Supplementary Materials for

WiChR, a highly potassium-selective channelrhodopsin for low-light one- and two-photon inhibition of excitable cells

Johannes Vierock et al.

Corresponding author: Johannes Vierock, johannes.vierock@charite.de; Peter Hegemann, hegemann@rz.hu-berlin.de

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The PDF file includes:

- Supplementary Text
- Figs. S1 to S15
- Legends for data S1 to S5
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Other Supplementary Material for this manuscript includes the following:

- Data S1 to S5
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Supplementary Text

The origin of B1ChR1 and B1ChR2

Two partial sequences from the stramenopile CCR clade, 1KP_LLXJ_2038500 and 1KP_LLXJ_2042068, were extracted by us previously from the transcriptome assemblies of the red alga *Chroodactylon ornatum* (1000 Plant transcriptomes [1KP] assembly LLXJ) and the green alga *Acrosiphonia* sp. SAG 127.80 (1KP assembly JIWJ) (4). Together with a third sequence related to *Cafeteria* ACRs (1KP_LLXJ_2001886 and 1KP_LLXJ_2001887), nearly identical ChRs were found in transcriptome assemblies of two additional unrelated algae: *Halochlorococcum marinum* (ALZF) and *Picochlorum atomus* (JQFK) signifying that none of the four algae were the source of the ChR genes. To clarify the origin of the ChRs, 18S rRNA fragments were extracted from the four assemblies by searching the transcriptomes with blastn from blast+ using *Homo sapiens* 18S as the query. The matched sequences longer than 250 nt were searched against the nr database and the only shared 18S gene shared by all four assemblies matched *Bilabrum latius* HFCC35 SSU rRNA (MN315515.1) with identity values ranging from 99-100% (Fig. S7B). Search against a collection of algal and protist protein database with ublast from usearch v. 11.0.667 (https://drive5.com/usearch/) with an e-value threshold of 1e-10 and recording best-matching taxa for each protein in the four assemblies indeed revealed a sizable fraction of transcripts of stramenopile origin (Fig. S7C). Thus, we attributed the source of the common contamination in the four algal cultures to a non-clonal *Bilabrum* sp. (Anoecida) and the ChRs sequences were labeled B1ChR1 (1KP_LLXJ_2038500), B1ChR2 (based on 1KP_LLXJ_2042068, see below) and B1ChR3 (1KP_LLXJ_2001886). Further support to the anoecid origin of B1ChR1-3 is provided by the close relationships of B1ChR1 to CbuChR1 (members of the stramenopile CCR clade) and B1ChR3 to CarACRs (collectively: anoecid ACRs) from the other anoecid *Cafeteria burkhardae* (Fig. S6A). The two species are the only currently known stramenopiles with genes from both of these ChR families.

The truncated 5’ end of the B1ChR1 transcript was extended by iterative read mapping with bowtie2 v. 2.3.5.1 (64) in --sensitive-local mode using the raw data in SRA run ERR2041128. The original transcript 1KP_LLXJ_2042068 and some of other B1ChR2 variants appeared to contain premature stop codons. Raw read mapping from the four source datasets revealed the presence of stop-free variants in SRA runs ERR2041020 and ERR3487419. Thus, the raw reads in ERR2041020 were mapped to the transcript 1KP_LLXJ_2042068 using bowtie2, duplicate reads were removed with MarkDuplicates from picard v. 2.26.5 (http://broadinstitute.github.io/picard/) and phased with samtools phase v. 1.11 (65). The haplotype with no premature stop codons was chosen and the majority-rule consensus sequence was taken to represent the gene.

