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Homology Cloning, Heterologous Expression and Characterization of a New Channelrhodopsin

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by

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THESIS

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Sing-Yi Hou

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Homology Cloning, Heterologous Expression and Characterization of a New

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Publication No. _____

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Channelrhodopsins are phototaxis receptors in the plasma membranes of motile unicellular algae. They function as light-gated cation channels and this channel activity has been exploited to trigger action potentials in neurons with light to control neural circuits ("optogenetics"). Four channelrhodopsins were identified in two algal species, *Chlamydomonas reinhardtii* and *Volvox carteri*, with known genome sequences; each species contains 2 channelrhodopsins, one absorbing at longer wavelengths and one at shorter wavelengths, named *Cr*ChR1 and *Cr*ChR2, respectively. Our goals are to expand knowledge of channelrhodopsin mechanisms and also to identify new channelrhodopsins from various algal species with improved properties for optogenetic use. For these aims we are targeting algae from extreme environments to establish the natural diversity of their properties. We cloned a new channelrhodopsin from the psychrophilic (cold-loving) alga, *Chlamydomonas augustae*, with degenerate primers based on the 4 known homologs. The new protein is 48% and 52% identical to *Cr*ChR1 and *Cr*ChR2, respectively. We expressed the channelrhodopsin in HEK293 cells and measured light-induced currents to assess their kinetics and action spectrum. Based on the primary

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structure, kinetics of light-induced photocurrents in HEK293 cells, and action spectrum maximum of 520 nm near that of the two previously found *Cr*ChR1, we named the new channelrhodopsin *Ca*ChR1. The properties of robust channel activity at physiological pH, fast on-and-off kinetics, and greatly red-shifted action spectrum maximum from that of *Cr*ChR2, make *Ca*ChR1 advantageous as an optogenetic tool. To know this new channelrhodopsin better, we expressed His-tagged *Ca*ChR1 in *Pichia pastoris* and the yield is about 6 mg/L. The purified His-tagged *Ca*ChR1 exhibited an absorption spectrum identical to the action spectrum of *Ca*ChR1-generated photocurrents. The future work will be measurement of the photocycles of *Ca*ChR1 by flash photolysis, crystallization of *Ca*ChR1 for the structure and mutagenesis of *Ca*ChR1 to find the critical amino acids accounting for red-shifted spectra, slow inactivation and rapid on-and-off kinetics.

Seven new channelrhodopsins including *Ca*ChR1 from different algal species have been cloned in our lab at this time, bringing the total known to 13. The work of cloning of these new channelrhodopsins along with the expression of *Ca*ChR1 was published in Photochemistry and Photobiology in January 2012 (1).

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Chapter I: Introduction of Channelrhodopsins

Light is essential to the behavior of photosynthetic microorganisms as well as their energy needs. These organisms have developed specialized ways to use light for gaining energy and for motility. Thirty years ago, archaeal rhodopsins, retinal-containing proteins in halobacteria, were found to control the photomotility behavior (phototaxis) in microorganisms (2-3). One decade ago, such retinal-containing rhodopsins were found to be also crucial for light control of motility (phototaxis, which is movement in response to light direction, and photophobic responses, which are sudden stops and turning reactions that help the cells avoid spots of intense light) of the phylogenetically distant green flagellate alga *Chlamydomonas reinhardtii* (4).

With the *C. reinhardtii* expressed sequence tag (EST) Project, it was shown that there were certain proteins homologous to the archaeal receptors (5). Knock-down experiments revealed that two rhodopsins in *C. reinhardtii* identified with the information above had the ability to be photoexcited and generate the transmembrane currents and drive photomotility behavior in their native algal cells. Based on their sensory role, they were named *Chlamydomonas* **s**ensory **r**hodopsins A and B (CSRA and CSRB) (4). Furthermore, these proteins were found to function as light-activated ion channels when expressed in *Xenopus* oocytes, and were therefore named as channelrhodopsins 1 and 2, abbreviated ChR1 and ChR2 (6-7). Alternative names are Acop-1 and Acop-2 (for **a**rchaeal type *Chlamydomonas* opsin 1 and 2) are also independently used in some publications (8). In consideration of the burgeoning amounts of new channelrhodopsins found in other different algae during recent years, I collectively called all channelrhodopsins here as ChR1 or ChR2 according to their properties and added the abbreviations with the initial letters of the native hosts' genus and species names to the

ChR1s and ChR2s in this thesis. For example, the first discovered ChR1 and ChR2 mentioned above are designated as *Cr*ChR1 and *Cr*ChR2, where *Cr* is referred to *C. reinhardtii* in this thesis. There are two similar proteins subsequently mapped in related algae *Volvox carteri and Mesostigma viride*: *Vc*ChR1, *Vc*ChR2 (9-10) and *Mv*ChR1, respectively (11).

Channelrhodopsins arouse great interests because they can be used in numerous fields of biomedical research, especially neuroscience. An emerging application is named "optogenetics", that is, to manipulate the cells by means of lights to activate expressed ChRs (12). This technique provides both temporal and spatial precision of stimulation in mammalian cells and living animals. The optogenetics have been used to demystify the function of normal brain and cardiac cells, stem cell differentiation, and an extensive range of mental and neurological diseases, such as depression, autism, schizophrenia, drug abuse, and Parkinson's disease (13). In addition, to achieve the goal of gene therapy for vision restoration, the trial experiments have been already applied in rodent models with promising results (14-15). Therefore, finding new channelrhodopsins and knowing the basic mechanism of channelrhodopsins in algal phototaxis is undoubtedly fundamental to further biomedical research.

*Cr*ChR1 and *Cr*ChR2 separately have advantages and disadvantages in certain ways when applied on optogenetics. The kinetics of the currents generated by *Cr*ChR1 is faster than those of the currents generated by *Cr*ChR2 (16-17), which is therefore capable for more frequent photostimulation. However, *Cr*ChR2-generated currents show slow inactivation, i.e., a decrease in the current amplitude upon continuous illumination, while the inactivation of *Cr*ChR1-generated currents is much more rapid (16-17). Scientists

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prefer using longer (redder) wavelength excitation when performing optogenetics, because long-wavelength excitation light decreases the scatter produced by biological tissues and avoids the absorption by hemoglobin. *Cr*ChR1 has an advantage here because the maximal spectrum of *Cr*ChR1-generated currents in native *C. reinhardtii* cells is at 505 nm, while *Cr*ChR2-generated currents have a maximal absorption at 470 nm (4). However, the long-wavelength spectral sensitivity of *Cr*ChR1 can only be observed at acidic pH in heterologous systems (16, 18), which is not ideal for application in physiological environments of human cells.

C. reinhardtii is the most widely-used and well-studied model organism of genus *Chlamydomonas* (http://www.algaebase.org) while there are still at least hundreds of other species in this genus. They can be found in soil, melting snow, temporary pools, and eutrophic lakes. In many other *Chlamydomonas* species besides *C. reinhardtii*, the rhodopsin-mediated photocurrents similar to *Cr*ChR1 and *Cr*ChR2 have been recorded (19) and our unpublished observations). These photocurrents strongly suggested that they also contain channelrhodopsin specific regions, but it was unclear if the similar sequences have the same function as *Cr*ChR1 and *Cr*ChR2. We hypothesized that channelrhodopsin variants from *Chlamydomonas* species in the cold area inhabiting special ecological niches may work differently from their *C. reinhardtii* counterparts, and may offer possibly alternative advantages for optogenetic applications. *C. augustae,* the subject in my thesis is known as "snow algae", which originally grows in areas seasonally or permanently covered with snow. We supposed that if we can find the channelrhodopsins in snow algae such as *C. augustae,* it has some distinctive properties compared to *Cr*ChR1 and *Cr*ChR2.

Including the new channelrhodopsin that I found in *C. augustae* (*Ca*ChR1), we have identified three new channelrhodopsin variants in these *Chlamydomonas* species by homology cloning and four other new ChRs from algae from other genera in our laboratory. My thesis focuses on the process of cloning, expressing and characterizing *Ca*ChR1, which was the first new channelrhodopsin found by homology cloning. The comparison of *Ca*ChR1 with the known channelrhodopsins *Cr*ChR1 and *Cr*ChR2 and other new-found channelrhodopsins is also described in this thesis to show the unique properties of this new channelrhodopsin.

Chapter II: Materials and Experimental Methods

Algal Strain and Cultivation Conditions:

Chlamydomonas augustae was purchased from Culture Collection Program of Algae (UTEX) at the University of Texas, Austin, TX and the National Center for Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, ME. *C. augustae* (UTEX SNO134) were grown on Bold 1NV medium plates under illumination of 14 µmol photon $\cdot$ m$^{-2}\cdot$ s^{-1} at 4℃ or 16℃ in enriched Bold's basal medium which contains three times the amount of nitrogen source and vitamins as described at the website of the Culture Collection of Algae and Protozoa (UK) under illumination of 5 µmol photon $\cdot$ m$^{-2}\cdot$ s^{-1} at 4℃. Illumination was set with a cycle of 16 h light, 8 h dark and cool-white fluorescent lamps are the light source.

Primer designation:

The degenerate primers for homology cloning were designed according to the conserved regions of the four earlier known channelopsins from *C. reinhardtii* and *V. carteri*, and to reduce their degeneracy, the primers were selected further according to the codon usage preference of *C. augustae* which was accessed from Codon Usage Database (http://www.kazusa.or.jp/codon/). The forward and reverse primers which eventually got the partial channelrhodopsin gene were designed at helix B and helix G, named as SYHB-F (5'-TGC GGN TGG GAG GAG RTN TA-3'), and SYOG-R (5'-AGR ATR TGC TCR TGR ATC-3'), respectively. The primers are synthesized by Sigma-aldrich (St. Louis, MO).

