1 Introduction

Behavior of higher organisms is regulated by signal inputs from nutrition, air or light and signal transduction via biochemical reactions in a complex nervous system. Nerve cell function relies on the controlled variation and directed propagation of changes in membrane potential. These changes are induced by opening ion channels in the cell membrane. Incorrect connections in the nervous network or dysfunction in ion channels can lead to severe neurological diseases like epilepsy or Parkinson's [1]. Up to now, medical treatment of these diseases involves implantation of electrodes. However, nerve stimulation by electrodes represents a massively invasive method.

In 2003, a significantly less-invasive alternative was found which allows triggering of nerve cell activity by light pulses [2]. This neurophysiological approach involves a light-induced, cation-selective channel, named Channelrhodopsin-2 (ChR2). ChR2 is a promising tool to control physiological reactions. It evolved to the major protein in the up-coming field of optogenetics, a successful attempt to treat neurological disorders in a light-controlled manner. Despite progressive application of ChR2 in optogenetics, many structural details and the functional mechanism are poorly understood. The open questions are specifically addressed in the present work in order to improve the neurobiological applications by a deeper molecular understanding of ChR2. Therefore, a variety of spectroscopic methods was applied which promise to elucidate yet unknown details about the structure and the function of ChR2.

A few months ago, the X-ray structure of C1C2 was resolved, giving new insights into its structure as a seven transmembrane helical protein [3]. C1C2 is a chimera consisting of the N-terminal part of ChR1, a close relative of the channelrhodopsin family, and the last two helices and the C-terminal part of ChR2. So far, the structure

of pure ChR2 could only be resolved by cryo-electron microscopy [4] or modeled on the basis of sequence homology to other microbial rhodopsins [5, 6]. Vibrational spectroscopy represents an optimal tool to gain lacking information on a molecular level.

This thesis is divided in several parts which aim at an atomistic understanding of ChR2 properties. As prerequisite to spectroscopic studies, ChR2 protein expression and purification need to be established and optimized which constitutes the first part of this work. The second part focuses on spectroscopic characterization of structure and function of ChR2. FTIR (Fourier transform infrared) difference absorption studies on ChR2 variants performed in this work aim at the identification of those amino acids which are involved in the gating mechanism of the channel. Flash photolysis using a pH-sensitive indicator is performed to follow proton uptake and release. Since little is known about the exact configuration of the retinal or proposed hydrogen bonds to the retinal Schiff base, resonance Raman spectroscopy is applied in this work which has a strong selectivity for signals of the chromophore.

As third part of this thesis, surface-supported methods are advanced in order to allow studies on proteins in a membrane monolayer. Use of nanostructured gold surfaces is known to enhance vibrational signals by generation of surface plasmons. Application of surface-enhanced FTIR absorption spectroscopy (SEIRAS) may lead to identification of those amino acids whose role in the functional mechanism of ChR2 depends on the overall membrane potential. Single-molecule force spectroscopy (SMFS) is applied in cooperation with Dr. F. Oesterhelt (Düsseldorf University) to determine the length of helices or inter- and intrahelical connections like disulfide bonds in ChR2. Finally, the sensitivity limit of doubly vibrationally-enhanced four-wave mixing (DOVE-FWM), a nonlinear spectroscopic technique, is tackled to be optimized for application to membrane protein monolayers. Experiments are carried out in the group of Prof. D. Klug (Imperial College London).

2 Channelrhodopsin-2

Rhodopsins are widely known as photosensory receptors or ion pumps. They are found in all domains of life. Retinylidene proteins with shared sequence homology, named type I rhodopsins, are distinguished from type II rhodopsins which represent the visual pigment of animals [7]. Prokaryotic type-I rhodopsins act as sensors (e.g. Sensory rhodopsins I and II (SRI and SRII) from *Halobacterium salinarum*) or ion pumps (e.g. Bacteriorhodopsin (BR) and Halorhodopsin (HR) from *H. salinarum*). The phototaxis of archaea is controlled by SRI/II [8]. SRI controls either a photophobic reaction caused by destructive UV light or a photophilic reaction towards advantageous yellow light [8]. SRII instead regulates a photophobic reaction to blue light [9]. Halobacterial transducer proteins and a cascade of chemoreceptor proteins are responsible for further signal transduction to affect flagellar movement [10].
Already in 1945, a retinylidene photoreceptor was assumed to control algal movement [11]. First in 2002, two photosensory receptors of *Chlamydomonas reinhardtii*, the so-called channelrhodopsins (ChR), were described to be part of the phototactic machinery localized in the eyespot of these flagellate unicellular green algae [12]. They represent the first eukaryotic microbial rhodopsins [12]. Photoexcitation leads to a current that depolarizes the plasma membrane which in turn changes the pattern of flagellar movement by a yet undefined signal cascade [13, 14]. It was shown that Channelrhodopsin-1 (ChR1) was expressed preferably under strong light whereas ChR2 was expressed under less intense light conditions [2]. Besides ChR1 and ChR2 from *C. reinhardtii* (reviewed in [2, 15–17]), four other ChRs have been identified so far, namely VChR1 and VChR2 from *Volvox carteri* [18, 19], MChR1 from *Mesostigma viride* [20] and DChR from *Dunaliella salina* which seems to be the evolutionary link

