraction reagent

KEY WORDS

Optogenetic

Channelrhodopsin-2

Light-activated channel

Mutagenesis

Neural activity

PALAVRAS CHAVE

Optogenética

Canal de rodopsina 2

Canal activado pela luz

Mutagénese

Actividade neuronal

SUMÁRIO

O sistema nervoso é constituído por diferentes tipos de neurónios que comunicam entre si formando uma rede complexa. A grande heterogeneidade desta rede proporciona um desafio à compreensão dos mecanismos e conexões neuronais. Desde o nascimento da neurociência, um dos grandes objectivos, foi e continua a ser, a possibilidade de exercer um controlo preciso sobre a actividade de diferentes tipos de neurónios para melhor perceber a sua função. A optogenética fornece uma estratégia para alcançar este objectivo. O termo "optogenética" refere-se à integração dos conceitos de óptica e genética para atingir ganho de funçao e perda de funçao de eventos específicos dentro de grupos de células particulares, embutidas em tecidos vivos, heterogéneos e complexos. Um papel-chave desta nova metodologia é desempenhado pelas *Channelrhodopsins*. Na natureza, estas proteínas transmembranares estão presentes na alga *Chlamydomonas reinhardtii*, onde são responsáveis pelo comportamento fototáxico deste microrganismo. Ao contrário de outras proteínas relacionadas, como por exemplo, a *Bacteriorhodopsin* (BR), *Halorhodopsin* (HR) ou rodopsinas sensoriais (SRs), que são conhecidas já desde 1980, a mais "recente" família de opsinas, as Channelrhodopsins, só foram descobertas em 2002. A *Channelrhodopsin-1* e *Channelrhodopsin-2* (ChR2) são membros da família de proteínas que ligam e usam o retinal (um aldeído da Vitamina-A) como cromóforo para responder à luz. A ChR2 é um canal activado por luz, que após iluminação com luz azul, permite a passagem passiva de catiões através da membrana celular. Quando expresso em neurónios, este canal permite assim a despolarização da célula. Esta propriedade faz com que este canal seja uma importante ferramenta para a optogenética. De facto, em 2005, um grupo de investigadores, expressou a ChR2 em neurónios de mamíferos e descreveu a capacidade de controlar, na ordem dos milissegundos, a transmissão sináptica excitatória e os potenciais de acção. A partir dessa altura, estudos adicionais foram apoiando a ideia do desenvolvimento de mais proteínas activadas pela luz, como um meio de controlar a excitabilidade neuronal e a transmissão sináptica em vário modelos *in vivo*, desde *Drosophila*, até murganhos transgénicos. Estes últimos em particular validaram a possibilidade de usar estas ferramentas para o mapeamento de circuitos neuronais complexos. Apesar do sucesso das ferramentas optogenéticas, as *Channelrhodopsins* apresentam ainda limitações, tais como, baixas correntes e forte inativação, o que não é ideal para certas aplicações biológicas, e uma absorção que normalmente não estende

para além de 520nm, o que limita a sua aplicação profunda em meios como o cérebro que exibem grande dispersão de luz.

A engenharia genética foi usada para obter variantes de *Channelrhodopsins* com algumas melhorias. O leque de ferramentas continua a ser expandido através do suplemento de novas variantes de *Channelrhodopsin* obtidas por várias estratégias (tais como, mutagénese dirigida, permute de domínios entre rodopsinas de diferentes espécies, modificações no N- e no C-terminal, e pesquisas em genomas para encontrar novas rodopsinas) que podem ser combinadas para aumentar o campo de desenvolvimento de ferramentas optogenéticas. Um contributo significativo para estas ferramentas ocorreu em 2012 com a cristalização e a descoberta da estrutura da *Channelrhodopsin*. Isto abriu a possibilidade de compreender a estrutura da proteína e evidenciar quais os aminoácidos envolvidos na formação do poro interno do canal e os que interagem com o cromóforo – o retinal. Mutações nos aminoácidos envolvidos na interação com o cromóforo, levantam a possibilidade de induzir um efeito de deslocação para o vermelho no espectro de absorção da *Channelrhodopsin*, e assim reduzir a sobreposição com o espectro da proteína não-mutada. Esta estratégia permite conseguir ferramentas para um controlo preciso de redes neuronais com conexões complexas.

O objectivo deste trabalho foi expressar, purificar e caracterizar novas variantes de ChR2. Começando por um estudo que prevê o efeito de mutações especificas no espectro de absorção de ChR2, foram selecionados dois mutantes da fenilalanina 269 (F269), um aminoácido muito perto da molécula de retinal. O primeiro passo foi a geração destes mutantes por mutagénese dirigida, usando *primers* específicos para a forma humanizada da ChR2. Após a confirmação dos resultados por sequenciação, os novos mutantes foram sub-clonados num vector para a expressão e purificação de proteínas num sistema de expressão heteróloga – a levedura *Pichia pastoris.* A sequência mutada foi integrada num vector contendo o promotor AOX1, que ao ser activado por metanol leva à expressão do gene de interesse. A transformação de *Pichia pastoris* por electroporação com o DNA de interesse originou um elevado número de transformantes. Apóa a electroporação, as células foram plaquedas em meio com concentrações crescentes de zeocina por forma a selecionar clones com múltiplos eventos de integração. As colónias selecionadas, foram depois crescidas ao longo de 24 horas em meio contendo metanol para induzir a expressão proteica.

Diversos protocolos foram testados com o objectivo de maximizar a recolha de ChR2 expressa. Dentro destes, o método de "*French Press*" foi o que apresentou maior eficiência. Finalmente foi também optimizado o sistema de purificação de proteína por cromatografia e a detecção proteica em gel pelo método do coloração com prata.

ABSTRACT

The nervous system consists in a complex network of interconnected cells. Its great heterogeneity provides a challenge for a deeper understanding of the neural mechanism and the connection in the brain. Since the birth of neuroscience, a main goal was, and still is, the possibility of having a precise control of the activity of specific types of neuron and to dissect their function. Optogenetics provides a good strategy to reach this purpose. The term "optogenetics" refers to the integration of optics and genetics to obtain gain or loss of function in well-defined events within specific cells in living tissue. A key role in the improvement of this new technique is played by Channelrhodopsins. These are transmembrane protein channels, naturally expressed in the algae *Chlamydomonas reinhardtii,* and are responsible for the phototaxis behavior in this microorganism. Unlike their "close relatives" Bacteriorhodopsin (BR), Halorhodopsin (HR) and Sensory rhodopsins (SRs), already known since the 1980s, the "latest" of the opsin family, Channelrhodopsins (ChRs), were discovered in 2002. Channelrhodopsin-1 and Channelrhodopsin-2 are members of the retylinidene family of proteins and, as all the protein belonging to this class, use retinal, a vitamin-A aldehyde, as chromophore to respond to light. Channelrhodopsin-2 is a blue-light activated channel that after illumination triggers a passive cationic conductance through the membrane. If expressed in neurons, the channel permits the depolarization of cell. This property makes this channel a very useful optogenetic tool. In fact, in 2005 a group of researchers, expressing ChR2 in mammalian neurons, described a reliable, millisecond-time scale control of neuronal spiking and excitatory synaptic transmission. From this point, additional studies supported the idea of developing light-activated signaling proteins to control neuronal excitability and modulate synaptic transmission *in vivo* models, from *Drosophila* to ultimately transgenic mice. These applications validate the power of ChR2 as an innovative tool to perform precise control over neuronal spiking and synaptic transmission and to have the possibility of mapping complex neural circuits. Despite this success as optogenetic tools, Channelrhodopsins still present some limitations. Small currents and strong inactivation properties are not ideal for some biological applications. Furthermore, an absorption spectra not normally extendible over 520 nm limits their use in high light-scattering environment such as the brain. Nevertheless, genetic engineering has been used to obtain Channelrhodopsin variants with improved features. This array of tools is continually expanded through the addition

of new Channelrhodopsin variants obtained by various strategies (site-directed mutagenesis, domain swapping between different Channelrhodopsin species, modification of N-termini and C-termini or genome mining to find new sequences) that can be combined to enhance the field of optogenetic tool development. A great contribute to the improvement of these tool came in 2012 with the publication of the crystal structure of Channelrhodopsin. This opened the possibility of a better comprehension of the protein structure, evidencing the amino acid residues involved in the formation of the inner pore, those responsible for the gating of the channel, and the amino acids that interact with the retinal, in the chromophore pocket. It seems that specific amino acid are involved in the interactions with the chromophore and that mutations of these amino acids may lead to the possibility of a shift in the absorption spectra of the channel, in order to reduce the overlapping of the wavelengths and to obtain a more precise control in the complex network of neural connections.

The aim of this work was to express, purify and characterize novel mutated variants of ChR2. Starting from a predictive study of mutations, we selected two mutants of the phenylalanine 269 (F269), an amino acid residue very close to the retinal binding pocket.

The first step was to perform a directed mutagenesis, using specific primers designed to induce the desired mutations in a humanized ChR2 sequence. After confirming the result through sequencing, we performed a ligation to subclone our novel mutants in a vector for the expression and purification of the proteins in the yeast *Pichia pastoris*. The mutated sequence was then integrated in frame with the promotor *AOX1*, which when activated by methanol induces the expression of our gene of interest.

We transformed the yeast with our mutated DNA through electroporation, yielding a high rate of transformants. After transformation, we plated the cells on Zeocin plates with varying levels of antibiotic concentration to select clones with multiple copy integration. The selected colonies were then grown in a liquid media with methanol for 24 hours, to induce the expression of the protein.

Different methods of protein extraction were tested in order to establish which one displayed most efficient extraction. We found that the French press yields the highest efficiency in protein recovery.

Finally we optimized the purification of the protein in a chromatography system and assess purity through silver staining.

CHAPTER 1 - INTRODUCTION

1.1. OPTOGENETICS

"And still we could never suppose that fortune were to be so friend to us, such as to allow us to be perhaps the first in handling, as it were, the electricity concealed in nerves, in extracting it from nerves, and, in some way, in putting it under everyone's eyes". With these words in 1791, Luigi Galvani, a professor of University of Bologna, introduced in his most famous publication, *De Viribus Electricitatis In Motu Musculari Commentarius*, his discovery: the first evidence of involvement of electricity in nerve conduction and muscular contraction (Piccolino, 1998). The pioneering experiments on frog by Galvani laid the first bases for the electrical stimulation of neurons (Piccolino, 1997). Although considerable progress has been attained, the need to have a precise control over the activity of specific neurons intermixed in the heterogeneous milieu of the brain tissue, is still a main goal in neuroscience. Neuronal stimulation with electricity, commonly used to study brain activity, is not able to completely reach this purpose because electrodes unspecifically stimulate all cells near the site of insertion. Another limitation is that direct stimulation cannot hyperpolarize cells and is therefore unsuitable to silence neurons (Deisseroth, 2010). Already in 1979, Francis Crick, wrote about the complexity of the brain and advanced the hypothesis that the major challenge for neuroscience is the need to precisely control the activity in one cell type while leaving the other unaltered (Crick, 1979). Crick later speculated that a good control tool for neuronal activity was light, but at that time there was no specific idea on how to make specific cells responsive to light (Deisseroth, 2011). This idea became real when it was investigated the possibility to express invertebrates photoreceptors, "receivers" of light, in neurons. Coexpression of a combination of *Drosophila* photoreceptor genes, called chARGe (formed by arrestin-2, rhodopsin and the α subunit of G protein) sensitizes vertebrate neurons to light (Zemelman et al., 2002). In parallel, researchers studying phototaxic microorganisms, in particular algae, found the molecular underpinnings of this phenomena, which years later would be of relevant importance because of their capacity to respond to light (Deisseroth, 2011). These organism express proteins that use light as source of energy and information about microbial environment. Later studies lead to identification of many members of this protein family that belong to the family of rhodopsins. The combination of these discoveries opened the way for a new technique known as "optogenetic". The term "optogenetic" refers to the integration of optics and genetic to

obtain gain or loss of function of well-defined events within specific cells of living tissue (Miesenbock and Kevrekidis, 2005, Deisseroth et al., 2006, Deisseroth, 2010, 2011, Miesenbock, 2011). The protagonists of this new technical approach are the rhodopsins, a class of membrane proteins responsible for the transduction of light stimulation. One well-characterized rhodopsin is the Bacteriorhodopsin (Oesterhelt and Stoeckenius, 1971), a single-component ion pump. Soon after, Halorhodopsin, a light-gated chloride pump was also discovered (Matsuno-Yagi and Mukohata, 1977, Bogomolni and Spudich, 1982, Schobert and Lanyi, 1982). These two rhodopsins can be considered the precursors of the entire class of new rhodopsins which have now been brought to the scientific knowledge. The latest discovery were Channelrhodopsins (Nagel et al., 2002, Nagel et al., 2003), light-gated channels activated by blue light that can be used in neuroscience as an optogenetic tool to mediate action potentials and control complex neuronal circuits (Hegemann and Nagel, 2013).

1.2. OPSIN PROTEIN FAMILY

Opsins are a group of light sensitive membrane receptors, belonging to the retinylidene protein family. Each opsin protein requires the incorporation of retinal, a vitamin-A aldehyde, as chromophore to enable light sensitivity. The complex opsin-retinal is known as rhodopsin. Different members of opsins are found in prokaryotic and eukaryotic organisms. The family can be divided into two big groups that, even if not homologous, share some sequences homology evolved towards proteins with similar forms and functions. These two groups are:

- Type I opsins or microbial opsins;
- Type II opsins or animal opsins.

Type I opsins genes are present in lower organisms such as prokaryotes, algae and fungi where they are responsible for different functions (phototaxis, energy storage, development and retinal biosynthesis) (Spudich, 2006). Type II opsins are mainly present in higher organism and are responsible for vision and some of this proteins seem to be involved in circadian rhythm and pigment regulation (Sakmar, 2002, Shichida and Yamashita, 2003). Both families encode for seven transmembrane (7-TM) proteins with an internal pocket where retinal binds to the protein. The retinal molecule is covalently fixed in the binding pocket and forms a protonated Shiff base with a lysine residue

located in the seventh transmembrane helices. One of the most important difference between type I and type II opsins is the retinal binding. Type I opsin genes encode for proteins that use retinal in all-*trans* configuration. After photon absorption retinal changes conformation to a 13-*cis* configuration and, once activated, it reconverts to the all–*trans* state without dissociating from the protein. Type II opsin genes encode for G–protein coupled membrane receptors and bind retinal in a 11-*cis* state. After illumination and photoabsorption, retinal isomerizes to an all-*trans* configuration and, after some changes in protein structure, it allows the beginning of a transducing cascade. In this case the retinal in all-*trans* configuration diffuses from the protein and a new 11-*cis* retinal is recruited for a new signaling cascade (Fenno et al., 2011). Another important difference between the two classes is in the functional mechanism: type I opsins are direct light-gated ion channels or ionic pumps (Figure 1.1), type II opsins are G-protein receptors (Zhang et al., 2011).

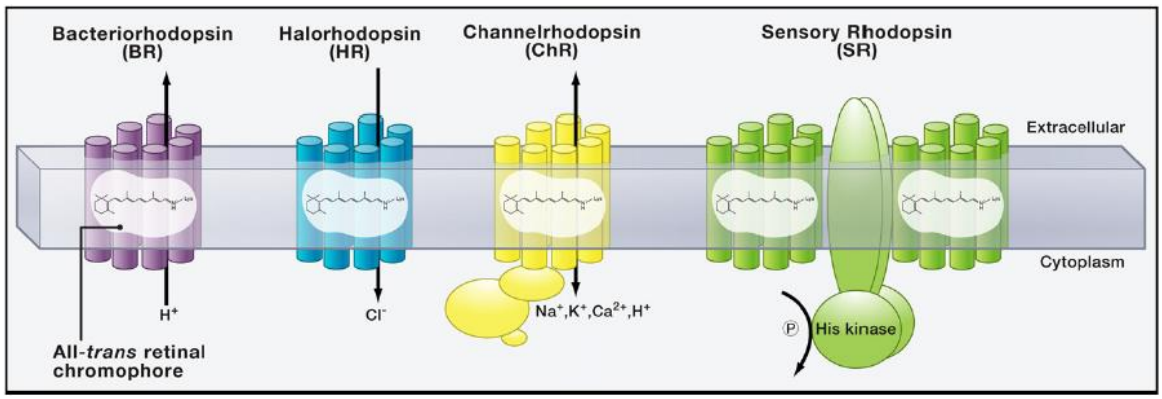

Figure 1.1. Schematic representation of the members of the type I opsins.
The figure represents several members of microbial type I rhodopsins formed by 7-TM segments. BR is a proton pump which transfer protons from the intracellular side to extracellular side of the membrane. HR is a chloride pump that transfer ions to the inner side of the cell. ChRs are channels that conduct cations across the membrane along the electrochemical gradient. SRs forms a complex with a transducer (Htr) and bind a His-kinase that controls a phosphorylation system that regulates cellular flagella. From (Zhang et al., 2011).