Homology cloning of new channelopsin sequences:

The fully growing algae were tranferred from plates into 25 mL liquid medium in 250 mL flasks and grown for 18 h at 16 ℃. Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA). Initial strand cDNA was made with the Transcriptor first strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) using oligo-d(T) primer. PCR fragments were then cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced by UT Sequencing Facility. For the fragments showing homology with channelopsins, 3' and 5' RACE (rapid amplification of cDNA ends) was performed using the SMARTer RACE cDNA amplification kit (Clontech Laboratories, Mountain View, CA).

The full-length cDNA were obtained with the specific forward and reverse primers redesigned according to the sequenced DNA. And the full-length was cloned into the pGEM-T Easy vector and sequenced. The sequence encoding for the 7TM domains (residues 1–352) according to the prediction were inserted flanked by BamHI and NotI sites to exchange the existing VcChR1 sequence in the pcDNA3.1∕VcChR1-EYFP mammalian expression vector (a gift from Dr. K. Deisseroth, Stanford University). The vector map and sequence can be downloaded at the Optogenetics Resource Center website. The presence of EYFP is not expected to involve channelrhodopsin properties, as has been shown in previous publication by quantitative comparison of currents generated by other fluorescent tags such as YFP-, mCherry- and myc-tagged CrChR2 (27).

Whole-cell patch clamp recording in HEK293 cells:

HEK293 cells were transfected with the TransPass COS/293 transfection reagent (New England Biolabs, Ipswich, MA). All-trans-retinal (Sigma) was fresh-made and solved in ethanol at the final concentration of 2.5 μM. After 2-3 days transfection, the cells were measured with an Axopatch 200B amplifier (Molecular Devices, Union City, CA). The signals were digitized with a Digidata 1440A and data were obtained with pClamp 10 software (both from Molecular Devices).

Patch pipettes with resistances 2–5 MΩ were made from borosilicate glass and filled with solution as follows: 126 mM KCl, 2 mM $MgSO_4$, 0.5 Mm $CaCl_2$, 5mM EGTA, 25 mM HEPES, pH 7.2. On the other hand, the bath solution contained: 150mM NaCl, 1.8 mM $CaCl_2$, 4 mM KCl, 1mM $MgCl_2$, 5mM glucose, 10 mM, HEPES, pH 7.4, unless otherwise indicated. However, in the experiments at pH 9, Tris was used to replace HEPES in the base solution to stabilize the higher pH. The holding potential was -60 mV except the IV curve measurements. Light excitation was provided by a Polychrome IV light source (T.I.L.L. Photonics GMBH, Grafelfing, Germany) pulsed with a mechanical shutter (Uniblitz Model LS6; Vincent Associates, Rochester, NY; half-opening time 0.5 ms). The light intensity was tuned manually with neutral density filters or with built-in polychrome system. Maximal quantum density at the focal plane of the 40× objective lens was ca2 $\times 10^{22}$ photons$\times m^{-2}$.

Channelopsin expression and purification from *Pichia*:

The 7TM domain of *Ca*ChR1 (1–352 residues) was cloned into the pPIC9K vector (Invitrogen, Carlsbad, CA) flanked by EcoRI and NotI sites. There are two TEV protease sites added at the N-terminus and before the C-terminal 6 His-tag. The 20 μg plasmids

was linearized with BspEI and transformed into the *P. pastoris* SMD1168 (*his4, pep4*) strain by electroporation according to the manufacturer's instructions. First, since transformants can grow on histidine-deficient plates because the genes are integrated in the genome, we selected the colonies which grow on the His select plates. Second, their geneticin resistance has a positive correlation with the amount of DNA insertion. Say, the colony which could grow on 4 mg/mL geneticin is speculated to have 7-10 copies of the insert. A starter culture was inoculated into 500 mL minimal methanol yeast medium containing additional 100 mM phosphate buffer, pH 6. Expression was induced by 0.5% methanol every 24 h in the presence of 5 μM all- trans-retinal. Cells were grown for 2 days, collected by low-speed centrifugation and disrupted by a bead beater (Biospec Products Inc., Bartlesville, Ok). Membrane fragments were harvested by ultracentrifugation for 1 h at 230 000 g and solubilized by incubation with 2% dodecyl maltoside (DDM) for overnight. The His-tagged protein was then partially purified with a Ni-NTA agarose column (Qiagen, Hilden, Germany).

Absorption spectroscopy

Absorption spectrum of the partially purified *Ca*ChR1 in the UV/Visible range was measured on a Cary 4000 spectrophotometer (Varian, Palo Alto, CA).

Chapter III: Homology Cloning the Channelrhodopsin Gene in *Chlamydomonas augustae*

Low-degeneracy Primer designing

When we aligned the amino acid sequences of *Cr*ChR1, *Cr*ChR2, *Vc*ChR1 and *Vc*ChR2, we found there are several regions with high conservation which may be good targets for designing the degenerate primers (Fig 1). We started to use the degenerate primers in the regions of helix C and F with degeneracy 384 and 512, respectively (Fig 1and Table 1). Using cDNA of *C. reinhardtii* as a positive control, we got channelopsin gene ("channelopsin" is the name of the apoprotein, which is called channelrhodopsin when it is complexed with retinal) as a PCR product with these primers. However, we could not get any PCR products from cDNA of *C. augustae and C. nivalis.* Considering that there may be some secondary structures in the cDNA of helix C and F or other problem, we looked for other regions of conservation and switched the targeting regions to helix B and the region beyond helix G and re-designed the primers (named SYHB and SYOG, respectively. Fig.1 and Table2)

To reduce the degeneracy, we looked up the codon usage of the Chlamydomonas genus and selected the possible of codons of *C. augustae and C. nivalis* (Table 2). The final degeneracy was narrowed down to 32 and 16.

With these new primers, we cloned a 600-bp PCR product from cDNA of *C. augustae,* which has 30-52 % similarity compared to the amino acid sequences of *Cr*ChR1, *Cr*ChR2, *Vc*ChR1, and *Vc*ChR2. With RACE PCR technique, we obtained the full length (713 aa) channelopsin homolog (Acc. No. JN596951). In addition, with these primers and the methods of reducing the degeneracy, we also got the channelopsin homologs

from *C. yellowstonensis* (717 residues, Acc. No. JN596948) and *C. raudensis* (635 residues, Acc. No. JN596949).

Helix A

```
           60            *        80            *        100
ChR2  : FVTNPV-VVNGSVLVPE-DQCYCAGWIESRGTNGAQTASNVLQWLAAGFSIL :  65
VChR2 : GSSYTN-LNNGSIVIPS-DACFCMKWLKSKGSPVALKMANALQWAAFALSVI :  60
ChR1  : QTSYTLENNGSVICIPNNGQCFCLAWLKSNGTNAEKLAANILQWITFALSAL : 104
VChR1 : IVRYPTDLGNCTVCMPR-GQCYCEGWLRSRGTSIEKTIAITLQWVVFALSVA :  60
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Helix B

```
          *       120            *       140            *
ChR2  : LLMFYAYQTWKSTCGWEEIYVCAIEMVKVILEFFFEFKNPSMLYLATGHRVQ : 117
VChR2 : ILIYYAYATWRTTCGWEEVYVCCVELTKVVIEFFHEFDEPGMLYLANGNRVL : 112
ChR1  : CLMFYGYQTWKSTCGWEEIYVATIEMIKFIIEYFHEFDEPAVIYSSNGNKTV : 156
VChR1 : CLGWYAYQAWRATCGWEEVYVALIEMMKSIIEAFHEFDSPATLWISSGNGVV : 112
```

Helix C   Helix D

```
           160           *       180            *       200
ChR2  : WLRYAEWLLTCPVILIHLSNLTGLSNDYSRRTMGLLVSDIGTIVWGATSAMA : 169
VChR2 : WLRYGEWLLTCPVILIHLSNLTGLKDDYNKRTMRLLVSDVGTIVWGATAAMS : 164
ChR1  : WLRYAEWLLTCPVILIHLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALS : 208
VChR1 : WMRYGEWLLTCPVILIHLSNLTGLKDDYSKRTMGLLVSDVGCIVWGATSAMC : 164
```

Helix E   Helix F

```
          *       220            *       240            *       260
ChR2  : TGYVKVIFFCLGLCYGANTFFHAAKAYIEGYHTVPKGRCRQVVTGMAWLFFV : 221
VChR2 : TGYIKVIFFLLGCMYGANTFFHAAKVYIESYHTVPKGLCRQLVRAMAWLFFV : 216
ChR1  : KGYVRVIFFIMGLCYGIYTFFNAAKVYIEAYHTVPKGICRDLVRYLAWLYFC : 260
VChR1 : TGWTKILFFLISLSYGMYTYFHAAKVYIEAFHTVPKGICRELVRVMAWTFFV : 216
```

Helix G