Distribution of ChRs among stramenopiles

Systematic search for ChR genes in various algal and protist groups (see Suppl. Dataset S1) revealed that the KCRs reported from *Hyphochytrium catenoides* (HcKCR1, HcKCR2 and HcChR3) are part of a clade (Fig. S6A and S7A). The other stramenopiles with members of this ChR group are marine heterotrophic flagellates from the clade of Opalozoa: *Wobblia lunata* and Placidida sp. Caron Lab Isolate (Placidida) and *Cafeteria burkhardae* and *Bilabrum* sp. (Anoecida) (Fig. S6A and S7A). Additional related sequences were obtained from metatranscriptomic
datasets. Given the fact that the successfully characterized members of the clade are cation (potassium) channels, we designate the family as stramenopile CCRs. Closely related to stramenopile CCRs are cryptophyte CCRs and a novel clade of uncharacterized ChRs from colpodellid alveolates. A more basal position is occupied by dinoflagellate channels whose selectivity is not yet known (5) and a yet another clade of ChRs from colpodellids. The opposite branch of the ChR tree is occupied by all of the known ACR families including three previously characterized families from Stramenopiles and a singleton ChR from MAST-22 and the monophylum uniting prasinophyte ACRs and green algal CCRs (Fig. 3B, S6A). It is curious to note that among Stramenopiles only the anoecids appear to have ChRs from both the CCR clade and an ACR clade while the other groups possess only one ChR type each. Given the presence of related ChR families in the other phylum of the SAR supergroup, the alveolates (dinoflagellates and colpodellids), it is tempting to hypothesize that the stramenopile CCRs were present in the last common ancestor of the phylum and were lost many times, most notably in the lineage of photosynthetic stramenopiles (Ochrophyta), although lack of strong phylogenetic signal precludes robust testing of this hypothesis. The obtained clustering pattern of the stramenopile CCRs might indicate that the common ancestor of Anoecida and Placidida had two CCR genes and that \textit{H. catenoides} acquired its ChRs from Opalozo, although this remains to be replicated in the future on a bigger sample of species and proteins. As for other ChRs in general, physiological role of the different ChRs in stramenopiles is hypothesized to be in phototactic reactions in flagellated life stages (swarmers in the case of labyrinthulids (66) and \textit{H. catenoides}).
Fig. S1. K⁺-selectivity changes of HcKCR1 during continuous illumination. (A) Photocurrent traces recorded at 4 selected voltages. (B) I(E) relation for the three time points $I_1$, $I_2$, and $I_3$ either 5 ms, 100 ms or 2000 ms after light on and (C) the three determined $E_{rev}$ values. Note that the selectivity adapts during the first 100 ms, whereas the current amplitude continues to decline for another 2 seconds until $I_3$. Data show mean ± SD with $n = 5$. 
Fig. S2. HcKCR1 photocurrent adaptation and recovery in darkness. (A) A set of 14 double pulse experiments with increasing delay between pulses. (B) Recovery kinetics as the peak ratio of Peak2 over Peak1 plotted against the second pulse delay (mean ± SD, n = 5). Photocurrents were measured at 0 mV with 110 mM Na\(^{+}\) and 110 mM K\(^{+}\).
Fig. S3. Representative photocurrent traces at either high $K^+$(red) or $Na^+$(blue) and $I(E)$ relations of HcKCR1 pore mutants measured at 530 nm illumination. Boxes show enlarged signals with the given magnification factor. Most mutations have no or little influence on the $K^+$ selectivity except D87L and N99L. All data show mean ± SD.
Fig. S4. Photocurrent traces at either high K$^+$ (red) or Na$^+$ (blue) and $I(E)$ relations of HcKCR1 pore mutants upon 530 nm illumination (continued). W102 mutations to Q, H and P as well as D116N and Y222A show drastic K$^+$ selectivity loss. All data show mean ± SD.