1.3. MICROBIAL RHODOPSINS

Since their discovery, microbial rhodopsins have elicited significant interest and have been intensively studied. The first microbial rhodopsin discovered was the Bacteriorhodopsin (BR) isolated for the first time in the *Halobacterium salinarum* (Oesterhelt and Stoeckenius, 1971). Bacteriorhodopsins, in contrast to animal visual rhodopsins pigments (which are G protein-coupled receptor), are light-activated pumps

capable of protons translocation from the intracellular side to the extracellular side, maintaining in this way a proton gradient across membrane to permit ATP synthesis and recovery of energy under low-oxygen conditions (Michel and Oesterhelt, 1976). Starting from BR, additionally retinal-binding protein were identified and described as behaviorally relevant photosensors in *Halobacterium salinarum*. In fact, between the late 1970s and the half of the 1980s other three members of this class of protein were identified: Halorhodopsin (HR) (Matsuno-Yagi and Mukohata, 1977, Schobert and Lanyi, 1982), Sensory Rhodopsin I (SRI) (Bogomolni and Spudich, 1982) and Sensory Rhodopsin II (SRII) (Wolff et al., 1986). Several studies focusing on haloarchaeal rhodopsin produced significant insight into the mechanistic aspect of these proteins. Presently, bacteriorhodopsins are some of the best characterized membrane-embedded proteins. Nevertheless, and despite showing significant structural similarities, these proteins display different functions. Halorhodopsin is a chloride pump that transport chloride ions from the extracellular side to the internal part of the cell (Schobert and Lanyi, 1982), while sensory rhodopsins I and II are phototaxis receptors controlling the cell's swimming behavior in response to light intensity and color (Hoff et al., 1997). Until 1999 four different microbial retynilidene proteins, identified in *Halobacterium salinarum,* were known: Bacteriorhodopsin and Halorhodopsin, which function as ion pumps, generating an ion gradient across the membrane, and Sensory Rhodopsin I and Sensory Rhodopsin II, which act as photoreceptors to find favorable light conditions (Spudich et al., 2000). It was initially thought that these rhodopsins were present only in Archaea, but with the genome sequencing and analysis of several microorganisms it became apparent the widespread presence of proteins homologous to archaeal rhodopsins in organisms of the other domains of life, Bacteria and Eukarya (Bieszke et al., 1999, Sineshchekov et al., 2002, Jung et al., 2003). Microorganisms containing rhodopsin genes are present in many environments and comprise a wide phylogenetic range of microbial life, including haloarchaea, proteobacteria, cyanobacteria and green algae. In all these classes of organism, rhodopsins share common structural characteristics and in particular they show a strong conservation of amino acid residues, particularly in the retinal-binding pocket. This homology defines the large phylogenetic class, called type I rhodopsins, that distinguish in functions from the type II rhodopsins class in which are included visual pigments and related retinylidene proteins of higher organisms (Spudich et al., 2000).

1.4. CHANNELRHODOPSINS

Channelrhodopsins are light-gated membrane channels belonging to the protein family of microbial type rhodopsins, which include rhodopsins of several organisms such as archaea, eubacteria, cyanobacteria, fungi and algae. They are the first light-gated ion channels known in nature, identified for the first time in the green alga *Chlamydomonas reinhardtii*. These channels are photoreceptors that promote ion transport across the membrane, leading to different behavioral responses of the microalga to the light.

1.4.1. The discovery of ChR2

Chlamydomonas reinhardtii and other green algae show two different motility responses to light: phototaxis and photophobic response (Witman, 1993, Hegemann, 1997). Phototaxis is the capacity of an organism, depending on the light intensity of the environment, to move away or towards a light source thanks to molecular mechanisms used for detection of light direction. In algae, phototaxis is defined as the oriented swimming of cells along the direction of a light beam. The photophobic response is defined as the reorientation of swimming induced by an abrupt increase of light intensity (Witman, 1993). This behavior response in microalgae, unicellular flagellated organisms, is possible thanks to a system of photoreception called the eyespot (Hegemann, 1997) (Figure 1.2).

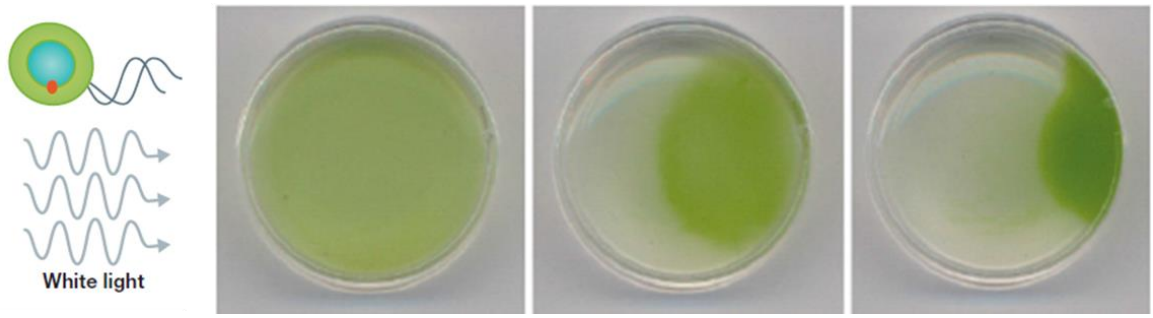

Figure 1.2: Phototaxis response of *Chlamydomonas reinhardtii*
Phototaxis behavior of *C. reinhardtii* wild type strain in culture exposed to a light source. Adapted from (Hegemann and Nagel, 2013)

In *Chlamydomonas*, the eyespot is directly connected to the flagella. Usually the alga has a rotational movement around his own axis and when the eyespot detects the light it signals to the flagella to alter the beating plane leading in this way to a change of the position from perpendicular to parallel respect to the light (Schaller et al., 1997). Later studies demonstrated involvement of ion fluxes in this behavioral responses, proving an involvement of Ca^{2+} in regulation of flagella beating (Schmidt and Eckert, 1976). Peter Hegemann and his group clarified this mechanism by demonstrating that there are two photocurrents, one that occurs in the eyespot (photoreceptor current) and one in the flagella (flagellar current), triggered by Ca^{2+} flux. Hegemann showed that in both cases, photocurrents are mediated by Ca^{2+} and are suppressed by Ca^{2+} channel inhibitors, suggesting that the photoreceptor and the flagellar current are carried by Ca^{2+} and are part of a rhodopsin-regulated signal transduction chain responsible for the cellular behavior under different light conditions (Harz and Hegemann, 1991, Holland et al., 1996, Holland et al., 1997). Together, these studies lead to the idea that photocurrents are mediated by rhodopsins acting as photoreceptor and to the direct coupling model (DCM) which propose a directly linkage of the photoreceptor with the ion channel and the formation of a single protein complex that can became permeable to ionic species upon illumination (Hegemann, 2008). In this context, the discovery of the Bacteriorhodopsin (BR), the first single-component light-driven proton pump, was relevant (Oesterhelt and Stoeckenius, 1971). Presently, Bacteriorhodopsin is considered one of the best-understood rhodopsin. Nagel et al in 1995 studied this rhodopsin in animal cells using oocyte of *Xenopus laevis* and with a voltage-clamp technique they obtained the analysis of light induced photocurrents of the pump (Nagel et al., 1995). Then, using improvements in cDNA data banks and sequencing DNA, genomes of several microbial-new types rhodopsins were discovered. Among these new identified sequences there are two long sequences from *Chlamydomonas* very similar to those of Bacteriorhodopsin. These sequences codified for two type-I opsins later named Channelrhodopsins-1 (ChR1) and Channelrhodopsins-2 (ChR2) that act as light–driven channels, differently from the other type-I opsins that act as light – driven ion pumps (Bacteriorhodopsins and Halorhodopsins). In 2002 a single-protein membrane channel responsive to blue light was discovered by Nagel et al. (Nagel et al., 2002). This was the first Channelrhodopsin described. Searching in a *C. reinhardtii* database, a cDNA sequence that encoded for an opsin-related protein was found. This protein, named Channelopsin-1 (Chop1), showed significant sequence similarity with sensory

rhodopsins (SRs), Bacteriorhodopsins (BRs) and Halorhodopsins (HRs), and high conservation of amino acid residues forming the retinal-binding pocket. The expression of the cRNA encoding Chop1 in *X. laevis*, in the presence of *all*-trans retinal demonstrated this protein is a selective channel for protons that opens after absorption of light (acting as a combination of photoreceptor and ion channel) and mediates a H^+ current (Nagel et al., 2002). In 2003 Nagel and colleagues performed studies using Channelrhodopsin-2 to probe if they were able to obtain a functional rhodopsin channel. They expressed the Chop2 sequence from *C. reinhardtii* genome database, in *Xenopus laevis* oocytes in the presence of retinal. Testing full-length ChR2 and a ChR2 fragment (comprising only amino acid 1-315, i.e. missing part of the intracellular C-terminal tail) their results established that in the presence of these proteins, there are photocurrent not normally present in non-injected oocytes. This photocurrent varied in size and direction with membrane potential and with the content of cations in solution, suggesting that light triggers a cationic conductance of ChR2. Expressing the ChR2 fragment (a.a. 1-315) in HEK293 and BHK cells they found the same properties of conductance independently from the host system (Nagel et al., 2003). In these two types of cells, ChR2 produced a passive cation conductance after illumination in a manner similar to the conductance produced in *Xenopus* oocytes. They also showed that ChR2 directly mediates the passive cation conductance without involving any soluble or downstream messenger as demonstrated by the fact that after photostimulation there is a rising in photocurrent within a few milliseconds, suggesting that the activation of the channel is independent of a diffusible transmitter and that ChR2 is itself a cation channel (Nagel et al., 2003). These studies were the starting point to better understand Channelrhodopsins and reveal their functions and biophysical characteristics, the authors showed that the expression of ChR2 in mammalian cells may be used as tools for depolarization of cells upon illumination.

1.4.2. Structure

All rhodopsins are retinylidene proteins that share similar structural characteristics. They are composed of an opsin apoprotein and a retinal chromophore (Spudich et al., 2000). Channelrhodopsin apoproteins are constituted of 737 amino acids. The N-terminal part is composed of ~300 a.a. arranged in seven transmembrane helices. These seven transmembrane domains form an internal pore where retinal is bound. This

binding is responsible for the light-gated properties of the channel. The remaining ~ 400 a.a. are in the C-terminal regions which does not influence channel functions (Nagel et al., 2003). For this reason these 400 a.a. are normally omitted in almost all the variants of ChRs used for optogenetic purposes. The first structural information about Channelrhodopsins were obtained through homology modelling studies based on known structures from microbial rhodospisns , as Bacteriorhodopsin and Halorhodopsin (Plazzo et al., 2012, Watanabe et al., 2012). In 2005, soon after the complete sequencing of *C.reinhardtii*, researchers detected overlapping partial cDNA sequences encoding for opsin-related proteins, named Channelopsins (Chop1 and Chop2), which had a core region with sequence similarity (15 – 20%) to the Archeal sensory rhodopsins and the ion transporters bacteriorhodopsin and halorhodopsin (figure 1.3).

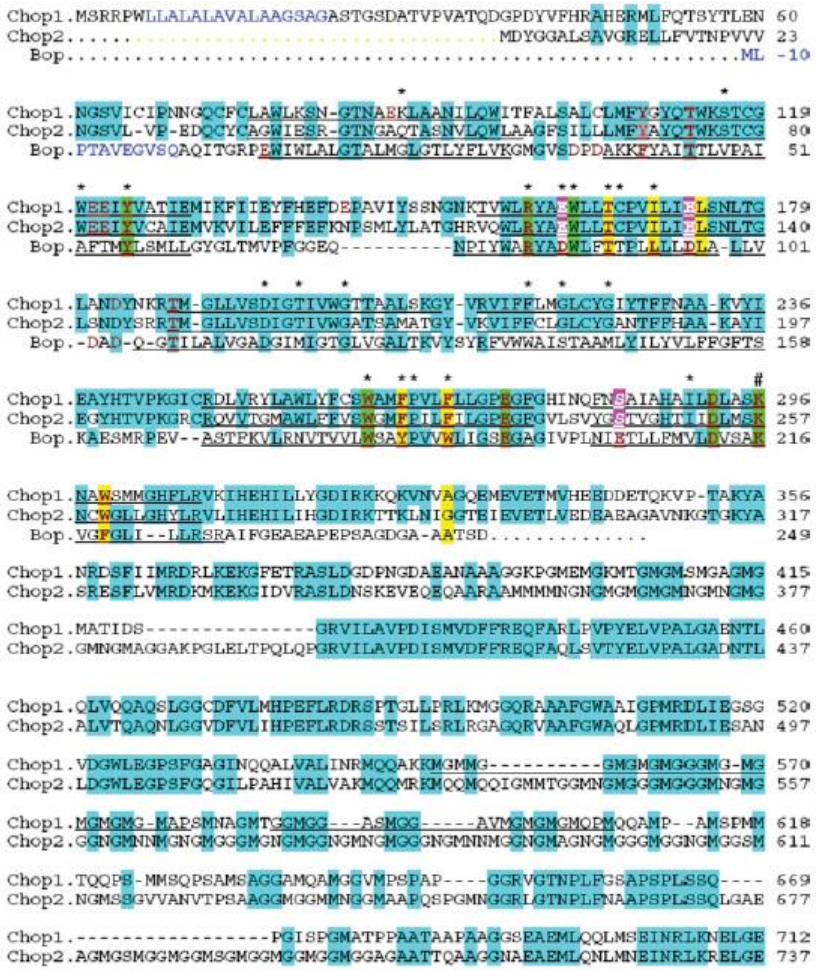

Figure 1.3. Sequence comparison of Chop1, Chop2 and Bop.
Alignment and comparison of the amino acid sequences of three related proteins: Chop1 and Chop2 from *C. reinhardtii*, and Bop of bacteriorhodopsin from *H. Salinarium.* Amino acids of BR that are known to interact with retinal are indicated with an asterisk. Amino acids highly conserved in most microbial rhodopsin are highlighted in green, the one that are functionally

homologous in microbial opsin sequences are highlighted in yellow and other identities are highlighted in blue. Amino acid that contributes to H^+-conduction in BR are shown in red. The key substitutions are shown in white on purple background. Underlined regions indicate hypothetical or identified 7-TM regions. Adapted from (Nagel et al., 2005b)

The consensus motif LDXXXKXXW suggests that Lys^{296} in Chop1 and Lys^{257} in Chop2 are amino acid residues that bind the retinal molecule. Of the 22 amino acid that are in contact with the retinal in Bacteriorhodopsin, 60% are identical or conservatively exchanged in Chop1 and Chop2 (Nagel et al., 2005b). In 2011, the first experimental determination of a Channelrhodopsin structure was resolved by electron crystallography at 6Å resolution. The projection map of ChR2, obtained by cryo-electron microscopy of two-dimensional crystals grown from pure, heterologously expressed protein revealed that ChR2 consists of seven transmembrane helices similar to the other microbial rhodopsins, as shown by the comparison with projection map of Bacteriorhodopsin (Muller et al., 2011). Finally in 2012, the first atomic structure of ChR was published (Kato et al., 2012). This work highlighted the structural characteristics of a novel chimeric construct consisting of the last two TMs of ChR2 and the first five TMs of ChR1, again excluding the C-terminal part of ~400 a.a. This construct was named C1C2. The truncated chimaera C1C2 (residues 1-342) is composed of an N-terminal domain in the extracellular side of the membrane, seven transmembrane domains (TM1-TM7) connected by three cytoplasmic loops (ICL1-ICL3) and three extracellular loops (ECL1-ECL3), and the C-terminal intracellular domain. It was also found that two C1C2 protomers associate to form a dimer through interactions in the N-terminal regions that form inter-protomer disulphide bonds (figure 1.4a). The authors found that within each C1C2 protomer there are 6 lipids and 43 water molecules. The crystal structure of C1C2 revealed also the presence in the N-terminal domain of β-sheets that might contribute to dimer stabilization (Kato et al., 2012). The primary sequence of ChR is similar to BR and the other microbial rhodopsins and in accordance with this similarity, C1C2 structure superimposes well on the BR structure (Figure 1.4b), nevertheless, the end part of TM7 is shifted towards the central axis of the monomer (Kato et al., 2012). The functional aspect of this characteristic is still unclear but it might contribute to stabilize the intracellular C-domain through hydrogen-bonds. Another important distinct feature is that extracellular ends of TM1 and TM2 are tilted outward in respect those of BR. These tilts enlarge the cavity formed by the seven

transmembrane motif, allowing water influx for the transport of cations (Kato et al., 2012).