between the other channelrhodopsins and microbial rhodopsins [6]. In 2002, it was discovered that the channelrhodopsins are light-gated ion channels which are selective for cations [16]. All ChRs share sequence homology and similar functionalities but differ in spectral sensitivity, photocurrent and desensitization which is the required relaxation time prior to the next possible excitation. In order to put more emphasis on certain properties, variants of ChR2 and chimeras were produced [21] as described later in this chapter. Due to the promising option to use even the wild-type form of ChR2 in medical approaches, the present work directs the scientific focus to the elucidation of functional and structural details of ChR2, exclusively.

2.1 Structure of Channelrhodopsin-2

Channelrhodopsin-2 from *C. reinhardtii* is an 80 kDa light-sensitive membrane protein consisting of 737 amino acids. As displayed in fig. 2.1, the two domains of ChR2 divide the protein into the membrane-spanning N-terminal part nChR2 (amino acids 1-307) and the cytosolic, C-terminal part cChR2 (amino acids 307-737). nChR2 has structural and sequence-related similarity to regions of type-I rhodopsins which are comprised of seven transmembrane helices [17] whereas cChR2 is not related to any known protein family [17]. Expression, purification and investigation of full-length ChR2 have not been successful so far.

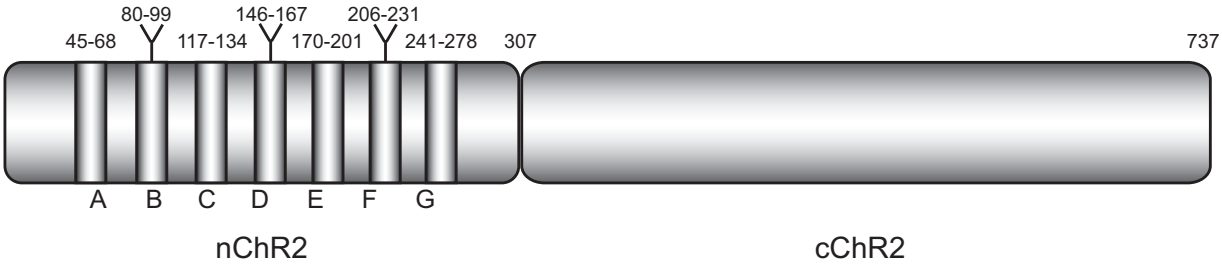

Figure 2.1: Schematic view of the structure of ChR2 from *C. reinhardtii*. The left part represents nChR2 with its seven transmembrane helices at their respective position according to the X-ray structure of the ChR1-ChR2 chimera C1C2 (see fig. 2.3 [3]). The right part shows the 430 amino acids-containing cytosolic cChR2 domain.

2.2 The N-terminal domain of Channelrhodopsin-2

The N-terminal domain of ChR2, nChR2, shows a rather small sequence identity to archaeal rhodopsins like BR from *H. salinarum* (35 % identity and 51 % similarity), but an 85 % identity to the sequence of the putative ion pathway of BR [22]. A sequence alignment to BR is given in the supplementary information (chapter A.1). Like all microbial rhodopsins, nChR2 consists of a seven-transmembrane-helix domain in which the membrane-spanning helices are connected via loops on the extracellular and cytosolic part with each other. The embedded retinal chromophore is covalently bound to a lysine residue (K257 in nChR2) via the retinal Schiff base (see fig. 2.2).