```
          *       280            *       300            *
ChR2  : SWGMFPILFILGPEGFGVLSVYGSTVGHTIIDIMSKNCWGLLGHYLRVLIHE : 273
VChR2 : SWGMFPVLFLLGPEGFGHLSVYGSTIGHTIIDILSKNCWGLLGHFLRLKIHE : 268
ChR1  : SWAMFPVLFLLGPEGFGHINQFNSAIAHAILDIASKNAWSMMGHFLRVKIHE : 312
VChR1 : AWGMFPVLFLLGTEGFGHISPYGSAIGHSILDIIAKNMWGVLGNYLRVKIHE : 268
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           320           *       340            *       360
ChR2  : HILIHGDIRKTTKLNIGGTEIEVETLVEDEAEAGAVNKGTGKYASRESFLVM : 325
VChR2 : HILLYGDIRKVQKIRVAGEELEVETIMTEBAPD-TVKKSTAQYANRESFLTM : 319
ChR1  : HILLYGDIRKKQKVNVAGQEMEVETMVHEEDDE-TQKVPTAKYANRDSFIIM : 363
VChR1 : HILLYGDIRKKQKITIAGQEMEVETLVAEEEDD-TVKQSTAKYASRDSFITM : 319
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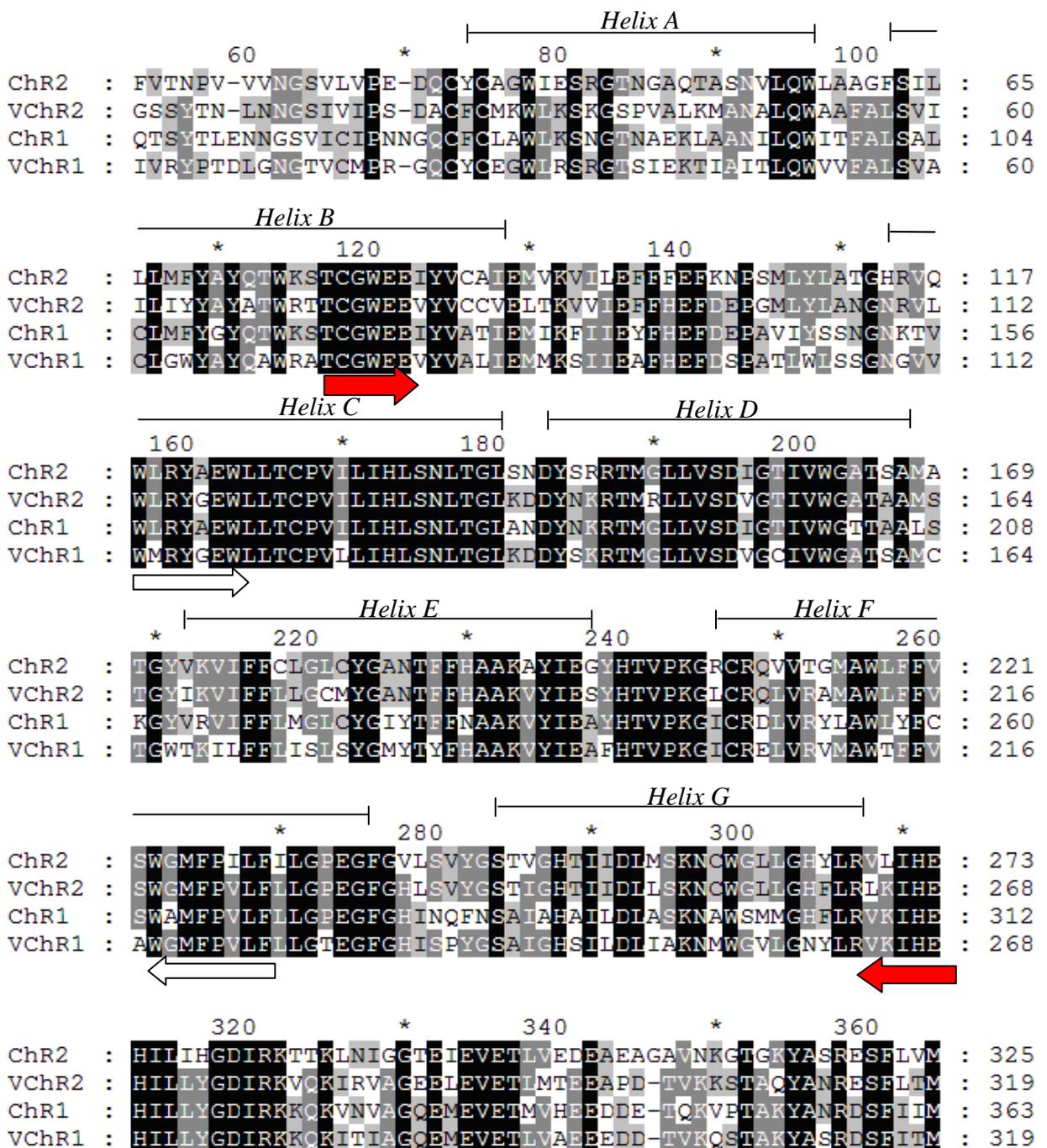Figure 1: The alignment of amino sequences of *Cr*ChR1, *Cr*ChR2, *Vc*ChR1 and *Vc*ChR2. The total identical amino acids are shown in black blocks. The white arrows of helix C and F are the primers that failed to get any PCR products from cDNA of new *Chlamydomonas* algae. The red arrows at helix B and the region beyond G are the primers (SYHB and SYOG) that we successfully used for cloning the partial gene of *Ca*ChR1.

Primer names	Helix C: W <u>L</u> <u>R</u> <u>Y</u> <u>A</u> E W	Degeneracy
chlr_deg_87s	tg<u>ght</u><u>nmg</u><u>nta</u><u>ygs</u>agaatgg	384
chlr_deg_88s	tg<u>ght</u><u>nmg</u><u>nta</u><u>ygs</u>tgaatgg	384
chlr_deg_89s	tg<u>ght</u><u>nmg</u><u>nta</u><u>ygs</u>cgaatgg	384
chlr_deg_90s	tg<u>ght</u><u>nmg</u><u>nta</u><u>ygs</u>ggaatgg	384
chlr_deg_91s	tg<u>ght</u><u>nmg</u><u>nta</u><u>ygs</u>agagtgg	384
chlr_deg_92s	tg<u>ght</u><u>nmg</u><u>nta</u><u>ygs</u>tgagtgg	384
chlr_deg_93s	tg<u>ght</u><u>nmg</u><u>nta</u><u>ygs</u>cgagtgg	384
chlr_deg_94s	tg<u>ght</u><u>nmg</u><u>nta</u><u>ygs</u>ggagtgg	384

Primer names	Helix F: W <u>A</u> M <u>F</u> <u>P</u> <u>V</u> L F	Degeneracy
chlr_deg_95a	aa<u>nar</u><u>nay</u>nggr<u>aaca</u>tagccca	512
chlr_deg_96a	aa<u>nar</u><u>nay</u>nggr<u>aaca</u>ttgccca	512
chlr_deg_97a	aa<u>nar</u><u>nay</u>nggr<u>aaca</u>tcgccca	512
chlr_deg_98a	aa<u>nar</u><u>nay</u>nggr<u>aaca</u>tggccca	512
chlr_deg_99a	aa<u>nar</u><u>nay</u>nggr<u>aaca</u>taccca	512
chlr_deg_100a	aa<u>nar</u><u>nay</u>nggr<u>aaca</u>ttcccca	512
chlr_deg_101a	aa<u>nar</u><u>nay</u>nggr<u>aaca</u>tcccca	512
chlr_deg_102a	aa<u>nar</u><u>nay</u>nggr<u>aaca</u>tgcccca	512

Table 1: The primers of helix C and F, which have high degeneracy.

```
UUU   9.9(     7)   UCU 11.3(     8)   UAU   5.7(     4)   UGU   0.0(     0)
UUC 31.2(    22)   UCC 19.8(    14)   UAC 24.1(    17)   UGC 22.7(    16)
UUA   1.4(     1)   UCA   4.2(     3)   UAA   5.7(     4)   UGA   0.0(     0)
UUG 11.3(     8)   UCG   1.4(     1)   UAG   1.4(     1)   UGG   9.9(     7)

CUU   9.9(     7)   CCU 15.6(    11)   CAU   8.5(     6)   CGU 21.2(    15)
CUC 11.3(     8)   CCC 21.2(    15)   CAC 15.6(    11)   CGC 35.4(    25)
CUA   2.8(     2)   CCA   1.4(     1)   CAA   2.8(     2)   CGA   8.5(     6)
CUG 26.9(    19)   CCG   0.0(     0)   CAG 31.2(    22)   CGG   0.0(     0)

AUU 12.7(     9)   ACU 19.8(    14)   AAU   0.0(     0)   AGU   5.7(     4)
AUC 34.0(    24)   ACC 22.7(    16)   AAC 41.1(    29)   AGC 15.6(    11)
AUA   0.0(     0)   ACA   1.4(     1)   AAA   2.8(     2)   AGA   1.4(     1)
AUG 15.6(    11)   ACG   0.0(     0)   AAG 93.5(    66)   AGG 21.2(    15)

GUU 25.5(    18)   GCU 41.1(    29)   GAU 19.8(    14)   GGU 15.6(    11)
GUC 31.2(    22)   GCC 39.7(    28)   GAC 21.2(    15)   GGC 28.3(    20)
GUA   1.4(     1)   GCA 14.2(    10)   GAA   0.0(     0)   GGA 19.8(    14)
GUG 19.8(    14)   GCG   5.7(     4)   GAG 55.2(    39)   GGG   1.4(     1)
```

Table 2: The codon usage frequencies of *Chlamydomonas* genus.

The numbers after triplets are the frequency per thousand genes while the numbers in

parenthesis are the amounts.

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Primer names	Helix B **T C G W E E I**	Degeneracy
SYHB-F	**Tgcgg<u>n</u>tgggaggag<u>r</u>t<u>n</u>ta**	**32**
	Out of Helix G **I H E H I L**	
SYOG-R	**ag<u>r</u>at<u>r</u>tgctc<u>r</u>tgratc**	**16**

Table 3: The optimized primers of helix B and out of helix G, which have much lower

degeneracy compared to those in table 1.

Sequence comparison of *Ca*ChR1 with other channelrhodopsins

The new channelopsin protein from *C. augustae* shares some common features with all so far known channelopsins: a predicted seven transmembrane (7TM) domain responsible for light-gated channel activity and a C-terminal domain with various lengths and yet unknown function. When the 7TM domains are analysed separately from the rest of the domains or the overall sequence homology is concerned, ChR1s and ChR2s are roughly divided into two branches on the phylogenetic tree (Fig. 2a). The C-terminal domain sequence of new channelopsin shows closer homology to *Cra*ChR2/*Vc*ChR2 branch (Fig. 2b). However, the sequences of the new channelopsin from *C. augustae* and the new one found in *C. yellowsonesis*) are so close to each other that they always form an individual branch from other channelopsins.