Fig. S5. Spectral and electrophysiological properties of HcKCR1 and pore mutants. (A) Action spectra of normalized peak photocurrents following 10 ms light pulses of different color but equal photon density for HcKCR1-WT and central pore mutants (n = 6/6/7/5 for WT/Y106F/T109A/C232A at 0 mV) (B) for counter ion mutants (n = 6/6/5 for WT/D105N/D229N at 0 mV) and (C) for outer pore mutants (n = 6/5/9 for WT/W102Q/Y222A at -40 mV). (D) Peak-photocurrent densities at -40 mV holding voltage and (E) channel closure half time at -40 mV holding voltage. Photocurrents in (A-C) and (E) were recorded with high extracellular Na\(^+\) and in (D) with high extracellular K\(^+\). All data show mean ± SD.
Fig. S6. Phylogenetic analysis and diversity of the stramenopile CCRs and related ChRs. (A) Phylogeny of channelrhodopsins. ChR subfamilies are colored by the selectivity of the characterized members: blue for cation channels and red for anion channels. Highlighted in bold are stramenopile CCRs (HcKCR1, HcKCR2 (17), HcCCR (21), WlChR1 and B1ChR2). Species prefixes are as follows: B1 - *Bilabrum* sp., Cbu - *Cafeteria burkhardae*, Hc - *Hyphochytrium catenoides*, P1 - Placidida sp. Caron Lab Isolate, Wl - *Wobblia lunata*. The sequences and the tree are available as Suppl. Data File S2 and gene annotations for stramenopile CCRs as Suppl. Data File S3. (B) Residues involved in potassium selectivity in KCRs and the TM3 motif (105-109-116) across the subfamily of stramenopile CCRs and the related subfamilies of cryptophyte CCRs and uncharacterized proteins from Colpodellida.
Fig. S7. Stramenopile with ChR genes. (A) Distribution of ChR families among Stramenopiles. Stramenopiles in the groundplan are heterotrophic heterokont flagellates. Appearance of fungus-like forms is indicated for hyphochytrids, oomycetes and labyrinthulids and of photosynthetic forms for ochrophytes. Blue and red dots correspond to individual ChR genes (note that the number of the ACR genes per assembly among Labyrinthulidae varies). Numbers in parentheses indicate the number of species for which genetic data are available (for SAGs a conservative estimate is given). MAST (marine stramenopile) groups for which only single SAGs were available are omitted. Consensus cladogram according to (67-70). (B) and (C) ChRs B1ChR1, B1ChR2 and B1ChR3 appearing in transcriptome assemblies of four unrelated algae come from an anoecid stramenopile: (B) Incidence of SSU rRNA gene fragments in the four transcriptome assemblies. (C) Distribution of the best ublast hits among the protein sequences predicted for the four algae.
Fig. S8. Structure-based multiple sequence alignment of KCRs and ChRmine (see Suppl. Data File S4). Conservation for each position is represented as a sequence logo (y-axis reflecting bits of information). Secondary structure features are shown based on the structure of ChRmine (PDB: 7W9W): α-helical regions shown as cyan bars above and predicted transmembrane domains as green bars below. The alignment was trimmed at both ends to the rhodopsin domain proper with numbers in parentheses indicating the number of the residues trimmed from the N-terminus of each sequence.
Fig. S9. Membrane targeting of KCRs in ND7/23 cells (A) Confocal images of ChRmine and the potassium selective HcKCR1, WiChR, and B1ChR2 tagged with the named fluorescent proteins in ND7/23 cells. Scale bar: 10 µm. (B) Quantification of membrane targeting for the constructs visualized above. The targeting was calculated as the ratio between fluorescence density in the outer cell border and the enclosed intracellular fluorescence density.
Fig. S10. AlphaFold tertiary and 3D pore structures (A) ChRmine based on an in silico equilibrated Cryo-EM structure (PDB-ID 7SFK) and K⁺-selective CCRs (B) HcKCR1, (C) WiChR and (D) B1ChR2 based on equilibrated AlphaFold2 models. Amino acids involved in cation selectivity and conductance in HcKCR1 and their homologues in other BCCRs are represented as licorice, those important for K⁺ selectivity are highlighted in color. The outer pore in K⁺ selective BCCRs is smaller and more separated from the bulk compared to ChRmine. In HcKCR1, a hydrophilic and water-filled cavity between helices 3, 4, 6 and 7 is delimited from the central gate by W102 and Y106 and from the bulk water by W210, Y222 and N99. Geometrical RMSD calculations of aromatic residues in HcKCR1 