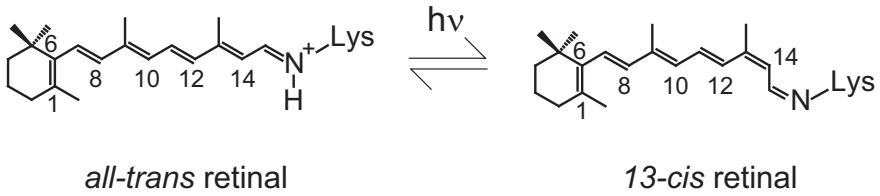

Figure 2.2: Formula of retinal bound to lysine via a Schiff base with official numbering. According to the reaction mechanism of microbial rhodopsins, the retinal isomerizes from *all-trans* (left formula) to 13-*cis* (right) upon light excitation and relaxes to *all-trans* in ground-state type I rhodopsins.

The retinal binding pocket is widely conserved among the other microbial rhodopsins. In analogy, the retinal was proposed to isomerize from *all-trans* to 13-*cis* configuration after light excitation [23]. Fig 2.2 displays the different isomeric retinal forms and the nomenclature of carbon numbering. Isomerization leads to a cyclic photoreaction including conformational changes of the protein moiety as known from other rhodopsins. Fig. 2.3 A shows the nChR2 sequence with the Schiff base lysine K257 marked in yellow and positions of point mutations used in this work (C128, D156, E123, S245) marked in cyan.

For a long time, structural details of ChR2 remained experimentally inaccessible due to missing X-ray crystallographic or NMR results. Recently, the helical arrangement of ChR2 was published when cryo-electron microscopy was used to probe 2D crystals of ChR2 [4]. The microscopic results prove a dimeric structure of ChR2 with helices C and D facing the opposing monomer. Dimeric structures like this are already known from sensory rhodopsins. More detailed insights into the molecular structure were obtained when Watanabe *et al.* developed a structural ChR model on the basis of different molecular computational approaches addressing sequence patterns of all six identified channelrhodopsins [6].

Finally in 2012, Kato *et al.* published the X-ray structure of C1C2, a chimera formed of the first five helices from ChR1 and the last two helices from ChR2 [3]. Therefore, the resulting structure does not reflect all properties of ChR2 but gives first ideas about its atomic structure (fig. 2.3). For better comparison, a sequence alignment of ChR2 and C1C2 is given in the supplementary information (chapter A.1). The crystal structure confirms earlier findings that ChR2 forms a dimer. Fig. 2.3 B displays the structure of a C1C2 monomer with the seven transmembrane helices in cartoon shape. The *all-trans* configurated retinal and its linkage to the apo-protein are shown as sticks. Additionally, the positions of point mutations relevant for this work are displayed as sticks.

2.2.1 The N-terminal domain of Channelrhodopsin-2 *in vitro*

Upon blue-light illumination, truncated nChR2 undergoes a cyclic photoreaction (for details on the mechanism see chapter 2.2.2). As a consequence to retinal isomerization, a pore is formed which allows light-gated ion channeling [16]. This pore is presumably an electronegative channel between transmembrane helices A, B and G in which residues E90, E97, E101, E123 and D253 (ChR2 numbering) are located [3]. These residues and especially those on helix B define ion conductance and selectivity as shown by mutational studies. Besides the mentioned electronegative side chains, the following polar residues are also located in the central part of the pore: Q56, T59, S63, E83, K93, T246 and N258 [3]. The putative restriction site is probably formed by S63, E90 and N258 [3]. Alternatively, Y70 (helix A) may close the pore via its unpolar side chain [3].
For the conductive channel, a low photocurrent was observed as indicator for the activity of ChR2 [15]. The conductance was estimated to be about 50-250 fS [2, 21, 24, 25]. After closure, the channel remains in a non-conductive, so-called desensitized state. This is synonymous to a transient inactivation of the protein limiting the maximal frequency of light stimulation. Desensitization under continuous illumination is expressed by a photocurrent reduction towards a steady-state level.
Electrophysiological experiments yielded information about the selectivity of the ion channel and its behavior at varying light intensity. The permeability for protons was found to be 10^6 times higher than for sodium cations [2]. In addition, ChR2 is only half as permeable for potassium as for sodium. Passage of Ca^{2+} as bivalent cation is preferred whereas magnesium ions are passed much less efficiently [2]. Patch-clamp