Although the overall sequence of the new channelopsin from *C. augustae* seems closer to *Cra*ChR2/*Vc*ChR2, it has two molecular determinants which are exclusively conserved in *Cr*ChR1/*Vc*ChR1 sequences: 1) Glu87 (*Cr*ChR1 numbering) in the predicted helix A is accounting for pH-dependent color tuning and fast channel inactivation of *Cr*ChR1, when compared to *Cr*ChR2 (19); 2) Tyr226 (*Cr*ChR1) in the predicted helix E, as compared to Asn187 to *Cr*ChR2, results in differences of spectral sensitivity, inactivation and kinetics between *Cr*ChR1 and *Cr*ChR2 (22). According to these criteria, the new channelopsin of *C. augustae* along with the one of *C. yellowsonesis* are supposed to belong to the *Cr*ChR1/*Vc*ChR1 class (Fig. 2). Their red-shifted spectra are the further evidences to classify them into the *Cr*ChR1/*Vc*ChR1 group (see below), as compared to the *Cr*ChR2/*Vc*ChR2 class (9,16). Thus, we named the new channelopsins as *Ca*ChR**1** and *Cy*ChR**1**, respectively. In contrast, according to the two

above-mentioned molecular determinants, the sequence from *C. raudensis* (*Cra*ChR2) should belong to the *Cr*ChR2/*Vc*ChR2 class. However, this placement is only tentative because we so far cannot see any photocurrents generated by *Cra*ChR2 in HEK cells.

With a look of the 7 TM domain of *Ca*ChR**1** sequence more carefully, we found some other conserved residues at the critical sites which may form the retinal-binding pocket and are coincident with other known rhodopsins/channelrhodopsins. These include: 1) His at the position of the Schiff base proton donor (Asp96 according to bacteriorhodopsin (BR) numbering); 2) Glu in the position of the Schiff base proton acceptor (Asp85 in BR); 3) five Glu residues in or near the predicted helix B; 4) Cys128 and Asp156 (*Cr*ChR2 numbering) that form a predicted hydrogen bond between the helices C and D (20-22). 5) Tyr57, Gly122, Trp182, Asp212 and Lys216 (BR numbering) are conserved in *Ca*ChR**1**, while Tyr185 and Trp189 (BR numbering) are occupied by Phe residues. These are the biggest differences between previous known channelopsins and other microbial rhodopsins.

The function of C-terminal domains of channelrhodopsins remains unknown, although *Ca*ChR1 and *Cy*ChR1 again show close similarity (Fig. 2b). No helices are predicted in the C-terminal domains of *Ca*ChR**1** in contrast to *Cr*ChR1 (4). Interestingly, as in previously known channelrhodopsins, the C-terminal domains of *Ca*ChR1 contain highly conserved regions which are unique and with no homology to any other known proteins. These conserved regions are composed of repeats that vary in amino acid compositions and length among different channelopsins. In *Ca*ChR**1**, the features are long stretches of Gly-Met repeats, Gln repeat and Met-rich regions. Such repeats/ regions are predicted to form homopolymeric oligomers (24), which occur in many eukaryotic proteins and may

also be involved with protein-protein (24) or protein-membrane (25) interactions. At the very end of the C-terminal domains, there is a highly conserved region of about 40 residues which can be found in all so far known channelrhodopsins except *Mv*ChR1. This 40 a.a residue has homology to domains in fibrinogen and ABC transporters that account for protein multimerization and protein-protein interaction. Channelrhodopsins are located at the membrane in the eyespot (8,18) in algal cells and are attached to the acetylated microtubules of the four-membered flagellar rootlet (26). Therefore, the C-terminal domains are supposed to control subcellular localization of channelrhodopsins.

*Ca*ChR**1** has an extra large extracellular N-terminal region compared to other channelopsins. This region contains a conserved N-glycosylation site. The *Cr*ChR1 and several other channelopsins (except *Cra*ChR2) also have such possible glycosylation sites but at different positions in the N-termini (Fig. 3). The similar possible N-glycosylation sites are also located at the cytoplasmic end of helix C among all so far known channelopsins. The well-known protein which has N-glycosylation sites in the cytoplasmic domains of proteins is the α-subunit of mammalian Na^+, K^+-ATPase (27). The glycosylation of *Cr*ChR2 was confirmed by treatment with peptidyl N-glycosidase F when heterologously expressed. However, the positions and amount of glycosylation remain undetermined (28). The glycosylations are responsible for proper folding and targeting of channelrhodopsins. Thus it can be an explanation why several investigators have tried but did not succeed in getting the functionally expressed channelrhodopsin in *E. coli*. Unlike *Cr*ChR1, *Ca*ChR**1** sequences lack an additional α-helix predicted in the N-terminus which is predicted as a signal peptide.

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It is speculated that when *Chlamydomonas* algae exhibit phototaxis, channelrhodopsin phosphorylation is involved in the processes of adaption which allows the broad range of light intensities. Three phosphorylated residues of *Cr*ChR1 were identified by phosphoproteomics of the eyespot fractions. The phosphorylated sites were found in the cyplasmic loop next to the 7TM and this loop is highly conserved in so far known channelopsin sequences (except *Mv*ChR1). Therefore, Ser 366 and Ser 384 in *Ca*ChR1 (Ser 359 and Ser 377 in *Cr*ChR1) are suggested to be the phosphorylated residues. However, Thr374 is unique for *Cr*ChR1 because none of other channelopsins has this phosphorylated residue (Fig.3).

Since *C. augustae* grows at the low temperature, and we expect its channelrhodopsin has some unique properties compared to other channelopsins, it is also important to examine whether *Ca*ChR1 has some special amino acid composition like other psychrophilic (cold-loving) organisms. There are some features of psychrophilic organisms such as decreased percentages of Pro, Arg and Ala residues, and an increased percentage of Ile residues (29, 30) in *Ca*ChR1. But no increased percentage of Gly can be found in *Ca*ChR1, another psychophilic protein indicator.
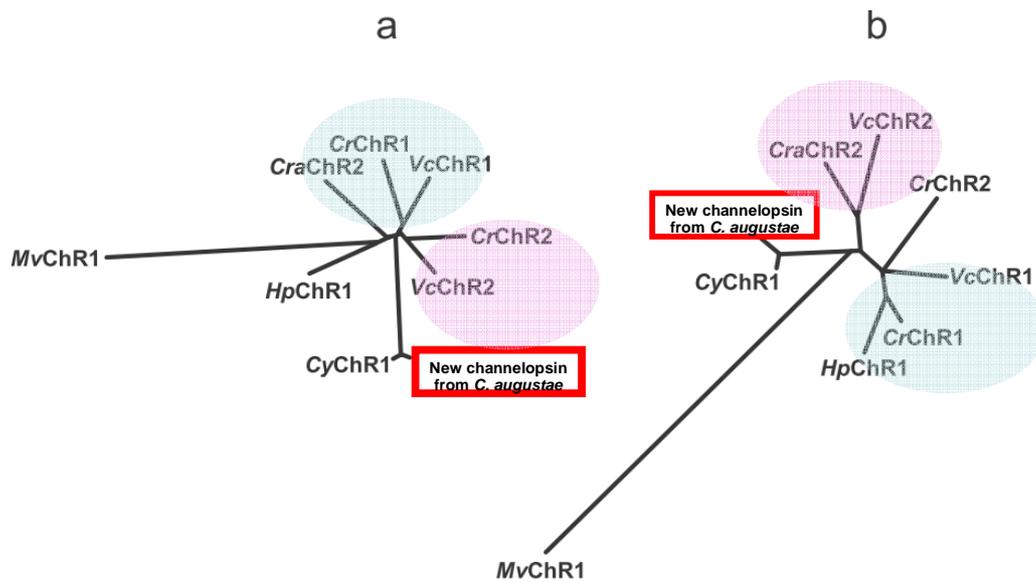
Figure 2: Phylogenetic trees of the 7TM domains (a) and the C-terminal domains (b) of the so far known channelopsins constructed by the neighbor-joining method. The abbreviations of channelrhodopsins are as follows: *Cr*ChR1 = channelrhodopsin 1 from *C. reinhardtii*; *Cr*ChR2 = channelrhodopsin 2 from *C. reinhardtii*; *Vc*ChR1 = channelrhodopsin 1 from Volvox carteri; *Vc*ChR2 = channelrhodopsin 2 from *V. carteri*; *Mv*ChR1 = channelrhodopsin 1 from *Mesostigma viride*; *Cy*ChR1 = channelrhodopsin 1 from *C. yellowstonensis; Cra*ChR2 = channelrhodopsin 2 from *C. raudensis*; *Hp*ChR1 = channelrhodopsin 1 from *Haematococcus pluvialis*.