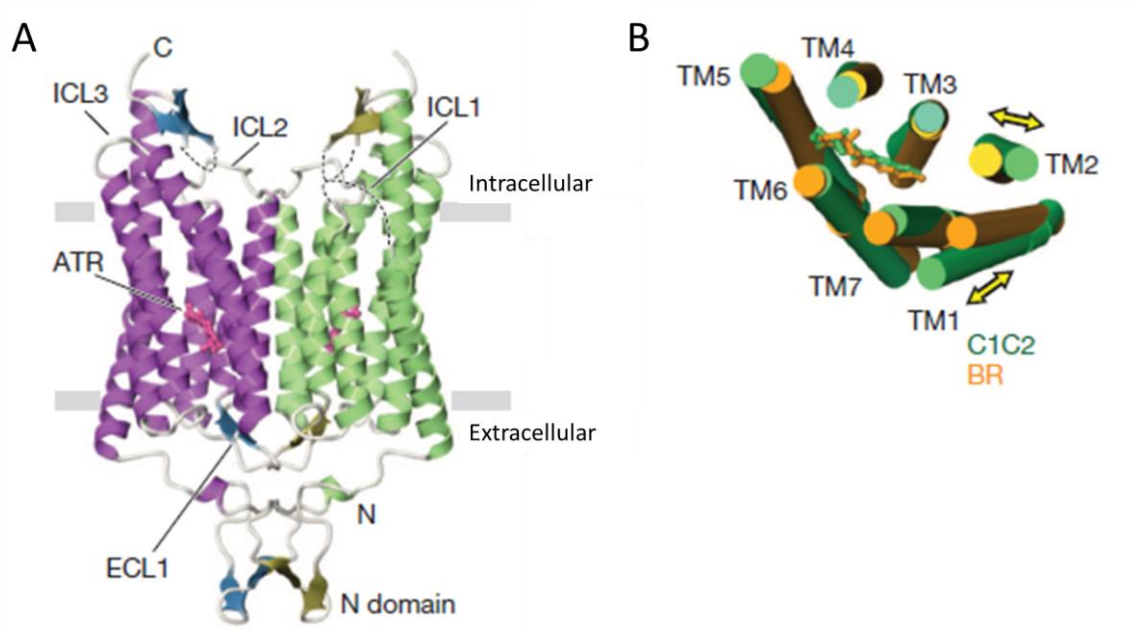

Figure 1.4. Structure of C1C2 and comparison with BR.
(A) Crystal structure of C1C2 dimer subunits with cromophore (ATR). (B) Superimposition of C1C2 transmembrane segments (green) and BR domain (yellow). Shifts of TM1 and TM2 are indicated with double arrows, adapted from (Kato et al., 2012)

1.4.2.1. Electronegative ionic pore

The seven transmembrane domains of the functional part of the channel are arranged to form an inner pore responsible of the intrinsic ion transport activity. Channelrhodopsins are cation transporters. The permeability to cations is due to the electrostatic interactions that occur within the pore. Structure modeling studies predicted the presence of residues that may contribute to the formation of a cavity in the channel (Watanabe et al., 2012). The cavity expands between TM1, TM2, TM3 and TM7 forming an intruding electronegative pore that is rich in charged and polar residues. The major contribute to the pore comes from five glutamic acid residues of TM2 (Glu82, Glu83, Glu90, Glu97 and Glu101) (Watanabe et al., 2012). The crystal structure published by Kato et al confirmed this model showing that 12 polar residues are aligned in the channel forming a hydrophilic electronegative surface. These residues, especially those on TM2, define ion conductance and selectivity as shown by some recent mutational studies (Ruffert et al., 2011). Also, the importance of the negative amino

acid residues disposed along the pore was underlined by several point mutations (Kato et al., 2012). These mutants showed loss in ion selectivity and conductance. Besides the mentioned electronegative side chains, other polar residues are also located in the central part of the pore (Q56, T59, S63, E83, K93, T246, N258 and Y70). These residues form two constriction sites in the cytoplasmic side of the channel and are responsible for the gating of the channel through their interactions (Kato et al., 2012).

1.4.2.2. Retinal binding pocket

Retinal is a polyene chromophore derived from vitamin A. It is also known as retinaldehyde or vitamin A aldehyde. All proteins of retinylidene family share the common feature of binding a retinal molecule. As in all the microbial-type rhodopsin, *all-trans* retinal is covalently linked to a lysine residue (Lys296) in TM7, via a protonated Schiff base, connecting to the protein through a N=C bond. Other five aromatic residues (Trp163, Phe217, Trp262, Phe265 and Phe269) are located around the Lys296 forming a hydrophobic pocket for the retinal, whereas Cys128, Thr198 and Ser295 form a less-hydrophobic pocket that may contribute to the color shift of retinal (Figure 1.5) (Kato et al., 2012). This structure is the light–sensing moiety of the channel and the retinal is responsible of the absorption properties of the protein. Usually, when isolated, retinal absorb in the UV range (λ_{max}=380 nm) but this values can change depending on the environment around the retinal, inducing an effect called opsin shift. Even if not yet completely understood, it seems that the shift in spectra absorption and the wavelength regulation is a result of conformational manipulation of the chromophore and electrostatic interactions that occurs in the retinal binding pocket (Welke et al., 2011), as demonstrated by a study on human retinol binding proteins (hCRBPII) mutants (Wang et al., 2012), it is possible to a shift the absorption profile of the *all-trans* retinal by more than 200 nanometers over across the visible spectrum.

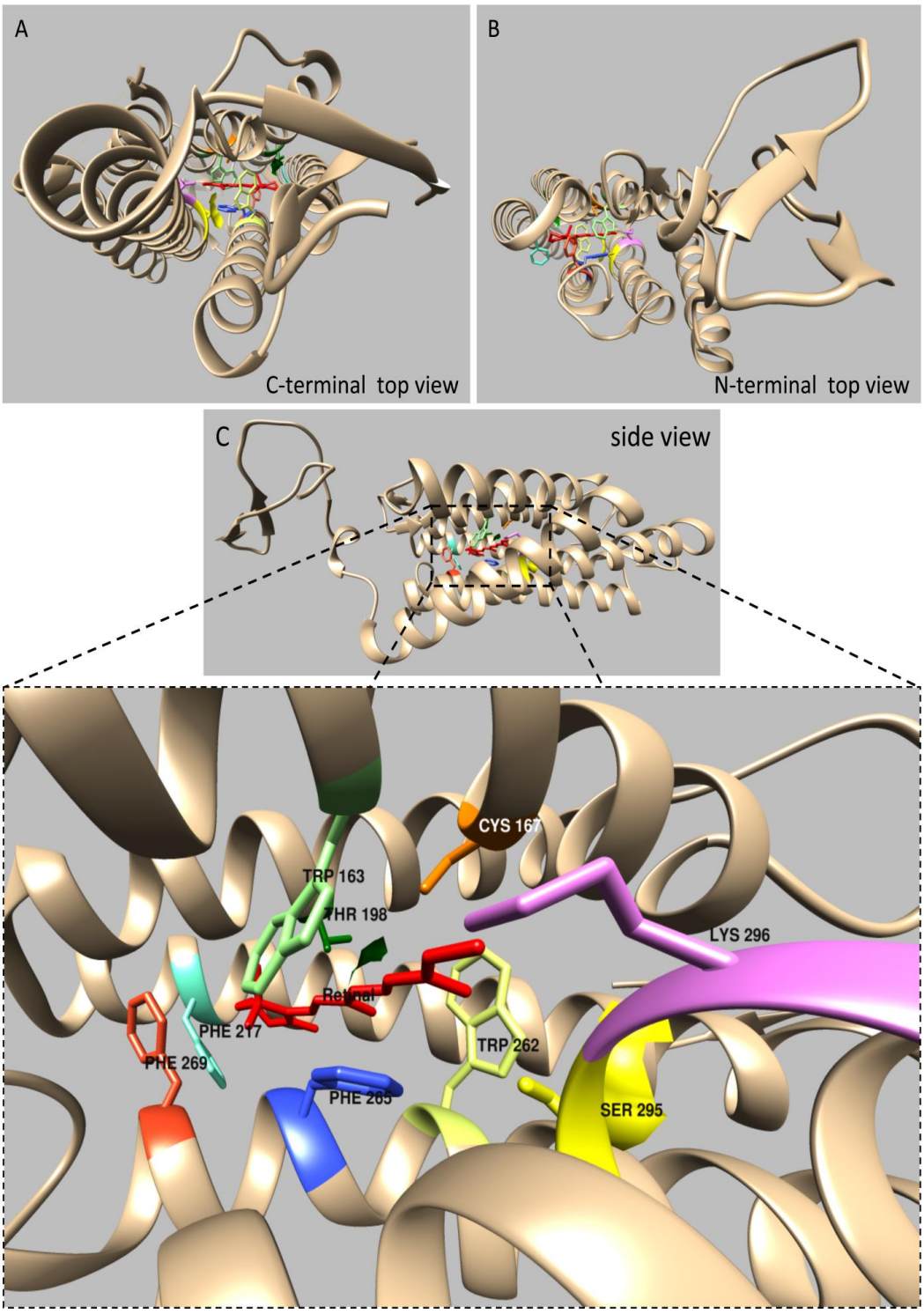

Figure 1.5. 3D structure of the C1C2 chimaera.
(A) and (B) Top view of the C-terminal and N-terminal domain. (C) Global side view of the chimaera structure. The retinal binding pocket is highlighted in the inset (C),. The amino acids forming the pocket and their respective structures are depicted in different colors. The retinal structure is represented in red.

When present in type I opsins, after light illumination retinal isomerizes and changes its configuration from *all-trans* to 13-*cis* which for all the microbial rhodopsins relatives is the first event in light response (Figure 1.6).

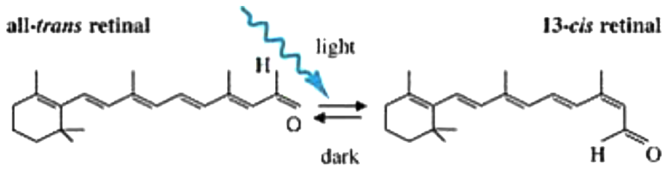

Figure 1.6. Isomerization of *all-trans* retinal.
Activation by photoisomerization of all-*trans* retinal to 13-*cis* retinal at wavelengths of 470 nm. After photoisomerization, the covalently bound retinal spontaneously relaxes to *all-trans* in the dark, providing closure of the ion channel and regeneration of the chromophore. From (Wong et al., 2012)

Isomerization of retinal is accompanied by structural changes of the protein which in the case of Channelrhodopsin leads to its activation and opening of the channel with a consequent influx of cations and the change in membrane potential. Finally, it was also showed that C1C2 chimeric protein conserves the same arrangement as BR, in which a water molecule receives a proton from the protonated Shiff base and donates this proton to an amino acid near the binding pocket. In C1C2 this proton acceptor is Asp253 for its proximity to the Lys257, as confirmed also by mutational studies (Kato et al., 2012).

1.4.3. Channelrhodopsin-2 photocycle

Even before the discovery and publication of the crystal structure of ChR2, several studies focused their attention on the mechanisms of the channel (Hegemann et al., 2005, Bamann et al., 2008, Nikolic et al., 2009). In fact, one of the main question in respect of light-gated ion channels concerns the coupling between light activation and protein action and how the spectral properties can be correlated with the opening and the closing of the channel (Bamann et al., 2008). This correlation between the states of the channel and the spectral absorption characteristics is known as “photocycle”, which is a representation of the molecular mechanisms controlling changes in the structure and permeability of the channel. It is now understood that the triggering event of this cycle is the absorption of a photon leading to light-induced isomerization of retinal from *all-*

is the absorption of a photon leading to light-induced isomerization of retinal from *all-trans* to 13-*cis* and its thermal isomerization to the initial *all-trans* state (Hegemann and Moglich, 2011). This cyclic reaction contains a series of thermally unstable intermediates and the entire reaction is governed by light energy absorption and storage of part of this energy in the protein. Part of this energy is used to drive the photocycle, and the other part is transformed in force necessary for ion transport (Haupts et al., 1997). For the well-known light-driven pump bacteriorhodopsin, the proton translocation is strictly coupled to the photocycle (Bamann et al., 2008). During the photocycle, the Schiff base plays a central role (Lanyi, 2004). After illumination and isomerization of retinal, Schiff base deprotonates and releases the proton toward the extracellular side of the membrane. As result, a blue-shifted state with an absorbance of 410 nm is observed. Then there is a reprotonation of the Schiff base from the intracellular side with the formation of red-shifted state, absorbing at 570 nm (Bamann et al., 2008). Similar intermediates are formed in the photocycles of SRI and HR (Haupts et al., 1997). On the basis of this photocycle, multiple studies were performed to better understand the ChR2 photocycle, that is acknowledged to be significantly different from other photocycles in most type I opsins. In the case of ChRs, light-induced processes after illumination are not directly linked to the ion transport but are responsible for changes in the protein structure. It is thought that light absorption by retinal induces the isomerization of *all-trans* to 13-*cis* form and this light-induced processes at the retinal site is transduced to a conformational change in the protein structure leading to the open state and to the passive flow of cations through the membrane (Bamann et al., 2008). In 2003, in parallel with functional expression studies demonstrating that ChR2 is a light-gated ion channel, the first model of for its photocycle was also proposed (Nagel et al., 2003). Starting from this first proposal of a three-state hypothetical model for ChR2 cycle (Nagel et al., 2003), several others models have been suggested (Hegemann et al., 2005, Nikolic et al., 2006, Ernst et al., 2008). Even if not yet thoroughly understood, the cyclic mechanism that presently has gained more traction is the six-state model (Figure 1.7) (Ritter et al.). According to this model, it is possible to identify three different states of ChR2: the non-conductive but excitable state; the active or conductive state; and the non-conductive, desensitized state. When in the dark, the channel is in a dark-adapted state that is a non-conductive but excitable state, with an absorption spectra between 450 and 470 nm. This state is called D470 and its absorption spectra indicates that the Shiff base between the retinal

and the protein is protonated. After light excitation the dark state is converted (~ 1,5 ms) to a conductive state via two fast intermediates: P500 and P390. P500 is formed in few nanoseconds (~ 50 ns) after illumination, but still maintains the protonated Shiff base. After only 25 µs, as a result from the deprotonation of the Shiff base, there is the conversion to the P390 state. The P390 state decays with a time constant of ~ 1 ms into a third photoproduct named P520. This photointermediate corresponds to the conductive state of the channel, as demonstrated by the fact that its formation and relaxation are in parallel with the rising and the falling of the photocurrent (Bamann et al., 2008). P390 and P520 states are in a pH-dependent equilibrium. P520 converts after 10 ms in a P480 intermediates, which relax in less than one minute back to the dark adapted state. Since the reconversion to the dark-adapted state is the slowest step of the photocycle, lasts intermediates can accumulate under particular light conditions (continuous light) leading to light-adapted forms of ChR2. The P480 state is the non-conductive and desensitized state. In reality, the recovery to the dark state proceeds via two P480 sub-intermediates: $P480_a$ and $P480_b$. The formation and decay of these two subspecies of P480 depend on changes in hydrogen bonding of Glu90. $P480_a$, formed from P520, cannot be directly reactivated by light. On the other hand $P480_b$ is photoreactive and respond to 480 nm light. In this last step, in $P480_b$, the major structural rearrangements take place and lead to the reformation of the initial dark-adapted state. Although widely studied, the photocycle is still not fully understood and some aspect are unknown. Some steps can be skipped under defined light conditions with some shortcuts, such as the conversion of $P480_b$ directly in P500 without the reformation of the D470 or the direct formation of the dark state from P520. These modification of the cycle led to the possibility of having ChR2 variants or mutants that show specific adapted state, as the SFO (step-function opsins) mutants which are able to remain in the P520 state for a longer period.