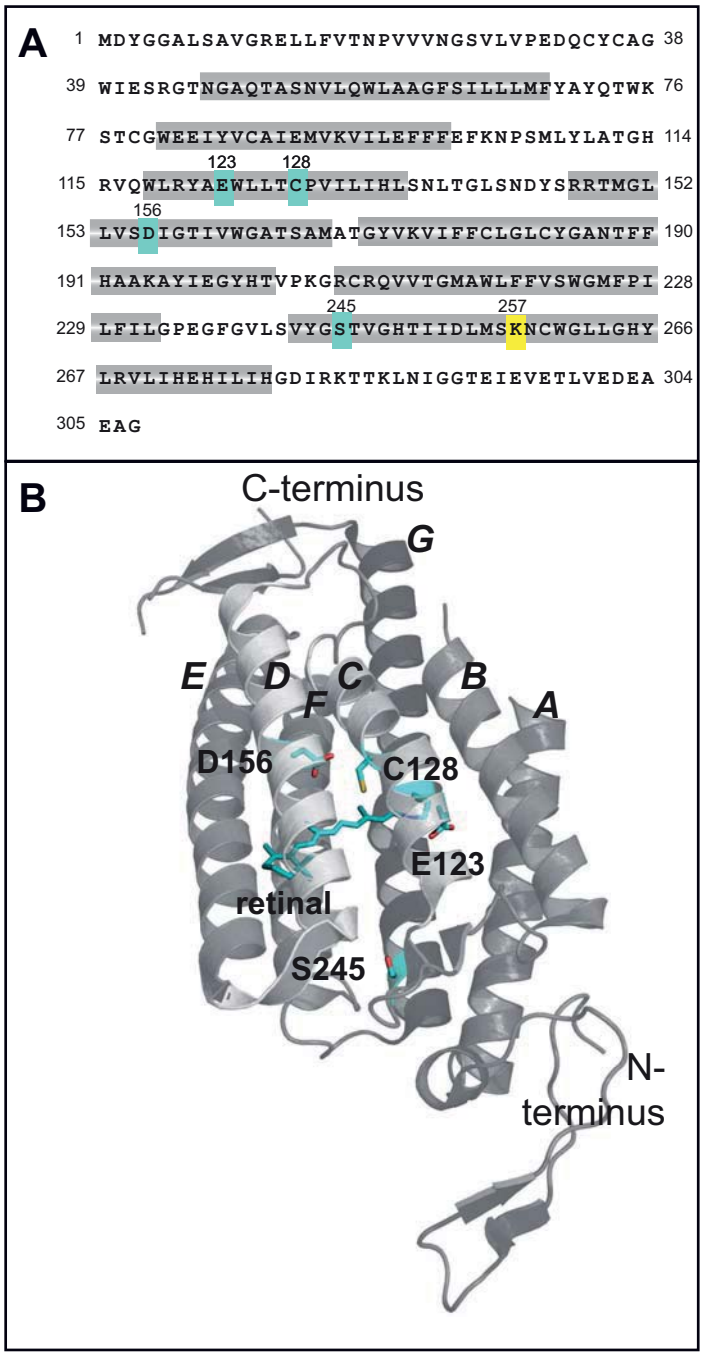

Figure 2.3: Primary sequence and structural arrangement of ChR2 from *C. reinhardtii*. A) Sequence of nChR2 in one letter code. The lysine to which the retinal chromophore is bound via a retinal Schiff base is marked in yellow; positions of mutations used in this work are marked in cyan. B) Crystal structure of one monomer of C1C2 (pdb entry 1GU9 [3]). Black letters mark the N- and C-termini and helices A to G. Residues which were mutated for spectroscopic analysis are shown as sticks (cyan: carbon, blue: nitrogen, red: oxygen, yellow: sulfur).

experiments provided evidence for the influence of the desensitization of nChR2 by the membrane potential and the bulk pH [2]. Recovery from inactivation is reported

to be slower at high extracellular pH [2, 26]. Additionally, influence of a Ca^{2+} gradient was observed. Hence, nChR2 is said to be voltage- and Ca^{2+}-driven. The unique property to act as a light-driven cation-selective channel makes ChR2 a valuable tool for neurobiological applications, called optogenetics. This promising and cutting-edge field of research is described in section 2.2.3.