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CaChR1   FNVTITIGGGHHGGHAGPVDNSIVIGGIDGWIAIPAG-DCYCAGWYVSHGSSFEATFAHVCQWSIFAVCILSLLWYAWQYWKATCGWEEVYVCCIELVFI   140
CyChR1   HNVTLLIAPPHAGGHACPTDTSQQITGIDGWIAIPAG-DCYCAGWYVSHGSSFEAIFAHVCQWSIFAVCVLSLLWYAQYWKATCGWEEVYVCCIELVFI   141
CrChR1   VATQDGDVYFHRAHERMLFQTSYTLENNGSVICIPNNGQCFCLAWLKNGTNAEKLAANILQWITFALSALCLMFYGYQTWKSTCGWEEIYVATIEMIKF   133
CraChR2  LTPYGCLNDFYCNPAYGLADAGYNYCYVQSAYGKLAIVQTDQLSWLYSHGSSGAKAASIAFDATAVIGLMFYAWDTWKATTGWEEVYVCTIELIKV    138
CrChR2   ----MDYGGALSAVGRELLFVTNPVVVNGSVLVPED--QCYCAGWIESRGTNGAQTASNVLQWLAAGFSILLLMFYAYQTWKSTCGWEEIYVCAILEMVKV   94
BR       -----------QAQITG--------------------RPEWIWLALGTALMGLGTLYFLVKGMGVSDPDAKKFYAITTLVPAIAFTMYLSMLL----    62

CaChR1   CFELYHEFDSPCSLYLSTANIVNWLRYSEWLLCCPVILIELSNVTGLSDDYGRRTMGLLVSDIATIVFGITAAMLVSWPKIH-EYLLGFTMCCYTFYLAA   239
CyChR1   CFELYHEFDSPCSLYLSTSNVVNWLRYSEWLLCCPVILIELSNVTGLSDDYGRRTMGLLVSDIATIVFGVTAAMLVNWPKIH-EYLIGFTMCCYTFFLAA   240
CrChR1   IIEYFHEFDEPAVIYSSNGNKTVWLRYAEWLLTCPVILIHLSNLTGLANDYNKRTMGLLVSDIGTIVWGTTAALSKGYVRVH-FFLMGLCYGIYTFFNAA   232
CraChR2  LIEIEKEEEIPCSLYIPTGNWVLWLRYAEWLLTCPVILIHLSNITGLKDDYNKRTMRLLVSDIGCIVWGVTSAMTVGYLKWI-FFAIGLLYGSNTYFHSA   237
CrChR2   ILEFFFEEKNPSMLYLATGHRVQWLRYAEWLLTCPVILIHLSNLTGLSNDYSRRTMGLLVSDIGTIVWGATSAMATGYVKVI-EFCLGLCYGANTFFHAA   193
BR       ------GYGLTMVPFGGEQNPIYWARYADWLFTTPLLLLDLALLVDAD---QGTILALVGADGIMIGTGLVGALTKVYSYRFVWWAISTAAMLYILYVLF   153