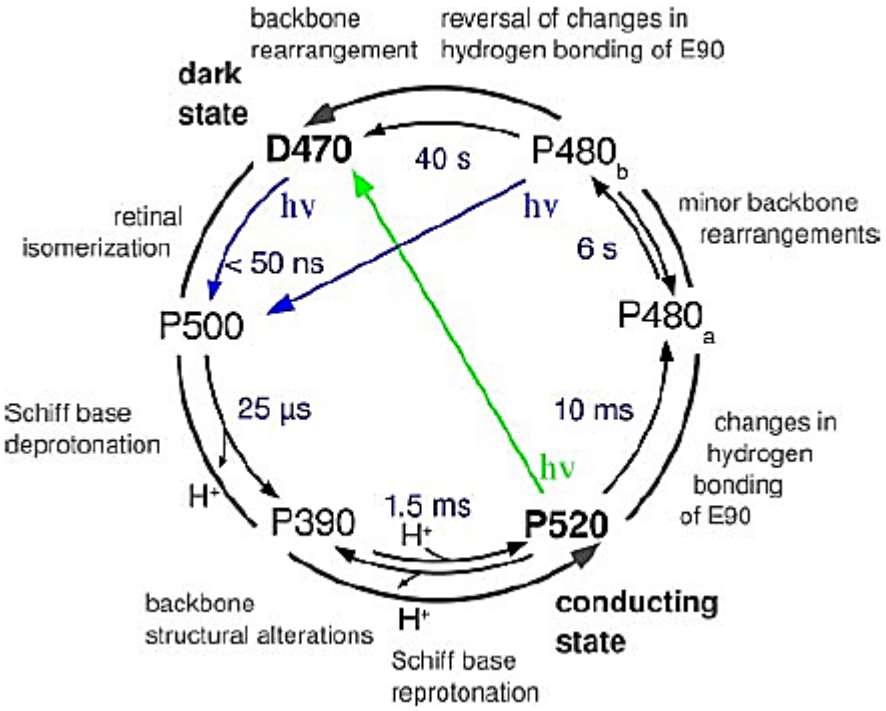

Figure 1.7. Six-state photocycle model.
The D470 dark state is converted, after light excitation and isomerization of retinal into the intermediate P500, that after deprotonation of the Schiff base, it is converted into the P390 state. This state decays in a third photoproduct, P520, that correspond to the conductive state of the channel. From the P520 state, the recovery to the dark state proceedes via two intermediates, $P480_a$ and $P480_b$, sub-intermediates of the P480 state. In $P480_b$, the major structural rearrangements take place and lead to the reformation of the initial dark-adapted state. Shortcuts of the photocycle are indicated with the arrows. The dark state can be directly recovered from P520 with green light (green arrow) and $P480_b$, which is a photoreactive intermediate, can be converted by light to the P500 intermediate. From (Ritter et al., 2008)

All the information about Channelrhodopsins from crystal structure study of Kato et al and from the six-state model of photocycle from Ritter et al brought a better understanding of the structure and the functional mechanisms of the channel, creating even greater interest in this tool.

1.5. APPLICATION TO NEUROSCIENCE

Progress with ChRs have led to more studies reaching a deeper knowledge of this class of proteins and dispersing the idea of using this channel as tool in science and neuroscience. One key advantage of application using optogenetic strategies derives from the possibility of introducing engineering channels in neurons with the aid of

engineered viruses. The neurons expressing the channel acquire the capacity to respond to light under a strict control. In fact, many modified optogenetic tools are becoming commonly used for specific control of spatially restricted or genetically defined locations in the brain. The first evidence of the introduction of Channelrhodopsin-2 gene into mammalian neurons dates back to the summer of 2005, when Boyden and colleagues described a reliable, millisecond-time scale control of neuronal spiking and excitatory synaptic transmission thanks to the activity of ChR2 (Boyden et al., 2005). Additional work, following this seminal paper appeared in subsequent years to support the idea that it was possible to develop light-activated signaling proteins to control neuronal excitability and modulate synaptic transmission (Li et al., 2005). Optogenetic tools were engineered for delivery to cultured hippocampal neurons (Boyden et al., 2005), followed by mammalian cell lines (Ishizuka et al., 2006) until finally in live primate brain (Han et al., 2009), in which the expression of ChR is a valid, promising and potentially non-invasive alternative to unspecific cell stimulation with electrodes. At the same time, it provide a methodology for investigating the causal role of specific cell types in neural computation, cognition, and behavior, opening up the possibility of a new ultraprecise neurological study. Several additional models validate the capacities of ChR2 as innovative tool to a deeper study and understanding of the complex mechanisms at the basis of the brain and the neural transmission. In fact, ChR2 photoactivated strategies were implemented in different *in vivo* model systems. One example are studies on *C. elegans*, in which the expression of ChR2 in excitable cells (neurons and muscles) can elicit strong responses (Nagel et al., 2005a). In this study, a ChR2 mutant fused with YFP was expressed in muscle and mechanosensory neurons and when transgenic animals were illuminated with blue-light they showed strong and simultaneous contractions of all muscle cells causing a visible shrinking of the body. Technical approaches using ChR2 has proven to be versatile and suitable also for other organisms, such as *Drosophila*, in which ChR2 was used to investigate behavioral response of the insect to specifics odors and to understand which neurons are involved in the attractive and repulsive responses to odors (Schroll et al., 2006, Suh et al., 2007). Another evidence of the capacity of ChR2 in the interpretation of neural mechanisms came from Zebrafish studies (Douglass et al., 2008). In this case ChR2 was expressed in somatosensory neurons involved in the encoding of tactile stimuli and was showed that a light-pulse induced an escape behavior. A limitation in these experiments was the *all-trans* retinal, which is not naturally produced by invertebrates, had to be provided in

food or in the environment (Nagel et al., 2005a, Zhang et al., 2007b). On the other hand, most vertebrates present enough all-trans retinal to trigger reliable photostimulation of the ChR2, without adding any chemical or gene (Zhang et al., 2006). Motivated by these findings, studies on the application of ChR2 in neurobiology progressed to the generation of transgenic mice expressing the construct ChR2 fused to yellow fluorescent protein (YFP) in a specific subset of neurons of the central nervous system. This opened the way to use the power of ChR2 to map neural connectivity in brain tissue for in vivo activation and mapping of neuronal circuits (Arenkiel et al., 2007). Another paper demonstrated that the same engineering transgenic can be used to map neural connectivity in mammalian brain and visualize the spatial distribution of synaptic circuits (Wang et al., 2007). All these results demonstrated the potential of using optical tools in better understanding neural activity and transmission in brain. Channelrhodopsins therefore provided a single-component strategy to activate or inhibit defined neural pathway with potential implications in studies of neurological disease. Its success led to testing other types of rhodopsin, such as *Natromonas pharaonis* halorhodopsin (NpHR), a light driven chloride importer and membrane hyperpolarizer activated by yellow light. This pump act as suppressor of action potentials and can be co-expressed with ChR2 to perform bidirectional control of neurons and neural pathway with cell-type specificity, high temporal precision and rapid reversibility (Figure 1.8) (Zhang et al., 2007a). Threfore, NpHR and ChR2 form a complete system for the multimodal, high-speed, genetically targeted, all-optical interrogation of living neural circuits (Zhang et al., 2007b).

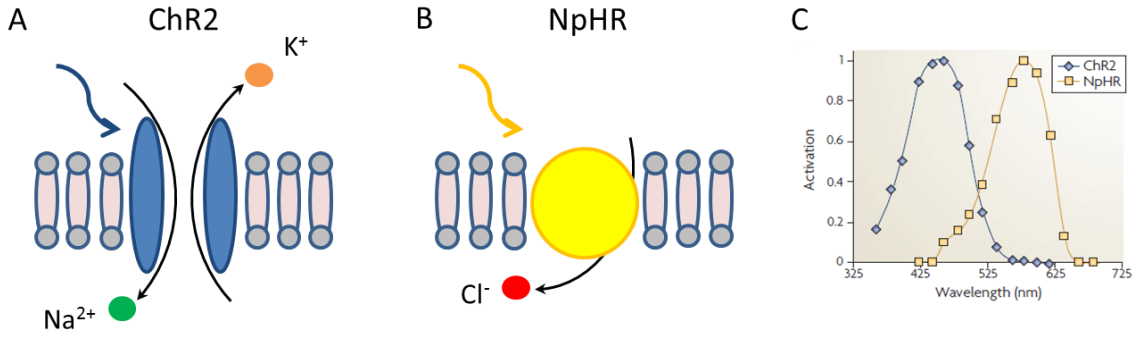

Figure 1.8. ChR2 and NpHR.
(A) and (B) Schematic cartoon of the optogenetic tools ChR2 and NpHR. ChR2, activated by blue light, allows entry of cation, mostly Na^{2+}, into the cell. NpHR, activated by yellow light, allows the entry of Cl^-. (C) Action spectra for ChR2 and NpHR. The absorption peak of ChR2 is ~470 nm, the one of NpHR is ~580 nm. The two excitation maxima are separated by ~100

nm,so the two opsins activate independently with different light. Adapted from (Zhang et al., 2007a).

1.6. CHANNELRHODOPSIN VARIANTS: A TOOLBOX IN EXPANSION

Despite the success that Channelrhodopsins obtained recently as optogenetic tools, they present small currents and strong inactivation properties that are not ideal for some biological applications. Moreover, the natural absorption spectra of Channelrhodopsin does not normally extend over 520 nm, limiting in this way their use in high light-scattering medium as the brain (Hegemann and Moglich, 2011). In recent years different strategies were used to obtain Channelrhodopsin variants with improved features to overcome the limitations in Channelrhodopsin's biological applications. Nowadays the set of single-component optogenetic tool available for neuroscientists is wide enough to contain several tools of different categories: fast excitation, fast inhibition, bi-stable modulation and control of biochemical signaling in neurons and other cells. This array of tools is continually expanded through the addition of new Channelrhodopsin variants with improved features obtained using various strategies (site-directed mutagenesis, domain swapping between different Channelrhodopsin species, modification of N-terminal and C-terminal regions, genome mining to find new sequences) that can be combined to enhance the field of optogenetic tool development (Hegemann and Moglich, 2011). All the tools result from molecular engineering and allow experimental manipulation tuned for the desired physiologic effect, the desired kinetic properties of the light-dependent modulation and the required wavelength, power and spatial extent of the light signal to be deployed (Yizhar et al., 2011). Currently, several Channelrhodopsin variants are designed using sequencing data from new algal genomes or applying molecular engineering to known opsins to improve their usability in a wider range of organisms. The newly discovered variants aim to improve channel proprieties, resulting in higher conductance, faster or slower kinetics, different times of desensitization and modified permeability (Lin, 2011). For example, the H134R mutants, in which histidine-134 is switched for an arginine, has a modest reduction in desensitization, an increase in light selectivity and slower channel closing. All these changes lead to an increase in the photocurrent, but the slower channel-closure kinetic defines a less precise temporal precision (Nagel et al., 2005a). Similarly, other

modifications, as T159C (Berndt et al., 2011) or L132C (Kleinlogel et al., 2011), result in an increase in the photocurrent and a slower closure kinetic of the channel. In other variants, called ChETA, modifying ChR2 glutamine 123 in threonine or alanine, was found to accelerate the closure kinetic with a consequent faster photocycle, increasing the accuracy of a fast optogenetic control (Gunaydin et al., 2010). Another class of ChR variants comprises mutants with a modification of cysteine 128 with threonine, alanine or serine. These "step-function opsins" (SFO) or "bi-stable opsins" enable bi-stable, step-like control of neuronal membrane potential with the rising of a stable photocurrent which can be terminated with a pulse of yellow light (Berndt et al., 2009). Thus, until now, these "ultrafast" and "ultraslow" variants, obtained with a mutation of some specific amino acid residues, offer the possibility of control over the timing and duration of stimulation (Deisseroth, 2010). More optogenetic devices were added when in 2008, investigations on the genome of another algal specie, *Volvox carteri*, produced a different Channelrhodopsin (VChR1), which respond to yellow light (Zhang et al., 2008). A Channelrhodopsin with similar characteristic was also reported in *Mesostigma viride* algaes (Govorunova et al., 2011). The discovery of VChR1 was the starting point of the idea to expand the absorption spectrum of the Channelrhodopsins, not only scanning the natural world to find novel types of proteins but also mutating amino acid residues that are closer or inside the retinal binding pocket of the already known ChR2. One good characteristic of retinal-binding proteins is the versatility of absorption bands and the fact that the absorption features can be shifted using molecular techniques to confer different spectral characteristics to the protein (Hegemann and Moglich, 2011). Rapidly accelerating genomic and molecular engineering efforts are continually made to expand the optogenetic toolkit in a range that goes over the visible spectra. To all these approaches can be also added the genesis of chimeras and the combination of different mutants to cover the entire set of possibility of improving many and various characteristics of this class of proteins. In the Figure 1.9 all the known variants are disposed in a graph that correlate the peak of absorption and the closure time constant.

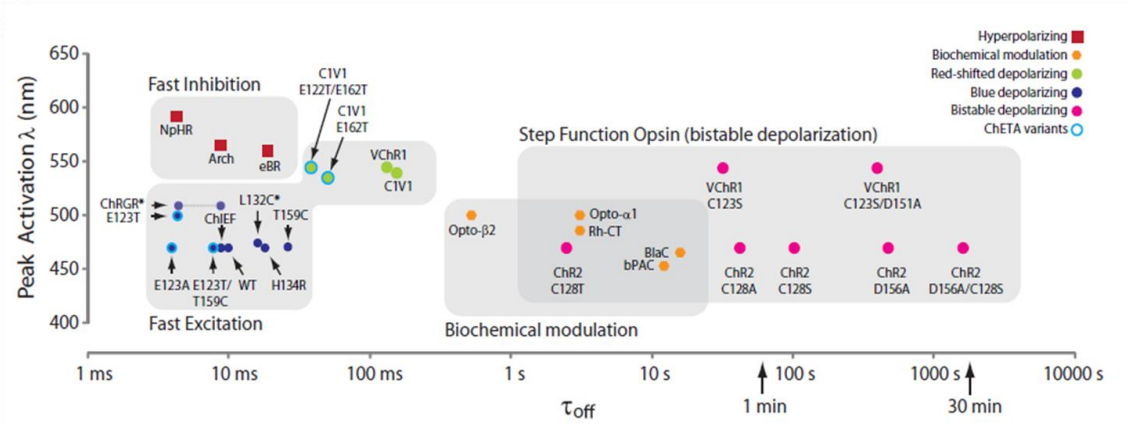

Figure 1.9. Schematic representation of the known single-component optogenetic tools. Peak activation wavelenghts plotted against kinetics of decay of single-component optogenetic tools. From (Yizhar et al., 2011).