2.2.2 Functional mechanism of nChannelrhodopsin-2

According to electrophysiological observations, three states are distinguishable for nChR2: the non-conductive but excitable state, the active, conductive state and the non-conductive, desensitized state. A more detailed and mechanism-related information is obtained by time-resolved UV/Vis spectroscopy [25–27] leading to a modified model with four identified photointermediates (fig 2.4, described in [28] as follows): Ground-state nChR2 is non-conductive and has an absorption maximum of 470 nm. Upon blue-light iIllumination, intramolecular changes in the protein are induced which lead to P1, the first intermediate state, within 50 ns [25]. Ultrafast kinetic studies on ChR2, however, proved the first intermediate to appear with a time constant of 3 ps [29]. The rise of P1 is indicated by a red shift of the absorption maximum to 500 nm. The following intermediates are formed by thermal relaxation processes. P1 decays with a time constant of 4 µs to P2 [26, 30], the second intermediate (maximal absorption at 390 nm). This strong blue shift is already known from formation of the M intermediate in the intensively studied BR photocycle. In analogy to BR, P2 was concluded to comprise a deprotonated retinal Schiff base. The third intermediate P3 (λ_{max} = 520 nm) arises with 150 µs [25]. Correlating UV/Vis kinetics with electrophysiological measurements, P3 formation is found to be associated with channel opening. As part of the back reaction, P4 (λ_{max} at 480 nm) defines the already non-conductive, desensitized state. Full recovery of the initial ground-state channel takes place with 5 s [25]. For comparison, an alternative three-state model is proposed for the photocycle of ChR2 from *V. carteri* also by use of flash photolysis [26]. This photocycle presents a fast intermediate appearing about 4 µs after excitation whereas the model proposed by Bamann *et al.* lacks this intermediate [25].

Although UV/Vis spectroscopy allows identification of different photointermediates, complementary techniques like FTIR difference spectroscopy are needed to monitor conformational changes of the retinal protein, as already shown by Radu *et*

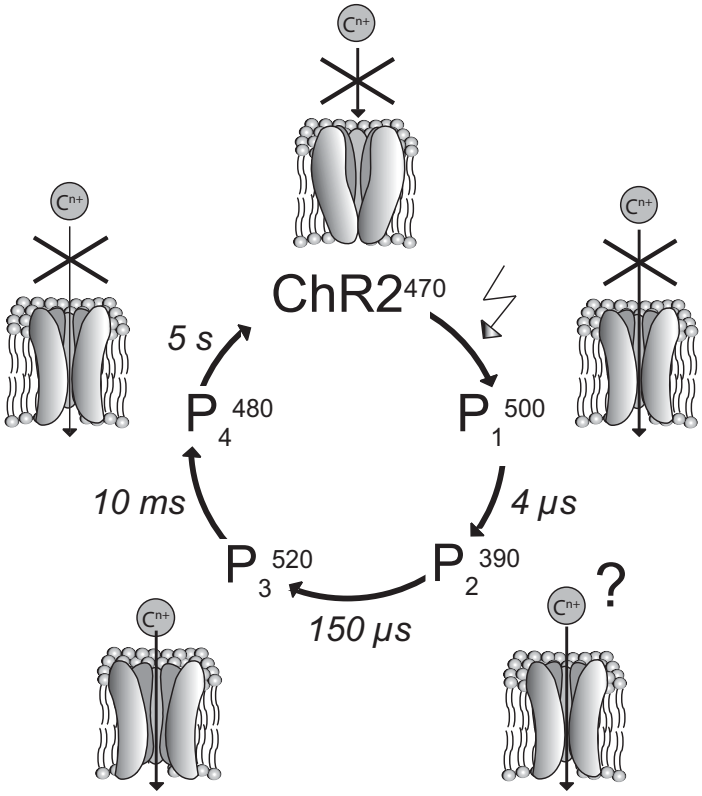

Figure 2.4: Model of nChR2 photocycle as derived from time-resolved UV/Vis and FTIR difference spectroscopy. Upon illumination (indicated by flash symbol), dark state ChR2 transforms into the intermediate P1. Due to thermal relaxation, the intermediate states P2-P4 are passed through. Relaxation times of the different states were determined by flash photolysis. The conductivity status of each photointermediate was derived from electrophysiological measurements. The conductivity status of P2, however, is still unknown [25]. The figure is modified from [30].

al. [30]. They present structural and functional details of the mechanism obtained by FTIR difference and resonance Raman spectroscopy which cannot be elucidated by UV/Vis spectroscopy. It was found that P1 and P4 show strong alterations in the secondary protein structure, although both are known to comprise non-conductive states. As these data include results obtained in the course of this work, details about FTIR-based insights on the functional mechanism of ChR2 are given in chapter 4.3.2.