CaChR1   KVLIESFHQVPKGICRHLVKAMAITYYVGWSFFPLIFLFGQSGEKKISPYADVIASSFGDIISKN   304    ANRGSFIVMRGNMKAQGIDVRASLD   386
CyChR1   KVLIESFHQVPKGICRHLVKAMAITYFVGWSFFPLIFLFCQSGEKKISPYADVIASSFGDIISKN   305    ANRGSFIVMRDKMKEQGIDVRASLD   387
CrChR1   KVYIEAYHTVPKGICRDLVRYLAWLYECSWAMFBVLFLLGPEGFGHINQFNSAIAHAILDLIASKN 297    ANRDSFIIMRDRLKEKGFEIRASLD   379
CraChR2  KVYIEAYHTVPKGRCRVIVRLMAYCFYLAWTMFPILFALGPEGMGQMSAYMSTILTTHADVLSKQ   302    ANRESFVHMAEQMKKNGIEVRATYD   384
CrChR2   KAYIEGYHTVPKGRCRQVVTGMAWLFFVSWGMFPILFILGPEGFCVLSVYGSTVGHTIIDLMSKN   258    ASRESFLVMRDKMKEKGIDVRASLD   371
BR       FGFTSKAESMRPEV-ASTFKVLRNVTVVLWSAYPVVWLIGSEGACIVPLNIETLLFMVLDVSAKV   217
```
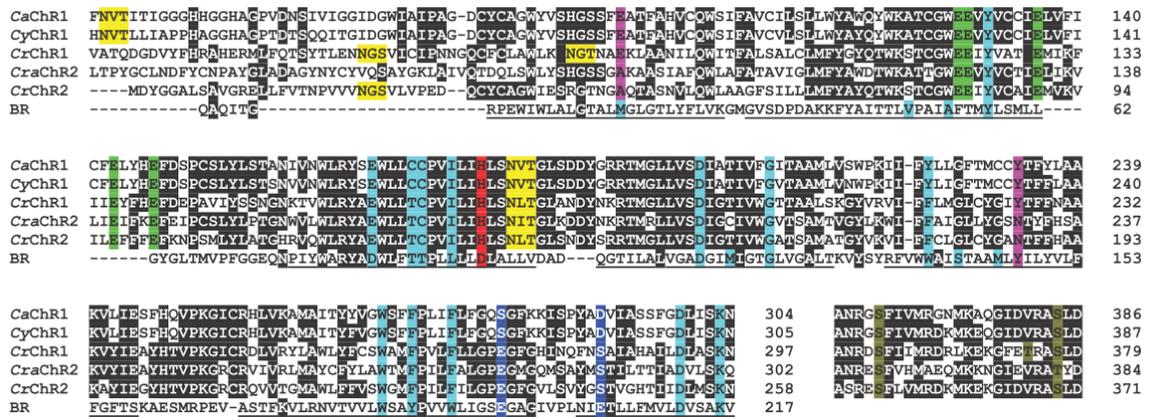
Figure 3: Partial alignment of *Chlamydomonas* channelopsin and bacteriorhodopsin (BR) sequences.

Black background: highly conserved identical residues.

Turquoise background: positions of the residues that form the retinal-binding pocket in BR.

Green background indicates: Glu residues in the predicted helix B.

Magenta background: molecular determinants that differentiate *Cr*ChR1 ∕ *Vc*ChR1 from *Cr*ChR2 ∕ *Vc*ChR2.

Red background: residues in the position of the proton donor in BR.

Blue background: residues in the positions of Glu194 and Glu204 in BR.

Yellow background: predicted glycosylation sites.

Olive background: conserved residues known to be phosphorylated in *Cr*ChR1 or *Cr*ChR2.

Underlined characters: the regions that form transmembrane helices in BR.

Chapter IV: Heterologous Expression of the Channelrhodopsin Gene in HEK cells and Electrophysiological Characterization

Functional characterization of new channelrhodopsins

The fluorescence of EYFP-tags followed by 7TM domains of *Ca*ChR1 indicated that the expression in the plasma membrane of HEK293 cells is successful. The typical signals recorded at the maximal light intensity under our standard conditions (holding potential (V_{hold}) -60 mV and bath pH 7.4) shows in Figure 4. The currents generated by *Ca*ChR1 and CyChR1 had similar features while they are quite different from the kinetics of the currents generated by *Cr*ChR1, that generated by *Ca*ChR1 were however quite unique. The currents generated by *Cr*ChR1 have a rapid initial rise with a time constant (τ) ~ 1 ms which is followed by a peak and a rapid (τ ~ 5 ms) decline under continued illumination, i.e., inactivated to a lower level (Fig. 4a, black line). The currents displayed a consequent slight increase with τ ~ 200 ms in many HEK cells. The *Cr*ChR1 also has similar behavior reported in a previous publication. (31). However, the rise of *Ca*ChR1 generated currents has two distinct phases. The first rapid phase resembled *Cr*ChR1-generated currents, but a slower rising phase with τ ~ 20 ms appeared later (Fig. 4a, red and green lines). After the peak, *Ca*ChR1-generated currents ended with an inactivation which is remarkably slow (τ ~500 ms).
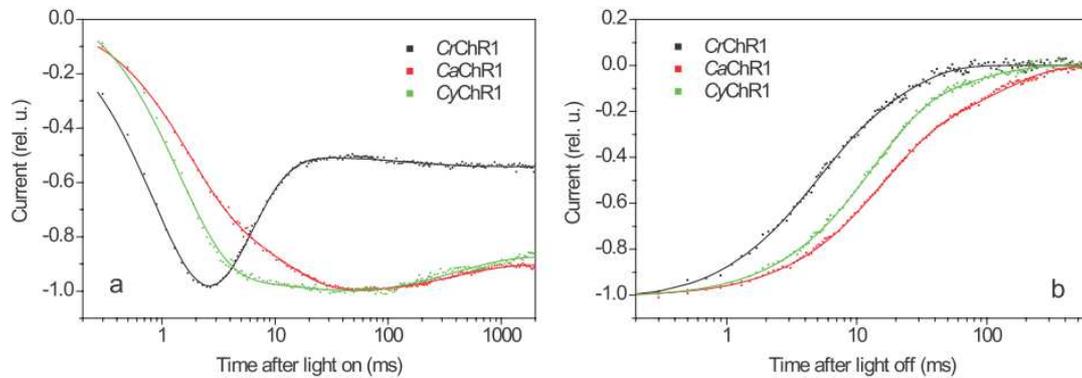
Figure 4: (a) Typical kinetics of light-induced currents generated by ChR1s in HEK293 cells: *Cr*ChR1 (black dots), *Ca*ChR1 (red dots) and *Cy*ChR1 (green dots). (b) Decay of the same ChR1 currents after 2 s illumination. The currents in (a) have been normalized to the peak amplitude, and the currents in (b) has been normalized to the plateau level and respectively fitted with three (a) or two (b) exponential functions (solid lines). The excitation wavelength was 520 nm, 520 nm and 480 nm for *Ca*ChR1, *Cy*ChR1, and *Cr*ChR1, respectively. These measurements are done under a series of light pulses with 30s time intervals. The cells homogeneously expressing EYFP fluorescence were selected for these measurements. Bath pH was 7.4, V hold was -60 mV.)

The *Ca*ChR1-generated currents have a bigger peak compared to the plateau level, which is measured at the end of the first light flash. The peak/plateau was not fully recovered even after 30 min dark interval, suggesting a very slow adaptation process, or an irreversible bleaching existed. The latter may due to the unstable pigments. However, the currents generated by the second and all succeeding flashes recorded with 30 s dark intervals had no obvious differences. The peak/plateau ratio of *Cr*ChR1 measured in our laboratory at the maximal light intensity was 1.7 ± 0.2 (mean $\pm$ SEM, n = 8), which was similar to the earlier reported results (32), while the peak/plateau ratio of *Ca*ChR1 was notably smaller: 1.2 ± 0.1 (mean $\pm$ SEM, n = 12 and n = 6, respectively). The absolute plateau amplitude was 101 ± 25 pA (mean $\pm$ SEM, n = 8) for *Cr*ChR1, while it was 64 ± 9 pA (mean $\pm$ SEM, n = 12) for *Ca*ChR1. After light was off, the currents decayed biexponentially with $\tau \sim 15$ and ~ 120 ms for *Ca*ChR1, which was both slower than that measured for *Cr*ChR1 (Fig. 4b), but similar to that for *Cr*ChR2 (17).

The most extensively used channelrhodopsin variant in optogenetics, *Cr*ChR2, produces huge enough currents in HEK cells even solely relying on the endogenous retinal (19) and our own unpublished observations), indicating the trace amount of *Cr*ChR2/retinal complex present in these cells is enough for working functionally. However, the currents in cells transfected with *Ca*ChR1or *Cr*ChR1 were significantly smaller with no additional exogenous retinal: their plateau levels dropped to only $\sim 8\%$ for *Ca*ChR1 and $\sim 26\%$ for *Cr*ChR1 compared to the results obtained in the presence of exogenous retinal.

*Cr*ChR1 and many channelrhodopsins displayed a typical dependence on the holding potential (V_{hold}), and *Ca*ChR1-generated current was also greatly influenced by

the holding potential. (Fig. 5a, and c.) As the same as that for *Cr*ChR1, the reversal

potentials (V_r) were near zero observed in our laboratory. In order to test if *Ca*ChR1 is a

highly proton-selective channel like *Cr*ChR1 (ref), we prepared different buffer for HEK

cells to change the external pH and tested proton permeability of *Ca*ChR1 by measuring

current-voltage relationships (I-V curves). Lowering the pH of the external buffer raised

the current amplitude at a given voltage and the reversal potential became more positive.

The degree of this shift for *Ca*ChR1 was like that for *Cr*ChR1 (Fig. 5a and c). Therefore,

we can conclude that *Ca*ChR1, like *Cr*ChR1 (6, 18), is highly selective for protons.

However, after the light is off, the rate of current decay for *Ca*ChR1 slightly increased

when the bath pH dropped from 7.4 to 5.4. (Fig.5d). In contrast, that for *Cr*ChR1

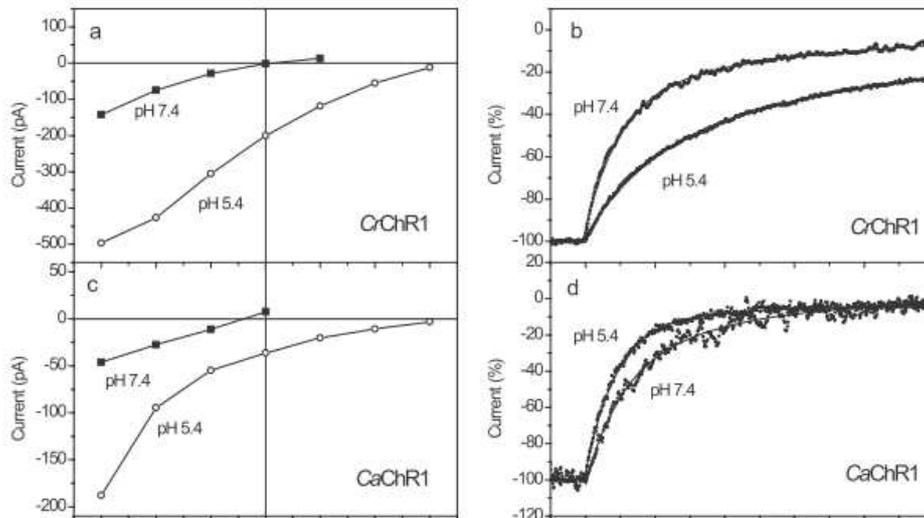decreased at acidic bath pH in the previous study (17).

Figure 5: (a and c) Typical current-voltage relationships (I–V curves) for the plateau level. The I-V curves were measured at the tail of a 2 s excitation light pulse with an increasing V_{hold} with 20 mV steps from -60 mV at the bath pH 7.4 and 5.4 (solid squares and open circles, respectively) in HEK293 cells expressing *Cr*ChR1or *Ca*ChR1. Corresponded to the spectral maxima of *Ca*ChR1 and *Cr*ChR1, the wavelength was 520 nm and 480 nm, respectively (b and d). Normalized current decay traces recorded from cells expressing *Cr*ChR1or *Ca*ChR1 at -60 mV as holding potential. The measurements at different pH 7.4 or 5.4 were subsequently recorded with the same cell. It is worth notice that the opposite effects of pH changes on the decay kinetics in *Ca*ChR1 and CrChR1. Zero time began from the end of a 2 s excitation light pulse. Excitation light was the same as in a and c. Experimental data (dots) were fitted with two exponential functions (solid lines).

The lower intensity light was used to measure the spectral sensitivity of photocurrents with the method described earlier for *Mv*ChR1-generated currents (11). The maximal sensitivity for *Ca*ChR1 was at 520 nm at pH 7.4 (Fig. 6a and b, black squares), which is 40 nm longer than both that of the action spectrum of *Cr*ChR1-generated currents in oocytes and the absorption spectrum of purified *Cr*ChR1 at neutral pH (17, 18). For *Ca*ChR1, the spectra almost stayed the same when measured at neutral (7.4) and acidic (5.4) pH (Fig. 6a and b, black and red symbols), while the spectrum of *Cr*ChR1 displayed a big red shift when the pH of the medium decreased (17, 18). However, when the pH increased from 7.4 to 9, there was a tiny blue shift (~10 nm) in the spectrum of *Ca*ChR1 (Fig. 6a and b, green solid triangles).
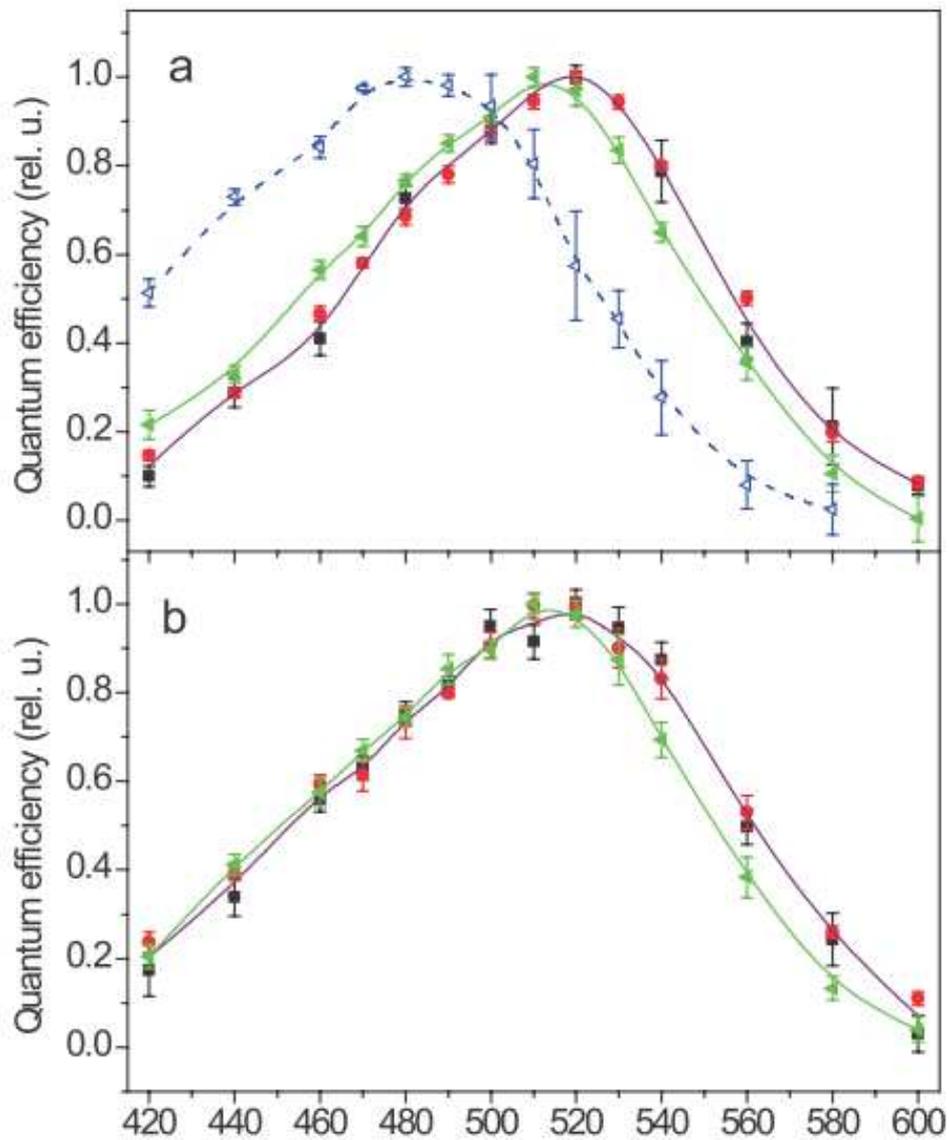