Since 2005, considerable efforts have been made to expand the capabilities and properties of microbial-opsins as optogenetics tool using genomic discoveries, molecular engineering, opsin targeting and optical-device development. The importance of opsins as a research tool grew rapidly, and they are used in more than 800 laboratories around the world (Deisseroth, 2011). Channelrhodopsin allows the manipulation of membrane potential in genetically defined cells with light, unlocking new possibilities in neuroscience and other fields of research. Channelrhodopsin is becoming an invaluable and helpful research tool, as indicated by the many studies that are focusing their attention on this channel and the increasing reports in the literature about this new analytical tool. Despite these good capacities, considerable information still needs to be unearthed to achieve a better understanding of Channelrhodopsins and their use. Therefore, while many ChR variants have been already developed, many features need to be exposed and some characteristics optimized to generate better tools. To reach this purpose new variants should be developed and compared with the already existent Channelrhodopsins to collect enough information and to produce novel variants facilitate the dissemination and applicability of this new tool.

OBJECTIVES

Channelrhodopsin-2 is a light-gated channel, discovered in the green alga *Chlamydomonas reinhardtii* (Nagel et al., 2003). When stimulated by light, the channel changes its conformation leading to the inflow of cations through the cell membrane. Since its discovery, Channelrhodopsin-2 (ChR2) has been used as a research tool to depolarize membranes of excitable cells using light. The first report of the introduction of ChR2 gene into neurons in culture (Boyden et al., 2005) opened the way to several new possibilities also employing ChR2. Neverhtless, and despite this success, ChR2 still presents several limitations in its application to neuroscience research. For this reason, molecular engineering approaches have introduced several ChR variants with improved features. Presently, a variety of mutants are already known (Yizhar et al., 2011), however, many features in the currently available tools require a further characterization or an optimization of their desirable properties. In this contest, a significant contribution was given by the recent discovery of the crystal structure of Channelrhodopsin (Kato et al., 2012). This data now allows for a deeper comprehension on the interactions between opsin and the retinal molecule.
Manipulations in the pocket environment where retinal is present, provides a strategy to modify the absorption spectra of the channel (Wang et al., 2012) and thus gives the possibility of modulating the receptivity of ChR2 to a wider variety of excitation wavelengths.

In this work, we investigated the possibility of generating new ChR2 variants through a directed mutagenesis techniques and expression in heterologous system. More specifically we performed:

- Directed mutagenesis in a specific site of the humanized ChR2 sequence to switch the codon codifying the phenylalanine 269 with a codon codifying aspartic acid (D) or histidine (H);

- Cloning of the mutants F269D and F269H in a vector (pPICZ A) suitable for heterologous expression of the protein in yeast;

- Optimization of expression and purification protocols for WT-ChR2.

CHAPTER 2 – MATERIALS AND METHODS

Primers		Sequence
F269D_mut_FW	Forward	5’ GTTCCCAATTCTCGACATTTTGGGGC 3’
F269D_mut_REV	Reverse	5’ GCCCCAAAATGTCGAGAATTGGGAAC 3’
F269H_mut_FW	Forward	5’ GTTCCCAATTCTCCACATTTTGGGGC 3’
F269H_mut_REV	Reverse	5’ GCCCCAAAATGTGGAGAATTGGGAAC 3’
Seq_FW	Forward	5’ CATTTTTTCACGCCGCCAAAGC 3’
Seq_REV	Reverse	5’ GGCTTCGTCTTCGACGAGAGTC 3’
yChR2_FW	Forward	5’GCCGAATTCAAAAATGTCTGACTATGGCG GCGCTTTGTC 3’
yChR2_REV	Reverse	5’ GCCGGTACCGGCGGCCGCTGGCACG 3’
AOX1_FW	Forward	5’ GACTGGTTCCAATTGACAAGC 3’
AOX1_REV	Reverse	5’ GCAAATGGCATTCTGACATCC 3’

Table 2.1. List of used primers.

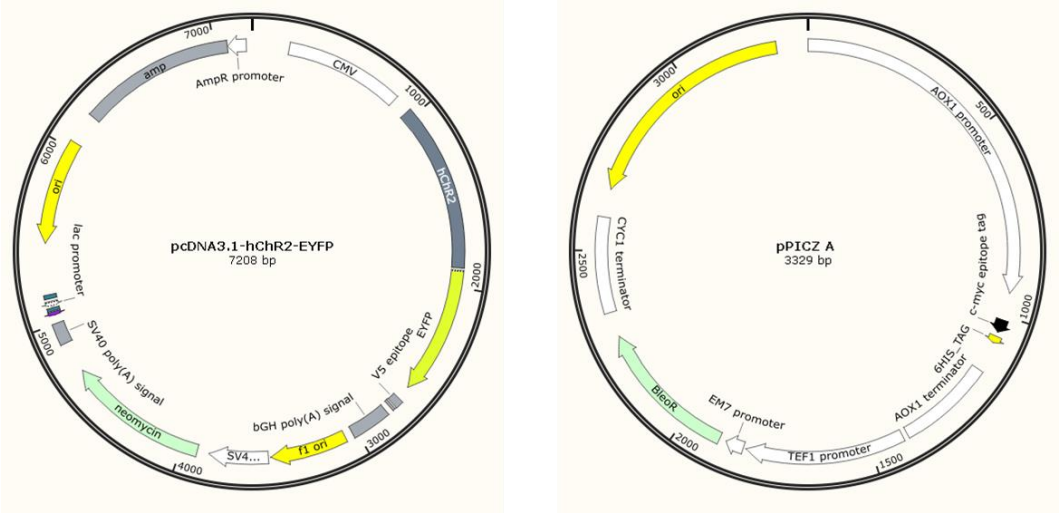

Figure 2.1. Maps of the used vectors.

2.1. DIRECTED MUTAGENESIS

Data about humanized Channelrhodopsin-2 protein sequence are accessible on the Protein Data Bank (PDB) with the accession number 3UG9. The target for the mutation is the phenylalanine amino acid in the position 269 (F269) that is close to the retinal binding pocket. One aim was to generate mutations that switch the phenylalanine with aspartic acid (D) and histidine (H), which are amino acids belonging to different groups.

2.1.1. Primers design

Primers were designed using the vector pcDNA3.1-hChR2-EYFP (addgene 20939) as DNA template (Figure 2.1), this vector contains the humanized version of ChR2. The "TTC" codon codifying for F269 is in the position 1650 of the plasmid sequence. The primers F269D_mut_FW, F269D_mut_REV, F269H_mut_FW and F269H_mut_REV used for site-direct mutagenesis (Table 2.1) were designed using PrimerX, specifying the codon target of mutation. The codon for phenylalanine, "TTC" was substituted with either a "CAC" codon codifying for aspartic acid or a "GAC" codon codifying for histidine. The primers were synthesized by Stabvida.

2.1.2. PCR reaction

For PCR reaction the Takara PrimeSTAR HS was used and optimized to amplify the pcDNA3.1/hChR2-EYFP plasmid (Figure 2.1). 8 reactions were performed for each mutation. Each reaction contained: 10 µl of 5x PrimeSTAR buffer (Takara), 4 µl dNTP mix, 1 µl of each primers (with a concentration of 0.2 µM), 1 µl of DNA template (pcDNA3.1-hChR2-EYFP, with a concentration of 4 ng/µl), 0.5 µl of PrimeSTAR HS DNA polymerase (2.5 U/µl; Takara) and dH_2O to a final volume of 50 µl.

REAGENTS	VOLUMES
5X PrimeSTAR buffer	10 µl
dNTP mix	4 µl
F269D_mut_FW	1 µl
F269D_mut_REV	1 µl
Template DNA	1 µl
DNA polymerase	0.5 µl
dH_2O	32.5 µl

Table 2.2. Reagents used for PCR for the mutation F269D.

REAGENTS	VOLUMES
5X PrimeSTAR buffer	10 µl
dNTP mix	4 µl
F269H_mut_FW	1 µl
F269H_mut_REV	1 µl
Template DNA	1 µl
DNA polymerase	0.5 µl
dH_2O	32.5 µl

Table 2.3. Reagents used for PCR for the mutation F269H.

PCR programs were used as follows:

- 98°C for 30 seconds
- 98°C for 30 seconds
- 65°C or 62°C for 30 seconds
- 72°C for 8 minutes and 30 seconds

} 18 cycles

PCR amplification was confirmed by running the products on an agarose gel (0.7%). Positive reactions were digested with *Dpn*I enzyme for 2 hours and 30 minutes to remove parental methylated DNA. Digested DNA was then analyzed on agarose gel (0.7%) to retrieve the positive PCR band. A Zymoclean Gel DNA Recovery kit (Zymoclean Research) was then used to recover digested DNA from agarose gel. The purified DNA was used to perform bacteria transformation.

2.1.3. Bacteria transformation

LB-agar plates were prepared to grow the transformed bacteria. The plates made, had LB-agar composition of 2.5% LB (Fisher scientific) and 1.5% agar (Fisher scientific). The medium was then autoclaved and let cool just enough to handle the flask and add Ampicillin (100 µg/ml) for the antibiotic selection. A thin layer of LB-agar (~10 ml) was poured in sterile Petri dishes that then were gently swirled in circular motion to spread the agar medium, avoiding the formation of bubbles. The plates were left at room temperature until LB-agar solidified and then stored at 4°C. The transformation was performed using the heat shock protocol using *DH5α E. Coli* cells as follows. After thawing the cells, 10 µl of purified DNA from mutagenesis PCR was added. After 30

minutes in ice, a heat shock was given at 42°C for 45 seconds. After that the cells were left in ice for 5 minutes. 900 µl of room temperature LB (or SOC) was added and the eppendorfs placed at 37°C for 60 minutes in an incubator with rotation (400 rpm). After a centrifugation, pellets were resuspended and spread on plates. Plates were left growing overnight at 37°C.

2.1.4. DNA extraction

After transformation and growing, colonies were picked from plates and grown overnight in LB plus ampicillin (100 µg/ml). Pure plasmid DNA was obtained with a NZYMiniprep kit (NZYtech), doing two steps of elution with 15 µl of dH_2O for each step.

2.1.5. Sequencing

DNA was sent to STABvida for sequencing using Seq_FW primer and Seq_REV (Table 2.1) primers.

2.2. CLONING INTO pPICZ A VECTOR

To express the ChR2 sequence in yeasts, a specific vector is necessary. The vector used in this work was the pPICZ A from Invitrogen (Figure 2.1).

2.2.1. PCR reaction to amplify Channelrhodopsin-2 sequence

A PCR reaction was performed to amplify the specific Channelrhodopsin DNA sequence with the desired mutations from the pcDNA3.1 vector (Figure 2.1.) and adding restriction sites to subclone hChR2 and our novel mutants into the pPICZ A vector (Figure 2.1.). A common set of primers, yChR2_FW and yChR2_REV (Table 2.1) was designed to add a 5' *EcoR*I and 3' *Kpn*I restriction sites and a yeast Kozac Sequence (AAAAATGTCTG) was added to the 5' region to increase expression. The PCR reaction mix contained: 10 µl of 5X PrimeSTAR buffer (Takara), 4 µl of dNTP mix 2.5 mM, 1 µl of forward primer 10 µM, 1 µl of reverse primer 10 µM, 1.5 µl of template DNA (~300 ng), 1 µl of PrimeSTAR HS DNA taq polymerase (Takara) and dH_2O to a final volume of 50 µl.

REAGENTS	VOLUMES
5X PrimeSTAR buffer	10 µl
dNTP mix	4 µl
yChR2_FW	1 µl
yChR2_REV	1 µl
Template DNA	1.5 µl
Taq polymerase	1 µl
dH_2O	31.5 µl

Table 2.4. PCR reaction mix.

Cycling conditions used were:

- 98°C for 30 seconds
- 98°C for 10 seconds
- 55°C for 10 seconds
- 72°C for 2 minutes

} 35 cycles

- 72°C for 5 minutes

1 µl of the reaction was run on 1% agarose gel to confirm the amplification. Then the reactions were digested with 1 µl of proteinase k (Invitrogen) for 30 minutes at 55°C and the DNA was finally purified and concentrated with the DNA clean and concentrator kit from Zymoclean (Zymo research), doing two steps of elution with 6 µl of dH_2O.

2.2.2. Digestion of vector pPICZ A and PCR products

To insert the ChR2 sequence in the vector pPICZ A, two digestion were performed: one for the vector and one for the PCR products. The vector was digested for 3 hours at 37°C. The mix used in the digestion contained: 4 µl of DNA of vector pPICZ A (~1 µg), 3 µl BSA (bovine serum albumin) 10X, 3 µl of NEB buffer 1 10X (New England Biolabs), 1 µl of the restriction enzyme *EcoR*I (New England Biolabs), 1 µl of the restriction enzyme *Kpn*I (New England Biolabs) and 18 µl of dH_2O. After the digestion, 10 µl of Cutsmart buffer (New England Biolabs), 1 µl of CIAP (Calf-intestinal alkaline phosphatase; New England Biolabs) and 59 µl of dH_2O were added to the previous mix and incubated for 2 hours at 37°C to remove phosphate groups from vector. The

plasmid DNA was then purified with a PCR clean and concentrator kit from Zymo research, doing two steps of elution with 10 µl of dH_2O.

REAGENTS	VOLUMES
DNA	~1 µg
BSA (10X)	3 µl
NEB buffer 1 (10X)	3 µl
*EcoR*I	1 µl
*Kpn*I	1 µl
dH_2O	up to 30 µl

REAGENTS	VOLUMES
Cutsmart	10 µl
CIAP	1 µl
dH_2O	59 µl

Tables 2.5. Mix of vector digestion and mix to remove phosphate groups.

DNA amplified by PCR was digested for 3 hours at 37°C using the following mix: 10 µl of DNA, 5 µl of BSA 10X, 5 µl of NEB buffer 1 10X (New England Biolabs), 1 µl of *EcoR*I (New England Biolabs), 1 µl of *Kpn*I (New England Biolabs) and dH_2O up to 50 µl. After the digestion the whole volume of the reaction was run on an agarose gel (1%). The band corresponding to the ChR2 sequence (~900 bp) was cut from the gel and the DNA extract with the Zymoclean Gel DNA recovery kit from Zymo Research, using two steps of elution with 6 µl of dH_2O.

REAGENTS	VOLUMES
DNA	10 µl
BSA (10X)	5 µl
NEB buffer 1 (10X)	5 µl
*EcoR*I	1 µl
*Kpn*I	1 µl
dH_2O	28 µl

Table 2.6. Reagents used in the digestion of DNA amplified.

2.2.3. Ligation

A reaction of ligation was performed to insert ChR2 sequence in the vector. This reaction was left overnight at 16°C and then at room temperature for 2 hours. The mix contained: 1 µl of vector DNA, 3 µl of DNA to insert, 1 µl of T4 ligase buffer 10X (NZYtech), 1 µl T4 ligase (NZYtech) and 4 µl of dH_2O.

REAGENTS	VOLUMES
Insert DNA	3 µl
Vector DNA	1 µl
T4 Ligase buffer 10X	1 µl
T4 Ligase	1 µl
dH_2O	4 µl

Table 2.7. Mix of ligation.

2.2.4. Transformation on LB + Zeocin plates

Low salt LB plates with Zeocin were prepared and used to isolate the positives clones from the ligation. Low salt LB plates were prepared with Lennox-LB (Fisher), supplemented with Zeocin antibiotic (100 µg/ml, Invitrogen).

2.2.5. Screening

A PCR reaction was used to screen for positive ligations. Reaction for each colony screened contained: 0.5 µl of Taq polymerase (NZYtech), 1 µl of AOX1_FW primer (10 µM) (Table 2.1), 1 µl of AOX1_REV primer (10 µM) (Table 2.1), 2.5 µl NZYtech buffer, 1.5 µl $MgCl_2$, 1.25 µl dNTP mix and 17.25 µl of dH_2O in a final volume of 25 µl. Positive clones were selected for sequencing.

REAGENTS	VOLUMES
Taq polymerase	0,5 µl
dNTP mix	1,25 µl
AOX_FW	1 µl
AOX_REV	1 µl
$MgCl_2$	1,5 µl
NZYtech buffer	2,5 µl
dH_2O	17.25 µl

Table 2.8. Reagents used for the PCR screening.