revealed high flexibility of F88 (2.00 Å), W102 (2.12 Å), Y106 (1.85 Å) and W210 (2.27 Å) side chains, while Y222 was more rigid (1.13 Å) due to predicted hydrogen bonds to N99 and Q218. (Fig. S10B) This flexibility would allow the formation of a continuous cation translocation pathway during the conducting state. While amino acids of the selectivity filter in KCRs are identical in B1ChR2, their orientation, especially of N98 and W101, was found to be vastly different due to modification of secondary residues. The same is true for counterion D104. Structurally, an overall high degree of conformity of HcKCR1 and WiChR, but not B1ChR2 was revealed. The KCR outer pore segments differ substantially from the ChRmine geometry, especially due to the abundance of aromatic amino acids resulting in different architectures of helices 2 and 3 and the extracellular loop 1. The model of B1ChR2 is an exception and its secondary structure has more similarity to ChRmine in that respect. Also, the
contacts between helices 1, 2, 3 and 7 in WiChR and B1ChR2 are more polar because HeKCR1-C29 is exchanged to WiChR-D47 and B1ChR2-N24, thus attracting water molecules.
Fig. S11. Two-photon holographic stimulation system. (A) Schematic of the microscope. The holographic stimulation is based on the use of a low (500 kHz) repetition rate femtosecond laser at $\lambda=1030$ nm. The laser beam is phase-modulated by a liquid crystal based Spatial Light Modulator (SLM) and then projected at the pupil of the microscope objective (OBJ). Arbitrary illumination shape can be reproduced at the sample plane. (B) Top and side view of the fluorescence generated by a circular holographic spot (12 $\mu$m diameter), typically used for single cell photostimulation. Top and side view are acquired with the upper and a lower inverted objective respectively (see Methods). On the right, the integrated fluorescence axial profile: experimental point (black) and Gaussian fit (red) with an axial FWHM of 29 $\mu$m.
Fig. S12. Neuronal membrane parameters as determined right after establishing whole-cell configuration and switching to current-clamp mode (no holding current) for non-expressing neurons, neurons expressing HcKCR1 and neurons expressing WiChR. At a significance level of 0.01 (two-sample t-test) no differences were observed between expressing and non-expressing cells for all parameters; Although we observed a large variance especially for the input resistance and the rheobase within the groups most likely indicative of different cell types. (A) Input resistance determined by injection of hyperpolarizing and depolarizing square currents (10 pA steps) and linear interpolation from the ΔV vs. ΔI curve. (B) Resting membrane potential determined within the first 30 s after break in. (C) Rheobase extracted as the first square current injection (1 s, 10 pA steps) that evoked an action potential.
Fig. S13. Inhibition with pulsed 2P holographic illumination and corresponding local temperature rise in the tissue. (A) Whole-cell current clamp recordings of HcKCR1 (top) and WiChR (bottom) with square current injections (1 s) of rising amplitude and an inhibition period of 500 ms in the middle. From left to right the duration of illumination is reduced from continuous for the whole 500 ms over 10 Hz and 5 Hz to 2 Hz pulsing of 5 ms illumination. All illumination protocols were compared at the same cell; power densities were the same for HcKCR1 and WiChR and the different frequencies (28-33 μW/μm²). (B) Extraction of the firing rates for all pulsed protocols (and non-illuminated neurons) before (250 ms), during (500 ms) and after (250 ms) the illumination period normalized to the first 250 ms. Firing rates are shown for current injection 50 pA (left) and 100 pA (right) above the respective rheobase; determined as the first spike during the first or last 250 ms without light. Values are shown as mean±SD (HcKCR1_50pA n=6, WiChR_50pA n=7, noLight_50pA n=8, HcKCR1_100pA n=4, WiChR_50pA n=3, noLight_50pA n=3). (C) Simulation of local heating (r = 0) induced by one holographic spot (12 μm diameter, 31 μW/μm², 1030 nm) for continuous