Figure 6: (a, b) The action spectra of photoelectric currents detected in HEK293 cells by *Ca*ChR1 (a) *or Cy*ChR1 (b) at the bath with neutral pH (7.4), acidic pH (5.4) or basic pH (9.0) in black squares, red circles and green solid triangles. respectively. In contrast, the action spectrum from *C. reinhardtii* measured at pH 7.4 is shown in Panel a (blue open triangles, dashed line).

Chapter V: Heterologous Expression of the Channelrhodopsin Gene in *Pichia pastoris* and Protein Characterization

To get the sufficient amount of new channelrhodopsin proteins to do functional assays such as absorption spectrum and photocycle measurements or obtain the structure by crystallization, we have tried different expression systems. Firstly, we tried heterologously expressed *Cr*ChR2, *Mv*ChR1, *Cy*ChR1 and *Cra*ChR1 in *E.coli* UT5600 or BL21, but none of them could be expressed in these prokaryotic systems. Since CrChR1, CrChR2, *Mv*ChR1, *Cy*ChR1, *Ca*ChR1 could all be expressed in mammalian HEK293 cells (some of the channelrhodopsins which can be expressed successfully in our lab are listed in table 4), we speculated that there may be several post-transcription sites such as glycosylation and phosphorylation sites in channelrhodopsin as predicted before, and that is the reason we can only get the channelrhodopsins in eukaryotic system.

Therefore, we chose a yeast system. We started from expressing *Ca*ChR1 in the *Pichia pastoris* strain GS115, but the expression level was very low, which is coincident with previous reported attempts (18). To improve the expression amount, we switched to the strain SMD1168, which is deleted for much of the PEP4 gene and may keep more proteins from degradation. With this host and the vector pPIC9K rendering multiple cloning integration, we successfully got 6.4 mg from 1 liter *Pichia* culture as estimated from absorbance at 520 nm. Subsequently, this method is also successful on expression of *Cr*ChR2 (Table 4).

With partial purification, we obtained the *Ca*ChR1 protein enough for the subsequent experiments. The absorption spectrum of *Ca*ChR1 partially purified from *Pichia* is perfectly coincident with the action spectrum of photocurrents generated by this pigment in HEK cells, which shows that its native state was essentially preserved in

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detergent (Fig. 7). The result suggests the *Ca*ChR1 folds and functions well with the

presence of all-*trans*-retinal in either yeast cells or mammalian cells, which may be the

model to determine how it works in the native algal cells.

	HEK cells	*E.coli*	*pichia* cells
*Cr*ChR1	V	N/A	X
*Cr*ChR2	V	N/A	V
VcChR1	V	N/A	N/A
*Hd*ChR1 (*Haematococcus pluvialis*)	X	N/A	N/A
*Mv*ChR1	V	X	X
*Ca*ChR1	V	N/A	V
*Cy*ChR1	V	X	N/A
*Cra*ChR2	X	X	X
*Ds*ChR1 (*Dunaniella salina*)	V	N/A	ongoing
*Ps*ChR1 (*Platymonas* subcordiformis*)	V	N/A	ongoing

Table 4: Some of the channelrhodopsins expressed with different expression systems in

our laboratory.
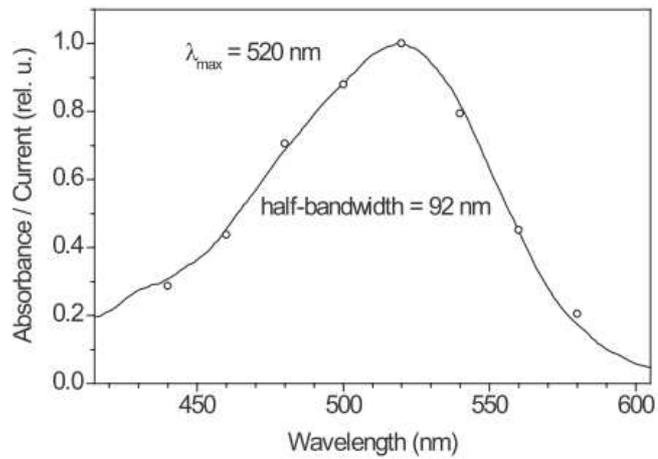
Figure 7: The absorption spectrum of partially purified *Ca*ChR1 in detergent (black

line) compared with the action spectrum of photocurrents (open circles) in HEK cells in

pH 7.4.

Chapter VI. Discussion and Future Perspectives

In the past decade, scientists keep looking for more efficient and various optogenetic tools. With this hope, they launched studies of molecular engineering with known channelrhodopsins, especially *Cr*ChR2 (e.g., (33-35)). However, this approach is limited because of the current minimal understanding of their structure and function.

In order to expand the toolbox of channelrhodopsin probes for optogenetic applications,meanwhile, we need to expand the basic knowledge of channelrhodopsin. We expect to find natural channelrhodopsin variants in other algal species, and analysis of accessible genomic and EST databases is a resourceful way to explore for new channelopsins. Sequences from *C. reinhardtii*, *V. carteri* and *M. viride* have been cloned by this method. *Mv*ChR1 from the latter is the most different variant so far which acts as a light-gated channel (11).

However, we can only obtain the handful of phototactic algal DNA sequences since there are only a few algal sequencing have been launched. Thus, the homology cloning of new channelopsins is required. The microbial opsin genes from the cryptophyte *Cryptomonas* and the glaucophyte *Cyanophora* had been successfully cloned by homology cloning in previous publications. (36-37). However, these genes are phylogenetically very far from known channelopsins of green algae and when they were expressed in HEK cells ion channel activity could not be observed. (with our unpublished observations).

In this thesis, I used homology cloning to identify new channelopsin sequences in *C. augustae* which has reasonably high homology with the earlier known channelopsin genes *CrChR1*. However, along with CyChR1 and CauChR2 which are cloned in our lab with the same strategy, we only got one channelopsin gene in one algal species, whereas

there are both two genes cloned from *C. reinhardtii* and *V. carteri*. In *C. reinhardtii*,

*Cr*ChR1 functions as a fast, high-saturating current component, whereas *Cr*ChR2 works

in a slow (delayed), low-saturating current fashion (4). We have tested almost all

flagellate species which generate rhodopsin-mediated photocurrents, there are two

components can be detected in native cells except *M. viride* (38). Therefore it is highly

likely that most algae, including *C. augustae*, *C. yellowstonensis* and *C. raudensis,* also

contain at least two channelrhodopsin homologs. To "fish" more possible

channelrhodopsin genes in the same organism, we may optimize the primer designing

according to the codon usage/preference in ChR1 and ChR2 in the future study.

Photocurrents generated by *Ca*ChR1 have different fashion from that of *Cr*ChR1

in the kinetics, inactivation and light dependence, suggesting at least quantitatively they

may have different photochemical reaction cycles. *Ca*ChR1 generated currents has a

dependence on the external pH, which may result from the residue in the position of

Glu94/Glu87 (*Ca*ChR1/*Cr*ChR1 numbering) in color tuning). *Mv*ChR1, in contrast, does

not display the spectral shift over the whole tested pH range from 5.3 to 9, maybe

because it contains a non-carboxylated residue in this position instead (11). This residue

was suggested to help the formation of the counterion of the protonated Schiff base,

which is a unique character of ChR1-like channelrhodopsins in contrast to ChR2-like

ones (17). Thus, protonation/deprotonation of this residue would influence on the

chromophore polarity and alter the absorption spectrum. Interestingly, both *Ca*ChR1 and

*Cy*ChR1 have the spectral transition at a higher pH than that in *Cr*ChR1.

In addition, *Ca*ChR1 is different from the *Cr*ChR1 in that the spectral maximum

of their protonated forms occurs at 520 nm (*Cr*ChR1 is at 497 nm) (18). The red-shifted

spectrum of *Vc*ChR1, which has a maximum at 530 nm, due to the residues in the positions of Ser141 and Ala215 (BR numbering), are unique from all other known channelrhodopsins (9). However, *Ca*ChR1 contains Gly and Ser which remains in all other known channelopsins except *Vc*ChR1. The other structural/spatial reasons may account for their red-shifted spectral sensitivity. We need additional structural data to define the influence on pH dependence of the spectral sensitivity with crystallization or Cryo-EM.

*Ca*ChR1 contains two unique residues found in the positions of Glu194 and Glu204 (BR numbering), which are different from *Cr*ChR1 and, in fact, from all other known channelrhodopsins. The residues Glu194 is conserved in all other known channelopsins, while *Ca*ChR1 has a Ser at this position. On the other hand all previously known channelopsins have a Ser at position Glu 204 (BR numbering), but it is Asp in *Ca*ChR1. In BR, Glu194 and Glu204 attribute partially to extracellular hydrogen-bonded network in the proton release pathway (39) and are known to account for pH dependence of spectral transitions (40). It is reasonable that these residues are also in charge of regulating the pH dependence and/or color tuning in channelrhodopsins. The further studies such as mutagenesis are required to test this hypothesis.

The advantages of *Ca*ChR1 on optogenetic application are its lesser inactivation of photocurrents and red-shifted spectral sensitivity. However, the currents generated by *Ca*ChR1 in HEK cells were much smaller than those generated by *Cr*ChR1 and *Cr*ChR2. If small currents of *Ca*ChR1 result from poor expression/targeting, these can potentially be optimized by the addition of ER export sequences and/or signal peptides. This method