2.2.3 Optogenetics

In 2002, the channeling activity of nChR2 was described for the first time [16]. Finding a cation-selective channel which can be simply controlled by blue light opens a new perspective for neurological research and makes nChR2 a valuable and very

promising tool in the so-called optogenetics. Optogenetics base on heterologous expression nChR2 in the outer membrane of target cells. Upon light excitation, the induced ion flux changes the membrane potential of transfected cells exclusively whilst leaving the surrounded cells unaffected. Selective expression of ChR in target cells allows behavioral control of single cells, tissue or even entire organisms. The optogenetic approach was mentioned first in 2005 [31] and is reported to be applied to mammalian cells first in 2008 [32]. So far, this method was applied to stem cells, muscle and heart cells and human neurons *in vitro*. Its potential to treat neurological disorders like epilepsy or Parkinson's disease [1] makes it a promising and non-invasive alternative to unspecific cell stimulation by electrodes being the only applicable method at present. Heterologous expression of ChR2 or the chloride pump Halorhodopsin (HR) from *H. salinarum* allows direct control of cell activity and inhibition, respectively. Fig. 2.5 shows the optogenetic control of nerve cells by blue-light stimulation.

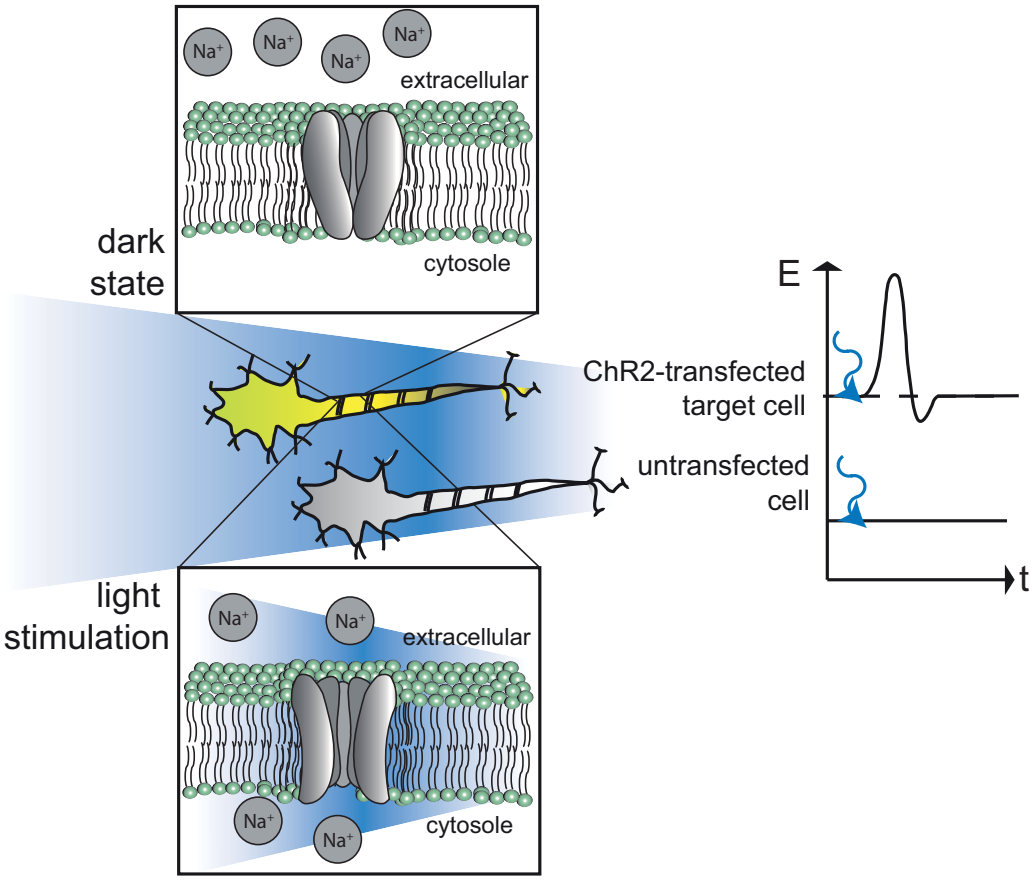

Figure 2.5: Illustration of optogenetic approach. Target cells (marked in yellow) are transfected with nChR2. Blue-light excitation (indicated by blue flash symbol on the right) leads to channel opening resulting in a sodium cation influx into the cells. As a consequence, action potentials are detectable for the ChR2-transfected cells, exclusively.