The cycle of the PCR was:

- 95°C for 2 minutes
- 95°C for 1 minute
- 52°C for 30 seconds
- 72°C for 1 minute

} 30 cycles

- 72°C for 5 minutes

2.3. ELECTROPORATION IN *Pichia pastoris*

The yeast *Pichia pastoris* was used as heterologous system for the expression of ChR2 mutants. Yeast strain and protocols used for expression are from the EasySelect *Pichia* Expression Kit (Invitrogen) and the integration of the mutated sequence in yeast genome was obtained with electroporation. This method induces the formation of pores in the membrane of yeast cells facilitating the integration of DNA in the host system. Before starting the electroporation procedure, a X-33 strain of *Pichia pastoris* (Invitrogen) was grown on YPD (1% yeast extract from, 2% peptone, 2% dextrose and 2% agar from Fisher scientific) plates. The plasmid DNA used for the electroporation was extracted using a PureLink Hipure Plasmid Filter Maxiprep Kit (Invitrogen) and linearized through enzymatic digestion to facilitate homologus recombination in *Pichia pastoris* genome. The reaction was as follows: 10-20 µg of DNA, 5 µl of CutSmart buffer (New England Biolabs), 2 µl of *Pme*I restriction enzyme (New England Biolabs) and dH_2O up to 50 µl. After 3 hours of digestion at 37°C, an extra 1 µl of *Pme*I was added to the reaction.

REAGENTS	VOLUMES
DNA	10-20 µg
CutSmart buffer	5 µl
*Pme*I	2 µl + 1 µl
dH_2O	Up to 50 µl

Table 2.9. Mix of linearization reaction.

The digestion then was left at 37°C for 2 hours. The enzyme was then inactivated at 65°C for 20 minutes and 1 µl of the reaction was run against uncut vector on 1% agarose gel to confirm the linearization. The DNA was purified with the clean and concentrator kit from Zymo research and used for the electroporation. Electrocompetent cells were prepared following the Invitrogen protocol. Briefly, a single *Pichia pastoris* colony was growth in 5 ml of YPD (1% yeast extract from, 2% peptone, 2% dextrose) overnight at 30°C with shaking. This culture was inoculated in fresh YPD and growth overnight to an $O.D._{600}$ = 1.3-1.5. The culture was centrifuged and cells were resuspended in ice-cold sterile water for two times. After another centrifugation, cells were resuspended in sorbitol (1M, Sigma aldrich) to a final volume of 1 ml. 80 µl of yeast cells were electroporated with 5-10 µg of DNA using a MicroPulser Electroporator from Biorad. After adding 1 ml of sorbitol (1M, Sigma aldrich), the cells were incubated for 2 hours at 30°C. Then electroporated yeast were plated on YPD-Zeocin plates with different concentration of antibiotic (1000 µg/ml and 100 µg/ml), as direct way to select multi-copy recombinants, and grown for 48 hours. Plates with Zeocin concentration of 1000 µg/ml were used to identify "superclones" integrating more copies of the vector with a resulting higher antibiotic resistance.

2.4. INDUCTION OF THE PROTEIN

The isolated ChR2 clone was first growth in Buffered Glycerol-complex Medium (BMGY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer at pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin and 1% glycerol) overnight at 30°C with shaking until $O.D._{600}$ between 4 and 6 was reached. Then the growth was induced in Buffered Methanol-complex Medium (BMMY; 1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer at pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin, 10 µM of retinal and 2.5% methanol). Methanol is the inducer of alcohol oxidase (AOX1) promoter that drives expression of the gene of interest encoding the desired heterologous protein. The cultures were induced for 24 hours and then centrifuged. The pellet were weighed and used for the extraction of the protein.

2.5. EXTRACTION OF THE PROTEIN

Different protocols were used to extract protein from yeast to establish the more efficient method. The methods tested were: acid-washed glass beads, french press, yeast protein extraction reagent and sonication.

2.5.1. Acid-washed glass beads

The pellets of induced cells were homogenized in a breaking buffer (11.3 mM NaH_2PO_4 at pH 7.4, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM PMSF) in a ratio of 1 ml of buffer for 0.35 g of cells. The final volume was split in several tubes and acid-washed glass beads (Sigma-Aldrich) were added to a ratio 2 g of beads for 1 ml of buffer. The cells were broken by vortexing (3000 rpm) for 30 seconds with 30 seconds on ice in between each mixing. This step was repeated 10 times. After 10 minutes on ice, the supernatant was transferred in a new tube and centrifuged to remove unbroken cells and cell debris.

2.5.2. French press

Each pellet of induced cells was resuspended with homogenization buffer (11.3 mM NaH_2PO_4 at pH 7.4, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM PMSF) to which was added *cOmplete ULTRA Tablets, Mini, EDTA-free, EASYpack* (protease inhibitor from Roche) in a ratio of 1 pill for 25 ml of buffer. For each 500 ml of culture, 10 ml of buffer were used. The homogenate was passed four times through a French press (Thermo Scientific) and then centrifuged at 8000 g for 15 minutes. The supernatant obtained with this last centrifugation was centrifuged at 180000 g for 1 hour to get membrane fraction. The pellet was resuspended at 4°C overnight in solubilization buffer (20 mM of sodium phosphate buffer at pH 7.2, 200 mM NaCl, 5% glycerol, 10 mM imidazole, 0.1 mM PMSF, 1% DDM, 250 mM arginine and 3 µM retinal).

2.5.3. Yeast protein extraction reagent (Y-PER)

The extraction method with Y-PER reagent (Thermo Scientific) was performed following the manufacturers protocol. The pellet was resuspended in an appropriate amount of reagent to which PMSF (1 mM) was added. After 20 minutes in agitation at room temperature, the mixture was centrifuged and the supernatant obtained was stored at -80°C to be used afterwards.

2.5.4. Sonication

For the sonication method, the pellet was resuspended in homogenization buffer (11.3 mM NaH_2PO_4 at pH 7.4, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM PMSF) and treated with ultrasound sonication, alternating 30 seconds of pulsing with 30 seconds of resting on ice. Then the mixture was centrifuged at 8000 g for 10 minutes and the supernatant, corresponding to the membrane fraction, saved to be used afterwards.

2.6. PROTEINS QUANTIFICATION

Pierce BCA Protein Assay Kit (Thermo Scientific) was used for protein quantification following the Thermo Scientific protocol and using 2 mg/ml albumin standard following the manufacturer's instructions.

2.7. SDS–PAGE

Protein samples were run on SDS-PAGE, in 12% polyacrylamide gels (1,5 M Tris-Cl pH 8.8, 40% acrylamide, 20% SDS, 10% APS and TEMED) and 4% acrylamide stacking gel (0.625 M Tris-Cl pH 6.5, 40% acrylamide, 20% SDS, 10% APS and TEMED). Three part of protein samples were diluted with 1 part of 4x Laemmli Sample Buffer to which β-mercaptoethanol was added. Protein samples were incubated overnight at 4°C in the denaturing buffer.

2.8. COOMASSIE STAINING

The gel was incubated for 10 minutes in a Coomassie staining solution (0.25% Brilliant Blue, 45% methanol, 10% acetic acid). Then the gel was washed first with water and then 3 times with a destaining solution (25% methanol, 5% acetic acid) and left in destaining overnight. The gel was imaged with a computer scanner.

2.9. WESTERN BLOT

Proteins were transferred to PVDF membranes in a Mini-trans blot system with a Tris-glycine buffer (0.025 M Tris base, 0.192 M Glycine) for 2 hours at 100 V. Membranes were activated with methanol, then used for the transfer and blocked for 1 hour in 5%

milk in TBS-T (0.01 M Tris, 0.15 M NaCl, 0.1% Tween). After the blocking, membranes were incubated overnight at 4°C with anti-HisTag primary antibody diluted (1:1000) in 5% BSA in TBS-T, then washed in TBS-T and incubated with an Anti-Rabbit secondary antibody diluted (1:10000) in 5% BSA in TBS-T. The membrane were finally revealed using Pierce ECL Western Blotting Substrate (Thermo scientific) at the STORM 860 Gel. Antibodies used were: His-Tag Polyclonal antibody (Cell Signaling technology) as primary antibody and Horseradish peroxidase Donkey anti-Rabbit IgG (Jackson ImmunoResearch laboratories) as secondary antibody.

2.10. PURIFICATION

Protein purification was performed in an AKTA Prime system using pre-packed nickel column (HisTrap FF crude, GE Healthcare). Before being used, the column was equilibrated with 10-15 column volumes of a solubilization buffer (20 mM of sodium phosphate buffer at pH 7.2, 200 mM NaCl, 5% glycerol, 10 mM imidazole, 0.1 mM PMSF, 0.03% DDM and 3 µM retinal) using a peristaltic pump. To proceed with the purification, protein extracts were first centrifuged at 180000 g for 1 hour and the supernatant was diluted 5 times with solubilization buffer (20 mM of sodium phosphate buffer at pH 7.2, 200 mM NaCl, 5% glycerol, 10 mM imidazole, 0.1 mM PMSF, 0.03% DDM, 250 mM arginine and 3 µM retinal). The soluble proteins were then transferred to the pre-packed nickel column. The column was then washed with solubilization buffer. The protein was finally eluted using increasing concentrations of imidazole (25 mM, 100 mM, 250 mM and 500 mM).

2.11. SILVER STAINING

To perform the silver staining the gel was first treated with a solution made of 25% MeOH and 5% acetic acid and then washed two times first with 50% ethanol and then with 30% ethanol. The gel was then incubated in Sodium Thiosulfate (0.2g/L) for 1 min. After two washing with water, the gel was incubated in Silver Nitrate (2g/L) for 20 min. After another washing in water, the gel was developed in a solution made of 30g/L Sodium Carbonate, 10mg/L sodium thiosulfate and 0.02% formalin. The developing reaction was stopped by removing the gel from the previous solution and incubating it

in Tris (50g/L) 2.5% acetic acid for 1 min or simply washing the gel several times with water.

CHAPTER 3 - RESULTS

3.1. PREDICTION OF PUTATIVE ChR MUTANTS

With the goal of obtain useful variants of hChR2, novel mutations were selected on the basis of previous TDDFT studies performed at the Center for Computational Physics, University of Coimbra. These calculations obtained a predictive variation on the absorption spectra of the channel due to specific mutations. The two predicted mutants selected for the work are F269D, a red-shift mutant, and F269H, a blue shifted mutant. The graphic depicted on Figure 3.1 shows the putative shift of Channelrhodopsin mutants based on the TDDFT calculations for these two mutations.

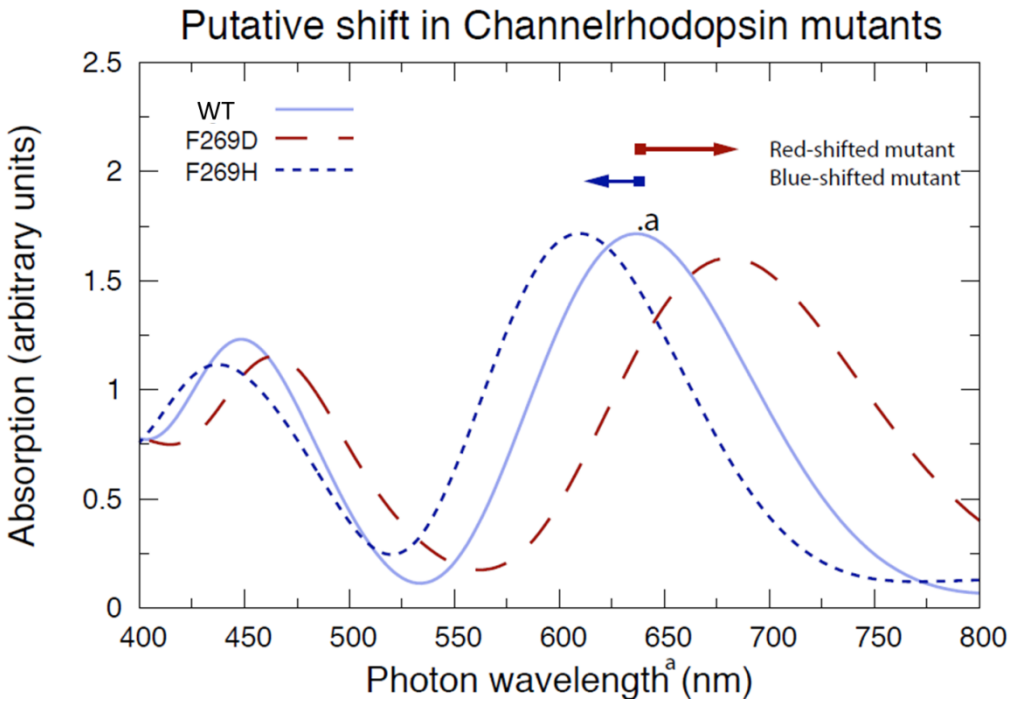

Figure 3.1. Putative shift in ChR mutants.
Predicted spectral characteristics of wildtype (WT) ChR2 (blue line) using TDDFT and two proposed point mutants (F269H and F269D). The F269D mutation is proposed to induce a significant red-shift in the absorption of the chromophore, while mutant F269H induces a blue-shift. Note (.a) wavelength output form TDDFT analysis is systematically right-shifted, e.g. the experimental absorption peak at pH 6.0 of dark adapted WT-ChR2 is centered at 480nm. These results are from Micael J.T. Oliveira, Bruce F. Milne and Fernando M.S. Nogueira (unpublished data)

3.2. SECTION 1

In the first section of this chapter we highlighted the supporting theoretical predictions that motivate the production and characterization of F269 ChR variants. The aim of this work was to perform mutagenesis in humanized ChR2 sequence and transfer the mutated sequence from a mammalian expression vector, to a vector that can be used to transform *Pichia pastoris* cells, which is the heterologous system selected for protein expression in this study.

3.2.1. Identification of F269 in protein structure

The structure of ChR2 (Figure 3.2) is available on PDB (accession number 3UG9). Starting from this information an analysis was performed to identify the amino acid residues targeted for mutagenesis. The key amino acid is the phenylalanine in position 269 (F269). This residue is very close to the retinal binding pocket and its mutation should produce a shift in the absorption spectra of ChR2 as predicted from the TDDFT study. The objective was to switch the F269 (Figure 3.2), a nonpolar amino acid, with an aspartic acid (D) or a histidine (H), which, in contrast to phenylalanine, are both polar residues (acidic and basic). This substitution should change the electric environment of the retinal binding pocket of ChR2 and produce a shift in the absorption spectra. The location of F269 in the 3D and the primary structure of the protein are represented in Figure 3.2 and Figure 3.3.

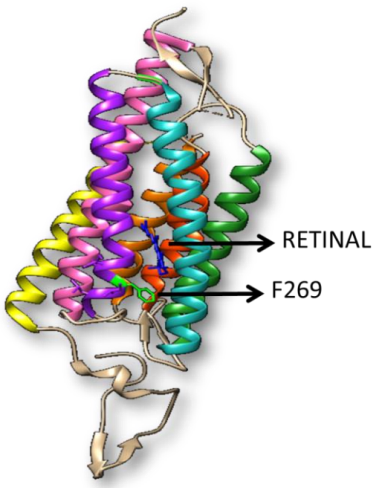

Figure 3.2. Structure of hChR2.
3D structure of the channel. The retinal molecule is highlighted in blue and F269in green.