has successfully worked on molecular engineering of Halorhodopsin for use as an

optogenetic tool (41).

Bibliography

1. Hou, S.Y., Elena G. Govorunova, Maria Ntefidou, C. Elizabeth Lane, Elena N. Spudich, O. A.Sineshchekov and J. L. Spudich (2012) Diversity of Chlamydomonas Channelrhodopsins. Photochemistry and Photobiology, 88: 119–128

2. Bogomolni, R. and J. L. Spudich (1982) Identification of a third rhodopsin-like pigment in phototactic Halobacterium halobium. Proc. Natl Acad. Sci. USA 79, 6250–6254.

3. Spudich, J. L. and R. A. Bogomolni (1984) Mechanism of colour discrimination by a bacterial sensory rhodopsin. Nature 312, 509–513.

4. Sineshchekov, O. A., K.-H. Jung and J. L. Spudich (2002) Two rhodopsins mediate phototaxis to low- and high-intensity light in Chlamydomonas reinhardtii. Proc. Natl Acad. Sci. USA 99, 8689–8694.

5. Govorunova, E. G., K.-W. Jung, O. A. Sineshchekov and J. L. Spudich (2004) Chlamydomonas sensory rhodopsins A and B: Cellular content and role in photophobic responses. Biophys. J. 86, 2342–2349.

6. Nagel, G., D. Ollig, M. Fuhrmann, S. Kateriya, A. M. Musti, E. Bamberg and P. Hegemann (2002) Channelrhodopsin-1: A light- gated proton channel in green algae. Science 296, 2395–2398.

7. Nagel, G., T. Szellas, W. Huhn, S. Kateriya, N. Adeishvili, P. Berthold, D. Ollig, P. Hegemann and E. Bamberg (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc. Natl Acad. Sci. USA 100, 13940–13945.

8. Suzuki, T., K. Yamasaki, S. Fujita, K. Oda, M. Iseki, K. Yoshida, M. Watanabe, H. Daiyasu, H. Toh, E. Asamizu, S. Tabata, K. Miura, H. Fukuzawa, S. Nakamura and T. Takahashi (2003) Archaeal-type rhodopsins in Chlamydomonas: Model structure and intracellular localization. Biochem. Biophys. Res. Commun. 301, 711–717.

9. Zhang, F., M. Prigge, F. Beyriere, S. P. Tsunoda, J. Mattis, O. Yizhar, P. Hegemann and K. Deisseroth (2008) Red-shifted optogenetic excitation: A tool for fast neural control derived from Volvox carteri. Nat. Neurosci. 11, 631–633.

10. Kianianmomeni, A., K. Stehfest, G. Nematollahi, P. Hegemann and A. Hallmann (2009) Channelrhodopsins of Volvox carteri are photochromic proteins that are specifically expressed in somatic cells under control of light, temperature, and the sex inducer. Plant Physiol. 151, 347–366.

11. Govorunova, E. G., E. N. Spudich, C. E. Lane, O. A. Sineshchekov and J. L. Spudich (2011) New channelrhodopsin with a red-shifted spectrum and rapid kinetics from Mesostigma viride. mBio 2, e00115-11.

12. Deisseroth, K. (2011) Optogenetics. Nat. Methods 8, 26–29.

13. Chow, B. Y., A. S. Chuong, N. C. Klapoetke and E. S. Boyden (2011) Synthetic physiology strategies for adapting tools from nature for genetically targeted control of fast biological processes. Methods Enzymol. 497, 425–443.

14. Kokaia, M. and A. T. Sorensen (2011) The treatment of neurological diseases under a new light: The importance of optogenetics. Drugs Today (Barc) 47, 53–62.

15. Tomita, H., E. Sugano, H. Isago and M. Tamai (2009) Channelrhodopsins provide a breakthrough insight into strategies for curing blindness. J. Genet. 88, 409–415.

16. Doroudchi, M. M., K. P. Greenberg, J. Liu, K. A. Silka, E. S. Boyden, J. A. Lockridge, A. C. Arman, R. Janani, S. E. Boye, S. L. Boye, G. M. Gordon, B. C. Matteo, A. P. Sampath, W. W. Hauswirth and A. Horsager (2011) Virally delivered channelrhdopsin-2 safely and effectively restores visual function in multiple mouse models of blindness. Mol. Ther. 19, 1220–1229.

17. Tsunoda, S. P. and P. Hegemann (2009) Glu 87 of channelrhodopsin-1 causes pH-dependent color tuning and fast photocurrent inactivation. Photochem. Photobiol. 85, 564–569.

18. Berthold, P., S. P. Tsunoda, O. P. Ernst, W. Mages, D. Gradmann and P. Hegemann (2008) Channelrhodopsin-1 initiates phototaxis and photophobic responses in Chlamydomonas by immediate light-induced depolarization. Plant Cell 20, 1665–1677.

19. Wang, H., Y. Sugiyama, T. Hikima, E. Sugano, H. Tomita, T. Takahashi, T. Ishizuka and H. Yawo (2009) Molecular determinants differentiating photocurrent properties of two channelrhodopsins from Chlamydomonas. J. Biol. Chem. 284, 5685–5696.

20. Radu, I., C. Bamann, M. Nack, G. Nagel, E. Bamberg and J. Heberle (2009) Conformational changes of channelrhodopsin-2. J. Am. Chem. Soc. 131, 7313–7319.

21. Berndt, A., O. Yizhar, L. A. Gunaydin, P. Hegemann and K. Deisseroth (2009) Bi-stable neural state switches. Nat. Neurosci. 12, 229–234.

22. Nack, M., I. Radu, M. Gossing, C. Bamann, E. Bamberg, G. F.von Mollard and J. Heberle (2010) The DC gate in channelrhodopsin-2: Crucial hydrogen bonding interaction between C128 and D156. Photochem. Photobiol. Sci. 9, 194–198.

23. Hancock, J. M. and M. Simon (2005) Simple sequence repeats in proteins and their significance for network evolution. Gene 345, 113–118.

24. Xiao, H. and K. T. Jeang (1998) Glutamine-rich domains activate transcription in yeast Saccharomyces cerevisiae. J. Biol. Chem. 273, 22873–22876.

25. Weiss, J. L., N. A. Evans, T. Ahmed, J. D. Wrigley, S. Khan, C. Wright, J. N. Keen, A. Holzenburg and J. B. Findlay (2005) Methionine-rich repeat proteins: A family of membrane-associated proteins which contain unusual repeat regions. Biochim. Biophys. Acta 1668, 164–174.

26. Mittelmeier, T. M., J. S. Boyd, M. R. Lamb and C. L. Dieckmann (2011) Asymmetric properties of the Chlamydomonas reinhardtii cytoskeleton direct rhodopsin photoreceptor localization. J. Cell Biol. 193, 741–753.

27. Hart, G. W. (2009) Nuclear and cytoplasmic glycosylation. In Essentials of Glycobiology (Edited by A. Varki, R. Cummings, J. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart and M. E. Etzler), pp. 171–182. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

28. Kirsch, T. (2007) Functional Expression of Channelrhodopsin 2(ChR2) in the Methylotrophic Yeast Pichia pastoris and its Biophysical Characterization. Ph. D. thesis, J.W. Goethe University, Frankfurt.

29. Violot, S., N. Aghajari, M. Czjzek, G. Feller, G. K. Sonan, P. Gouet, C. Gerday, R. Haser and V. Receveur-Brechot (2005) Structure of a full length psychrophilic cellulase from Pseudoalteromonas haloplanktis revealed by X-ray diffraction and small angle X-ray scattering. J. Mol. Biol. 348, 1211–1224.

30. Thorvaldsen, S., E. Hjerde, C. Fenton and N. P. Willassen (2007)

Molecular characterization of cold adaptation based on ortholog protein sequences from Vibrionaceae species. Extremophiles 11, 719–732.

31. Hegemann, P., S. Ehlenbeck and D. Gradmann (2005) Multiple photocycles of channelrhodopsin. Biophys. J. 89, 3911–3918. Photochemistry and Photobiology, 2012, 88, 12742.

32. Lin, J. Y., M. Z. Lin, P. Steinbach and R. Y. Tsien (2009) Characterization of engineered channelrhodopsin variants with improved properties and kinetics. Biophys. J. 96, 1803–1814.

33. Gunaydin, L. A., O. Yizhar, A. Berndt, V. S. Sohal, K. Deisseroth and P. Hegemann (2010) Ultrafast optogenetic control. Nat. Neurosci. 13, 387–392.

34. Kleinlogel, S., K. Feldbauer, R. E. Dempski, H. Fotis, P. G. Wood, C. Bamann and E. Bamberg (2011) Ultra light-sensitive and fast neuronal activation with the Ca^{2+}-permeable channelrhodopsin CatCh. Nat. Neurosci. 14, 513–518.

35. Berndt, A., P. Schoenenberger, J. Mattis, K. M. Tye, K. Deisseroth, P. Hegemann and T. G. Oertner (2011) High-efficiency channelrhodopsins for fast neuronal stimulation at low light levels. Proc. Natl Acad. Sci. USA 108, 7595–7600.

36. Sineshchekov, O. A., E. G. Govorunova, K. H. Jung, S. Zauner, U. G. Maier and J. L. Spudich (2005) Rhodopsin-mediated photoreception in cryptophyte flagellates. Biophys. J. 89, 4310– 4319.

37. Frassanito, A. M., L. Barsanti, V. Passarelli, V. Evangelista and P. Gualtieri (2010) A rhodopsin-like protein in Cyanophora paradoxa: Gene sequence and protein immunolocalization. Cell. Mol. Life Sci. 67, 965–971.

38. Sineshchekov, O. A. and J. L. Spudich (2005) Sensory rhodopsin signaling in green flagellate algae. In Handbook of Photosensory Receptors (Edited by W. R. Briggs and J. L. Spudich), pp. 25–42. Wiley-VCH, Weinheim.

39. Lanyi, J. K. (2006) Proton transfers in the bacteriorhodopsin photocycle. Biochim. Biophys. Acta 1757, 1012–1018.

40. Balashov, S. P., E. S. Imasheva, T. G. Ebrey, N. Chen, D. R. Menick and R. K. Crouch (1997) Glutamate-194 to cysteine mutation inhibits fast light-induced proton release in bacteriorhodopsin. Biochemistry 36, 8671–8676.

41. Gradinaru, V., K. R. Thompson and K. Deisseroth (2008) eNpHR: A Natronomonas halorhodopsin enhanced for optogenetic applications. Brain Cell Biol. 36, 129–139.

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