illumination (red) compared to pulsed 5 ms illumination (green) at different frequencies. Dashed lines indicate the peak temperature rise within 2 s. (D) Simulation of local temperature rise for r = 0 (center of the first spot; white) as in (C) with increasing number of holographic spots placed within a 100 x 100 μm square (left); white - spot 1; blue - spots 2 - 5; red - spots 6 - 10; orange - spots 11 - 20; light blue - spots 21 - 40. Middle: time course of temperature rise for continuous (dashed line) vs. 2 Hz (cont. line) illumination for 1, 5, 10, 20 and 40 spots placed as shown on the left; inset shows the first 30 ms. Right: Extraction of the peak temperature rise after 2 s for indicated number of holographic spots comparing continuous (blue) and pulsed illumination (green).
Fig. S14 Neuronal inhibition with 2P holographic stimulation of WiChR in mouse visual cortex in vivo. (A) Whole-cell patch clamp recordings for the demonstration of action potential suppression in three WiChR-expressing neurons from two different mice in layer 2/3 of visual cortex, here 153 µm, 105 µm, and 129 µm deep for cells 1-3 respectively. AP firing was induced by current injections above the rheobase of the respective neuron and for inhibition 10 Hz trains (5 ms) of temporally-focused 2P holographic illumination were targeted at the soma of the patched cell. For each cell, the membrane potential traces of two repetitions are shown and photostimulation is indicated in red; liquid junction potential of +11.9 mV was corrected. For each repetition, the firing rate before, during and after illumination was extracted and is plotted below, mean±SD across repetitions plotted in red. (B) Similar recording as in (A) but with different illumination frequencies of the 5 ms pulse, here 10, 20, and 30 Hz, showing inhibition can be increased by illuminating at higher frequencies. For each stimulation frequency two repetitions are shown and photostimulation is indicated in red. For each repetition, the firing rate before, during and after illumination was extracted and is plotted below, mean±SD across repetitions plotted in red.
Fig. S15 WiChR effects on the physiology of hiPSC-derived atrial cardiomyocytes (aCMs). (A) Representative photocurrent and membrane hyperpolarization trace from patch-clamp recordings of WichR-expressing aCMs. (B) Comparison of AP duration at 90% repolarization (APD$_{90}$) for control cells and cells before and after AP inhibition. (C) Comparison of maximum upstroke velocity for control cells and cells before and after AP inhibition. (D) Comparison of AP frequency for control cells and cells before and after AP inhibition. Before light: Average of ten AP before light application. After light: Average of 11$^{th}$ to 20$^{th}$ AP after light. Mann-Whitney test was used for group-wise statistical analyses.
Other Supplementary Materials

Data S1 List of searched stramenopile assemblies. Stramenopile transcriptome, genome and SAG assemblies recruited in the search for ChR genes and the detected ChR families, Excel spreadsheet.

Data S2 Channelrhodopsin phylogeny. Representative sequences of ChRs and outgroups used for the phylogenetic reconstruction in fasta format and the resulting phylogeny in newick format.

Data S3 Annotated ChR transcripts. Annotated sequences of the transcripts coding for stramenopile CCRs of Bilabrum sp., Placidida Caron Lab Isolate and Wobblia lunata in genbank format.

Data S4 Structure based alignment Trimmed structure-based alignment of ChRmine, HcKCR1, HcKCR2, WiChR1 and B1ChR2. Clustal alignment file.

Data S5 PDB files of the equilibrated AlphaFold2 models of HcKCR1, WiChR and B1ChR2.

Movie S1 WiChR activation inhibits spontaneous contractions of hiPSC-derived aCMs. Representative video shows camera-based imaging of hiPSC-derived aCMs before, during (blue circle) and after blue light application. Corresponding analysis is shown in Fig. 7B.

Table S1 Sequences of primers and genes Summery of primers for site-directed mutagenesis and human codon adapted DNA sequences of synthesized ChRs.
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