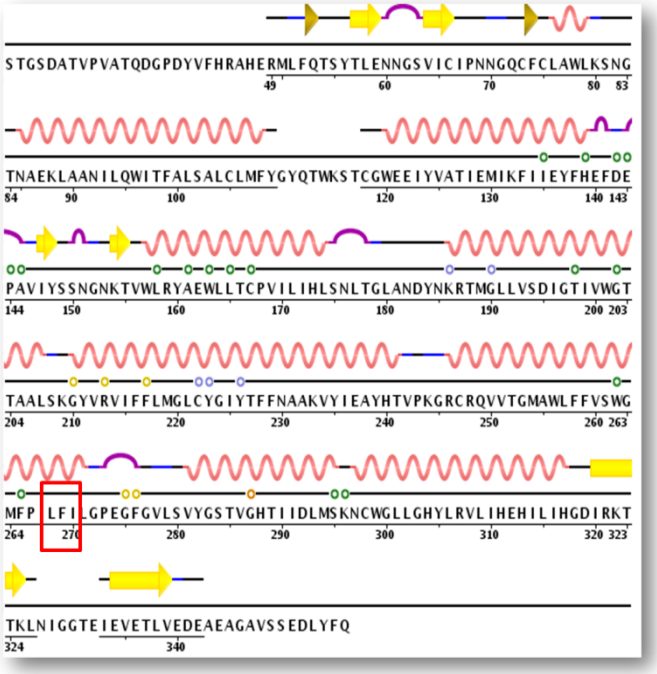

Figure 3.3. Sequence of ChR2.
Amino acid sequence of the channel with the corresponding α-helix and β-sheet domains. F269 is highlighted in the red square.

3.2.2. Direct mutagenesis of F269

The first step in this project was to perform directed mutagenesis on the plasmid containing the humanized sequence of ChR2 (available on www.addgene.org with the accession number 20939). This mutagenesis had as target the phenylalanine 269 (F269). The aim was to replace the TTC codon from F269 with a GAC codon which codifies for an aspartic acid and a CAC codon codifying for an histidine. PCR amplification was performed using specific primers to insert two mismatched bases for each mutation. The vector used as DNA template was the pcDNA3.1-hChR2-EYFP. In the PCR mix, different amounts of DNA were used and two annealing temperature (T_a), 65°C and 62°C. An agarose gel was made to verify the amplification. The expected size for the positive results was ~7000 bp. These mutations are named F269D and F269H. In Figure 3.4 an agarose gel shows the result of PCR amplifications products.

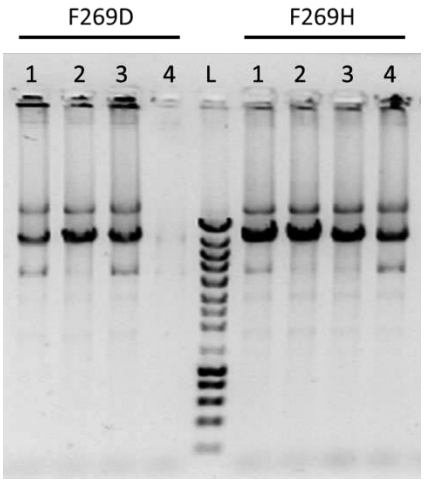

Figure 3.4. PCR product of direct-site mutagenesis.
0,7% agarose gel with F269D and F269H mutated DNA from amplification. For each mutation a PCR was performed with different amounts of DNA and two different annealing temperature (T_a). L: ladder III (NZYtech).

Two positives for the F269H (samples 2 and 3) and one positive for F269D (sample 2) were digested with *Dpn*I to eliminate the parental DNA and were screened on an agarose gel (Figure 3.5). Since *Dpn*I does not cut the PCR amplified vector, the size of the band correspond to a linear piece of DNA of ~7000 bp.

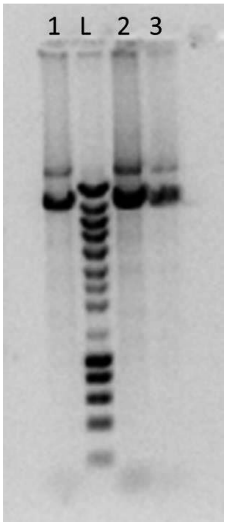

Figure 3.5. Digestion with *Dpn*I
0,7% agarose gel with products of mutagenesis. Lane1: F269D. Lane 2 and 3: F269H. L: ladder III (NZYtech).

After transformation in *E.Coli DH5α*, DNA was extracted and sent to sequencing (Figure 3.6).

A

	836	840	850
T7, ChR2, EYFP	836	TGTTCCCAATTCTCTTCATTTTGG	
hChR2	674	TGTTCCCAATTCTCTTCATTTTGG	
F269D (T7)	735	TGTTCCCAATTCTCGACATTTTGG	
F269D (Reverse)	806	TGTTCCCAATTCTCGACATTTTGG	

B

	836	840	850
T7, ChR2, EYFP	836	TGTTCCCAATTCTCTTCATTTTGG	
hChR2	674	TGTTCCCAATTCTCTTCATTTTGG	
F269H (T7)	732	TGTTCCCAATTCTCCACATTTTGG	
F269H (Reverse)	809	TGTTCCCAATTCTCCACATTTTGG	

Figure 3.6. Sequencing of F269D and F269H.
Alignment of the sequencing products of the mutagenesis and analysis performed in the Vector NTI software. (A) F269D mutations sequenced with a T7 primer and the reverse primer against the entire sequence of the plasmid sequenced with the T7 primer (T7,ChR2,EYFP) and hChR2 sequence (hChR2). (B) F269H mutations sequenced with a T7 primer and the reverse primer against the entire sequence of the plasmid sequenced with the T7 primer (T7,ChR2,EYFP) and hChR2 sequence (hChR2).

3.2.3. Construction of a vector containing mutated ChR2 to transform *Pichia pastoris*

To induce the expression of the mutated ChR2 sequences obtained by mutagenesis, a "shuttle" vector with the inserted mutated fragments was constructed. This final vector was prepared using the pPICZ A as the expression vector. The vector and the mutated DNA were treated for ligation, leading to the insertion of the mutated ChR2 in the pPICZ A vector. Briefly, the samples to be inserted were amplified by PCR using the primers yChR2_FW and yChR2_REV (Table 2.1). Using these primers, the resulting amplified fragments were flanked with *EcoR*I and *Kpn*I restriction sites. So the amplified fragments and the original pPICZ A vector were digested both with *EcoR*I and *Kpn*I and purified. The cut with these restriction enzymes creates "sticky ends" at the extremities of both constructs (vector and amplified DNA), ideal for the next step of the ligation. *EcoR*I and *Kpn*I digested pPICZ A and inserts were ligated. The ligation mix was used in transformation of *E.Coli DH5α* competent and supercompetent cells on Zeocin plates. Since the resistance for this antibiotic is carried on the pPICZ A vector, only positives clones which incorporate the product of the ligation grow on Zeocin plates. The colonies were picked and used directly for PCR amplification as screening for positive ligation result. In this PCR screen, AOX_FW and AOX_REV primers were

used (Table 2.1). This primers amplify the region in which the mutated sequence is inserted. Different reactions were prepared with two different insert:vector ratios. Positives clones correspond to a band of 1.2 kb (Figure 3.7). F269D shows all positives clones. F269H shows positives and negatives results (Figure 3.7). Positive clones 8, 18, 37 and 40 were selected and sent to sequencing to confirm our cloning result (Figure 3.8).

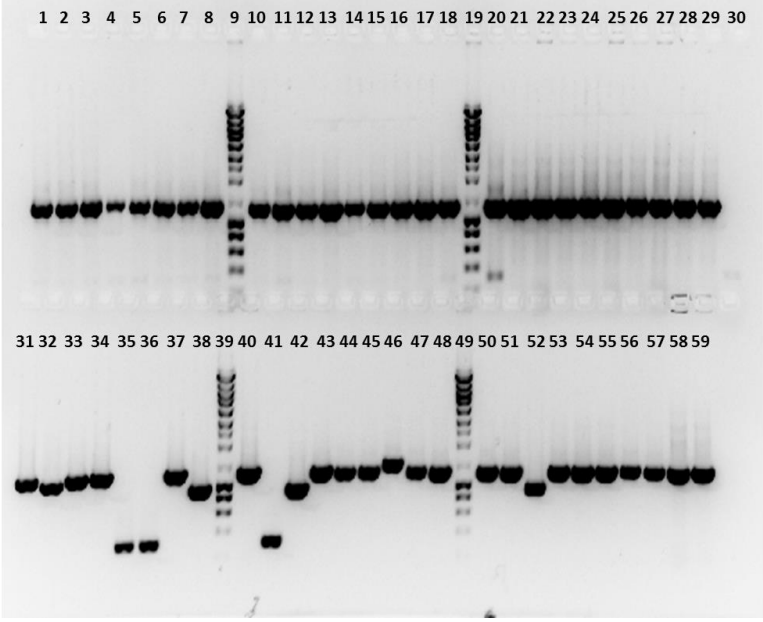

Figure 3.7.Screening of the ligation.
The figure shows a 1% agarose gel of the ligation products. In line 9, 19, 39 and 49 is the reference ladder III (NZYtech). Bands corresponding to 1.2 kb are the positives clones.

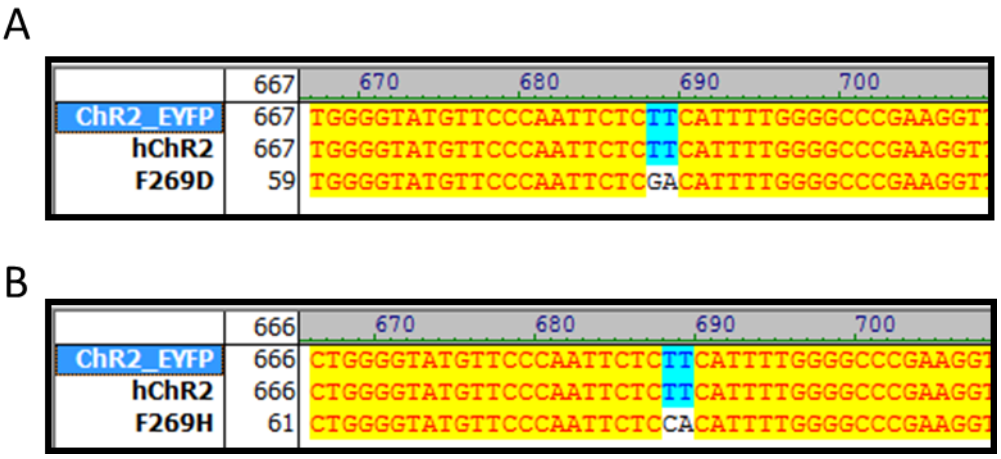

Figure 3.8. Sequencing of the products of the ligation.
Alignment of sequencing of products of the ligation performed with the software Vector NTI. (A) F269D sequence compared with ChR2 and hChR2. (B): F269H sequence compared with ChR2 and hChR2.

3.2.4. Linearization of the pPICZ A containing ChR2 mutated sequences

The vector pPICZ A with the inserted hChR2 obtained from the ligation was used to transform electrocompetent cells of *Pichia*. The DNA was digested with *Pme*I enzyme to linearize the vector. *Pme*I cut in the *AOX1* region of the plasmid. This step is necessary to increase the possibility of integration of the mutated DNA into the host 5'AOX1 genomic region. By linearizing the recombinant vector at *Pme*I restriction site located in the *AOX1* region recombinants were conveniently generate. The first step to facilitate the recombination was the linearization of the vector with the enzyme *Pme*I. Digested samples were run on agarose gel against the uncutted pPICZ A vector containing the WT-ChR2 (Figure 3.9).

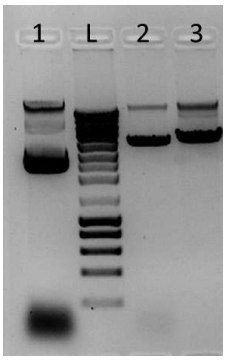

Figure 3.9. Linearization of the vector pPICZ A
1% agarose gel of linearized vectors against the non-linerized vector. In lane 1 pPICZ A containing WT-ChR2 not digested. In lane 2 the vector with the insert F269H and in lane 3 vector with the insert F269D.

3.3. SECTION 2

In the next section the second part of the work is presented. In this part results about the induction, expression and purification of the protein in *Pichia pastoris* are shown. Here we are showing results relatively only the wild-type hChR2. The reason is that the strategy was to optimize the protocol of induction, expression and purification of wild-type hChR2 and then apply this protocol to the mutants. In the next pages the wild-type hChR2 will be named WT-ChR2.

3.3.1. Electroporation of *Pichia pastoris*

Plasmid containing the WT-ChR2 was linearized with *Pme*I and *Pichia pastoris* X-33 cells were then transformed by electroporation using the linearized vector. Fresh electrocompetent cells from X-33 strains were prepared and used for electroporation. The electroporated cells were plated on YPD-Zeocin plates with different concentration of antibiotic (100 µg/ml and 1000 µg/ml), as direct selection of positives recombinants. This method gives the advantage of directly enriching for strains containing multiple copies of the expression vector. Since the resistance gene product is sensitive to the concentration of antibiotic, the transformants that contain more resistance product can resist to higher levels of the drug. The YPD-Zeocin plates were grown for 48 hours. After this time positive clones were visible on plates. "Superclones" are considered the colonies that integrated in their genome more copies of the vector through repeated cycles of homologous recombination. Therefore they contain not only multi-copies of the mutated DNA sequence but also from the Zeocin resistance cassette.

3.3.2. Induction of WT-ChR2

The positive clones were picked and grown in specifics media. The clones were first grown in BMGY, a medium containing glycerol, until an $O.D._{600}$ between 4 and 6 is reached, usually 5 is an optimal $O.D._{600}$. Clones grown in BMGY were centrifuged and resuspended in BMMY, a medium containing methanol, to induce the production of the protein. The methanol is the activator of the promoter *AOX1*, the first enzyme involved in the methanol utilization pathway of the yeast. If the homologous recombination occurs, the gene of interest, in this case the WT-ChR2 sequence, should be in front of the *AOX1* promoter and it will be expressed in the presence of methanol. The cultures were induced for 24 hours and then centrifuged to be used for the next steps.

3.3.3. Extraction of WT-ChR2 protein: comparison of different methods

After the induction the WT-ChR2 protein was extracted from yeast cells using different extraction methods, in order to establish the technique with the better yield of protein extract. The methods compared were: acid-washed glass beads, French press, Yeast Protein Extraction Reagent and Sonication. Yeast cells were first homogenized in a breaking buffer and then treated for the extraction. After the extraction the protein samples were centrifuged and the supernatant in which is present the membrane fraction

of the protein was saved. A western blot of this fraction was made to compare the efficiency of the different methods (Figure 3.10).

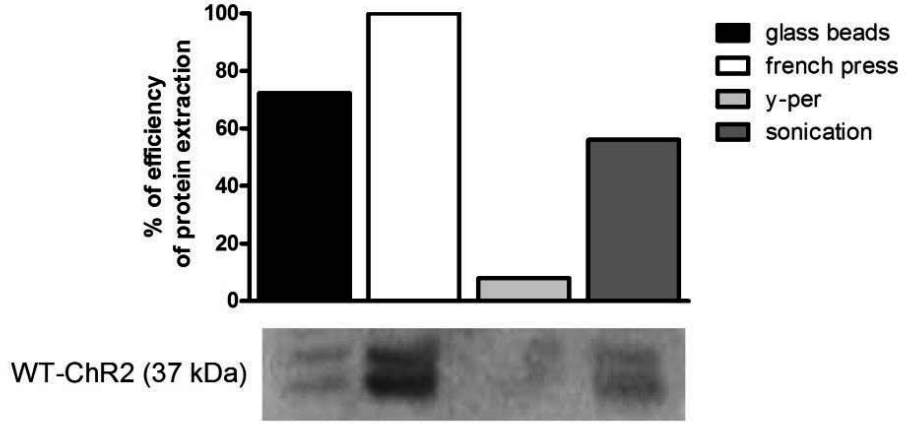

Figure 3.10. Comparison of the different methods of extraction.
Yeast cells were resuspended in homogenization buffer before the extraction. Proteins were extracted with different methods. Protein extracts were quantified with the BSA method and the efficiency was established considering the concentration of the protein for the volume of the extract. The results were confirmed with a western blot analysis. These experiments were performed with equal contribution from Bruno Cruz and Fabio Mazza and the image here represented was chosen for its exemplary nature.

3.3.4. Purification of WT-ChR2

Once established, the best method to get the protein extract, an affinity chromatography was performed to obtain purified WT-ChR2. The system used for chromatography was the AKTA system with pre-packed nickel columns, equilibrated with a solubilization buffer. The protein extract was diluted in solubilization buffer and then transferred in a nickel column, containing a resin with selectivity and high affinity for histidine-tagged proteins. Then the protein was eluted with increasing concentration of imidazole, used in affinity chromatography for the purification of His-tagged protein. In the image below is represented a cartoon of the chromatogram.

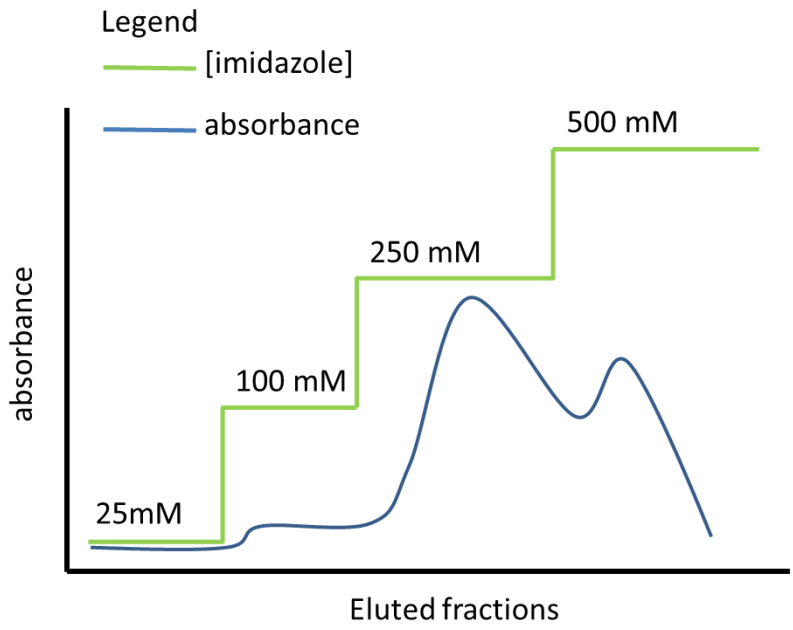

Figure 3.11. Schematic representation of the expected chromatogram of protein elution.
In the figure a cartoon of the chromatogram of the elution fractions of the protein. The protein was eluted with different concentration of imidazole (25 mM, 100 mM, 250 mM and 500 mM) and a trace of this concentration is given by the green line. The blue line corresponds to the absorption spectra of eluted protein.
Note: the real image of the system chromatogram was not representative due to a problem in the detector.

The fractions 17, 18, 23,24, 37, 38, 43 and 44 from chromatography were run on a SDS-PAGE and then the gel was stained with a silver staining to detect with more accuracy the WT-ChR2 purified (Figure 3.12). A good result is visible in the fractions 37, 38, 43 and 4, in which is visible a strong band between 33 kDa and 40 kDa.

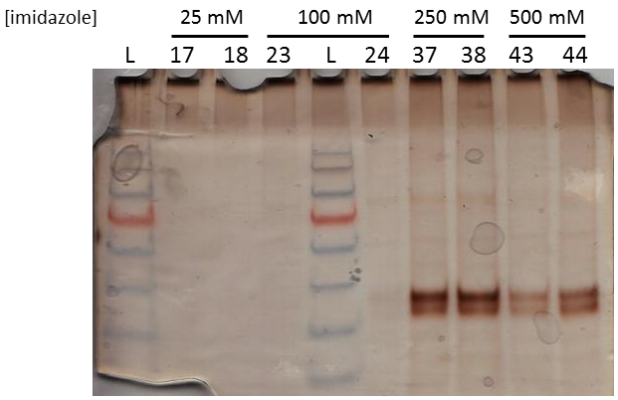

Figure 3.12. Silver staining of SDS-PAGE of eluted WT-ChR2.

In figure, SDS-PAGE gel stained with Silver staining. The purified protein is visible in lane 24, 37, 38, 43 and 44, corresponding to a band between 33 kDa and 44kDa.

3.3.5. Optimization of the WT-ChR2 induction

In order to obtain a larger amount of purified protein and try to obtain a better result of the purification, another *Pichia* strain was used to induce and express the WT-ChR2, instead of the X-33 wild type strain. We applied the same protocol of induction and the extraction of the protein with French press using the SMD1168 strain which is a protease-deficient strains that may be more suitable for the expression of recombinant protein.

Strain	Genotype	Application
GS115	*his4*	Selection of expression vectors containing *HIS4*
X-33	Wild type	Selection of Zeocin™-resistant expression vectors
KM71	*his4, aox1::ARG4, arg4*	Selection of expression vectors containing *HIS4* to generate strains with Mut^S phenotype
KM71H	*aox1::ARG4, arg4*	Selection of Zeocin™-resistant expression vectors to generate strains with Mut^S phenotype
SMD1168	*his4, pep4*	Selection of expression vectors containing *HIS4* to generate strains without protease A activity
SMD1168H	*pep4*	Selection of Zeocin™-resistant expression vectors to generate strains without protease A activity

Table 3.1. *Pichia pastoris* strains
In the table are illustrated the strains of *Pichia pastoris* available for expression of protein. The SMD1168 lacks of protease activity leading to a possible better expression of the protein. From http://www.lifetechnologies.com/order/catalog/product/C17500.

After the protein extraction, the protein samples were diluted 5X in homogenization buffer and used to perform a Western blot analysis. The result (Figure 3.13) shows that in the case of ChR2 the SMD1168 strain did not perform as well when compared with the X-33 strain.

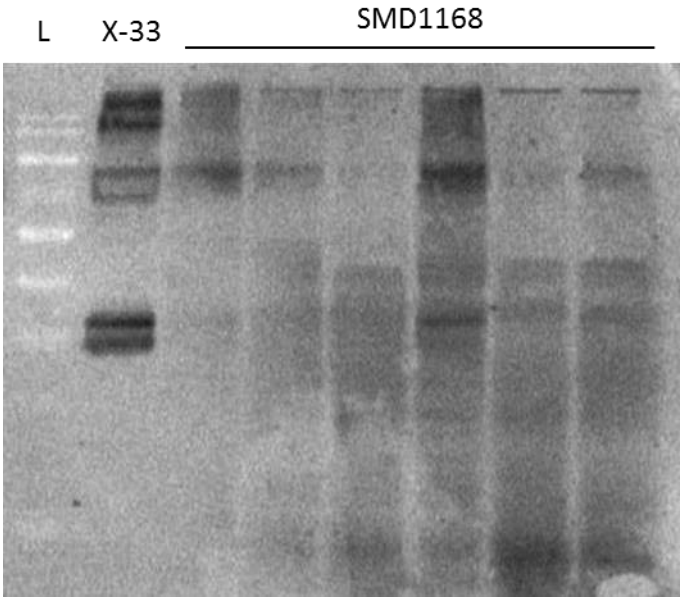

Figure 3.13. Blot of SMD1168 strain compared with the X-33 strain.
Yeast cells were resuspended in homogenization buffer before the extraction. Proteins were extracted with French press. Protein samples were diluted 5X in homogenization buffer and used to do a SDS-PAGE. Western blot confirms the expression of the protein. As is visible from the blot, the band corresponding to X-33 strain has greater intensity than the SMD1168, demonstrating a higher expression of the protein in X-33.

CHAPTER 4 – DISCUSSION AND CONCLUSIONS

Channelrhodopsins (ChR1 and ChR2) are membrane channels, gated directly by light, naturally expressed in the microalga *Chlamydomonas reinhardtii* (Nagel et al., 2002, Nagel et al., 2003). In the alga they are part of the eyespot, a simple vision system, and work as photoreceptor responsible of reaction to light (Hegemann, 2008).

Among the two Channelrhodopsin proteins, ChR2 has received more attention for its application in neuroscience. ChR2 is a light gated membrane channel, activated by blue-light, that, after photon absorption, rapidly opens to generate a flow of positive charged ions through the cell membrane (Nagel et al., 2003). The great interest in the field of neuroscience for this protein came from the evidences that its heterologous expression in mammalian brain allows a precise and selective activation and control of specific genetically targeted neurons, without needing to add exogenous factors. In fact several studies have demonstrated the power of ChR2 in mapping neural circuitry (Petreanu et al., 2007, Wang et al., 2007), inducing synaptic plasticity (Zhang and Oertner, 2007), restoring vision in rhodopsin-deficient animals (Bi et al., 2006) and inducing behavioral response in free-moving animals (Nagel et al., 2005a, Schroll et al., 2006).

Despite the wide application of the ChRs, there are still several limitations that often prevent the desired effect of the channel in the depolarization of neurons, such as low expression levels, rapid inactivation, inappropriate kinetics or small conductance. Moreover, an open challenge is to generate ChR variants with completely separated activation spectra in order to control two types of cells with light of different wavelength. For these reason in the last years, several strategies (site-directed mutagenesis and domain swapping between different Channelrhodopsin species) have been used to generate improved ChRs (Hegemann and Moglich, 2011). Currently, the available set of optogenetic tool for neuroscientist is wide enough to contain several tools of different categories, but is still necessary to discover new features and strategies to optimize and generate finer tools, in order to get more precise and noninvasive control of neural activity.

Nevertheless, in terms of spectral properties, few variants were generated and characterized. ChR2 variantes are mostly light-blue activated channels with an absorption peak around 480 nm, that can be shifted no more than 20 nm in the point-mutant variants known until now (Hegemann and Moglich, 2011).

In this work we investigated the possibility of creating novel Channelrhodopsin-2 variants, designing new ChR2 mutants that differs from the WT-ChR2 in their spectral

characteristics. Towards this, we used a TDDFT study from the Center for Computational Physics in the University of Coimbra (unpublished data), on putative shifted ChR2 variants, the mutants selected for the work were F269D and F269H, which are proposed to be responsible respectively for a red shift and a blue shift in the wavelengths of the spectra (Figure 3.1). With the availability of the crystal structure of ChR2 (Kato et al., 2012), it was revealed the essential molecular architecture of the channel, including the retinal binding pocket, the inner pore and the cationic conductance, opening the way to design novel ChR variants with improved features. In this structure, phenylalanine 269 is one of the five aromatic residues located around the retinal and involved in the formation of the retinal binding pocket (Kato et al., 2012). This proximity to the point where the retinal binds, make F269 a good candidate to develop novel ChR2 variants, since it has been already demonstrated that mutagenesis designed to alter the electrostatic environment in the binding pocket induce a shift in the absorption profile of *all-trans*-retinal (Wang et al., 2012).

On the basis of this considerations, we performed a site-directed mutagenesis to replace the TTC codon, encoding for the non-polar F269, with a CAC and a GAC codon, codifying respectively for polar amino acids histidine and aspartic acid. Once obtained, the mutants were confirmed by sequencing, in parallel we proceed with optimizing the purification of WT-ChR2 protein. On this scope, we subcloned the WT-ChR2 in a "shuttle" vector (pPICZ A) to transform the yeast *Pichia pastoris* and induce the heterologous expression and production of the protein. *Pichia pastoris* is a methylotrophic yeast species increasingly used in last years as promising heterologous expression system for integral membrane protein in an eukaryotic host (Ramon and Marin, 2011). One of the advantages of this yeast is the possibility for increased level of protein expression by inserting in the genome our gene of interest with a specific Kozak Sequence (Kozak, 1984), to improve the translation process and facilitating the recognition of the initiator codon by ribosome (Cigan and Donahue, 1987). For this reason, we amplified the fragment of WT-ChR2 with specific primers to add *EcoR*I restriction sites and the yeast Kozak Sequence at 5' end and *Kpn*I at 3'end and inserted the modified fragment in the pPICZ A vector for afterwards expression of the protein. Different studies also showed the capacity of the yeast to integrate multiple copies of expression vector providing a rapid and better means of obtaining multi-copy expression colonies via selection on plates with higher concentration of antibiotic (Clare et al., 1991, Romanos et al., 1998, Mansur et al., 2005). After establishing the most

efficient method of extraction, we purified the protein through affinity chromatography. Although the chromatography positive result, additional studies and procedures may be necessary to optimize the protocol and make it more effective, improving the rate of expression and obtain higher levels of the protein. In order to reach this objective, we may use a different strain of *Pichia pastoris*. One strain, widely used for protein expression, is the SMD1168, which lack key protease activity and might led to a higher expression of our protein (Yang et al., 2004, Shi et al., 2007). Nevertheless, in our work X-33 wild type strain proved more efficiency when compared to SMD1168.

The optimization of the protocol, improving expression and purification of the protein, will allow the production of functionally relevant mutants of the channel. To reach this purpose, the strategy used in this work was to prove the efficiency of the protocol on WT-ChR2, adapting it to the successive purification of the positive mutants obtained with the directed mutagenesis.

Additional functional assay are also necessary to study spectral characteristic of the channel and confirm the putative shift. Moreover our approach can be considered useful in the validation of the TDDFT theory. The detection of an absorption spectra corresponding to the predicted one will validate the theory, and give the attempt of a wider application of the TDDFT method.

The characterization of the spectra absorption of the mutants should also be followed by electrophysiological recordings, to study the properties of the mutated channels. This is needed to determine photocurrents and to investigate possible alterations in the functionalities of the protein. In fact the mutation of the F269 residue, even if selected on the basis of a predictive shift of absorption peak, might change also other features, such as conductance, permeability or kinetics, achieving a chance of adding novel variants to the optogenetic toolbox already described in the literature.

In conclusion the work described in this thesis moves the first step towards the future development of novel ChR2 variants with the future purpose of a replicable strategy to produce functionally relevant Channelrhodopsin mutants. In this case the idea is to engineer channels with a blue-shift and channels with red-shift in the absorption spectra leading to separation of the maxima peaks of the two mutants. This will generate two distinct peaks of absorption and avoid the overlapping of activation. The advantage of this properties is the possibility of a distinct activation of the two ChR2 mutant with different light, and if expressed in two different class of neurons the result is excitation

of specific type of neuron, resulting in the possibility of control a defined subset of cells intermixed in the multitudes of the neural tissue, as already demonstrated through the combination of ChR2 and NpHR, activated by two different light, respectively blue and yellow (Zhang et al., 2007a). The only known channels with a shifted spectra are the one derived from other algal genome, VChR1 from *Volvox carteri* (Zhang et al., 2008) and MChR1 from *Mesostigma viride* (Govorunova et al., 2011), but until now no point mutation are described to confer the same spectral characteristics to the *Chlamydomonas reinhardtii* channel. However many point mutations induce the improvement of the kinetic of the channel, as the SFO, bistable mutant with an increased time constant of deactivation (Berndt et al., 2009), and the ChETAs, mutations with accelerated closure kinetics (Gunaydin et al., 2010). On the basis of these evidences, the predicted mutations used in our work could represent channels with a combination of different improved features or could show a different characteristic from the one expected, leading however to a deeper current knowledge of the channel and consequently to the opportunity to manipulate the primary structure of the channel obtaining variants with desired properties.

However, our approach shows a strategy of genetically targeted engineering of ChR2 and obtain new ChR2 variants to add to the already known optogenetic tools. Future perspective for ChR2 and its engineered mutants are very wide. They open the possibility to study and to get a better comprehension of the neural mechanisms and in particular neural interconnection between neurons, leading to the understanding of the complex circuitry that is the basis of our brain consisting in a system for precise manipulation of neural activity with light in exploration of functional connectivity of complex neural circuits in vivo (Arenkiel et al., 2007, Wang et al., 2007).

Looking over, the possible application of ChR2 in neuroscience could be various and the channel and its mutated variants could become a commonly used analytical tool or a therapeutic device, as demonstrated by the last discoveries in the restoration of visual deficiency in mouse (Bi et al., 2006, Doroudchi et al., 2011), paving the way to novel application of ChR2. In this context an important role could be played from the mutants with a shift in the absorption spectra, thanks to their ability to respond to light and to be activated by different wavelength of the visible spectra.

Nevertheless, more studies have to be done to improve the functional properties of optogenetic tools and to extend the application of this new strategies in several fields such as in disease models and in the comprehension of physiological mechanisms.

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